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Effect of Human Cytomegalovirus on Invasive Capability of Early Pregnant Extravillous Cytotrophoblasts*

Tao LIU (刘 涛)[1, 2†], Xiaofei ZHENG (郑晓菲)[3†], Juanjuan CHEN (陈娟娟)[1], Nan WANG (王 楠)[1], Juan XIAO (肖 娟)[1], Dandan ZHANG (张丹丹)[1], Zongzhi YIN (尹宗智)[1], Wei LI (李 伟)[1], Suhua CHEN (陈素华)[1#]
[1]*Department of Obstetrics and Gynecology, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430030, China*
[2]*Department of Obstetrics and Gynecology, Tai'an Central Hospital, Tai'an 271000, China*
[3]*Department of Information Center, Tai'an Central Hospital, Tai'an 271000, China*

Summary: The effect of human cytomegalovirus (HCMV) on invasive capability of early pregnant extravillous cytotrophoblasts (EVTs) was investigated *in vitro*. Primary EVTs were obtained by complex phosphoesterasum digestion and gradient centrifugation from villous tissue aseptically taken from healthy pregnant women. Cytokeratin7 (CK7), vimentin (Vim) and c-erbB-2 were immunocytochemically detected to identify source of cells, and HCMVpp65 antigen was assayed to determine the infection state of primary EVTs by immunocytochemical staining. The EVTs were divided into two groups: control group and HCMV group, and the expression of c-erbB-2, matrix metalloproteinase-2 (MMP-2) and MMP-9 proteins was detected in two groups by immunocytochemistry and Western blotting. Enzymic activity changes of MMP-2 and MMP-9 were tested by gelatin zymography in primary EVTs infected with HCMV. The invasion of primary EVTs was detected by cell invasion assay *in vitro* after they were infected by HCMV. The cell source identification showed that the cells obtained were highly-pure primary EVTs, and primary EVTs could be infected by HCMV. Primary EVTs could express c-erbB-2, MMP-2 and MMP-9 proteins, and as compared with control group, the protein expression was decreased significantly in HCMV groups (P<0.05). Primary EVTs could secrete active MMP-2 and MMP-9 *in vitro*, and the activity of two MMPs was decreased significantly in HCMV groups (P<0.05). The *in vitro* cell invasion assay showed that the number of primary EVTs permeating Matrigel in HCMV group was decreased (P<0.05). We are led to conclude that HCMV can infect primary EVTs and inhibit their invasion capability, suggesting that the impaired EVT's invasion capability might be related to the abnormal expression of c-erbB-2, MMP-2 and MMP-9 proteins.
Key words: human cytomegalovirus; extravillous cytotrophoblast; c-erbB-2; MMP-2; MMP-9; invasion

Human cytomegalovirus (HCMV) is the most common pathogen causing interuterine infection. The underlying mechanisms by which HCMV infection results in abortion, stillbirths and fetal growth retardation have not been fully understood[1]. It is generally believed that, three weeks after fertilization, cytotrophoblasts differentiate into extravillous cytotrophoblasts (EVTs), invade into the decidual interstitium and remodel the maternal stroma and blood vasculature[2]. Lowered invasive capability of EVTs, impaired remodeling of the maternal vessels and reduced placental blood supply are important pathological bases of abortion, stillbirths, fetal growth retardation and hypertensive disorder complicating pregnancy (HDCP)[3]. c-erbB-2 protein is one of the markers of EVT and is involved in the invasion process of EVTs[4]. Matrix metalloproteinases (MMPs) in general, MMP-2 and MMP-9 in particular, are key enzymes that dictate the migration and invasion of EVTs. Currently, the effect of HCMV on the invasive capability of EVTs is little examined. In this study, we, from the perspective of EVT functions, investigated the mechanisms by which HCMV causes abortion, stillbirths and fetal growth retardation in molecular terms.

Tao LIU, E-mail: liutao770404@163.com
#Corresponding author, E-mail: csh@tjh.tjmu.edu.cn
†The two authors contributed equally to this work.
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1 MATERIALS AND METHODS

1.1 Materials

1.1.1 Sample Collection and Sources of Viruses Villi were collected from healthy pregnant women who, from March 2009 to July 2010, received induced abortion on their own accord at Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, for the reason of family planning. They had gestational age of 5–10 weeks and their peripheral blood was negative for HCMV IgM. The ethics standards concerning the medical tests on human beings formulated by the Ethics Committee were strictly followed during the whole process of the study. HCMV AD169 viral strain was provided by the Hubei Institute of Virology, Wuhan, Hubei, China, with the TCID50 being $10^{-5.46}$.

1.1.2 Major Reagents DMEM/Ham's F12, and Trypsin media were products of Gibco Co., USA. Standard fetal calf serum (FCS) was made by Hyclone, USA. DNase Ⅰ was bought from Sigma Co., USA. Rat tail tendon collagen type I was procured from Shanghai Canspec Scientific Instruments Co. Ltd., China. Other reagents used included Percoll solution (Amersham Pharmacia, USA), cytokeratin7 (CK7), vimentin (Vim) monoclonal antibody and SP immunohistochemical kits (Beijing Zhong Shan-Golden Bridge Biological Technology Co. Ltd., China), rabbit anti-HCMVpp65 polyclonal antibodies (Santa Cruz Co., USA), rabbit anti-MMP-2 and anti-MMP-9 polyclonal antibodies (Wuhan Boster Bioligical Technology Co. Ltd., Wuhan, China), Matrigel (BD Co., USA), total protein extraction kit and Bradfordprotein assay kit (BestBio Co., Shanghai, China), SDS-PAGE kit sample preparation kit (Wuhan Guge Co., Wuhan, China), PVDF membrances (Millipore Co., USA), ECL kit (AMRESO Co., USA) etc.

1.2 Methods

1.2.1 Isolation and Culture of EVTs EVTs were isolated and cultured by following the method reported by Handschuh *et al* with some modifications[5]. Briefly, villi and decidua in early human pregnancy were thoroughly washed in bacteria-free D-Hanks solution. The samples were removed of decidua and villous stroma and the remaining tissues were minced into 1-mm^3 pieces. Then complex enzymes containing 0.125% trypsin, 4.2 mmol/L $MgSO_4$, 25 mmol/L Hepes and 20 U/mL DNase Ⅰ were added. The digestion was terminated after 50 min incubation with shaking. The supernatant was collected, filtered through 80-mesh and then 300-mesh metal sieve, and centrifuged at 500 g for 10 min. The supernatant was then discarded, the cells were suspended in DMEM/Ham's F-12, and 35%, 40%, 45%, 50% Percoll gradient solution was added to the cell suspension. The sample was then centrifuged at 1000 g for 25 min. The cell suspension added with 40%–45% Percoll gradient solution was transferred into another centrifugation tube containing DMEM Ham's F12 with 10% FCS for washing (2 times). Afterwards, the cells were re-suspended and then seeded, at a density of 50 000 cells/cm^2, into a 48-well culture plate coated with rat tail collagen protein type Ⅰ (0.006 mmol/L solutions of rat-tail collagen in acetic acid, which was cultured at 37 °C in 5% CO_2 for 24 h. The plates were washed with D-Hanks three times and the culture medium was changed.

1.2.2 Grouping The cells in the HCMV group was infected with HCMV by culturing the cells in a 1:6 (v/v) mixture of 100 TCID50 HCMV and DMEM/Ham's F-12 medium containing 3% FCS. The cells in the control group were cultured with equal amount of PBS. The supernatant was discarded 2 h after the culture and the cells were washed with PBS three times. The culture with DMEM/Ham's F-12 medium lasted for 48 h. A portion of the cells were used for immunohistochemical staining and the other part was subjected to Western blotting after extraction of total protein.

1.2.3 Immunohistochemical Detection The cells in HCMV group were fixed with methanol-acetone (1:1) solution at room temperature for 30 min 48 h after culture with HCMV. CK7, Vim and c-erbB-2 were immunohistochemically determined as previously reported[5] for the determination of cell sources. pp65 antigen of HCMV was detected for assessing the status of HCMV infection. The expression of c-erbB-2, MMP-2 and MMP-9 proteins was detected for judging the invasive capability of the virus. The first antibodies were mouse anti-human CK7 or Vim monoclonal antibodies, rabbit anti-HCMVpp65, anti-c-erbB-2, anti-MMP-2 and MMP-9 polyclonal antibodies (at a dilution of 1:100). Under a light microscope, the positive staining presented as yellowish-brown or brown particles. HMIAS-2000 high definition color medical image analysis was used for the processing of the results of c-erbB-2, MMP-2 and MMP-9 detection. Ten visual fields were chosen at random under high power lens and the average absorbance (*A*) value was calculated and subjected to semi-quantitative statistical analysis.

1.2.4 Western Blotting Western blotting was employed for detecting the expression of c-erbB-2, MMP-2 MMP-9 proteins. Detection was conducted by following the kit instructions. The total protein was extracted and the protein concentration was determined. For each sample, 50 μg was taken for 10% SDS-PAGE and the sample was transferred to wet PVDF membrane. After blockade at room temperature with 5% defatted milk powder for 2 h, the rabbit anti-c-erbB-2, -MMP-2 and -MM-P9 polyclonal antibodies (at a dilution of 1:500) were added and then incubated at 4°C overnight. After washing of membrane with TBST, the secondary antibodies were added and the samples were incubated at room temperature for 2 h. Then the membrane was re-washed, visualized with ECL kits, developed with a gel imaging and analysis system. The resultant blots were analyzed by using Quantity One software package (Bio-Rad Laboratories, USA) for determining the *A* values of the proteins and internal control (β-actin) of each group. The protein levels were expressed and the ratio between the *A* value of a target band and that of the internal control band was calculated.

1.2.5 Gelatin Zymography Forty-eight h after cells in the HCMV group were infected with HCMV, the cells in both groups were cultured in serum-free medium for 24 h.

Then the supernatant was collected and centrifuged at 2000 r/min for 10 min. The precipitate was harvested and stored at –70°C. Bradford technique was employed for the determination of protein concentration in all groups. The loading dosage was made identical according to the protein concentration of each group. The sample was mixed with loading buffer at 1:1 and 20 μL sample was loaded and 10% SDS-PAGE (containing 1.0 mg/mL) electrophoresis was run at 4°C and 100 V for approximately 1.5 h. The gelantin was put into eluent two times for 45 min, rinsed two times for 20 min, incubated at 37°C for 42–48 h, stained and de-stained. MMP-2 (72 kD) and MMP-9 (92 kD) presented as brightbands against blue background. Gelantin imaging and analysis system was utilized to read the bands and calculate the area, width and gray-scale for statistical analysis.

1.2.6 *In Vitro* Invasion Assay *In vitro* invasion assay was used for determining the invasive capacity of EVT. The Transwell chamber coated with Matrigel was put into 24-well culture plates, and 400 μL medium containing conditioned medium and complete medium (1:1) was added to the chamber. With HCMV group, EVTs at 1×10^5/mL and 100 TCID50 HCMV 14.29 μL (with a total amount of 100 μL) was put into the Transwell chamber. For the control group, PBS of identical amount was used instead of HCMV solution. Each group had 4 duplicate samples. After incubation for 24 h, the sample was fixed with formaldehyde, hematoxylin-stained and observed under an inverted microscope for counting the number of the cells migrating through the micropore membrane. For each sample, 10 randomly selected high power fields were counted.

1.3 Statistical Analysis

All experiments were performed 3 times. SPSS 13.0 software package was used for statistical analysis and data were expressed as $\bar{x}\pm s$. Differences between groups were tested by independent t test. A $P<0.05$ was considered to be statistically significant.

2 RESULTS

2.1 Source of EVTs

Immunohistochemical staining showed that cultured cells were mainly mononuclear cells (MNC), which were, for the most part, triangle- and irregularly-shaped. Over 96% of the cells exhibited CK-7-positive and c-erbB-2-positive staining, with occasionally seen Vim-positive staining, suggesting that the primary cells isolated from villous tissues were highly-pure EVTs (fig. 1).

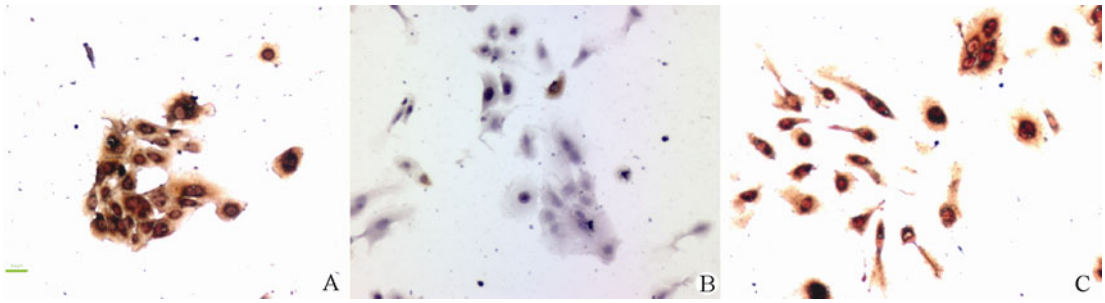

Fig. 1 Immunocytochemical detection of CK7 (A), Vim (B) and c-erbB-2 (C) antigens in primary EVT (SP method, ×100)

2.2 Detection of HCMVpp65

Immunohistochemical staining revealed HCMV pp65-positive staining in EVTs after culture with HCMV for 48 h (fig. 2A) while the cells in the control group were negatively stained (fig. 2B), indicating that HCMV could infect primary EVTs.

2.3 Expression Levels of c-erbB-2, MMP-2 and MMP-9 Proteins

Immunohistochemical staining and Western blotting showed that the EVTs in both HCMV group and control group had expression of c-erbB-2, MMP-2 and MMP-9 proteins but the expression levels of all the three proteins were significantly reduced ($P<0.05$) (fig. 3).

2.4 Zymographical Findings

Gelatin zymographic examination exhibited that primary EVTs could secrete MMP-2 and MMP-9 with enzymatic activity and after infection with HCMV, the enzymatic activity of both MMP-2 and MMP-9 was significantly reduced ($P<0.05$) (fig. 4).

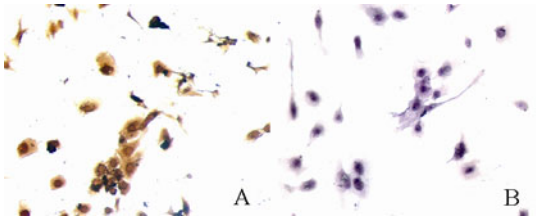

Fig. 2 Immunocytochemical detection of HCMVpp65 antigen in primary EVTs of HCMV group (A) and control group (B) (SP method, ×100)

2.5 *In Vitro* Cell Invasion Assay

EVTs in both groups penetrated the Matrigel and the number of cells going through the gel membrane was 49.2±3.27 in HCMV group and 57.0±3.61 in the control group, suggesting that the HCMV decreased the invasive capability of EVTs.

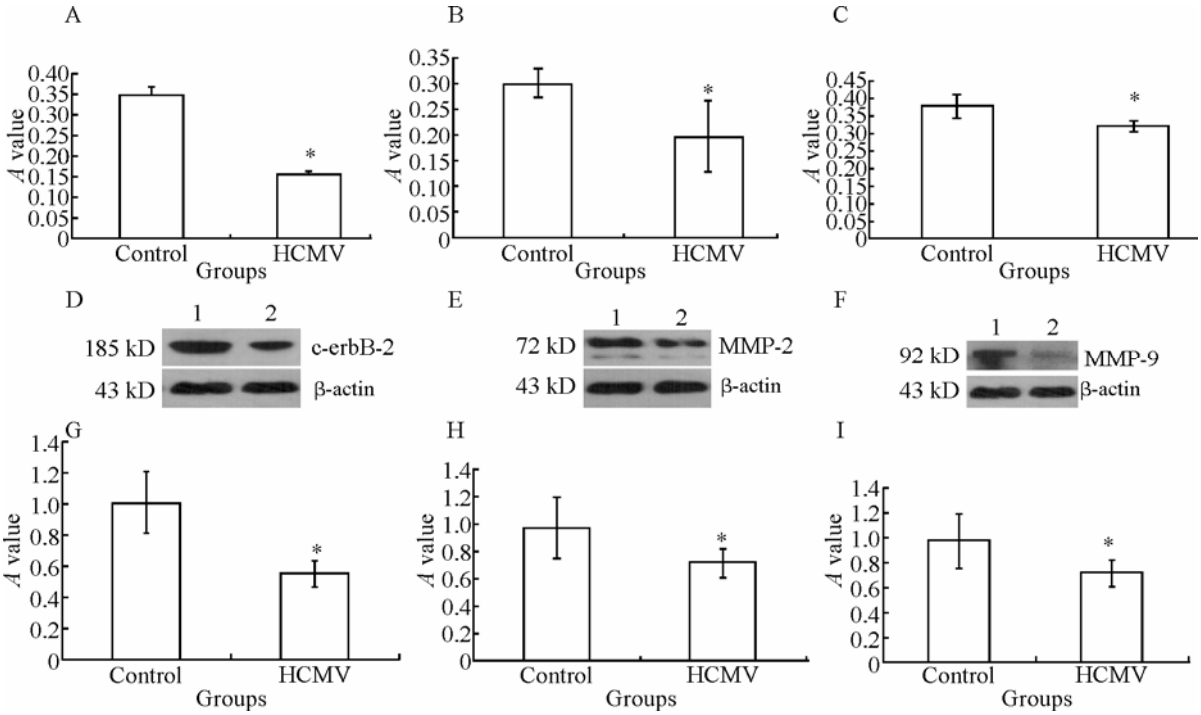

Fig. 3 The expression level of c-erbB-2, MMP-2 and MMP-9 proteins in two groups

Immunocytochemical detection of c-erbB-2 (A), MMP-2 (B), MMP-9 (C) proteins in two groups; results of Western blotting detection of c-erbB-2 (D), MMP-2 (E), MMP-9 (F) proteins in two groups; densitomeric quantification of the Western blotting results of c-erbB-2 (G), MMP-2 (H), MMP-9 (I) proteins in two groups; $^{*}P<0.05$ as compared with the control group

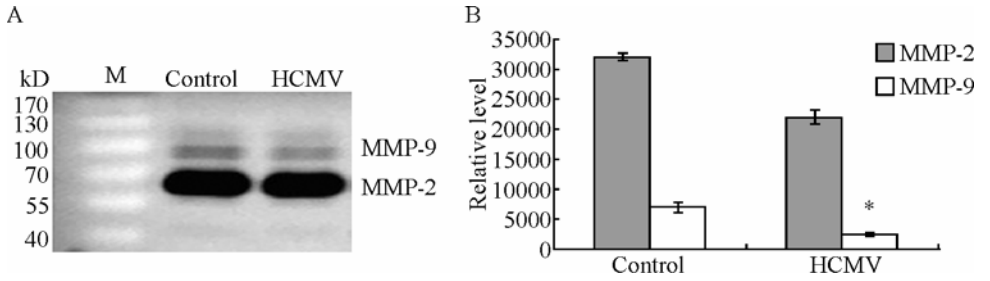

Fig. 4 Gelatin zymographic detection (A) of the activity of MMP-2 and MMP-9 of EVTs and the quantification (B)

M: Marker

3 DISCUSSION

Extravillous trophoblasts grow out 3 weeks after fertilization and remodel the maternal stroma and blood vasculature by penetrating into the endometrial stroma and gradually replacing endothelial cells of uterine spiral arteries[2]. Good remodeling of spiral arteries is not only essential for the substance exchanges of placenta, nutritional supply to fetus and discharge of metabolites but also a prerequisite for maintaining normal physiology of pregnancy and obviating pathological pregnancy. Impaired invasive capability of EVT, an important pathological basis of poor remodelling of spiral arteries, are intimately associated with abortion, stillbirths, fetal growth retardation, HDCP and poor development of offspring[3]. It is currently believed that HCMV is the most common intra-uterine pathogen and can cause abortion, stillbirth and the damage of central nerve system (CNS)[6]. The exact mechanism by which HCMV affects the development and growth of offspring remains poorly understood. So far, reports about the effect of HCMV on EVTs are scanty. In this study, the change in the invasive capability of EVTs after HCMV infection was examined by isolating primary EVTs from the healthy villous tissues.

Our study showed that cultured cells were mainly MNCs, which were mostly of triangle or irregular shape. CK-7-positive and c-erbB-2-positive staining was observed in virtually all cells, and Vim-positive staining was occasionally observed. EVTs specifically expressed CK-7 and c-erbB-2 but did not express Vim[7, 8]. Highly pure primary EVTs were obtained in this study, which laid foundation for further study of EVTs. Immunocytochemical study demonstrated that, 48 h after infection with HCMV, practically all EVTs in HCMV group exhibited HCMVpp65 staining while non-infected cells (controls) showed no viral signal (HCMVpp65 staining). HCMVpp65 antigen is expressed in endothelial cells of vessels and peripheral lymphocytes, mononuclear cells and polymorphonuclear leucocytes (PMN) and, therefore, is an indicator of early infection. This result showed that

HCMV could infect EVT cultured *in vitro*.

c-erbB-2 belongs to the family of human epidermal growth factor receptors and is closely involved in the fetal formation, tissue repair and regeneration. It is highly expressed in the primary EVTs cultured *in vitro* and its expression is highly coincident, both temporally and spatially, with the invasive capability of EVTs[4, 9–11]. MMP, especially MMP-2 and MMP-9, is one of key enzymes that mediate the migration and invasiveness of EVT and is specifically distributed in all kinds of trophoblast cells with some temporal characteristics and their activity is consistent with the change of EVTs invasive capability[12]. For instance, MMP-2 is expressed in the whole gestational period and is active only during early pregnancy and mid-trimester, while MMP-9 is highly expressed in EVTs during the early pregnancy and mid-trimester[13]. *In vitro* cell invasion assay showed that, under the induction of chemotactic agent, EVT went through Matrigel from serum-free upper chamber into the lower chamber containing serum. The number of cells penetrating the membrane can reflect the invasive capability of EVT[14]. Our study confirmed that，c-erbB-2, MMP-2 and MMP-9 proteins were all expressed in EVT and as compared with the control group, the expression levels of c-erbB-2, MMP-2 and MMP-9 proteins in HCMV group were significantly lower and the activity of MMP-2 and MMP-9 was significantly decreased and the number of cells going through the member was significantly reduced. These findings suggested that *in vitro* cultured primary EVT possesses good invasive capability but after infection with HCMV, its invasive capability is significantly impaired. The diminished invasive capability after HCMV infection might be related with decreeased expression of c-erbB-2, MMP-2 and MMP-9 proteins. The exact regulating mechanism, however, warrants further study.

This study, by means of *in vitro* test, confirmed that, at cellular level, HCMV could infect EVT *in vitro* and impair their invasive capability. The lowered invasive capability of EVT might have something to do with the aberrant expression of c-erbB-2, MMP-2 and MMP-9.

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