EFFECT OF HUMAN CYTOMEGALOVIRUS AND GLUCOSE ON ADHESION MOLECULES EXPRESSION IN CULTURED HUMAN ENDOTHELIAL CELLS

T.S. ALTANNAVCH[1*], K. ROUBALOVÁ[2], P. KUČERA[3], O. JUZOVÁ[3], M. ANDEL[1]

[1]Second Department of Internal Medicine, Third Faculty of Medicine, Charles University, Šrobárova 50, 100 34 Prague 10, Czech Republic; [2]National Institute of Public Health, Prague, Czech Republic; [3]Department of Immunology, Kralovske Vinohrady Hospital, Prague, Czech Republic

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Summary. – The aim this study was to investigate the effect of glucose on the induction of adhesion molecules by Human cytomegalovirus (HCMV) in endothelial cells *in vitro*. Primary cultures of human umbilical vein endothelial cells (HUVECs) pretreated with 16.5 mmol/l glucose for 24 hrs were infected with a HCMV strain with tropism for endothelial cells. Expression of adhesion nmolecules (ICAM-1, VCAM-1 and ELAM-1) was measured by flow cytometry. While high concentrations of glucose *per se* activated the expression of all three adhesion molecules tested, HCMV induced the expression of ICAM-1 only. Moreover, it potentiated the expression of ICAM-1 in glucose-pretreated HUVECs, while it did not affect at all or slightly suppressed the glucose-activated expression of VCAM-1 and ELAM-1. The modulatory effect of glucose and HCMV on the expression of adhesion molecules in endothelial cells may be applied in increased vulnerability to patients with diabetes mellitus or atherosclerosis.

Key words: Human cytomegalovirus; glucose; adhesion molecule expression; human endothelial cells

Introduction

HCMV is regarded as one of the risk factors of atherosclerosis. It can infect human endothelial cells both *in vivo* and *in vitro* and induce expression of adhesion molecules in endothelial cells which plays a key role in the development of inflammatory lesion in the vascular wall and may be mediated by the effect of some cytokines or high concentration of glucose.

Possible causes of endothelial dysfunction leading to atherosclerosis include elevated and modified low density lipoprotein (LDL), free radicals caused by cigarette smoking, hypertension, diabetes mellitus, genetic predisposition, elevated plasma homocysteine concentrations and infection. From infectious agents participating in this process, HCMV and *Chlamydia pneumoniae* are the best documented candidates (Ross, 1993; Vogel, 1997; Falk *et al.*, 1995; Vallance *et al.*, 1997; Leinonen, 1993).

HCMV is an ubiquitous human virus, which infects 50–80% of general population. Like other herpesviruses, it infects humans mostly in childhood and establishes a latent infection resulting in persistence of the virus in the organism throughout the whole life. There are both epidemiological and biological evidences for the association of HCMV with atherosclerosis: several case-control and cohort studies have documented elevated titers of antibodies to HCMV in atherosclerotic patients (Melnick *et al.*, 1990; Sorlie *et al.*, 1994; Benditt *et al.*, 1983; Hajjar *et al.*, 1986; Minick *et al.*, 1979). Recipients of heart transplant who experienced CMV infection are in higher risk of acute infection and transplant-associated atherosclerosis (Olivari *et al.*, 1990; Epstein *et al.*, 1996; Melnick *et al.*, 1983). HCMV can infect endothelium both *in vitro* and *in vivo* and both the viral antigens and DNA were found in atherosclerotic lesions (Drunen and Vossen, 1997;

*E-mail: altannavch@hotmail.com; fax: +4202-67162710.

Abbreviations: HCMV = Human cytomegalovirus; HUVEC = human umbillical vein endothelial cells; MAb = monoclonal antibody; PBS = phosphate-buffered saline; p.i. = post infection

Bruggeman *et al.*, 1988). *In vivo* it establishes latency in endothelial cells, which can be disrupted by activation of the cells (Van Geelen and Drunen, 1995).

It has been shown that HCMV induces expression of ICAM-1 adhesion molecules on the surface of infected endothelial cells (Shahgasempour *et al.*, 1997; Almeida *et al.*, 1994). Expression of adhesion molecules in endothelial cells is an important step in promotion of inflammation in atherosclerotic plaques.

It influences the adhesiveness of the endothelium with respect to leukocytes and platelets. The accumulation of leukocytes in atherosclerotic lesions results in endothelial injury due to production of proinflammatory cytokines, production of nitric oxid (NO) radicals or T cell-mediated cytotoxicity.

In another study we have found that the adhesion molecules ICAM-1, ELAM-1 and VCAM-1 can be induced in HUVECs also by cultivation at elevated concentrations of glucose.

In the present study we investigated if the glucose-induced expression of adhesion molecules can be further modulated by HCMV infection.

Materials and Methods

Virus. HCMV strain VHL/E, a gift from Dr. W. James Waldman, Ohio State University, Columbus, OH, USA), was propagated in HUVEC cultures.

Cell culture. Human umbilical vein endothelial cells (HUVECs) were isolated from an umbilical cord vein by collagenase treatment and cultured in gelatin-coated tissue culture flasks (Greiner) in medium 199 (BioWhittaker, USA) containing 20% of fetal calf serum, 20 ng/ml basic fibroblast growth factor, 10 ng/ml epidermal growth factor (all from Gibco-BRL), 100 U/ml penicillin, 0.1 mg/ml streptomycin (Sevapharma, Czech Republic), 25 μg/ml gentamicin (Gibco), and 2.5 mg/ml amphotericin (Fluka). The cultures were maintained at 37°C and 5% CO_2 and passaged once or twice a week by treatment of confluent monolayers with 0.125% trypsin, washing the cell suspension with phosphate-buffered saline (PBS) and resuspending the cells in double volume of fresh medium. Isolated endothelial cells were identified by indirect immunofluorescence staining using a monoclonal antibody (MAb) anti-CD31. For all experiments HUVEC cultures at passages 1–4 were used.

Treatment of HUVECs with a high glucose concentration and infection with HCMV. Confluent monolayers of HUVECs cultured in physiological concentration of glucose (5.5 mmol/l) or pretreated with 16.5 mmol/l glucose for 24 hrs were harvested by trypsinization, washed with medium 199 and inoculated with a multiplicity of infection of 25 ID_{50} per cell. Negative controls were prepared both from untreated and 16.5 mmol/l glucose-pretreated mock-infected HUVECs. After 2 hrs at 37°C the inoculum was removed, the cells were resuspended in complete culture medium with 5.5 mmol/l and 16.5 mmol/l glucose, respectively, and incubated in Falcon culture flasks at 37°C in 5% CO_2. After 24, 48 and 72 hrs of incubation, aliquots of the cultures were taken for measurement of adhesion molecules expression.

Measurement of adhesion molecules expression by flow cytometry. The cell cultures were rinsed with PBS, harvested with ice-cold 0.005% EDTA in PBS, and resuspended in a mixture of fluorescein labeled MAbs anti-ICAM-1, anti-VCAM-1 (both from B. D. Pharmingen, USA) and anti-ELAM-1 (Bender, Austria), and incubated for 15 mins at 37°C. At least 5000 cells were analyzed by fluorescence-activated flow cytometry on FACS Cytoronabsolute (Ortho-Diagnostic Systems, Johnson & Johnson).

Statistical analysis. The results were evaluated using, a method of variation analysis, the Anova test. Mean values ± SD are indicated. Values of $P \leq 0.05$ were considered statistically significant.

Results and Discussion

The effect of HCMV infection on expression of ELAM-1, VCAM-1 and ICAM-1 was examined by infecting the cells with HCMV in the presence of physiological (5.5 mmol/l) or elevated (16.5 mmol/l) glucose concentration. Expression of adhesion molecules was determined by flow cytometry at 24, 48 and 72 hrs post infection (p.i.) (Table 1).

It was found that HCMV infection substantially upregulates both the spontaneous and high glucose concentration-induced expression of ICAM-1 in infected cells. Moreover, significantly increased levels of ICAM-1 expression by HCMV in 16.5 mmol/l glucose-treated cells was detectable at 24 and 48 hrs p.i. At 72 hrs p.i. the stimulation of expression ICAM-1 was insignificant (Table 1).

On the other hand, the ICAM-1 expression was markedly different from that of ELAM-1 and VCAM-1 (Fig. 1). The HCMV infection had no significant influence on the

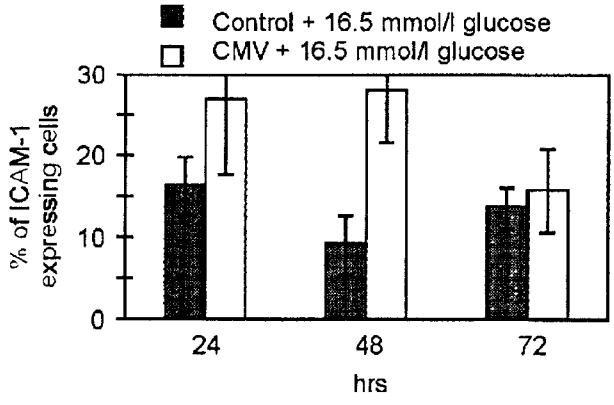

Fig. 1

Effects of 16.5 mmol/l glucose and HCMV infection on the expression of ICAM-1 in HUVECs

The cells pretreated with 16.5 mmol/l glucose were infected with HCMV, incubated in complete culture medium with 16.5 mmol/l glucose for 24, 48 and 72 hrs and measured by flow cytometry. The results are expressed as means ± SD obtained from at least eight measurements from three experiments.

Table 1. Induction of adhesion molecules expression by HCMV and a high glucose concentration in HUVECs

Adhesion molecules	% of positive cells					
	24 hrs	P	48 hrs	P	72 hrs	P
ELAM-1						
Control	4.9 ± 0.9		4.9 ± 2.3		5.8 ± 1.1	
HCMV	3.0 ± 1.5	IS[a]	2.7 ± 5.2	IS[a]	4.9 ± 0.9	IS[a]
Control + 16.5 mmol/l glucose	18.6 ± 5.7	<0.001[b]	13.9 ± 5.2	0.02[b]	9.1 ± 2.1	0.002[b]
HCMV + 16.5 mmol/l glucose	14.2 ± 2.7	IS[c]	8.6 ± 3.2	IS[c]	6.7 ± 4.3	IS[c]
VCAM-1						
Control	4.2 ± 1.9		4.8 ± 2.3		3.2 ± 2.1	
HCMV	4.5 ± 0.8	IS[a]	6.1 ± 3.4	IS[a]	2.3 ± 0.6	IS[a]
Control + 16.5 mmol/l glucose	18.6 ± 5.7	<0.001[b]	8.6 ± 3.2	0.02[b]	9.1 ± 2.1	0.002[b]
HCMV + 16.5 mmol/l glucose	15 ± 2.3	IS[c]	6.7 ± 1.5	IS[c]	8.4 ± 2.8	IS[c]
ICAM-1						
Control	6.1 ± 1.9		7.7 ± 3.3		9.3 ± 2.5	
HCMV	29 ± 6.9	<0.001[a]	36.8 ± 6.3	<0.001[a]	19.5 ± 3.2	≤0.05[a]
Control + 16.5 mmol/l glucose	16.4 ± 3.5	<0.001[b]	9.3 ± 3.6	<0.001[b]	13.7 ± 3.2	<0.01[b]
HCMV + 16.5 mmol/l glucose	27 ± 9.2	≤0.05[c]	28 ± 6.4	<0.001[c]	15.8 ± 5.1	IC[c]

Values are means ± SD obtained from at least eight separate measurements from three experiments.
[a]HCMV-infected vs. uninfected cells.
[b]16.5 mmol/l glucose-treated vs. untreated cells.
[c]16.5 mmol/l glucose-treated HCMV-infected vs. 16.5 mmol/l glucose-untreated HCMV-uninfected cells.
IS = insignificant.

spontaneous or high glucose-induced expression of ELAM-1 and VCAM-1. Moreover, the high glucose concentration-induced expression of these adhesion molecules in the cells pretreated with 16.5 mmol/l glucose was unaffected or even suppressed in HCMV-infected cells (Figs 2 and 3).

This finding may indicate a difference in the regulation of expression of the adhesion molecules. Stimulation of ICAM-1 but not ELAM-1 and VCAM-1 in HCMV-infected HUVECs has been observed also by Sedmak *et al.* (1994) and in HCMV-infected arterial and microvascular endothelial cells by Knight *et al.* (1999). Moreover, these authors have shown that the HCMV-infected HUVECs are refractory to tumor necrosis factor α (TNF-α)-mediated induction of ELAM-1 and VCAM-1, while the expression of ICAM-1 remains unchanged.

In our study the HCMV infection did not alter or slightly decreased the expression of glucose-induced ELAM-1 or VCAM-1, however, it exhibited synergistic effect on the

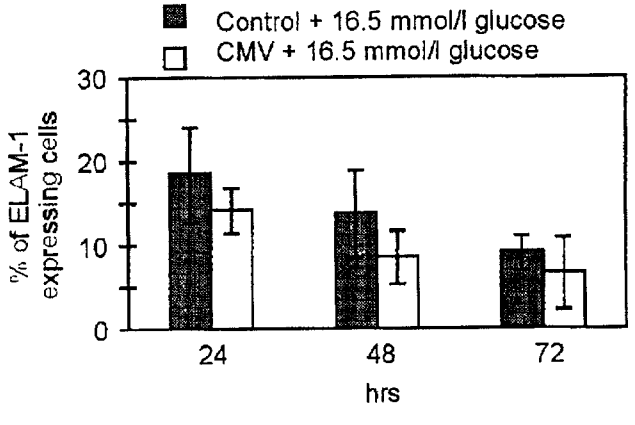

Fig. 2

Effects of 16.5 mmol/l glucose and HCMV infection on the expression of ELAM-1 in HUVECs

For the legend see Fig. 1.

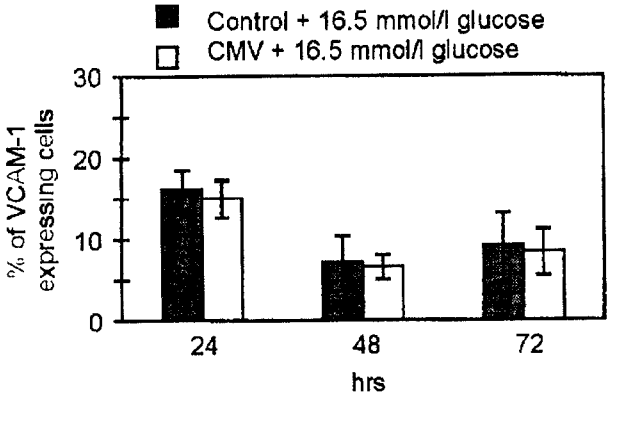

Fig. 3

Effects of 16.5 mmol/l glucose and HCMV infection on the expression of VCAM-1 in HUVECs

For the legend see Fig. 1.

glucose-induced expression of ICAM-1. The variation in the modulatory effect of HCMV on the TNF-α- and glucose-induced expression of adhesion molecules may be caused either by differences in signalling pathways sensitive to glucose and TNF-α, or, more probably, by differences in the sequence of stimulatory signals. In our system, HUVECs were pre-treated with glucose, while in the study of Knight *et al.* (1999) the HCMV infection preceeded the TNF-α induction.

From the presented study we cannot conclude whether the observed modulation of the adhesion molecules expression was caused by inflammatory cytokines present in the virus inoculum or by direct effect of the virus. Based on investigating the kinetics of expression of the ICAM-1 and the CMV immediate early genes (IE72) in HCMV-infected HUVECs, Burns *et al.* (1999) have suggested that the ICAM-1 expression is induced by the IE72-driven transactivation of the ICAM-1 gene. This hypothesis has been confirmed by transcription experiments of Guetta *et al.* (2001).

Effect of HCMV on the expression of ICAM-1 in endothelial cells may represent an important mechanism of the virus participation in the atherosclerotic process. The increased adhesiveness of endothelial cells may result in local accumulation of leukocytes. Their activation may trigger the virus life cycle in latently infected cells and the spread of infection into neighbor cells (Guetta *et al.*, 1995). The cellular immune response to viral antigens and the production of proinflammatory cytokines by virus-infected or activated immunocompetent cells can promote formation of atherosclerotic lesions.

The patients with diabetes mellitus are prone to atherosclerosis. The ability of HCMV to cooperate with high concentrations of glucose in modulation the adhesive molecules expression in endothelial cells may play some role in this process.

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