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Output จากโครงการวิจัย

1. manuscript เพื่อตีพิมพ์เผยแพร่
2. การนำผลงานวิจัยไปใช้ประโยชน์
   2.1 เชิงสาธารณะ นำไปสู่ความร่วมมือในการทำงานวิจัยระหว่างหน่วยงานและสาขาวิชา ได้แก่ ระหว่างสาขาที่เกี่ยวข้องกับโรคติดเชื้อ และโรคหลอดเลือดหัวใจ
   2.2 เชิงวิชาการ ข้อมูลที่ได้สามารถนำไปใช้เป็นส่วนหนึ่งของการสอน และเป็นข้อมูลเบื้องต้นสำหรับการทำวิทยานิพนธ์เพื่อการศึกษาในเรื่องโรคติดเชื้อและภาวะหลอดเลือดแดงแข็งต่อไป

ภาคผนวก

Herpes Simplex Virus 1 Induced Expression of LOX-1 Expression in an Endothelial Cell Line, ECV 304

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Abstract

Besides dyslipidaemia, smoking, hypertension and diabetes, infection has been proposed to be a risk factor for atherosclerosis. Microbes that have been shown to be involved in atherogenesis are *Chlamydophilia pneumoniae*, cytomegalovirus, herpes simplex virus and *Helicobacter pylori*. The existences of antibodies to those microbes and microbes found in plaques have been demonstrated to be associated with atherosclerosis.

It has been shown that herpes simplex virus I (HSV-1) could infect vascular endothelial cells and when endothelial cells were activated with oxidized LDL (oxLDL), adhesion molecule expression, oxygen radical production and apoptosis were increased which may lead to endothelial cell dysfunction and foam cell accumulation resulting in atherogenesis. LOX-1 is a major receptor for oxLDL on endothelial cells and its expression was increased in atherosclerosis.

We attempted to investigate whether HSV-1 infection increases expression of LOX-1 in endothelial cells. LOX-1 mRNA expression determined by RT-PCR and LOX-1 promoter activity measured by luciferase assay were increased in endothelial cells following HSV-1 infection. This suggests that one of the mechanisms by which HSV-1 is involved in atherogenesis is the enhanced uptake of oxLDL via the increased expression of LOX-1 in endothelial cells.

Keywords: LOX-1, herpesvirus, herpes simplex virus, atherosclerosis

Introduction

It is now well accepted that the inflammatory process is involved in atherosclerosis and it is hypothesized that hypertension, smoking and elevated levels of LDL cholesterol are factors leading to injury of vascular endothelial cells and this injury results in activation of the inflammatory process. However, atherosclerosis can develop in patients without those mentioned risk factors so other risk factors may be involved in atherogenesis. During the past decade, several recent studies have suggested that infections by microbes such as *Chlamydophilia pneumoniae*, *Helicobacter pylori*, Cytomegalovirus and Herpes simplex virus are potential pathogenic factors. Supporting evidence includes the finding of microbes, their structural components or their nucleic acids in atherosclerotic lesions compared with in non-atherosclerotic lesion and higher antibody titers against those microbes in patients than in control groups. In addition, antibiotic treatment prevented acceleration of atherosclerosis by infection in an animal model (1). The mechanisms by which infection can promote atherosclerosis may be the increase of coagulation, induction of endothelial dysfunction, and increased instability of plaques resulting in inflammatory process (2-9).

Herpes simplex viruses, members of the family herpesviridae, are responsible for diseases ranging from common, relatively benign cutaneous lesions to fatal HSV encephalitis (10). In addition to those symptoms, HSV has been implicated as an etiologic factor in pathogenesis of human atherosclerosis. It has been shown that greater amounts of saturated cholesterol esters and triacylglycerol accumulated in HSV-1 infected human and bovine arterial smooth muscle cells than in uninfected cells (11). This suggested that HSV-1 induced lipid accumulation in smooth muscle cells which is a characteristic feature of atherosclerosis. Existence of HSV-1 nucleic acid and antigen in atherosclerotic lesions has been demonstrated to be related to atherosclerosis compared with non-atherosclerotic tissues (12-14). The ability of HSV to replicate in endothelial cells has been demonstrated and it has been suggested that vascular endothelium may be a site of latent HSV infection and re-activation of virus infection may enhance atherosclerosis (15).

It has been shown that HSV infection of endothelial cells contributes to deposition of thrombin on atherosclerotic plaques and to the coagulant-necrosis state that characterizes HSV-infected mucocutaneous lesions (16). The adhesion of leukocytes to endothelium may be an

initial step in inflammation and one mechanism that promotes cell adhesion is expression of adhesion molecules. It has been shown that HSV-1 infection of endothelial cells increased adherence of leukocytes to endothelial cells and induced expression of adhesion molecule GMP140. The expression of adhesion molecules may be a pathogenic mechanism in HSV-1-induced cell injury and inflammation (17-18). In vascular cells, HSV-1 infection leads to lipid accumulation, attraction of leukocytes with subsequent inflammatory damage, activation of procoagulant on endothelium with increased thrombin generation and platelet adhesion. (Reviewed in 19)

Oxidative modification of low density lipoprotein (LDL) is involved in formation of macrophage-derived foam cells which are a typical feature of atherosclerotic lesions (20). Foam cells could be induced from macrophages after taking up oxidatively modified LDL (oxLDL) OxLDL is elevated in patients with acute myocardial infarction compared with healthy controls and it has been proposed to be a marker for coronary artery disease (21-24). Besides induction of macrophage foam cell formation, oxLDL induces endothelial cell dysfunction, resulting in impaired nitric oxide production and induction of proatherogenic genes, adhesion molecule expression, and smooth muscle cell growth factors. More than ten receptors for oxLDL referred to as scavenger receptors, have been cloned (25-27). LOX-1 (lectin-like oxidized low-density lipoprotein receptor) has been identified as a receptor for oxLDL. LOX-1 was initially identified from vascular endothelial cells by Sawamura T, et al 1997) (28) . Regulation and function of this receptor in atherosclerosis have been widely studied (review in 29-32).

Besides oxLDL, expression of LOX-1 can also be upregulated by various stimuli such as angiotensin II, TNF-α,PMA, lysophosphatidylcholine, interleukin-4, histamine, peroxisome proliferator-activated receptor (PRAP), norepinephrine, endothelin-1 and ischemia-reperfusion (33-44). Activation of LOX-1 induced activation of NF-kB, expression of adhesion molecules, chemokines, endothelin-1 and superoxide anion and decrease the release of superoxide anion and production of nitric oxide resulting in endothelial cell dysfunction (28,45-46). Specific genetic variations in LOX-1 are associated with coronary artery disease (47-48).

Although considerable evidence suggests that HSV infection is related to atherosclerosis, the molecular mechanisms are not clearly understood. Uptake of oxLDL is involved in promotion of atherosclerosis, and there is evidence suggesting that vascular endothelium may be the site of latent HSV infection. For these reasons, we investigated whether HSV-1 infection could lead to the induction of LOX-1 expression, possibly resulting in increased uptake of oxLDL

in endothelial cells. ECV304, an endothelial cell line was used for HSV-1 infection. LOX-1 mRNA expression induced by HSV-1 infection was demonstrated. The result of mRNA expression was confirmed by the activation of promoter of LOX-1 following HSV-1 infection.

Materials and Methods

Viruses and Cells

HSV-1 (KOS strain) and Vero cells (African green monkey kidney cell line) were kindly provided by Associate Professor Parvapan Bhattarakosol, Ph.D. Department of Microbiology Faculty of Medicine, Chulalongkorn University. Vero Cells were grown in growth medium M199 (GIBCO BRL, USA) supplemented with 10% fetal bovine serum (GIBCO BRL), 100 units/ml penicillin G, 100 µg/ml streptomycin (GIBCO BRL) and 0.01M HEPES (N-2-hydroxyethyl-piperaine-N′-2-ethan sulfonic acid) (GIBCO, BRL).

The human endothelium-derived cell line, ECV304, kindly provided by Professor Yasuyuki Sasaguri, Department of Pathology and Cell Biology, School of Medicine. University of Occupational and Environmental Health, Kitakyushu, Japan, was grown in M199 supplemented with 10% fetal bovine serum, L-glutamine, penicillin G (100 units/ml) and streptomycin (100 µg/ml).

RNA isolation

ECV304 cells (2.5×10^5 /ml/well) were plated in a 24-well plate at 37°C overnight. Cells then were infected with HSV-1 as indicated. RNA from ECV304 cells was isolated using Purescript (Gentra,USA) according to the manufacturer's instructions. Briefly, culture medium was removed and 300 ul of cell lysis solution were added onto cells in each well. Cell lysates were transferred into 1.5 ml tubes, 100 ul of protein-DNA precipitation solution was added and cell suspension was incubated on ice for 5 minutes before centrifugation at 14,000 rpm for 3 minutes. Supernatant containing RNA was transferred into new tubes and RNA was precipitated using 300 ul isopropanol. RNA pellets obtained by centrifugation were washed with 70% ethanol and then resuspended into RNA hydration solution. RNA concentration was measured at 260 nm.

RT-PCR

RNA (1 ug) was reverse transcribed into cDNA using ImProm-II Reverse Transcription System (Promega, USA) with oligo(dT) as a primer according to the manufacturer's instruction. Then, cDNA was amplified using primers : 5' TGC CTG GGA TTA GTA GTG ACC and 5'CCA GTT AAA TGA GCC CGA GG for LOX-1 mRNA (49) and 5'CTA CAA TGA GCT GCG TGT GG and 5' AAG GAA GGC TGG AAG AGT GC for β-actin mRNA (50). The thermal profiles used were 40 cycles at 94°C for 40 seconds, 57 °C for 1 minute and 68 °C for 1 minutes for LOX-1 and 20 cycles at 94°C for 40 seconds, at 60 °C for 1 minute and at 72 °C for 1 minute for β -actin.

PCR products were subjected to 1.5% agarose gel electrophoresis and visualized by ethidium bromide staining. The expected product sizes were 360 and 528 bp for LOX-1 and β- actin, respectively.

Sequecing of PCR product

The purified PCR product was sequenced using primers specific for LOX-1 (the same primers used in PCR amplification), ABI Prism Bigdye Terminator Cycle Sequencing Ready Reaction kit version 3.1 and the Perkin Elmer 9600 automated nucleic acid sequencer as followed. PCR amplification was done in total 20 ul reaction containing purified PCR product, Big dye terminator, and primers in kit buffer. PCR was performed for 25 cycles in the following condition : rapid heat 96 °C, heat denaturation at 96 °C for 10 seconds, and primer annealing at 50 °C for 5 seconds, and DNA extension at 60 °C for 4 minutes. The product was further processed for sequencing using automated nucleic acid sequencer. The sequence obtained was alignment with Clustal X program and homology Blast search in Genbank database.

Transfection and Luciferase assay

A plasmid containing the LOX-1 promoter region upstream of luciferase gene (pLOX-1Luc) and pGL3 plasmid, a promoter-less luciferase reporter plasmid were kindly provided by Professor Yasuyuki Sasaguri, Department of Pathology and Cell Biology, School of Medicine. University of Occupational and Environmental Health, Kitakyushu, Japan (39).

pLOX-1Luc or pGL3 were transfected into ECV304 cells using the liposome reagent, TransFast Reagent (Promega) according to the manufacturer's instruction. ECV cells were plated onto a 24-well plate at a concentration of 1 x 10^5 cells /ml/well and incubated at 37°C for 24 hours. Cells were washed with PBS and the plasmid/liposome mixture (the combination of plasmid and TransFast Reagent in M199 serum free medium) was added. Cells were incubated for 1 hour before one ml of M199 medium containing 10% FBS was added in each well. The incubation was continued for 48 hours before HSV-1 was added and further incubated at the indicated times.

Luciferase assay (Promega) was done using Luciferase Assay System (Promega) according to the manufacturer's recommendation. Briefly, culture medium was removed from transfected-ECV304 cells infected with HSV-1. Cells were washed with PBS and lysed with 50 ul of luciferase lysis buffer. Lysed cells were transferred to microcentrifuge tubes and cell lysates were obtained by spinning at 14,000 rpm for 15 seconds. The supernatant was transferred to a new tube and stored at –80°C or processed for luciferase activity measurement. The activity of luciferase was determined by mixing 20 ul of cell lysates with 100 ul of luciferase assay reagent and measured (Reader 50 Luminometer). Transfection and luciferase assay were done in triplicate. Luciferase activities were measured as relative light unit (RLU) and expressed as fold induction relative to the sham-treated cells transfected with pGL3.

Results

HSV-1 infection induced LOX-1 mRNA expression

ECV304 cells were infected with 5 MOI of HSV-1 for 2, 4 and 8 hours. Cells were then harvested and RT-PCR was performed from isolated RNA as mentioned in Materials and Methods. As shown in Figure 1A, HSV-1 induced LOX-1 mRNA expression by 2 hours (Lane 2) and the expression could still be observed at 8 hours post infection (Lane 4). Amount of RT-PCR product using primers specific for β-actin was similar in all Lanes (Figure 1, Lanes 5-8).

Sequencing of PCR product was performed in order to confirm the amplification of LOX-1 cDNA. The DNA seqeunce alignment indicated that the product was LOX-1 cDNA (GenBank AB010710)(data not shown).

Induction of LOX-1 mRNA expression was dose dependent

ECV304 cells were infected with HSV-1 at 0.5, 5 and 10 MOI for 4 hours. RT-PCR for LOX-1 expression was performed. LOX-1 expression was observed when 0.5 MOI of HSV-1 was used (Figure 2A, Lane 2) and the expression was increased when the amount of viruses was increased to 5 MOI (Lane 3). There was no difference in LOX-1 expression when 10 MOI of viruses was used compared with 5 MOI (Lanes 3 and 4). Amount of RT-PCR product using primers specific for β-actin was similar in all Lanes (Figure 2B, Lanes 1-4).

HSV-1 induced activation of LOX-1 promoter activity

In addition to detection of LOX-1 mRNA expression, the activation of LOX-1 promoter following HSV-1 infection was also investigated. Various amounts of plasmid (0.25, 0.5, 0.75 and 1.0 ug) were tested for transfection of ECV304 cells according to the manufacturer's suggestion. The plasmid at 0.5 ug was selected for further use since there was no difference in luciferase activity when 0.5 and 0.75 ug of plasmid was used and the activity was lower at 1.0 ug plasmid (Figure 3). Higher amount of plasmid gave lower reading possibly due to its toxicity to cells.

ECV304 cells transfected with pLOX-1Luc or pGL3 were infected with 5 MOI of HSV-1 for 4 and 8 hours. The experiment was done in triplicate. Cell lysates were collected and assayed for luciferase activity. As shown in Figure 4, the significant induction of luciferase activity was observed at 8 hours following infection.

Activation of HSV-1 promoter activity by HSV-1 was dose dependent

Various amounts of HSV-1 (0.5, 5 and 10 MOI) were tested and the results showed that the induction of luciferease activity implying the activation of LOX-1 promoter was dose dependent. Corresponding to the result of RT-PCR, there was no significant difference in fold induction between infection with 5 and 10 MOI of HSV-1 (Figure 5).

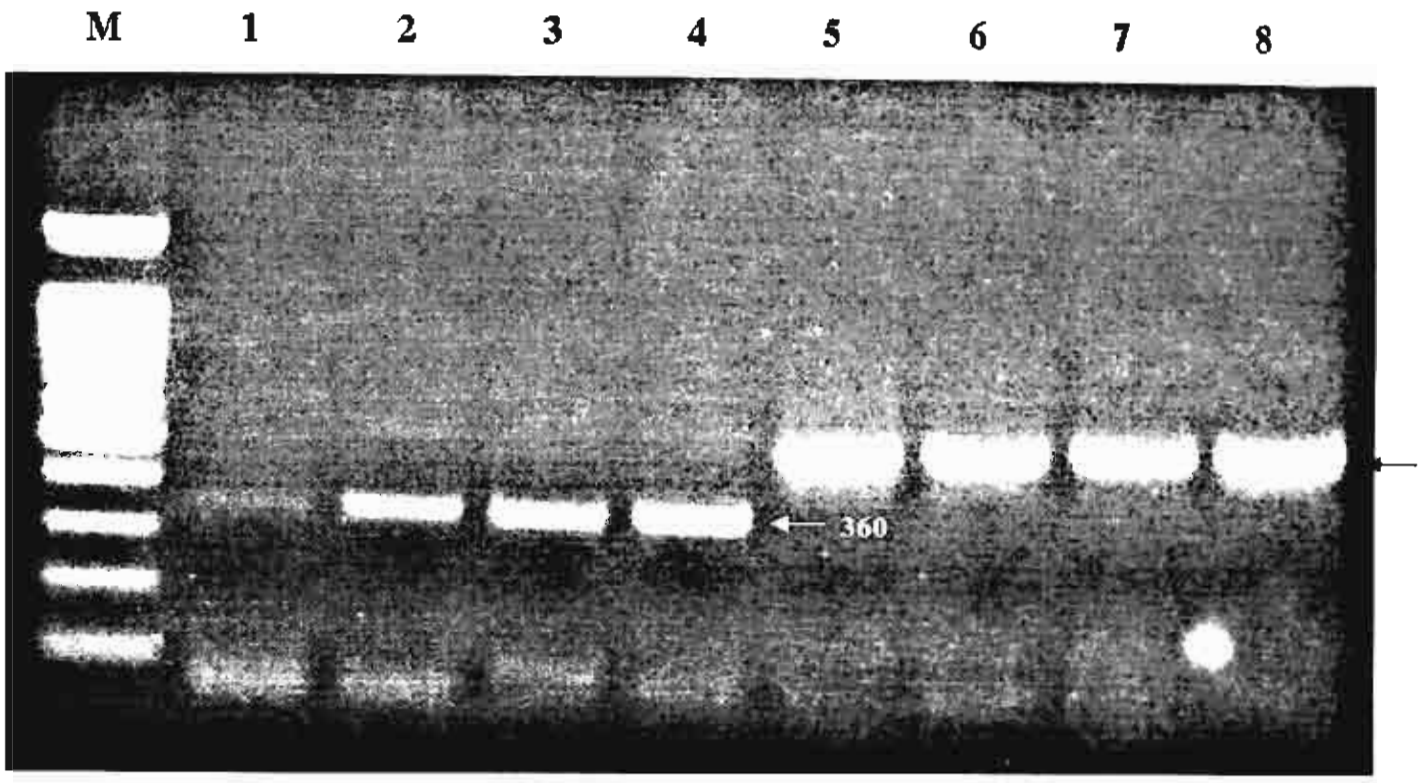

Figure 1. Induction of LOX-1 mRNA expression by HSV-1. ECV304 cells were infected with 5 MOI of HSV-1 for 2, 4 and 8 hours. RNA was isolated and reverse transcription was performed. cDNA was used for PCR with primers specific for LOX-1 (Lanes 1-4) or for β-actin (Lanes 5-8). Lanes 1 and 5 are from non-infected cells, Lanes 2 and 6 from 2-hour infection, Lanes 3 and 7 from 4-hour infection and Lanes 4 and 8 from 8-hour infection. Lane M is 100-bp markers. The arrows indicate the PCR products which are 360 and 528 bp for LOX-1 and β-actin, respectively. This experiment is the representative of 3 similar experiments

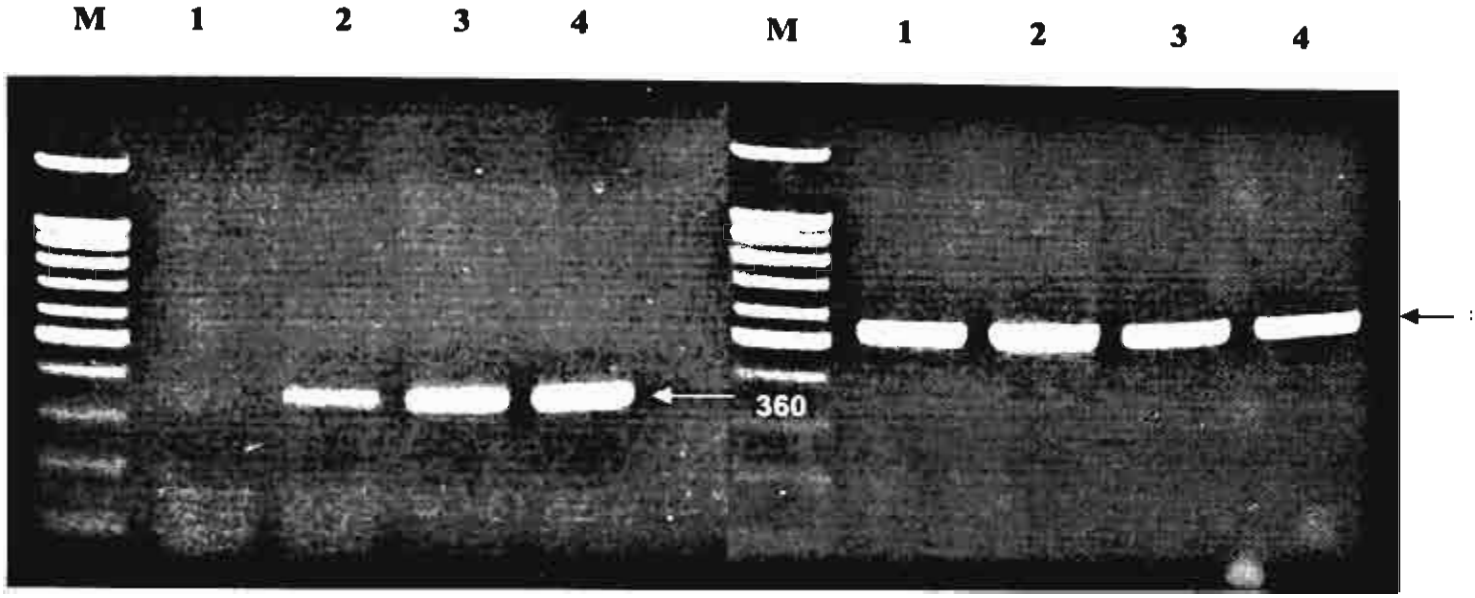

Figure 2. Various amounts of HSV-1 on LOX-1 mRNA expression. ECV304 cells were infected with 0.5, 5 and 10 MOI of HSV-1 for 4 hours. RNA was isolated and reverse transcription was performed. cDNA was used for PCR with primers specific for LOX-1 (Figure 2A) or for β-actin (Figure 2B). Lane 1 is from non-stimulated cells, Lanes 2-4 are from cells infected with HSV-1 at 0.5, 5 and 10 MOI, respectively. The arrows indicate the PCR products which 360 and 528 bp for LOX-1 and β- actin, respectively. This experiment is the representative of 3 similar experiments

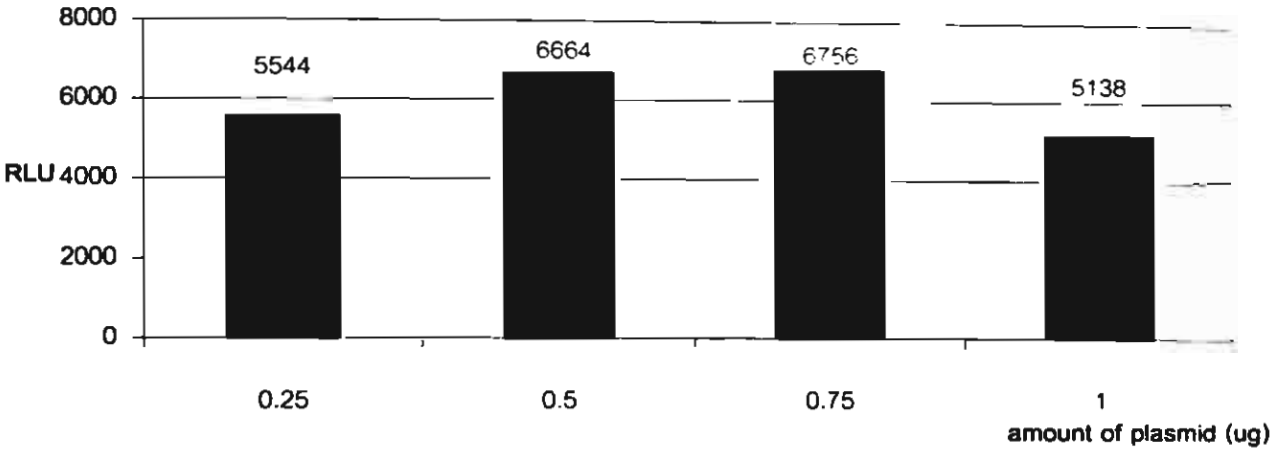

Figure 3. Determination of an optimal amount of DNA for transfection. Various amounts of pLOX-1Luc (0.25, 0.5, 0.75 and 1.0 ug) were used for transfection of ECV304 cells. Luciferase activities were measured and expressed as relative light units (RLU).

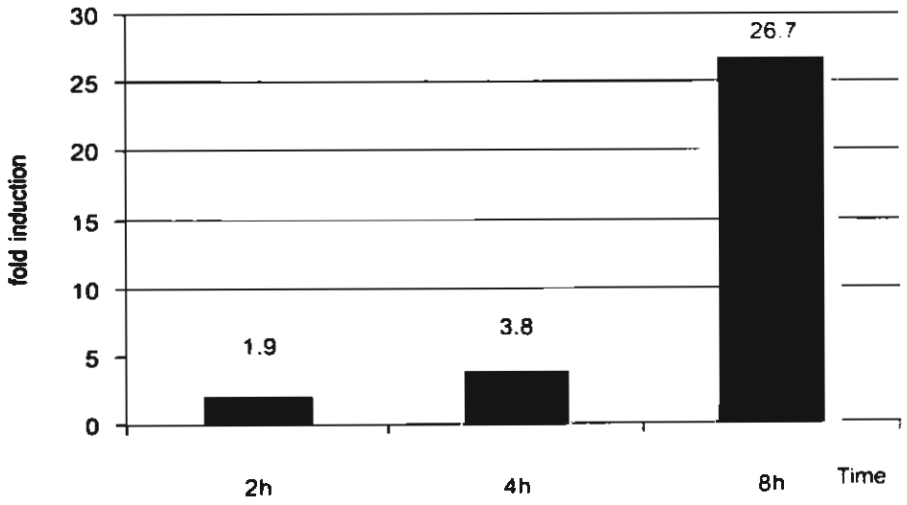

Figure 4. Activation of LOX-1 promoter activity by HSV-1. LOX-1Luc or pGL3 (0.5 ug) were transfected into ECV304 cells as mentioned in Materials and Methods. Transfected ECV304 cells were infected with 5 MOI of HSV-1 for 2, 4, and 8 hours. Cell lysates were harvested and luciferase activities were measured. The induction of luciferase activities was expressed in fold induction compared with non-stimulated cells. The experiment was done in triplicate

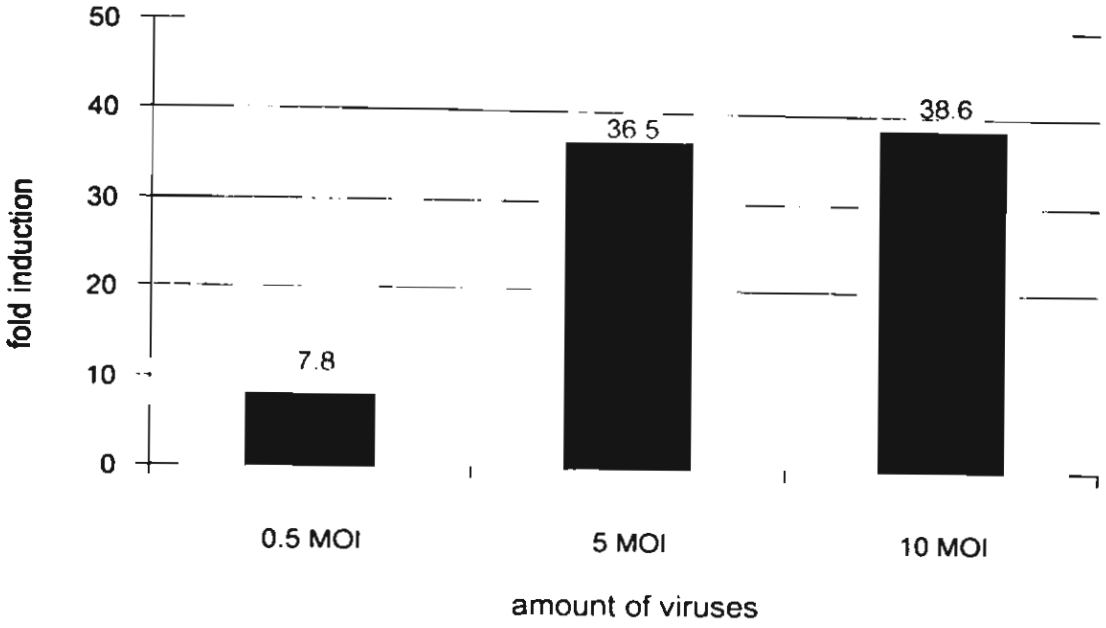

Figure 5. Effects of varying MOI of HSV-1 on activation of LOX-1 promoter activity. LOX-1Luc and pGL3 were transfected into ECV304 cells. Transfected cells were infected with 0.5, 5 and 10 MOI of HSV-1. Cell lysates were collected and luciferase activities were measured. The induction of luciferase activities was expressed in fold induction compared with non-stimulated cells. The experiment was done in triplicate.

Discussion and Conclusion

Members of herperviruses such as cytomegalovirus and herpes simplex viruses have been proposed to be etiologic agents of atherosclerosis. HSV was selected for our studies since we are interested in the mechanisms of HSV immune evasion. We have obtained data supporting the hypothesis that HSV induced apoptosis in T lymphocytes and the induction was caspase-dependent (manuscript in preparation). Molecular mechanisms of HSV infection in T lymphocytes and molecular mechanisms of HSV-1 involved in atherogenesis should be compared and hopefully, can provide additional useful information. HSV-1 was used in this study since there is more evidence on HSV-1 than HSV-2 in atherogenesis. However, other infectious agents proposed in atherosclerosis are in our plans for future work.Since HSV

infections are very common , not only in atherosclerotic patients but also in general population, it is difficult to interpret results from epidemiological studies for explanation of atherogenesis by this infectious disease. Further studies in molecular mechanisms of HSV infection in both atherosclerotic patients and in the general population should help to delineate how infections by HSV is involved in atherosclerosis.

Because LOX-1 has been shown to be induced in various stimuli as mentioned earlier, we attempted to investigate whether HSV-1 infection induced expression of this receptor. Since there is no antibody to LOX-1 commercially available at this time our project was proposed, we observed LOX-1 expression at mRNA and promoter activity levels. Our data demonstrate that HSV-1 increased expression of LOX-1 on ECV304, an endothelial cell line, suggesting that one of the mechanisms that HSV-1 induces atherogenesis is the induction of LOX-1 expression. In order to confirm our observation, the uptake of oxLDL induced by HSV-1 infection and LOX-1 protein expression will be our interest for future experiments.

Since the uptake of lipoprotein and release of reactive oxygen species and immune mediators contribute to atherosclerosis, the inhibition of lipid accumulation and reduction of inflammatory response may be of therapeutic value in prevention of coronary artery disease. Moreover, if infections have been strongly supported to be involved in atherogenesis, antiobiotic treatment and vaccination to infections may be of interest in management of patients with atherosclerosis and in new approaches of treatment. Since there is an increase in incidence of HIV infection, opportunistic infections by herpesviruses are also increased. Understanding the mechanisms of how herpesviruses cause infectious diseases or atherosclerosis will facilitate not only the treatment of patients with persistent infections by herpesviruses but also HIV patients with opportunistic infections.

In conclusion, we believe that further studies should delineate the involvement of HSV infection and atherosclerosis. Etingin et al (1991) demonstrated that HSV infection of endothelial cells increased expression of an adhesion molecule, GMP140, and this expression requires expression of HSV glycoprotein C. Expression of GM140 was suggested to increase adhesion of circulating blood cells in endothelial cells and may be a mechanism in virus-induced vascular injury and inflammation. Our proposed mechanism implies that HSV-1 infection increased uptake of modified LDL by increasing expression of its receptor, LOX-1 and this contributes to endothelial dysfunction and inflammation. This study has drawn HSV-1 infection close to the mechanism of lipid accumulation in vascular endothelium which is the main factor

for atherogenesis. Experiments in normal human endothelial cells will need to be done to confirm whether similar phenomenon as seen in ECV304 cells can be observed.

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