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ORIGINAL ARTICLE

Detection of Human Cytomegalovirus in Amniotic Fluid and Villous Tissues

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Background/Purpose: Human cytomegalovirus (HCMV) is the most common cause of intrauterine infection. Endothelial and/or epithelial cells in uterine tissue are major sites of latent or persistent HCMV infection in women, and the uterus may play an important role in viral dissemination. To elucidate the pathomechanisms of intrauterine infection by HCMV, we examined the presence of the HCMV genome, antigen and infectious virion in amniotic fluid and villous tissues.
Methods: The isolation of infectious HCMV in amniotic fluid was detected using the MRC-5 human embryonic fibroblast cell line. The HCMV genome was detected by polymerase chain reaction. Expression and distribution of HCMV antigens and mRNA transcripts in villous tissues were examined by immunostaining using primary antibodies specific for an immediate early nuclear antigen and for a late cytoplasmic antigen of HCMV, and *in situ* hybridization using an antisense probe specific for the phosphoprotein 71 (pp71) region of HCMV, respectively.
Results: The HCMV genome was detected in 46 (26%) out of 176 amniotic fluid specimens. Moreover, the infectious virus was isolated in nine of these specimens (5.1%), with viral immediate early and late antigens being detected in the cultured cells of amniotic fluid. In the villous tissues of all examined cases (7/7), viral mRNA and antigen were detected, and 48% of the pregnant women whose cervical smears were positive for the HCMV genome by polymerase chain reaction delivered babies with positive urinary samples.
Conclusion: These results suggest that transplacental transmission of HCMV to the fetus occurs more frequently than has been previously reported. Further studies are necessary to precisely clarify the mechanisms of intrauterine HCMV infection.

1. Introduction

In the late 19th century, large "protozoan-like" cells were noted by several researchers.[1–4] This was followed by numerous reports of cases with cytomegalic inclusion disease and isolation of the agent, human cytomegalovirus (HCMV). HCMV is one of eight herpes viruses known to infect humans. It is a widespread human pathogen that has a minor clinical impact on healthy individuals, but causes various organ diseases in immunosuppressed patients and neural damage in fetuses infected *in utero*,[5,6] and HCMV persists as a lifelong infection. In addition, HCMV is frequently activated in immunocompromised individuals such as AIDS or organ

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transplant patients, causing severe morbidity and eventual mortality.[5,7,8]

HCMV is the most common cause of intrauterine infection, affecting 0.2–2% of liveborn infants. Between 10–15% of infants infected with congenital HCMV exhibit the clinically apparent or symptomatic form of the disease.[9,10] Intrauterine transmission occurs transplacentally during maternal viremia. The placenta acts as a portal of entry for the virus, but it also acts as a barrier during maternal primary infection.[11,12] Primary infection in the mother and intrauterine transmission during the first 16 weeks of pregnancy have a much greater clinical impact on the fetus than non-primary infections and infections occurring during the last trimester of pregnancy.[11,13] Approximately 15% of women with primary infections during early pregnancy abort spontaneously.[14] In such cases, the placenta, but not the fetus, shows evidence of infection, which suggests that placental involvement is important in its own right and precedes viral transmission to the fetus.[15,16] HCMV can be transmitted *in utero* from both primary and reactivated maternal infections. This accounts for the inordinately high incidence of congenital HCMV infection in comparison with other intrauterine viral infections.[17,18]

We have previously detected high rates of HCMV transcripts and antigens in both cervical smears and uterine tissues.[19,20] We hypothesized that endothelial and/or epithelial cells in uterine tissue are a major site of latent or persistent HCMV infection in women, and that the uterus plays an important role in viral dissemination.[20,21] In the current study, to further elucidate the pathomechanisms of intrauterine HCMV infection, we used polymerase chain reaction (PCR), *in situ* hybridization (ISH), immunostaining (IMS) and virus isolation to investigate whether HCMV DNA, mRNA transcripts, antigens and virions are present in amniotic fluid and villous and decidual tissues.

2. Methods

2.1. Amniotic fluid

A total of 176 amniotic fluid specimens were randomly collected from amniocentesis samples of pregnant women (gestational ages between 11 and 30 weeks). These samples had been sent to the Division of Human Genetics, Medical Research Institute, Kanazawa Medical University, Japan, from university hospitals, institutes, hospitals and clinics all over Japan. After centrifugation at 5000 rpm for 10 minutes, the supernatant was divided into two parts; one part was subjected to virus isolation and the other was stored at –80°C for subsequent PCR analysis. In six cases, the packed cells were cultured in Eagle's minimum essential medium alpha (Gibco Laboratories, Life Technologies, Inc., Grand Island, NY USA) and supplemented with 10% fetal calf serum (Bocknek Ltd., Rexdale, Canada). The medium was changed once a week until the cells became sufficiently abundant for analysis. Cells were then scraped and cultured on cover slips, after which adherent cells were fixed and processed as described below, and subjected to IMS for HCMV antigens. Culture medium was also subjected to virus isolation.

2.2. Villous and decidual tissues

Villous and decidual tissues were obtained from the placentas of seven pregnant women who had artificial abortions or premature deliveries due to fetal abnormalities at gestational ages from 7 to 15 weeks. Four serum specimens from the seven cases in this study were investigated for the presence of HCMV IgG antibody by indirect immunofluorescent testing, as described previously.[22] Tissues were fixed in a 10% solution of commercial formalin in water at room temperature. Fixed tissues were dehydrated in a graded ethanol series and were embedded in paraffin. Serial sections (4 μm) were then prepared and mounted on 3'-aminopropyltriethoxysilane-coated glass slides for ISH and IMS.

2.3. Urine and cervical smear specimens

Urine and cervical smear specimens from neonates and their mothers were kindly supplied by the Division of Obstetrics and Gynecology, Ishikawa Prefectural Central Hospital. Urine collected within 24 hours after birth was centrifuged at 2500 rpm for 10 minutes, and the supernatant was diluted fivefold in distilled water. Cotton swabs rubbed over the cervix were immediately rinsed in 1 mL of phosphate-buffered saline (PBS). The diluted supernatant and the rinsed PBS served as urine and cervical smear specimens, respectively, and were stored at –80°C until use.

2.4. Virus isolation

The human embryonic lung fibroblast cell line MRC-5[23] for viral isolation was grown in Dulbecco's modified Eagle's minimal essential medium (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) supplemented with 10% fetal calf serum, L-glutamine (0.3 mg/mL; Nacalai Tesque Inc., Kyoto, Japan), streptomycin (100 μg/mL; Nacalai Tesque Inc.) and penicillin (100 units/mL; Nacalai Tesque Inc.). All cell cultures were maintained in a humidified incubator at 37°C in the presence of 5% carbon dioxide. Amniotic fluid supernatant or cell culture medium was then inoculated onto a monolayer of MRC-5 cells. Culture medium was changed once a week and cells were observed for cytopathic effects characteristic of HCMV for up to 3 weeks after inoculation.

2.5. PCR

The protocol for PCR and hybridization was essentially the same as described previously.[19] For amplification