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Original Article

Pathogenesis of Congenital Rubella Virus Infection in Human Fetuses: Viral Infection in the Ciliary Body Could Play an Important Role in Cataractogenesis

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ABSTRACT

Background: Development of congenital rubella syndrome associated with rubella virus infection during pregnancy is clinically important, but the pathogenicity of the virus remains unclear.
Methods: Pathological examination was conducted on 3 aborted fetuses with congenital rubella infection.
Findings: At autopsy, all 3 aborted fetuses showed congenital cataract confirmed by gross observation. Rubella virus infection occurred via systemic organs including circulating hematopoietic stem cells confirmed by immunohistochemical and molecular investigations, and major histopathogical changes were found in the liver. It is noteworthy that the virus infected the ciliary body of the eye, suggesting a possible cause of cataracts.
Interpretation: Our study based on the pathological examination demonstrated that the rubella virus infection occurred via systemic organs of human fetuses. This fact was confirmed by immunohistochemistry and direct detection of viral RNA in multiple organs. To the best of our knowledge, this study is the first report demonstrating that the rubella virus infection occurred via systemic organs of the human body. Importantly, virus infection of the ciliary body could play an important role in cataractogenesis.

1. Introduction

Rubella disease is now prevented by vaccines, but remains poorly controlled in developing countries including Southeast Asia. Rubella is an acute infectious disease that normally follows a mild clinical course. However, infections during pregnancy, especially before week 12 of gestation, can cause severe birth defects known as congenital rubella syndrome (CRS) (Banatvala and Brown, 2004; Duszak, 2009). Clinical signs of CRS include cataract, glaucoma, heart disease, loss of hearing, brain dysfunction, and pigmentary retinopathy. These illnesses are clinically important, yet the pathogenesis of rubella virus (RV) infection in fetuses/newborns remains obscure due to the lack of a suitable animal model for this purpose. There have been very few reports in the literature of histopathological studies on CRS in humans and most appeared in the period from late 1960 to early 1970 (Töndury and Smith, 1966; Brookhouser and Bordley, 1973; Menser and Reye, 1974).

In Vietnam, rubella epidemics occurred during the period from 2011 to 2012. Through this outbreak, our investigation based on molecular epidemiology showed that RV RNA was detectable in the placenta from all of 10 aborted fetuses and 10 newborns from pregnant women with rubella (Pham et al., 2013). Importantly, all newborns and aborted fetuses were found by gross examination to have congenital cataracts. To obtain more detailed information, we conducted further histopathological and immunohistochemical examinations in the aborted fetuses and herein discuss the pathogenicity of RV infection in human fetuses.

2. Patients and Methods

2.1. Patients

We examined 3 aborted fetuses. All mothers of the 3 aborted fetuses had a history of rubella with a rash, fever and lymph node swelling at weeks 5–6 of gestation. Ultrasound imaging in these mothers showed hyperechogenic lesions in the liver, kidney and bowel of all fetuses. Abortion was carried out at weeks 23, 22 and 13 of gestation, respectively.

2.2. Ethical Approval

This study conforms to the ethical guidelines and was approved by the ethics committees of the Hung Vuong Hospital, Ho Chi Minh City,

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Vietnam. Written informed consent in this study was obtained from all of the mothers.

2.3. Histopathological and Immunohistochemical Examination

Tissue samples obtained at the autopsy were divided into two portions. One portion was used for conventional histological examination and the other for viral RNA detection. In the former, tissue samples were fixed in 10% buffered formalin and embedded in paraffin blocks for histopathological and immunohistochemical examinations. In the latter, tissue samples were frozen and stored at −80 °C until use.

To examine distribution of RV-related antigen in multiple organs, thin sections of formalin-fixed paraffin-embedded tissues were stained immunohistochemically by an avidin–biotin complex immunoperoxidase method (LSAB2 kit/HRP/DAB; Dako Cytomation, Copenhagen, Denmark) using a mouse monoclonal antibody against RV capsid protein (Abcam Ltd., Cambridge, UK). Furthermore, to identify hematopoietic stem cells in tissues, human CD34 antigen was immunostained using a mouse monoclonal antibody (CD34 Class II, Dako Cytomation). Normal mouse serum as the primary antibody was used for the negative control.

2.4. Detection of RV Gene by Nested Reverse-transcriptase (RT)-PCR

Total RNA was extracted from frozen tissue specimens using the RNA extraction kit (^{NK}RNAPREP kit, Nam Khoa Biotek Co., Ho Chi Minh City, Vietnam). Viral cDNA was synthesized with mixture of random primer and oligo(dT) primer using iScript reverse transcriptase (Bio-Rad Laboratories, CA, USA) with the following condition: 25 °C, 5 min, 42 °C, 30 min and 85 °C, 5 min. For RV gene amplification, hemi-nested PCR was carried out with primers designed from the E1 gene of RV. RV gene fragment was amplified by the primer combination of RV8537F (5′-GGG TAC GCG CAG CTG GCG TC-3′; sense, nt 8537–8556) and RV9117R (5-CAY TTG CGC GCC TGM GAG CC-3′; antisense, nt 9117–9098) for the outer primer pairs (581 bp) and RV8633F (5′-AGC GAC GCR GCS TGC TGG GG-3′; sense, nt 8633–8652) and RV9117R for the inner primer pairs (485 bp). Nucleotide position is based on rubella virus vaccine strain wistar RA 27/3 (accession # FJ211587).

Five μl of cDNA product was placed in PCR buffer containing Platinum Taq and 360 GC Enhancer (20% v/v; Applied Biosystems, Foster City, CA, USA) due to the RV genome having an extremely high GC-rich sequence. Amplification conditions included pre-incubation at 95 °C, 5 min, followed by 40 cycles consisting of 94 °C, 30 s, 60 °C, 30 s and 72 °C, 1 min for the 1st round PCR and 94 °C, 30 s, 65 °C, 30 s and 72 °C, 1 min for the 2nd round PCR.

In addition, we also examined to detect negative-strand RV RNA, which indicates the replicative form of the positive-strand RNA virus in infected cells. To detect the negative-strand RV RNA, viral cDNA was synthesized with RV-specific sense primer (RV8537F) using iScript reverse transcriptase with the following condition: 25 °C, 5 min, 42 °C, 30 min and 95 °C, 5 min. Obtained viral cDNA was amplified by the hemi-nested PCR with the same condition as described above.

Amplicons were analyzed by electrophoresis on 2% agarose gels staining with ethidium bromide and recovered using the Promega Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA). The amplicons were subjected to direct sequencing using the ABI PRISM™ Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems), on a capillary sequencer model 3130 (Applied Biosystems).

3. Results

At autopsy, all 3 aborted fetuses showed a congenital cataract confirmed by gross observation. Histologically, the following findings were observed. In the liver, congestion, necrotizing and inflammatory changes accompanied with hemorrhage, apoptotic hepatocytes (so called acidophil bodies), expansion of the portal area with mild to moderate inflammatory infiltrate (rarely with piecemeal necrosis), giant cell formation of hepatocytes, mild deposition of bile pigments into the hepatocytes and vacuolar degeneration of epithelial cells in the intrahepatic bile ducts were observed (Fig. 1a–d). Interestingly, active erythrophagocytosis by Kupffer cells suggesting virus-associated hemophagocytic syndrome was seen (Fig. 1e). The kidney showed mild nephritis accompanied by partial hemorrhage (Fig. 1f). In the lung, all cases had mild pneumonia accompanied by congestion, alveolar hemorrhage and interstitial edema (Fig. 1g, h). The spleen and lymph node from one case showed hypoplasia with hemorrhage (Fig. 1i, j). The heart exhibited mild myocarditis accompanied by interstitial edema (Fig. 1k). In the central nervous system (CNS), no remarkable change suggesting viral encephalitis was found.

The immunohistochemical examination revealed localization of RV capsid antigen in multiple organs from all 3 cases tested (Table 1). In the liver, which showed necrotizing and inflammatory changes, virus antigen was localized on the surface of hematopoietic mononuclear cells produced in the fetal liver (Fig. 2a, b). These mononuclear cells tested positive for cell surface marker CD34 by immunohistochemical examination, suggesting that they were derived from hematopoietic stem cells. Similar RV infected hematopoietic stem cells expressing CD34 were also observed in the systemic organs including the spleen, kidney, lungs, heart, CNS and eyes.

Virus antigen was also localized in the epithelial cells of the glomerulus and proximal tubules of the kidney, bronchioles and alveolus in the lungs, myocardial cells in the heart, spleen cells, lymphoid tissue, and nerve cells in the cerebral cortex (Fig. 2c–j). Furthermore, an important finding in the eye was the presence of virus antigen in the epithelial cells of the ciliary body and the lachrymal glands (Fig. 2k, l). RV capsid antigen was prominent in the cytoplasm of all infected cells. No positive reactions using normal mouse serum as the negative control were seen.

By the nested RT-PCR assay using tissues from multiple organs, positive-strand RV RNA was detectable in nasal swabs, placenta and the lens of the eye in all 3 cases tested. Furthermore, viral RNA was also detectable in all of the major organs including the liver, kidney, spleen, heart, lungs, the eye and CNS (consisting of the cerebral cortex, cerebellum and brain stem) obtained from all fetuses examined (Table 1). In addition, our examination detected negative-strand RV RNA, which indicates the replicative form of the positive-strand RNA virus in infected cells. This result showed that all samples tested were positive for the negative-strand RNA of the virus.

To confirm the sequence identity of the virus genes, amplicons detected in liver tissues from 3 cases were sequenced. The result showed that nucleotide sequences of RV from the liver tissues were identical among all 3 cases. Furthermore, these 3 strains isolated in the tissues also showed identical nucleotide sequences to those of RV strains identified in the rubella outbreak season during the period from 2011 to 2012.

4. Discussion

Clinically, the most important illness during congenital rubella infection is the development of malformations in the fetus (Banatvala and Brown, 2004; Duszak, 2009). In particular, it is known that the fetus of early pregnancy is at greater risk than at later stages. So far, many epidemiological studies on RV infection have been reported from many countries, but there are very few reports of pathological studies on rubella (Töndury and Smith, 1966; Brookhouser and Bordley, 1973; Menser and Reye, 1974). For this reason, the pathogenesis of RV infection in human fetuses remains unknown. In addition, the lack of appropriate animal models for rubella infection impedes efforts to elucidate this issue.

In this study, we analyzed the pathological features of RV infection in human fetuses having CRS. Our results presented here indicate that the route of RV infection was via the systemic organs of the human fetuses. This fact has been confirmed by immunohistochemistry and direct detection of viral RNA in multiple organs. This was also demonstrated by the detection of negative-stranded RNA of RV, which indicates the replicative form of positive-stranded RNA virus in infected cells.