Stem Cells International

Phases I–III Clinical Trials Using Adult Stem Cells

Guest Editors: Jeffrey M. Gimble, Bruce A. Bunnell, Louis Casteilla, Jin Sup Jung, and Kotaro Yoshimura

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doi:10.4061/2010/604713

Editorial

Phases I–III Clinical Trials Using Adult Stem Cells

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Received 31 December 2010; Accepted 31 December 2010

Mesenchymal progenitor/stromal/stem cell (MSC) research has made substantial progress during the past decades. Investigators have published a wealth of basic science and preclinical data documenting the potential utility, efficacy, and safety of MSCs. The majority of this work has focused on the bone marrow- or adipose-derived MSCs. Since this body of evidence has set the stage for clinicians to advance from the bench to the bedside, *Stem Cells International* set out to publish a special issue devoted to the topic of *Phases I–III Clinical Trials Using Adult Stem Cells*. The result is a collection of ten outstanding articles submitted by investigators representing ten countries across Asia, Europe, and North America. In all cases, the methods section of each manuscript included a statement documenting that the clinical investigations were performed following institutional review board approval and/or that patient informed consent had been provided prior to the conduct of the study.

Bieback et al. in Germany (*Translating research into clinical scale manufacturing of mesenchymal stromal cells*) and B. Philippe et al. in France (in "*Culture and Use of Mesenchymal Stromal Cells in Phase I and II Clinical Trials*") have focused on the process of MSC isolation and expansion with respect to current Good Manufacturing Practices (cGMP). These authors highlight the state of the art as well as the challenges facing the development of clinical grade cells for therapeutic applications.

From a regulatory perspective, the simplest application of MSC is to use them in the context of their tissue of origin. S. Akita et al. in Japan (in "*Noncultured Autologous Adipose-Derived Stem Cell Therapy for Chronic Radiation Injury*") have exploited adipose-derived stem cells for soft tissue regeneration. They describe their clinical experience using autologous adipose-derived stem cells in combination with growth factors and scaffold to treat ten patients suffering from dermal radiation lesions. A series of three review papers from Belgium ("*Phase 1–3 Clinical Trials Using Adult Stem Cells in Osteonecrosis and Nonunion Fractures*" by J. –P. Hauzeur and V. Gangji), the USA and China ("*Mesenchymal Progenitor Cells and Their Orthopedic Applications: Forging a Path towards Clinical Trials*" by D. S. Shenaq et al.), and the Netherlands ("*Clinical Applications of Human Mesenchymal Stromal Cells for Bone Tissue Engineering*" by Chatterjea et al.) have examined the use of bone marrow-derived MSC for orthopedic applications. Each work provides a unique perspective on the published clinical trials using MSC to treat cartilage and tendon repair, critical sized defects, metabolic bone diseases, nonunion fractures, and/or osteonecrosis.

From a public health perspective, MSC applications for common cardiac and central nervous system disorders could

have substantial health and societal benefits. R. Sanz-Ruiz et al. in Spain (in "*Phases I–III Clinical Trials Using Adult Stem Cells*") have emphasized the critical importance of randomized clinical trials to assess the utility of MSCs as they focus on the clinical evidence supporting MSC-based treatment of cardiac disease. Furthermore, they review the guidance documents on future clinical trials provided by a task force convened by the European Society for Cardiology. P. A. Walker et al. in the USA (in "*Progenitor Cell Therapy for The Treatment of Central Nervous System Injury: A Review of the State of Current Clinical Trials*) provide a similar perspective relating to central nervous system disorders. They evaluate the use of bone marrow-derived MSCs for ischemic stroke, spinal cord injury, and traumatic brain injury and conclude that there remains a need for further evidence to support these clinical applications.

There are potential MSC applications for metabolic disorders as well. A. C. Piscaglia et al. in Italy (in "*Stem Cell Therapies for Liver Disease: State of the Art and New Perspectives*) have reviewed the limited number of clinical trials using hematopoietic stem cell (HSC), MSC infusion, or cytokine (G-CSF) therapy to reverse hepatic injury due to cirrhosis, drug exposure, or other causes of end stage liver disease. Likewise, A. V. Vanikar et al. from India (in "*Cotransplantation of Adipose Tissue-Derived Insulin Secreting Mesenchymal Stem Cells A Novel Therapy for Insulin-Dependent Diabetes Mellitus*") describe their experience combining adipose- and bone marrow-derived MSC to treat a cohort of 11 diabetic subjects for a period of up to 23 months. They report improved hemoglobin A1C levels as well as decreased daily requirements for exogenous insulin.

Uniformly, these authors highlight both the promise and the challenges faced by this emerging field of medicine. Their manuscripts identify the critical need for additional prospective, randomized controlled clinical trials evaluating all cell-based therapies, regardless of the underlying disorder. In summary, this *special issue* provides a snapshot of the current status of MSC-based clinical trials across the globe. Hopefully, this publication will provide a benchmark for future meta-analyses evaluating a far greater body of clinical evidence regarding the safety and efficacy of MSC therapies.

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SAGE-Hindawi Access to Research
Stem Cells International
Volume 2010, Article ID 193519, 11 pages
doi:10.4061/2010/193519

Review Article

Translating Research into Clinical Scale Manufacturing of Mesenchymal Stromal Cells

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Received 16 April 2010; Revised 26 November 2010; Accepted 17 December 2010

Academic Editor: Jin Sup Jung

It sounds simple to obtain sufficient numbers of cells derived from fetal or adult human tissues, isolate and/or expand the stem cells, and then transplant an appropriate number of these cells into the patient at the correct location. However, translating basic research into routine therapies is a complex multistep process which necessitates product regulation. The challenge relates to managing the expected therapeutic benefits with the potential risks and to balance the fast move to clinical trials with time-consuming cautious risk assessment. This paper will focus on the definition of mesenchymal stromal cells (MSCs), and challenges and achievements in the manufacturing process enabling their use in clinical studies. It will allude to different cellular sources, special capacities of MSCs, but also to current regulations, with a special focus on accessory material of human or animal origin, like media supplements. As cellular integrity and purity, formulation and lot release testing of the final product, validation of all procedures, and quality assurance are of utmost necessity, these topics will be addressed.

1. Mesenchymal Stromal Cells in Cellular Therapies

The vision for cellular therapies in regenerative medicine seems obvious: to replace diseased, dying, or missing cells or tissues with healthy cells [1]. The role of stem cells in this respect is under intense scrutiny, to define principles of organ regeneration and to develop innovative novel methods to treat organ failure. Mesenchymal stem or stromal cells (MSCs) emerge as key candidates for cellular therapies, covering regenerative and immune therapies.

MSCs have a great appeal for cell and immune therapy and tissue engineering for numerous reasons:

(1) they are relatively easy to procure from a variety of tissues [2];

(2) they expand rapidly in cell culture [3];

(3) they show only minor spontaneous differentiation during *ex vivo* expansion [4];

(4) they are multipotent [4, 5];

(5) they form supportive stroma for hematopoiesis and support hematopoietic stem cell engraftment [6];

(6) they seem to be largely immunologically inert, paving the way for allogeneic transplantations [7];

(7) they are immunosuppressive [8];

(8) they secrete numerous trophic factors which modulate inflammation, remodelling, and apoptosis [9].

Based on the initial work of Friedenstein and Caplan, bone marrow-derived MSCs (BM-MSCs) are the ones best described and most advanced in clinical settings. In most comparative studies, BM-MSCs serve as gold standard [2]. For therapeutic applications, easily accessible and highly abundant sources are advantageous. Adipose tissue (AT), most often obtained as lipoaspirate, has emerged as an alternative tissue because cells occur at high frequency and the procurement is less invasive than that of BM aspiration [2]. Blood is obviously the most accessible adult tissue source for cells. Peripheral blood, however, does not contain MSCs in a nonpathological setting, at least not in numbers relevant for clinical scale manufacturing [10]. The same findings still

hold true for umbilical cord blood (CB) [2, 11]. An inverse correlation between the gestational age and yields of MSCs is described [12]. Thus MSCs in full term CB are present at only low frequencies which hamper the isolation success [2, 11, 13]. In contrast to CB, isolation success is guaranteed when using the umbilical cord matrix or Wharton's jelly [14]. Fetal tissues are interesting as they seem to contain comparatively immature MSCs expressing pluripotency markers like SSEA-3, and 4, Oct-4, Sox-2 and Nanog [15].

2. Towards Clinical-Scale Manufacturing of MSCs

MSCs are increasingly used in many preclinical as well as in some clinical settings for immunomodulation or tissue repair as summarised in more detail in the adjoining sections of this special issue. It is important to note that up to date administration of MSCs proved to be safe and also efficacious in a variety of disorders [16]. Several of these disorders are characterized by both inflammation and tissue defects. Often it cannot be dissected whether efficacy of MSCs is due to their production of trophic factors which stimulate endogenous repair mechanisms, their direct differentiation into various cell types or their immunomodulatory effects. In some of the models, factors released by the MSCs are obviously sufficient to mount a substantial part of the effect [9]. Thus whether efficacy of MSCs requires long-term persistence of MSCs remains to be elucidated.

Translating the rapid progress in stem cell science into innovative cellular therapies led to early and late stage clinical trials. However the complexity of the translational process ranging from the conception, to advanced clinical testing and finally new cell therapeutic drugs revealed a lag behind of standards and guidelines and often suffer from a regulatory burden. Increased scrutiny by the regulatory authorities is mutually dependent from the increasing numbers of cell therapy clinical trials. A variety of organisations responded to that threat to propose minimal set of standards or consensus guidelines [17–21].

Controversies about characteristics and potencies exist most probably due to the fact that different laboratories employ not only different tissue sources, but also extraction methods, culture protocols, and characterisation tools. Any variation may result in isolation and expansion of different subpopulations of cells or may change characteristics of the cells [22]. Thus Dominici et al. proposed minimal criteria defining MSCs [17], namely,

(1) adherence to plastic under standard culture conditions,

(2) expression of CD105, CD73, and CD90, and lack of expression of CD45, CD34, CD14, or CD11b, CD79alpha or CD19 and HLA-DR surface molecules,

(3) differentiation to osteoblasts, adipocytes, and chondroblasts *in vitro*.

Given the fact that even MSCs used in clinical trials are produced and characterised by a variety of different protocols, reproduction or interpretation of the clinical results may be hampered [21]. Accordingly, standardized protocols have to be developed assuring that the manufactured cells behave solely in the clinically intended purpose and do not exert adverse effects by, for example, uncontrolled differentiation or transformation. Typically MSC can be cultured for 40–50 population doublings until the growth rate declines significantly and cells undergo replicative senescence [23]. Under certain conditions, however, MSCs have been observed to transform spontaneously. Discrepancies were resolved, when laboratories reported that transformation of MSCs was caused by cross-contamination with tumor cell lines [24, 25].

Therefore, an essential requirement is that all steps in MSC manufacturing from starting material up to potency testing for the intended indication have to be highly standardized to assure a required and reproducible cellular quality and potency. The challenge for scientists aiming at producing MSCs for clinical trials is to define optimal cell culture conditions to efficiently isolate and *ex vivo* expand homogenous MSCs while maintaining cellular qualities required for the intended clinical application and minimising risks of adverse events. For example, we recently verified whether AT-MSCs isolated and expanded in human serum (HS) share characteristics with cells cultivated in fetal bovine serum (FBS) [22, 26]. Although all criteria defined in [17] have been fulfilled, minimal differences were obvious regarding cell size and gene expression profiles. By comparing two human supplements to FBS, we observed that FBS alters gene expression slightly, but in genes categorized to differentiation and adhesion/extracellular matrix [22]. Current studies focus on dissecting in detail whether the supplements alter cellular behaviour in a way affecting the intended therapeutic application.

The therapeutic aim is to repair cell or tissue damage but without the risk of inducing tumors, severe immune reactions, or unwanted tissue development. Thus both safety and efficacy measures shall be considered in the establishment of the manufacturing process. Scarcity of MSCs often requires *ex vivo* expansion; extensive expansion in consequence may lead to ineffective or degenerated cells [23]. Thus it is important to understand and carefully control the production process and accordingly to define measures that reliably predict safety and efficacy of cell therapeutics.

3. Regulatory Frameworks

The employment of adult stem cell types in clinical studies, in general, necessitates formal approval by the respective regulatory body. This approval requires manufacturing, processing, and testing of cellular products according to the current national regulations, including current good tissue practice (GTP), good manufacturing practice (GMP) and good clinical practice (GCP). All cell-based products shall comply with these rules to ensure the product is safe, pure, and potent. GTP and GMP refer to common standards, regulating facilities, personnel, equipment, reagents and supplies, procedures and finally controls (process, final product and laboratory controls). These standards should

be considered as soon as the development of a cellular product begins. For that reason, regulatory authorities offer investigators their advice to fine-tune the process from the early beginning on.

In Europe, MSCs are classified as advanced therapy medicinal products (ATMPs) [27, 28]. ATMPs include gene therapy medicinal products, somatic cell therapy products (as defined in Directive 2001/83/EC), and tissue-engineered products. Cells fall under this regulation in case they have been subjected to substantial manipulation, resulting in a change of their biological characteristics, physiological functions or structural properties relevant for the intended therapeutic application, for example, regeneration, repair, or replacement. ATMP refers to cells or tissues that are not intended to be used for the same essential functions in the recipient as in the donor. This means that MSCs can be considered as somatic cell therapy products or tissue-engineered products depending on the indication and the manipulation during the manufacturing process. Concerning clinical trials with MSCs the rules set out in Article 6(7) and Article 9(4) and (6) of Directive 2001/20/EC shall apply. In the EU the responsible body for clinical trials approval are the health authorities at national level. This is in contrast to USA, where the NIH takes over this part [28, 29]. In the EU, GMP and GCP are more interrelated than in USA. The European Regulation No. 1394/2007 is effective since December 2008 and is binding in its entirety and directly applicable in all Member States. (Regulation (EC) No 1394/2007 of the European Parliament and of the council http://ec.europa.eu/health/human-use/advanced-therapies/index_en.htm. The main elements are (i) a centralised marketing authorisation procedure, (ii) the committee for advanced therapies (CAT) as multidisciplinary scientific committed to review the quality, safety and regulatory aspects of ATMP, (iii) technical requirements adapted to particular ATMP characteristics incentives for small- and medium-sized enterprises.) It is in compliance with the 2004/23/EC directive on donation, procurement and testing of human cells and tissues and with the directive 2002/98/EC on human blood and blood components. It is amending the Guideline on cell-based medicinal products (EMEA/CHMP/410869/2006) which focuses on the manufacturing and quality control of cell-based medicinal products as well as their nonclinical and clinical development.

In the US, the FDA (Food and Drug Administration) announced in 1997 the "Proposed Approach to Regulation of Cellular and Tissue-Based Products" (21 CFR 1271). (http://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/CFRSearch.cfm?CFRPart=1271. Sec. 1271.1: "The purpose of this part,..., is to create a unified registration and listing system for establishments that manufacture human cells, tissues, and cellular and tissue-based products (HCT/P's) and to establish donor-eligibility, current good tissue practice, and other procedures to prevent the introduction, transmission, and spread of communicable diseases by HCT/P's".) This became effective in 2005 as rules for Human Cells, Tissues, and Cellular and Tissue-Based Products (HCT/Ps). Although only one cell-based product (Carticel, autologous cell-based product for cartilage repair manufactured by Genzyme) has been licensed by the FDA, this does not reflect the actual number of trials with cell-based products. Those cell-based therapeutics do not require FDA-approval "that are minimally manipulated, labeled or advertised for homologous use only, and not combined with a drug or device" as specified by Parson, [30]. In contrast, manipulated autologous cells for structural use meet the definition of somatic cell therapy products and require an "investigational new drug" (IND) exemption or the FDA-license approval. In 2007 the "Guidance for Industry: Regulation of Human Cells, Tissues, and Cellular and Tissue-Based Products (HCT/Ps)—Small Entity Compliance Guide" and in 2009 the "Guidance for Industry on Current Good Tissue Practice (cGTP) and Additional Requirements for Manufacturers of Human Cells, Tissues and Cellular and Tissue-based Products" (http://www.fda.gov) have been released. The FDA provides recommendations to support manufacturing establishments of HCT/Ps to better understand and comply with the regulatory framework. Clinical studies employing MSCs underlie the IND mechanism. Accordingly investigator have to make an IND application, which necessitates detailed study protocols, describing the clinical plan as well as the preparation and testing of the therapeutic cell product [31].

Both regulatory frameworks in the EU and USA are to assure safety and thus they require a thorough analysis of all critical steps and aspects in advance. Although there are still differences [19, 28, 29], the authorities are in contact to further harmonise them. Thus it can be expected that by serving the requirements of one community, the chance is high to fulfil the others as well.

In the following parameters relevant for the manufacture of MSCs are exemplified.

4. Manufacturing Process

The manufacturing process is highly fragmented as exemplified in Figure 1, illustrating a GMP-compliant MSC manufacturing process with processing and testing steps. Thus it should be well established and validated before initiating pivotal clinical trials because changes in the manufacturing process may confound clinical trial results.

4.1. Tissue Procurement. In general, the starting material is a critical issue and includes common donor eligibility criteria, like age and viral testing. MSCs have been applied in autologous and allogeneic settings and derived from various tissue sources. Due to an immuno privileged status, a single allogeneic MSC donor may serve for multiple recipients raising the demand for well-defined eligibility criteria [32].

The most often used cellular source to obtain MSCs is BM, followed by AT and then other tissue sources, where we will herein focus on perinatal tissues. BM-derived MSCs are harvested via BM aspiration after puncture of the donors iliac crest. Aspiration strategy and volume impact the yield of MSCs, so that multiple aspirations from the same site and low aspiration volume (<8 mL) should be avoided [33].

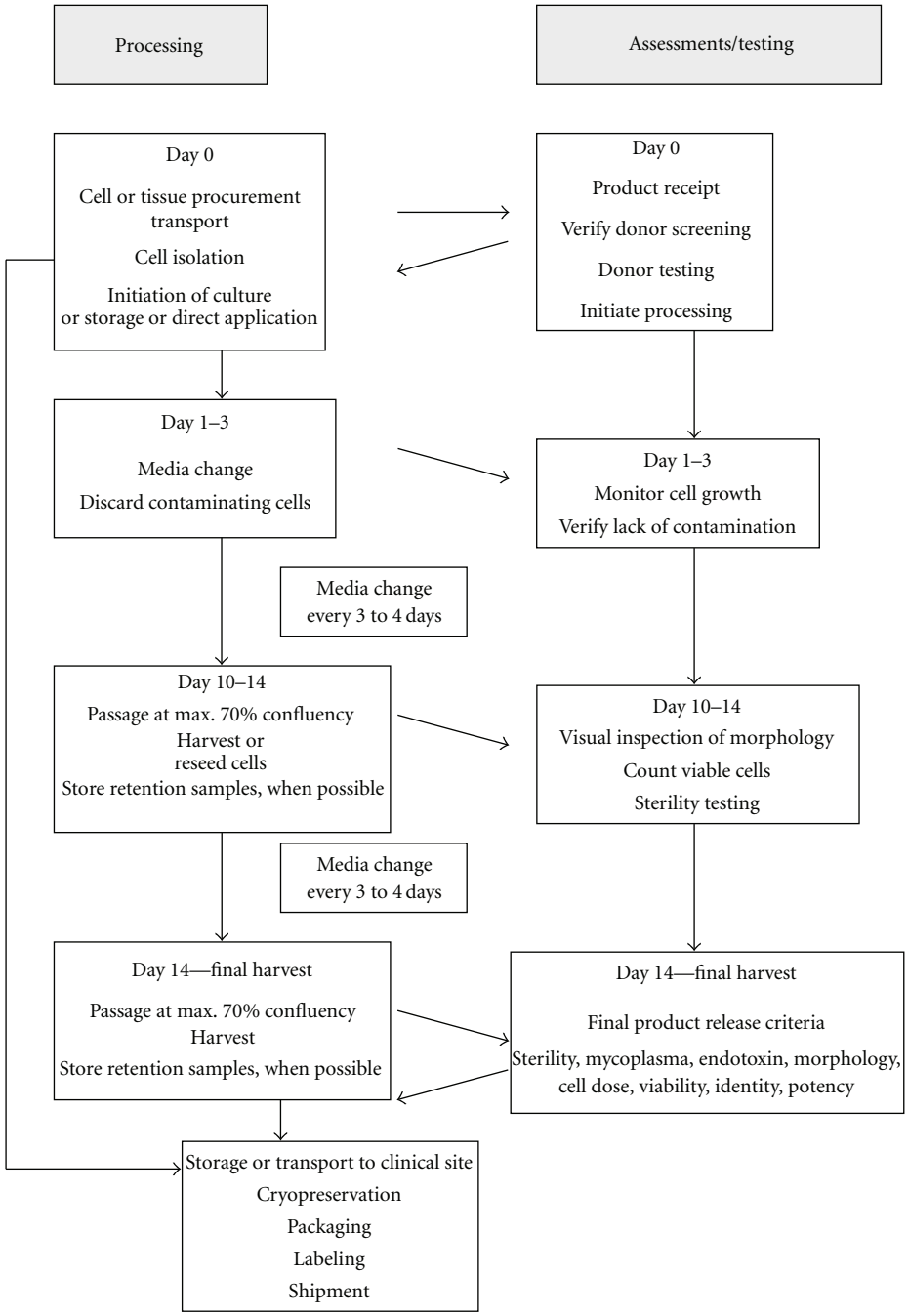

Figure 1: Flow chart illustrating essential processing and testing steps during MSC manufacture. This scheme summarises a GMP-compliant manufacturing process of MSC expansion separated into processing and related testing activities. On day zero the tissue is harvested and transported to the processing lab. Here donor eligibility criteria are checked again and donor testing/reception control initiated (viral, bacterial, blood group, condition, weight, if applicable cell counting, etc.). Verified reception control is a prerequisite for starting processing in the GMP facility. Here cell isolation is performed and expansion cultures initiated or the product is directly applied to the patient. Predefined in-process controls shall be taken at any critical processing step to verify cellular qualities and sterility. If expansion is initiated, normal protocols include a medium exchange step concomitantly depleting contaminating cells. Cell growth can be monitored visually as well as potential contamination. Passaging of the cells can be performed within the next 14 days involving controls for morphology, viability, and sterility. Assuming day 14 for the day of harvest and product release, cells have to be specified against predefined final product release criteria. The product can then be cryopreserved allowing for additional potency assays or directly transported to the recipient. Packaging, labeling, and shipment conditions again have to follow GMP rules.

Further hints indicate that donor age impacts the cell yield and the differentiation potential of MSCs [34, 35]. Aspirated BM volume can be a critical issue as high volumes can result in dilution with blood, too low volumes however demonstrated low to highly heterogeneous yields of MSCs [33]. Some data indicate that MSCs can be isolated from the marrow filter washouts dedicated for hematopoietic stem cell procurement [36]. For isolation, most protocols employ density gradient centrifugation, although the necessity for this step is debated and still under optimisation [37]. Mononuclear cells are then cultivated in MSC culture media until fibroblastoid cells show outgrowth.

AT represents an accessible source of MSCs, often referred to as adipose stem cells (ASCs) [38]. AT can be procured by different techniques, including excision or aspiration, from regions of the body where it is largely present (abdomen, trochanter region, groin, knee). MSCs can be isolated from the tissue by collagenase digestion and centrifugation and cultivation of the stromal vascular fraction to give rise to MSCs [2]. Various studies analysed the impact of harvesting conditions to the cell yield. The yield of ASC does not seem to be affected by the aspiration technique comparing syringe-based or pump-assisted liposuction [39], still there seem to be significant differences between the harvesting sites concerning the adipogenic properties [40] and their susceptibility to apoptosis [41]. Regarding the yield of the harvested ASC, there are controversial results: some report a richer yield of nucleated cells and colony forming units in hip versus abdominal liposuction [39], some others a superior yield of colony forming units after abdominal liposuction compared to hip liposuction [42].

In studies comparing liposuction versus tissue excision, liposuction method turns out to be superior as the cell yield in aspiration material remains stable even after 24 h storage in contrary to the decreasing cell yield of excisates. In contrast to these results a latter study reveals a higher cell yield and viability after excision [43].

Concerning the influence of the negative pressure during liposuction negative pressure of −350 mmHg leads to a greater cell yield than lower pressure of −700 mmHg [44].

Postnatal gestational tissues inherit numerous advantages over MSCs derived from aged adult tissues. Early focus on perinatal tissues harbouring stem cells arose from HSC and MSCs identified in CB [11, 45]. Subsequently fetal liver, lung, brain, but also villous placenta, fetal membranes as well as amniotic fluid were identified to host MSCs [13, 15]. Not discussing abortal tissues, in the majority of cases perinatal tissues are discarded at birth, thus cells harvestable without any risk for the baby or its mother. Consequently, there is an unlimited supply, easy access, and minimal ethical/legal issues associated with perinatal tissues. Tissues can be stored for autologous use or allogeneic settings as fetal cells have been demonstrated to be immunoprivileged. Hence CB storage is one strategy widely followed in numerous countries, not only for allogeneic, but also for potential autologous applications [46].

4.2. Manufacture at the Bedside: Volume Reduction and Direct Application. Some therapeutic applications, for example, in cardiac cell therapy, use solely minimally processed tissues, like volume reduced mononuclear cells which can be performed at the patients bedside [47]. This suggests an attractive and probably cost and time reducing option for autologous therapeutic settings and fall under different regulations (the ATMP so-called "hospital exemption") ("Any advanced therapy medicinal product, as defined in Regulation (EC) No 1394/2007, which is prepared on a nonroutine basis according to specific quality standards, and used within the same Member State in a hospital under the exclusive professional responsibility of a medical practitioner, in order to comply with an individual medical prescription for a custom-made product for an individual patient." (EU regulation 1394/2007)). Due to the high precursor frequency especially AT can be processed at the patients' bedside [48]. Still there are different aspects that have to be considered in order to minimize the risks of cellular therapy [49, 50]. But minimally processed tissues contain a large heterogeneous mixture of stem, progenitor cells, and mature cells, thus of suboptimal composition [51]. A few studies comparing volume reduced to expanded AT-derived cell preparations revealed different results [52, 53]. Consequently, to the current date all clinical applications specifying the use of MSCs have been using culture expanded cells.

4.3. Manufacture in a GMP-Facility

4.3.1. Isolation and Expansion. In order to fulfil the regulatory standards the manufacture of a cell product requires the use of safe and pure components and materials. If possible, licensed or GMP-grade reagents should be used, in case of research-grade reagents additional in-house testing may be required to ensure safety and quality. Reagents, including supplements, cytokines, growth factors, used for expansion or differentiation of MSCs should be controlled and documented. Several parameters for *ex vivo* expansion of MSCs are critical to ensure both good expansion rates as well as maintenance of multipotency of MSCs. These include, for example, starting material, methods used for enrichment or separation, plating density, devices used for MSC culture, media, supplements and growth factors as well as passage number or population doublings [54].

4.3.2. Cell Seeding. Plating densities have emerged as a critical issue for MSC isolation and expansion. Low seeding densities in primary culture seem to be associated with the emergence of more immature progenitor subsets [3]. Moreover, seeding at low densities allows higher expansion rates. For scale-up, accordingly, two different protocols are proposed. In one protocol cells are seeded at nearly clonal levels. This allows expanding the cells to high cumulative population doublings within one passage, but requires large culture area [55]. The other protocol seeds cells at higher concentrations. Due to reduced possible population doublings, this procedure necessitates a second passage, which, however, facilitates efficient depletion of contaminating cells [56].

4.3.3. Media and Supplements. Culture conditions shall retain or even accelerate regenerative and trophic properties of MSCs. The variety in protocols is immense and a standard has not yet been defined. The classical media composition consists of a basal medium (DMEM or alpha-MEM) and 10%–20% supplement, most often FBS, which is available in GMP grade allowed for clinical use. The ongoing debate regarding xenogenic, especially ruminant proteins in pharmaceuticals also applies to MSCs. FBS bears the risk of transferring xenogenic, potentially infectious, or immunogenic proteins. Immunogenicity against FBS proteins has demonstrated to compromise the therapeutic benefit [57, 58]. Thus although GMP-compliant FBS batches are available and used in clinical grade manufacturing the regulatory authorities ask to replace FBS with a nonxenogeneic alternative if possible for the manufacturer.

Up to now no completely serum-free media formulation in clinical-grade is available which allows both isolation (critical issue: attachment factors) and expansion of MSC [59]. Serum proteins provide not only nutrients but also essential attachment factors. Several laboratories have proposed the use of human components to supplement MSC growth medium. Here either autologous or allogeneic HS or platelet-derived factors have been evaluated [26, 54, 59, 60]. HS as well as platelet lysate are very crude protein cocktails. Essential growth factors for optimal MSC culture have not yet been defined. PDGF, EGF, TGF-β, and IGF have been subjected to investigation. Basic FGF has demonstrated most promising effects in expanding MSCs whilst maintaining stem cell properties and reducing replicative senescence [61]. Recently, Pytlík et al. described a HS and growth factor supplemented clinical-grade medium which allowed for high cell expansion mediated by loss of contact inhibition [62].

Any significant change in the production process may affect cellular functions. Accordingly, it is necessary to analyse the qualities of MSCs in comparability studies to ensure that cellular qualities are not compromised. Within a variety of publications, pooled human platelet lysate emerged as suitable alternative for BM-MSC isolation and expansion [26]. Our own data indicate that for ASC in contrast pooled HS has better effects on expansion [60]. But as specified above, it has to be determined whether therapeutic qualities are modified, improved or impaired. It is conceivable that depending on the clinical setting different protocols (cellular sources, manufacturing protocols, quality control/potency assays) come into place to derive the optimal product.

4.3.4. Devices for Expansion. MSCs grow as adherent cells until reaching confluency and then further expand by serial passaging. Therefore the number of cells which can be harvested in an *ex vivo* expansion culture is determined by the surface area. Typically MSCs are cultivated in conventional monolayer cultures. In order to achieve a large surface area multilayered cell factories are used [55, 56]. This approach is labour intensive and money consuming. Also by using bioreactors it became possible to expand MSCs [63, 64]. As closed systems should be preferred in a GMP-setting, Rojewski et al. report a fully automated bioreactor allowing large-scale GMP-compliant manufacturing [65].

A critical issue is the proliferative age of MSCs: MSCs have a restricted lifespan and reach a senescent state in which cellular functions become diminished and the risk for accumulating mutations rises [32, 66]. Most often proliferative capacity is expressed by passage numbers. Passage numbers in contrast to population doublings do not describe the de facto proliferation history which is critical when reaching a certain—not yet well—defined time point (maximum 30 population doublings) [66].

4.3.5. Storage or Cryopreservation. After isolation, volume-reduced cells or *ex vivo*-culture expanded cells can be transplanted directly or stored for long term under low temperature conditions. A variety of studies investigated the effects of storage conditions and cryopreservation methods and media [67–69] demonstrating that MSCs can be cryopreserved and thawed without loss of function [70]. Cryopreservation gives the only opportunity to perform time-consuming release tests prior to clinical application of the cells, hardly possible to achieve when the cell product is intended for immediate release.

4.4. Product Specification. As always it is necessary to weigh carefully the risk against the potential benefits of stem cell therapy. Potential risks can be reduced when applying appropriate release tests capable of ensuring safety, efficacy and consistency of the product. Cell-based products require special considerations on the manufacturing process, especially when they have to be applied immediately. In this situation it is impossible to obtain results of laboratory tests prior to the cell application, thus a limited set of controls has to ensure that the product fulfils all the predefined quality criteria. For cellular products in general sufficient numbers of viable, high quality cells are required. These can be easily documented by simple, rapid cell viability tests.

Cell-based products cannot be sterilized to avoid transferring infectious diseases. By using human and/or xenogenic material, there is a potential for adventitious agent contamination, thus testing for bacteria, fungi, mycoplasma and viruses should be performed. Regardless of the use in autologous or allogeneic settings an increased attention to assure aseptic processing is mandatory.

As indicated above, safety might be affected as prolonged *ex vivo* culture can accumulate aberrations. But only anecdotal studies indicate that MSCs may undergo spontaneous transformation, associated with chromosomal aberrations, induction of oncogenes and tumorigenicity after transplantation [23, 71]. Clinical experience indicates that cells, when harvested before onset of senescence, demonstrate extremely low probability of tumor formation [66]. Current testing systems including karyotype analyses, FISH, comparative genomic hybridization, or PCR to check for tumor marker expression may not be sensitive enough to detect the expected low proportion of affected cells [72], but even occurrence of alterations, like aneuploidity does not predict transformation, as recently demonstrated by

Tarte et al. These data nevertheless helped to refine control assays, easily to perform, to control cell cycle/senescence and transformation pathways by, for example, PCR for p14, $p16^{Ink4a}$, p21, p53, hTERT, and oncogenes like c-myc.

Further clinical safety concerns relate to possible ectopic tissue formation or other adverse events in the recipient. Despite the fact that in general no adverse reactions have been recorded, this possibility cannot be neglected yet. Mice treated locally with MSCs for myocardial infarction developed calcifications [73]. Further the relatively big cell size has been observed at times to cause pulmonary sequestration and embolism after intravascular transplantation [74]. Furthermore the protocol for cell application can affect transplantation as recently demonstrated when comparing different cell suspension media [75]. Finally, although therapeutically intended in some clinical settings, for example, to prevent or treat GvHD, the immunomodulatory capacities may also favour tumor growth or formation of metastasis, as observed in animal models [76–79].

For the EU and USA, the criteria for test procedures differ. Test procedures, for example, for sterility test, have to be approved by the FDA or the national regulatory authorities in Europe. But as the requirements are not identical, the international conference of harmonisation (ICH) intends to harmonise them (for more information, see respective homepages).

4.5. Identity and Impurities. The identity of cell-based products can be ensured where necessary by genotypic or phenotypic analysis. In MSC cultures, the fraction of cells displaying identity markers (mesenchymal markers) and the identification of contaminating cells (hematopoietic markers) can be easily and rapidly quantified by flow cytometric analyses [17, 80]. Above this morphological assessment of fibroblastic phenotype and proliferation can be easily documented in expansion cultures.

Product-related impurities have to be determined and specified, including endotoxin testing [81]. Where appropriate, impurities relating to, for example, degradation products from structural or matrix components shall be specified, as well as process-related impurities derived from added bioactive components.

4.6. Potency. Product characterisation has to consider the functional capacities related to the intended clinical use. The minimal criteria of the ISCT require to control the capacity of MSCs to adhere to normal plastic culture surfaces, to generate cells with a fibroblastic phenotype, which express or fail to express a typical set of surface markers, and to exhibit multilineage differentiation potential, at least into the osteo-, adipo-, and chondrogenic lineage [17]. Although every laboratory employs these assays, the assays are time consuming and far from being standardized yet, so comparison between laboratories and also the read-out of clinical data is hampered [80]. As mentioned before development of preclinical efficacy tests in the investigated indication are highly desirable as MSCs appear to employ different modes of function according to the intended use. Depending on the clinical intention, the following assays can be performed to assess potency, but it has to be kept in mind that none of these assays has been directly correlated to therapeutic efficiency [32].

Clonogenicity. The CFU-F assay is a suitable but not standardized tool to quantify precursor frequencies. Analysis demands for appropriate dilution to clonal levels as CFU-F frequencies do not follow a linear regression correlated to the input cell number [54, 55].

Differentiation Potential. The multilineage differentiation potential is a hallmark of MSCs, but discussed in detail elsewhere [4, 5, 82]. *In vitro* assays can be performed using self-made or commercially available induction media. However, it is increasingly discussed whether and to which extent *in vitro* data correlate to *in vivo* differentiation potential [82, 83].

Immunomodulatory Capacities. The perspective of modulating immune responses against allo- and possible also autoantigens has rendered MSCs an attractive population of cells for immune therapies. *In vitro*, assays have been established to quantify the expression of surface molecules, such as HLA class I and II and costimulatory molecules. In cocultures with peripheral blood mononuclear cells, MSCs do not elicit an alloreactive response. Furthermore, when added as third party in mixed lymphocyte reactions or mitogen-driven cultures, MSCs dose dependently inhibit immune cell responses. Very low concentrations of MSCs however can stimulate immune responses [84]. While this has not yet been observed *in vivo*, too low numbers of MSCs transplanted may accelerate the immune response rather than mitigate it in GvHD or autoimmune settings.

Hematopoiesis/Stromal Support. The beneficial effects of cotransplanting MSCs in haematological settings have already been demonstrated [85]. This effect can be assayed *in vitro* in coculture experiments using hematopoietic stem cells and MSCs and thus may be an adequate quality control system for this therapeutic indication [86].

Trophic Support. In a variety of settings MSCs showed promising therapeutic effects even though the transfused cells were—if at all—only barely detectable in the injured organs. Recent data further demonstrated that especially secreted factors actively modulate debilitating local inflammatory reactions. Reduction of apoptosis, and fibrotic tissue remodelling as well as recruitment of local resident regenerative cells contributed to the beneficial effects [9, 87]. Accordingly some studies already demonstrate therapeutic effects when infusing MSC conditioned medium instead of cells [88, 89]. Depending on the therapeutical setting, analysing the secretome by quantifying levels of chemo- or cytokines may subsequently emerge as additional potency assay [26, 60].

4.7. Validation. Safety and efficacy of a cellular product has to be demonstrated prior to their administration in humans. MSC application in clinical settings has progressed fast with 128 hits entering "mesenchymal stem cells" as search term under http://www.clinicaltrials.org. Nevertheless, there is a trend to go back from bedside to bench to better characterise and improve MSCs and importantly to standardise protocols for isolation, expansion, and finally characterisation. Changes *in vitro* necessitate translating them into clinical protocols. Thus it is a critical point to coordinate the clinicians needs with the researchers option in close interaction with the manufacturing laboratories, who overview the margin framed by the regulatory authorities. Any significant changes in the manufacturing protocols require validation *in vitro* and in appropriate *in vivo* animal models to assure safety and efficacy [28] as well as absence of toxicity (related to dedifferentiation or unwanted differentiation, migration to unwanted sites). All animal models have inherent limitations, like, for example, the application of human cells in a xenogenic milieu [82]. This requires the use of severely immunocompromised small animals preventing contrariwise analysis of immunological reactions. Furthermore for a variety of disease models, for example, in orthopaedics, small animals are not capable of modelling the disease. Consequently, one has to define a compromise between all requirements: clinical, regulatory, and laboratory to agree on appropriate validation strategies.

5. Conclusion

In the recent years numerous advancements led to the employment of MSCs in a variety of therapeutic indications raising expectations and hope. Although numerous clinical trials have been initiated worldwide (http://clinicaltrials.gov), standardized protocols for isolation, expansion, and characterisation, especially those on GMP-grade, seem to lag behind. The sum of clinical studies has supported safety and efficacy by demonstrating absence of major side effects associated with success reports. The lack of conformity between manufacturing protocols however is considered as potential threat to further development of the field. The heterogeneity of isolation, expansion, and characterisation protocols remains as obstacle. Thus to ensure the success of MSC-based therapies, we regard as a major critical issue to standardise and harmonise translational protocols in order to develop manufacturing processes along-side with developing therapies and not thereafter.

Acknowledgments

This work was supported by research funds of the German Federal Ministry of Education and Research (01GN0531 and 01GN0939) and additionally by a project commissioned by the European Community ("CASCADE" HEALTH-F5-2009-223236).

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SAGE-Hindawi Access to Research
Stem Cells International
Volume 2010, Article ID 503593, 8 pages
doi:10.4061/2010/503593

Review Article

Culture and Use of Mesenchymal Stromal Cells in Phase I and II Clinical Trials

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Received 9 June 2010; Revised 16 August 2010; Accepted 19 September 2010

Academic Editor: J. Gimble

Present in numerous tissues, mesenchymal stem cells/multipotent stromal cells (MSCs) can differentiate into different cell types from a mesoderm origin. Their potential has been extended to pluripotency, by their possibility of differentiating into tissues and cells of nonmesodermic origin. Through the release of cytokines, growth factors and biologically active molecules, MSCs exert important paracrine effects during tissue repair and inflammation. Moreover, MSCs have immunosuppressive properties related to non-HLA restricted immunosuppressive capacities. All these features lead to an increasing range of possible applications of MSCs, from treating immunological diseases to tissue and organ repair, that should be tested in phase I and II clinical trials. The most widely used MSCs are cultured from bone marrow or adipose tissue. For clinical trial implementation, BM MSCs and ADSCs should be produced according to Good Manufacturing Practices. Safety remains the major concern and must be ensured during culture and validated with relevant controls. We describe some applications of MSCs in clinical trials.

1. Introduction

From the end of the 1960s to the beginning of the 1970s, a Soviet scientist, Alexander Friedenstein, discovered a population of adherent cells in bone marrow (BM) that could differentiate into osteoblasts, chondrocytes, and hematopoietic stromal supportive cells [1]. The cells had a fibroblast shape, and when seeded at low density, some could form clonal colonies, which suggested the presence of precursor cells, the colony-forming unit-fibroblasts. Because the cells were capable of differentiating into various lineages of the mesoderm, they were named mesenchymal stem cells (MSCs) [2]. The stemness status and particularly the long-term self-renewal potential of MSCs has not been definitely established, so the preferred term is multipotent mesenchymal stromal cells [3], both terms being abbreviated to MSCs. In general, MSCs refer to native stem cells present *in vivo* in BM and to derived cultured cells.

Cultured MSCs are a mix of cells ranging from progenitors to mature stromal cells. Besides their differentiation potential, MSCs have an immunosuppressive effect both *in vitro* and *in vivo* by acting on all immune effectors [4]. However, the role of MSCs in tissue repair is not restricted to their differentiation potential or immunosuppressive effects. Indeed, MSCs have consistent trophic effects mediated by the wide range of growth factors and cytokines they produce [5].

These biological properties of MSCs rapidly led to investigation of their use in cell-based therapy by the middle of the 1990s. Caplan's team was the first to intravenously inject autologous, cultured MSCs in patients during a safety assessment trial [6]; up to 50×10^6 MSCs could be safely injected in humans. Later, injected MSCs were used in clinical trials to treat diseases such as osteogenesis imperfecta [7], metachromatic leukodystrophy [8], acute myocardial infarction [9], and graft-versus-host disease (GVHD) [10]. MSCs were also implanted to treat bone defects [11, 12].

More than 50 clinical trials related to MSCs have been reported at http://www.clinicaltrials.gov.

MSC populations with similar properties are found in almost all tissues in mammals and humans [13, 14]. Among them, adipose tissue is the most promising source of MSC-like cells suitable for clinical trials. Indeed, since the description of adipose-derived stromal cells (ADSCs) by Zuk and colleagues, in 2001, the large amount of data generated has shown adipose tissue to be the richest source of mesenchymal progenitor cells (ADSCs are at least 100 times more abundant in adipose tissue than are MSCs in BM). ADSCs and MSCs share many characteristics [15] but also differences in protein and function [16] (Table 1). For example, ADSCs have greater angiogenic potential than do MSCs [17]. ADSCs were used in clinical trials as soon as 5 years after their description [18, 19] and more than 10 clinical trials have been reported at http://www.clinicaltrials.gov. The clinical use of MSCs from other sources, especially the fetus, is far less advanced.

In this paper, we report on the current experience in the use of MSCs and ADSCs. First we discuss culture requirements and safety concerns, and then describe several ongoing clinical trials.

2. Culture Requirements: From Bench to Bedside

2.1. Culture Medium. The proof of concept and original data obtained *in vitro* and *in vivo* in the field of cell therapy are generally acquired with cells derived from culture protocols established for research use and do not necessarily follow Good Manufacturing Practices (GMP) rules. Thus, before injecting cells in humans, the first step is to replace research reagents with products suitable for human use. At this step, the two main problems are to accurately adapt the culture protocol with appropriate reagents and to prove that the resulting human cell culture exhibits all the properties described in the proof of concept.

No well-established rule exists to adapt the culture protocol, but the adaptation must be progressive (change one parameter at a time), with a careful validation of the therapeutic properties of the cells at each step. Because animal experiments must be sparingly used (referred to the 3R statement of the European Community: reduce replace refine), *in vitro* validation tests must be preferred and developed in order to limit animal experimentation. For example, angiogenic properties can be estimated by the dosage of angiogenic factors released in the culture medium and/or by measurement of *in vitro* vessel-like structure formation. Whatever the tests, they must be closely related to the expected *in vivo* effects of the cells. At the end of the adaptation phase, the cell product must be tested *in vivo* in small-animal models and, if possible, large-animal models. We discuss later the relevance and validity of the animal model.

Special attention should be paid to the risk of infectious disease transmission to humans by components in the culture medium. Consideration of this risk must also include the new variant of Creutzfeld-Jakob disease. Moreover, by using a culture medium containing proteins from an animal origin, MSCs may retain in their cytoplasm a substantial amount of xenogenic proteins. A standard preparation can represent 7 to 30 mg of fetal calf serum (FCS) proteins per 100×10^6 MSCs [20]. This level of proteins may elicit immunologic responses *in vivo* that could explain some of the failures in MSC cell therapy [7, 20]. Such data led to an effort to decrease or eliminate the use of xenogeneic components in the culture media for clinical MSC preparations. Replacing FCS with human autologous or AB serum has been proposed, but both appear to be less effective than FCS. However, supplementing the medium with human AB serum and fibroblast growth factor 2 can overcome this deficit [21]. Alternatively, FCS can be replaced with human plasma enriched with growth factors contained in platelets, such as platelet-derived growth factor, endothelial growth factor, and vascular endothelial growth factor. These cytokines are strongly mitogenic for MSCs and have been used since the 1980s [22, 23]. They can be obtained by activation of platelets with thrombin or simply by a cycle of freezing/thawing of the plasma that disrupts the platelets and releases growth factors [24, 25]. In our experience, this substitute for FCS is effective for MSC and ADSC expansion *in vitro*: it reduces the cell doubling time by at least 30% and the cells retain their morphology and functions.

The need to cultivate MSCs in a defined medium is important for homogeneity between cell production processes. In the 1990s, at least two teams described formulations allowing for cultivation of MSCs in a defined medium [26, 27]. Since then, some companies have developed similar defined media for MSC culture that unfortunately do not contain molecules inducing MSC adhesion. Thus, the culture process requires treatment to adsorb an attachment protein (fibronectin, collagen) on the culture surface. In addition, the formulation often is not disclosed, which prevents its use in clinical trials. Furthermore, these formulations do not include growth factors, which must be added to the medium, with the concern that they have not been produced with GMP. The following describes the culture of MSCs.

2.2. Culture Device: Closed Systems, a First Approach. The production of MSCs, which are adherent with contact inhibition requires substantial culture surfaces. As soon as the clinical trial requires more than 10^8 MSCs, surface areas of culture exceeding 2000 cm^2 are needed, which corresponds to at least seven 300-cm^2 culture flasks; these are time consuming to handle and imply a nonnegligible risk of contamination. When a large number of cells are required, for example, for treating GVHD ($[3 \text{ to } 8] \times 10^8$ MSCs per patient), the flask solution will become unmanageable because the required surface area exceeds 6000 cm^2. With some culture containers, reaching this surface area with a few units is impossible. Possible solutions are the CellStacks (Corning, the USA) and CellFactory (Nunc, Denmark) systems, which start from a unit surface area of 635 cm^2 and offer the possibility of 2, 5, 10, and 40 stages per container. These devices can also be connected by tubes for performing various operations (e.g., culture initiation, medium exchange, cell harvesting) in a simple and protected way. In this case, all fluids must be contained in a form that

Table 1: Features of bone-marrow derived mesenchymal stem cells (MSCs) and adipose-derived stem cells (ADSCs).

Feature	MSCs	ADSCs
Tissue sampling	Adult General anesthesia	Adult Local anesthesia
Cell purification process	No proteolytic digestion	Proteolytic digestion, generate different cell subsets
Phenotype	CD49a, CD73, CD90, CD105 CD271 and negative for CD34	CD49a, CD73, CD90, CD105 (CD271?) and CD34 in early passage
Frequency (CFU-Fs)	0.005	0.05
Potency	Hematopoietic support Classic mesenchymal lineage (adipo-, chondro- and osteogenesis) Immunosuppressive properties Precommitted towards osteogenesis (cultured MSCs)	Hematopoietic support Classic mesenchymal lineage (adipo-, chondro- and osteogenesis) Immunosuppressive properties Precommitted towards adipogenesis Good angiogenic potential

CFU-F: colony-forming unit-fibroblasts.

ensures simple handling (sterile bags with suitable connections). We have partnered with a pharmaceutical company (MacoPharma, France) to develop a simple connection system in which the basic medium is prepared in sterile bags (Figures 1, 2, and 3). This system, adapted for 2- or 5-stage containers, ensures easy and rapid manipulation. Ten-stage containers and the associated fluid bags are so huge that they are cumbersome for a single technician to manage. All these systems that require connection steps cannot be strictly assimilated into a real closed system: they must always be handled in a very clean environment (class B zone) under a class A flow hood. A bioreactor that automates all the steps of the culture and allows for a real closed system can overcome these limitations. Such a machine must be versatile and allow for the elimination of unwanted nonadherent cells, as well as medium exchange and cell removal.

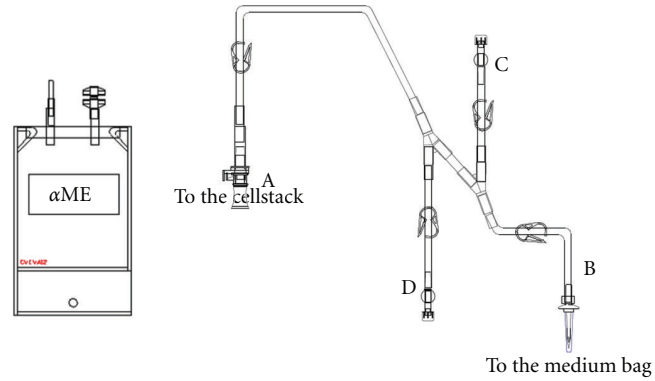

Figure 1: Set of tubing plus medium bag for cell seeding operation.

2.3. Safety Requirements: The Risk of Transformation. Regarding safety, the risk of transformation of MSCs during the culture process remains a major concern. Since the first report of spontaneous transformation of adipose-derived human MSCs, in 2005 [28], another team reported the same events with human BM MSCs [29]. In the first study, the transformation process required a long culture time and seemed to involve a mesenchymal-epithelial transition [30]. Concerning the second study of human BM MSC transformation, surprisingly, the process seemed shorter, with high frequency of transformation. However, for the first report, all the data concerning the transformation were later found to be related to contamination by an epithelial cancer cell line during the experimental procedures [31]. A similar retraction is under publication for the second report [32]. During the same time, using different techniques of controls, karyotype, and comparative genomic hybridization, numerous teams reported on genetically stable MSCs during culture [33, 34]. Moreover, in France, we used two different clinical-grade culture protocols, with aneuploidy features in a few productions, in two different clinical trials to study the significance of these features. We used karyotype and fluorescent *in situ* hybridization but also looked deeper at the molecular mechanisms involved in senescence and transformation. Clinical-grade cultured human BM MSCs, with or without aneuploidy, did not have any selective advantage, did not transform in culture and reached senescence with the normal evolution of adult cells [35]. In addition, tumors did not develop in immunocompromised mice injected with these MSCs. Finally, we demonstrated that the BM MSCs were not prone to genetic instability and did not easily transform during the normal culture process. These data and the retractions described previously strongly suggest that MSCs can be produced safely. However, these studies led to an improvement in controls for a focus on more accurate, relevant, and sensitive targets—investigating the expression and epigenetic status of the main genes of senescence and transformation pathways such as p53, p21, $p16^{ink4a}$, hTERT, and c-myc.

2.4. Animal Models. As described previously, animal models are necessary for demonstrating safety and efficacy. In addition to causing potential lack of efficacy that could be encountered with chemical drugs (e.g., absence of the target, differences in metabolism, pharmacokinetics), human cell-based products elicit an immune reactive response in the host that rejects these cells. Thus, the cells must be tested in immunodeficient animals, which means rodents and most often mice. Nude or severe combined immunodeficiency (SCID) mice have been extensively used for this purpose,

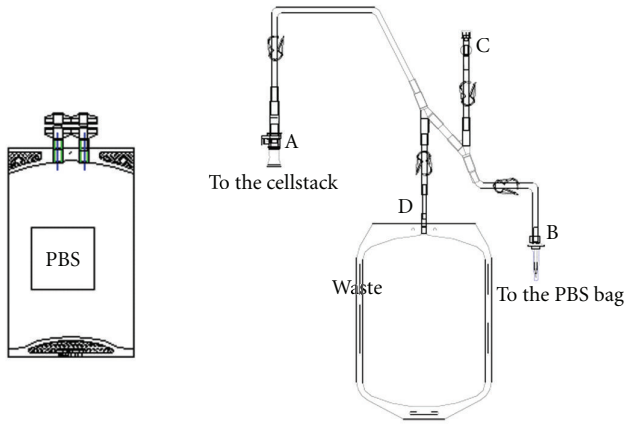

Figure 2: Set of tubing plus phosphate-buffered saline and waste bag for medium exchange.

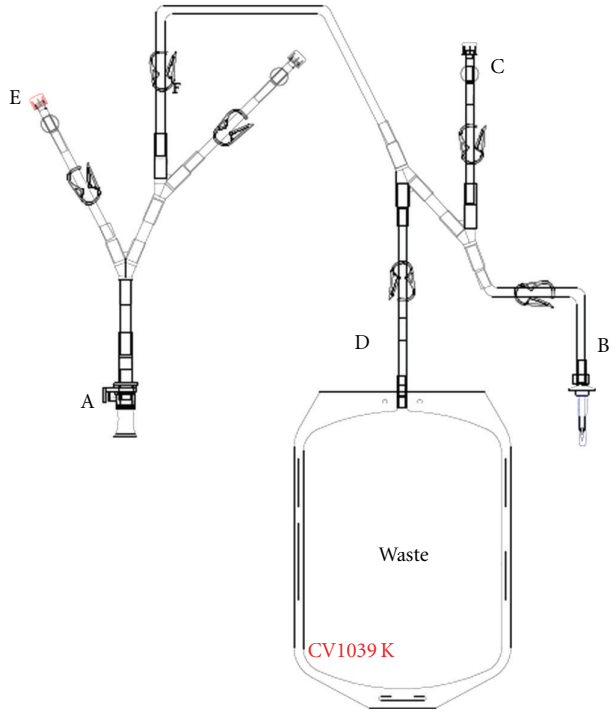

Figure 3: Set of tubing with waste bag for cell detachment.

but they bear residual immune cells that could interfere with the human cells and thus bias the results. Thus, NOG mice or other very immunocompromised mice must be used, although the cost and difficulty of the experiments increase. Immunocompromised mice represent an aberrant immune context for many applications of cell-based therapy. Furthermore, the mouse may not be a relevant pathological model with their reduced life span as compared with humans and their size, which leads to difficult functional evaluation. For example, the heart rate in mice is about 300/s but only 80/s in humans; thus, intramyocardial injections may be problematic. Another solution could be to use a large-animal model and inject animal cells produced according to the same conditions as for human cells. Again, this solution is not completely satisfactory because of the real "active compound" (i.e., human cells are not tested, which implies a specific-cell production protocol because animal cells cannot be produced in the same rooms where human cells are produced). Despite these limitations, regulatory agencies require such validations. Thus, the validation experiments must be a compromise agreed upon by these authorities and must be different and specific to the treated condition.

3. Clinical Trials with MSCs and ADSCs

3.1. Acute Graft-Versus-Host Disease (aGVHD). Because of the great immunomodulatory effects of MSCs *in vivo* and *in vitro* and since the first report by Le Blanc et al. [36], MSCs have been mainly used for treating or preventing aGVHD during allogeneic hematopoietic stem cell (HSC) transplantation. The efficiency of such therapy is greatest in liver and gut GVHD and in children [10, 37]. Some studies have suggested that cotransplantation of MSCs with HSCs can reduce the incidence of aGVHD [38]. In 2007, the Société Française de Greffe de Moelle et Thérapie Cellulaire (SFGM-TC) started a phase II, randomized placebo-controlled trial of corticosteroid-resistant aGVHD in patients receiving allogeneic HSC transplantation with or without coinjection of BM MSCs. MSCs were cultured according to a process developed by the SFGM-TC and validated by the French regulatory authority (AFSSaPS). The trial planned to enroll 78 patients. At month 5 and after two cell productions, karyotype analysis revealed clones with aneuploidy features not related to transformation [35], which led to suspension of the trial. Before the trial suspension, 11 patients were enrolled. This low number of patients did not allow for analysis of efficacy, but no enrolled patients receiving the cultured MSCs showed deleterious effects, even the one who received MSCs with aneuploidy. Potential late adverse events, including tumors, were never demonstrated. These observations are consistent with the lack of adverse side effects (transformation of MSCs, allosensitization by mismatched MSCs, increased incidence of infections) reported elsewhere during or immediately after infusion of MSCs [10, 38].

Further studies addressing use of MSCs with a randomized, large cohort of patients are under way [39]. Of note, treating aGVHD with MSCs for an immunosuppressive effect could lead to an increased frequency of relapse [40]. MSCs could be an efficient and safe way to treat and prevent aGVHD, but new studies should be implemented to focus on patients at risk for grade 2 to 4 aGVHD and for patients with visceral organ involvement.

3.2. Heart Failure. Cardiac failure after myocardial infarction represents poor prognosis and is associated with increased morbidity and mortality. Treatments for cardiac insufficiency have evolved well in recent years, but their purpose is to improve symptoms and prevent aggravation of the disease. Without possibility of revascularization of the myocardium, other therapies must be considered, such as injection of MSCs. The hope is to regenerate the cardiac muscle or

to limit the ventricular remodeling by alternative mechanisms that call on the paracrine release of growth factors. Both small- and large-animal models have demonstrated the usefulness of MSC injection after acute and chronic myocardial infarction [41, 42]. Although many clinical trials have used uncultured BM cells, results of at least two clinical trials involving MSCs have been reported. In the first, a Chinese team harvested BM 8 days after the myocardial infarction, cultured it for 10 days, then reinjected the cells intracoronarily [43]. No side effects were observed, and the cardiac wall velocity and left ventricle ejection fraction values (LVEF) were improved in the treated group as compared with a control group. In the second randomized, double-blind, placebo-controlled clinical trial of 60 patients, 39 received cryopreserved allogeneic MSCs injected intravenously 1 to 10 days after myocardial infarction [44], with no adverse events or ectopic tissue formation observed. The group receiving MSCs showed significantly less arrhythmia. The LVEF was significantly improved in the subgroup with anterior myocardial infarction.

Because the risk of cardiac failure is high after myocardial infarction, we have begun a phase I clinical trial to evaluate the feasibility and safety of intramyocardial injection of BM MSCs in patients with ischemic cardiopathy. We include patients with myocardial infarction and stable symptomatic cardiac insufficiency (LVEF < 45%) of ischemic origin, without possibility of revascularization. From these patients, we harvest 15 ml BM, from which MSCs are selected by adherence to plastic and are amplified in culture during 17 days. The culture is done in devices that allow all steps to be performed in a near-closed system (see following discussion). The MSCs are injected (6×10^7 MSCs) in the border zone of the infarction location. The main outcome of the trial is feasibility and safety of MSC intramyocardial injection at 1 month. The secondary outcomes are clinical, biological, and morphological effectiveness during 2-year follow-up. We aim to validate, for the first time, the feasibility and safety of intramyocardial injection of MSCs to improve the contractile function of the left ventricle and decrease morbidity of patients with cardiac insufficiency. Two patients have received the cells and have shown no adverse events.

3.3. Limb Ischemia. Critical limb ischemia (CLI) is a peripheral arterial disease with different grades described by Fontaine. Grades III and IV are characterized by chronic, ischemic pain at rest and ischemic skin lesions, either ulcers or gangrene. This clinical diagnosis can be confirmed by hemodynamic parameters such as ankle or toe systolic pressure. The frequency is around 32.5‰ before 40 years of age and 71‰ above 50 years. The estimated annual incidence of CLI ranges from 500 new cases per million in the European Union to 1 000 per million in the United States, with diabetes being the greatest risk factor. About 60% of patients should undergo revascularization whenever technically possible to prevent limb loss. The amputation rate for patients is about 20%. Because of the inefficiency of therapeutics when revascularisation is not possible, additional limb-saving strategies, including cell therapy, are required [45].

Results of a single clinical trial of CLI involving autologous BM mononuclear cells have been published. In total, 46 patients received intramuscular injections of (0.9 to 2.8) $\times 10^9$ BM mononuclear cells retrieved from patients under general anesthesia. The results were encouraging: 70% of patients showed improved oxygen tension, a significant decrease in pain and increased mobility [46]. Half of the patients reported no pain. The first positive effects appeared as soon as week 4, with stabilization at 6 months after treatment. Follow-up revealed significant improvement in leg pain, ulcer size, and pain-free walking distance maintained during at least 2 years after the therapy, although the ankle brachial index and transcutaneous oxygen pressure value did not change significantly [47].

From our preclinical data obtained from animal models, we began a phase I monocentric clinical trial to assess the feasibility and safety of intramuscular injections of autologous ADSCs for patients with CLI of the leg with no possibility of revascularization. We aimed to investigate the possibility of preventing amputation and more largely, decreasing morbidity. With patients under local anaesthesia, 30 g of adipose tissue is sampled by liposuction. After digestion and centrifugation of samples to separate mature, floating adipocytes from stromal cells, ADSCs are selected by adherence to plastic and are amplified in culture in a near-closed system for 2 weeks. The ADSC are injected (10^8 cells) intramuscularly at 45 points in all the muscles of the leg by use of a grid.

The main outcome is feasibility and safety as assessed by observations of local necrosis, thrombosis, local and general infection or inflammation up to month 6 after ADSC injections. The secondary outcomes are wound healing, lack of amputation, oxygen tension at the toe or ankle, and pain. The trial aims to validate, for the first time, the feasibility and safety of the intramuscular injection of ADSCs to improve vascularity in the leg. Three patients have received ADSC injections and have shown no serious adverse events. This trial needs to include 6 more patients.

4. Future Directions

4.1. Allogeny. MSCs are well known to have an inhibitory effect on immune cell proliferation *in vitro* and *in vivo* [48, 49]. This observation has opened new perspectives concerning the use of MSCs in the context of allogenicity, for creating cell banks and for treating disease when there is no time to purify and expand cells. The results obtained with allogeneic cells are similar to those obtained with autologous cells. For the heart, intramyocardial injections and systemic delivery of allogeneic MSCs have been found to preserve myocardial viability and improve local and global heart function in rodents and pigs [50–52]. This finding is associated with decreased scar size and fibrosis and increased angiogenesis [53]. Most of the effects seem to be due to paracrine activity [44] and modulation of proinflammatory, proangiogenic, and immunomodulatory molecules by the peri-infarcted myocardium [54, 55]. However, the presence of transplanted cells and these effects seem to be transient [44, 54, 55]. The immunosuppressive properties of MSCs were also

tested for allograft tolerance in a model of allogeneic heart transplantation, with contrasting results, including increased rejection depending on cell dose and treatment [56, 57]. These opposite conclusions are confusing, and a definitive answer in such a complex field requires further experiments.

The immunomodulatory effects of ADSCs have been described *in vitro* and *in vivo* [58–62]. These effects could also be due to secreted factors such as transforming growth factor beta, hepatocyte growth factor, prostoglandin E2, and IDO [62] and in some areas, different from MSC effects. Indeed, ADSCs inhibit immunoglobulin production but also suppress this B-cell function to a much greater extent than MSCs [63]. So the use of ADSCs could not be a simple and direct translation of MSC uses and needs dedicated experiments.

4.2. Cell Administration. Cells are transplanted most frequently by intratissue delivery, but massive cell death is frequently observed after the injection. To overcome such limitations, alternative cell-delivery systems should be considered. One solution could be to render the cells more resistant to the accompanied stress of the injection. For example, we have shown that preconditioning with melatonin increases the survival, paracrine activity, and efficiency of MSCs when injected intraparenchymally [64]. This effect is a consequence of higher resistance to oxidative stress and secretion of proangiogenic factors. Another promising approach could be to engineer biological cell sheets to efficiently transplant MSCs by respecting host tissue structure. This approach was successful with use of undifferentiated ADSCs in the heart [65]. After transplantation, the engrafted sheet gradually grew to form a thick stratum that included newly formed vessels, undifferentiated cells, and a few cardiomyocytes. Cardiac wall thinning was reversed in the scar area and cardiac function improved.

Finally, the possibility of targeting cells to various damaged sites by bloodstream delivery is attractive and seems suitable for these cells because they can be distributed to any tissue with no clonal or extensive proliferation at their final destination site [66]. This route of delivery has been investigated in the heart, and pharmacological agents can modulate its efficiency [67].

5. Culture Medium

Although increasing reports, including those of clinical trials, have established that MSCs are good candidates for cell-based therapy, aspects of this therapy should be improved. Regarding new therapeutic targets, central nervous system diseases, autoimmune diseases, and lesions of the skin or cornea, phase I and II trials should be developed. Moreover, GMP conditions, particularly relevant safety controls and closed systems, must be fully implemented, and phase I and II trials should be performed with GMP-produced MSCs. If MSCs are effective with this process, phase III trials can be implemented and cell products finally licensed. Our own experience is consistent with this conclusion, and in the next few years, cell therapy with MSCs or ADSCs will be validated and can help treat a large number of diseases ranging from nonunion fracture, to limb ischemia and heart failure, to autoimmune diseases.

Acknowledgments

This work was supported by FRM (Program: Vieillissement Cardiovasculaire normal et Pathologique Ref DCV20070409252), PHRC MESAMI, the CHU of Toulouse, and the 7th FP of the European Commission: CASCADE (no. 223236; HEALTH-F5-2009-223236).

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SAGE-Hindawi Access to Research
Stem Cells International
Volume 2010, Article ID 532704, 8 pages
doi:10.4061/2010/532704

Research Article

Noncultured Autologous Adipose-Derived Stem Cells Therapy for Chronic Radiation Injury

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Received 7 June 2010; Revised 4 August 2010; Accepted 28 September 2010

Academic Editor: Kotaro Yoshimura

Increasing concern on chronic radiation injuries should be treated properly for life-saving improvement of wound management and quality of life. Recently, regenerative surgical modalities should be attempted with the use of noncultured autologous adipose-derived stem cells (ADSCs) with temporal artificial dermis impregnated and sprayed with local angiogenic factor such as basic fibroblast growth factor, and secondary reconstruction can be a candidate for demarcation and saving the donor morbidity. Autologous adipose-derived stem cells, together with angiogenic and mitogenic factor of basic fibroblast growth factor and an artificial dermis, were applied over the excised irradiated skin defect and tested for Patients who were uneventfully healed with minimal donor-site morbidity, which lasts more than 1.5 years.

1. Introduction

There is an increasing worry on radiation injuries probably caused by nuclear power plant (NPP) reactor accidents, therapeutic irradiation for malignancy, and interventional radiology (IRV) of unexpectedly prolonged fluoroscopic procedures for cardiovascular diseases such as arrhythmia, ischemic heart diseases, or nuclear medicine of overdose intake of the radioactive for nuclear medicine of internal radiation therapy. The problems are concerning chronic radiation injury as well as how to heal such local and systemic injures acutely. Local chronic radiation injury is resistant to conventional therapeutic modalities such as flap coverage or skin grafting because the deteriorated margins are sometimes indistinguishable from normal intact tissue, and thus sufficient enough debridements are not obtained with surgeons' naked eyes.

These conditions should be treated properly for the sake of life saving and improvement of local wound healing [1]. However, data of total evidence-based clinical analysis were not established yet. Authors' institute, Nagasaki University, is selected as a global strategic center for radiation health risk control by the Japan's Ministry of Education, Culture, Sports and Technology from FY 2007 to 2011 and exploring to establish such therapeutic regimens, to prevent the radiation injuries, and possibly to regenerate medical and surgical therapy for radiation injuries by using patients' own adipose tissue-derived stem cell therapy.

Often seen chronic radiation injuries are well handled by sufficient enough blood supply to the radiated tissues, especially in the cartilage, bare bone, and hardened scar tissues. For this purpose, local, distant, and microsurgical vascularized flaps are applied. Recent development of microvasculature of the skin and soft tissues including the connective tissues plays major roles in attributing to accelerate local wound healing. Also, externally administered angiogenic growth factor such as basic fibroblast growth factor (bFGF) together with temporal wound coverage of artificial skin substitute is very effective for those patients with severe injuries, patients with comorbidities, who are

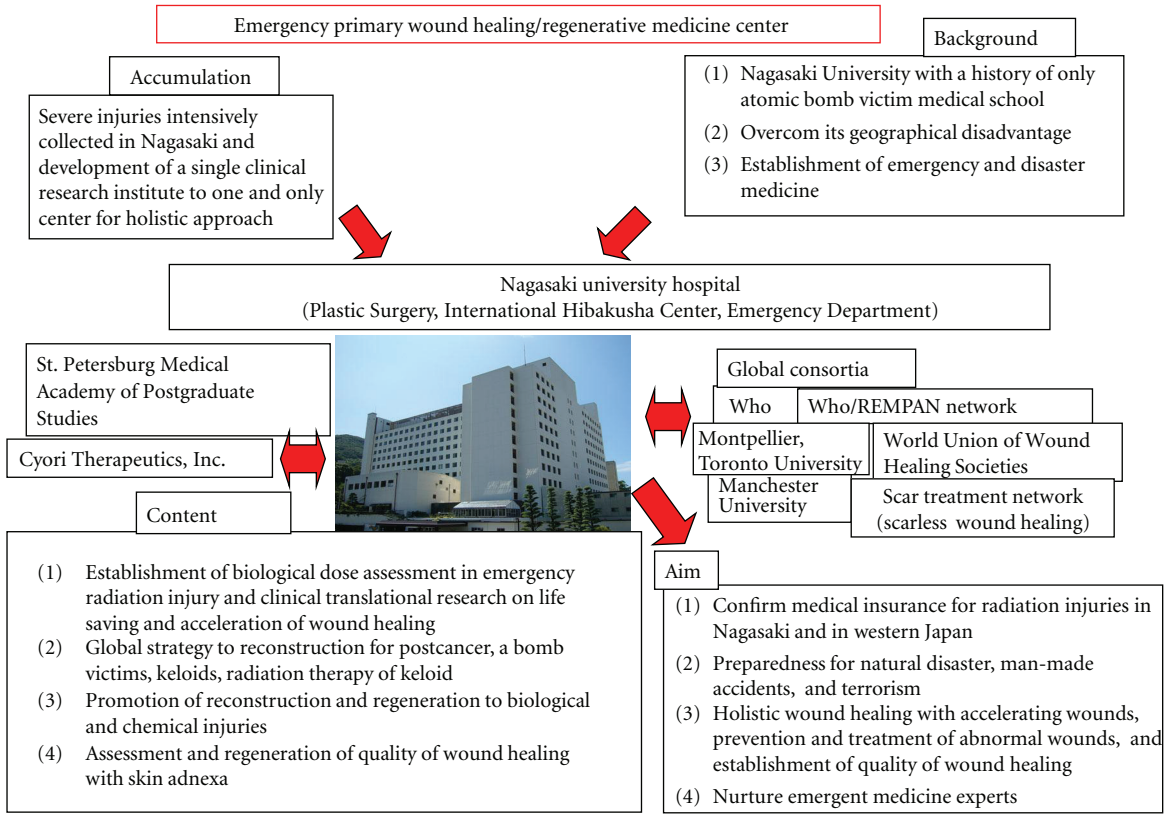

Figure 1: Strategy of emergency radiation injury. Collaborative work with highly established international centers and organ is proposed.

intolerant to the extensive and long surgeries [2]. Here, chronic radiation-injured wounds are tested with non-cultured autologous adipose-derived stem cells and clinical implications are discussed.

2. Materials and Methods

2.1. Treatment of Chronic Local Radiation Injury with Conventional Methods and Stem Cells. Often experienced in radiation therapy for malignancy, cardiovascular modalities should be categorized as difficult wounding with poor vasculature or less healing potentials.

From January 1990 to April 2007, 10 (8 females and 2 male) patients who demonstrated chronic radiation injuries such as telangiectasia, xerosis, epidermal atrophy karatoses, and fibrosis as well as deep ulcers in the costal ribs and sternum by adjuvant radiation therapy after mastectomy and prolonged fluoroscopic procedures for cardiovascular diseases were surgically treated.

Other selective clinical cases used angiogenic growth factor namely human recombinant basic fibroblast growth factor (rh-bFGF), which is clinically approved and widely used for clinical wounds in Japan with skin substitutes, which are also clinically available not only in Japan but also in many other nations including USA, the majority of EU nations, and several Asian counties, and the effectiveness of using the artificial skin substitutes in the chronic radiation injuries is temporal coverage and sustainability of both internal and external cells and growth factors. Therefore, combined use of bFGF and artificial skin substitute leads to improved quality of wounds (scar tissue) as well as facilitated wound healing [3].

One case was treated with non-cultured autologous adipose-derived stem cell (ADSC) for chronic sacro-coccygeal radiation ulcer in 2008, which was caused by a therapeutic radiation at fractionate 50 Gy at 40 years previously.

2.2. Methods. This study was approved by the Ethics Committee of the Nagasaki University Hospital, and written informed consent was obtained from all patients (approved no. 08070296) and partly supported by the Global COE (Center of Excellence) Program E08, Global Strategic Center for Radiation Health Risk Control, and it was funded by the Japan Society for the Promotion of Science. This national research grant enables us to investigate 3 main themes related to radiation health risk: (1) atomic-bomb disease followup cohort research with over 60-year continuous research history, (2) radiation basic science, and (3) international radiation health research. Especially, this radiation regeneration research was involved in further international collaboration framework under international organizations such as WHO (World Health Organization) and IAEA (International Atomic Energy Agency) (Figure 1).

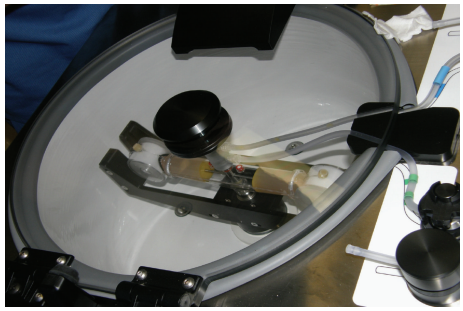

Figure 2: The Adipose-derived stem cells are processed in a closed-circuit machine within 1.5 hours.

2.3. Harvesting of Adipose Tissue by Liposuction and Isolation of ADSCs. 3–5 mm incisions, two incisions in the abdomen, four incisions in the thigh, and two incisions in the gluteal region, were made on the abdominal region, the thigh, and the gluteal region. The subcutaneous layer was infiltrated with a lactated Ringer's solution with addition of 0.5 mL of epinephrine and 25 mL of 1% lidocaine per 500 mL. Adipose tissue was suctioned using an 18-G Becker cannula with a 50 mL syringe. Total 250 gram-fat tissues, 120 grams from the abdominal region, 80 grams from the gluteal regions, and 50 grams from the thighs were harvested.

ADSCs were isolated from the suctioned adipose tissue by using the Celution system. (Cytori Therapeutics, Inc., USA). Briefly, the suctioned adipose tissue was introduced into the Celution cell-processing device, which automatically and aseptically extracts and concentrates the mononuclear fraction of adipose tissue and removes unwanted or deleterious cells, cell and matrix fragments such as lipids. By using the Celution system, a 5 mL solution is added to isolated ADSCs in about one and a half hour (Figure 2). The whole procedure is in a closed circuit and this reduces the chance of the contamination.

The small portion of processed ADSCs was used for the ex vivo cell culture and confirmed the proliferation and differentiation potential. The ADSCs-rich fraction was then plated onto collagen type-I-coated plastic culture flasks in a serum-free medium for primate embryonic stem cells (Primate ES medium, RiproCELL, Tokyo), and the cells, clonally expanded, were collected and stored in Liquid Nitrogen as the primary ADSCs. ADSCs were subcultured when they reached to 80% confluence. Cells were treated with trypsin/EDTA solution, neutralized with tripsin-neutralizing solution, and collected by centrifugation for 5 minutes at 1,200 rpm. The pellects were resuspended in a fresh medium; the number of cells was counted, and 3×10^5 cells were plated into T25 flasks (25 cm^2) for subculture while the rest of the cells were stored in liquid nitrogen.

2.4. Adipose-Derived Stem Cell Grafting and Postoperative Management. For the scaffold purpose, we used the artificial dermis (Terudermis, Olympus-Terumo Biomaterials Co., Ltd., Japan) (Figure 3). The Terudermis is composed of two layers: a lower layer of bovine atelocollagen and an upper layer comprising a silicone sheet which protects against infection and dryness from the outside. After minimum debridement, the Terdermis was multilayered and stacked over freshly debrided wounds. The silicone sheets were removed except top Terudermis. The two-thirds of isolated ADSCs alone were injected; around the debrided wounds, at the base of the wounds, and into Terudermis. Another one-third of ADSCs was mixed with the autologous adipose which was rinsed with a lactated Ringer's solution. In the Celution system, after isolating ADSCs, the disposable cell collection plastic case one was again used to mix the suctioned fat, which is rinsed separately in the 50-cc syringe and repeated until the oil droplets are removed. After being mixed, it was injected into a zone of hard fibrotic tissue around the debrided wounds in 2-cm width in all directions.

2.5. Angiogenic Growth Factor and Basic Fibroblast Growth Factor (bFGF). Genetically recombinant human bFGF (Fiblast, Trafermin) was purchased from Kaken Pharmaceutical Co., Inc (Tokyo, Japan). The Freeze-dried bFGF was dissolved in 5 mL of benzalkonium chloride containing solution right before the first use and stored at 4°C for one day, with 300 μL sprayed over 30 cm^2 area from 5 cm distance, and 0.3 mL per day of this solution was applied over the wound. One week after removing the silicone layer, human recombinant fibroblast growth factor (bFGF: Fiblast, Kaken Co., Ltd., Japan) (Figure 4) was sprayed. The wound was covered with nonadherent occlusive foam dressing.

3. Results

3.1. Treatment of Chronic Local Radiation Injury with Conventional Method. All wounds were healed after several surgical modalities. None of the cases was healed with single procedure (2 to 6 surgeries, mean 4.3).

Of our cases, one breast-cancer patient was treated by a standardized Halsted method with major and minor pectoralis muscle, radical neck, and axillary and internal mammary lymph node dissections. This patient has undergone 50-Gy fractionate radiation therapy postoperatively. The radiated area showed chest fistula deep to the pleura with surrounding unhealthy hardened scar tissue and chronic inflammation.

The whole affected area was sequentially excised in 3 reconstructive surgeries, starting with rectus abdominis musculocutaneous flap, then latissimus dorsi musculocutanous flap, and finally with groin-free flap. In the course after each surgery, the margin of the flap was partially dehiscent and necrotized, which required further touchups? The total number of the reconstructive surgery was 6 (Figure 5).

3.2. Treatment of Chronic Local Radiation Injury with Adipose-Derived Stem Cells. Regeneration method with patient's own non-cultured ADSCs was planned for a patient underwent 50-Gy fractionate radiation therapy for uterine cancer 40 years ago. The pigmented sacrococcygeal region appeared with central intractable wound. Necrotized bone and fascia muscle along with malodour were observed. The ADSCs-treated chronic radiation wounds underwent

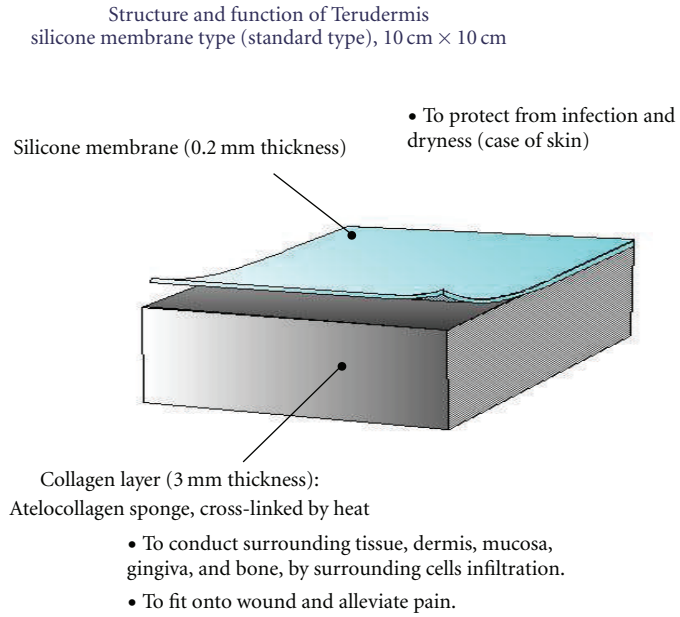

Figure 3: Freeze-dried bilayer artificial dermis made of bovine dermis. The outer membrane of silicone layer is easily removed and easily soaked with cell-containing solution.

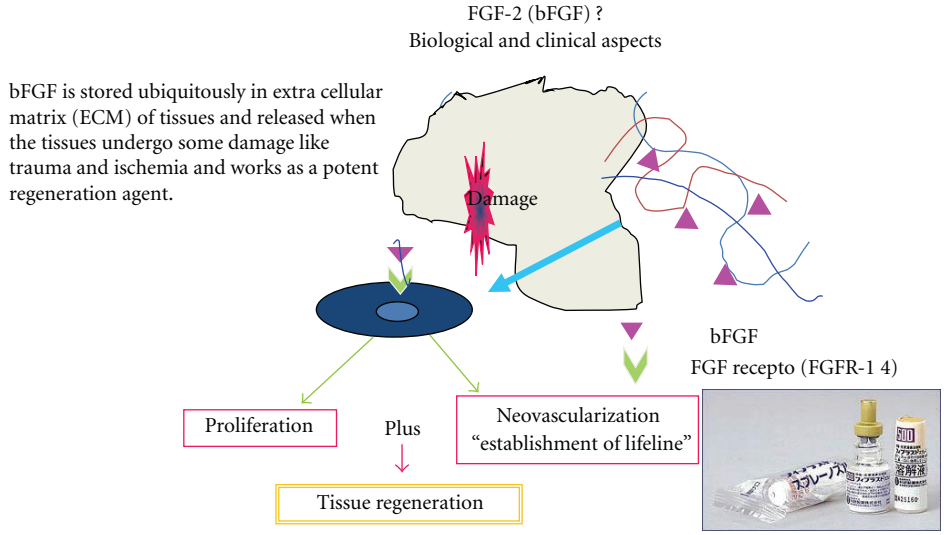

Figure 4: Commercially available growth factor and basic fibroblast growth factor (bFGF). Mode of action is explained and the mechanism is proposed.

debridement to remove unhealthy superficial necrotized bone, fascia, periosteum, and muscle. 3.8×10^7 cells in 5-mL of final volume from 250 mL of subcutaneous aspirated fat obtained from nonradiated area were used. Some ADSCs were directly injected in wound bed and margins; others were soaked with the artificial dermis. In a few days postoperatively, the silicone upper layer of the artificial dermis (Terdermis) was removed, and bFGF was sprayed over the regenerated wound for three weeks. There was no significant adverse effect neither in donor site or treated wound. The wound was healed uneventfully by day 82 and no sign of recurrence appeared, but the regenerated tissue developed mature in 1.5 years (Figure 6).

4. Discussion

Local radiation injuries caused during medical therapy for malignant tumors [4] and heart disease [5] may be accompanied with systemic symptoms of hematologic, neurologic, and gastrointestinal symptoms such as neutropenia, thrombopenia, fatigability, nausea, and diarrhea by contact to the

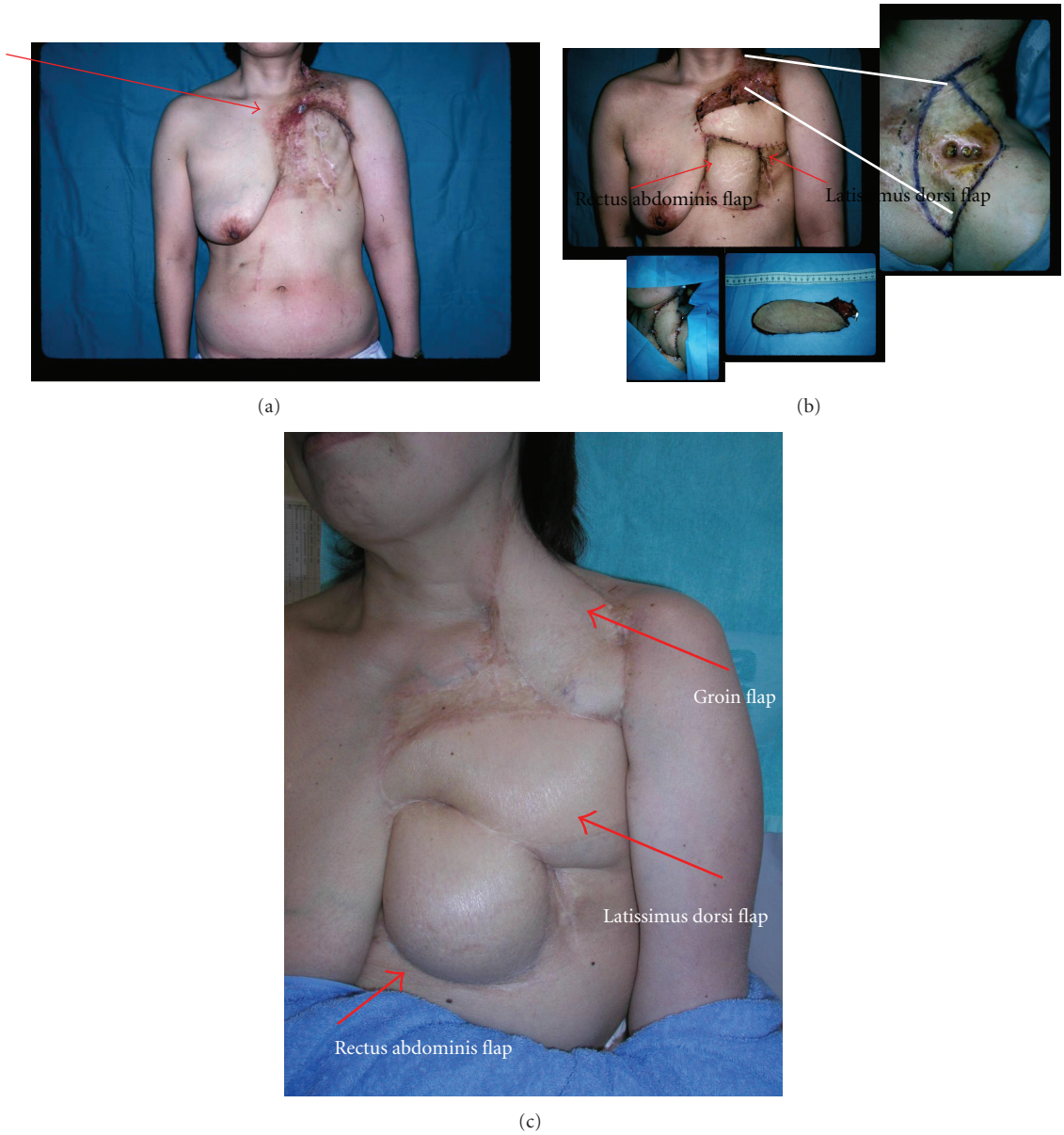

Figure 5: 55-year-old woman underwent a left breast cancer surgery by a standardized Halsted methods, followed by 50-Gy fractionate radiation therapy 15 years previously. (a) The chest demonstrates fistula to the costal rib and adjacent to the pleura as the arrow depicts, and the surrounding tissues were firm and various-degree inflammation existed. (b) Sequential three major flaps (rectus abdominis, latissimus dorsi, and free groin flap) are used for total coverage. (c) In 7 years postoperative view. There is irregularity of the scar margins.

scrap yard radioactive wastes without notice [6] or exposure to the radiation accidents [7] by touching gammagraphy radioactive source by mistake [8]. Since locally radiated tissues show decreased or insufficient vascularity and tissue damage, demonstrating erythema, teleangiectasia, pigmentation, or dermal atrophy, once wound is developed, it is often intractable and further leading to tissue necrosis, infection, and later fibrosis in demonstrating chronic radiation injury syndrome [9]. Therefore, radiation-injured wounds tend to persist for a long time, show impaired healing, and be prone to recurrence even by minor trauma. Radiated wounds are treated by adequate debridement both in the depth and in the width and covered with well-vascularized tissues or by cultured bone-derived mesenchymal stem cells [8]; however, the long-term outcome is not warranted, and donor-site morbidity and the duration for treatment are sometimes concerned, especially for the aged patients or patients who somehow have problems in harvesting the donors or being limited due to the coexisting diseases. As seen in our reconstructive cases, the surgical modalities constantly required multiple surgeries partly due to the definitive damage-free margins of the affected tissue.

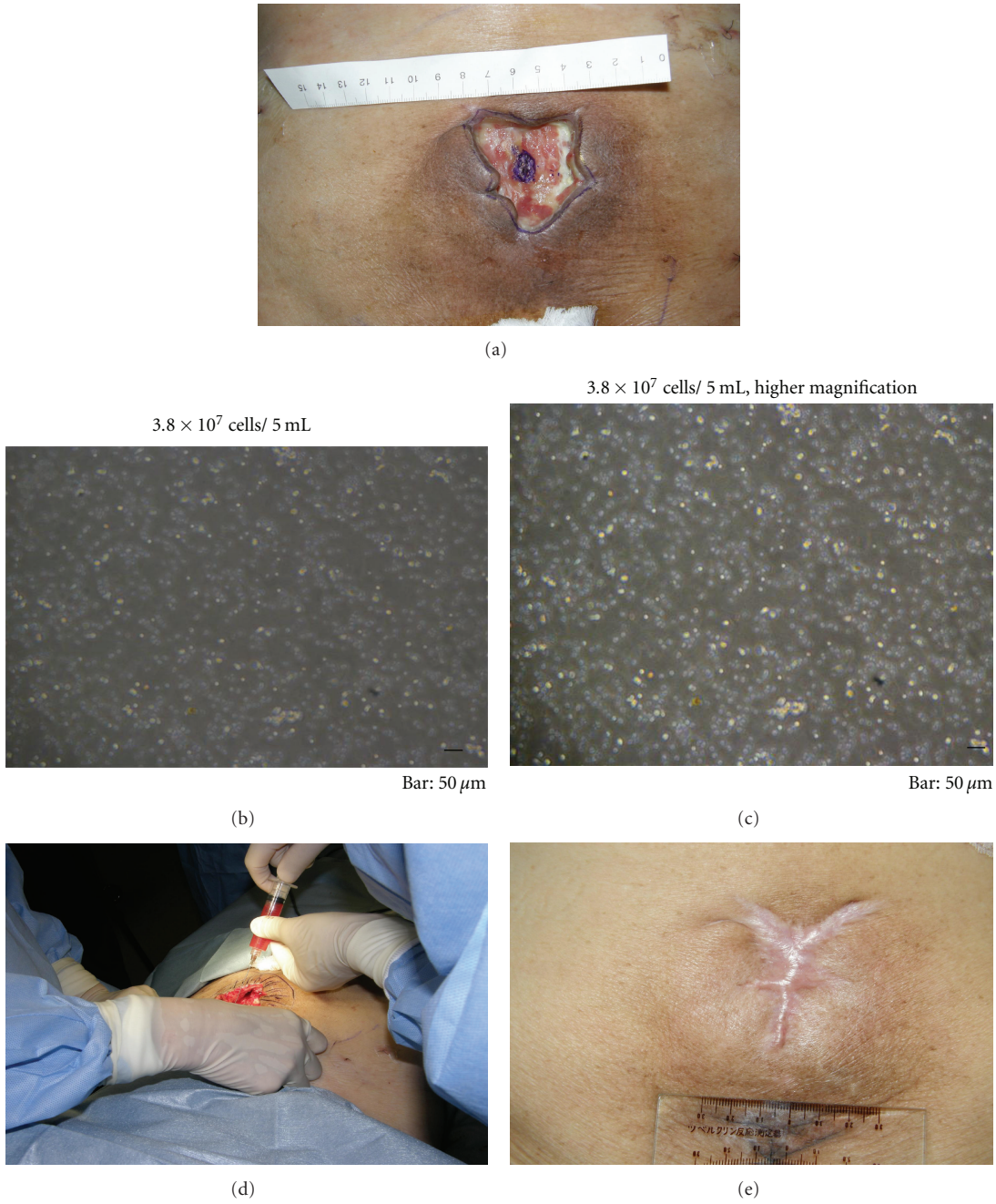

Figure 6: 89-year-old woman underwent a uterine cancer surgery followed by 50-Gy fractionate radiation therapy 40 years previously. (a) In 10 × 10 cm area of radiation, 5 × 10 cm area was exposed. Bone, fascia, and muscle as well as skin and fat were affected. (b, c, d) After careful debridement, 3.8×10^7 cells/5 mL were applied over the wound bed and margins and soaked with artificial dermis. In a few days postoperatively, bFGF was sprayed over the peeled-off inner regenerated tissue for 21 days. (e) In 1.5 years postoperative view. The regenerated tissue remained durable, soft, and pliable.

Application of Stem cell therapies for repair and regeneration has recently been investigated at a clinical level in variously defected or injured tissues, among which stem cells and adipose-derived stem cells (ADSCs) can be harvested with a minimally invasive procedure by liposuction procedure through a small incision. Similar to our method but in detail very different, Clinically purified autologous lipoaspirates were used as treatment for radiotherapy tissue damage of consecutive 20 patients. Indirectly, induced ADRCs have potential in cell therapy for radiation injury due to increasing neovascularization and retention of the fat property [10].

This enables us to adopt this regeneration method for patients with severe comorbidity such as elderly systemic disease and physical wasting state (data not shown). The

ADSCs contain several types of stem and regenerative cells, including endothelial and smooth muscle cells and their progenitors and preadipocytes [11]. The ADSCs have the capacity to differentiate into multiple lineages and cell types including mesodermal tissues such as fat, bone, cartilage, endothelial cells of endodermal origin, and neurons and epidermis of ectodermal origin as seen in the mesenchymal stem cells [12].

Management of radiation injuries composes two major parts. One is localized injuries and the other is of systemic injuries. Among localized radiation injuries, chronic injuries are more common in the medical field after caner radiation therapy. Usually management of these chronic wounds is well handled by well-vascularized tissue transfers as various plastic surgical procedures have proved. In consideration of each patient general condition and preference, the choice of therapeutic selections should be performed. On the other hand, when the local radiation injuries are encountered in an acute phase, there are high chances for innovative procedures using autologous stem cells. The hMSCs are resistant to radiation. We have previously demonstrated *in vitro* cell proliferation curve and are also able to produce protein avoiding cell apoptosis [13]. And the application of cultured bone-derived mesenchymal stem cells successfully healed severe local radiation wounds. However, the cultured stem cell therapy takes longer period as long as 16 days before cell therapy and required multiple (5 times) cell injections as well as 2 skin grafting, 2 flaps, and 1 artificial dermis coverage [14]. Also, increasing evidences demonstrate that ADSCs are similar to hMSCs in cell properties and characteristics both *in vitro* and *in vivo* [11]. ADSCs are highly yielding and less invasive for donor sites. The acute myocardial infarction porcine models by improving left ventricular function, perfusion, and remodeling [15]. When localized radiation was distant enough from the donor sites adipose tissues, immediate debridement and regeneration happens using adipose-derived stem cells, which are available for processing within 1.5 hours simultaneously in the same operation theater without cell culture since adipose tissues (fat tissues) are abundant in adult humans compared to other stem cell sources. In the limited clinical circumstances of high-risk patients such as elderly and chronic local infection, there is still opportunity of harvesting and processing the patient's own fat-derived stem cells successfully as seen in our case. Practically for emergency radiation injury cases, more abundant cell sources such as fat are the primary candidate for this purpose. The cell property and characterization of ADSCs are discussed and discussed either fresh or cultured [16]. The results from the clinical trial for acute myocardial infarction are expected and may be applicable for acute radiation injury treatment.

For treatment of systemic radiation injuries, stockpiled stem cells should be globally available through medical assistance network system under WHO-REMPAN, in which Nagasaki University is highly involved in its activity, or other international frameworks. Early resurfacing of the damaged skin and subcutaneous tissues is as important as hematological and intestinal system resuscitation [17].

Also, therapeutic guidelines for systemic radiation injuries are anticipated from practical and regulatory view points. Highlighting innovative technology and devices as well as currently existing medicines and devices is expected for the sake of preparing to treat "systemic" radiation injuries most effectively.

Therapeutic regimens of radiation injuries used to be dependent on each subspecialty in the medical filed such as internal medicine, radiology, and surgery.

Recent establishment of wound care specialty was mostly led by plastic surgeons, but other supporting specialists such as nurses, dermatologists, and gastrointestinal physicians and surgeons may be practically handling these rare but of significant impact "radiation injuries" as a interdisciplinary approaches. Therefore, more specialization for "radiation injuries" may be required.

Acknowledgment

Nagasaki University Global COE Program, "Global Strategic Center for Radiation Health Risk Control," by Japan Society for the Promotion of Science.

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SAGE-Hindawi Access to Research
Stem Cells International
Volume 2010, Article ID 410170, 4 pages
doi:10.4061/2010/410170

Review Article

Phases 1–3 Clinical Trials Using Adult Stem Cells in Osteonecrosis and Nonunion Fractures

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Received 10 May 2010; Accepted 10 October 2010

Academic Editor: Kotaro Yoshimura

Nonunion fractures and aseptic bone necrosis are two pathological conditions having some impairment of the cellular part of the repair: a reduction of MSC and of the osteoblastic activation. Both are good candidates for cell-based therapies using stem cells. We made a review of the published human trials. Only autologous bone marrow aspirate implantation was until now used. In Nonunion, a direct injection—15 to 150 ml—was made in 4 case series studies. In another, the bone marrow aspirate was concentrated before injection. The results were good. In bone necrosis, only one level 1 study was published. The results at 24 months were positive in terms of reduction of the necrosis and appearance of collapse. In 3 case series studies, a treatment with concentrated bone marrow aspirates was deemed useful with good results in 76 to 96%. These results are interesting but need confirmation by controlled studies.

1. Introduction

The physiological bone repair process is impaired in delayed or nonunion (NU) fractures [1] and aseptic bone necrosis (ON) [2].

Although the physiopathological factors are different, in both diseases, bone lesions are not repaired in the right time nor in the right manner.

Bone healing is produced by a cellular mechanism including mesenchymal stem cells (MSCs). The MSCs need to be recruited in the pathological area. These non-hematopoietic progenitor cells are able to be differentiated in osteoblasts under the influence of growth factors such as bone morphogenetic proteins (BMPs), platelet-derived growth factor, transforming growth factor beta, insulin-like growth factor, fibroblast growth factor, and PTH.

MSC can be found mainly in bone marrow, but also in fat tissue, synovium, periosteum, skeletal muscles, and umbilical cord. Some recent data suggest that the osteogenic differentiation capability of MSC from bone marrow and from periosteum is higher than MSC from adipose tissues [3].

Several methods could be used to increase MSC population and its osteogenic differentiation in the pathological area:

(i) a local injection of bone marrow aspirates,

(ii) a preliminary culture of the bone marrow aspirate to increase the number of MSC cells,

(iii) a preliminary culture of the bone marrow aspirate to produce an expansion and an osteogenic differentiation of the MSC,

(iv) a genetic modification of the injected MSC to increase the secretion of growth factors like BMP and VEGF [4, 5].

In nonunion, the etiology is not clearly understood. Excessive mechanical instability of the fracture, a reduction of bone vascularity, and smoking are cited. Furthermore, some genetic predisposition could exit. In atrophic NU sites, osteoblast progenitor cells are significantly reduced [6]. In bone marrow from the iliac crest of atrophic NU bone marrow-derived mesenchymal stem cells are in smaller number and have a reduction of their proliferative capacity [7].

In nontraumatic ON, apoptosis of osteocytes and cancellous bone lining cells in the necrotic lesion and also at some distance from the lesion, in the proximal femur [8] are increased. The replicative capacities of osteoblastic cells obtained from the intertrochanteric area of the femur are reduced in patients with ON [9]. The number and the activity of fibroblast colony-forming units, reflecting the number of mesenchymal stem cells that could potentially give rise to mature osteoblasts have been shown to be decreased in ON [10, 11]. Moreover, the capillaries serving as a conduit for the stem cells and bone cells needed in bone repair in addition to providing blood supply could be altered by emboli or thrombosis in ON [12].

In both pathological conditions, some impairment of the cellular part of the repair could exist: a reduction of MSC and of the osteoblastic activation.

The best treatment remains to be found in both conditions. Among the different developed approaches, the cell-based therapies to improve bone repair are presented and seem to be promising. They are based on the concept of the regenerative medicine and aim to recover an optimal bone repair process.

This paper summarizes a review of the trials published in this field.

2. Clinical Trials in Nonunion Fractures

A recent review of the current technologies in bone-healing and repair didnot find any human study of level-I evidence concerning bone marrow aspirates, nor gene therapy [13]. Only a few studies support the therapeutic use of bone marrow transplantation in human [2].

A systematic review was conducted using Pub Med, Medline. This research was completed checking references cited in listed articles. The key words were "bone marrow", "stem cells", "nonunion fractures", and "cell-based treatment".

Unlike animals, in humans, only bone marrow (BM) aspirates implantations were until now used.

2.1. BM Aspirate. Connolly and coauthors should be the first to report results in a case of infected NU of the tibia [14]. In a further report of the use of marrow graft for osteogenesis from 1986 to 1995 including 100 patients having a tibial NU, a good response was found in 80% [15]. No complications were reported. The method used is made under general anesthesia. The patient was placed in a prone position and the marrow was aspirated in 3–5-ml aliquots. Simultaneously with the marrow aspiration, a second marrow needle was inserted into the site of the nonunion to directly inject the BM aspirate. The total injected volume was 100–150 ml. In 2 cases a second injection was done. No reason for this second injection was mentioned by the authors. The healing time ranged from 6 to 10 months

In 1990, Healey et al. published good results in 7/8 cases of NU after BM aspirates injection in situ [16]. These cases were all failure of osseous reconstruction (autogenous iliac crest bone grafting) after lower-extremity resections for sarcoma affecting bone. The bone marrow, 56 ml at the beginning of the series to 3 ml at the end, was aspirated from the iliac crest under general anaesthesia, and directly injected in NU, until a total of 50 ml has been grafted. No heparin was used to avoid potential impairment of bone healing associated with heparin [17, 18]. In 4 cases a second injection was made when no healing process was seen on review of serial roentgenogram. The healing time ranged from 4 to 36 weeks (mean 18).

In 1993 Garg et al. applied a technique they tested earlier in rabbits [19]. They grafted bone marrow percutaneously in 20 ununited long bone fractures (15 in the tibia, 3 in the humerus and 2 in the ulna). Under general anesthesia, 15–20 ml of bone marrow aspirates (3-4 aspirations of 5 ml) from the posterior iliac crest was directly injected into the NU sites twice, with an interval of 3 weeks. All cases were immobilized in a plaster cast. In 17/20 cases a bone fusion was observed after 5 months [6–10] .

In 2005 Goel et al. presented results of BM grafting in tibial NU [20]. Under local anesthesia, 3–5 ml of marrow was aspirated from the anterior iliac crest and injected immediately percutaneously into and about the nonunion site. Subsequent aspirations were performed 1 cm posterior to the previous site until a maximum of 15 ml of marrow was injected. Injections were repeated at 4–6 weeks if there was no radiological evidence of callus formation. The procedure was considered a failure if there was no clinical and radiological union at 6 weeks following the third injection. The results revealed clinical and radiological bone union in 15 out of 20 patients (75%), with an average time to union following the first injection of 14 weeks. Four patients (20%) showed no evidence of union and were considered a failure. There were no cases of infection following the injection, and no complications at the donor site.

2.2. Concentrated BM Aspirate. Only one trial using a concentration of the BM aspirate was published. In 2005, Hernigou et al. reported the results of a retrospective study including 60 tibial NU [21]. Under general anesthesia, 300 ml BM were aspirated from both anterior iliac crests, then filtered and concentrated by centrifugation on a cell separator. The 50 ml concentrated bone marrow was injected in NU. Weight bearing was not allowed during minimum 1 month until a callus appeared. Failure was considered when no healing existed after 6 months. In 53/60 patients, bone union was obtained in mean 12 weeks (range 4–16 week). They quantified the number of injected MSC and found a significant lower count of MSC in the negative cases.

2.3. Other. There was until now no human study using gene modified MSC, expanded MSC or differentiated MSC in osteoblasts. Only a recent publication concerns the effect of autologous osteoblast (OB) to improve the fracture healing [22]. The autologous OB cells were obtained from a 4 weeks culture of 3–5 ml bone marrow aspirate. A mixture with 0,4 ml (12×10^6 cells) and fibrin was prepared and injected under local anesthesia into the fracture area. In this randomized study, a significant fracture healing acceleration was shown.

3. Clinical Trials in Osteonecrosis

A systematic review was also conducted using Pub Med, Medline. This research was completed checking references cited in listed articles. The key words were "bone marrow", "stem cells", "osteonecrosis", "bone necrosis", "avascular bone necrosis", and "cell-based treatment".

In 2002, Hernigou and Beaujean reported the results of a noncontrolled study of femoral head osteonecrosis [23]. The patients were followed up from 5 to 11 years with a mean of 7 years. When patients were treated before collapse, hip replacement was done in 9 of the 145 hips. Total hip replacement was necessary in 25 hips among the 44 hips operated after collapse. The authors classified this study in an evidence level III. But the study didnot have any control! The evaluation was only based on a comparison with the estimated natural evolution of cases published in other studies. The correct level of evidence seems to be level IV. The method for implanting the bone marrow aspirate in the necrotic area was the same as described for the same author in NU. The volume of BM aspiration made under general anesthesia was 300 ml. A filtration and a concentration by cell separator were performed. The final volume to inject into the necrotic area was 50 ml.

In 2004, Gangji et al. published a controlled, double blind, prospective study including 18 femoral head ON before collapse treated by core decompression using a 5 mm trephine with or without concentrated BM aspirate [24]. The method to obtain and to prepare BM was the Hernigou's method. After 24 month followup, there was a significant reduction in pain and joint symptoms within the BM graft group ($P = .021$). At 24 months, five of the eight hips in the control group had deteriorated with appearance of a collapse of the femoral head, whereas only one of the ten hips in the BM graft group had progressed to this stage ($P = .016$). Survival analysis showed a significant difference in the time to collapse between the two groups. In addition, in the BM graft group, the volume of the necrotic lesion decreased by 35%.

In 2008, a publication in Chinese presented a retrospective study using another method of treatment [25]. A 3-tunnels core decompression was performed in the femoral head to allow implantation of bone marrow MSC and decalcified bone matrix. Among the 87 patients (103 hips), the average rate of excellent and good results (based on clinical and radiological evaluation) were deemed to be 75, 7% after a followup of mean 26 months. No more details were given.

In 2009, Wang et al. reported the results of 59 ON of the femoral head (before or after collapse) in a prospective noncontrolled study [26]. The 100–180 ml BM aspirate was concentrated to 30–50 ml. The implantation into the necrotic area was done through 2-3 holes made using a trocart with a 3.5 mm outer diameter. The followup was mean 27 month (range: 12–40). Clinically, the overall success was deemed in 80% and hip replacement was made in 7/59 hips (11,9%).

4. Discussion

In NU, this paper shows that the therapeutic effect of MSC is only supported by some studies using BM aspirate, concentrated or not, of evidence level IV. Several differences between these studies must be noted. The type of NU and the therapeutic methods were not the same. Different methods to harvest and to inject bone marrow were used. The volume and the number of injected MSC (when evaluated) were quite variable.

Good results were found in all. With small volume (15–20 ml) and without any concentration they were 83% [19] and 75% [20]. With larger volumes (300 ml) and after concentration, the good results increased slightly to 88% [21]. Clearly, the question of the best method, and the interest of larger BM aspirate volumes are not resolved.

An additional question is the interest of an injection of large volume in lesions having a smaller volume. What is about homing and proliferation of injected MSC? Is the bone repair boosted by the injected MSC or by other components of the BM aspirate like growth factors? Trials using BMP have proven their efficacy in 7 studies with level-1 evidence [13].

In ON, the effect of BM implantation was tested in one trial level-II evidence [24] and 3 trials level-IV [23]. The method for harvesting and concentrating the bone marrow as the injected volume as the method of implantation was the same in 2 studies but used 2 or 3 tunnels core in the 2 others. Such results are very promising, but need to be confirmed in larger randomized control studies. The same answer about the relationship between the injected volume and the lesion volume needs to be studied.

Finally we do not find data to confirm that the therapeutic effect of BM aspirate should be due to its cellular part, especially MSC and not to growth factors.

In conclusion, these reviews confirm that BM aspirate could induce bone repair in NU and ON. But the data are very preliminary and a lot of questions remain to be clarified.

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SAGE-Hindawi Access to Research
Stem Cells International
Volume 2010, Article ID 215625, 12 pages
doi:10.4061/2010/215625

Review Article

Clinical Application of Human Mesenchymal Stromal Cells for Bone Tissue Engineering

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Received 17 May 2010; Revised 13 July 2010; Accepted 11 September 2010

Academic Editor: J. Gimble

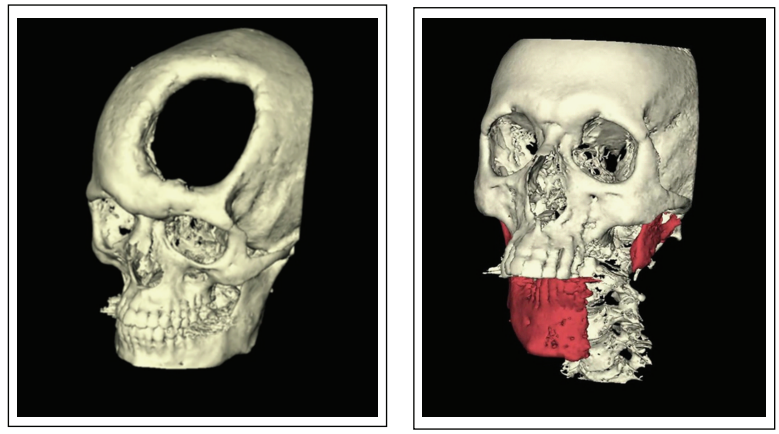
The gold standard in the repair of bony defects is autologous bone grafting, even though it has drawbacks in terms of availability and morbidity at the harvesting site. Bone-tissue engineering, in which osteogenic cells and scaffolds are combined, is considered as a potential bone graft substitute strategy. Proof-of-principle for bone tissue engineering using mesenchymal stromal cells (MSCs) has been demonstrated in various animal models. In addition, 7 human clinical studies have so far been conducted. Because the experimental design and evaluation parameters of the studies are rather heterogeneous, it is difficult to draw conclusive evidence on the performance of one approach over the other. However, it seems that bone apposition by the grafted MSCs in these studies is observed but not sufficient to bridge large bone defects. In this paper, we discuss the published human clinical studies performed so far for bone-tissue regeneration, using culture-expanded, nongenetically modified MSCs from various sources and extract from it points of consideration for future clinical studies.

1. Introduction

Bone lesions/defects caused by, for example, trauma, bone resection due to ablative surgery, or correction of congenital deformities are a common problem in clinical practice. In the majority of the cases, the body's self-healing capacity is able to repair the defect. Yet every year, in roughly 1 million cases of skeletal injury, the defect size is too big or conditions not optimal to allow healing (Figures 1 and 2). In these cases, external help is required in the form of bone graft procedures to achieve union [1].

The most frequently used sources of bone grafting are autologous and allogeneic bone [2]. Autologous cancellous bone grafts are most successful in the present clinical scenario, because in addition to being osteoconductive and osteoinductive, they are safe, cheap, and easily available to the surgeons. However, obtaining autologous grafts requires the patient to be subjected to additional surgery, thus introducing extra morbidity at the donor site and increasing surgical costs [3–5]. Besides, the amount of graft material is limited and chances of complications such as infections, instability, and paraesthesia at the donor site can affect up to 30% of patients [6–8]. An alternative is allogeneic bone grafting, which can be obtained from authorized tissue banks which collect and store bone tissues from human cadavers [7]. By this approach, problems associated with harvesting and quantity of graft material are bypassed. To avoid problems associated with immunogenicity, donor grafts can be devitalized via processes such as irradiation and freeze drying. Unfortunately, this processing also eliminates the cellular component, thus reducing the graft's osteoinductivity, thereby resulting in a slower rate of new bone formation as compared to autologous grafts [9].

As an alternative to autologous or allogeneic bone grafts, surgeons may use scaffolds made of synthetic or natural biomaterials that promote the migration, proliferation, and



Skull defect (a)

Mandibular defect (b)

Figure 1: 3D reconstruction of a skull and mandibular defect in trauma patients. Surgeons are often faced with patients having large defects in the bone which do not heal spontaneously. The gaping hole in the skull and the area highlighted in red in the mandible are examples of large-sized defects in real patients. Though autografts are the gold standard treatment for such patients, the amount of graft material required is often the limiting factor. Tissue regeneration using synthetic or natural scaffolds seeded with mesenchymal stem cells can be an alternative solution for such patients.

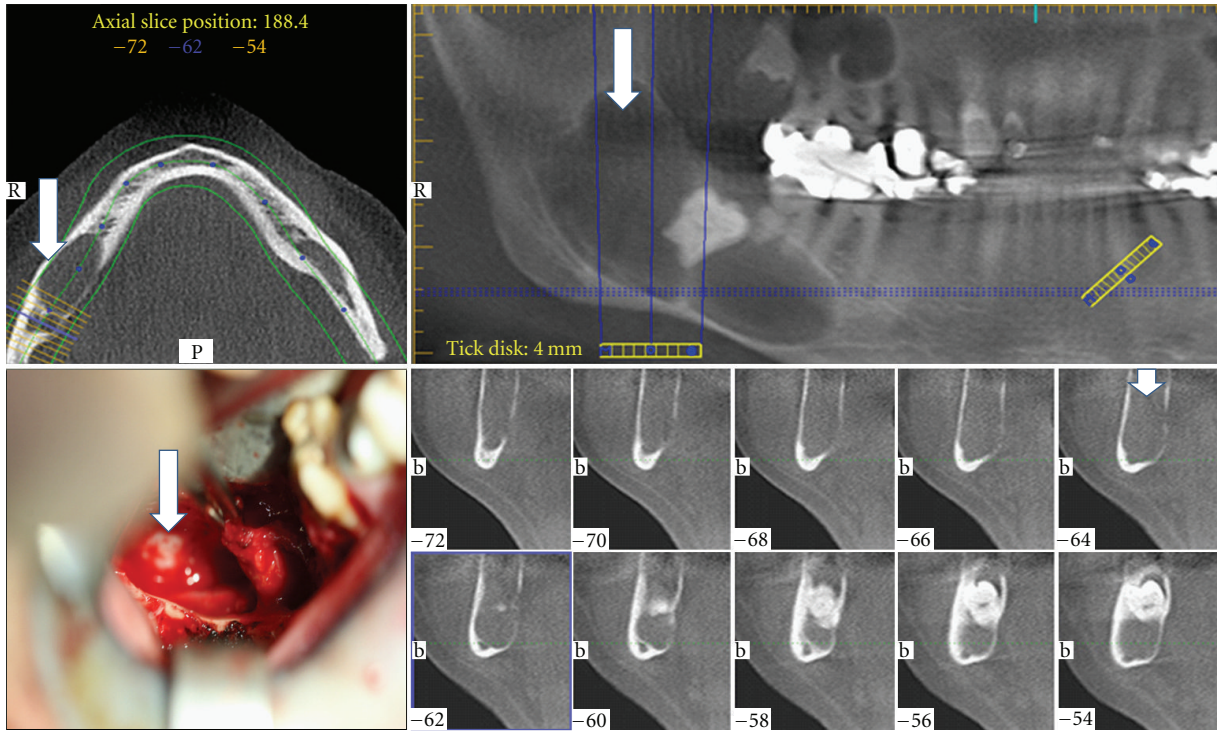


Figure 2: Mandibular defect following cyst. CT scan of huge cyst in the mandible (see white arrows). The clinical picture represents the situation after removing the cyst revealing the alveolar nerve positioned at the bottom of the cavity (black arrow).

differentiation of bone cells. In the last decade, a large number of publications have illustrated the osteoinductive and osteoconductive properties of scaffolds such as synthetic hydroxyapatite (HA) [10–12], coralline HA [13–15], β-Tricalcium phosphate and porous biphasic calcium phosphate [16–19], calcium phosphate cements [20], chemically treated titanium [21], and glass ceramics [22]. However, the degree of osteogenic and osteoinductive properties provided by the osteoprogenitor cells, as present in the autografts, exceeds that of the scaffolds. To improve osteoinductivity, scaffold materials can be loaded with osteoinductive growth factors such as bone morphogenetic proteins (BMPs).

The drawback of the growth factor approach are the supraphysiological concentrations needed to obtain the desired osteoinductive effects, their high costs, and more importantly, potential ectopic bone formation [23, 24].

Alternatively, scaffolds can be loaded with osteoprogenitor cells in order to generate a living bone graft *in vitro*, an approach referred to as bone-tissue engineering. Various possible sources for osteoprogenitor cells have been considered. Osteoblasts obtained from autologous bone biopsies and then expanded *in vitro* were an obvious first choice due to their nonimmunogenicity. However, the relatively low number of cells obtained after dissociation of the biopsy specimen, the time-consuming nature of the whole process and the problems associated with obtaining osteoblasts from patients with bone-related diseases prompted continuation of the search for better options [1, 25]. Mesenchymal stromal or stem cells (MSCs) which can be obtained from various tissue sources, like bone marrow, adipose tissue, umbilical cord, or placenta provide an alternative source of osteoprogenitor cells. MSCs were first identified in the bone marrow by Friedenstein and coworkers in 1966 [26] and were subsequently named mesenchymal stem cell (MSCs) by Caplan [27]. They are very attractive to researchers as they can be extensively expanded *in vitro* to obtain numbers sufficient to treat large bone defects [28], and they have immunosuppressive effects *in vivo*, which may make them suitable for allogeneic transplantations [29, 30]. MSCs isolated from different sources share many phenotypical and functional characteristics. However, depending on the tissue source and the isolation methods employed, their differentiation potential varies [31]. The varied tissue sources and isolation methods make it difficult to determine if the resulting cells are sufficiently similar to allow for a direct comparison. Therefore, the International Society for Cellular Therapy proposed a set of minimal criteria to label a cell as a MSC [32]. These include: (1) cells must be plastic adherent when maintained in standard culture conditions; (2) they must express CD105, CD73, and CD90 and lack expression of CD 45, CD34, CD14, or CD11b, CD79α or CD 19 and HLA-DR surface molecules; (3) they should differentiate into osteoblasts, adipocytes, and chondroblasts *in vitro*. Haynesworth et al. were the first to combine human MSCs from adult bone marrow with ceramic scaffolds to successfully generate bone *in vivo* upon ectopic implantation into immunodeficient mice [33]. This provided proof-of-principle on the feasibility of using hMSCs in bone-tissue engineering. Since then a lot of interest has been generated in the field of tissue engineering, resulting in *in vitro* and *in vivo* studies with different scaffold/cell combinations. The proof of concept for repair of critically sized bone defects using tissue-engineered bone graft substitutes has been provided by a number of animal studies [30, 34–44], and several clinical studies have been conducted to assess the safety and efficacy of this approach in man. Nevertheless, bone-tissue engineering did not find its way to routine clinical practice.

Here, we present an overview of all published human clinical studies performed so far to generate bone using constructs seeded with culture expanded, autologous, non-genetically modified MSCs obtained from various human cell sources, suggest possible recommendations for future design of clinical studies, and describe future research directions. Cells from the periosteum have not been included in this paper because no studies have been performed to determine the MSC nature of the periosteal cells used in the clinical studies. There are previous reports which indicate that the periosteal cells fulfill the minimal criteria to be labeled as an MSC [45–48]. However, there are differences in the isolation and expansion protocols used in these studies and in the studies employing the cells for clinical applications [49–52]. Studies using the mononuclear fraction of the bone marrow or adipose tissue have also been excluded from this paper. Although MSCs are present in the mononuclear fraction, other populations of cells also form a large part of this fraction.

2. Clinical Studies in Humans Using Autologous MSCs from Various Cell Sources for Bone Tissue Engineering

Prior to market release of tissue-engineered products, an investigational new drug application (NDA) may have to be submitted to accredited regulatory bodies such as the Food and Drug Administration (FDA) or the European Medicines Agency (EMEA). Following this, clinical trials have to be enrolled as phase 1, phase 2, or phase 3 trials. In phase 1, evidence is obtained about the safety of a particular approach in a selected group of patients. Generally, these are small trials with a number of patients recruited being less than 30. In phase 2, more patients are included to evaluate effectiveness on the possible applications. Phase 3 clinical trials involve multicentre trials on 300–3000 patients and are a definitive assessment of the concerned treatment in comparison with the current gold standard. Following completion of all phases of clinical trials, the regulatory body reviews the results, before making a final decision on the release of the tissue engineered product in the market. A search on *clinicaltrials.gov* using search terms, "Mesenchymal stromal cells", "autologous MSCs," and "bone tissue engineering", provided 2 relevant studies.

(1) "Treatment of osteonecrosis of the femoral head with implantation of autologous bone marrow cells, a pilot study."
This is a phase 1 study, which started in January 1999 and was completed in September 2008. However, no publications describing the study results are currently available in literature.

(2) "Autologous implantation of Mesenchymal stem cells for the treatment of distal tibial fractures". This is an ongoing, phase 1/phase 2 study. The study started in April 2009 and the expected primary completion date is April 2011. No results from this study have yet been published in the literature

Designs of clinical trials vary from randomized control trials (RCT), replicated single subject experiments, cohort

outcome studies, systematic case studies, and case reports. In general, the more rigorous the design of a clinical trial, the greater the credibility that can be attached to the conclusions derived from the outcome of a study. Based on the methodological rigour applied, RCTs are generally considered at the top of the hierarchy as randomization in selection of patients for inclusion in the various treatment groups ensures negation of the selection bias while inclusion of controls help rule out the effects of the confounding factors that may have an effect on the treatment outcome. However, due to practical and ethical issues involved in conducting RCTs, most of the trials conducted on human patients and described in literature for bone-tissue engineering are at the level of cohort outcome studies or case reports. Cohort studies provide information on the percentage of patients which respond positively to a given therapeutic technique while case reports describe the effects of using a particular tissue-engineered graft in a single patient. The observed results in the latter can be thus idiosyncratic to the specific patient being evaluated and systematic replications of the experiment would be necessary prior to obtaining conclusive evidence. Absence of controls in cohort studies is a major drawback of such a study design. However, these preliminary attempts also have an important role in the development of scientific research because they generate information that can provide some clues to the safety and potential therapeutic effects of the treatment option and may stimulate researchers to perform the more elaborate, time consuming, and costly RCTs in the future. In this paper, we list all human clinical studies, including case reports that have been published in literature, using autologous, culture expanded, nongenetically modified, human MSCs for purpose of bone tissue engineering. None of these studies have obtained approval from institutions such as FDA or EMEA. The ethical approval for conducting these studies has been provided by their respective local university/hospital ethics committees.

The first clinical case series demonstrating feasibility of using tissue-engineered constructs (TEC), as an alternative to autologous bone grafts for treating long bone defects, was reported by Quarto et al. [53]. In 2001, they described the preliminary results of 3 patients (27, 16, and 15 months respectively postsurgery) suffering from various segmental defects (Figure 3). The patients were chosen because conventional surgical therapies such as Ilizarov's technique which excludes graft transplant, had failed. The Ilizarov's technique relies on the bone regeneration potential to fill the gap created artificially via osteotomy of the affected segment while maintaining the periosteum intact and then distraction of the two separated halves fixed apart used ring fixators [54]. Autografts were technically difficult to perform because the degree of bone loss would leave the patient with serious donor site morbidity. The first patient was a 41-year-old female with a 4 cm large segmental bone defect in the right tibia, the second a 16-year-old female suffering from a traumatic loss of a 4 cm segment of the distal diaphysis of the right ulna, while patient 3 was a 22-year-old male, who missed a 7 cm segment of the right humerus. For all the patients, macroporous 100% hydroxyapatite (HA)

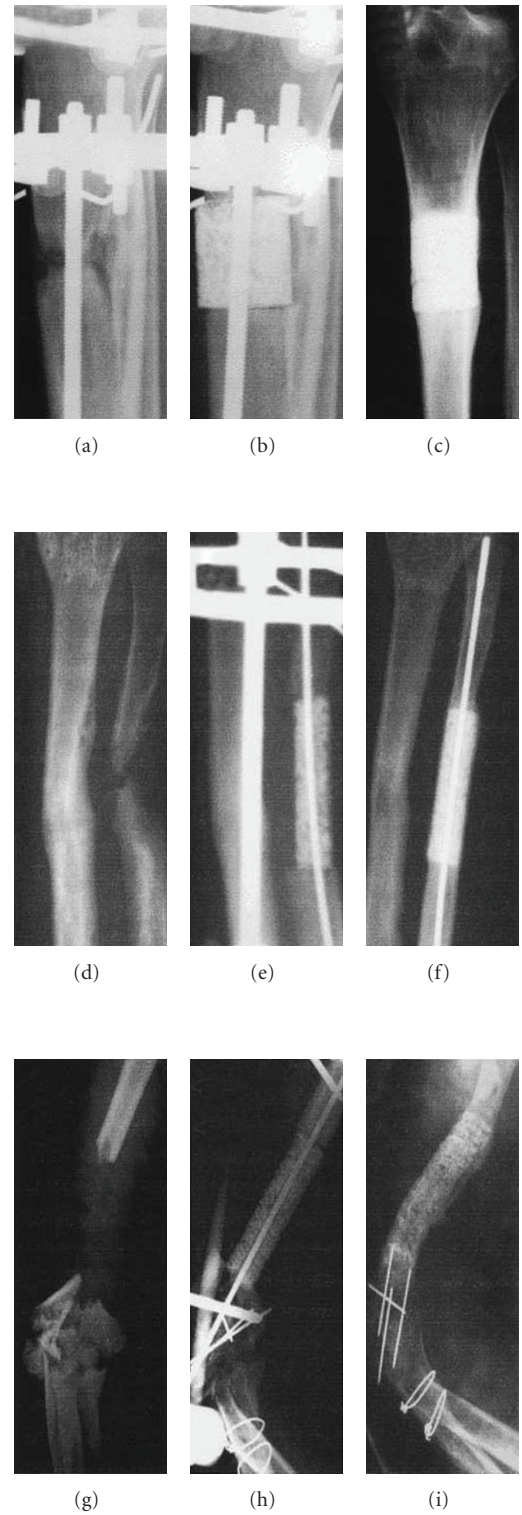


Figure 3: Radiographs obtained before and after the repair of large bone defects in three Patients from the study by Quarto et al. panels (a)–(c) show films obtained from Patient 1 before, immediately after, and 18 months after surgery, respectively. Panels (d)–(f) show films from Patient 2 before, immediately after, and eight months after surgery, respectively. Panels (g)–(i) show films from Patient 3 before, immediately after, and 15 months after surgery, respectively. All the films obtained at the last time point demonstrate bridging of the defect with newly formed bone.

scaffolds were custom made to fit the shape and size of the defect. These were then loaded with *ex vivo* expanded hMSCs isolated from their own bone marrow. All 3 patients were monitored with radiographs and CT scans, which revealed abundant callus formation by the second month postsurgery and good integration of the implants with peri-implant bone formation by the sixth month after surgery. A followup report 6-7 years after surgery reported that the implants displayed good osseointegration with no further complications. Angiographic evaluation performed in patient 3, 6.5 years after surgery also indicated vascularization of the grafted zone suggesting presence of vital bone at the graft site. However, no controls were included in this study, and initial followup was based only on radiological evaluation which the authors admit was not optimal because the high mineral density of the scaffolds used made it difficult to differentiate the new bone from the preexisting scaffold [55]. Nevertheless, the study showed that the procedure is safe to perform.

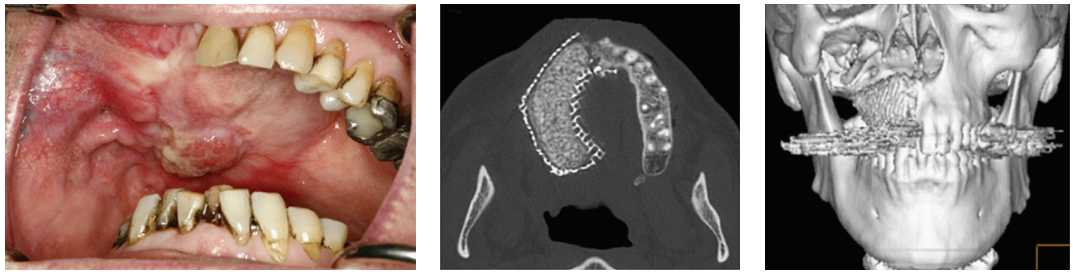
In the years after the initial trial, case studies involving single patients treated with tissue-engineered constructs were reported in the literature. In 2007, Krecic Stres et al. treated 1 patient with a comminuted fracture femur using a combination of TEC and autologous cancellous bone in a ratio of 2 : 1 [56]. The TEC was generated by seeding bone marrow derived MSCs on porous calcium-triphosphate granules. Clinically, the researchers claim that the patient has been recovering well. However, the combination of autologous bone with the TEC makes it difficult to draw conclusive inferences on the feasibility of using TEC alone for bone-tissue engineering as it would be impossible to determine the individual contributions of the TEC and the autologous bone. Moreover, the investigators only relied on clinical evaluation and X-rays to determine new bone formation. No controls or biopsies were planned for the patient. Also, the actual defect size was not mentioned. This is essential as the size of the graft has been found to be crucial in determining the survival of the cells within the core of the graft.

Hibi et al. reconstructed an alveolar cleft defect by injecting culture expanded and osteogenically-induced bone marrow derived MSC mixed with autologous platelet rich plasma [57]. This study provided a novel approach of using autologous platelet rich plasma as the scaffolding material for the cells. The patient was followed up postoperatively with serial CT scans which showed the regenerated bone extending from the cleft walls after 3 months and bridging the cleft after 6 months. It remains unclear whether the defect is filled by bone tissues produced by the implanted cells, or it is formed due to osteoconduction from the border of the cleft defect.

In 2010, Lee et al. described a successful reconstruction followed by dental implant placement of a 15 cm jaw defect as a result of segmental mandibulectomy due to central hemangioma in a 14-year-old boy [58]. Three reconstructive surgeries were performed. In the first surgery, autologous resected mandible obtained during the hemimandibulectomy was used as a tray into which osteogenic-differentiated autologous bone marrow stem cells and fibrin glue was injected. Due to lack of adequate mandibular bone for dental implant placement and recovery of dentition, the second surgery involved vertical distraction osteogenesis with injection of autologous osteoblastic-differentiated MSCs. The third and final surgery was 7 months later for implant placement. At the time of implant placement biopsies were taken from the implant site and histological evaluation of the biopsies revealed newly formed viable lamellar bone. Dental CT images taken 4 months after the implant placement confirmed continued presence of mineralized bone at the augmentation zone.

In 2009, Mesimäki et al. reconstructed a major maxillary defect in an adult patient using autologous adipose-derived MSCs (ASCs) combined with rhBMP-2 and β-TCP granules in a microvascular reconstruction surgery [59]. After isolating the ASCs from abdominal subcutaneous fat in autologous serum using GMP class clean room facilities, the cells were seeded on β-TCP scaffolds. Prior to combining with cells, the scaffolds were incubated for 48 hours in basal medium supplemented with rhBMP-2. This medium was discarded when the cells were added and fresh medium without rhBMP-2 was added. The cell scaffold combination was kept in culture for 48 hours prior to their placement in a titanium cage and subsequent implantation in a pouch prepared in the patients left rectus abdominus muscle. The vascular supply of the muscle was not disturbed. 8 months later, the rectus abdominus muscle pouch was opened, and the titanium cage filled with the TCP granules and ASCs was macroscopically examined. The new bone formed in the cage was clinically confirmed to be vital and rigid. A biopsy taken from the newly formed bone revealed histology of normal mature bone. Subsequently, the vessels were disconnected from the rectus abdominus muscle and the muscle flap together with the tissue engineered bone was placed in the maxillary defect. The abdominal vessels were reanastamosed with the facial vessels. The muscle was left to epithelialize intraorally. The patient was followed up with CT scans. Within two months of the surgery, the muscle flap had almost completely epithelialized, and the shape and normal bone density was achieved in the reconstructed maxilla (Figure 4). Four months after placement of the graft, dental implants were placed and their primary stability was reported to be excellent. The implants osseointegrated without any reported adverse effects. This study was the first clinical case where ectopic bone was produced using autologous ASCs in a microvascular reconstruction study. It demonstrated the feasibility and safety of using ASCs for bone regeneration. However, the relative contribution of the rhBMP-2 and the ASCs in the new bone formed remains to be determined.

In 2007, a study was performed by Sbayesteh et al. for posterior maxillary sinus augmentation involving 6 patients [60]. In this study, the cell source was the bone marrow from the iliac crest and the carrier material was hydroxyl apatite/β-tricalcium phosphate (HA/TCP) particle. After 3 months, biopsies were taken and results showed a mean bone formation of 41%. Although biopsies were taken, no information of the bone distribution in the scaffold or the source of the newly formed bone (donor or recipient) was provided.



(a) (b) (c)

Figure 4: Two months postoperative results of the study by Mesimaki et al. Mesimaki et al. reconstructed a major maxillary defect in an adult patient using autologous adipose-derived MSCs (ASCs) combined with rhBMP-2 and β-TCP granules in a microvascular reconstruction surgery. Two months postoperative results indicate that (a) the rectus abdominis muscle has atrophied nearly totally and epithelialized almost completely. Only a small area in the molar region was nonepithelialized. A well-formed buccal sulcus is also noted. Axial (b) and 3D CT scans (c) show the shape and normal bone density of the new maxilla.

Another clinical study was reported by our group in order to test the potency of bone-tissue engineering using bone marrow-derived MSCs seeded onto hydroxyapatite particles in 6 patients, requiring reconstruction of bony jaw defects prior to dental implant placement (Figure 5) [61]. Culture expanded bone marrow-derived MSCs were seeded on hydroxyapatite particles varying in size from 1–4 mm^3. Similar to the work of Schimming et al., the cells were grown on the scaffolds for another 7 days in order to allow further osteogenic differentiation and extracellular matrix deposition and then placed under the mucoperiosteal flap in the defect site. In this study, both the *in vitro* osteogenic capacity and the *in vivo* bone-forming potential of the constructs was assessed using representative samples of cells and constructs, respectively. The *in vitro* potential was tested by performing alkaline phosphatase staining, while the *in vivo* bone-forming capacity of the constructs was confirmed by implanting representative constructs, prepared in an identical fashion to the constructs actually used for the defects, in subcutaneous locations in nude mice. Although no quantification of the bone formed by these hMSCs in the mouse subcutaneous model has been performed, we noted that all the constructs with cells implanted in the nude mice showed bone formation. Four months after application of the construct in the jaw of the human subjects, and before placement of the implant, a biopsy was taken from the operation site. Bone formation was evaluated histologically in the human patients, and in 3 of them, no new bone formation was observed. Of the remaining 3, in 2 patients bone tissue in the scaffolds was observed in close contact with the preexisting bone of the bony defect. This can likely be attributed to migration of osteoblasts from the surrounding bone tissue. In only 1 patient, bone formation was observed more than 7 mm from the preexisting bone tissue. We consider this to be strongly suggestive for *de novo* osteogenesis induced by the implanted cells. An overview of the above mentioned clinical trials are presented in Table 1.

3. Experimental Design of Clinical Studies

The clinical studies conducted so far have demonstrated that it is safe to use hMSCs in bone grafting procedures. None of the reports mention adverse effects such as inflammation or excessive tissue growth, despite the fact that there are *in vitro* studies which suggest that MSCs which have been extensively cultured (4-5 months) can develop genomic instability, which can be an indicator of malignant transformation [62–64]. For most clinical applications, a 6–8-week expansion phase provides sufficient cell numbers. This may account for the fact that no malignant potential of the TECs has been observed in the clinical cases performed so far. However, to ensure safety for the patient, we propose that in future clinical studies, chromosomal analysis of implanted cells is assesed. Secondly, most of the clinical studies published have a short followup period. We recommend using longer followup periods to obtain data on the long term safety of TEC.

The data presented in the clinical studies make it likely that the grafted hMSCs were able to contribute to bone regeneration, which provides proof of concept for the potential use of tissue-engineered grafts in bone regeneration. However, the lack of "gold standard" controls and objective evaluation measures such as bone quantification using histology makes it difficult to draw strong conclusions. The studies where biopsies have been used to evaluate the percentage of bone formed seem to suggest that the contribution of the grafted cells is very limited and certainly not sufficient to bridge critical-sized defects. Thus, in order to be able to normalize the efficacy of a given bone TE strategy with respect to that of other trials, we recommend the use of a reference for the bone-forming potential of a tissue-engineered graft. Given that immune-deficient mice have been used by many researchers in the field, we would like to propose that the tissue-engineered grafts to be implanted in patients will be evaluated in mice in parallel and bone formation will be quantitatively assessed.

Future studies should attempt to include comparisons of the TECs with autologous bone grafts for the same

Table 1: Overview of the clinical studies performed on humans using-tissue engineered constructs.

Principal investigator	Year	Cell source	Scaffold	Patients	Area of reconstruction	Salient features	Evaluation	Reported outcome
R.Quarto	2001	Bone marrow	100% hydroxyapatite	3	Long bone defects (1 tibia, 1 ulna, 1 humerus)	(1) First clinical trial in humans using hMSCs (2) Patients with long bone defects selected (3) Patients had good clinical recovery (3) No side effects even after 6-7 years followup	Radiology CT scan Angiography	No quantification of new bone formed. Good integration of the host bone with the implanted scaffolds
H. Hibi	2006	Bone marrow	Platelet gel	1	Alveolar cleft defect	(1) First study using platelet gel as the scaffolding material	Serial Ct scans	Comparable bone formation to that described in literature with autolgous bone grafts
Y.Soleymani	2007	Bone marrow	HA/TCP	6	Maxillary sinus augmentation	(1) Good bone formation in all scaffolds (2) Mean amount of new bone regenerated was 41.3% (3) When compared to the Vacnati study, stark difference in the amount fo bone formed, probably due to location of defect or cell source	Radiology Biopsy	Reported successful with mean bone regenerate as 41.34% and good osseointegration
H.Krecic-Stres	2007	Bone marrow	Porous calcium triphosphate granules	1	Femoral defect	(1) autologous bone graft was mixed with TECs made with MSCs and scaffolds in ratio of 1 : 2 to fill the defect	Radiology	Good clinical recovery. No bone quantification performed
Gert Meijer	2008	Bone marrow	Hydroxyapatite scaffolds	6	Intraoral osseous defects	(1) Only study which performed a biopsy to not just to quantify the amount of bone formed but also the location of bone on the scaffold. This helped identify if the bone was formed due to osteoconduction alone or as a result of osteo conduction with de novo bone sytnthesis. (2) Demonstarted the donor donor variation with hMSCs	Radiology Biopsy	5 patients had no new bone
K. Mesimaki	2009	Adipose tissue	β-TCP	1	Maxillary reconstruction	(1) First clinical study to use autologous MSCs derived from adipose tissue and expanded employing good manufacturing protocols (GMP) to heal a bone defect. (2) Use of rhBMP-2 to promote bone formation *in vivo*. (3) Use of a microvascular flap reconstruction surgery for bone tissue engineering	Radiology biopsy	8-month followup indicated presence of mature bone. No quantification of the amount of bone formed is provided. Good clinical course

Table 1: Continued.

Principal investigator	Year	Cell source	Scaffold	Patients	Area of reconstruction	Salient features	Evaluation	Reported outcome
Jun Lee	2010	Bone marrow	Freeze dried Autologous cancellous bone	1	Mandible reconstruction	(1) Repair of a large segmental defect (15 cm) (2) Injection of MSCs with fibrin glue into the defect site. (3) Use of autologous cancellous freeze dried bone as a tray to hold the MSCs in place.	Biopsy radiology	New bone formation after 4 months. No quantification provided. Goo clinical recovery

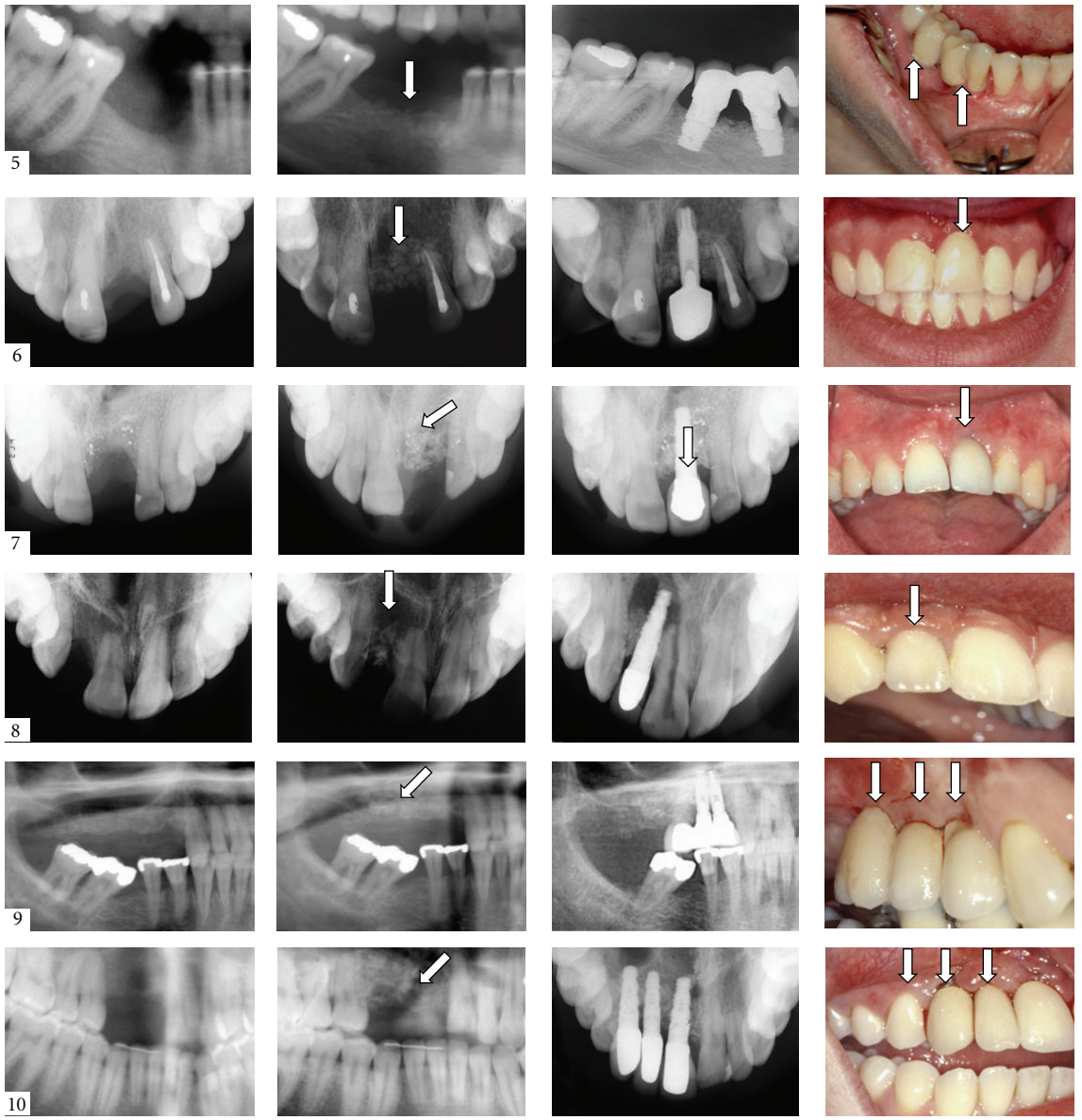


Figure 5: Overview for patients 5–10 from the study by Meijer et al. to reconstruct a maxillary defect and placement of dental implants. First column; radiographs showing the alveolar defects. Second column; showing the reconstruction (arrow) by augmentation (5–8) and by sinus elevation procedure (9 and 10). Third column; radiographs showing the dental implants and the prosthetic construction (crown or bridge). Fourth column; clinical situation at the end of the rehabilitations (arrow).

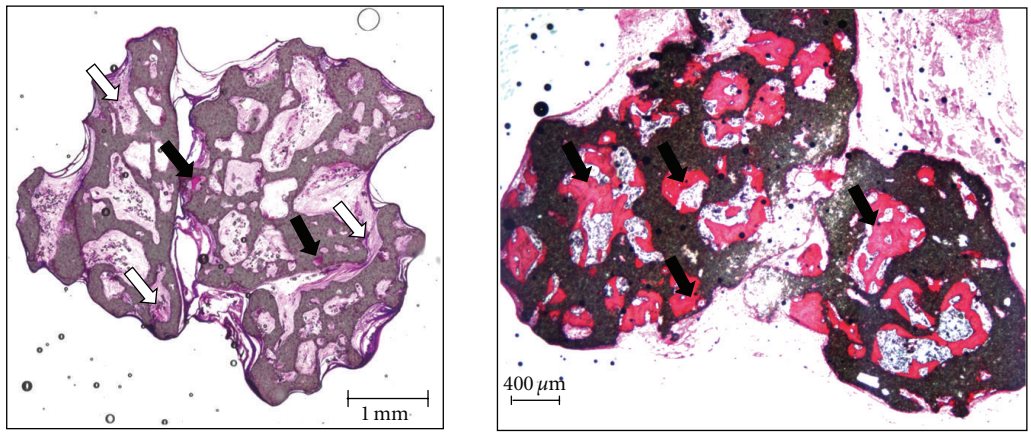


Figure 6: Representative section of scaffold seeded with human bone marrow compared to that seeded with rat bone marrow. Calcium phosphate ceramic scaffolds were seeded with equal number of cells derived from either human or rat bone marrow and implanted subcutaneously in nude mice for 6 weeks. Almost all the pores of the scaffold seeded with rat cells are filled with newly formed bone while the pores of the scaffold seeded with human cells have only one pore with a small amount of bone while the rest of the pores are filled with fibrous tissue. The sections are stained with basic fuschin and methylene blue. The newly formed bone is stained red with basic fuschin (black arrows) while the remaining fibrous tissue is stained pink (white arrows). The black areas represent the scaffold.

application. This type of study design can thus provide conclusive evidence on the efficacy of the new treatment method as compared to the established standard treatment option. When possible, the two types of implants should be implanted in the same patient. A possible situation when this can be performed without raising ethical issues is when a patient with bilateral defects needs quantities of autologous bone graft which may be difficult to obtain without putting the patient at high risk of complications and morbidity. In such cases, the autograft can be used to treat one defect while the other defect is treated simultaneously with the TEC.

Objective evaluation methods should be used to determine the amount of new bone formed. The sample size of the patients should be large enough to allow statistical analysis of the data obtained. We also recommend choice of a surgical site or a tissue-engineered scaffold which allows quantification of bone-tissue formation without added inconvenience to the patient. For instance, we implanted tissue-engineered grafts in the jaw, where we were able to obtain a biopsy in the routine course of the procedure. Other possibilities include tissue-engineered grafts where MRI, microCT or other noninvasive imaging strategies can be applied to quantify bone formation.

In all the clinical studies described, culture-expanded MSCs have been combined with a scaffolding material to generate TECs. Expansion of hMSCs can have unfavorable effects on their differentiation potential [64, 65]. For instance, Banfi et al. demonstrated that as early as after the first passage, the bone forming potential was reduced by about 36 times as compared to fresh marrow [66]. Future studies should employ methods to generate TECs which completely bypass the expansion phase of MSCs on plastic. Studies by Warnke et al. [67], Wongchuensoontorn et al. [68] Gan et al. [69], and Aslan et al. [70] have already demonstrated the feasibility of seeding either mononuclear or enriched populations of MSCs obtained on scaffold material for enhancing the osteogenic potential of the cells.

4. Concluding Remarks

Bone-tissue engineering may alleviate problems associated with the current standard treatment used to heal bone defects. However, the success with TECs generated using human MSCs is currently limited. In the majority of the cases, the human MSCs fail to produce clinically relevant amounts of bone while MSCs from other species convincingly generate sufficient bone volume (Figure 6). It is therefore necessary to identify donors with good osteogenic potential and invest research efforts in improving the bone forming capacity of the obtained hMSCs to the level of those obtained from the other species using the widely available ectopic mouse models before embarking on future clinical studies.

Identification of a donor having cells with good osteogenic potential still poses a major hurdle for bone-tissue engineering. So far, no literature evidence of a positive correlation between hMSC osteogenesis *in vitro* and bone-formation *in vivo* has been reported [71]. Our group isolated hMSCs from 62 donors and assessed the *in vitro* lineage differentiation capacity with gene expression signature and *in vivo* bone forming capacity. We are currently investigating a gene which we believe could be used as a reliable diagnostic marker for *in vivo* bone-forming capacity (unpublished data).

This is especially attractive as the knowledge that MSCs lack certain surface markers responsible for the host T-cell response opens up possibilities for using such allogeneic cells

with proven bone forming potential [72, 73]. In addition to being a ready source of guaranteed bone-forming cells the patient would also have the benefit of not having to undergo immunosuppressive therapy. Moreover, combining allogeneic cells with scaffolds would then make it possible to have a standardized off the shelf bone-tissue engineering product which then can be routinely applied to the clinic.

Other areas of preclinical research focus should include identification of more potent subfractions of hMSCs, *in vitro* and *in vivo* studies with MSCs isolated from "waste" tissues such as umbilical cord, human placenta, amniotic fluid, and aborted fetuses, alternative seeding strategies to avoid the unphysiological expansion of MSCs on plastic and genetic manipulations of MSCs [74–76] to enhance the expression of osteogenic genes and priming of MSCs using growth factors such as BMPs [77–79] or compounds such as cAMP [43] or vitamin D [80] to enhance the bone-forming capacity while maintaining acceptable costs and safety profile. When the stage is set again for clinical studies, attempts should be made to optimize the experimental design. With the imminent need for bone graft substitutes and the good results obtained with animal-derived MSCs, bone-tissue engineering using human MSCs is likely to reenter the clinic once their biological performance is enhanced.

Acknowledgments

The authors gratefully acknowledge the support of the TeRM Smart Mix Program of the Netherlands Ministry of Economic Affairs and the Netherlands Ministry of Education, Culture, and Science.

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SAGE-Hindawi Access to Research
Stem Cells International
Volume 2010, Article ID 519028, 14 pages
doi:10.4061/2010/519028

Review Article

Mesenchymal Progenitor Cells and Their Orthopedic Applications: Forging a Path towards Clinical Trials

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Received 17 May 2010; Revised 7 July 2010; Accepted 28 September 2010

Academic Editor: Jin Sup Jung

Mesenchymal progenitor cells (MPCs) are nonhematopoietic multipotent cells capable of differentiating into mesenchymal and nonmesenchymal lineages. While they can be isolated from various tissues, MPCs isolated from the bone marrow are best characterized. These cells represent a subset of bone marrow stromal cells (BMSCs) which, in addition to their differentiation potential, are critical in supporting proliferation and differentiation of hematopoietic cells. They are of clinical interest because they can be easily isolated from bone marrow aspirates and expanded in vitro with minimal donor site morbidity. The BMSCs are also capable of altering disease pathophysiology by secreting modulating factors in a paracrine manner. Thus, engineering such cells to maximize therapeutic potential has been the focus of cell/gene therapy to date. Here, we discuss the path towards the development of clinical trials utilizing BMSCs for orthopaedic applications. Specifically, we will review the use of BMSCs in repairing critical-sized defects, fracture nonunions, cartilage and tendon injuries, as well as in metabolic bone diseases and osteonecrosis. A review of www.ClinicalTrials.gov of the United States National Institute of Health was performed, and ongoing clinical trials will be discussed in addition to the sentinel preclinical studies that paved the way for human investigations.

1. Introduction: What Are Stem Cells?

The popularity of stem cells in the clinical arena has significantly increased, given the rapid improvement in our understanding of their biology. Classically, stem cells are defined by their capacity to retain an undifferentiated state for a prolonged period while retaining the potential to differentiate along one lineage (unipotent), multiple lineages (multipotent), or into all three germ layers (pluripotent) [1]. These cells can be broadly categorized into two major classes: embryonic and adult stem cells. Embryonic stem cells (ESCs), isolated from the inner cell mass of the blastocyst, are pluripotent cells with the potential of differentiating into tissues from all three germ layers [2, 3]. While ESCs have significant regeneration capacity, their clinical application has been limited as a result of multiple factors including: (1) a propensity to form teratomas, (2) ethical concerns with isolation, (3) rejection by the host immune system

after transplantation, and (4) the use of a feeder layer to retain an undifferentiated state in vitro [4–6]. Recently discovered, another source of pluripotent stem cells are induced pluripotent stem (iPS) cells, derived from somatic cells treated with few defined factors [7–11]. While iPS cell-based therapy has the potential to revolutionize the field of regenerative medicine, many obstacles must be overcome before their clinical application can be realized [12].

Furthermore, naturally occurring adult stem cells have also been identified and categorized into either hematopoietic stem cells (HSCs), a source of various hematopoietic cell lineages, and nonhematopoietic stem cells, which can give rise to cells of mesenchymal origin [13]. Many reports have suggested that these nonhematopoietic stem cells, also known as mesenchymal progenitor cells (MPCs), can be isolated from various tissues including blood, adipose, skin, mandible, trabecular bone, fetal blood, liver, lung, and even the umbilical cord and placenta [14, 15]. Upon harvest, these cells can be expanded in vitro with high efficiency without sacrificing differentiation capacity [16–20]. While these multipotent progenitor cells share many similar characteristics, they can be differentiated based on their expression profile and differentiation propensity along various lineages [21]. Amongst the various sources, MPCs isolated from the bone marrow, a subset of Bone Marrow Stromal Cells (BMSCs) are considered to have the greatest potential for multilineage differentiation and have been the most characterized [22, 23].

BMSCs were initially described by Friedenstein and colleagues more than 40 years ago as adherent cells, with a fibroblast-like appearance capable of differentiating into osteoblasts, chondroblasts, adipocytes, and tenocytes [22, 24, 25]. Unlike ESCs, BMSCs provide the flexibility of autologous transplantation, circumventing ethical concerns or immunologic rejection [26].These cells also play a sentinel role in proliferation and differentiation of hematopoietic cells [27]. Mankani et al. illustrated that the formation of both hematopoiesis and mature bone organ is correlated with the high local density of BMSCs [28]. Additionally, BMSCs are considered to be immune privileged and have the capacity for allogenic transplantation a property that has been used in the clinical setting for the treatment of various autoimmune diseases [29–31]. While many studies have suggested that MPCs are immunoprivileged and do not undergo rejection, others have cast doubt on this notion, showing that in certain scenarios, MPCs induce immune rejection [32]. More investigations should be conducted to provide further insight into the specific interaction between these progenitor cells and the host immune system.

Considerable effort has been put forth to identify specific surface markers that characterize MPCs, yet disagreement within the literature has prevented the creation of definitive standards. The minimal criteria identified by the International Society for Cellular Therapy for identifying BMSCs requires the cells: (1) to be plastic adherent while maintained in cell culture, (2) to express CD73, CD90, and CD 105 and lack expression of CD11b, CD14, CD19, CD34, CD45, CD79-alpha, and HLA-DR, and (3) to differentiate into osteoblasts, adipocytes, and chondroblasts in vitro [33]. Additional studies have also suggested that CD146 is considered an important marker of bone marrow progenitor cells [34, 35]. These guidelines were set in place to enable a unified approach for comparison amongst different studies.

Bone marrow is generally considered a milieu plentiful for various cell types, collectively referred to as stromal cells. Amongst these, the multipotent subset of BMSCs comprises a small fraction (<0.01) [36], yet despite their small numbers, the relative ease with which BMSCs can be harvested has propelled their experimental use. Researchers have pioneered the creation of stable animal models aimed at mimicking human conditions to study the therapeutic capacity of these bone marrow-derived cells [37]. Because of their ubiquity, tolerance of expansion, paracrine capabilities, and multipotency, the potential for clinical applications of BMSCs in the orthopaedic realm is countless.

In this paper, we will focus on the development of human clinical trials utilizing BMSCs for orthopaedic applications. The path towards the creation of such trials beginning from sentinel animal investigations will be highlighted. Specifically, the progenitor subset of BMSCs used in the context of critical-sized defects, fracture nonunions, cartilage and tendon repair, metabolic bone disease, and osteonecrosis will be discussed. A review of www.ClinicalTrials.gov of the United States National Institute of Health was performed to underscore the status of ongoing clinical trials.

2. Modes of Use and Preparation

As previously mentioned, MPCs have been successfully harvested from a variety of tissues; however, most clinical trials utilize BMSCs for therapeutic applications. These cells are harvested from the iliac crest and expanded ex vivo in supplemental media. Cell expansion is currently a time-consuming process, generally requiring 3–5 weeks to obtain sufficient number for therapeutic application [38]. This paper will touch upon the different strategies for MPCs preparation and use within the clinical arena, and therefore, a prior overview of such approaches should be outlined. As the most commonly used cell type for clinical application, BMSCs can be administered through either autologous or allogenic transplantation. In clinical trials, fresh autologous BM and passaged BMSCs have been utilized for cellular therapy [39]. Freshly harvested aspirates can further undergo centrifugation to concentrate BMSCs prior to percutaneous or intravenous injection [40–42]. When expanded ex vivo, various cell-specific characteristics are utilized to enrich progenitor cells and separate them from other cells in the bone marrow. One such characteristic that enables their selection is BMSC preferential adherence to the plastic surface of the culture dish [43, 44]. Immunoselection for previously discussed MPC markers also enables further identification [45–47]. After expansion and selection, the harvested cells can be utilized for various therapeutic applications.

Subsequent to ex vivo expansion, BMSCs can be cultured in the presence of a scaffold-enabling colonization and cell differentiation prior to material grafting at the affected site [48]. Preconditioning of the graft-scaffold composite can be performed in vivo. For example, the composite can be first

inserted into a heterotopic, richly vascularized area in order to stimulate angiogenesis within the intended implant. After adequate growth, the newly vascularized tissue can be excised and inserted into the desired site, thus increasing the chance for cell survival [49]. Mankani and colleagues impregnated BMSCs into a hydroxyapatitie/tricalcium phosphate (HA/TCP) matrix and subsequently observed the generation of mature bone with similar histologic and mechanical properties to the bone formed with standard transplant techniques in animal models [50]. The above-mentioned modes of administration are only a few of the many methods that various clinical trials have utilized to provide innovative therapeutic strategies for many diverse clinical applications.

3. Critical-Sized Bone Defects

Critical-sized defects are osseous defects beyond certain size where complete healing will not occur during the lifetime of the organism. Sixty years ago, the idea of autografting arose as a solution for repairing large defects that would otherwise not bridge in the normal environment. It was clear then that tissue progenitors were necessary for effective defect repair. Classically, grafting involves the harvest of unaffected pieces of tissue from remote sites and their implantation into affected areas. Yet, autograft use is limited by the lack of sufficient donor tissue to fill large defects and the morbidity afflicted to the donor site. These clinical drawbacks have led to the search for cells that could be induced to differentiate and grow into the desired tissue type [51].

The concept of using cells to repair tissues is continually evolving. The idea that MPCs possess the capacity for bone regeneration in vitro and ectopically in vivo has been tested and reaffirmed in various studies [52]. Promising results spurned the development of animal models as building blocks for the eventual clinical use of stem cells in humans. These models can mimic (1) normal fracture healing, (2) critical-sized segmental defects, and (3) non-critical-sized defects, as in the case where healing is prevented by fracture nonunions [51]. A variety of animal models including mice, rats, dogs, sheep, and goats have been utilized to study the role of MPCs in promoting repair in critical-sized defect. These studies have also investigated critical size defects of different anatomical locations including: the femur, tibia, metatarsal, mandible, and calvaria [53].

In a pilot study performed on a sheep model, Kon et al. reported the use of autologous bone marrow stromal cells in conjunction with hydroxyapatite ceramic (HAC) carriers and demonstrated faster bone repair in the BMSC-treated group as compared to HAC alone [54]. Potentially, this combination could be used clinically for the treatment of significant long bone defects. In 2003, Arinzeh et al. further expanded upon earlier trials and found that autologous and allogeneic MPCs could repair critical-sized bone defects in the canine model without immunosuppressive treatment [55]. Kuznetsov and colleagues demonstrated that BMSCs have the capacity to provide long-term bone augmentation of the mandible [56]. In an earlier study, a similar group concluded that BMSCs in conjunction with HA/TCP can successfully close craniofacial bony defects in a mouse model [57]. Biomechanically, the newly formed bone demonstrates similar properties as the normal bone surrounding the defect site [58]. Encouraging results from these animal studies and others have paved the way for investigations involving humans.

Quarto et al. provide one of the first clinical reports involving the repair of segmental diaphyseal defects in 4 patients using culture-enriched ex vivo expanded progenitor cells [59]. Autologous bone marrow stromal cells were placed on macroporous hydroxyapatite (HA) scaffolds, with their size and shape dependent on the characteristic of the specific bony defects. These grafts were stabilized via external fixation. Integration at the bone-implant interface was observed one month postoperatively and complete consolidation was evident after 5–7 months. All patients recovered limb function with no complications associated with the implants. At last followup (6 to 7 years postoperatively in patients 1 to 3), a good integration of the implants was maintained without evidence of late fracture [60].

Ohgushi et al. have expounded upon earlier studies by pioneering the production of bone matrix ex vivo. After expanding marrow-derived MPCs, the investigators then subcultured the osteoprogenitor cells to allow for the fabrication of osteoblasts/bone matrix onto various substrata. The in vitro cultured regenerative bone was then delivered to hospitals for surgical use. Care was taken to assure the risk of bacterial/fungal contamination is minimized. Since 2002, Ohgushi and colleagues have been utilizing these techniques clinically for the treatment of chronic skeletal diseases (i.e., OA and benign bone tumors) [61].

The use of scaffolds or inert materials impregnated with MPCs to generate three-dimensional implants is a branch of tissue engineering which is rapidly growing. By utilizing scaffolds, researchers attempt to create an in vivo environment that favors the development of the desired tissues for implantation. Seeding these composites with the progenitor cells creates a potential for increased bony regeneration because it enhances the repair process by supplying progenitors that secrete factors. Perhaps one of the greatest examples of this mode of tissue engineering for bony regeneration is the case report by Vacanti et al., where the authors replaced the avulsed phalanx of a 36-year-old man with periosteal progenitor cells harvested from the distal radius seeded onto a natural coral (porous HA) scaffold. This procedure resulted in the functional restoration of a biomechanically sound thumb of normal length, without the comorbidity associated with harvesting bone grafts [62].

Promising preclinical studies and case reports have provided support for the therapeutic role of culture-expanded osteoprogenitor cells and their application in conjunction with different scaffolds for healing of long bone defects in clinical trials. Hoping to take advantage of this tissue-engineering approach, Emory University gained approval in March 2009 to conduct a Phase II/III randomized, single-blinded control trial with 50 participants utilizing the allograft substance Trinity in the repair of bony deficits in patients with benign disease (NCT00851162). Trinity is currently FDA approved for use in traumatic bony defects including the spine. MPCs, along with an allograft carrier,

can incorporate and induce bone formation. The MPCs are preimmunodepleted and, therefore, do not stimulate local T-cell proliferation but instead are activated to act as osteoblasts promoting bone formation. As this study is ongoing, no results have been made available.

The therapeutic potential of MPCs has also been reported in the treatment of critical-sized defects of the craniofacial skeleton. Lendeckel et al. reported improved healing in a 7-year-old girl suffering from widespread traumatic calvarial defects, who was treated with autologous adipose-derived MPCs [63]. While CT-scans demonstrated new bone formation, whether the regenerate was a direct outcome of donor cell differentiation or rather a paracrine effect induced by local cells, remains to be elucidated. In another case report, tissue-engineered osteogenic material was injected into a patient undergoing distraction osteogenesis utilizing a fibular flap for mandible reconstruction. The material was comprised of autologous, culture-expanded MPCs that were induced towards osteogenesis via platelet-rich plasma (PRP) activated by thrombin and calcium chloride. Full consolidation of the regenerate was observed after 3 months. Post injection, the regenerated ridge became thicker, and thus aided in bridging a gap between the native mandible and distracted fibula [64].

In case reports, other groups have also reported the efficacy of BMSC transplantation in combination with PRP for bone regeneration during distraction osteogenesis [65]. Ueda et al. used this mode of tissue engineering by combining PRP and beta-tricalcium phosphate as grafting materials for maxillary sinus floor augmentation with simultaneous implant placement in 6 patients. A mean increase in mineralized tissue height of 7.3 ± 4.6 mm was evident when comparing the pre- and postsurgical radiographs. Thus, in these cases, injectable tissue-engineered bone provided stable and predictable results in terms of implant success [66].

4. Non/Delayed Unions

During normal fracture healing, undifferentiated MPCs are recruited to injury sites and under the influence of regulatory cytokines (e.g., BMPs), and they proliferate and differentiate into chondrocytes and osteoblasts to repair the defect. While many fractures under appropriate conditions heal properly, some fail to heal due to a variety of causes associated with the host, the surgical technique, inadequacy of the vasculature, and infection amongst other causes. At the cellular level, non-union is defined as the cessation of both periosteal and endosteal healing without bridging [67]. Nonunions complicate approximately 10%–20% of 6 million fractures occurring each year in the United States [68] whereas the incidence varies by fracture location. While it remains a common clinical concern, management of nonunions remains a challenge.

In 1978, Salama and Weissman described the first attempt to utilize the osteogenic potential of BMSCs to manage delayed fracture healing in the clinical arena [69]. The authors combined bone xenografts with autologous bone marrow aspirates for grafting in 28 patients for a variety of clinical indications. The treatment of tibial nonunions with autologous marrow injections was expanded further by Connolly et al. who combined this method with either cast immobilization or intramedullary nailing in 20 successful cases over a five-year period [70]. In a followup series of 100 patients, Connolly also employed this technique in the treatment of delayed unions and nonunions of fractures, arthrodeses, and bone defects [71]. Since the original report by Salama et al., numerous researchers have begun utilizing bone marrow aspirates as adjuncts in the treatment of non- or delayed unions with promising results [72].

Hernigou et al. further emphasized the potential role of progenitor cells in bony healing in a study of 60 tibial atrophic nonunions. Using percutaneous injection of a concentrated buffy coat obtained from the centrifugation of autologous iliac crest bone marrow aspirates, the authors noted a positive correlation between the volume of the mineralized callus and the number and concentration of progenitor cells in the aspirate. Analysis of the buffy coat revealed the presence of progenitor cells and other mononuclear cells, most likely providing osteogenic and angiogenic influences. In the 7 patients where union was not accomplished, the concentration of stem cells injected appeared significantly lower than in patients with osseous union ($P = .001$ and $P < .01$). Perhaps a limitation in this study is the lack of a control group with placebo treatment [41]; however, the data demonstrates that successful treatment of nonunion with percutaneous bone marrow grafting is dependent upon the number and concentration of progenitor cells.

In 2005, Goel et al. embarked on a prospective clinical study to evaluate the efficacy of percutaneous bone marrow grafting in 20 patients with established tibial nonunions with minimal deformity, while they waited for open surgical procedures [73]. Three to five milliliters of marrow was aspirated from the iliac crest and injected immediately into and about the site of non-union. Subsequent aspirations were performed 1 cm posterior to the previous site until a maximum of 15 ml of marrow was injected. Clinically and radiologically, bony union was documented in 15 out of 20 patients (75%), with an average time to union following the first injection of 14 weeks. Four patients (20%) showed no evidence of union and were considered a failure. There were no cases of infection following the injections, and no complications at the donor site. Based on these promising results, the authors concluded that percutaneous bone marrow grafting can be considered a safe, simple, and reliable technique for managing non-unions**.** This minimally invasive method of treating tibial non-unions without deformity can potentially allow the avoidance of major surgical reconstruction in qualified patient populations.

Maneerit et al. conducted one of the first prospective randomized clinical trials examining the use of bone marrow in the treatment of non- or delayed unions over a 2.5-year period [74]. They compared outcomes between percutaneous bone grafting and open bone grafting of tibial shaft fractures or high-energy tibial fractures which required early prophylactic bone grafts. Subjects were randomized to either percutaneous bone graft ($n = 15$) or open bone graft ($n = 15$). Percutaneous bone graft technique was associated with significantly less blood loss ($P < .01$) and shorter operative

time ($P < .01$) although one patient in the percutaneous group had posterior tibial nerve palsy postoperatively with complete recovery after 6 weeks. No differences in rate of union, healing time of the successful cases, postoperative pain and hospital stay were observed, indicating that the percutaneous technique has effective results similar to the open technique in promoting union of tibial fractures.

Entering the realm of clinical trials marks an important milestone on the pathway to approval of MPC therapy for bony non-unions. In October 2003, Aastrom Biosciences enrolled 36 patients who had failed previous surgical intervention with long-bone atrophic non-unions from type IIIA or IIIb fractures with fracture gaps of <6 cm in a multicenter, nonrandomized, open-label uncontrolled, single-group phase I/II clinical trial (NCT 00424567) [52]. Subjects were treated with open reduction and internal fixation and an allograft bone matrix graft extender plus BMSCs expanded from autologous iliac crest aspirates. In April 2009, the Hadassah Medical Organization in Israel began a randomized, open-label, single-group Phase I/II clinical trial for the treatment of distal tibial fractures (NCT00250302). Twenty-four patients with distal tibial fractures without joint involvement will undergo autologous implantation of MPCs loaded onto carriers at fracture sites to determine the safety and efficacy of this mode of treatment.

5. Cartilage Repair

Chondral defects secondary to accidental trauma, necrosis of subchondral bone tissue, or arthritis have become some of the more common conditions today [79]. Approximately 15% of the world's population reportedly suffers from joint diseases. However, despite ongoing research, repair and regeneration of cartilage defects remains a challenge in orthopaedic surgery. Human cartilage is characteristically avascular and depends on diffusion from cyclical loading during joint movement for nutrient acquisition [80]. Given their unique microenvironment, chondrocytes have adapted to a low basic metabolic rate and have limited potential to increase their metabolic activity to allow for tissue repair. As a result, articular cartilage is considered a tissue with minimal intrinsic repair capacity in vivo.

Currently, many of the treatment modalities including drug therapy, arthroscopy, and prosthetic joint replacement provide symptomatic relief but do not directly address the underlying pathophysiology [81]. Other treatment modalities for cartilage repair, which aim to address the underlying molecular cause, range from bone marrow stimulation, mosaic plasticity, and autologous chondrocyte implantation (ACI). ACI is a method first reported by Brittberg et al. in 1994 [82]. In this technique, chondrocytes are isolated from the cartilage of nonweight bearing sites and expanded ex vivo. These expanded cells are subsequently injected into the defect sites and covered with an autologous periosteal flap to ensure cell adherence. Initial clinical trials of ACI showed promise [83]; however, this treatment requires the extraction of chondrocytes directly from the patient and thus causes trauma to healthy articular cartilage. Other disadvantages of ACI include: leakage of transplanted cells, periosteal hypertrophy, loss of the chondrocyte phenotype in the expanded cells in monolayer culture, lack of applicability to large lesions, and decreased efficacy in patients over 40 years due to low cellular activation levels. Additionally, the newly regenerated cartilage often consists of fibrocartilage, rather than the desired hyaline cartilage within the joint space [84].

Researchers tried to address these initial pitfalls with the development of a second generation ACI procedure. This method employs various biomaterials such as collagen type I gel, hyaluronin-based scaffolds, and collagen type I/III membranes to recreate a 3D environment ideal for expression of chondrogenic phenotypes and to secure cells within the defect site in lieu of a periosteal flap [85–87]. However, prospective clinical studies comparing first and second generation ACI failed to show significant difference in short-term clinical outcomes [88].

Although current technologies may improve morbidity associated with local cartilage defects, they still fall short in the treatment of systemic arthritic disease. BMSC therapy for cartilage disorders is principally sound since progenitor cells are typically harvested from the iliac crest, circumventing the need to damage healthy articular cartilage. Furthermore, the number of successfully cultured cells is larger due to their excellent proliferation capacity enabling abundant supply.

The chondrogenic potential of MPCs was first reported by Ashton et al. in 1980 [89], and since then, many researchers have focused on delineating the mechanism underlying chondrogenic differentiation [90–92]. Currently, only one prospective clinical trial of BMSC transplantation for repair of cartilage defects has been published. Wakitani et al. recruited 24 patients with knee osteoarthritis who underwent a high tibial osteotomy to examine the effects of a cellular versus noncellular impregnated scaffold on cartilage defects in the medial femoral condyle [76]. For the cellular arm, bone marrow-derived MPCs were suspended in a type I collagen gel and transplanted with an autologous periosteal flap into the defect site. The control group also received a periosteal flap but with a cell-free scaffold. No clinically significant improvement was observed in the cell-treated group versus controls, but the arthroscopic and histological scores were higher in the BMSC transplanted subjects. Furthermore, in a later study, the same group investigated the efficacy of autologous BMSCs for repair of cartilage defects and demonstrated clinical and histological improvement in the treated patients [75].

Case reports from the same research group also document improvement in clinical symptoms following BMSC transplantation [93], but comparative clinical trials with other surgical methods must be performed to assess the utility of this type of tissue engineering in humans. Black et al. published the first randomized, double-blind, multicenter control trial examining the effectiveness of stem cell therapy in dogs [94]. They explored the effects of adipose-derived stem cells (ASCs) on lameness in dogs with chronic osteoarthritis of the femoral joint. Dogs treated with ASCs showed significantly improved scores for lameness pain, and range of motion compared with control dogs. While these studies show promising results, it is important to identify

the paracrine potential of ASCs and their effect on clinical outcomes.

Numerous clinical trials are ongoing to examine the utility of MPC therapy in the treatment of cartilage defects. Cairo University School of Medicine has undertaken a Phase II/III clinical trial which began in December 2006 to examine whether implanting autologous, culture-expanded MPCs, obtained from patients with early OA, cartilage defects, or osteochondral joint disease, is effective in treatment of such conditions. Twenty-five subjects will undergo bone marrow aspiration from the iliac crest with implantation of the ex vivo expanded MPCs into defect sites via open surgery or arthroscopy (NCT00891501). Starting in August 2008, The Royan Institute of Tehran University of Medical Sciences enrolled 6 patients in a Phase I study aimed at investigating the efficacy and safety of autologous transplantation of BMSCs mixed with a collagen I scaffold in cartilage defects and osteoarthritis of the knee (NCT00850187). Ullevaal University Hospital in Oslo, Norway began a Phase I clinical trial in April 2009, enrolling 50 patients, to compare the treatment efficacy of autologous MPCs versus chondrocytes implanted in a commercially available scaffold in patients with cartilage defects (NCT00885729).

In an in vivo study of osteoarthritis in a caprine model, Murphy et al. demonstrated that intra-articular injection of BMPCs at sites of meniscal injury resulted in engraftment of those cells and regeneration of meniscal tissue to produce a chondroprotective effect [95]. Based on results from this in vivo trial, Osiris Therapeutics completed a Phase I/II placebo-controlled, RCT in February 2008 of 60 patients to examine the safety and efficacy of hMPCs suspended in a solution of hyaluronic acid (Chondrogen) for the repair of meniscal tissue following meniscectomy. In Chondrogen treated subjects, surgically removed meniscal tissue was regenerated, cartilage surface was protected, and joint damage was decreased in comparison to control subjects. These benefits persisted for at least one year. As a followup study, in June 2008, Osiris Therapeutics began a second randomized, placebo-controlled Phase I/II clinical trial to examine the long-term safety of high and low-dose BMSCs (Chondrogen) in the repair of meniscal tissue following meniscectomy (NCT00702741). In this 3-year followup study, the treatment group will receive a single intra-articular injection of 50 million donor-derived hMPCs (low dose) or 150 million donor-derived hMPCs (high dose) in suspension with commercial sodium hyaluronan and will be compared to control patients receiving an injection of a vehicle (diluted hyaluronan) alone.

Although bone marrow provides a good source of stem cells, interest in MPCs derived from the umbilical cord blood (UCB) has recently emerged. Some studies have suggested that the prevalence of MPCs is much higher in preterm UCB as compared to samples obtained from term fetuses [96]. Various studies have suggested that MPCs isolated from UCB have the highest level of activity among all adult stem cells [97]. Using umbilical cord blood for the harvesting progenitor cells also forgoes the ethical implications associated with harvesting embryonic stem cells. With this in mind, in February 2009, Medipost Co Ltd. began a randomized, open-label, multicenter and Phase 3 clinical trial of 104 patients to compare the efficacy and safety of allogeneic-unrelated umbilical cord blood-derived MPC product (Cartistem) to that of a microfracture treatment in patients with articular cartilage defects or injuries (NCT01041001). MPCs were isolated from umbilical cord blood, cultured with semi solid polymer, and surgically administered into lesion sites in order to stimulate the regeneration of defective cartilage tissue.

6. Osteonecrosis (ON)

The treatment of osteonecrosis of bone is another arena where cell-based therapies can play a pivotal role. These injected bone marrow cells most likely secrete cytokines that promote angiogenesis and subsequent osteogenesis [98].

Hernigou proposed the use of bone marrow transplantation for treatment of osteonecrosis of the humeral head in 1997 in the case of a 13-year-old patient with sickle cell disease [99]. The donor was an HLA-identical sibling who was heterozygous for sickle cell anemia. Marrow transplantation by intravenous infusion was performed after administration of chemotherapy and total lymphoid irradiation. Three months post transplantation, pain and range of motion was significantly improved, and radiographs revealed rapid reconstruction of the left proximal humerus epiphysis, with a tendency toward normalization of the marrow signal apparent via T1-weighted MR imaging. Thereafter, many clinical studies followed to examine the role of bone marrow aspirates in the treatment of ON in various skeletal locations, demonstrating promising results for the early stages of disease (Table 1) [100].

In January 2009, Fuzhou General Hospital in China approved a study to enroll 30 patients in a clinical trial to examine the safety of expanded autologous MPCs infused into the femoral artery of patients with osteonecrosis of the femoral head (NCT00813267). Patients will undergo MPC infusions at the start of the study on day 0 followed by subsequent injections at 6-month intervals. They will be evaluated using digital subtraction angiography, X-ray examination, and CT and MRI scanning.

7. Osteogenesis Imperfecta (OI)

OI is a heterogeneous group of inherited disorders characterized by abnormal production of type I collagen by osteoblasts leading to osteopenia, multiple fractures, severe bony deformities and short stature [101–103]. It has variable clinical phenotypes, ranging from subclinical presentation and normal life expectancy to osteopenia or death [104]. Currently, pharmacological management is the treatment of choice. Many studies have demonstrated the utility of bisphosphonate therapy in OI for increasing bone density, strength, and reducing the number of fractures [105]; however, these effects must be confirmed by double blind, randomized control trials. Many researchers also worry about the potential adverse effects of bisphosphonates on the child skeleton, and therefore, raised concern regarding long-term outcomes [106].

Table 1: Clinical studies of bone marrow aspirates in osteonecrosis.

Authors	Year	Study	Description	Main findings
Hernigou et al.	1997	Case report	13-year-old with SSA and ON of humeral head treated with allogenic bone marrow transplantaion	Improvement in pain and motion range; X-Rays at 3 months showed significant regeneration of the proximal humerus and epiphysis
Hernigou et al.	2002 2005	Prospective noncontrol	189 hips in 116 patients were treated with core decompression and with ABM harvested from anterior iliac crests; the aspirated marrow was reduced in volume by concentration and injected into the femoral head after core decompression with a small trocar; followup of 5–10 years	In 145 of the stage I/II patients, hip replacement was required in 9; total hip replacement was necessary in 25 of 44 hips operated on at stage III/IV patients with greater number of OPGr cells transplanted had better outcomes
Gangi et al.	2002	Prospective control	Treatment of 18 hips with stage 1 or 2 (ARCO) femoral head ON with either core decompression alone or in combination with ABM injection	Significant reduction in pain and joint symptoms at 2 years; 5 of 8 hips in control group deteriorated to stage 3 compared to 1 hip in treatment arm
Yan et al.	2006	Prospective noncontrol	Treatment of 44 hips with stage 3 or 4 (ARCO) femoral head ON with ABM injection	Mean Harris hip score improved from 58 (46–89) to 86 (70–94) at 2 years; no complications
Kawate et al.	2006	Case reports	Treatment of 3 patients with steroid-induced ON of femoral head (ARCO Stages 4A–4C) by transplantation of autologous MSCs cultured with beta-tricaclium phosphate ceramics and free vascularized fibulas	Early bone regeneration observed but radiographic progression was seen in 2 of 3 patients at mean followup of 34 months
Lee et al.	2009	Case reports	Three patients with bilateral, large lesions (0.32 of femoral width) of ON of the femoral condyles treated by decompression, debridement, and ABM grafting using the Cellect DBM System to increase the number of OPGr cells from the aspirate	Cellect provided graft matrix enriched with a 3-fold to 4-fold increase in OPGr cells; no complications at 2 years with all 3 patients achieving near-normal function and activity levels

ABM: autologous bone marrow; ARCOL: association research circulation osseous; OPGr: osteoprogenitor; SSA: sickle cell anemia.

Table 2: Examples of the therapeutic applications of MPCs in humans.

Indications	Source	Mode of administration	Outcome
Fracture nonunion	Autologous BM	100% hydroxyapatite macroporous ceramic scaffolds with MPCs	X-ray & CT evidence of bone formation: recovered limb function [60]
	Autologous BM	Subcutaneous	Correlation between volume of mineralized callus and concentration of progenitor cells in the aspirate [40]
Cartilage defect	Autologous BM	Direct site transplantation	Improved clinical symptom and coverage of defect [75]
	Autologous BM	Cells embedded in collagen gel transplanted at site of cartilage defect	Improvement in arthroscopic and histologic grading [76]
Osteogensis imperfecta	Fetal MSC	Intrauterine transplantation	Osteoblastic differentiation and reduced fracture [77]
	Gene-marked Allogenic MPCs	IV infusion $\times$ 2	5 out of 6 patients demonstrated bone engraftment and increase in bone velocity [78]
Critical size defect	Autologous BM	Scaffold loaded	Faster full recovery of limb function than bone graft [59]
Craniofacial defect	Autologous Adipose-derived MPCs	Local administration of cells with fibrin glue	CT scans showed new bone formation and near complete calvarial continuity 3 months postoperatively [63]

MPC: mesenchymal progenitor cell, BM: bon marrow, IV: intravenous.

An alternative treatment is the transplantation of BMSCs, which in principle should alleviate or resolve a genetic disorder of bone. In fact, preclinical experiments carried out in animal models revealed that transplanted marrow stromal cells can migrate and incorporate into bone of recipient animals [107]. Horwitz et al. have been utilizing allogeneic bone marrow transplantation for the treatment of severe OI in children since 1999 [108]. In their latest case report, the authors described the use of gene-marked, donor marrow-derived mesenchymal cells to treat 6 children who underwent standard bone marrow transplantation for severe OI. Each child received two infusions of the allogeneic cells.

One patient developed an urticarial rash immediately following the second infusion, but otherwise, no clinically significant toxicity was reported [78].

Taking the concept of bone marrow transplantation for OI one step further, Le Blanc and colleagues performed an intrauterine transplantation of a female fetus diagnosed with severe OI at 32 weeks of gestation with MPCs isolated from a HLA-mismatched male fetus [77]. At 9 months of age, bone histology showed regularly arranged and configured bone trabeculae. Patient lymphocyte proliferation against donor MPC was not observed in coculture experiments performed in vitro after MPC injection. During the first 2 years of life, three fractures were noted, and at 2 years of corrected age, psychomotor development was normal, and growth followed the same channel. Given these findings, the authors concluded that allogeneic fetal MPCs can engraft and differentiate into bone in a human fetus even when the recipient is immunocompetent and HLA-incompatible.

Encouraged by previous clinical experiences, Horwitz et al. have begun a Phase I, nonrandomized control trial of 12 patients with Type II or III OI in order to evaluate the safety and effectiveness of repeated BMSC infusions (NCT01061099). Group A will consist of patients who have previously undergone bone marrow transplant whereas those in Cohort B lack a history of bone marrow transplantation. All participants will receive BMSC infusions approximately every 4 months to complete a total of 6 infusions over a 20-month period and will be followed for 4 months after their last infusion.

Drexel and Wayne State University underwent a Phase I pilot study from July 1999 to January 2008 of 8 patients with osteodysplasia who had undergone a previous bone marrow transplantation (NCT00186914). They were infused twice with ex vivo expanded, allergenic, gene marked donor BMSCs. The first dose was given at least 6 months post transplant and the second at 14 to 21 days after. Since the stromal cells were obtained from the original stem cell donor, no conditioning was required. Study results are still pending.

8. Tendon Repair

Currently, experimental animal models are in progress to establish convincing evidence for enhanced tendon repair with progenitor cell therapy, but advancements in the field have not yet reached the level of clinical trials. Investigations to date have demonstrated the utility of Type I collagen combined with autologous, expanded BMSCs for improving the biomechanical properties of injured rabbit tendons although differences in microstructure have yet to be seen [109].

Using an equine model, Crovace et al. injected and compared cultured bone marrow mesenchymal stem cells (cBMSCs) and bone marrow mononucleated cells (BMMNCs) versus placebo for treatment of collagenase-induced tendonitis in the horse. At 21 weeks, histological analysis and immunohistochemical stains with H&E and Herovici for collagen type I and III revealed mature type I collagen with normal architecture in tendons treated with cBMSC and BMMNC, while random collagen type III organization was observed in the placebo group. These results suggest that cBMSC and BMMNCs have the potential to promote tendon regeneration in an equine collagenase-induced tendonitis model [110]. Authors have also used BMSCs seeded onto various scaffolds and found that introducing autogenous mesenchymal progenitor cells onto composites significantly improved tendon repair compared to the use of a composites alone in the rabbit model [111].

One potential pitfall of BMSC therapy for tendon repair is the potential for ectopic bone production at the site of injury. Harris et al. observed this phenomenon in 28% of BMSC-treated rabbit tendons and concluded that better control of the differentiation pathway with additional in vitro testing is necessary prior to embarking on clinical trials with MPC therapy in tendon repair [112].

9. The Future of Stem Cells

Much attention has been engendered for combining the principles of stem cell therapy with those of gene therapy to engineer cells that can complement cellular function in genetic disorders, as discussed previously. Gene therapy can be executed ex vivo with the gene of interest introduced to the progenitor cells followed by its readministration back into the patient, thereby replacing the missing factor in the host. To date, these approaches have only been studied in animals but with great success.

Prior to embarking on clinical trials, the vectors used to deliver genes of interest must be optimized. Recombinant forms of bone morphogenic proteins (rhBMPs), for example, have been used in animal models to promote and hasten osteogenesis [113]. Based partly on this in vivo work, the US Food and Drug Administration approved the use of the rhBMP-2 and rhBMP-7 in spinal fusions and tibial non-unions, respectively. Although promising and seemingly effective, rhBMPs have multiple disadvantages, namely, the requirement of supraphysiologic concentrations and low biological activity due to high rates of clearance from the defect sites [114]. In addition, high costs and difficulty of production are potential factors limiting the use of rhBMPs in clinical practice.

An alternative mode of applying BMPs is through their expression using adenoviral vectors. This form of gene therapy is able to deliver recombinant BMP DNA to cells at the defect sites [115]. Treated cells can then synthesize and secrete their own endogenous BMPs and supply the extracellular environment with a continuous concentration of osteoinductive signaling factors without the need of reapplication [114]. These adenoviral vectors provide a short but high level expression of the gene of interest, which is sufficient to promote osteoblastic differentiation and subsequent bone formation [116]. Baltzer et al. used adenoviruses expressing BMP-2 (AdBMP-2) to induce healing of critical-sized bone defects in rat femurs [117], and other investigators have followed suit [118]. Our laboratory focuses in particular on the role of adBMP-9 in osteogenic differentiation of mesenchymal progenitors [119].

Table 3: Ongoing progenitor cell-therapy in orthopaedic patients.

Indications	Sponsors	Phase	Age range	Study type	Intervention	Source	Route of administration	Clinical Trial ID no.	Status
Bone defects	Emory University	II + III	11 years and older	Interventional	Trinity MPC & DBM	Commercial	Direct filling of bone defects with progenitor cells	NCT00851162	Not yet recruiting
Fracture nonunion	Aastrom Biosciences	I + II	18 years and older	Interventional	Fracture surgery + Cultured BM tissue	Autologous BM	Direct adminimistration to site of fracture	NCT00424567	Completed
Dital tibial fracture	Hadassah Medical Organization	I + II	18–65 years	Interventional	Autologous "MSC" implantation	Autologous BM	Cells loaded onto a carrier and implanted locally at the fracture site	NCT00250302	Recruiting
Knee Cartilage defects osteoarthritis	Royan Institute	I	45–60 years	Interventional	Autologous "MSC" implantation	Autologous BM	Cells loaded on collagen I scaffold are implanted locally to the cartilage defect site	NCT00850187	Recruiting
Osteochondral defects	Cairo University	I + II	15–55 years	Interventional	Autologous "MSC" implantation	Autologous BM	Cell pellets will be implanted into osteochondral defect via open surgery or arthroscopically	NCT00891501	Recruiting
Cartilage Defects	Ullevaal University Hospital	I	18–50	Interventional	Autologous "MSC" implantation versus chondrocyte implantation	Commercial	Cells loaded on commercially available scaffolds and implanted locally at the cartilage defect site	NCT00885729	Recruiting
Meniscectomy	Osiris Therapeutics	I + II	18–60	Interventional	Chondrogen versus placebo	Chondrogen (commercial)	Intra-articular injection	NCT00702741	Ongoing but not recruiting
Chondral defect	Medipost Co Ltd.	III	18 years and older	Interventional	Cell therapy versus microfracture	UCB	Culture expanded cells mix semisolid polymer will be implanted locally at the site of chondral defect	NCT01041001	Recruiting
Osteonecrosis of the femoral head	Fuzhou General Hospital	I + II	12–60 years old	Interventional	Autologous MSC	N/A	Direct cellular infusion through tubes inserted into MFCA, LFCA, and OA	NCT00813267	Not yet recruiting
OI Type I + II	Children's Hospital of Philadelphia	I	≤19 years	Interventional	Infusion Haploidentical MSCs in patients with history of VM transplant versus No history of transplant	Donor BM	Repeat infusion of MSCs in sub	NCT01061099	Recruiting
Osteodysplasia	St. Jude Children's Research Hospital	I	N/A	Interventional	Infusion of ex vivo expanded gene marked BMSC following allogenic BM transplantation	Donor BM	IV Infusion	NCT00186914	Completed

MPC: mesenchymal progenitor cells, DBM: demeneralized bone matrix, BM: bone marrow, MSC: mesenchymal stem cell, MFCA: medial femoral circumflex artery, LFCA: lateral femoral circumflex artery, OA: obturator artery, IV: intravenous, UCB: umlical cord blood, N/A: not available.

However, in vivo transfer of genes utilizing viral vectors provokes the possibility of immune reaction, which will prevent treatment efficacy. And thus, because of the strong potential for adverse reaction, utilizing adenovirus in human trials remains distant. Current strategies underway involve ex vivo genetic modification of autologous BMSCs via adenoviral vectors followed by their reimplantation in vivo. This approach avoids the transfer of viral particles or DNA directly into the patient and would most likely forgo the immune response associated with direct viral administration [120]. To date, however, ex vivo adenoviral gene therapy has been utilized only in animal models.

Concern surrounding the use of viral vectors as vehicles for gene delivery has prompted the utilization of other methods to genetically modify MPCs. Nucleofection is a modified electroporation technique that has been used by many different groups for genetic modification of BMSCs [121–123]. FuGENE 6, a cationic polymer-based commercially available transfection reagent, has also been successfully used to genetically engineer MPCs [124–126]. In addition, liposome-mediated transfection of MPCs has also been reported [127]. While these novel nonviral methods of transfecting cells are promising, significant research is necessary to delineate their effectiveness.

Genetic engineering of BMSCs is ideal, as it eliminates the requirement of large amounts of cells for implantation and culture expansion. Although the morbidity associated with bone marrow aspiration for the accrual of MPCs is less than previous methods of defect repair that utilized autologous bone grafting, researchers remain interested in developing more minimally invasive means of harvesting progenitor cells. One such modality which has gained popularity is utilizing adipose tissue as a source of progenitor cells. Given the ease of harvest and the availability of adipose tissue, many researchers are currently interested in understanding the mechanisms underlying the differentiation capacity of adipose-derived progenitor cells (APCs) [128]. While adipose-derived MPCs show significant promise as an important source of MPCs, further research into their characteristics as progenitor cells is necessary to harness their utility in the clinical setting.

10. Conclusion

The therapeutic capacity and safety of BMSCs have been documented in numerous animal experiments in vivo. Currently, 107 clinical trials utilizing exogenous BMSCs to treat a wide range of conditions are registered with ClinicalTrials.gov (Tables 2 and 3). Although many are also underway to examine the role BMSCs in orthopaedic associated tissue regeneration, limited evidence is currently available to support routine use. Results from large-scale multicenter clinical trials must be completed and analyzed prior to reaching the final destination of FDA approval for utilizing cell-based therapy to manage orthopaedic patients in the clinic. Given the abundance of ongoing investigations, however, we can expect a profuse amount of new clinical data in the near future. Science has progressed infinitely since Friedenstein's pioneering studies in the 1960s, and with the continuation of human trials, we move one step closer towards applying BMSC therapy as a novel paradigm.

Acknowledgments

This work was supported in part by research grants from the Brinson Foundation (T. C. He), the National Institutes of Health (nos. CA106569, AR50142, and AR054381 to T. C. He, R. C. Haydon, and H. H. Luu), The 863 Program of the Ministry of Science and Technology of China (no. 2007AA2z400 to L. Chen, T. C. He), the Natural Science Foundation of China (no. 30901530 to X. Luo, no. 30800658 to J. Luo), and the Natural Science Foundation Project of Chongqing Science and Technology Commission no. 2008BB5396 (L. Chen) and no. 2009BB5060 (J. Luo).

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SAGE-Hindawi Access to Research
Stem Cells International
Volume 2010, Article ID 579142, 12 pages
doi:10.4061/2010/579142

Review Article

Phases I–III Clinical Trials Using Adult Stem Cells

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Received 22 June 2010; Revised 6 August 2010; Accepted 30 August 2010

Academic Editor: Louis Casteilla

First randomized clinical trials have demonstrated that stem cell therapy can improve cardiac recovery after the acute phase of myocardial ischemia and in patients with chronic ischemic heart disease. Nevertheless, some trials have shown that conflicting results and uncertainties remain in the case of mechanisms of action and possible ways to improve clinical impact of stem cells in cardiac repair. In this paper we will examine the evidence available, analyze the main phase I and II randomized clinical trials and their limitations, discuss the key points in the design of future trials, and depict new directions of research in this fascinating field.

1. Concept and Types of Randomized Clinical Trials

A randomized clinical trial (RCT, also clinical study) is a research study in human volunteers to answer specific health questions. In other words, it is a rigorously controlled test of a new drug or a new invasive medical device on human subjects, in order to evaluate their effectiveness and safety by monitoring their effects on large groups of people.

In the present state of clinical research, carefully conducted RCT are the fastest and safest ways to find treatments that work in people and ways to improve health. Interventional trials determine whether experimental treatments or new ways of using known therapies are safe and effective under controlled environments. Observational trials address health issues in large groups of people of populations in natural settings.

All RCT must be conducted according to strict scientific and ethical principles. Every clinical trial must have a protocol, or action plan that describes what will be done in the study, how it will be conducted, and why each part of the study is necessary; including details such as the criteria for patient participation, the schedule of tests, procedures, and medications, and the length of the study.

RCT are conducted in a series of steps, called phases. Each phase is designed to answer a separate research question.

(1) Phase I: researchers test a new drug or treatment in a small group of people for the first time to evaluate its safety, determine a safe dosage range, and identify side effects.

(2) Phase II: the drug or treatment is given to a larger group of people to see if it is effective and to further evaluate its safety.

(3) Phase III: the drug or treatment is given to large groups of people to confirm its effectiveness, monitor side effects, compare it to commonly used treatments, and collect information that will allow the drug or treatment to be used safely.

(4) Phase IV: studies are done after the drug or treatment has been marketed to gather information on the drug's effect in various populations and any side effects associated with long-term use.

2. Clinical Research in Stem Cell Therapy: Same Methodology with a New Objective

Recent advances in reperfusion strategies have dramatically reduced early mortality after acute myocardial infarction

(AMI), but as a result there is a higher incidence of heart failure among survivors. Optimal medical therapy and device implantation can improve the prognosis and the quality of life of these patients. Nevertheless, mortality and rehospitalization rates are still high and entail an overwhelming cost.

The field of cardiac cell therapy has emerged as a new alternative in this situation, and has made rapid progress. Its final goal is to repair the damaged myocardium and to restore cardiac function. Nevertheless, this is a real therapeutic challenge, given the facts that the loss of cardiomyocytes after an AMI is in the order of 1 billion cells, that supporting cells have to be supplied together with cardiomyocytes and that environmental signals which guide stem cells to the cardiac lineage or to the secretion of paracrine factors might be absent in such a damaged tissue [1].

Studies evaluating this new approach during the last 15 years have overall succeeded to a greater or lesser extent, and evidence available so far is encouraging. Phase I and II RCT indicate that cell therapy is a safe treatment which can improve cardiac function after AMI and in the chronic phase of coronary artery disease (CAD). Trial results are not uniform, however, probably due (1) to a lack of standardization and optimization of cell isolation and delivery protocols, (2) to a lack of a universally accepted nomenclature and imprecise use of terminology, and (3) to the large number of stem cell types under investigation in different clinical settings. These persisting mechanistic uncertainties about stem cell therapy should not preclude continuing clinical trials, which often provide the unique opportunity of identifying issues missed by our suboptimal preclinical models.

Moreover, these inconsistencies can be avoided or reduced if classical scientific methodology is followed. Although considered a relatively new field of research, stem cell experimentation must invariably walk on the path of the scientific method. Since Erasistratus of Chios in the third century before Christ and then after Aristotle's time, scientific method has been used as a way to ask and answer scientific questions by making observations and doing experiments. It includes a series of steps, that is, (1) asking a question, (2) doing background research, (3) constructing a hypothesis, (4) testing the hypothesis by doing an experiment, (5) analyzing the data and drawing a conclusion, and (6) communicating the results.

In the case of stem cell therapy, RCT started questioning if there was a possibility of repairing the heart after different types of tissular damage. Background evidence has already demonstrated that this possibility exists through stem cell administration in several preclinical models of cardiomyopathy. Thus, the key points in the design of present and future RCT in humans are (1) to formulate an adequate hypothesis, (2) to select the ideal population, cell type, and delivery method, and (3) to develop a correct and precise protocol. These decisions must be made in the light of previous evidence and with a translational mentality, in which experimental/preclinical data should help to design RCT, and, inversely, results of human studies should transfer new questions and hypothesis to the laboratory/bench side.

3. Clinical Scenarios in Stem Cell Therapy: Evidence Available

Stem cell therapy has accumulated growing evidence in different physiopathological conditions in small and large animal models, but human research has been almost limited to CAD. In this paper we will focus on *randomized* placebo-controlled clinical trials in humans (Tables 1, 2, 3, and 4). Nonetheless, we will comment only on the largest and most relevant ones, as a way to analyze procedure-related variables that could have determined treatment outcomes and to address their limitations.

Natural history of CAD can be divided into acute (AMI) and chronic phases (chronic ischemic heart disease). In the latter stem cell therapy has been investigated in the subset of (1) ischemic heart failure (ventricular dysfunction) and (2) chronic myocardial ischemia (refractory angina).

In patients where restoration of contractile function is the clinical goal—such as those with end-stage ischemic heart failure or those early postinfarction—delivering cells with contractile potential may be of high priority. Under these conditions, naturally myogenic cells (i.e., skeletal myoblasts, cardiomyocytes, or any progenitor cell driven down a muscle lineage) appear to be a better first choice. However, on the one hand, formation of new myocardial mass has only been strictly established for embryonic stem cells (ESC), and is a process that has been achieved in very few trials and in small percentages with adult stem cells. And on the other hand, most of the studies after AMI have used bone marrow mononuclear cells (BMMCs) as an easily accessible source of adult stem and progenitor cells.

In conditions where chronic ischemia prevails, the angiogenic potential of the cells seems a more reasonable approach. In this case, BMMC, endothelial progenitor cells (EPCs), vascular progenitor cells or blood-derived multipotent adult progenitor cells and mesenchymal stem cells (MSCs) may be better choices than myogenic precursors.

3.1. Stem Cell Therapy after Acute Myocardial Infarction. Several trials have evaluated stem cell therapy after AMI, some with positive results and some with neutral ones. All of them used the intracoronary route, once the patency of the infarct-related artery was restored, and most of them with the mononucleated fraction of the bone marrow (Table 1). Four main RCT have been published with positive findings so far. In the BOOST trial [3], BMMC were proved to improve left ventricular (LV) contractility in the infarct border zone and global LV ejection fraction (LVEF) by 6%. However, only patients with larger infarcts showed maintained benefits in terms of LVEF at long followup (18 months). In the REPAIR-AMI trial [4], infusion of BMMC promoted an increase in LVEF of 2.8% at 12 months. The FINCELL trial [8] reported an improvement of 5% in LVEF after BMMC delivery. Finally, in the REGENT trial [12], patients treated with BMMC and with $CXCR4^+/CD34^+$ BMMC showed an increase of 3% in LVEF which was not observed in the control group, but these differences were not significant between treated and control patients at 6-month followup. This trial was

Table 1: Randomized clinical trials with stem cells in patients with acute myocardial infarction (intracoronary delivery).

Trial (year)	n	Cell type	Cell count	Days after AMI	Primary endpoint (followup)	Comments
Chen [2] (2004)	69	MSC	9×10^9	18	Improved LVEF at 6 m	LVEF by echocardiography
BOOST [3] (2004)	60	BMMC	2×10^9	6 ± 1	Improved LVEF at 6 m	Effect diminished after 18/61 m
REPAIR-AMI [4] (2006)	187	BMMC	2×10^8	3–6	Improved LVEF at 4 m	LVEF by ventriculography
Janssens [5] (2006)	66	BMMC	2×10^8	1	No change LVEF at 4 m	Improved regional contractility and reduction in infarct size
ASTAMI [6] (2006)	97	BMMC	7×10^7	6 ± 1	No change LVEF at 6 m	LVEF ↑8% by SPECT, ↑1% by MRI
TCT-STAMI [7] (2006)	20	BMMC	4×10^7	1	Improved LVEF at 6 m	LVEF by echocardiography
FINCELL [8] (2008)	77	BMMC	4×10^8	3	Improved LVEF at 6 m	LVEF by ventriculography
Meluzin [9] (2006)	66	BMMC	1×10^7 (low d) 1×10^8 (high d)	7	Improved LVEF at 3 m in high dose group	LVEF by SPECT
Penicka [10] (2007)	27	BMMC	3×10^9	9	No change LVEF at 4 m	LVEF by echocardiography
HEBE [11] (2008)	189	BMMC versus PBC	—	3–8	No changes in global or regional LV function	Final results pending
REGENT [12] (2009)	117	BMMC (unselected, $CD34^+/CXCR4^+$)	2×10^8 (unsel), 2×10^6 ($CD34^+$)	3–12	Improved LVEF with both cell types	LVEF by MRI (in 117/200 patients)

MSC: mesenchymal stem cells (bone marrow origin); BMMC: bone marrow mononuclear cells; PBC: peripheral blood cells; LVEF: left ventricular ejection fraction; LV: left ventricle; SPECT: single-photon emission computed tomography; MRI: magnetic resonance imaging.

limited by imbalances in baseline LVEF and by incomplete followup.

On the other hand, three RCTs resulted in neutral findings. Janssens et al. [5] reported no changes in LVEF after BMMC infusion, but a reduction in the infarct volume and an improvement in regional contractility in the greatest transmural infarct cases were observed in treated patients. In the ASTAMI trial [6] no significant effects on LVEF, LV volumes, or infarct size were observed after BMMC administration. The smaller number of cells and differences in the cell isolation protocol were invocated to explain these findings. Finally, in the HEBE trial [11], presented at the AHA Scientific Sessions in 2008, no changes in global or regional LV systolic function were reported after BMMC and mononucleated cells isolated from peripheral blood.

So far, no safety concerns after BMMC intracoronary infusion have emerged. The risk of a higher rate of instent restenosis was not confirmed in the FINCELL trial [8] and in two recent meta-analyses [13, 14]. Moreover, none of the trials reported an increased incidence of malignant arrhythmias with BMMC [1].

Two trials have used MSC after AMI. The study by Chen et al. [2] demonstrated an improvement in LVEF and perfusion with intracoronary infusion of these cells, but these results have not been duplicated. Hare et al. [15] intravenously administered allogeneic MSC after an AMI with no higher rate of MACE and some benefits in terms of LVEF.

New types of cells are also being explored, like adipose-derived stem cells (ADSCs) (Figure 1). No evidence is available to date, but the first-in-man RCT with intracoronary administration of freshly isolated ADSC after AMI (the APOLLO trial) has been recently completed.

Another approach for stem cell therapy after AMI is cell mobilization from the bone marrow with the administration of granulocyte colony-stimulating factor (G-CSF). Several RCTs have been published, but results have been somehow less encouraging (Table 2). Only three trials have reported positive results. In the FIRSTLINE-AMI trial [16], the RIGENERA study [17], and in the study by Takano et al. [18], significant improvements in LVEF were observed. The rest of the trials showed negative findings.

Finally, the MAGIC trials used a combination of G-CSF and intracoronary injection of peripheral blood progenitor cells. In the first trial no differences in LVEF were noted, and an increase in instent restenosis rate was observed (G-CSF administration before bare-metal stent implantation) [19]. Then the investigators changed the design and used

Table 2: Randomized clinical trials with granulocyte colony-stimulating factor in patients with acute myocardial infarction (subcutaneous).

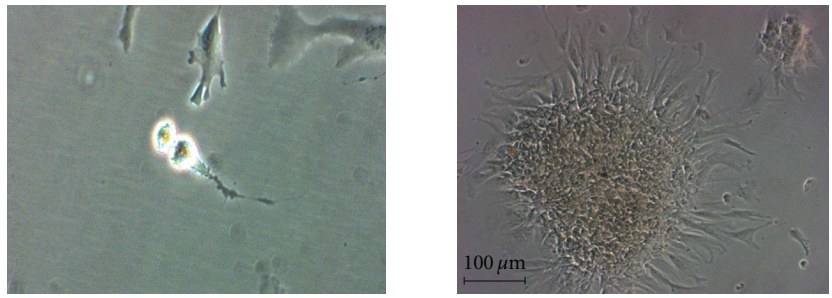
Trial (year)	*n*	Dose	Timing after AMI (PCI)	Followup	Comments
Valgimigli [28] (2005)	20	5 μg/kg × 4 d	1 d	No change LVEF at 6 m	LVEF by SPECT
FIRSTLINE-AMI [16] (2005)	50	10 μg/kg × 6 d	90 min	Improved LVEF at 4 m	LVEF by echocardiography
REVIVAL-2 [29] (2006)	114	10 μg/kg × 5 d	5 d	No change LVEF at 5 m	LVEF by MRI
STEMMI [30] (2006)	78	10 μg/kg × 6 d	28 h	No change LVEF at 6 m	LVEF by echocardiography and MRI
G-CSF-STEMI [31] (2006)	44	10 μg/kg × 5 d	35 h	No change LVEF at 3 m	LVEF by MRI
Ellis [32] (2006)	18	5 μg/kg × 5 d (low d), 10 μg/kg × 5 d (high d)	<30 h	Improved LVEF at 30 d	LVEF by echocardiography
RIGENERA [17] (2007)	41	10 μg/kg × 5 d	5 d	Improved LVEF at 6 m	LVEF by echocardiography
Takano [18] (2007)	40	2,5 μg/kg × 5 d	1 d	Improved LVEF at 6 m	LVEF by SPECT
MAGIC [19] (2004)*	27	10 μg/kg × 4 d; PBC: 1 × 10^9	1 d	No change LVEF at 6 m	LVEF by SPECT
MAGIC 3-DES [20] (2006)*	50	10 μg/kg × 3 d; PBC: 2 × 10^9	1 d	Improved LVEF at 6 m	LVEF by MRI

* MAGIC trials used a combination of indirect mobilization (G-CSF) and direct intracoronary injection of peripheral blood cells (PBC); LVEF: left ventricular ejection fraction; SPECT: single-photon emission computed tomography; MRI: magnetic resonance imaging.

Table 3: Randomized clinical trials in patients with chronic ischemic heart failure.

Trial	*N*	Cell type	Delivery	Timing	Primary endpoint	Comments
MAGIC [21]	97	SM	transepi	>4 weeks	No change LVEF	Reduction in LVEDV/LVESV
Dib [22]	23	SM	transendo	>10 years	Improved LVEF and viability	—
SEISMIC	47	SM	transendo	chronic	No change LVEF	—
TOPCARE-CHD [23]	58	BMMC versus CPC	ic	81 ± 72 months	Improved LVEF w/BMMC	—

SM: skeletal myoblasts; BMMC: bone marrow mononuclear cells; CPC: circulating progenitor cells; transepi: tranepicardial; transendo: transendocardial; ic: intracoronary; LVEF: left ventricular ejection fraction; LVEDV: left ventricular end-diastolic volume; LVESV: left ventricular end-systolic volume.



(a) (b)

Figure 1: Autologous adipose-derived mesenchymal stem cells during mitosis (a) and growing in colonies in the 6th day of culture (magnification ×10, (b)). These cells were expanded from the adipose tissue stroma-vascular fraction under good manufacturing practice (GMP) conditions in our Cell Production Unit (Hospital Gregorio Marañón, Madrid).

Table 4: Randomized clinical trials in patients with chronic myocardial ischemia.

Trial	*N*	Cell type	Delivery	Timing	Primary endpoint	Comments
Losordo [24]	24	$CD34^+$	transendo	chronic	Improved angina parameters	No clear perfusion benefit
PROTECT-CAD [25]	28	BMMC	transendo	chronic	Improved angina parameters	Improved LVEF and perfusion
Van Ramshorst [26]	50	BMMC	transendo	chronic	Improved angina parameters	Improved LVEF and perfusion

BMMC: bone marrow mononuclear cells; LVEF: left ventricular ejection fraction.

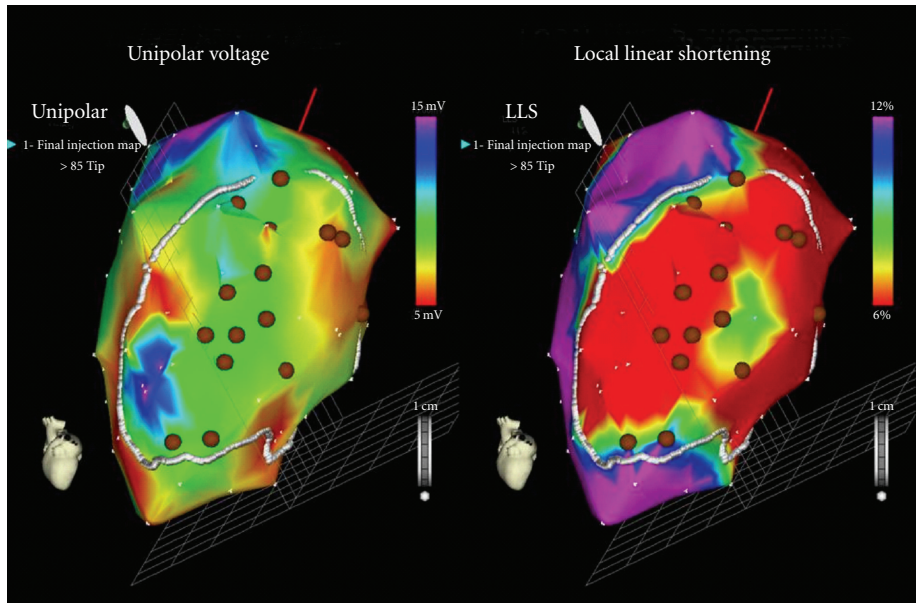


Figure 2: Electromechanical mapping of the left ventricle with the NOGA XP System (BDS, Cordis Corporation, Johnson and Johnson) from a patient enrolled in the PRECISE trial in our centre. Myocardial areas with low contractility and impaired endocardial voltage are identified as viable and targeted for cell injection (brown dots).

drug-eluting stents. In the MAGIC 3-DES trial, positive results in terms of LVEF were found after mobilization and intracoronary injection of isolated cells [20].

3.2. Stem Cell Therapy for Chronic Ischemic Heart Disease

3.2.1. Ischemic Heart Failure. Skeletal myoblasts and BMMC have been used in heart failure (HF) patients (Table 3). The MAGIC trial [21], with transepicardial injection of SM during coronary artery bypass grafting (CABG) surgery, reported no changes in global or regional contractility. However, a reduction in LV end-diastolic and end-systolic volumes was observed in the high-dose group. Moreover, a trend towards a higher incidence of ventricular arrhythmias was noted. Dib et al. [22] reported an improvement in LVEF and viability after SM transendocardial injection, in contradiction with the SEISMIC trial (presented by Serruys at the 2008 ACC meeting) which showed no benefit of the same procedure at 6 months.

In the TOPCARE-CHD trial [23], BMMC intracoronary delivery into the coronary artery supplying the most dyskinetic LV area showed an increase in LVEF of 2.9%, whereas progenitor circulating cells infusion and controls did not show any positive change. No major adverse cardiac events (MACE) were reported in this trial.

3.2.2. Chronic Myocardial Ischemia. Patients with advanced CAD and no further options of revascularization ("no-option" patients) have also been studied in stem cell therapy trials (Table 4). Three RCT have been completed using the transendocardial route after electromechanical mapping of the LV, with BMMC or blood-derived progenitor cells. Losordo et al. [24] studied peripheral $CD34^+$ cells isolated after G-CSF injections. Angina frequency and exercise time were improved, but no clear effects on myocardial perfusion were observed. In the PROTECT-CAD trial [25], BMMC injections improved NYHA functional class, exercise time, LVEF, wall thickening, and stress-induced perfusion defects. Finally, Van Ramshorst et al. [26] reported better LVEF, myocardial perfusion, angina functional class, exercise capacity and quality of life after BMMC administration.

ADSC have also been studied in this type of patients. The PRECISE trial is a prospective, double blind, RCT that has randomised 27 patients with end-stage CAD not amenable for revascularization and with moderate-severe LV dysfunction to receive freshly isolated ADSC or placebo

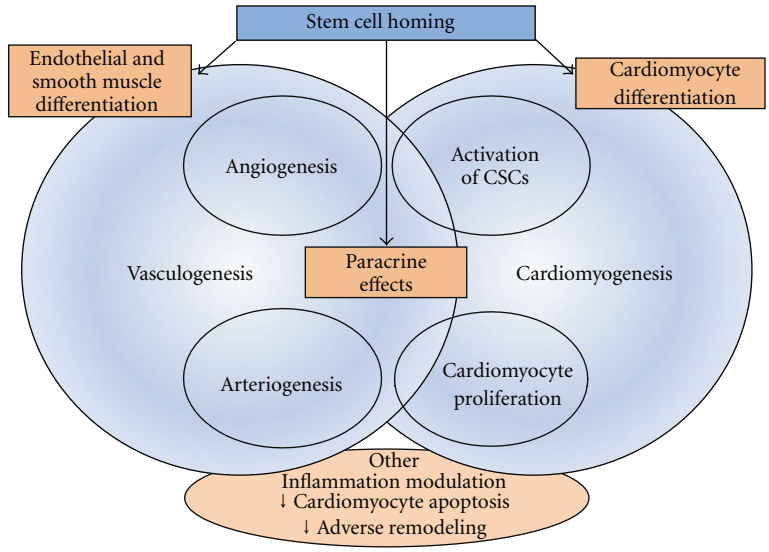


Figure 3: Proposed mechanisms of stem cell function after homing into the damaged heart. Note that differentiation processes and paracrine effects activate a cascade of events that interact actively to create new blood vessels and cardiomyocytes, with the final objective of functional cardiac repair. CSCs: cardiac stem cells.

in a 3 : 1 ratio. The cells were delivered via transendocardial injections after LV electromechanical mapping with the NOGA XP delivery system (BDS, Cordis Corporation, Johnson and Johnson) (Figure 2), and results are still waiting for publication.

4. Key Issues for the Design of Future Stem Cell Therapy Trials

4.1. Ethical Considerations. As with any novel medical intervention, with stem cell therapy there is an ethical dichotomy between the need for new therapeutic approaches and rigorous scientific evidence regarding the safety and efficacy of the procedures.

Due to its highly innovative nature, stem cell therapy should focus on reducing risks and providing rigorous evidence of efficacy and safety. Fundamental ethical requirements in this case include an acceptable balance of benefits and risks, informed and voluntary consent and equitable selection of subjects [27]. With transplantation of pluripotent cells (i.e., ESC and induced progenitor cells), additional safeguards are warranted because of the innovative nature of these treatments, differences between animal and human physiology, limited experience with these cells in humans, and the high hopes of desperate patients for whom no alternative effective treatment currently exists.

Some specific ethical recommendations have been given for RCT with stem cells [27]. They include the following.

(1) Phase I-II trials should enroll participants in late stages of serious illness, such as persons with advanced or refractory disease, but not so ill that they are at greatly increased risk for adverse events.

(2) Use a proper control group, in order to evaluate the positive effects of treatment and to ascribe culpability to any MACE seen with cell therapy. Then stem cells can be offered to the control group at the conclusion of the trial if the results show short-term benefit ("cross-over").

(3) Use clinically meaningful endpoints (see below, Section 4.5).

(4) Coordinate scientific and ethical review, judging the potential clinical benefit of the treatment and assessing the scientific justification for the trial, including proof-of-principle and preclinical data on safety and dosage.

(5) Verify that participants clearly understand the features of the trial. Since a comprehensive informed consent form may not prevent misconceptions about the trial, additional information should be given to those patients with significant misunderstandings. Participants should appreciate that researchers may not know whether or not the stem cell treatment will be beneficial, that animal studies might not predict effects of the cells in humans, and that unexpected adverse events may occur.

(6) Ensure publication of results, even negative ones. For the interest of patients, researchers, and sponsors, negative findings cannot be withheld from publication.

4.2. Patient Selection and Delivery Methods. Patients with larger AMI or with severely depressed baseline LVEF and stroke volumes, or those with transmural extent of the infarct seem to benefit the most after BMMC treatment [1]. Conversely, patients with microvascular obstruction may not respond to intracoronary infusion of cells. Therefore, patient selection before conducting a RCT must take into

account the pathophysiologic basis of the disease and baseline characteristics of the patients. For instance, it is well known that age, cardiovascular risk factors, and previous heart failure have a negative impact on the potentiality and functional capacity of the cells.

On the other hand, exploration of new delivery methods is mandatory, due to the low rate of cell retention, engraftment, and survival in the myocardium with the present routes of administration. New devices include transcoronary arterial injection into the perivascular space, improvements in transendocardial injection needle design, and the fusion of different imaging techniques for a more precise delivery (i.e., X-ray/MRI suites used in conjunction with electroanatomic maps of the LV).

4.3. Host Tissue and Cell-Related Issues. The two main determiners of cardiovascular repair are stem cells and injured myocardial tissue in which these cells are delivered. Both play the central role that will establish the efficacy of the treatment, and knowledge of the molecular/cellular changes and interactions between them is crucial when designing new RCT.

After AMI, if blood flow is not restored quickly, cell death and myocardial necrosis are definitive. This activates a complement cascade with free radical and cytokine generation that recruits leukocytes and initiates the inflammatory response. Inflammation, while potentially detrimental to surviving cardiomyocytes, is necessary to clear away the debris (clearance of necrotic cells) and orchestrate downstream healing events. Chronic inflammatory cells such as macrophages and mast cells secrete cytokines and growth factors, which in turn activate fibroblasts to proliferate and synthesize collagen, a major component of the scar that replaces cardiomyocyte loss. Neovascularization is also stimulated by the release of growth factors from the inflammatory cells. Scar remodeling may continue for months to years, depending on the extent of the initial ischemic event [33].

LV remodeling, defined as post-AMI changes in wall structure, chamber geometry, and pump function, is mainly caused by changes in extracellular matrix (ECM). Cardiac ECM not only supports and aligns cardiomyocytes, thereby preserving a fundamental mechanical relationship by which sarcomeric shortening is translated to muscle force contraction, but also has signaling functions. Indeed, ECM is a storage depot for growth factors, hormones, and cytokines, and uses integrins to communicate with cells [33]. All these functions are lost after myocardial ischemia due to the release from inflammatory and endogenous cells of matrix metalloproteinases (MMP) and cytokines. MMP degrade ECM, disengage integrins, and stimulate reparative fibrosis. Cytokines like tumor necrosis factor α (TNF-α) and interleukins like IL-1 and IL-6 induce MMP synthesis and are related to the development of LV dysfunction, pulmonary edema, endothelial dysfunction, and cardiomyocyte apoptosis [34].

These cellular and signaling processes that constitute the proliferative phase of infarct healing in the myocardium influence and determine the fate of implanted stem cells. Ischemic myocardium constitutes an inflammatory hostile environment for stem cells, which is devoid of nutrients and oxygen and lacks survival signals from the ECM and cell-to-cell interactions. Indeed, only a small fraction of them survives in such adverse conditions. Nevertheless, some studies have shown that certain implanted stem cells may improve or counteract this situation. Intramyocardial transplantation of EPC after AMI induces significant and sustained increase in angiogenic, antiapoptotic, and chemoattractant factors, that are upregulated in both transplanted and host cells (i.e., vascular endothelial growth factor-A [VEGF-A], fibroblast growth factor-2 [FGF-2], angiopoietin-1 [Ang-1], angiopoietin-2 [Ang-2], placenta growth factor [PIGF], hepatocyte growth factor [HGF], insulin-like growth factor-1 [IGF-1], platelet-derived growth factor-B [PDGF-B], and stromal cell-derived factor-1 [SDF-1]) [35]. These humoral factors provide an additional favorable milieu for neovascularization and repair or regeneration of ischemic myocardium. Furthermore, there is a cross-talk between the heart and the bone marrow mediated by humoral effects that may improve this therapeutic effect: it has been proved that EPC transplantation further mobilizes endogenous BMMC into peripheral circulation, recruiting them into the ischemic myocardium [35].

Having these considerations in mind, new lines of research are being developed to improve cell survival rates in the ischemic myocardium, between them [1] are the following.

(1) Preconditioning of the myocardium to retain a higher number of cells: low-energy shock waves, ultrasound-mediated destruction of microbubbles in the coronary circulation, and extracorporeal shock wave treatment have proved to increase retention of EPC, BMMC, and MSC. The last of these techniques is undergoing clinical testing in the Cellwave trial.

(2) Activation or increase of chemotactic factors to attract cells to the damaged area: high mobility group box-1 (HMGB-1), SDF-1 or its receptor CXCR4, β_2 integrin and endothelial nitric oxide synthase can be activated to increase the rate of homing of different types of stem cells (i.e., progenitor blood cells, EPC).

Regarding stem cells administered to the myocardium, their functional activity is determined by age and cardiovascular risk factors. As a consequence, future phase II-III RCT will explore cell enhancement strategies intended to increase their therapeutic potential. Several strategies are currently under investigation [1].

(1) Pretreatment of the patients with drugs to stimulate cell potentiality: statins, rosiglitazone, and nitric oxide synthase enhancer AVE9488 can improve the migratory, invasive, and neovascularization capacity of EPC.

(2) Strategies to prolong cell survival: between them, the use of a combination of growth factors to stimulate the expression of cardiomyocyte genes in MSC (currently under clinical investigation in the C-Cure trial), the use of heat shock to increase

the resistance of cells to external stressors and the pretreatment of ESC-derived cardiomyocytes with heat shock and a cocktail of survival factors, are being explored.

(3) Genetic modification of the cells prior to administration: overexpression of antiapoptotic genes or genetic manipulation to maintain cell's functionality (i.e., capacity to secrete paracrine mediators, to connect with host myocardium, or to differentiate into specialized cardiac cell types) can be achieved through genetic cell engineering.

(4) Combined injection of cells and biomaterials: BMMC encapsulation within scaffolds (epicardial patches) or peptide nanofibers represents another strategy that needs further investigation.

4.4. Mechanisms of Action. Nowadays, it is believed that stem cell therapy could lead to successful cardiac regeneration or repair by any or a combination of three main general mechanisms (Figure 3): (1) differentiation of the administered cells into all of the cellular constituents of the heart (i.e., cardiomyogenesis and vasculogenesis processes), or, less probably, fusion of the administered cells with those, (2) release of factors capable of paracrine signaling from the administered cells, and (3) stimulation of endogenous repair by injected cells, through stem cell cardiac niches activation [36].

4.4.1. Cardiomyogenesis and Vasculogenesis. While in the classic studies of the beginning of the decade (trans) differentiation of BMMC into cardiomyocytes, smooth muscle cells, and endothelial cells was postulated as the main mechanism that might explain the cardiac recovery resulting from stem cell therapy, this phenomenon has been demonstrated in low proportions in more recent studies. Regarding cellular fusion of administered cells with host myocardial ones, to date there is little evidence to support this hypothesis.

4.4.2. Paracrine Actions. Given that differentiation debate is still ongoing and that the number of newly generated cardiomyocytes and blood vessels is too low to explain significant functional improvements, the paracrine hypothesis is now considered the most plausible. According to this idea, the functional benefits of stem cells might be related to secretion of soluble factors that, acting in a paracrine fashion, protect the heart, attenuate pathological LV remodeling, induce neovascularization, and promote regeneration [37]. BMMC and MSC have been extensively studied and proved to produce and secrete a broad variety of cytokines, chemokines, and growth factors, between them VEGF, FGF, HGF, IGF, adrenomedullin, thymosin β4 (TB4), SDF-1, PDGF, and angiopoietin. These paracrine mediators are expressed/released in a temporal and spatial manner exerting different effects depending on the microenvironment after injury. In addition, these released factors may have autocrine actions on the biology of stem cells themselves [37].

The paracrine factors may influence adjacent cells and exert their actions via several mechanisms, including what follows.

(1) Myocardial protection: MSC and BMMC in an ischemic environment release cytoprotective molecules that increase cardiomyocyte survival (VEGF, FGF, HGF, IGF-1, TB4, SDF-1, PDGF, and IL-1).

(2) Neovascularization: BMMC, MSC, and EPC can give rise to vascular structures. The molecular processes leading to angiogenesis and arteriogenesis involve mediators such nitric oxide, VEGF, SDF-1, FGF, HGF, and angiopoietin.

(3) Cardiac remodeling: paracrine factors released by transplanted stem cells may alter the ECM (i.e., inhibiting cardiac fibroblast proliferation and types I and III collagen synthesis), resulting in more favorable post-AMI remodeling and strengthening of the infarct scar. Stem cells (MSCs) may also produce molecules that limit local inflammation, thus reducing the remodeling process.

(4) Cardiac contractility and metabolism: it has been demonstrated that stem cell therapy limits infarct size and prevents LV dysfunction. On the other hand, MSC and BMMC secrete inotropic factors (i.e., IGF-1) that positively modulate cell contractility, and these cells attenuate the profound bioenergetic abnormalities found in the border zone of myocardial infarction.

(5) Cardiac regeneration: as we have seen, differentiation and cell fusion with native cardiomyocytes occur in very low rates after stem cell administration. Therefore, it is now believed that exogenous stem cell transplantation may activate resident cardiac stem cells (CSCs) and/or stimulate cardiomyocyte replication via paracrine action. Factors secreted by BMMC, MSC, and EPC, including HGF, SDF-1, VEGF, and IGF-1, enhance proliferation, mobilization, differentiation, survival, and function of CSC or even restoration of cardiac stem cell niches.

4.4.3. Endogenous Repair. Finally, clonogenic and self-replicating endogenous CSCs have been isolated and cultured from human hearts. These CSCs—located in cardiac stem cell niches—have the capacity to differentiate into endothelial cells, smooth muscle myocytes, and cardiomyocytes. Though insufficient for a complete repair of the myocardium after any kind of insult, these cells can be activated by extracardiac delivered cells. Thus, administered allogeneic MSCs participate in maintaining stem cell niches, and through cell-to-cell interactions—apart from paracrine effects—may not only restore lost cellular constituents (differentiation) but also these niches with an ongoing and regulated self-replicating capacity [36].

4.5. Endpoints. Endpoints in RCT can be divided in hard and surrogate. The former include clinical endpoints such

as survival, disease free survival or improvement in objective measures of disease-related functions, and are the ones that can change medical practice and present guidelines. The latter are defined as parameters or physical signs used as substitutes for an endpoint with clinical meaning that measures the quality of life or mortality [38]. In other words, they are intermediate markers (i.e., laboratory measurements) or response variables that can substitute for a "true" endpoint for the purpose of comparing specific interventions or treatments. Specifically, such response variables provide some additional information on true endpoint occurrence times for study subjects having censored values for such times.

Hard or clinical endpoints have a direct relation with prognosis and survival. They include all-cause death, cardiac death, reinfarction, the need for further revascularizations, readmission due to heart failure and stroke, taken individually or as combined (composite) endpoints. Stem cell therapy will join the therapeutic spectrum for cardiovascular diseases only if it can be confirmed to improve quality of life and survival time with precise and accurate evidence of its influence on these parameters. Indeed, clinical variables have to be invariably the primary endpoints in large future phase III RCT.

The only trials that have explored the effect of stem cells in hard endpoints have been the REPAIR-AMI trial [4] and the BALANCE trial [39]. Both studies were not powered to detect differences in this kind of parameters (they were considered as secondary endpoints), and the latter was not even randomized. In the REPAIR-AMI trial the cumulative endpoint of death, recurrent AMI or necessity for revascularization was significantly reduced in the BMMC group compared with that in the placebo group after 12 months. Likewise, the combined endpoint of death, AMI and hospitalization for heart failure was also reduced. In the BALANCE study, BMMC transfer after AMI was associated with a significant reduction in mortality after 5 years of followup. Moreover, in two large meta-analyses [13, 14], a trend towards a beneficial effect of BMMC on death, reinfarction and rehospitalization for heart failure has also been reported.

Having said that, surrogate endpoints have been a necessity with stem cell therapy RCT, because the sample size required to have statistical power to detect differences in mortality in these studies has been impractical [40]. For this reason, and although they have been used in phase I studies in order to do smaller and shorter trials, they should not be the primary endpoints in phase II-III RCT.

Parameters used as surrogate endpoints to assess the effects of stem cell therapy on survival in ischemic heart disease have to meet three requirements: (1) they must be closely correlated with survival, (2) changes in the parameters must reflect changes in the prognosis, and (3) there must be a pathophysiological basis to account for both relations [41]. Thus, there are several variables that have been used as surrogate endpoints, basically imaging (metabolism, perfusion, and contractility parameters) and laboratory measurements.

4.5.1. Imaging Parameters. Many of the surrogate endpoints used in clinical research on heart disease are parameters obtained using imaging techniques, being LVEF the most frequently used. Since sample size depends on the standard deviation of the surrogate endpoint to be measured (which varies according to the variability of the imaging technique), the higher the spatial and temporal resolution of a given technique, the smaller the variability, especially if quantification is automatic [38]. Thus, techniques of choice to quantify LVEF in stem cell RCT are magnetic resonance imaging (MRI), single-photon emission computed tomography (SPECT), and contrast-enhanced echocardiography. The same techniques are the ideal ones for a proper evaluation of regional contractility (wall motion score index). Less accurate tools include simple echocardiography, computed tomography, and ventriculography.

However, if the study is conducted in patients who have had AMI, it is essential to calculate infarct size and thickness and thickening of the infarcted wall. For this purpose, MRI (through delayed-enhancement of gadolinium sequences) and SPECT are the best tools. If patients with depressed LVEF are included, we should use imaging techniques that appropriately measure LVEF and ventricular end-systolic and end-diastolic volumes (i.e., MRI and contrast echocardiography).

Finally, if the study group consists of no-option patients, imaging techniques should be employed that appropriately assess myocardial perfusion (i.e., MRI and SPECT). In all stages of ischemic heart disease metabolism can be precisely studied by positron-emission tomography (PET) in combination with SPECT data, but MRI also offers relevant information.

Another more experimental surrogate parameters include the evaluation of microcirculation by gadolinium delayed-enhancement, inotropic responsiveness with stress (dobutamine) MRI, assessment of angiogenesis and arteriogenesis (first-pass of gadolinium), and new techniques of metabolic function, such as detection of high-energy phosphate metabolism and blood-oxygen tension determination using blood-oxygen-level-dependent (BOLD) MRI [40]. Again, in all these variables MRI plays a central role.

Nevertheless, despite all these robust imaging armamentarium, current stem cell therapies (RCTs) are still limited by the inability to perform imaging at the cellular level and track the fate of the cells (being radionuclide, MRI and reporter gene techniques the most used). Thus, the development of molecular imaging (with the use of MRI, PET, SPECT, computed tomography and echocardiography) and better techniques at the cellular level that allow 3-dimensional imaging will be necessary for clinical applications in the future [42]. Finally, it is clear that only RCT with hard clinical endpoints (i.e., cardiac death, reinfarction, rehospitalization, revascularization, and stroke) will definitively establish the role of stem cell therapy in ischemic heart disease. In this regard, we must not forget that imaging cannot provide a substitute for clinical outcome data.

4.5.2. Laboratory Parameters. Several laboratory measurements have been used as surrogates in stem cell therapy RCT. Markers of inflammation, myocardial damage, and

heart failure are the most frequently used. Between them, C-reactive protein (CRP), creatine-phosphokinase (CPK), T and I troponin, and probrain natriuretic peptide (pro-BNP) are universally available. Cytokines such as interleukins, and growth factors have also been used.

4.6. Followup. Another key point is final followup. Long-term followup should be carried out and specified in all RCT protocols in stem cell therapy, in order to precisely define the safety of the procedure (i.e., oncogenesis, restenosis, late adverse events).

4.7. The Consensus of the Task Force of the European Society of Cardiology for Future Trials: Next Directions. The Task Force of the European Society of Cardiology on stem cells and repair of the heart was created in 2006 to investigate and regulate the role of progenitor/stem cell therapy in the treatment of cardiovascular disease. It was almost four years ago that this group of experts and opinion leaders stated the type of studies needed [43].

(1) Further large, double-blind, controlled RCT for the use of autologous BMMC in the treatment of AMI. The patient population should be all those presenting within 12 hours of AMI and treated with immediate revascularization, be it primary angioplasty or fibrinolysis.

(2) Double-blind, controlled RCT for the use of autologous BMMC in the treatment of AMI in those patients presenting late (>12 hours) or who fail to respond to therapy (candidates for "rescue" angioplasty). Although, these groups may represent a small proportion of all patients with AMI, their prognosis remains poor.

(3) Double-blind, controlled RCT for the use of autologous BMMC or SM in the treatment of ischemic heart failure. At some stage, the role of autologous stem/progenitor cells in the treatment of cardiomyopathies (in particular, dilated cardiomyopathy) will need to be examined.

(4) A series of well-designed small studies to address safety or mechanism to test specific hypotheses (i.e., studies with labelled cells or to investigate paracrine or autocrine mechanisms). Such hypotheses would have arisen from basic science experiments.

(5) Studies to confirm the risk/benefit ratio of the use of cytokines alone (i.e., G-CSF) or in conjunction with stem/progenitor cell therapy.

This Task Force also underlined the necessity for studies with hard clinical endpoints, MACE, subjective benefit and economic gain [43]. Another key point is standardization, both in outcome measures and in the processing of cells (better achieved in specialized centers following Good Manufacturing Procedure routines), in order to derive meaningful comparisons. Since these trials will need to include thousands of patients, they should be multicentre and ideally pan-European. An example of this new phase III large-scale trials is the RCT conducted by Zehier's group in Frankfurt, that will enroll up to 1200 patients with extensive AMI (LVEF <40%) and with a combined primary endpoint of cardiac death, reinfarction and rehospitalization due to heart failure. On the other hand, the Task Force stated that small uncontrolled trials with BMMC should be avoided, as they are unlikely to add anything new to the field.

Finally, next directions of cardiac cell therapy include what follows.

(1) The study of the array of bioactive molecules that are secreted by stem cells, which have been demonstrated to induce neovascularization, modulate inflammation, fibrogenesis, cardiac metabolism, and contractility, increase cardiomyocyte proliferation and activate resident stem cells. The exhaustive analysis of this "secretomes" of BMMC, MSC, or EPC would lead to a better understating of the mechanisms of action of the cells and to a hypothetical protein-based therapy (off-the-shelf, noninvasive, systemic, and repetitive administration).

(2) The use of different sources of pluripotent stem cells, like ESC, spermatogonial stem cells and oocytes. A new era has been initiated with the possibility of reprogramming adult cells (skin fibroblasts) to a pluripotent state by retroviral transduction [44, 45]. These "induced-pluripotent stem cells" (iPSs) show the characteristics of ESC and can differentiate to cardiomyocytes. New retroviral vectors and even nonviral vectors have been developed to reduce the risk of mutagenesis, and genetic modification of cells with suicide genes have been proposed to reduce the risk of tumor formation.

(3) The creation of bioartificial hearts after a process of decelularization with detergents, obtention of the underlying extracellular matrix (cardiac architecture), and stem cell repopulation [46]. The "acellular" heart can then be reseeded with cardiac stem cells or EPC, showing contractile activity in animal models. This new approach of tissue bioengineering has opened a fascinating era in cardiovascular medicine.

5. Conclusions

Although mixed results have emerged from the first stem cell therapy RCT in cardiovascular medicine, the overall data suggest that these procedures are feasible and safe in both acute and chronic scenarios of ischemic heart disease. After phase I-II RCT, it is clear that BMMC transfer after AMI has the potential to improve the recovery of LV systolic function beyond what can be achieved by current interventional and medical therapies. In chronic ischemic heart disease, SM and BMMC have proved to improve myocardial perfusion and contractile performance.

New types of cells (including ADSC and iPS), improvements in delivery and imaging methods, strategies to enhance cell potentiality or to improve the myocardial proinflammatory microenvironment, and the creation of

bioartificial hearts are the main new directions of research in the near future.

Finally, large-scale, phase III, double-blind, controlled RCT performed under rigorous safety standards are being initiated to prove unequivocal clinical benefits, including improved survival. These trials will definitively establish the effectiveness of stem cell therapy in improving clinical outcomes, confirming the real potential of cardiac regenerative therapy.

Conflicts of Interest

None of the authors has conflicts of interest to declare.

Acknowledgments

The authors thank the Spanish Cooperative Cardiovascular Disease Research Network (RECAVA, Red Temática de Investigación Cooperativa en Enfermedades Cardiovasculares) and the Spanish Cell Therapy Research Network (TERCEL, Red Temática de Terapia Celular from the Instituto de Salud Carlos III, ISCIII) for their sponsorship. They also appreciate the efforts of the members of the Task Force of the European Society. They would also like to thank the research nurses (María José Lorenzo, Alexandra Vázquez and Lucía Fernández) and Ana Fernández-Baza for her work, enthusiasm, and invaluable help.

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SAGE-Hindawi Access to Research
Stem Cells International
Volume 2010, Article ID 582382, 5 pages
doi:10.4061/2010/582382

Research Article

Cotransplantation of Adipose Tissue-Derived Insulin-Secreting Mesenchymal Stem Cells and Hematopoietic Stem Cells: A Novel Therapy for Insulin-Dependent Diabetes Mellitus

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Received 1 April 2010; Revised 10 June 2010; Accepted 25 November 2010

Academic Editor: Bruce A. Bunnell

Aims. Insulin dependent diabetes mellitus (IDDM) is believed to be an autoimmune disorder with disturbed glucose/insulin metabolism, requiring life-long insulin replacement therapy (IRT), 30% of patients develop end-organ failure. We present our experience of cotransplantation of adipose tissue derived insulin-secreting mesenchymal stem cells (IS-AD-MSC) and cultured bone marrow (CBM) as IRT for these patients. *Methods*. This was a prospective open-labeled clinical trial to test efficacy and safety of IS-AD-MSC+CBM co-transplantation to treat IDDM, approved by the institutional review board after informed consent in 11 (males : females: 7 : 4) patients with 1–24-year disease duration, in age group: 13–43 years, on mean values of exogenous insulin requirement of 1.14 units/kg BW/day, glycosylated hemoglobin (Hb1Ac): 8.47%, and c-peptide levels: 0.1 ng/mL. Intraportal infusion of xenogeneic-free IS-AD-MSC from living donors, subjected to defined culture conditions and phenotypically differentiated to insulin-secreting cells, with mean quantum: 1.5 mL, expressing Pax-6, Isl-1, and pdx-1, cell counts: $2.1 \times 10^3/\mu$L, $CD45^-/90^+/73^+$:40/30.1%, C-Peptide level:1.8 ng/mL, and insulin level: 339.3 IU/mL with CBM mean quantum: 96.3 mL and cell counts: $28.1 \times 10^3/\mu$L, $CD45^-/34^+$:0.62%, was carried out. *Results*. All were successfully transplanted without any untoward effect. Over mean followup of 23 months, they had a decreased mean exogenous insulin requirement to 0.63 units/kgBW/day, Hb1Ac to 7.39%, raised serum c-peptide levels to 0.38 ng/mL, and became free of diabetic ketoacidosis events with mean 2.5 Kg weight gain on normal vegetarian diet and physical activities. *Conclusion*. This is the first report of treating IDDM with insulin-secreting-AD-MSC+CBM safely and effectively with relatively simple techniques.

1. Introduction

The incidence of diabetes mellitus (DM) has been increasing in an epidemic-like fashion in the last two decades globally. India is expected to become the world capital of DM by year 2030 [1–3]. Insulin dependent diabetes mellitus (IDDM) is the second most common chronic disease of childhood believed to be autoimmune in nature and characterized by irreversible destruction of insulin-secreting pancreatic β islet cells. Symptoms of the disease appear when insulin-making β cell mass gets reduced by approximately 90% leading to severe insulin deficiency and hyperglycemia. At present the only therapeutic options for management are life-long exogenous insulin preparations. Sporadic reports of autologous hematopoietic stem cell transplantation (HSCT) have been reported with limited success [4].

We present our experience of insulin replacement therapy (IRT) by co-transplantation of insulin-secreting adipose tissue derived mesenchymal stem cells (IS-AD-MSC) and cultured-bone-marrow- (CBM-) derived HSCT in 11 IDDM patients.

2. Study Design (Figure 1)

This was a prospective nonrandomized open-label clinical trial conducted from October 2007 to September 2008 to test the efficacy and safety of combined IS-AD-MSC and HSCT as IRT in IDDM patients. HSC co-transplantation with IS-AD-MSC was designed to augment the effect of the later. Omental vein infusion was carried out so that the cells would get trapped in hepatic microcirculation and the liver, being tolerogenic organ, would not reject them. The institutional Review Board approved of consent forms and clinical trial.

Inclusion criteria were patients between 5 to 45 years of age, of any gender, with confirmed diagnosis of IDDM at least for 6 months, with low levels of serum C-peptide levels (<0.5 ng/mL).

Exclusion criteria were positive serology for HIV/HbSAg/HCV and underlying hematologic, nephrologic, cardiac, psychiatric, or hepatic diseases, and pregnancy.

Healthy nondiabetic donors from family of recipients having matching blood group with patients, who were willing to donate fat and bone marrow (BM) were approved as donors in this research protocol after their informed written consent.

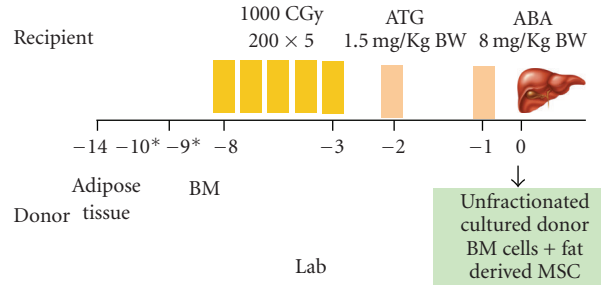

Figure 1: Ahmedabad paradigm of Cotransplantation of insulin secreting and hematopoietic stem cells for IDDM.

3. Methods

3.1. Adipose Tissue and BM Procurement from Donor. Adipose tissue (approximately 2 gm) was resected from anterior abdominal wall of donors on day −14, sutures taken after hemostasis were achieved and sent to stem cell lab for culture in appropriate transport medium to derive MSC and further differentiate them into insulin-secreting cells. On days −10 and −9, donors were stimulated with injection granulocyte colony-stimulating Factor (G-CSF), 7.5 μg/kg BW/ day subcutaneously followed by BM aspiration from their posterior superior iliac crest under local anesthesia, in which 60 mL BM was collected on day −8.

3.2. Isolation of MSC from Adipose Tissue. The resected adipose tissue was transported to the lab in self-designed proliferation medium with Dulbecco's modified eagle's medium (DMEM, Sigma, USA) (high glucose), 20% human albumin (Reliance Life Sciences, India), Fibroblast growth factor: 2 ng/mL, 1% Sodium pyruvate, and appropriate antibiotics which included penicillin, streptomycin, cefotaxime, and fluconazol and minced with knife into tiny pieces in Collagenase type I (10 mg/10 mL) solution. The entire contents of the medium were processed in culture dish and after mincing they were placed in incubator at 37°C with shaker arranged with 35 RPM for 1 hour, and subsequently transferred to 15 mL centrifuge tubes and centrifuged at 780 RPM for 8 minutes. After centrifugation the supernatant and pellets were separately cultured in proliferation medium on 100 sq.cm and 25 sq.cm cell+ plates (Sarstedt, USA), respectively, at 37°C with 5% CO2 under humid conditions for 10 days. Medium was replenished every other day.

3.3. Culture and Differentiation of h-AD-MSC into Insulin-Secreting Cells. On the 10th day of culture in proliferation medium, the cells were washed in phosphate buffered saline (1 N). The cells were harvested by means of trypsinization (0.25% Trypsin EDTA solution, Hi Media, India) and checked for viability using trypan blue, sterility (Bactec, USA) and counts in modified Neubauer chamber. For flow cytometric analysis of cells, CD 45(Per CP) negative and CD90 (PE)/CD73 (PE) (Becton, Dickinson, USA) positive tests were carried out. They were also stained by Giemsa and further subjected to differentiation in to insulin-secreting cells using differentiation medium with DMEM (glucose-17.5 mM), DMEM: F 12, Nicotinamide, Activin A, Exendin 4, Pentagastrin, hepatocyte growth factor, B-27, N-2 serum supplement, and antibiotics. This cocktail upregulates gene expression, nourishes the cells, and prevents their further proliferation. No xenogenic material was used.

The cells were kept in this medium for 3 days for differentiation and then subjected to isolation on Ficoll Hypaque by density gradient.

3.4. Testing for Molecular Marker Characterization of Insulin Secreting MSC. Cell pellet was then diluted with equal amount of medium and after testing for sterility, viability, and cell counts, subjected to immunofluorescence test for expression of transcription factors, paired box 6 (Pax-6), marker for glucagon production, isl-1, key regulator for normal islet cell development, which is the gene upregulating expression of insulin, and pancreatic duodenal homeobox gene (pdx-1) which is the regulator of β-cell specific gene expression, function, and for self-renewal of β progenitor cells [5, 6].

C-Peptide and Insulin. from supernatant of cultured cells were measured by chemiluminescence.

3.5. Glucose Challenge Assay. Cells were further incubated in 6 well plates at the concentration of 5 cells/cm sq. without glucose, in glucose, (90 mM), 5 mL, and 10 mL respectively for 2 hours and insulin and c-peptide levels were measured at the end.

3.6. Culture of BM. The aspirated BM was subjected to invitro expansion under self-designed medium using

DMEM:F12 (1 : 1) with 20% human albumin, Erythropoietin (V.H.B. Life Sciences, Inc, India), 10 μL/100 mL, G-CSF (Gennova Biopharma, India), 10 μL/100 mL, Mitomycin C, 2 μL/100 mL, nonessential amino acids, 1 mL/100 mL, Ascorbic acid, 10 μL/100 mL, and antibiotics in CO2 incubator at 37°C with 5% CO2 under humid conditions. No xenogenic material was used. Medium was replenished every other day for 8 days.

3.7. Recipient Conditioning. Nonmyelo-ablative low-intensity conditioning included target specific irradiation to subdiaphragmatic lymph nodes, spleen, part of pelvic bones, and lumbar vertebrae (200 cGY × 5 days) from day −8 to −3 of transplantation. Anti-T cell antibody, 1.5 mg/kg BW and anti-B cell antibody, 6 mg/kg BW were administered intravenously on days –2 and −1, respectively, to prevent rejection and facilitate grafting of transplanted cells. No immunosuppressive medication was used post-transplantation.

3.8. Cell Transplantation. Cell cocktail was transplanted into recipient on day 0 under general anesthesia as per our own technique of omental cannulation by mini-laparotomy in which a mid-line incision of 3 cm was made 5 cm above umbilicus, omental vein was cannulated using 20 guage needle and cells were infused at the rate of 6–8 mL/min. Omental vein was ligated with silk after infusion, hemostasis checked and wound closed with vicryl 2/0 stitches and subcuticular stitches were taken using 3/0 monocryl [7].

3.9. Posttransplant Patient Monitoring. Patients were monitored closely for 4 hourly blood sugar levels for 3 days after transplantation following 12 hourly monitoring for 1 week. Fasting and postprandial (PP) blood sugar (BS) levels were checked weekly for the 1st month and fortnightly for next the 2 months. Serum c-peptide levels were measured daily for the 1st week and with mixed meal tolerance weekly for the 1st month. Insulin administration was made on sliding scale with an objective of maintaining FBS <150 mg% and PPBS around 200 mg%. Glutamic acid decarboxylase (GAD) antibodies were monitored by ELISA technique (Euroimmun -Medizinische Labordiagnostika AG, UK) before and 3 months after infusion. Recipient monthly body weight, and number of diabetic ketoacidosis (DKA) episodes were monitored and evaluated before and after infusion. Glycosylated hemoglobin (Hb1Ac) (reference range: normal: <8.3%, good control: 8.3 to 9%, fair control: 9-10%, poor control: >10%, Erba diagnostics Mannheim, GmbH, Germany) levels were measured at 2-month intervals post-transplantation.

Key endpoints of study were morbidity, mortality, untoward side effects from stem cell transplantation, and changes in exogenous insulin requirements (daily dose/duration). Secondary endpoints were serum C-peptide levels with mixed-meal tolerance test at monthly intervals, GAD antibodies and Hb1Ac following stem cell transplantation.

3.10. Patients. Eleven patients (7 males, 4 females) with mean age 21.1 years (range: 13–43 years) with mean disease duration of 8.2 years (range: 1–24 years) were subjected to co-transplantation with IS-AD-MSC+HSCT. Seven out of 11 patients had associated DKA episodes (1 to 5 episodes). Mean Hb1Ac levels were 8.47% (range: 6.2–10.3%) and mean insulin requirement was 1.14 units/kgBW/day (range: 0.42–2.4 units per day).

4. Results

Donors were parents and siblings, in 4 patients cousin in 1, and uncles in 2 patients.

4.1. Stem Cells. Mean total cell quantum transplanted was 96.3 mL (range: 92 to 118 mL) with nucleated cell counts of CBM: $28 \times 10^3/\mu$L (range: 12.2 to $62.7 \times 10^3/\mu$L) and MSC-$1.2 \times 10^3/\mu$L (range: 0.5 to $2.1 \times 10^3/\mu$L), mean $CD34^+$, 0.62% (range: 0.06 to 2.01%), mean CD 45^-, $90^+/73^+$ counts, and 39.99% (range: 16.6 to 81.4%)/30.1% (range: 14.1 to 65.7%). All of them expressed transcription factors pax-6, pdx 1, and isl-1 (Figure 2). Mean C-peptide level of cell inocula was 1.84 ng/mL (range: 1.15–3.6 ng/mL) and insulin level was 339.3 μ IU/L (range: 118 to 739 μ IU/L).

4.2. Patient Values. Mean pretransplant serum C-peptide levels of 0.1 ng/mL (range: 0.02 to 0.3 ng/mL) increased gradually to mean 0.37 ng/mL (range: 0.1 to 1.8 ng/mL) (normal range: 0.7 to 1.9 ng/mL by Monobind Inc, USA) and mean pre-transplant exogenous insulin requirement of 1.14 units/kg BW/day (range: 0.42–2.1 units/kg BW/day) decreased to 0.63 units/kg BW/day (range: 0.09–1 unit/kg BW/day) (Figure 3(a)). It was observed that there was gradual fall in exogenous insulin requirement over the first 2 to 4 months which then remained steady (Figure 3(b)). Mean befor transplant Hb1Ac of 8.47 % (range: 6.22 to 10.3 %) decreased to 7.39 % (range: 5.72 to 8.98 %).

GAD antibodies between 10 and 210 IU/mL in 5 patients decreased to values between 4 to 180 IU/mL and in 3 patients where values were >2000 IU/mL befor transplantation remained the same. No functional correlation was observed between insulin requirements/c-peptide levels and GAD antibody levels.

4.3. Statistical Analysis. Insulin requirement, Hb1Ac, and serum C-peptide levels were subjected to Student's paired t-test and change in insulin requirement was found to be the most significant with $P = .009$, Hb1Ac showed $P = .03$ and c-peptide values showed $P = .05$.

There was no adverse/untoward side effect related to stem cell infusion or administration of induction therapy. Over a mean followup of 7.3 months (range: 2.2 months to 1 year) all patients continued to have a feeling of well-being after infusion and are physically more active, alert, on normal vegetarian diet, and better rehabilitated in their professional and personal lives. There was an impressive absence of DKA episodes in all of them. Pretransplant weight of 54.6 kg (range: 23.5 to 82 kg) increased marginally to 55.2 kg (range: 25 to 82 kg).

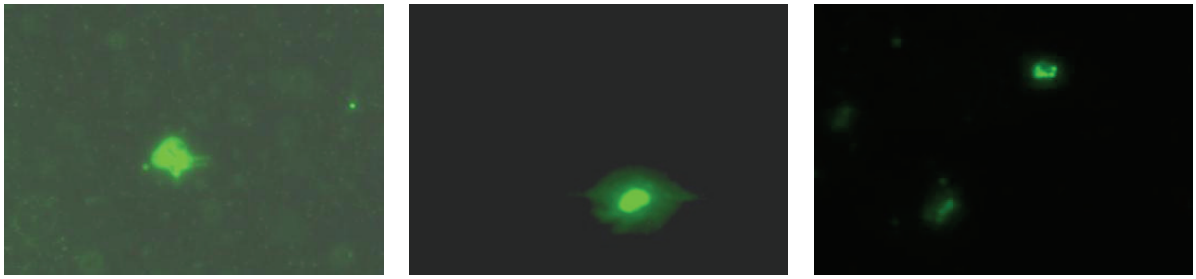

Figure 2: Indirect immunofluorescence demonstrating pax-6, isl-1, and pdx-1, from left to right, ×100.

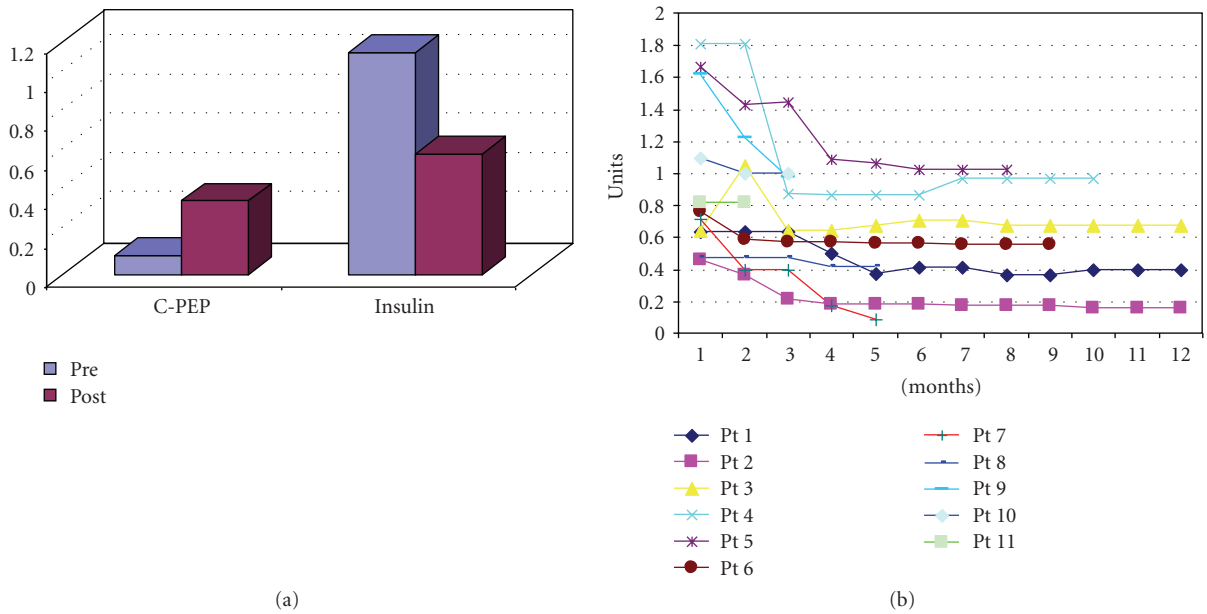

Figure 3: (a) Comparison of S.C-peptide levels and insulin requirement pre and post (7 months) stem cell transplantation. (b) Followup of exogenous insulin requirement (units/KgBW/Day).

5. Discussion

Therapeutic strategies for addressing immune dysregulation of IDDM include nonactivating monoclonal antibodies against CD3, gene therapies, autologous HSCT, infusion of dendritic cells, T-regulatory lymphocytes, umbilical cord cells, embryonic or adult stem cells, and allogenic BM transplantation [4, 6]. Invitro rodent models have shown MSC derived from BM and spleen with capability of insulin-secretion to treat hyperglycemia [8]. Human BM-derived and adipose tissue-derived MSC have been found to be phenotypically identical cell populations as those of rodents [9]. We have generated in vitro MSC from human adipose tissue which qualify the definition standardized by the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy. MSC have been defined as the cells having plastic-adherence when maintained under standard culture conditions, must have the ability of osteogenic, adipogenic and chondrogenic differentiation, must express CD73, CD90, and CD105, and must lack expression of hematopoietic lineage markers c-kit, CD14, CD34, CD 45, CD 11b, CD 29, CD79α, and HLA-DR [10]. Our cells fulfill these criteria. We further differentiated them to insulin secreting cells under defined culture conditions phenotypically identical to pancreatic β cells [11]. These cells expressed transcription factors pdx-1, pax-6, and isl-1, all three are central controlling genes capable of reprogramming nonpancreatic cells to surrogate β cell functions. Again our technique is a shortcut to reprogramming non-pancreatic cells as compared to vector-based gene transfer techniques [12].

Our results support the contention that combination of these three transcription factors represents the establishment of ectopic mechanisms to secrete insulin. These exciting results raise intriguing questions; whether this differentiation involves epigenetic reprogramming, or nuclear content of the MSC has become permissive to allow activation of this transcription program for β cell function. Whatever may be the reason this strategy has worked even in distantly related cells of origin.

6. Conclusion

This is the first report of successfully treating IDDM with co-transplantation of insulin-secreting adipose tissue derived MSC and HSCT. Easy and repeatable access to subcutaneous adipose tissue provides a clear advantage over isolation of MSC from BM. Isolation and culture techniques are simple and easy to perform.

Abbreviations

BM:	Bone marrow
CBM:	Cultured bone marrow
DKA:	Diabetic keto acidosis
DM:	diabetes mellitus
DMEM:	Dulbecco's modified eagle's medium
GAD:	glutamic acid decarboxylase
G-CSF:	Granulocyte colony stimulating factor
Hb1Ac:	Glycosylated hemoglobin
HSCT:	Hematopoietic stem cell transplantation
IDDM:	Insulin dependent diabetes mellitus
IRT:	Insulin replacement therapy
IS-AD-MSC:	Insulin secreting adipose tissue derived mesenchymal stem cells.

Acknowledgments

The authors are thankful to Yazdi Wadia and Priyadarshini Shah for statistical analysis and typing of the paper. C. N. Patel, J. V. Patel, A. G. Bhargava, B. N. Patel, and P. N. Bhavsar have carried out all the laboratory tests including flowcytometry analysis of the cases mentioned in the paper.

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SAGE-Hindawi Access to Research
Stem Cells International
Volume 2010, Article ID 259461, 10 pages
doi:10.4061/2010/259461

Review Article

Stem Cell-Based Therapies for Liver Diseases: State of the Art and New Perspectives

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Received 27 May 2010; Accepted 4 July 2010

Academic Editor: Jin Sup Jung

Millions of patients worldwide suffer from end-stage liver pathologies, whose only curative therapy is liver transplantation (OLT). Given the donor organ shortage, alternatives to OLT have been evaluated, including cell therapies. Hepatocyte transplantation has been attempted to cure metabolic liver disorders and end-stage liver diseases. The evaluation of its efficacy is complicated by the shortage of human hepatocytes and their difficult expansion and cryopreservation. Recent advances in cell biology have led to the concept of "*regenerative medicine*", based on the therapeutic potential of stem cells (SCs). Different types of SCs are theoretically eligible for liver cell replacement. These include embryonic and fetal SCs, induced pluripotent cells, annex SCs, endogenous liver SCs, and extrahepatic adult SCs. Aim of this paper is to critically analyze the possible sources of SCs suitable for liver repopulation and the results of the clinical trials that have been published until now.

1. Cell Therapies for Liver Diseases

Liver pathologies affect hundreds of millions of patients worldwide. The most common causes of hepatopathy are chronic hepatitis C and B, alcoholism, nonalcoholic fatty liver disease, autoimmune, and drug-induced hepatic disorders. Many of these conditions can be prevented or treated, but if not, they can lead to progressive liver injury, liver fibrosis and ultimately cirrhosis, portal hypertension, liver failure, and, in some instances, cancer. There are currently more than 5 million people in the United States suffering from end-stage liver pathologies, whose only curative therapy is liver transplantation (OLT). More than 5,000 liver transplants are performed in the United States each year (including more than 500 in children). About 20,000 people are waiting for OLT, but only 7,000 transplants are performed annually and as many as 1,500 patients die yearly while on the waiting list [1].

Given the donor organ shortage, various alternatives to OLT have been evaluated, such as split-liver and related living-donor liver transplantation. These procedures are still limited by the donor scarcity, the high costs, and the lifelong immunosuppressive treatments that they all require [2, 3]. Thus, the development of cell therapies for the treatment of end-stage hepatic diseases is currently under investigation all over the world. A cell therapy can be defined as "*the use of living cells to restore, maintain, or enhance tissue and organ function*" [4]. Cell therapies in hepatology have numerous potential advantages when compared to OLT, since transplantable cells can be (1) *in vitro* expanded and cryopreserved, abolishing the limit of organ shortage; (2) genetically manipulated, to correct inborn errors of metabolism; (3) cryopreserved for future use; (4) infused without major surgery; (5) obtained from the same patient, avoiding risk of rejection and need for lifelong immunosuppression [5].

One possible cell therapy source to restore the liver functional mass is represented by adult hepatocytes, that represent a particularly appealing tool, because they are mature and fully functional hepatic cells. Since the first successful hepatocyte transplantation in a rodent model of Crigler-Najjar syndrome, many preclinical studies and

clinical applications of this technique have been made to cure metabolic liver disorders and end-stage liver diseases [6]. In most instances, hepatocyte transplantation has been able to grant a clinical improvement for up to 12 months [7]. In patients with liver failure, hepatocyte-based therapies have also included the use of human or porcine hepatocytes in bioartificial liver devices [8]. Despite some encouraging results, the interpretation of these studies is hampered by the limited number and heterogeneity of patients, the lack of controls, the variety in terms of experimental design, outcome parameters, and follow-up duration. The evaluation of the efficacy of hepatocyte transplantation and bioartificial liver support systems is further complicated by the shortage of human hepatocytes. Indeed, it is ethically difficult to assign cadaveric livers to these experimental protocols, while many patients still die on the OLT waiting list. Moreover, primary cultured hepatocytes are hard to expand *in vitro* and cryopreserved cells are easily damaged during the freezing-thawing procedure. As a consequence, alternative solutions are being examined in the hepatic cell therapy field. Among these, of particular interest is the so-called "*regenerative medicine*", based on the therapeutic potential of stem cells (SCs) [5, 9].

SCs are undifferentiated cells, able to give rise to diverse mature progenies and to self-renew, through the alternation of symmetrical and asymmetrical divisions. SCs exist in all multicellular organisms and play a central role in tissue genesis, regeneration, and homeostasis, by providing new elements to increase tissue mass during pre- and postnatal growth, and by replacing cell loss due to senescence or damage [10, 11]. SCs possess a hierarchy of potentialities: from the totipotency of the zygote and its immediate progeny, to the pluripotency of embryonic stem cells (ESCs), to the multi/unipotency of tissue-specific adult SCs (ASCs). The latter persist in terminally differentiated tissues, allowing for their renewal and regeneration [12–15]. SCs colocalize with supporting cells in a physiologically limited and specialized microenvironment, or "*niche*", that varies in nature and location depending upon the tissue type [16]. The reciprocal interactions between SCs and their microenvironment, through cell-cell and cell-matrix connections as well as the secretion of soluble factors, influence SC behavior [17, 18].

SCs are already leaving the bench and reaching the bedside, despite an incomplete knowledge of the genetic control program driving their fate and plasticity [5]. In hepatology, the first attempts to translate SC basic research into new clinical strategies have been made. Aim of this review is to summarize the state of the art on SC-based therapies in hepatology. In particular, we will discuss the possible sources of SCs suitable for liver repopulation and will highlight both the benefits and the potential risks of these new tools for the treatment of liver pathologies.

2. Embryonic, Fetal, and Annex Stem Cells

2.1. Embryonic and Fetal Liver Stem Cells. ESCs and their derivatives might constitute an easily available source to obtain a large number of transplantable cells for regenerative treatments. ESCs are pluripotent cells derived from the inner cell mass of the blastocyst and can generate any differentiated phenotype of the three primary germ layers (endoderm, mesoderm, and ectoderm), as well as germ cells [15]. ESCs can be indefinitely maintained in an undifferentiated state, though they seem to develop karyotypic abnormalities over long periods in culture [5]. It has been demonstrated that ESCs can differentiate *in vitro* towards the hepatic lineage by simple removal of factors that prevent their differentiation, and/or through the exposure to appropriate growth factors, as reviewed elsewhere [7, 19]. Moreover, in several animal models of hepatic disease, ESC-derived hepatocyte-like cells were able to colonize the injured liver and function as mature hepatocytes [7, 19]. Fetal liver SCs, also named "*hepatoblasts*", appear when the hepatic endoderm has been specified and the liver bud is growing. Hepatoblasts are bipotent, being able to give rise to both hepatocytes and bile duct cells, and coexpress biliary and hepatocytic markers, such as albumin, alpha-fetoprotein (AFP), and CK19. Studies in diverse model organisms have revealed evolutionarily conserved inductive signals and transcription factor networks that elicit the differentiation of liver SCs, as reviewed elsewhere [19]. Murine hepatoblast cell lines have been established by various research groups and their capacity to repopulate the liver upon transplantation in animal models has been extensively proved, as discussed elsewhere [20]. In contrast to adult liver, ESCs and fetal liver SCs are thought to be highly proliferative, less immunogenic and more resistant to cryopreservation. However, ethical issues and the possibility of immune rejection and teratoma/teratocarcinoma formation in the recipients explain why their use is currently reserved to preclinical studies [21].

2.2. Induced Pluripotent Stem Cells. The recently described induced pluripotent stem cells (iPSs) might circumvent the ethical concerns and the risk of rejection related to embryonic and fetal liver SCs. Indeed, iPSs are embryonic-like SCs derived from somatic cells by forced expression of reprogramming factors (Oct3/4 and Sox2 along with either Klf4 or Nanog and Lin28). Theoretically, iPSs could be obtained from the same patient and used for tissue replacement or gene therapy. It is not yet clear how precisely the known developmental signals must be orchestrated to properly program hepatic cells at will, but detailed studies of the activated signaling pathways and their cross-regulatory interactions during embryogenesis will be informative. The first step of hepatic development from iPSs is the induction of definitive endoderm by using activin A. Further treatment with BMP-4 and bFGF can then direct cells towards the hepatic lineage [19]. Nowadays, iPS-based cell therapies have been applied in several animal models of pathologies, with encouraging results, and human iPS cells have been demonstrated to possess a hepatocyte-lineage differentiation potential comparable to that of ESCs [22]. Even if some limitations still remain (i.e., the potential for teratoma formation), iPS-derived hepatocytes are a very promising population for cell therapies in hepatology.

2.3. Annex Stem Cells. Another promising source for SC-based treatments in hepatology may be represented by cells established from placental/cordonal tissues, which do not seem to form teratomas or teratocarcinomas in humans, and have higher proliferation and differentiation potential than ASCs. Several studies indicated that umbilical cord and umbilical cord blood, placenta and amniotic fluid are an easily accessible source of pluripotent SCs, which may be readily available for transplantation, or for further expansion and manipulation prior to cell therapies [18, 23]. These cells can be extensively expanded without loss of potency and have a broad differentiation potential, since they can generate progenies of all three germ layers. These pluripotent annex SCs can be forced to differentiate into hepatocyte-like cells *in vitro* and are capable of liver repopulation *in vivo*, upon transplantation in animal models [7, 24]. We demonstrated that human umbilical cord blood SCs were able to colonize the liver and differentiate into hepatocytes after acute toxic liver damage in NOD/SCID mice and in immunocompetent rats [25, 26]. Moreover, microarray analysis led us to the identification of genes whose modulation strongly correlated with a more efficient process of liver repair after SC injection, proving the ability of these cells to positively influence the hepatic microenvironment and enhance the endogenous hepatic regeneration process [27]. The plasticity and accessibility of cord blood SCs have given the rationale for the creation of cord blood unit banks, where these cells can be collected and stored for future use.

3. Endogenous Adult Liver Stem/Progenitor Cells

Regenerative processes in postnatal liver parallel those occurring in development and involve populations of SCs and progenitor cells that can be identified by anatomic, antigenic, and biochemical profiles [28]. In particular, the liver has an extensive regenerative potential in response to parenchymal loss, mainly granted by mature hepatocytes, which can re-enter the cell cycle to restore the liver mass. This is a very efficient system and, after 2/3 partial hepatectomy (PH) in rats, proliferation of hepatocytes and cholangiocytes, followed by stellate and endothelial cells, can regrow the remnant to the original mass in less than 2 weeks [29]. However, whenever the replication ability of hepatocytes is experimentally inhibited or impaired by advanced chronic injury, liver regeneration can still be accomplished by the activation, expansion, and differentiation of the so-called "*hepatic progenitor/stem cells*". These cells are thought to be responsible for the human ductular reaction, which corresponds to the oval cell activation seen in specific rodent models of liver injury [30].

The term "*oval cells*" (OCs) was introduced to describe small proliferating cells with oval nuclei observed in rat livers following certain carcinogenic regimens [17, 31, 32]. OCs are an extremely heterogeneous population, which includes various fractions with different stemness potential, depending upon the experimental protocol and the animal model under investigation [33]. OCs are bipotent (able to give rise to both cholangiocytes and hepatocytes) and coexpress biliary and hepatocytic markers, such as CK19, alpha-fetoprotein, and albumin [34]. Over the past thirty years, several studies have described the isolation, culture differentiation and *in vivo* transplantation of liver OCs. A variety of surface antigens—including OV6, CD44, EpCAM and hematopoietic markers (i.e., CD34, CD133, c-kit)—have been used to identify and isolate OCs, as reviewed elsewhere [5, 28, 35]. Recently, Grompe's group has generated a new collection of monoclonal antibodies by immunization of Fischer rats with enzymatically dispersed nonparenchymal cells from the livers of adult mice treated with 3,5-diethoxycarbonyl-1,4-dihydrocollidine, to produce cell surface reactive reagents more specific for the oval cell response. Differential activity was observed on normal liver cells and at different stages of oval cell activation, indicating potential utility for progenitor cell identification [36]. Recently, Kamiya et al. were able to enrich and culture $CD133^+/CD13^+/CD49f^+$ bipotent progenitor cells from noninjured postnatal livers [37], while Sackett et al. have proposed the winged helix transcription factor Foxl1 as a potential marker of OCs in mice [38].

In humans, the counterpart of OCs is represented by the *intermediate hepatobiliary cells,* or *ductular hepatocytes,* or *hepatic progenitor cells* (HPCs), which can be seen in several hepatopaties, such as chronic cholestasis, submassive necrosis, alcoholic liver disease, focal nodular hyperplasia, and liver allograft failure [39]. In such conditions, intermediates between hepatic SCs and hepatoblasts and between hepatoblasts and adult parenchyma are observed and amplification of one or both pluripotent cell subpopulations can occur [28]. A frequent trigger of ductular reaction is the presence of chronic damage, resulting in hepatocyte senescence. Like OCs, HPCs are bipotent, coexpress biliary and hepatocytic markers, and also express hematopoietic progenitor cell antigens [40, 41]. In the last years, numerous studies have been published on the isolation, characterization and differentiation of putative liver progenitor cells from human livers, but the identification of a specific marker of HPCs awaits further investigation [5, 33, 37, 42–44].

Overall, whether OCs/HPCs fulfil the criteria to be considered true LSCs is still controversial. Some authors believe that OCs and HPCs may represent transit-amplifying cells derived from a more primitive LSC [32]. Given the difficulty of identifying unique LSC markers, it has been suggested to consider *stem-cellness* as a function instead of an entity: marking resident SCs based upon their quiescence might differentiate true SCs from their rapidly dividing derivatives [5]. By using a label-retention assay following acetaminophen-induced liver injury in mice, Kuwahara et al. found 4 possible LSC populations [45]: (a) replicating hepatocytes at the parenchymal/stromal interface; (b) ductular cells of the canal of Hering (CoH); (c) cholangiocytes of the intralobular bile ducts; and (d) periductular *null cells* (devoid of hepatocytic and biliary markers). The asymmetrically dividing cells that populate these sites might represent some form of lineage hierarchy within the LSC population: periductular null cells might give rise to the cytokeratin-positive cells of the CoH, which in turn could give rise

to the intraductular cells and periductular hepatocyte-like cells [46]. Recently, De Alwis et al. have demonstrated that regenerating hepatocytes arise from a LSC population in the CoH and move outward into the liver parenchyma, as in the *streaming liver hypothesis*. Interestingly, this observation seems to indicate that the LSC population is active also in healthy livers and contributes to the hepatic turnover [47]. In conclusion, LSC participation to hepatic repair is probably more complicated than in organs with normally rapid cell turnover and multiple liver cell populations might function as LSCs, depending on severity, location, and chronicity of injury.

The existence of multiple populations of liver cells with stemness potential implies the existence of multiple LSC niches, that can be activated depending upon the mechanism and location of injury [45, 46]. Presumably, the damaged liver releases molecules that stimulate the activation of OCs/HPCs and mediate their subsequent proliferation, migration and differentiation into mature hepatic phenotypes. Up to date, an extensive number of growth factors and cytokines which regulate the various phases of the OCs/HPCs response have been described, including SCF, HGF, EGF, FGF, Hedgehog and Wnt signalling pathways, SDF-1/CXCR4 axis, tumor necrosis factor, interleukin-6, interferons, transforming growth factors, and transforming growth factor like weak inhibitor of apoptosis (TWEAK) [48, 49]. Noteworthy, the responses of OCs/HPCs to HGF *via* cMet and the potential autocrine loops with TGF-alpha, EGF and FGF are similar to the patterns expressed by hepatocytes during liver regeneration, despite the fact that hepatocytes and LSCs do not tend to proliferate contemporaneously. This might be due to the modulatory effects of inflammatory cells within the niche, producing a range of cytokines and chemokines (such as TWEAK, TGF-beta and INF-gamma) that could influence the LSC response [50]. Another molecule of growing interest in the field of liver regeneration is the granulocyte-colony stimulating factor (G-CSF), a cytokine involved in mediating hematopoietic stem cells (HSCs) mobilization from the bone marrow (BM) into the peripheral blood [51]. In the last decade, several studies have indicated that G-CSF may be effective in mobilizing BM cells that contribute to liver repair [5, 51]. In 2007, we elucidated the double mechanism of action of G-CSF during OC-mediated liver regeneration in rats: G-CSF is able to contribute to liver repair by increasing the BM-derived liver repopulation *(vide infra)*, and also by activating the endogenous OCs, that express G-CSF receptor (G-CSFR) [52]. The upregulation of G-CSFR subsequent to G-CSF administration has been recently observed in a small-for-size liver model, confirming a direct receptor-mediated effect of G-CSF on the hepatic parenchyma [53]. These data expand the knowledge regarding the spectrum of actions of G-CSF on ASCs [51, 52]. In fact, it has been demonstrated that G-CSF and its receptor are widely present in neurons and adult neural stem cells. This expression is induced by ischemia, and both counteracts neuronal degeneration and contributes to long-term plasticity after damage. Similarly, G-CSF administration following myocardial infarction in mice induces G-CSFR expression in cardiomyocytes and results in the prevention of cardiac remodeling.

In summary, despite the efforts toward the characterization of human LSCs and of their niches, in view of a possible use for cell therapies, the microenvironmental factors driving LSC fate and LSCs themselves need to be univocally defined prior to attempting any clinical application. The identification of endogenous LSCs and of the signals that govern their proliferation and differentiation to mature hepatocytes might lead to the development of clinically feasible methods to induce liver repopulation from these endogenous cells and/or to allow maturation of stem/progenitor cells to hepatocytes *in vitro* and *in vivo*.

4. Extrahepatic Adult Stem Cells with Hepatogenic Potential

Liver regeneration is mainly an endogenous process, driven by hepatocytes and resident hepatic stem/progenitor cells. However, it has been observed that certain populations of extrahepatic ASCs can migrate into the liver and contribute to its repopulation and turnover [5]. A particularly high degree of plasticity has been shown by bone marrow stem cells (BMSCs), which can give rise to a wide range of phenotypes, including hepatocyte-like cells. Since the pioneering study by Petersen et al. in 1999 [54], numerous reports and reviews have been published on BM contribution to liver regeneration, often with contradictory conclusions [23, 55, 56]. It is generally agreed that BM represents a possible source of LSCs, even if the frequency of colonization, in the absence of a strong selective pressure, is very low, unlikely sufficient *per se* to achieve a significant contribution to hepatic repopulation. However, the few BM-derived cells which do engraft may play an important role in modulating the endogenous repair mechanisms within the hepatic stem cell niche [5, 27, 57]. As clearly demonstrated by Petersen's and our group in 2007, BMSCs may or may not play a critical role in liver regeneration, depending upon the experimental setting [58]. Based on the already cited model of lineage hierarchy within the LSC population [46], we can postulate that the periductular null cells might, at least in part, originate from extrahepatic SCs of BM origin and then give rise to the ductular cells of the CoH. These cells, in turn, can differentiate into intraductular cells and periductular hepatocyte-like cells.

Regarding the mechanisms underlying BMSC plasticity, upon engraftment BMSCs might either transdifferentiate into parenchymal cells or fuse with resident cells in the host tissue. Fusion phenomena between BMSCs and other cell types (i.e., Purkinjie cells, cardiomyocytes and hepatocytes) have been shown both *in vitro* and *in vivo* [5]. Cell fusion is a physiological phenomenon in certain districts, such as liver and muscle, and it may or may not play a prominent role in SC plasticity, depending on the model of injury and the host phenotype [59]. It has been also proved that fusion and transdifferentiation can coexist and produce therapeutically beneficial results [60, 61].

In order to initiate a BM response, the injured liver must signal to the responding cell types. A pivotal role in BMSC recruitment is played by SDF-1. BM-derived liver-committed SCs expressing SDF-1 receptor (CXCR4) are present in the peripheral blood and may colonize the damaged liver by following a SDF-1 gradient [62, 63]. Other molecules secreted by the injured hepatic milieu that can contribute to BMSC recruitment and homing into the liver are the hepatocyte growth factor (HGF), some fibrosis mediators, such as matrix metalloproteinase-9 (MMP9), and the G-CSF [64–66]. The mechanisms underlying the adhesion and retention of BMSCs to human liver compartments have been only in part elucidated [67].

Adult BM comprises two main populations of ASCs able to convert into hepatic cells, either by fusion or transdifferentiation: hematopoietic SCs (HSCs) and mesenchymal stem cells (MSCs).

HSCs are responsible for the renewal of blood cells and can be also isolated from umbilical cord blood and peripheral blood. It is generally accepted that the most primitive and long-term human HSCs are characterized by the expression of CD133, Thy1 (CD90) and VEGFR2 and by a variable expression of CD34 and CD38 [68]. BM-resident HSCs can be mobilized into the peripheral blood under specific stimuli such as tissue injury or administration of G-CSF [5]. Mobilized HSCs can colonize extramedullar sites and participate to their regeneration, by promoting the immune response and/or by transdifferentiating into ASCs within peripheral tissues [5, 18, 62].

MSCs, also called *stromal stem cells, mesenchymal progenitors, mesenchymal stromal cells, colony-forming unit-fibroblastic cells,* are highly proliferating, adherent cells, that reside in a perivascular BM niche and also in the wall of blood vessels within most organs [69]. Numerous studies have demonstrated that MSCs are able to differentiate into a variety of mesodermal cell lineages (osteoblasts, chondroblasts, adipocytes, myocytes, and cardiomyocytes), as well as nonmesodermal cells (such as hepatocytes and neurons), depending upon the microenvironment in which they reside [70]. MSCs might become a more suitable source for SC-based therapies than HSCs, because of their immunological properties: MSCs are less immunogenic and can induce tolerance upon transplantation [71]. Moreover, MSCs showed the highest potential for liver regeneration compared with other BM cell subpopulations in an animal model of hepatic injury [72].

A more recently identified SC population within the BM, the so-called *multipotent adult progenitor cells* (MAPCs), seems to be endowed with an impressive plasticity and has shown liver differentiation potential both in culture and in animal models [57]. These cells could potentially copurify with HSCs or MSCs and contaminate these cell populations investigated in liver repopulation studies. According to this hypothesis, rather than being a source of liver-committed SCs, BM could act as a hide out for recirculating pluripotent SCs that might be deposited early during development in BM and could be a source for tissue/organ regeneration [62, 73]. Therefore, the present distinction between HSCs and MSCs may become obsolete, given the heterogeneity and possible overlaps of these various BMSC populations, which could share a common *stem cellness* core [5].

As a closing remark, it is worth a note that adipose tissue has been reported as a rich source of easily accessible MSCs (*adipose tissue stromal cells*, ATSCs) capable of hepatic differentiation *in vitro* and *in vivo* [74, 75]. ATSCs are similar to BM-MSCs in terms of surface antigen marker profile and differentiation potential, and ATSCs have been reported to exert an even higher proliferative capacity *in vitro* [76, 77]. We have recently achieved the hepatogenic conversion of ATSCs, using a two-step protocol with sequential addition of growth factors. In order to understand the molecular events involved in ATSC hepatic transdifferentiation, the full genome expression profiles of ATSC-derived hepatocyte-like cells *versus* undifferentiated ATSCs were compared. We identified several targets that depict the numerous biological functions exerted by the liver, including protein metabolism, innate immune response regulation, and biodegradation of toxic compounds. Moreover, we showed that ATSC differentiation into hepatocyte-like cells might be caused by a mesenchymal-to-epithelial transition [78].

Overall, despite an incomplete knowledge of their biological properties, the plasticity and accessibility of HSCs and MSCs from BM and adipose tissue render these ASCs an attractive tool for the regenerative medicine.

5. Stem Cell-Based Therapies in Hepatology

As previously discussed, different types of SCs with hepatic differentiation potential are theoretically eligible for liver cell replacement. These include ESCs, iPSs, hepatoblasts, annex SCs, and adult SCs, such HPCs, HSCs, and MSCs. Despite encouraging results *in vitro*, the use of hepatocyte-like cells derived from these stem/progenitor cell populations is still confined to preclinical studies, given the scarce tissue-specific functionality and, up to now, the lack of evidence of strong liver repopulation levels in animal models. Nowadays, the most promising source for SC-based therapies is represented by the intraportal or intrahepatic infusion of freshly isolated or *in vitro* expanded HSCs [79]. Another appealing option is represented by the administration of mobilizing/proliferating agents, such as G-CSF, that is able to both enhance the HSC mobilization into the peripheral blood and facilitate the endogenous LSC activation [5]. G-CSF administration could also exert beneficial immunomodulatory effects in presence of liver failure, since it can reverse the neutrophil defects and the status of *immune paralysis* associated with severe hepatic insufficiency [49, 50, 80].

5.1. Bone Marrow Stem Cell Transplantation. BMSCs seem to be physiologically involved in liver repair in humans. A spontaneous mobilization of $CD34^+$ cells has been reported following liver resection in patients with primary liver cancer or metastasis [81]. Similarly, a significant increase in the percentage of $CD133^+$ cells has been found by Gehling et al. in blood samples of healthy living liver donors and further *in vitro* investigations have demonstrated that the mobilized cells were indeed liver committed [82]. Recently, the same

authors observed that liver cirrhosis is associated with an intermittent mobilization of various populations of liver-committed cells of putative BM origin into the circulation [83].

The possible therapeutic interest of BMSCs in hepatology was firstly investigated in 2005: intraportal autologous transplantation of $CD133^+$ BMSCs in patients with liver cancer undergoing portal embolization before extensive liver resection (LR) achieved some clinical improvement [84]. Similar results were obtained two years later by Fürst et al. who concluded that in patients with malignant liver lesions a combination of portal vein embolization and $CD133^+$ BMSC administration increased the degree of hepatic regeneration in comparison with embolization alone [85]. In 9 patients with cirrhosis, who received portal vein infusion of unsorted autologous BMSCs, an improvement in Child-Pugh score and albumin levels was reported [86]. Recently, a significant increase of liver function postLR has been documented in patients with cirrhosis and hepatocellular carcinoma, following autologous BMSC transplantation prior to surgery [87]. So far, only one negative result regarding BMSC-therapies for end-stage liver disorders has been reported: in a phase-I clinical trial on decompensated cirrhotic patients, the infusion of autologous $CD34^+$BM cells through the hepatic artery was unsafe and ineffective in improving the liver function [88].

5.2. Circulating Hematopoietic Stem Cell Mobilization, Collection and Reinfusion. Other SC-based therapies in hepatology have been based upon the collection from peripheral blood by leukapheresis and subsequent reinfusion of circulating HSCs, mobilized by G-CSF administration. The feasibility, safety, and pattern of BMSC mobilization with G-CSF in patients affected by cirrhosis has been evaluated [89]. Yannaki et al. described boost infusions of mobilized $CD34^+$ cells after a standard G-CSF regimen in two patients. The procedure was safe, well tolerated and associated with a lasting amelioration in the clinical course of the disease during the follow-up (1 month) [90]. A significant biochemical and histopathological improvement was achieved by intraportal administration of mobilized $CD34^+$ BMSCs following G-CSF exposure in one patient affected by drug-induced acute liver failure [91]. In a phase-I clinical trial on 5 patients with acute on chronic liver failure, G-CSF administration, followed by collection and intraportal or intrahepatic reinfusion of circulating $CD34^+$ cells, resulted in an improvement of the hepatic function in more than 50% of the cases, without significant side effects during a follow-up of 60 days [92]. The same patients were then monitored for up to 18 months: the procedure was safe in short and over long term, by absence of tumor formation and the beneficial effects lasted around 12 months [93]. Recently, in 9 patients with alcohol-related cirrhosis, the reinfusion into the hepatic artery of CD34+ BM-derived cells, collected after G-CSF mobilization and *in vitro* expanded, was well-tolerated and produced a clinical and biochemical improvement [94]. In another trial, a total of 40 patients with HBV-related cirrhosis were randomized to receive G-CSF alone or in combination with leukapheresis and reinfusion of peripheral blood monocytes into the hepatic artery. During a follow-up of 6 months, a significant biochemical and clinical improvement was observed in both groups, even though the subjects receiving G-CSF *plus* SC infusion obtained the greater and longer-lasting beneficial effects [95].

5.3. G-CSF Treatment. Given its beneficial role in hepatic regeneration, G-CSF alone has been employed for the treatment of end-stage liver diseases in humans. Gaia et al. treated 8 patients affected by severe liver cirrhosis with G-CSF 5 μg/Kg bid for three days: the treatment was well tolerated in all patients during a follow-up of 8 months, and a mobilization of BMSCs coexpressing epithelial and stem markers was noted [89]. Our group administered G-CSF (5 or 15 microgr/Kg/day) for 6 days to 24 patients with severe liver cirrhosis. This procedure was safe, resulted in a dose-dependent mobilization of BMSCs, but did not achieve any significant clinical improvement [96]. Similarly, Lorenzini et al. treated 18 nondecompensated cirrhotic patients with G-CSF, obtaining a good $CD34^+/CD133^+$ cell mobilization, despite the absence of clinical improvement [97]. Spahr et al. recently published the largest randomized trial, conducted on 24 patients with alcoholic cirrhosis, randomized to standard care associated with G-CSF or standard care alone. G-CSF was safe and able to mobilize $CD34^+$ cells and increase HGF; however, the study was too small to make any comment regarding survival or efficacy. Interestingly, G-CSF was associated with the induction of HPC proliferation within 7 days of administration [98].

Most of the above-mentioned clinical trials share common limits, being conducted on small groups of patients, without controls, and using outcome parameters easily subjected to be biased, as reviewed elsewhere [5, 7, 23, 99–103]. Overall, the use of BMSCs for the treatment of end-stage liver diseases holds several advantages, such as easy accessibility, unlimited supply and no risks of rejection or need for immunosuppressive therapies, when autologous cells are employed. Nonetheless, some conceptual issues still limit the diffusion of such treatments in the clinical practice.

(1) On the basis of the previously reported preclinical data, BM cells seem to facilitate liver regeneration mainly by a microenvironment modulation, which is likely to be transitory. In such a case, multiple treatments would presumably be required to achieve significant and lasting clinical results; technical issues that need to be addressed regard the surface antigens used for HSC purification, the route of delivery, the amount of infused cells and the timing of infusions.

(2) It is not clear at present whether mobilization with G-CSF without leukapheresis and reinfusion of HSCs would suffice to gain a significant clinical benefit. Moreover, G-CSF therapies need to be standardized in terms of dosage, timing, and eventually association with other cytokines or interventions.

(3) The possibility of cell fusion and the risk of malignant transformation of the transplanted cells, especially if *in vitro* pre-expanded before reinfusion, cannot be excluded and impose a careful evaluation and longer follow-up periods for assessing the safety and efficacy of SC-based treatments.

(4) BMSCs have the potential to differentiate into endothelial cells and fibroblasts within the liver, and, as such, they might exert a profibrogenic effect [57]. Therefore, it is mandatory to examine the involvement of the infused SCs in the reconstitution of hepatic nonparenchymal cells.

Until these open questions can be properly answered, through an intense collaborative effort from basic cell biologists, translational scientists and clinicians, SC-based therapies for liver diseases should be limited to well designed and adequately powered clinical trials.

6. Conclusions

SCs are promising tools at the service of regenerative medicine for the treatment of degenerative disorders, inborn errors of metabolism, and organ failure. In hepatology, the first attempts to translate SC basic research into new clinical strategies for the treatment of acute and chronic hepatopathies have been made. In particular, HSCs transplantation and G-CSF infusion are an attractive option for the treatment of end stage liver pathologies, as we already handle G-CSF and transplant HSCs in clinics, for hematologic and oncologic disorders. Right now, the major role for stem cell therapy is as a bridge to transplantation or as a way of maintaining those patients who are not eligible for OLT. Nonetheless, critical aspects need to be further addressed, including the long-term safety, tolerability, and efficacy of these SC-based treatments, as well as their carcinogenic potential. As a consequence, it is paramount to conduct larger and well-designed clinical trials to fully establish the safety profile of such therapies and to define the target patient groups with efficacy assessed by standardised protocols.

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SAGE-Hindawi Access to Research
Stem Cells International
Volume 2010, Article ID 369578, 8 pages
doi:10.4061/2010/369578

Review Article

Progenitor Cell Therapy for the Treatment of Central Nervous System Injury: A Review of the State of Current Clinical Trials

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Received 11 May 2010; Accepted 10 June 2010

Academic Editor: Bruce A. Bunnell

Recent preclinical work investigating the role of progenitor cell therapies for central nervous system (CNS) injuries has shown potential neuroprotection in the setting of traumatic brain injury (TBI), spinal cord injury (SCI), and ischemic stroke. Mechanisms currently under investigation include engraftment and transdifferentiation, modulation of the locoregional inflammatory milieu, and modulation of the systemic immunologic/inflammatory response. While the exact mechanism of action remains controversial, the growing amount of preclinical data demonstrating the potential benefit associated with progenitor cell therapy for neurological injury warrants the development of well-controlled clinical trials to investigate therapeutic safety and efficacy. In this paper, we review the currently active or recently completed clinical trials investigating the safety and potential efficacy of bone marrow-derived progenitor cell therapies for the treatment of TBI, SCI, and ischemic stroke. Our review of the literature shows that while the preliminary clinical trials reviewed in this paper offer novel data supporting the potential efficacy of stem/progenitor cell therapies for CNS injury, a great deal of additional work is needed to ensure the safety, efficacy, and mechanisms of progenitor cell therapy prior to widespread clinical trials.

1. Introduction

Central nervous system (CNS) insults including ischemic stroke, traumatic brain injury (TBI), and traumatic spinal cord injury (SCI) represent a major burden to the health-care system worldwide. Approximately 5 million people are burdened by the long-term physical, cognitive, and psychosocial deficits associated with TBI in the United States with 40% of patients reporting unmet needs 1 year post injury [1]. Furthermore, a recent analysis of stroke patients has shown a lifelong health burden of 9.5 quality adjusted life years associated with an initial cerebrovascular accident [2]. Overall, the economic impact of traumatic and acute CNS insults reaches several billion dollars in the United States alone [3, 4].

Preliminary research is currently underway to evaluate the potential efficacy of adult tissue progenitor (stem) cell therapies for the treatment of CNS injury. Adult tissue progenitor cells are located in select microenvironments (niches) which protect against overproliferation and depletion as well as regulate progenitor cell involvement in resident tissue repair and regeneration [5]. By definition, progenitor cells are multipotent with the capacity of self-renewal [6] making them prime candidates for the development of

novel therapies. Our paper will focus on progenitor cell populations derived from the bone marrow and umbilical cord blood niches including mesenchymal stromal cells (MSCs) and hematopoietic stem cells (HSCs). There are currently no clinical trials utilizing embryonic stem cells or induced pluripotent stem cells for TBI, stroke, or SCI.

2. Therapeutic Mechanism

Recent preclinical work investigating bone marrow derived-progenitor cell therapies for CNS injury has shown potential neuroprotection after TBI [7], ischemic stroke [8], and SCI [9]. While initial research indicated engraftment and transdifferentiation into neural cells could explain the observed benefit [10], the exact mechanism remains controversial. Potential mechanisms currently under investigation include engraftment and transdifferentiation, modulation of the locoregional inflammatory milieu, and modulation of the systemic immunologic/inflammatory response.

2.1. Engraftment and Transdifferentiation. Preliminary research completed in the Chopp laboratory showed both motor and cognitive improvement after the intravenous injection of MSCs in a rodent TBI model. The injected MSCs were found to migrate towards the site of injury, engraft, and display neuronal (neuronal nuclei (NeuN)) and astrocytic (glial fibrillary acidic protein (GFAP)) markers [11]. Hayase et al. have successfully induced MSCs to form neural spheres *in vitro* with subsequent implantation into rodent cortex after focal ischemic injury. The implanted progenitor cells were found to display neural cell markers and engraft up to 100 days after injury with associated behavioral recovery [12]. The Ha laboratory implanted umbilical cord blood-derived progenitor cells (HUCBCs) into the injury site after spinal cord contusion in a rodent model. The HUCBCs were found to engraft at the site of injury and differentiate into neural cells as evident by GFAP and microtubule-associated protein 2 (MAP2) staining. Locomotor testing showed functional improvement for all time points tested up to 8 weeks after SCI [13].

Despite the promising research showing engraftment and transdifferentiation of transplanted progenitor cells into neural cells, the importance of engraftment and frequency of transdifferentiation remain extremely controversial [14, 15]. The implantation of HSCs into murine striatum [16] and injury zone of spinal cord contusion [17] showed differentiation into macrophages or microglia but failure to transdifferentiate into neurons. Furthermore, the implantation of MSCs into demyelinated spinal cord showed migration into normal tissue and failure of transdifferentiation associated with collagen deposition and further axonal injury [18]. While the direct implantation of progenitor cells with transdifferentiation into neurons could afford neuroprotection, the low frequency of engraftment and differentiation may limit this pathway as a mechanism for functional recovery.

2.2. Modulation of the Locoregional Inflammatory Response. Progenitor cell migration towards the site of injury and interaction with resident microglia could modulate the locoregional inflammatory response leading to enhanced neuroprotection. Harting et al. investigated the local intracerebral inflammatory response after TBI by completing a series of rodent cortical injuries followed by the harvest of intracerebral fluid from 6 to 72 hours after injury. Multiplex cytokine analysis of the intracerebral fluid showed an increase in the proinflammatory cytokines IL-1α, IL-1β, IL-6, and TNFα in the direct injury and penumbral areas [19]. The observed increase in cytokine production offers an attractive target for novel cell therapies.

Coculture of human MSCs with immunologic cells (dendritic cells and T cells) was associated with an increase in the anti-inflammatory cytokines interleukin 4 (IL-4) and interleukin 10 (IL-10) in accordance with a decrease in the proinflammatory cytokine interferon gamma (IFN-γ). An increase in the proportion of T regulatory cells with MSC coculture was also observed [20]. Walker et al. directly implanted MSCs into the cortex of rodents after TBI and found an increase of interleukin 6 (IL-6) in brain tissue supernatants. Subsequently, a series of *in vitro* MSC and neuronal stem cell (NSC) cocultures showed activation of the NSC NFκB pathway leading to a decrease in apoptosis [21]. The Wen laboratory has shown increased intracerebral IL-10 with decreased tumor necrosis α (TNFα) leading to improved cognitive performance after the direct implantation of MSCs using a rodent ischemic stroke model [22]. These promising preliminary studies have shown that modulation of the locoregional postinjury proinflammatory environment could afford neuroprotection.

2.3. Modulation of the Systemic Immunologic Response. Recent work completed in the Pennypacker laboratory has shown that the adrenergic output associated with rodent ischemic stroke leads to the release of immunologic cells (T cells) from the spleen into the systemic circulation causing a reduction in splenic mass and an increase in cavity volume. Treatment with the panadrenergic blocker carvediol prevented the loss of splenic mass and decreased cavity volume [23]. Using a similar model, Verdrame et al. have shown that the immunologic cells released into the systemic circulation are mainly composed of cytotoxic CD8+ T cells potentially exacerbating neurological injury observed with ischemic stroke. The intravenous injection of HUCBCs in the stroke model also prevented the loss of splenic mass and decreased cavity size likely via a reduction in CD8+ cell release [24].

Additional research investigating the potential interaction between transplanted progenitor cells and lung immunologic cells is currently underway. Using a murine sepsis model, the Mezey laboratory has shown that the intravenous injection of MSCs is associated with decreased mortality and improved organ function. The observed benefit was derived from interactions between the injected MSCs and lung macrophages leading to increased IL-10 production via a prostaglandin E2-dependent mechanism. Furthermore, the beneficial effect was eliminated by the administration of antibodies to either IL-10 or the IL-10 receptor thereby confirming the importance of anti inflammatory cytokine production in therapeutic efficacy [25].

There is limited data on the interaction of implanted adult progenitor cells with other organ systems in the setting of neurologic injury. Distribution studies have demonstrated localization of implanted cells to liver and kidney in addition to the more commonly described spleen and lung after intravenous or intra-arterial administration [26–29]. To our knowledge, there is no published data on the liver and/or kidney acting as potential bioreactors for cytokine/growth factor secretion after progenitor cell therapy for neurologic injury.

Preliminary preclinical work investigating the potential role of progenitor cell therapeutics for CNS injury has shown promise. The mechanism of the observed benefit remains controversial; however, more recent data questions the frequency and clinical significance of transdifferentiation as well as the volume of cells reaching the injury site due to a significant pulmonary first-pass effect [30]. We believe that a more plausible explanation is that for some types of cell-based therapies, the transplanted cells are interacting with distant organ systems leading to alteration in the systemic inflammatory/immunologic response. Progenitor cells could interact with resident lung macrophages and splenic T cells leading to an increase in anti inflammatory cytokine production. The observed increase in systemic anti inflammatory cytokine concentrations may affect the resident brain microglia accounting for the observed therapeutic benefit.

3. Clinical Trials

The growing amount of preclinical data showing the potential benefit associated with progenitor cell therapy warrants the development of well-controlled clinical trials to investigate therapeutic safety and efficacy for central nervous system insults such as ischemic stroke, SCI, and TBI. Below we review the preliminary clinical trials investigating progenitor cell therapy for CNS insults completed to date.

3.1. Ischemic Stroke. Stroke is a leading cause of long-term disability in the United States [31]. Intravenous tissue plasminogen activator (tPA) is the only proven treatment for acute ischemic stroke within the first three hours of symptom onset [32]. Cell-based therapies have emerged as a novel and highly promising investigational approach to enhance recovery after stroke in animal models [33–37]. The encouraging preliminary results have led several investigators to launch clinical trials evaluating the safety of cell-based therapies in stroke patients. The safety, feasibility, ideal cell type, optimal dosage, and most favorable delivery method of cells are currently unknown.

Savitz et al. are currently conducting a prospective, Phase 1 trial (http://www.clinicaltrials.gov/ Identifier: NCT00859014) evaluating the safety of bone marrow aspiration and infusion of bone marrow mononuclear cells (BMMCs) in adults within 24–72 hours of ischemic stroke. Primary outcome measures include a series of short- and long-term safety assessments with a secondary evaluation of neurological function as measured up to 90 days after injury. Autologous BMMCs are administered via peripheral intravenous injection followed by serial measurements of hemodynamic variables to assess immediate postinfusion safety. Selected inclusion criteria are middle cerebral artery (MCA) territory infarct, age between 18 and 80 years, and National Institutes of Health Stroke Scale (NIHSS) between 6 and 20.

Lin et al. (China University Hospital, Taichung, Taiwan) have recently completed a Phase 1 clinical trial ensuring the safety of the direct intracerebral transplantation of CD34+ progenitor cells in stroke patients. The CD34+ progenitor cells were obtained from peripheral blood of patients with MCA strokes occurring within the past 6 to 60 months. Currently, a Phase 2 trial is recruiting patients to determine the potential efficacy of CD34+ cell implantation (http://www.clinicaltrials.gov/ Identifier: NCT00950521). The treatment group is to receive conventional rehabilitation as well as the direct implantation of CD34+ progenitor cells with the control group receiving conventional rehabilitation alone. The primary outcome is NIHSS scores collected serially for up to 12 months. Inclusion criteria are patients between 35 and 70 years old and an NIHSS between 9 and 20. The investigators plan to enroll 60 patients and is currently underway.

Hernández et al. (Hospital Universitario Central de Asturias, Asturias, Spain) are currently enrolling patients for a Phase 2 trial investigating the safety and efficacy of the intra arterial delivery of autologous CD34+ progenitor cells into the MCA after ischemic stroke. Cellular harvest and injection occurs between 5 and 9 days after the onset of stroke symptoms. Patient hemodynamics and neurologic status are monitored in the acute setting with follow up exams up to 6 months after treatment. Adverse events are classified as any worsening of the neurologic exam. Therapeutic efficacy is determined via serial physical, laboratory, and radiographic exams. Selected inclusion criteria are symptoms and signs of clinically definite MCA acute stroke (http://www.clinicaltrials.gov/ Identifier: NCT00761982).

André et al. (Federal University of Rio de Janeiro, Rio de Janeiro, Brazil) are conducting a Phase 1 clinical trial investigating the intravenous and intra arterial injection of the autologous bone marrow-derived mononuclear cell fraction within 90 days of MCA stroke. A cell dosage of up to 500×10^6 mononuclear cells will be used. Patients will be monitored with serial physical and radiographic exams up to 4 months after treatment. Adverse events will be recorded as any worsening in neurologic exam. Transcranial doppler will be used during intra arterial injection to ensure adequate blood flow in the middle cerebral artery. Improvement in neurologic deficits and neuroimaging will be recorded as secondary outcome measures during the study time period. Patients who are between 18 and 75 years old with an MCA infarct documented on imaging, and NIHSS scores between 4 and 20 are eligible (http://www.clinicaltrials.gov/ Identifier: NCT00473057).

Habib et al. (Imperial College London, London, England) are completing an additional Phase 1 trial investigating the safety of intra arterial injection of the autologous bone marrow CD34+ progenitor cell population into the MCA of patients with acute anterior circulation strokes. Safety is to be assessed by physical exam and laboratory parameters.

Table 1: Listing of location and details of current clinical trials being completed to investigate the potential role of bone marrow-derived progenitor cell therapeutics for the treatment of ischemic stroke.

Location of Study	Study Design	Deliver Route	Sample Size	Cell Type	Inclusion Criteria	Outcomes	Time Window
United States (The University of Texas in Houston)	Single arm	IV	10	Autologous BMMCs	- MCA stroke - 18–80 yo - NIHSS 6 to 20	Safety and feasibility	24 to 72 hrs
Taiwan (The China Medical University Hospital)	Randomized (cell infusion versus conventional treatment)	IC	30	Autologous peripheral blood CD34+ cells	- Stable deficits hemiplegia - 35–70 yo - NIHSS 9 to 20	Safety and efficacy	6 months to 5 years
Spain (Hospital Universitario Central de Asturias)	Single arm	IA	20	Autologous CD34+ bone marrow cells	- MCA stroke - 18–80 yo - NIHSS $\geq$ 8	Safety	5 to 9 days
France (University Hospital of Grenoble)	Randomized (Control versus 2 treatment groups)	IV	30	Autologous bone marrow derived progenitor cells	- Carotid territory stroke - 18–65 yo - NIHSS > 2	Feasibility and tolerability	6 weeks
United Kingdom (Imperial College London)	Single arm	IA	10	Autologous CD34+ bone marrow cells	- MCA stroke - 30–80 yo - Severe stroke conforming to the TACS phenotype (weakness, homonymous hemianopia and a focal cognitive deficit	Safety and tolerability	7 days
Brazil (Federal University of Rio de Janeiro)	2 arms (non randomized: 10 IA/5IV)	IV/IA	15	Autologous BMMCs	- MCA stroke - 18–75 yo - NIHSS 4 to 20	Safety	3 hrs to 90 days

IV: intravenous; IA: intra-arterial; IC: intracerebral; BMMC: bone marrow mononuclear cells; MCA: middle cerebral artery; NIHSS: National Institutes of Health Stroke Scale; TACS: total anterior circulation stroke.

Improvement in clinical function as assessed by the Modified Rankin Score and NIHSS is secondary outcome that will be evaluated. Selected inclusion criteria include a clinically definite acute stroke with known onset time, ability to start treatment within 7 days of onset, and patients between 30 and 80 years old. Data collection for the trial is set to be completed in May 2010 (http://www.clinicaltrial.gov/ Identifier: NCT00535197).

Preliminary clinical trials investigating the role of cell therapeutics for ischemic stroke have been limited to date and powered only to evaluate safety (Table 1). The majority of these studies are restricting enrollment to patients with MCA infarcts territory and do not assess the role of these cells in other areas of the brain. No optimal method of delivery has been established, and it is unclear whether intravenous, intra-arterial, or other approaches may be safer and lead to better outcomes. Additionally, the studies employ different outcome measures limiting the ability to compare results among trials. While these preliminary studies have yielded some data to support the safety of cellular transplantation, additional trials need to be completed prior to controlled multicenter trials. A recent consensus conference (STAIR) was convened to discuss the future of clinical trials in stroke. The consensus highlighted the need for well-designed clinical trials with cell therapy being an excellent candidate [38].

3.2. Traumatic Brain Injury. A search of the Clinicaltrials.gov database identified 279 ongoing or recently completed clinical trials for patients with TBI (search performed 3/31/2010). The treatments were focused on both acute therapy, as well as ongoing or chronic therapy, and included (but were not limited to) medications (i.e., amantadine, carbamazepine, oxycyte, or erythropoietin), hyperbaric oxygen, hypothermia, educational interventions, and physical rehabilitation. Using the search terms "stem cell" and "brain" identified nearly 300 studies, including studies evaluating the use of adult stem cells (administered via various routes, in various numbers, and using various cell types) to treat hypoxic/ischemic encephalopathy, cerebral palsy, multiple sclerosis, amyotrophic lateral sclerosis, neuronal ceroid lipofuscinosis, Parkinson's disease, and others.

A single Phase I study using bone marrow-derived mononuclear cells in children after isolated TBI has recently been completed. In this study, 10 children age 5–14 years with a Glasgow coma scale score of 5–8 were treated with 6×10^6 bone marrow-derived mononuclear cells per kg body weight delivered intravenously within 48 hours of an isolated TBI. To determine the safety of administration, systemic and cerebral hemodynamics, laboratory parameters, chest radiographs, and serial clinical assessments were monitored. Additionally, serial cerebral magnetic resonance imaging,

neuropsychologic evaluation, and functional outcome measures were obtained as preliminary measures of efficacy.

There were no identifiable adverse events with close monitoring of the neurologic, pulmonary, renal, hepatic, and hematologic systems. Functional and neuropsychological testing, including the Glasgow Outcome Scale, the Pediatric Injury Functional Outcome Scale, and the Wechsler Abbreviated Scale of Intelligence, revealed recovery consistent with (or improved from) expected baselines. Magnetic resonance imaging volumetric data revealed no significant change in grey matter, white matter, intracranial volume, or CSF space at 1 and 6 months as measured relative to expected norms [39].

This study should open the door for translation of cell therapies, particularly among patients with neurologic diseases and among pediatric patients. Given the apparent safety of this study, the development of larger, multicenter studies to further assess dosing and efficacy of autologous cell therapy for TBI is underway. Additionally, similar (more dispensable) progenitor cell populations, such as cord blood cells, may be safe and efficacious as well and warrant further study.

3.3. Spinal Cord Injury

3.3.1. Human Trials Using Autologous BMMC Delivered by Intravenous or Intra-Arterial Infusion. A trial comparing autologous BMMC intravenous transplantation plus physical therapy to physical therapy only in patients with chronic SCI has been reported from Al-Azhar University Military Medical Academy in Cairo, Egypt. No outcomes data has been published on this trial.

Syková et al. treated two groups of patients with autologous BMMC via either intravenous infusion or intra-arterial infusion through the vertebral artery. In the first group (subacute group), cell therapy was delivered between 10 and 33 days following SCI. The second group (chronic group) was treated between 2 and 18 months after SCI. Of the eight subacute patients, four were treated intravenously and four via an intra-arterial route. Two of the chronic patients were treated intra-arterially and the remaining ten patients were treated intravenously. Patients were evaluated 3, 6, and 12 months post BMMC treatment. All four of the subacute patients treated via the intra-arterial route and one of the four treated intravenously experienced improvement in the American Spinal Injury Association (ASIA) score. One of two chronic patients treated via an intra-arterial route experienced an improvement in ASIA score, but none of the remaining 10 chronic patients treated with intravenous administration of BMMCs improved [40].

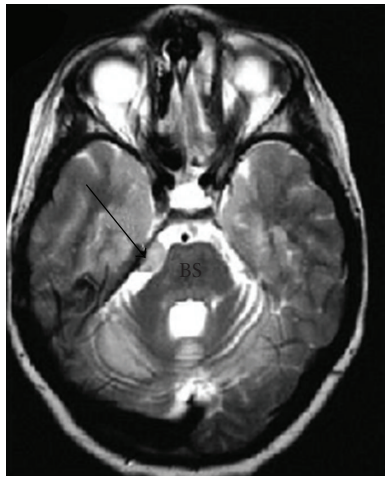
Cristante et al. treated 39 patients with chronic SCI using peripheral stem cell mobilization (granulocyte monocyte colony stimulating factor (GM-CSF) treatment) and subsequent collection by apheresis. The patients all had complete SCI of two or more years duration. At least 2.5×10^6 CD34+ cells per kg body weight were collected. Cells were delivered intra-arterially into the anterior spinal artery at or near the level of their SCI. Patients were followed with serial somatosensory-evoked potential (SSEP) testing over 30 months. Overall 66.7% of patients experienced improved latency on SSEP evaluation. No difference in response rates was identified between paraplegic or quadriplegic patients [41].

Our group has recently obtained an investigational new drug (IND) application to treat chronic (greater than 6 months post injury) pediatric SCI with autologous BMMC via intravenous infusion. Patients will receive pre- and serial posttreatment neurologic examinations, ASIA Scale ratings, SSEP testing, cystometrogram (CMG) testing, and spinal magnetic resonance imaging with diffusion tensor tractography. We expect to begin enrolling patients by late summer 2010.

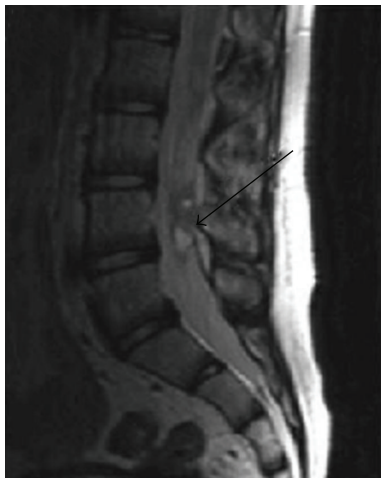
3.3.2. Human Trials Using Autologous BMMC Delivered by Lumbar Puncture. Callera et al. treated a total of 10 patients with established SCI. Patients were pretreated with GM-CSF for 5 days and then treated with 100×10^6 autologous BMMC by lumbar puncture (LP). Patients underwent repeat LP 7 days posttreatment and the repeat LPs were reported to be normal. No mention of functional outcome was reported [42]. The same group of investigators treated 16 cases of chronic SCI with either autologous bone marrow-derived CD45+ cells labeled with iron nanoparticles (10 patients) or iron nanoparticles only (6 patients) by lumbar puncture. Serial magnetic resonance imaging scans performed following treatment demonstrated cell migration to the edges of the SCI in 5 of the cell-treated patients but none of the nanoparticles only treated patients [43].

3.3.3. Human Trials Using BMMC Delivered by Direct Injection or Surgical Implantation into the Injured Spinal Cord. Deda et al. treated 9 patients with complete SCI (ASIA Grade A) using a processed autologous bone marrow preparation (cells harvested in Turkey, shipped to Ann Arbor, Michigan for processing, and returned to Turkey for cell infusion). Cells were delivered by intravenous infusion, direct injection into the spinal cord above and below the injury site, and by a cell-infused matrix implanted surgically into the injury site. The authors reported improvement to ASIA Grade B of C in treated patients, improved SSEP latencies in treated patients and no adverse events [44].

Yoon et al. treated a total of 35 patients with SCI using direct injection of BMMCs into six sites surrounding the spinal cord injury. Patients were divided into acute (treated within 2 weeks of injury), subacute (treated between 2 and 8 weeks from injury), and chronic (treated greater than 8 weeks from injury) treatment groups. All treated patients also received GM-CSF treatment for 5 months after treatment. A control group of 13 patients who underwent surgery without BMMC or GM-CSF treatment was included in the study. Neurologic improvement (ASIA A to ASIA B or C) was reported in roughly 30% of the acute and subacute treatment groups, but not in in the chronic treatment control groups. Neurologic improvements were greater in patients with the greatest leukocytosis following GM-CSF treatment. Neuropathic pain occurred in a third of the subacute and chronically treated patients but in only one of sixteen acutely treated patients. One control group patient developed neuropathic pain [45].



(a)



(b)

Figure 1: (a) Brain MRI demonstrating a lesion (arrow) based on the tentorium next to the brain stem (BS). (b) Spinal-lumbar MRI (T2) showing an intradural lesion (arrow) at the level of the L4 vertebra. Reproduced with permission.

3.3.4. Human Trials Using Embryonically Derived Stem Cell Products. Considerable regulatory caution has been exercised when human trials using embryonically or fetally derived stem cell products are proposed. Although these more immature cell types have the theoretical advantage of pluripotency, they have also been associated with tumor formation. A case report from Israel describing the development of multifocal CNS glioneuronal tumors following treatment of a child with ataxia telangiectasia using fetal neural stem cells (obtained from multiple human fetuses) has caused researchers and regulators to move cautiously in this area. The tumors were shown to have developed from the transplanted fetal tissue (Figure 1) [46].

Geron Corporation (Menlo Park, Calif, USA) has started a United States Food and Drug Administration (FDA) approved trial using embryonically derived oligodendroglial precursor cells (identical to those used by Kierstadt) to replace myelin-forming cells within injured spinal cords. The cell preparations are injected directly into the spinal cords at the lesion site. The treatment population is restricted to adults with complete thoracic (T3–T9) level SCI. Patients must undergo treatment within 7 to14 days following injury. Preclinical data showing that SCI animals treated with Geron's cell line developed cysts at the level of treatment caused the FDA to put a hold on the clinical trial. Geron and the FDA have reached an agreement to allow the trail to move forward if subsequent preclinical studies provide satisfactory outcome.

4. Conclusions

Prior to large, multicenter clinical trials investigating the potential efficacy of progenitor cell therapies for CNS insults, a number of issues need to be addressed. Further research into optimal cell dosing, cell delivery method, and techniques for *in vivo* cell tracking need to be completed to ensure the safety of potential trials while affording them the best possible chance at success. Additional preclinical work to more clearly delineate the progenitor cell mechanism of action would also aid in the planning of quality-controlled clinical studies. Overall, while the very preliminary clinical trials reviewed in this paper offer novel data supporting the potential efficacy of cell therapeutics for CNS injury, a great deal of additional work is needed to ensure the safety and efficacy of progenitor cell therapy prior to widespread clinical trials.

Acknowledgments

This work was supported by Grants nos. NIH T32 GM 08 79201; M01 RR 02558; Texas Higher Education Coordinating Board; Children's Memorial Hermann Hospital Foundation.

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