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Study of MMP-2 and FAK Expression in HSV-1 Infected Human Corneal Epithelial Cells

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Abstract: To investigate Matrix Metallo Proteinases-2 (MMP-2) and Focal Adhesion Kinase (FAK) expression in Herpes Simplex Virus-1 (HSV-1) infected human corneal epithelial cells. Human corneal epithelial cells were infected with HSV-1 *in vitro* to establish cell model of viral infection. The expression of MMP-2 and FAK were detected by RT-PCR, Western blot, Immunohistochemical and Immumofluorescence Method at 2, 20 and 40 h post-infection. Results at 2, 20 and 20 h post-infection, the mRNA of MMP-2 and FAK expression were significantly increased compared with uninfected cells ($p<0.01$, $p<0.05$). At 2 h post-infection, the expression of MMP-2, FAK and Phosphorylation FAK (P-FAK) protein were not significantly different with uninfected cells ($p>0.05$). At 20 and 40 h post-infection, the expression of MMP-2, FAK and P-FAK protein were significantly increased compared with uninfected cells ($p<0.05$). At the initial stage of HSV-1 infected, P-FAK plays an important role in the process of virus invading and MMP-2 activation.

Key words: MMP-2, FAK, HSV-1 keratitis, virus, cells

INTRODUCTION

The Matrix Metalloproteinases (MMPs) are a family of protein-cleaving enzymes that degrade extracellular matrix and basement membrane components which play central roles in some physiological processes such as wound healing, tissue repair and remodeling in response to injury and in progression of diseases such as arthritis, cancer and chronic tissue ulcers (Nagase *et al*., 2006). The previous researches show that MMPs were also implicated in some pathological conditions of corneal tissue such as corneal melting, corneal ulceration and perforation (Fini *et al*., 1998; O'Brien *et al*., 2001). One (the) previous study shows that MMP-2/9 produced by resident corneal cells and the inflammatory cells invading the cornea may possibly play an important role in early epithelial keratitis and ulcerative process in the late phase after corneal HSV-1 infection. The ratio of MMPs to TIMPs (tissue inhibitors of metalloproteinases) may be important for the course of necrotizing HSV keratitis (Yang *et al*., 2003). Although, the latest researches also support that the activities of MMPs are regulated by TIMPs (Yang *et al*., 2003), the mechanism of MMPs expression, secretion and activation in HSV-1 infection corneal is still unclear.

FAK was originally identified as a tyrosine kinase localized to focal adhesions which are multiprotein structures that link the extracellular matrix to the actin cytoskeleton through integrin receptors. FAK participates in the the regulation of cell cycle progression, cell survival, vascularization and cell migration (Mon *et al*., 2006; Cheshenko *et al*., 2005). More recent studies indicate that FAK was also involved in the regulation of MMPs expression, release and activation (Hu *et al*., 2006; Segarra *et al*., 2005).

Cheshenko *et al*. (2005) reported that the activation of FAK signaling was found in human cervical epithelia and colonic epithelia infected by Herpes Simplex Virus (HSV). FAK phosphorylation may involve in delivery of viral capsids to the nuclear pore. So, researchers hypothesize that the FAK maybe play a key role in the development of HSK through up-regulation of MMP-2 expression and promoting the HSV replication in the human corneal epithelial cells.

MATERIALS AND METHODS

Human corneal epithelial cells culture: The remaining rim of the cornea from corneal transplantation surgery was used for the present experiments. The human tissue was used in strict accordance with the basic principles of the Declaration of Helsinki. The cornea were separately with surgical microscope to provide a human corneal epithelial cells. The cells were cultured in DMEM/H supplemented with 10% FBS and they were used for the present studies after four to six passages. The purity of the cultures was

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judged on the basis of both cell morphology and reactivities with mouse Monoclonal antibody to Cyto Keratin 3 (CK 3).

Human corneal epithelial cells HSV-1 infection: After cells were grown to 80-90% confluence, cells were infected with HSV-1 (F) at a Multiplicity of Infection (MOI) of 5. After 1 h of adsorption at 37°C with 15 min intermittent rocking, the inoculum was removed and the medium was replaced with serum-free DMEM/high glucose. The cells were incubated at 37°C in a 5% CO_2-95% air incubator. At the indicated times, cells were harvested for further experiments. The expression of EGFP green fluorescence indicated that the HSV-1 were successfully infected into HCE cells. Count the number of positive cells to confirm virus infection ration (EGFP positive cells/total cells×100%).

Detect the mRNA expression of MMP-2 and FAK by PCR: HCE cells were harvested and washed with PBS after 2, 20 and 40 h of HSV-1 infection. Total RNA was isolated with TRIzol reagent according to the manufacturer's instructions. RNA was reverse-transcribed into cDNA then amplified using the specific primers listed as follow. The sequence of the primer sets used was: forward 5'-GTCCACCGCAAATGCTTCTA-3', reverse 3'-TGCTGTCACCTTCACCGTTC-3' for β-actin (190 bp); forward 5'- CCCTATGGTGAAGGAAGTCG-3', reverse 5'-TGCCATCTCAATCTCTCGGT-3' for FAK (106 bp); forward 5'-AGTGACGGAAAGATGTGGTGTG-3', reverse 5'-CTTGGTGTAGGTGTAAATGGGTG-3' for MMP-2 (182 bp).

The PCR products were electrophoresed in GoldView stained 2% agarose gels. Quantification of the bands was performed using a Geliance 200 Imaging System (Perkin Elmer, USA) and Gel-Pro Software. The level of mRNA was expressed as the ratio of Integrated Optical Density (IOD) of specific PCR products over β-actin IOD.

Detect the protein expression of MMP-2, FAK and p-FAK by Western-blot: HCE cells were harvested and washed with PBS after 2, 20 and 40 h of HSV-1 infection. And aliquots of cell extracts (total proteins) were separated on a 8-12% SDS-polyacrylamide gel. Then, proteins were transferred to a polyvinylidene difluoride membrane and incubated overnight at 4°C with the mouse monoclonal antibody anti-MMP-2, FAK and p-FAK (Santa Cruz, USA), respectively. Membranes were then washed and incubated with HRP conjugated secondary antibodies in confining liquid for 1 h and developed using ECL Test kit (Beyotime, China) on the film. The density of the bands on the film were scanned and analyzed with an image analyzer.

Detect the histopathology: HCE cells were cultured on a glass coverslip in 6 well chamber dishes and infected with HSV-1 as described in study. Slide-mounted cells were blocked by endogenous peroxidase-blocking solution and followed by goat serum (each for 15 min at 37°C). After blocking nonspecific binding, cells were incubated with mouse anti-human monoclonal antibodies that recognize MMP-2, FAK, p-FAK (Santa Cruz, USA) at 4°C overnight. Cells were then incubated with the biotin-rabbit anti-goat antibody (Santa Cruz) at 37°C for 30 min. Samples were counterstained with hematoxylin and eosin. Negative control specimens were taken by staining without the primary antibodies. The MMP-2, FAK and p-FAK positive cells were analyzed in the study per high-power field (x200).

Indirect immunofluorescence: HCE cells were cultured on a glass cover slip in 6 well chamber dishes and infected with HSV-1 as described in study. Slide-mounted cells were blocked by endogenous peroxidase-blocking solution and followed by goat serum (each for 15 min at 37°C). After blocking nonspecific binding, cells were incubated with mouse antihuman monoclonal antibodies that recognize MMP-2, FAK (Santa Cruz, USA) at 4°C overnight. Cells were then incubated with FITC-conjugated secondary goat anti-rabbit IgG (DAKO, Japan) at 37°C for 60 min. Prior to mounting, cells were stained with PI for 10 min then observed using a confocal laser scanning microscope (Carl Zeiss, Germany). Cells incubated with PBS (instead of the first antibody) were used as negative controls.

Statistical analysis of data was performed by one-way ANOVA and a Student-Newman-Keuls test to determine statistically significant differences ($p<0.05$).

RESULTS AND DISCUSSION

The infection efficiency of HSV-1: The EGFP was used as an maker to detect whether the HSV-1 were successfully infected into HCE cells. Count the number of green fluorescence cells and total cells at same visual fields by fluorescence microscope to confirm virus infection ration. The infection efficiency is shown in Fig. 1 which is approach to 92% at a Multiplicity of Infection (MOI) of 5 (Fig. 1).

Expression of MMP-2 and FAK in HSV-1 infected HCE cells: The mRNA level of MMP-2 and FAK in HCE cells infected with HSV-1 was estimated using RT-PCR (Fig. 2a, Table 1). The mRNA level of MMP-2 increased significantly at 2, 20 and 40 h when compared with uninfected cells ($p<0.05$) but there was no difference at the various time point in statistics. Interestingly , the mRNA level of FAK in infected cells at 2, 20 and 40 h increased significantly compared with normal control ($p<0.05$) which was similar to MMP-2 expression and there was also no difference at the various time point in statistics.

To further verify the results of RT-PCR, Western-blot was used to observe the changes of p-FAK, FAK and MMP-2 protein expression in HCE cells infected with HSV-1 (Fig. 2b, Table 2). After 2 h infected with HSV-1, there was no obvious change in the p-FAK protein

Table 1: The relative gray values of Matrix Metallo Proteinase-2 (MMP-2) mRNA electrophoretic bands in infected and non-infected cells at various times, obtained from Western blot

Parameters	2 h	20 h	40 h	F-values	p-values
Non-infected cells	0.47±0.05	0.45±0.02	0.43±0.07	0.771	0.485
Infected cells	1.22±0.02	1.23±0.09	1.25±0.10	0.190	0.830
t-values	31.142	18.918	15.021	-	-
p-values	0.0000	0.0000	0.000	-	-

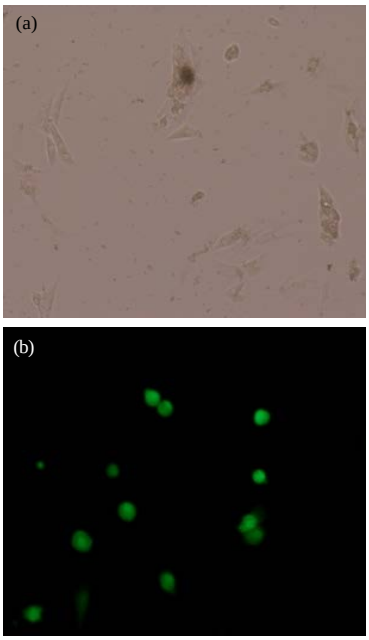

Fig. 1: The virus infection efficiency of type I Herpes Simplex Virus (HSV-1) infection in Human Corneal Epithelial (HCE) cells

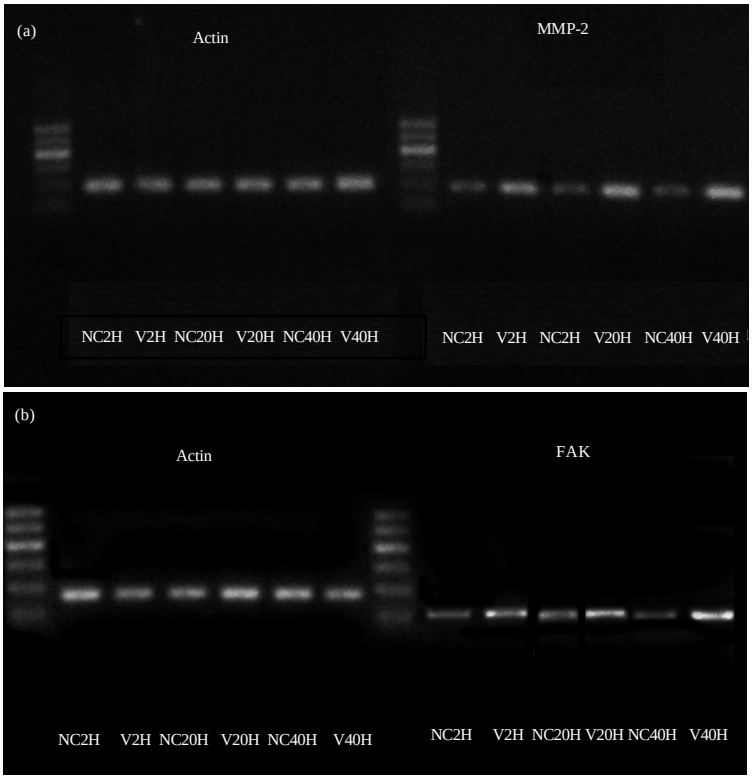

Fig. 2: a) Changes in the expression of MMP-2 mRNA in HCE cells after HSV-1 infection and b) changes in the expression of FAK mRNA in HCE cells after HSV-1 infection

expression of HCE cells while the p-FAK protein remarkably increased after 20 and 40 h compared with normal control cells. The similar result was found in FAK protein. The MMP-2 protein expression was consistent with FAK protein after HSV-1 infection but interestingly the MMP-2 protein of uninfected cells remarkably decreased after 40 h which was inconsistent with results from RT-PCR (Fig. 3).

P-FAK expression in HCE cells detected by Western blotting assays: P-FAK expression in HCE cells as detected by Western blotting assays at 2, 20 and 40 h after HSV-1 infection is shown in Fig. 4. No statistically significant difference was observed between infected and non-infected cells at 2 h after infection ($p>0.05$). At 20 and 40 h after infection, P-FAK expression differed significantly between infected and non-infected cells ($p<0.05$). P-FAK expression in the infected cells at 2 h after infection was significantly different from the level detected at 20 and 40 h ($p<0.05$). Over time, P-FAK expression of non-infected cells did not change significantly ($p>0.05$). FAK expression in HCE cells detected by Western blotting at 2, 20 and 40 h after HSV-1 infection (Fig. 4, column 2) was in line with the aforementioned P-FAK expression. MMP-2 expression in HCE cells detected by Western blotting assays 2, 20 and 40 h after HSV-1 infection is shown in Fig. 4 (column 1). No statistically significant difference was observed between infected cells and non-infected cells at 2 h after infection ($p>0.05$). At 20 and 40 h after infection, MMP-2 expression was significantly greater in infected than in non-infected cells ($p<0.05$). MMP-2 expression in the infected cells at 2 h after infection was significantly lower than that at 20 and 40 h ($p<0.05$). At 40 h, MMP-2 expression in non-infected cells was significantly different from that in the same cells at 2 and 20 h after infection ($p<0.05$).

The results of histopathology and immunofluorescence in infected HCE cells: The MMP-2, FAK and p-FAK positive cells infected with HSV-1 showed blue nucleus and brown cytoplasm by immunostaining. There was consistent results by immuno-fluorescence (Fig. 4).

Cells with positive immunofluorescence staining for MMP-2 shows green fluorescent light, cells with positive

Table 2: The relative gray values of the Focal Adhesion Kinase (FAK) mRNA electrophoretic bands in infected and non-infected cells at various times, obtained from Western blot

Parameters	2 h	20 h	40 h	F-values	p-values
Non-infected cells	0.40±0.03	0.43±0.06	0.41±0.08	0.321	0.731
Infected cells	0.87±0.05	0.87±0.04	0.93±0.04	3.610	0.079
t-values	18.024	13.644	13.000	-	-
p-values	0.000	0.000	0.000	-	-

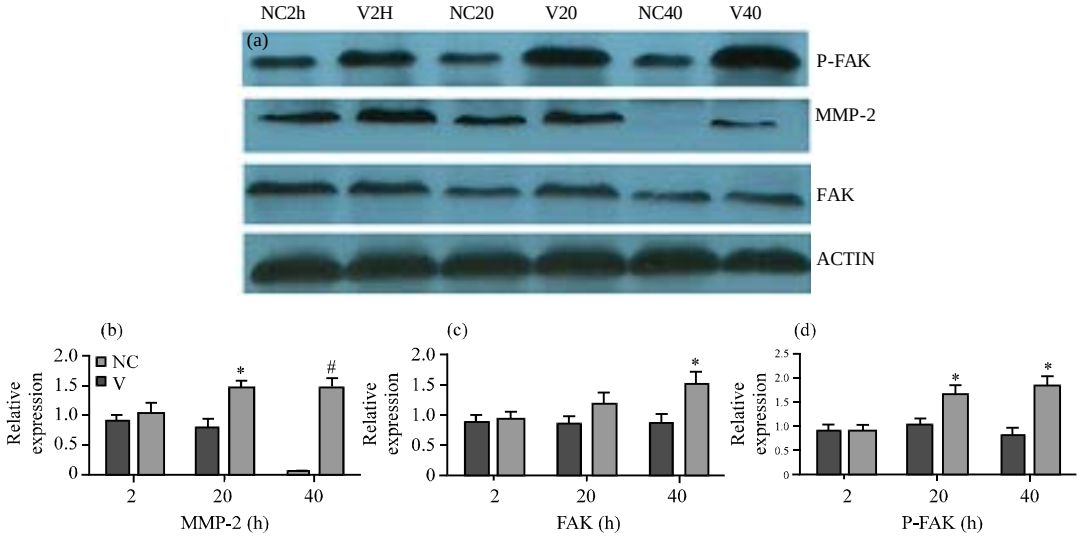

Fig. 3: Changes in the expression of matrix metalloproteinase-2 (MMP-2), Phosphorylated Focal Adhesion Kinase (P-FAK) and FAK proteins in Human Corneal Epithelial (HCE) cells after type I Herpes Simplex Virus (HSV-1) infection; a) changes in the expression of MMP-2, P-FAK and FAK protein in HCE cells after HSV-1 infection; b) histogram of the relative expression of MMP-2; c) FAK and d) P-FAK protein in infected (V) and Negative Control (NC) cells in panel A. HCE cells infected with HSV-1 showed an increasing trend in protein expression of MMP-2, P-FAK and FAK with the increase in infection time, $p<0.05$; $^{\#}p<0.01$

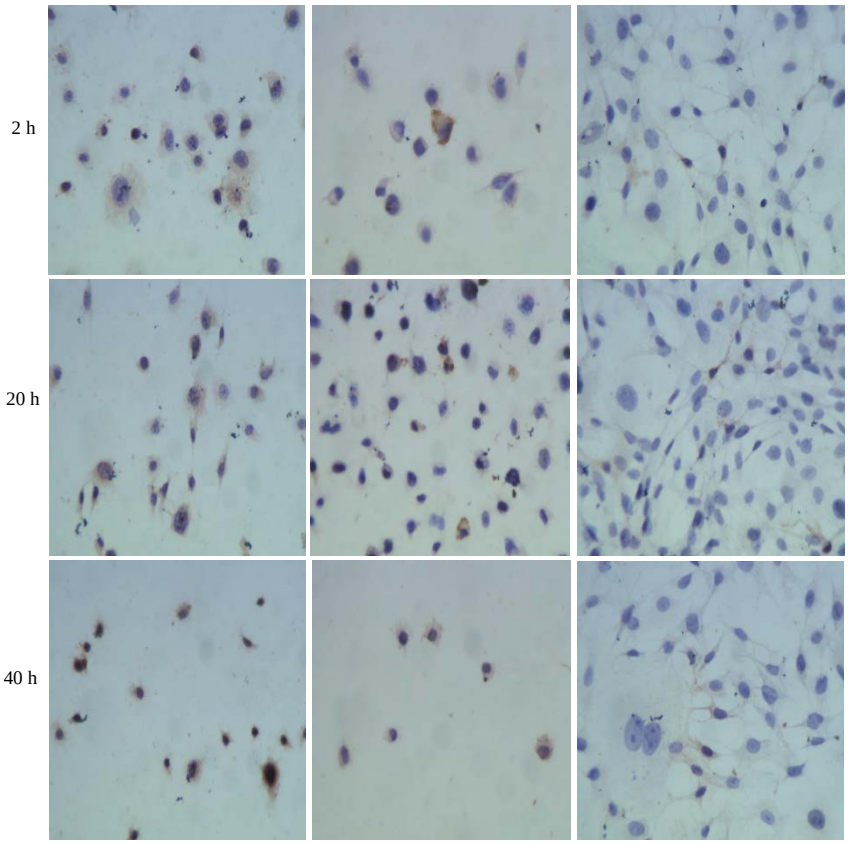

Fig. 4: Detection of Matrix Metallo Proteinase-2 (MMP-2), focal adhesion kinase FAK and Phosphorylated FAK (P-FAK) protein expression using immunohistochemical staining (x400)

immunofluorescence staining for FAK shows red fluorescent light, cells with negative immunofluorescence staining for all shows blue fluorescent light. The number of positive cells was increased along with the infected time elongation (Fig. 5).

In this research, HSV-1 was successfully transfected into HCE cells *in vitro*. The MMP-2 expression was detected in early phase. The results showed that the mRNA level of MMP-2 remarkably increased after 2 h of HSV-1 infection compared with normal control and lasted until 40 h of HSV-1 infection. The MMP-2 protein expression remarkably increased until 20 h of HSV-1 infection which fall behind the change of mRNA level. The result was consistent with other researches.

Yang *et al.* (2003) reported that MMP-2 protein was highly expressed in the cornea of HSV-1 keratitis mice model at day 2/14 after HSV-1 infection. It was mainly appeared in the sub-epithelial stroma and the superficial substantia propria layer. At day 7, the MMP-2 protein expression was reduced when the corneal healed. At day 14, there was significantly increased staining in the whole stroma but especially in the area of necrosis and close to the ulcer. Strong staining was detected in the neutrophils.

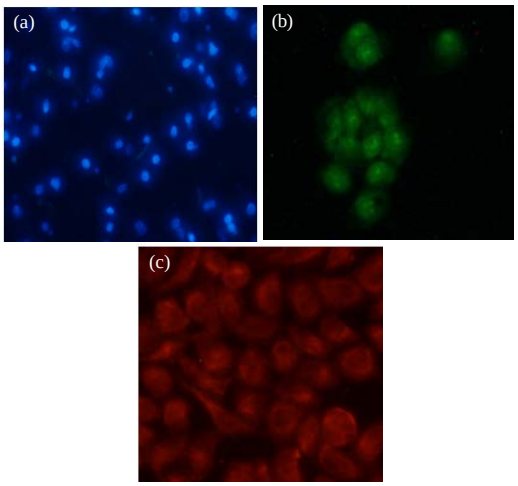

Fig. 5: The immunofluorescence staining for virus transfection cells

These data suggest that MMP-2 produced by resident corneal cells and PMNs may possibly play a role in early epithelial keratitis and in the ulcerative process in the late phase after corneal HSV-1 infection.

Cheshenko *et al.* (2005) reported that the activation of FAK signaling was found in human cervical epithelia and colonic epithelia infected by Herpes Simplex Virus (HSV). FAK phosphorylation may involve in delivery of viral capsids to the nuclear pore. Infection was reduced by >90% in FAK knockout cells relative to control cells. In this research, we found the mRNA level of FAK highly expressed in HCE cells after 2 h of HSV-1 infection and the expression of FAK and p-FAK proteins remarkably increased after 20 h of HSV-1 infection which was consistent with the expression of MMP-2 protein.

Hsia *et al.* (2003) reported that the activation of FAK lead to formation of a FAK-Src-p130Cas-Dock 180 signaling complex, elevated Rac and c-Jun NH_2-terminal kinase activation and increased matrix metall oproteinase expression and activity. Recent research found that the activity of MMP-2/8/9 obviously elevated at day 2 post-infection in the cornea of HSV-1 Keratitis Mice Model by gelatin zymogram (Yang *et al.*, 2003).

Accordingly, it was supposed that the phosphorylation and activation of FAK may play an important role in delivery of HSV-1 capsids to the nuclear pore and activation of MMP-2 which promoted the the course of cornea ulcer in HSV keratitis.

CONCLUSION

In the present research, researchers investigated the presence and enzymatic activities of FAK and MMP-2 in the human corneal epithelial cells with HSV-1 infection and identified the relationship between FAK and MMPs and their role in the development of HSK. The observations suggest that MMPs produced by resident corneal cells and by the inflammatory cells invading the cornea may participate in the destruction of the cornea after HSV-1 corneal infection and that TIMPs are expressed during the healing process.

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