CYTOMEGALOVIRUS INFECTION INCREASES THE EXPRESSION AND ACTIVITY OF ECTO-ATPASE (CD39) AND ECTO-5' NUCLEOTIDASE (CD73) ON ENDOTHELIAL CELLS

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Submitted

Abstract

Cytomegalovirus infection severely influences the function of endothelial cells. In the present study we describe the enhanced expression of ecto- ATPase or CD39 and ecto-5' nucleotidase or CD73, two enzymes essential in the homeostasis of platelet activation and platelet aggregation, on CMV infected endothelial cells. The expression levels as well as the enzymatic activity of ecto-ATPase and ecto-5' nucleotidase were significantly increased on CMV infected endothelial cells compared to uninfected endothelial cells. Addition of ATP, ADP or AMP to supernatants of CMV-infected or uninfected endothelial cell layers, revealed an increased turnover of AMP mediated by CMV infected endothelial cells. The superoxide production of stimulated polymorphonuclear cells was inhibited in the presence of CMV-infected endothelial cells as compared to uninfected endothelial cells, which was probably due to the enhanced activity of ecto-5' nucleotidase.

It is hypothesized that increased production of adenosine as reflected by upregulation of ecto-5' nucleotidase, may prevent local activation of platelets counteracting procoagulant activity of endothelium induced by CMV. In addition, the anti-inflammatory effect of adenosine may inhibit the activity of proinflammatory cells, also supporting putative evasion of the virus from nonspecific host defense.

Introduction

Human cytomegalovirus (CMV) infections in immunocompromised patients may cause CMV disease, which involves a variety of organs and tissues [1]. In vivo, endothelium is one of the targets of CMV. Infection with CMV has considerable impact on the physiological function of endothelial cells. CMV infection affects many responses of EC during inflammation, including those concerning to the coagulant state of endothelial cells.

Together with ecto-ATPase [2] (CD39) [3], ecto-5' nucleotidase (CD73) has an important role in regulation of platelet aggregation. During platelet activation, ATP and ADP are released from the platelet dense granula. ADP is essential for platelet recruitment and activation and ATP is able to activate polymorphonuclear cells (PMN) to release toxic oxygen products through stimulation of purinergic receptors present on these cells [4]. A rapid turnover of these extracellular nucleotides (i.e. ADP and ATP) to AMP is able to control both, microthrombus formation as well as release of oxygen radicals in the inflammatory microenvironment. The degradation product of this enzyme activity, i.e. adenosine, is a powerful inhibitor of platelet activation; moreover adenosine is a scavenger of oxygen radicals and is able to act as a vasodilator in certain microvascular beds [5].

As it has been shown previously that the expression of these endothelial ectoenzymes may vary in pathological conditions, such as for instance local ischemia [6], investigation of the expression levels of ecto-ATPase and ecto-5'nucleotidase at endothelial cells infected with CMV, may help to a better understanding of the pathomechanisms of CMV mediated effects at the vessel wall.

Materials and Methods

Cell culture

Human umbilical vein endothelial cells (HUVEC) were isolated from human umbilical cord veins [7,8] and grown in endothelial cell growth medium (RPMI1640 supplemented with 20% fetal calf serum, 50 μg/ml endothelial cell growth factor, 5 U/ml heparin, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin) using culture flasks precoated with 1% gelatin. HUVEC were used at passage 1 to 3.

Virus

The endotheliotropic CMV clinical isolate TB42 [9] was used to infect endothelial cell cultures. Viral infection of EC was achieved by seeding trypsinized CMV-infected EC together with uninfected EC at a ratio 1:10 in culture flasks. After 5 days more than 80 % of the EC were infected. The percentage of infection was determined by indirect immunofluorescent staining with monoclonal antibody E13 (Seralab, Sussex, UK) directed against CMV immediate early antigens.

Histochemistry

Immunostaining and enzyme histochemistry were performed to demonstrate the presence of ecto-ATPase and ecto-5' nucleotidase. All stainings were performed upon monolayers of CMV infected and uninfected HUVEC grown on Cookeslides. Immunostaining of ecto-5' nucleotidase was performed with a rabbit polyclonal IgG against human 5'-nucleotidase (prepared in our own laboratory). In a next step, cells were incubated with peroxidase-conjugated goat-anti-rabbit antibodies. Ecto-ATPase was detected with a mouse monoclonal antibody against apyrase [10], followed by peroxidase-conjugate goat-anti-mouse antibodies. 3-amino, 9-ethylcarbazole (Sigma Chemical Co, St Louis MO, USA) was used to visualize the reaction product.

Enzyme histochemistry to detect ecto-5' nucleotidase (CD73) activity was carried out according Wachstein and Meisel [11], using lead as a capture ion. Briefly, cells were fixed in acetone and incubated with 1.0 mg/ml AMP in 0.08 M Tris-maleate buffer in the presence of 0.12% $PbNO_3$ for 45 minutes at 37°C. Lead was visualized by staining with 2% $NaSO_3$ for 30 seconds, yielding a brown precipitate. Detection of ecto-ATPase (CD39) was performed using the cerium based method as described previously [12,13]. Incubation was done with 1.5 mg/ml ATP, 5 mM $Mg(NO_3)_2$ and 0.6 mM $CeCl_2$ for 45 minutes at 37°C. The reaction product (cerium phosphate) was visualized by 0.05% H_2O_2 and 0.5 mg/ml 3', 3'-di-amino-benzidine in 0.1 M Trisbuffer resulting in a brown precipitate. In some experiments, the histochemical reaction was followed by a staining with monoclonal antibody E13 (Seralab, Sussex, UK). Indirect detection of E13 was with alkaline phosphatase-coupled goat-anti-mouse antibodies and Fast Blue (Sigma Chemical Co, St Louis MO, USA) as substrate.

Cytochrome C test

To determine the effect of CMV infected endothelial cells at activation of polymorphonuclear granulocytes (PMN) we used the ferricytochrome C reduction assay according to Pick and Mizel with minor modifications [14,15]. The superoxide anion production of PMN stimulated with N-formyl-methionyl-leucyl-phenylalanine (fMLP) was measured in the presence or absence of either CMV- infected or uninfected HUVEC. The assay was performed in 96-well microtiter plates containing monolayers of CMV infected or uninfected endothelial cells or with culture medium without cells. PMN were isolated from heparinized blood samples obtained from healthy individuals by centrifugation on a Lymfoprep^{TM} (Nycomed Pharma AS, Oslo, Norway, d = 1.077 g/cm^2) density gradient. Contaminating erythrocytes were lysed with ice-cold ammoniumchloride (155 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM $Na_2EDTA.2H_2O$). After two washes with ice-cold Hanks' balanced salt solution (HBSS) without calcium and magnesium, cells were adjusted to 10^6/ml in HBSS with calcium and magnesium and the temperature was adjusted to 37°C. PMN were pre-treated with cytochalasin B 1 μg/ml (Serva, Heidelberg Germany) for 5 min., followed by activation with 2 ng/ml TNF-α for 15 minutes. Endothelial cells were washed with HBSS, 0.2% BSA. Thus, PMN (2×10^5) were incubated with approximately 2×10^4 HUVEC in HBSS containing 0.850 mg/ml Cytochrome C (Ferricytochrome C: type VI from horse heart, Sigma Chemical Co, St Louis MO, USA), with or without 59 U/ml superoxide dismutase (SOD) (Sigma Chemical Co, St Louis MO, USA) and, with or without 2×10^{-7}M fMLP (Sigma Chemical Co, St Louis MO, USA). All reactions were performed in quadruple. The optical density (OD) at 550 nm was scanned in an automated microplate reader (Thermomax, Molecular Devices, USA) at 5, 15, 30, 45, 60 and 90 minutes. Between measurements plates were kept at 37°C. The superoxide anion production is expressed as the difference in OD (ΔOD 550) between values obtained in the presence of SOD versus OD values obtained without SOD. The results are expressed as mean differences measured after various incubation times.

Adenine nucleotide production

Confluent monolayers of CMV infected and uninfected HUVEC were grown in 6 well tissue culture plates. The cells were washed with serum-free medium (RPMI supplemented with 0.2 mg/ml bovine serum albumin) followed by incubation with serum-free medium containing 250 μM $MgCl_2$ and either 250 μM ATP (Boehringer, Mannheim, Germany), ADP (Sigma Chemical Co, St Louis MO, USA) or AMP (Serva, Heidelberg Germany). Supernatant samples were analyzed for ATP, ADP, AMP and adenosine contents by gradient ion-pair reversed-phase high performance liquid chromatography (HPLC) using a Nova-Pak C_{18} column as described by Olinga et al [16]. The adenine nucleotide concentrations were corrected for differences in cell numbers per well.

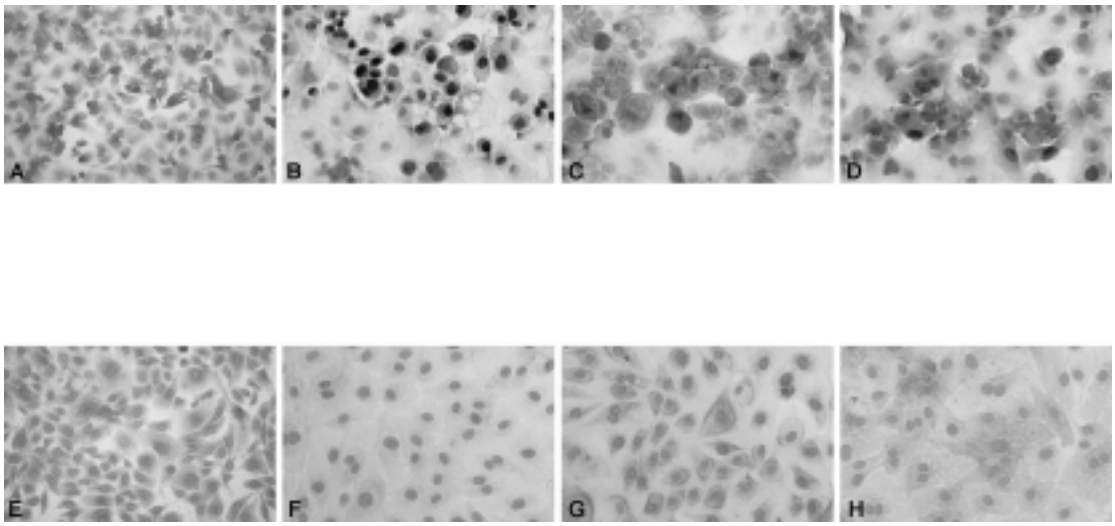

Figure 1

Expression and enzyme activity of ecto-ATPase (A, B, E, F) and ecto-5' nucleotidase (C, D, G, H) on CMV infected endothelial cells (A-D) and uninfected endothelial cells (E-H).

Antigen expression of ecto-ATPase and ecto-5'nucleotidase is shown in the Figures 1A, E, C, G and enzyme activity are shown in Fig. 1B, F, D, H.

CMV infected endothelial cells shown in Fig. 1B and Fig. 1D are additionally stained with MoAb E13 directed against CMV immediate early antigens (dark blue nuclear staining)

Results

CMV infected endothelial cells expressed markedly increased expression levels of ecto-5' nucleotidase compared to uninfected endothelial cells (Fig. 1C, G). The infected cells had a pattern of perinuclear cytoplasmic expression in combination with an enhanced membrane expression. Especially the endothelial cells that were not yet fully cytomegalic expressed high levels of ecto-5' nucleotidase. Furthermore, using a lead-based histochemical reaction, we showed that the enzymatic activity of ecto-5' nucleotidase was also enhanced in the CMV infected endothelial cells (Fig. 1D, H). Endothelial cells highly positive for CMV-IE proteins in the nucleus but also endothelial cells with lower levels of CMV IE-expression had converted the AMP substrate.

Antigen levels (Fig. 1A, E) as well as enzyme activity (Fig. 1B, F) of ecto-ATPase were increased in CMV infected cells (Fig. 1A, B) as compared to uninfected cells (Fig. 1 E, F). The endothelial cells that showed enzyme activity for ATP, were almost all positive for CMV IE-proteins (Fig. 1B).

Next, we measured the enzymatic activity of both molecules in monolayers of CMV infected or uninfected cells.

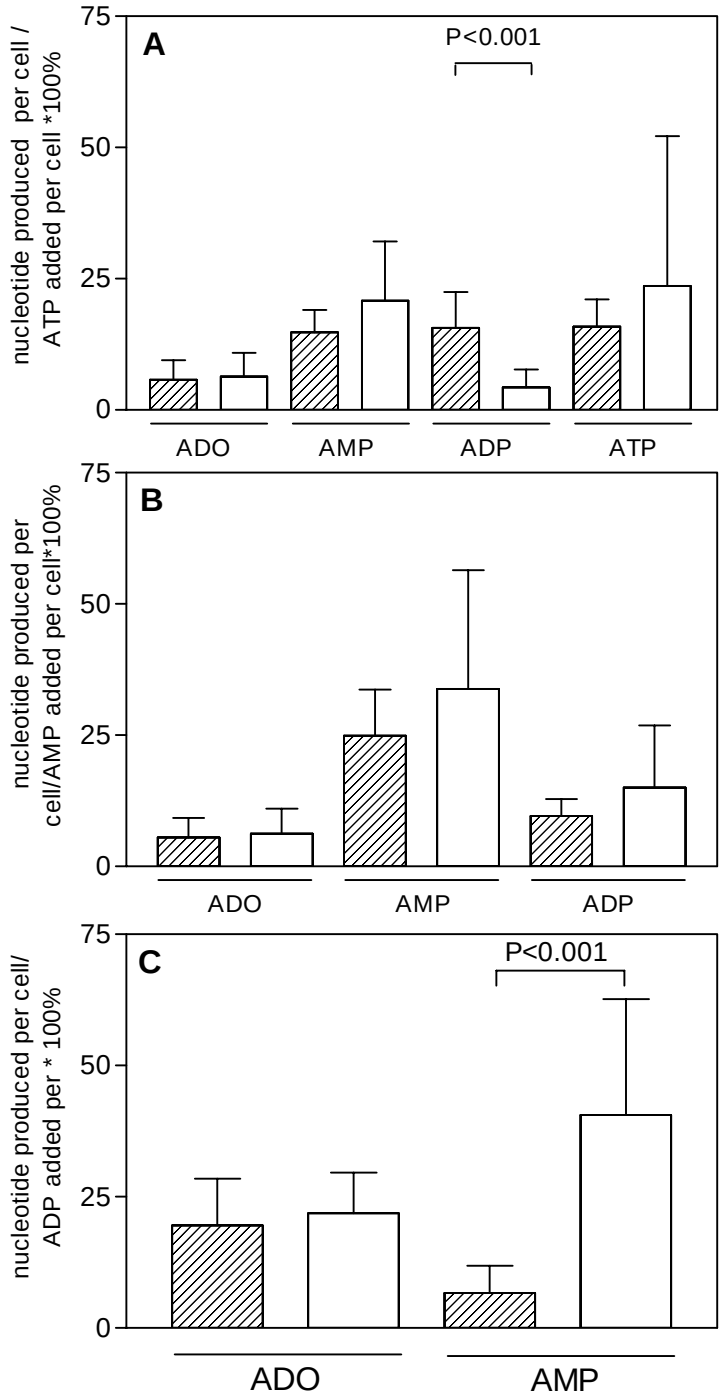

Figure 2

Addition of ATP, ADP or AMP to monolayers of CMV infected or uninfected endothelial cells. 250 μM of ATP (A), ADP (B) or AMP (C) was added for 1 hour to monolayers of CMV infected (hatched bars) and uninfected endothelial cells (white bars). The supernatant was harvested and the adenine nucleotide content was analyzed by HPLC. Data represent the extracellular adenosine (ADO), AMP, ADP, ATP concentration per cell per total added. Data represent mean ± SD of eight experiments.

Addition of ATP to infected and uninfected cell cultures resulted in higher extracellular ADP levels in the wells containing CMV infected cells compared to uninfected endothelial cells, whereas no differences were observed in finally concentrations of ATP, AMP and adenosine between CMV infected and uninfected cells. Addition of ADP did not result in different concentrations of ADP, AMP and adenosine between CMV infected or uninfected endothelial cells after a 1-hour incubation period.
In contrast, addition of AMP resulted in significantly lower levels of extracellular AMP in the wells with infected cells ($P<0.001$) (Fig. 2).
To investigate whether ecto-5' nucleotidase might have an anti-inflammatory role during CMV infection we used the cytochrome C reduction assay, which is a functional assay. Monolayers of CMV infected endothelial cells inhibited the oxygen radical production by PMN after stimulation with fMLP ($P<0.001$), whereas uninfected EC did not inhibit the oxygen radical production. The incubation of PMN with endothelial cells without fMLP had no stimulatory effects at oxygen radical production (Fig. 3).

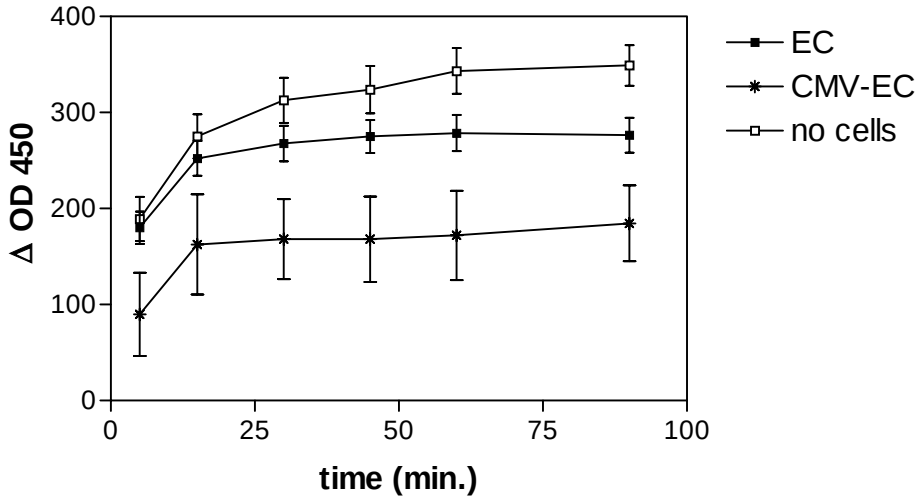

Figure 3
Activity of activity of activated PMN in the presence of CMV infected or uninfected endothelial cells. Superoxide production of PMN stimulated with fMLP in the absence (open circles) or presence of CMV infected endothelial cells (closed triangles) or uninfected endothelial cells (closed squares).
Data represent the mean ± SEM of four independent experiments performed in quadruple.

Discussion

In the present study we describe the enhanced expression and activity of ecto-ATPase (CD39) and ecto-5' nucleotidase (CD73) by CMV infected endothelial cells. These enzymes are involved in the extracellular adenine nucleotide metabolism. Functionally, the increased expression of ecto-5' nucleotidase after CMV infection was demonstrated in supernatants of infected cells by a higher turnover of exogenously added AMP. Furthermore, stimulated PMN produced lower amounts of oxygen radicals in the presence of CMV infected endothelial cells than in the presence of uninfected endothelial cells. This is an effect probably due to the anti-inflammatory features of adenosine.

The metabolism of extracellular nucleotides is an important regulatory tool in the homeostasis of platelet aggregation. Three main anti-aggregatory systems are involved in the control of platelet reactivity: prostaglandin production, nitric oxide and ecto-nucleotidase activity, respectively. The role of ecto-nucleotidases in platelet aggregation is known for many years [17], and is well studied in rat models [18]. Recently CD39-deficient mice, which had prolonged bleeding times and platelet hypofunction, clearly confirmed the importance of ecto-ATPase [19]. Under resting conditions, the efficient turnover of ATP and ADP to AMP prevents onset of an amplification cascade of platelet recruitment and activation induced by ADP and thus the formation of a hemostatic plug. In turn, ecto-5' nucleotidase may enhance the role of ecto-ATPase by converting AMP to adenosine, a known inhibitor of platelet aggregation. In an inflammatory environment, such as in the inflammatory model for minimal change disease in rats, the expression and activity of ecto-ATPase is severely reduced [10]. The reduction of ecto-ATPases during inflammation depended on sensitivity for oxygen radicals and was observed in rats [20] as well as in activated human endothelial cells [21]. The expression of ecto-5' nucleotidase is influenced by ischemic conditions as well and result in the upregulation of this enzyme [5]. *In vivo,* the pattern induced by hypoxia, i.e. a decreased ecto-ATPase expression in combination with increased ecto-5' nucleotidase expression was for instance observed in biopsies of kidney transplant recipients with chronic graft failure [6]. A delayed onset of graft function after transplantation resulted in a decreased expression of ecto-ATPase as well [22]. Obviously, these patients had suffered from considerable reperfusion damage with release of reactive oxygen species (ROS).

We describe the enhanced expression of ecto-ATPase and ecto-5' nucleotidase on CMV infected endothelial cells. Obviously, these molecules are not induced by activation of the endothelial cells; otherwise ecto-ATPase would be downregulated [23]. It seems more likely that CMV infection of the cells interferes with the endogenous expression of ecto-ATPase and ecto-5' nucleotidase. Whether this is a host defense reaction of the endothelial cell to procoagulant effects induced by CMV infection or a regulatory effect of CMV in the expression pathway of these molecules in the endothelial cell is currently unknown.

As a consequence of the upregulation of ecto-ATPase and ecto-5' nucleotidase, the production of adenosine is probably enhanced. Both neutrophils as well as shear forces can enhance the production of adenosine by endothelial cells [5]. We did not measure the adenosine concentration in the cytochrome reduction assay, but the inhibitory effect on the radical production by PMN is presumably due to the increased production of adenosine. In the experiments with external added nucleotides, adenosine did not accumulate in the supernatant of infected endothelial cells. Whereas ATP, ADP and AMP are relatively stable in the extracellular milieu, adenosine is rapidly taken up inside cells; it is processed to inosine and hypoxanthine or it can bind to purine receptors [24,25]. Therefore to maintain the anti-aggregatory and anti-inflammatory effects of adenosine in vivo a constant rate of production is required.

In the immediate microenvironment of adenosine production, the increased adenosine levels may affect several mechanisms. Adenosine has an anti-aggregatory influence on platelet reactivity. Whereas most herpesviruses [26], including CMV {van Dam Mieras, Muller, et

al. 1992 ID: 271}, induce an enhanced procoagulant environment at the surface of endothelial cells, the increased expression of ecto-5' nucleotidase at the same cell surface, as observed during CMV infection, may restore the delicate balance of haemostasis.

Furthermore, adenosine has a modulatory role in the inflammatory response. Binding of ATP to purine receptors on the PMN has a pro-inflammatory effect. It increases the PMN adherence and production of oxygen radicals. However, adenosine has potent anti-inflammatory capacities and causes inhibition of PMN transmigration and activation [4]. Thus, an increased production of adenosine may moderate the severity of the inflammatory reaction and thus the immune response against the virus.

In addition, a higher availability of adenosine locally at the endothelial cell surface may enhance the barrier function of endothelial cells. Proinflammatory cytokines increase the permeability of the endothelial layer. The binding of adenosine to A_2B type purine receptors of EC triggers an intracellular signal, which can restore the increased permeability of endothelial cells [28]. After infection with cytomegalovirus the endothelial cells develop a cytomegalic morphology and can detach from the basal membrane. CMV patients occasionally have cytomegalic endothelial cells in the blood stream [29]. Possibly, the increased production of adenosine contributes to the restoration of the integrity of the endothelial layer. Whether this affects the lesions induced by detachment of CMV infected endothelial cells remains to be investigated.

In conclusion, the increased expression of ecto-5' nucleotidase and ecto-ATPase at CMV infected endothelial cells may serve as a novel viral evasion strategy.

The higher production of adenosine may prevent activation and aggregation of platelets. This may counteract the procoagulant properties of infected endothelial cells. The anti-inflammatory effect of adenosine may inhibit the function of activated PMN. Finally, the increased levels of adenosine might contribute to restoration of disturbances at the endothelial surface by increase of the barrier function.

Acknowledgements

We thank Juul Baller and Patrick Lockhorst for technical assistance and Roelie van Wijk and Henk Moorlag for assistance in endothelial cell culture.

Grant support: Dutch Kidney foundation (C94.1386), European Commission Grant (ERB BMH4CT-0471 (DG12-SSMA)).

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