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Pregnancy Loss Following Coxsackievirus B3 Infection in Mice during Early Gestation Due to High Expression of Coxsackievirus-Adenovirus Receptor (CAR) in Uterus and Embryo

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Abstract: Coxsackieviruses are important pathogens in children and the outcomes of neonatal infection can be serious or fatal. However, the outcomes of coxsackievirus infection during early gestation are not well defined. In this study, we examined the possibility of vertical transmission of coxsackievirus B3 (CVB3) and the effects of CVB3 infection on early pregnancy of ICR mice. We found that the coxsackievirus and adenovirus receptor (CAR) was highly expressed not only in embryos but also in the uterus of ICR mice. CVB3 replicated in the uterus 1 to 7 days post-infection (dpi), with the highest titer at 3 dpi. The pregnancy loss rate in mice infected with CVB3 during early gestation was 38.3%, compared to 4.7% and 2.7% in mock-infected and UV-inactivated-CVB3 infected pregnant mice, respectively. These data suggest that the uterus and embryo, which express abundant CAR, are important targets of CVB3 and that the vertical transmission of CVB3 during early gestation induces pregnancy loss.
Key words: CAR, coxsackievirus, early gestation, pregnancy loss

Introduction

Many intrauterine viral infections are associated with abortion, congenital anomalies, fetal death, and perinatal infection. Well-known causative agents of intrauterine infection include rubella virus, cytomegalovirus, herpes simplex virus, parvovirus B19, and varicella-zoster virus [30]. Clinical evidence is emerging that intrauterine coxsackievirus B (CVB) infection during late gestation or the perinatal period leads to life-threatening diseases such as neonatal myocarditis, meningitis, hepatitis, encephalitis, long-term neurological deficits, or sudden death [4, 11, 16, 19, 24, 27, 32, 33, 40]. Despite the significant morbidity and mortality associated with perinatal CVB infection, limited data about the clinical outcomes of CVB infection during early gestation are available [3, 18]. More than 30% of all pregnancies in healthy women are spontaneously aborted during the early gestational period and the causes of these spontaneous abortions in many cases are unknown [41]. In addition, the causes of 40–50% of stillbirths are unknown. CVB infection is thought to be one of the causes

of spontaneous abortion; however, it is difficult to diagnose because it often goes undetected in adults, being either subclinical or producing non-specific symptoms.

To infect a cell, CVB can use the coxsackievirus-adenovirus receptor (CAR) and the decay accelerating factor (DAF) receptor [5–7]. CAR is a member of the immunoglobulin superfamily and a component of tight junctions [5, 13]. In the process of CVB3 infection, the binding of the virus to host cells leads to the abrogation of CAR expression at the cell surface [12]. Normally, CAR is highly expressed in the developing brain and essential for early embryonic cardiac development; however, CAR is naturally diminished during the neonatal period [2, 15, 20]. The present study was undertaken to investigate the hypothesis that embryos would be very susceptible to CVB3 infection and damaged if CVB3 infection occurred during early embryonic development, due to impairment of the normal physiological functions of CAR. Therefore, we examined CAR expression and CVB3 replication in embryos and the uterus to investigate the vertical transmission of CVB3 and the outcomes of CVB3 infection during early gestation.

Materials and Methods

Cells and viruses

CVB3 (Nancy Strain, ATCC No. VR-30) was propagated in HeLa cells and stored at −80°C. HeLa cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), penicillin (100 μg/ml), and streptomycin (100 μg/ml) (Lonza, Rockland, ME, USA) at 37°C in a humidified atmosphere containing 5% CO_2. Inactivation of CVB3 was performed under UV light for 30 min at room temperature. To confirm the viral activity, mock (NC), CVB3 (CVB), or UV-inactivated-CVB3 (UV) were infected to HeLa cell. Inactivation was confirmed by cytopathic effect (CPE), RT-PCR (Fig. 1a) and Western blot analysis (Fig. 1b).

Animals

Six-week-old, specific pathogen-free, ICR mice were purchased from Hyochang Science (Daegu, Korea). Animal experiments were approved by the Animal Institutional Review Board of Yeungnam University Medical School. Mice were acclimated for 1 week prior to experiment in a specific pathogen-free animal facility. Non-pregnant female ICR mice were inoculated intra-
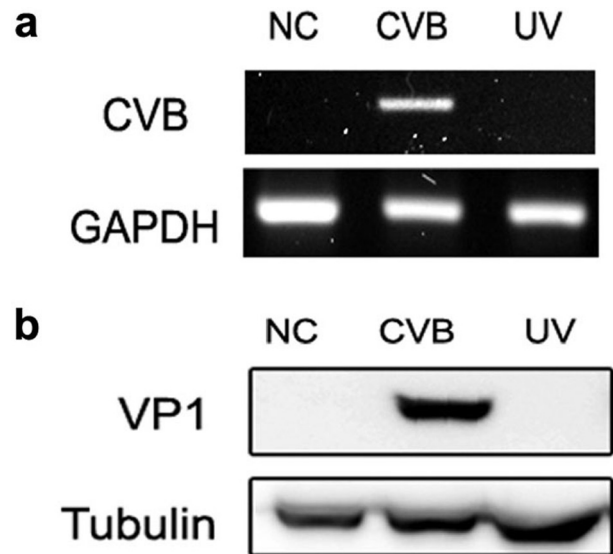

Fig. 1. Confirmation for UV inactivation of CVB3. HeLa cell was infected with mock (NC), live CVB3 (CVB), or UV-inactivated CVB3 (UV). Virus replication ability was confirmed by RT-PCR (a) and Western blot (b).

peritoneally with 0.5 ml of 1×10^4 PFU of CVB3 and then were anesthetized with ether to sacrifice at 1–7 days post-infection (dpi). Pregnant female ICR mice were divided into three groups: CVB (CVB3-infected), UV (UV-inactivated-CVB3-infected), and NC (mock-infected). Within 5 days post-conception (dpc), mice were inoculated intraperitoneally with 0.5 ml of 1×10^4 plaque forming units (PFU) of CVB3 (n=17), UV-inactivated-CVB3 (n=15), or DMEM (n=15) for the CVB, UV, and NC groups, respectively. All pregnant mice were observed daily throughout gestation. Pregnant mice were anesthetized with ether, sacrificed between 12 and 16 dpc, and tissue samples were immediately fixed for histology or frozen at −80°C until further use. Pregnant loss rate was expressed as the percentage of dead embryos from the total number of embryos.

RNA extraction and RT-PCR

Total RNA from each organ and cells was obtained using an Easy-BLUE™ total RNA extraction kit (INtRON Biotechnology, Seoul, Korea) according to the manufacturer's instructions. cDNA synthesis was performed using a Maxime RT PreMix kit and PCR was performed using a Maxime PCR PreMix kit (INtRON Biotechnology). Primer sequences were as follows. Primers for CVB3 (that can detect any of the B serotypes of coxsackievirus [35]) were: sense 5′-ccc cgg act gag

tat caa ta-3′ (position 180–199) and antisense 5′-gca gtt agg att agc cgc at-3′ (position 460–479)(Genbank No. M16572). Primers for *CAR* were: sense 5′-cga tgt caa gtc tgg cga-3′ (position 449–466) and antisense 5′- gaa ccg tgc agc tgt atg-3′ (position 787–804)(Genbank No. NM_001025192). Primers for beta-actin were: sense 5′-act ctt cca gcc ttc ctt c-3′ (position 830–844) and antisense 5′-atc tcc ttc tgc atc ctg tc -3′ (position 977–996) (Genbank No. BC138611). After amplification, products of the PCR reaction were separated on a 1.5% agarose gel containing ethidium bromide.

Western blot analysis

Cells and tissues were lysed with lysis buffer (50 mM Tris, 150 mM NaCl, 5 mM EDTA, 0.5% NP-40, 5 mM dithiothreitol (DTT), 0.2 mM sodium orthovanadate (Na3VO4), 100 mM NaF, 1 mM phenylmethanesulfonylfluoride, 10 μg/ml leupeptin). Western blotting was performed as described previously [9]. The primary antibodies were rabbit anti-CAR (Santa Cruz Biotechnology, Santa Cruz, CA, USA), mouse anti-γ-tubulin (Sigma-Aldrich, St. Louis, MO, USA), and mouse anti-Enterovirus (NovoCastra Laboratories, Newcastle, UK) which reacts with an epitope on VP1 peptide highly conserved within the enterovirus group such as coxsackievirus, echovirus, and poliovirus. The secondary antibody was horseradish peroxidase (HRP)-conjugated anti-rabbit IgG and anti-mouse IgG (Santa Cruz Biotechnology).

Histology

Organs were fixed in 4% formalin, and then embedded in paraffin. Four micrometer-thick sections were placed on a glass slide and stained with hematoxylin and eosin (H&E) for histology. Immunohistochemistry (IHC) for CAR was performed as previously described [34]. Briefly, the paraffin sections were deparaffinized. Heat-induced epitope retrieval was performed at 120°C for 10 min in citrate buffer, pH 6.0. The endogenous peroxidase activity was inactivated by incubation with 3% hydrogen peroxide for 10 min. After washing the sections in Tris-buffered saline, sections were then incubated with a 1:70 dilution of the primary antibody, rabbit anti-CAR (Santa Cruz Biotechnology), for 60 min at room temperature, and then treated with an EnVision detection kit (DAKO, Glostrup, Denmark) according to the manufacturer's instructions. The slides were imaged using a ScanScope® scanner (Aperio Technologies, Vista, CA, USA).

Plaque assay and plaque reduction neutralization assay

Frozen tissues were homogenized and freeze-thawed, with debris then cleared by centrifugation. CVB3 titers were determined by a plaque assay using a procedure modified from previous studies [22, 38]. Samples were serially diluted in 10-fold steps and aliquots of each dilution were used to inoculate HeLa cells. After an 1 h incubation, the medium was removed and 3 ml of DMEM containing 0.5% agar was added to each well. The cells were incubated at 37°C for 48 h and stained with 0.5% crystal violet in 20% methanol. Plaques were counted and viral concentrations were calculated as PFU per uterus or per embryo. To measure CVB3 antibody titers in mouse plasma, a plaque reduction neutralization assay was performed using a procedure modified from Kim *et al.* [21]. Blood was collected from the heart under anesthesia. Plasma was incubated at 56°C for 30 min to inactivate complement and then serially diluted in two-fold steps in FBS-free DMEM. Diluted plasma was combined with an equivalent amount of DMEM containing 1×10^3 PFU of CVB3. Briefly, the samples were applied to HeLa cells, incubated for 90 min at 37°C, then cells were overlaid with DMEM containing 0.5% agar. The remaining steps were the same as those used for the plaque assay procedure. The neutralizing antibody titer was defined as the plasma dilution that resulted in 50% plaque reduction.

Statistical analysis

Data analyses were performed using a Mann-Whitney U test in SPSS 18.0. A value of *P*<0.05 was considered to be significant.

Results

CAR expression patterns in embryos and the uterus of female mice

To examine the possibility of CVB3 infection in the uterus and embryos, *CAR* mRNA and protein expression were examined in the uteri of 8 week-old non-pregnant mice and embryos of embryonic stage at 12 days using RT-PCR and IHC. The hearts of the 8 week-old mice were used as positive controls. *CAR* mRNA was well expressed in the mouse heart, uterus, and embryo (Fig. 2a). CAR protein was localized in the endometrial epithelium, uterine glands, and uterine serosa, but not in the stroma or myometrium of the uterus (Fig. 2b, upper panel). In the heart, CAR protein was expressed in the
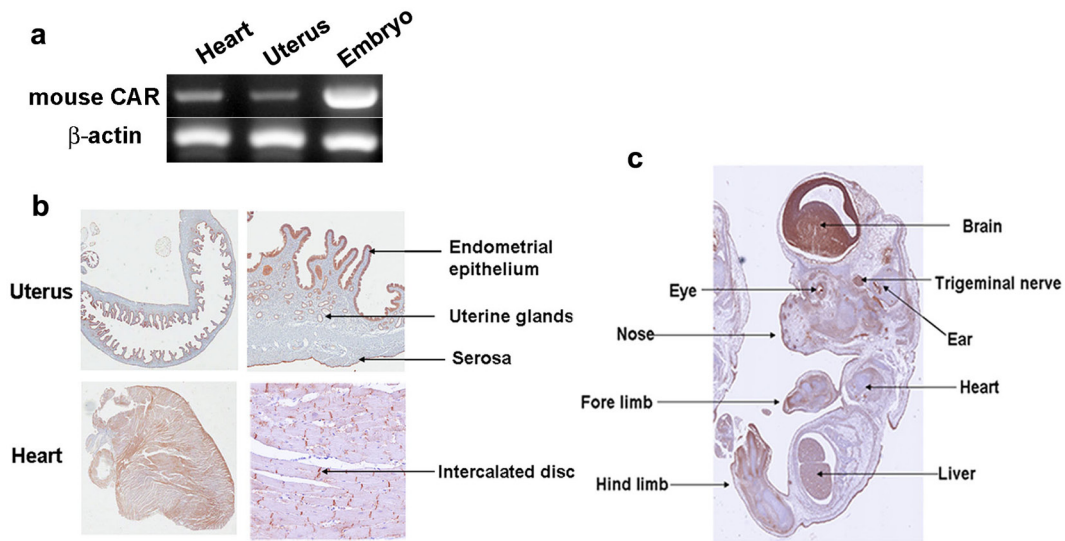
Fig. 2. CAR mRNA and protein expression in mouse tissues. RT-PCR was performed with heart and uterus of 8-week-old, non-pregnant, female ICR mouse and day 12 embryo (a). IHC using a CAR antibody was also performed on uterus (b, upper panel), heart (b, lower panel), and day 12 embryo (c).
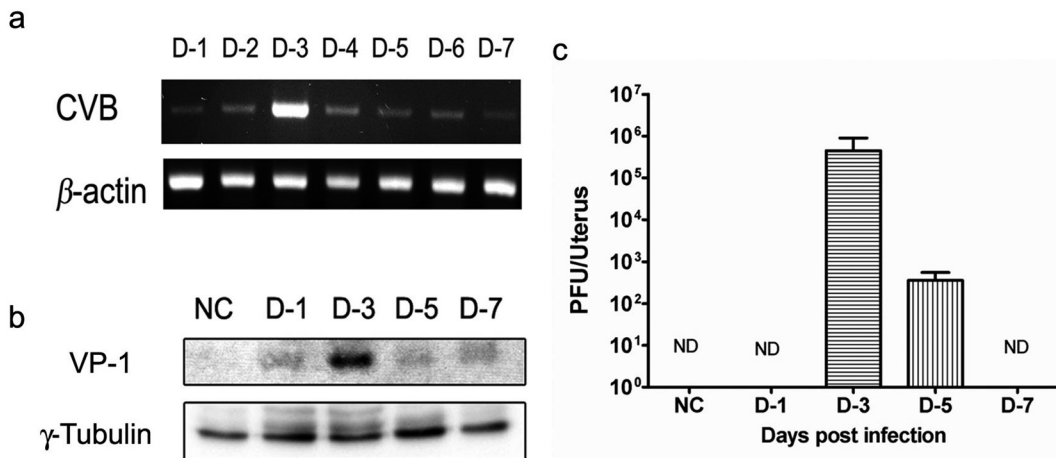

Fig. 3. CVB3 replication in uteri of CVB3-infected mice. CVB3 mRNA was detected with RT-PCR (a) and the enterovirus VP1 capsid protein was detected by Western blot analysis (b). CVB3 titers were determined by the plaque assay (c). The values of PFU/uterus are the means ± standard deviations of two to four uteri per group. ND: Not detected.

intercalated discs and striations of cardiac muscle, and in the pericardium (Fig. 2b, lower panel). In mouse embryos, CAR protein was expressed in neural tissues (especially high in the brain), the eye, inner ear, nostril hair follicles, liver, epithelia, and cells surrounding digit cartilage (Fig. 2c).

CVB3 replication in the uteri of non-pregnant mice

CVB3 replication in uteri was confirmed by RT-PCR, Western blot analysis, and a plaque assay at 1–7 dpi in non-pregnant mice. CVB3 RNA and VP1 capsid protein were detectable at 1 dpi and continued to 7 dpi. Both RNA and protein levels were highest at 3 dpi, gradually decreasing thereafter (Figs. 3a and b). CVB3 was isolated in uteri at 3 and 5 dpi with titers of 9.0×10^5 and 3.6×10^2 PFU/uterus, respectively (Fig. 3c). However, we could not detect CVB3 in uterus by IHC because the commercial antibodies strongly cross react with uterine muscle or uterine gland even though they did not cross react with heart muscle (data not shown).

CVB3 induced pregnant loss during early gestation

Three groups of pregnant mice were inoculated intraperitoneally with 1×10^4 PFU of live CVB3 (CVB), UV-inactivated CVB3 (UV) or 0.5ml of DMEM (NC), within 5 dpc. The number of mice included in the data

Fig. 4. Number of live embryos (per dam) for NC, UV, and CVB groups. The horizontal bar represents the median value of each group.

was 15–17 in each group. The number of live embryos per dam varied between groups, with 11–16 (median: 14) in the NC, 11–15 (median: 13) in UV groups, and 0–16 (median: 7) in the CVB group (Fig. 4). Pregnant loss rates were 4.7%, 2.7%, and 38.3% in the NC, UV, and CVB groups, respectively (Table 1).
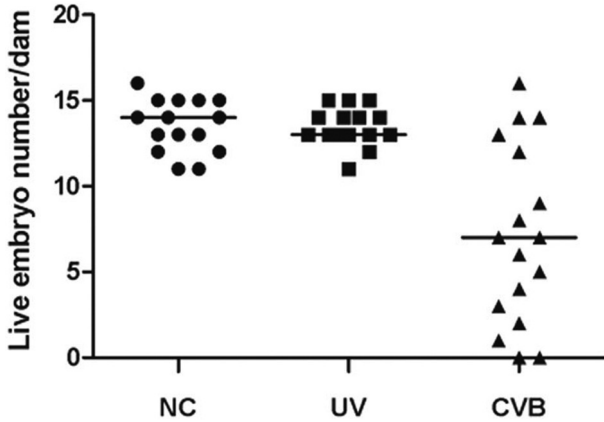
CVB3 replication in embryos and the placentas of pregnant mice

CVB3 replication was confirmed by RT-PCR and plaque assays. The CVB mRNA was not detected in embryos and placentas of mice from NC and UV groups (Fig. 5a). However, the mRNA was detected in most

Table 1. Pregnancy loss rates in mice during early pregnancy

Group	Dams	Total embryos	Dead embryos	Pregnant loss rate (%)
NC	15	213	10	4.7
UV	15	223	6	2.7
CVB	17	193	74	38.3*

*Significantly different from NC and UV groups ($P<0.05$).
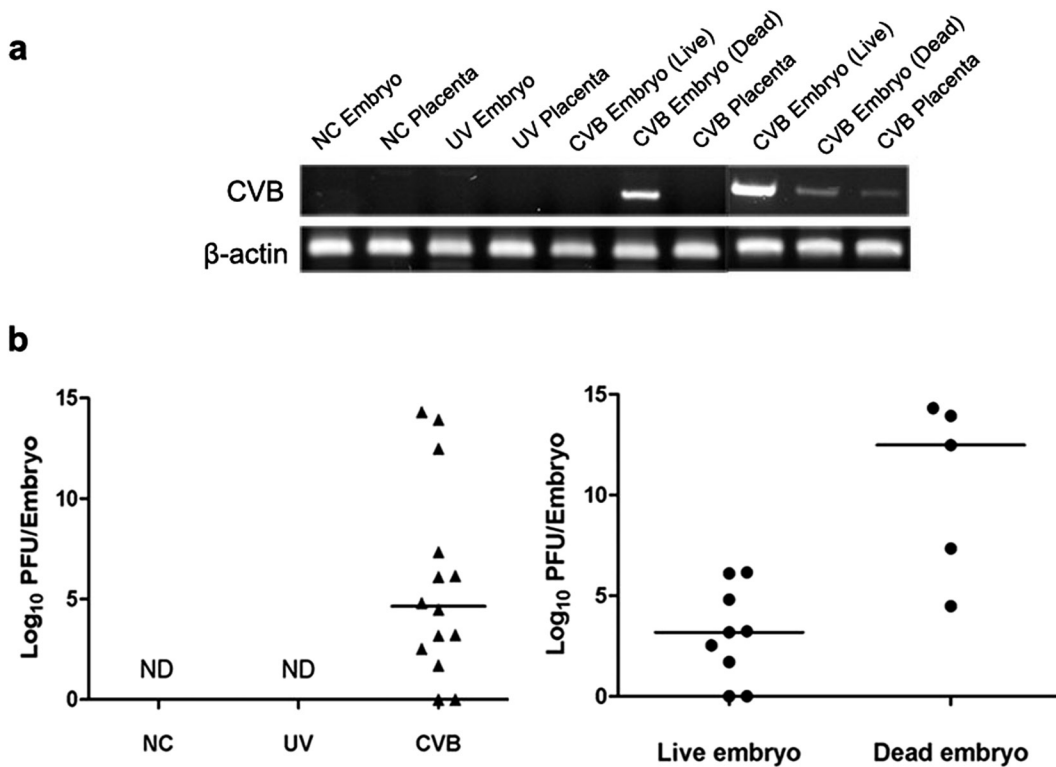

Fig. 5. Virus replication in embryos. RT-PCR was performed with embryos and placentas from NC, UV, and CVB groups (a). The amounts of live virus in embryos were measured by the plaque assay (b). Viral titers from embryos in NC, UV, and CVB groups (b, left). Viral titers between live and dead embryos were compared in the CVB group (b, right). The horizontal bar represents the median value. ND: Not detected.
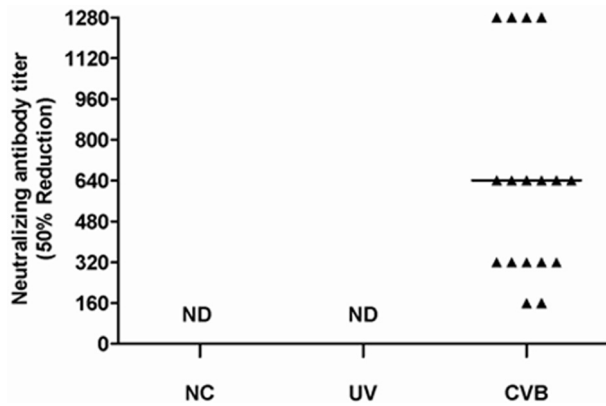
Fig. 6. Maternal neutralizing antibody titers were determined by the plaque reduction neutralization assay. The neutralizing antibody titer was defined as the plasma dilution that resulted in 50% plaque reduction. ND: Not detected.

embryos and placentas of the CVB group, with some exceptions (Fig. 5a). No plaques were observed for embryos from NC and UV groups (Fig. 5b, left). Fourteen embryos from the 13 CVB infected dams were used for the plaque assay. The range of virus titers in the CVB group was 0 to 2.1×10^{14} PFU/embryo and the median value was 4.75×10^4 PFU/embryo (Fig. 5b, left). When live and dead embryos in the CVB group were considered separately, the ranges of virus titers were 0 to 1.4×10^4 PFU/embryo (median: 1.5×10^3 PFU/embryo) and 3×10^4 to 2.1×10^{14} PFU/embryo (median: 3×10^{12} PFU/embryo) for live and dead embryos, respectively (Fig. 5b, right). Maternal neutralizing antibody against CVB3 was detected in the CVB group mice sacrificed between 7 to 11 dpi; titers varied from 160 to 1,280 and the median titer was 640 (Fig. 6).

Morphological features of embryos and the abortive uteri with CVB infection

Embryos from NC and UV mice displayed normal characteristic features of their embryonic stage at 12.5 dpc (Theiler stage 21) (Fig. 7a upper and middle panels). In contrast, most embryos from the CVB group were very fragile, especially in the brains and hearts (Fig. 7a lower panel). A massive infiltration of inflammatory cells was observed in the abortive uteri from the CVB group (Fig. 7b). There were large amounts of necrotic debris, with pyknotic nuclei and nuclear dust in the fibrinoid exudates and large numbers of neutrophils surrounding the fibrinoid exudates (Fig. 7b right). However, no inflammatory cells were observed in uterus and egg cyl-

inder of NC group (Fig. 7c).

Comparison of CAR expression in embryos of NC, UV and CVB groups

To identify the protein expression pattern and amounts of CAR in embryonic stage at 12.5 days embryos, IHC and Western blot analysis were performed. The expression pattern and amount of CAR in developing embryos was not significantly different in NC, UV, and CVB groups (Figs. 8a and b).

Discussion

Maternal coxsackievirus infection during the third trimester or delivery can lead to serious neonatal infection [4, 11, 16, 24, 27, 32, 33, 40]. However, limited data are available on the outcomes of coxsackievirus infection during early gestation because infections often remain undiagnosed. From the late 1960s to the 1970s, a few experimental data was published relating coxsackievirus infection to spontaneous abortion and congenital anomalies in mice [1, 25, 26, 33, 36]. Additionally, an association between CVB3 or CVB4 infection during the first trimester and a greater incidence of congenital heart defects in infants was demonstrated [8]. Until the 1980s, most studies of coxsackievirus infection during pregnancy have relied on indirect maternal serological evidences (usually after birth, without virological or molecular confirmation). From the 1990s, some clinical case reports have provided virological evidences that maternal CVB infection in the first trimester of pregnancy is related to spontaneous abortion and congenital heart disease [3, 18, 40]. Watson *et al.* [40] isolated CVB1 from the amniotic fluid of a fetus with a congenital heart anomaly and Axelsson *et al.* [3] reported that CVB infection was found more frequently in women suffering from a miscarriage than women receiving a voluntary termination before 13 weeks of gestation.

In 1997, CAR was found as a common receptor for adenovirus and CVB [5] and during the last decade CAR has been revealed as a critical molecule for the development of the embryo [2, 15, 20]. Previously, Koi *et al.* [23] suggested that adenovirus infection would be very susceptible during the first trimester because CAR is expressed in both villous trophoblast cells and extravillous trophoblast cells of the human placenta at 11 weeks. Because CVB uses the same receptor with adenovirus, CVB infection would also be susceptible during the first
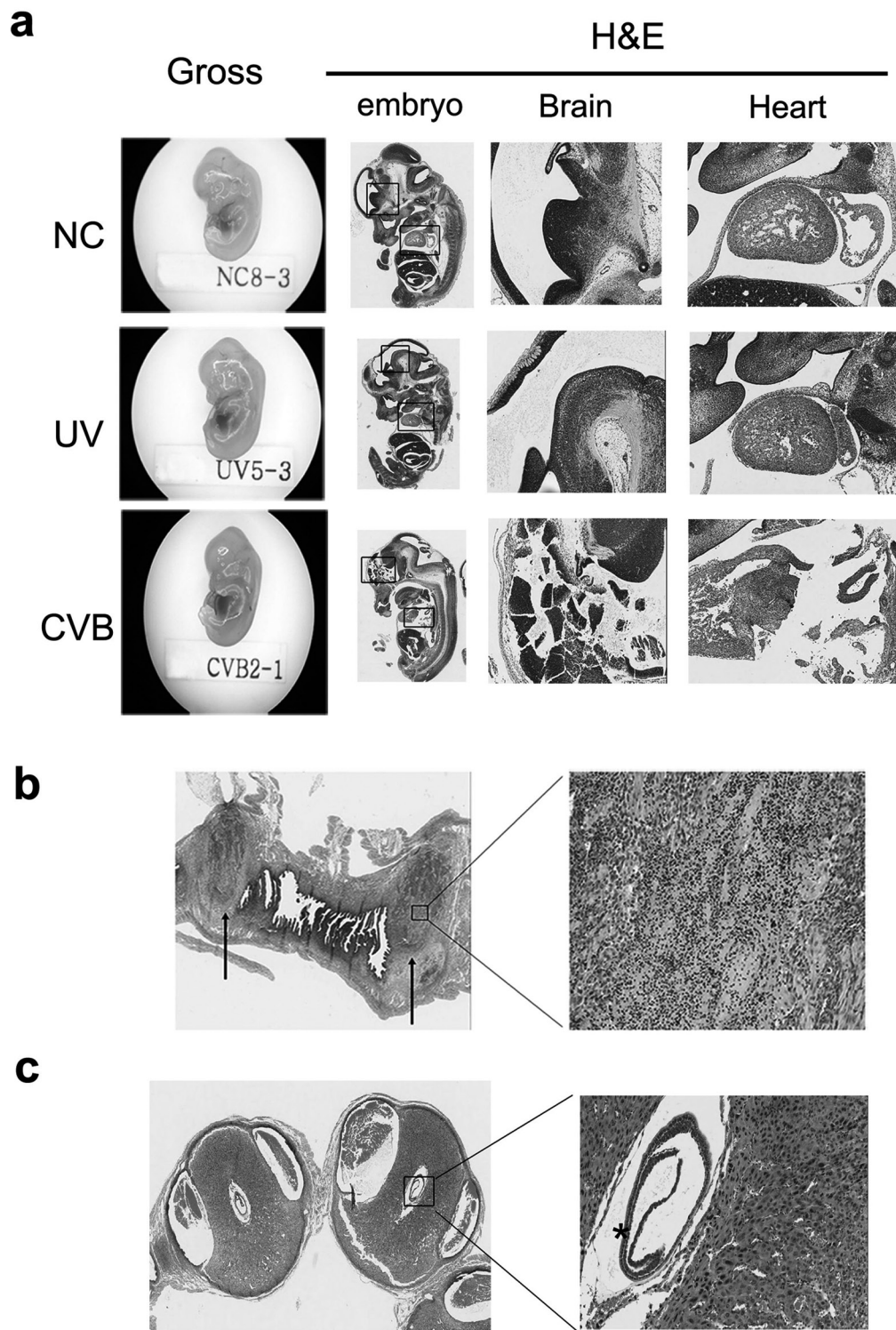
Fig. 7. Morphological features of embryos in NC, UV, and CVB groups and abortive uterus. Gross appearance and H&E staining of embryos at 12.5 days (a). Histology of an aborted uterus from a CVB3-infected dam (b) and uterus and egg cylinder from NC group at 6 days post coitus (c). Arrows indicate the implantation site. Star indicated egg cylinder. H&E-stained slides were scanned by ScanScope® at magnifications of 1×, 5×, and 10×.
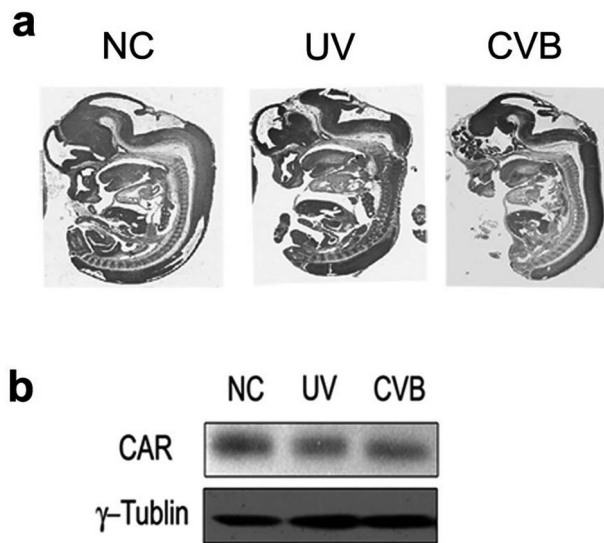
Fig. 8. CAR expression patterns in 12.5 days embryos of NC, UV, and CVB groups. Immunohistochemistry was performed using the CAR antibody (a). Western blot analysis of CAR protein expression in embryos (b).

trimester and might lead to adverse pregnancy outcomes.

The main purpose of this study was to examine the effects of CVB3 infection during early pregnancy. Although there has been some understanding of CAR expression in the placenta [15, 23], there has not been enough data on CAR expression in the uterus [31]. We found that CAR was highly expressed in the endometrial epithelia and uterine glands, thus confirming the uterus as a possible target for CVB. Furthermore, the CVB3 genome and proteins were detected from 1–7 dpi, with viral titer highest at 3 dpi in the uterus.

CAR is already known to be involved in the tissue organization of the developing central nervous system and heart [2, 20]. As heart development begins at embryonic day 8 in the mouse, CVB3 was inoculated no later than 5 dpc to mice to focus on CVB3 replication period in the uterus. In the present study, the pregnancy loss rate was approximately eight times higher in the CVB group than in the NC group. Many resorbed implantation sites were found and some abortive uteri showed inflammatory reactions in CVB3 infected mice.

Several mechanisms are likely involved in the termination of pregnancy in CVB3 infected mice during early gestation. As a maternal factor, impaired implantation is possible. Inflammation of the uterus may hinder the implantation of fertilized eggs. On the embryonic side, inappropriate organogenesis, especially in the brain

and heart, usually cause abortions. CAR has important roles in organ development and function. CAR knock-out mice die at embryonic day 11 because of heart anomalies [2, 10, 15]. CAR also regulates cardiac remodeling and electrical conductance between the atria and ventricles [17, 29]. CAR is also predominantly expressed in the developing brain and neuronal cells, followed by a rapid down-regulation at early neonatal stages [20, 42]. Even though the functions of CAR in the brain and neuronal cells are still under investigation, we at least know they are strongly dependent on the developmental stage of the embryo. Therefore, CVB3 infection during critical periods of heart development or brain formation could induce abortions.

The brains and hearts of many live embryos from the CVB group were very fragile. Interestingly, the amounts of CAR expression in live embryos were not significantly different among the three groups examined. In the process of CVB3 infection, CVB3 interacts with CAR, forming a complex that invaginates into the cytoplasm. However, CAR does not recycle to the plasma membrane [12]. Thus, although the amounts of CAR were not different, CAR disappears from the cell surface by internalization into the cytoplasm and normal cellular functions might be changed. Blocking of CAR with CAR antibodies disrupts the attachment and neurite extension of neural cells on extracellular matrix glycoproteins [28]. In epithelial cells, CAR is expressed in tight junctions and regulates the permeability of ions and macromolecules [14, 28, 31, 37]. Soluble CARs, adenovirus fibers, and anti-CAR antibodies disrupt tight junctions and increase epithelial permeability [39]. Therefore, the fragility of the embryos might be due to inappropriate CAR functions that alter cellular attachment to the extracellular matrix (or other cells) and cell permeability.

In addition, several factors may be involved in determining the outcomes of virus infection. For example, the susceptibility of the pregnant dam to CVB3, the amount of virus that reaches the uterus or embryos, and the developmental stage of embryos might affect outcomes. Overall, maternal CVB3 infection during early pregnancy induced a high rate of pregnancy loss. In the future, it is necessary to examine the prevalence of CVB3 infection in miscarriage patients. If CVB3 will be confirmed as an important causative agent in human, vaccine development against CVB3 will help to prevent of pregnant loss.

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