Cytomegalovirus infection increases the expression and activity of ecto-ATPase (CD39) and ecto-5′nucleotidase (CD73) on endothelial cells

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Received 27 October 2000; accepted 3 January 2001

First published online 6 February 2001

Edited by Hans-Dieter Klenk

Abstract We describe enhanced expression and enzymatic activity of ecto-ATPase and ecto-5′nucleotidase on CMV infected endothelial cells as compared to uninfected cells. These ectoenzymes play a major role in modulation of platelet activation and aggregation. Furthermore, adenosine has a modulatory effect upon inflammation. Addition of ATP, ADP or AMP to cultures of CMV infected or uninfected endothelial cells revealed increased turnover of AMP in CMV infected endothelial cells. In addition, the superoxide production by stimulated polymorphonuclear cells was inhibited in the presence of CMV infected endothelial cells as compared to uninfected cells, probably due to the enhanced activity of ecto-5′nucleotidase and associated to production of adenosine.

Key words: Cytomegalovirus; Endothelium; Ecto-ATPase; CD39; Ecto-5′nucleotidase; CD73

1. Introduction

Human cytomegalovirus (CMV) infections in immunocompromised patients may cause CMV disease, which involves a variety of organs and tissues [1]. The endothelial cell is one of the cell types that becomes infected with CMV. This has considerable impact on the physiological function of endothelial cells. Particularly the coagulant properties of EC are affected by infection with CMV.

In the coagulation cascade ecto-ATPase (CD39) [2,3] and ecto-5′nucleotidase (CD73) have 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 while 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. ATP and ADP) to AMP is able to control both microthrombus formation as well as release of oxygen radicals in the inflammatory microenvironment. The major degradation product of this enzyme activity, i.e. adenosine, inhibits platelet activation [5]. Moreover, adenosine scavenges oxygen radicals and acts as a vasodilator in microvascular beds [6].

Most herpesviruses [7], including CMV [8], induce enhanced procoagulant activity at the surface of endothelial cells. An increased expression of ecto-5′nucleotidase at the same cell surface could contribute to the delicate balance of hemostasis. We hypothesize that CMV infection of endothelial cells upregulates the expression levels of ecto-ATPase and ecto-5′nucleotidase to counteract procoagulant activity induced by CMV infection.

Furthermore, adenosine has a modulatory role in the inflammatory response [4]. Hence, an increased production of adenosine may moderate the severity of the inflammatory reaction and thus the immune response against the virus.

The present study was designed to evaluate alterations of expression or activity of ecto-ATPase and ecto-5′nucleotidase upon endothelial cells after CMV infection in vitro. Moreover, coculture experiments were conducted to evaluate the activity of inflammatory cells in vitro following contact with either CMV infected or uninfected endothelial cells.

2. Materials and methods

2.1. Cell culture

Human umbilical vein endothelial cells (HUVEC) were isolated from human umbilical cord veins [9,10] and grown in endothelial cell growth medium (RPMI 1640 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 passages 1–3.

2.2. Virus

The endotheliotropic CMV clinical isolate TB42 [11] 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.

2.3. Immunostaining and 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

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0014-5793/01/$20.00
PII: S0014-5793(01)02085-3

against human ecto-5′nucleotidase (prepared according to standard methods). Subsequently, cells were incubated with peroxidase-conjugated goat anti-rabbit antibodies. 3-amino, 9-ethylcarbazole (AEC) (Sigma Chemical Co, St. Louis, MO, USA) was used to visualize the reaction product. Ecto-ATPase was detected with a mouse monoclonal antibody against apyrase [12], followed by in order peroxidase-conjugated goat anti-mouse antibodies and AEC.

Enzyme histochemistry to detect ecto-5′nucleotidase (CD73) activity was carried out according to Wachstein and Meisel [13], 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 min at 37°C. Lead was visualized by staining with 2% $NaSO_3$ for 30 s, yielding a brown precipitate. Detection of ecto-ATPase (CD39) was performed using the cerium based method as described previously [14,15]. Incubation was done with 1.5 mg/ml ATP, 5 mM $Mg(NO_3)_2$ and 0.6 mM $CeCl_2$ for 45 min 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 Tris buffer (pH 7.6) resulting in a brown precipitate. In some experiments, the histochemical reaction was followed by staining with monoclonal antibody E13 (Seralab, Sussex, UK). Indirect detection of E13 was done with alkaline phosphatase-coupled goat anti-mouse antibodies and Fast Blue (Sigma Chemical Co, St. Louis, MO, USA) as substrate.

2.4. Cytochrome c test

To determine the effect of CMV infected endothelial cells on activation of polymorphonuclear granulocytes (PMN) we used the ferricytochrome *c* reduction assay according to Pick and Mizel with minor modifications [16,17]. 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 as well as in the presence of conditioned medium from CMV infected or uninfected HUVEC. Medium was harvested after 48 h. 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™ (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 $Na_2EDTA{\cdot}2H_2O$). 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 pretreated with 1 μg/ml cytochalasin B (Serva, Heidelberg, Germany) for 5 min, followed by activation with 2 ng/ml TNF-α for 15 min. Endothelial cells were washed with HBSS, 0.2% BSA. Cocultures were carried out by incubation of 2×10^5 PMN 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 min. Between measurements plates were kept at 37°C. The superoxide anion production is expressed as the difference in OD ($\Delta OD_{550\,nm}$) 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.

2.5. Release of adenine nucleotides of HUVEC after infection with CMV

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. [18]. The adenine nucleotide concentrations were corrected for differences in cell numbers per well.

3. Results

3.1. Immunostaining and histochemistry

CMV infected endothelial cells expressed markedly increased levels of ecto-5′nucleotidase (CD73) compared to uninfected endothelial cells (Fig. 1C,G).

The infected cells showed a pattern of perinuclear cytoplasmic expression in combination with enhanced membrane expression. In particular, the endothelial cells which were not fully cytomegalic yet, showed increased staining of ecto-5′nucleotidase. Furthermore, using enzyme histochemistry the enzymatic activity of ecto-5′nucleotidase was also shown to be increased (Fig. 1D,H). In contrast, No significant differences of ecto-5′nucleotidase activity could be detected in cells with high versus low CMV IE staining. As can be seen from Fig. 1A,E membrane expression 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. 1E,F). En-

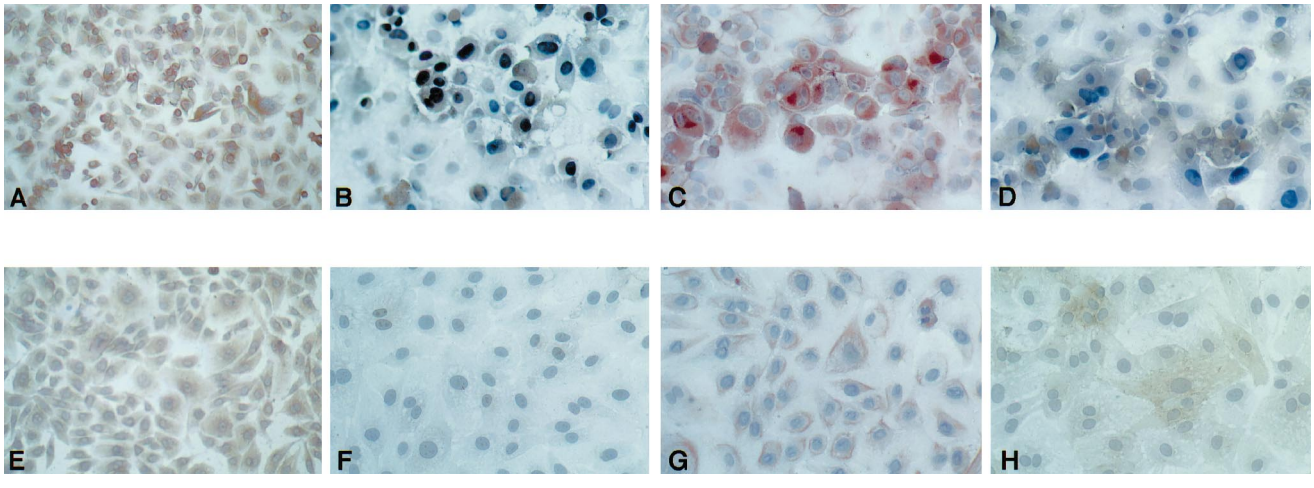

Fig. 1. Expression and enzyme activity of ecto-ATPase (A, B, E, F) and ecto-5′nucleotidase (C, D, G, H) on CMV infected endothelial cells (A–D) and uninfected endothelial cells (E–H). Deposition of reaction product reflecting ecto-ATPase and ecto-5′nucleotidase activity is shown in A, E, C, G. Enzyme activity is shown in B, F, D, H. CMV infected endothelial cells as depicted in B, D are stained with MoAb E13 directed against CMV immediate early antigens (dark blue nuclear staining).

dothelial cells showing enzyme staining for ATP were over 85% positive for CMV IE proteins (Fig. 1B).

3.2. Conversion of supplemented nucleotides to CMV infected HUVEC

Addition of ATP to infected and uninfected cell cultures resulted in higher extracellular ADP levels in supernatants of CMV infected cultures as compared to uninfected endothelial cells, whereas no differences were observed in the final concentration of ATP, AMP or adenosine in CMV infected versus 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 h 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).

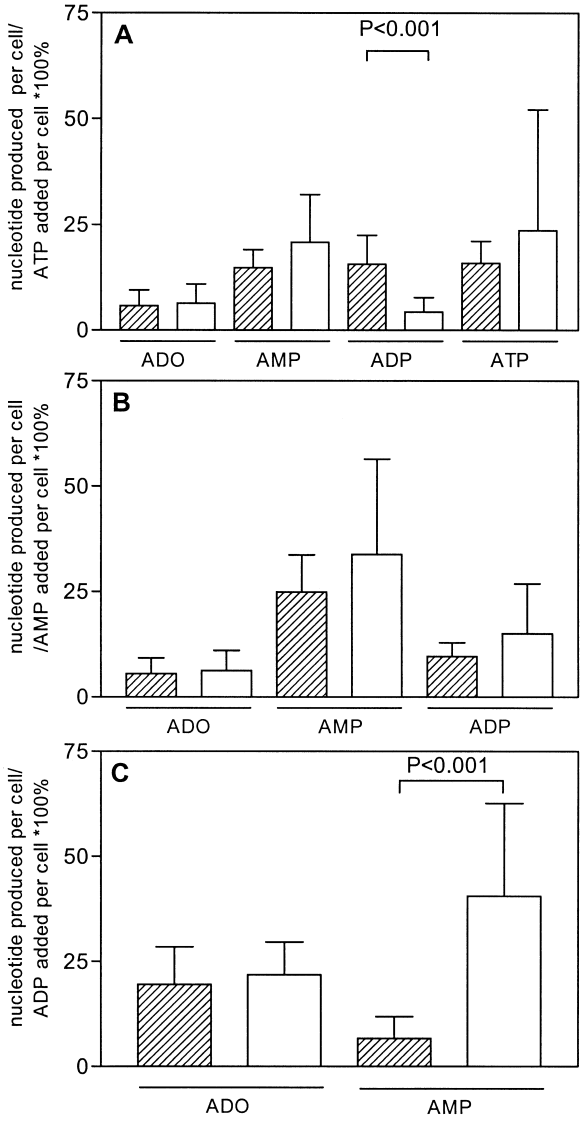

Fig. 2. Addition of ATP, ADP or AMP to monolayers of CMV infected or uninfected endothelial cells. 250 μM of ATP (A), ADP (B) or AMP (C) was added for 1 h to monolayers of CMV infected (hatched bars) and uninfected endothelial cells (white bars). The supernatant was harvested and adenine nucleotide content was analyzed by HPLC. Data represent extracellular adenosine (ADO), AMP, ADP, ATP concentration per cell per total added. The mean ± S.D. of eight experiments are presented.

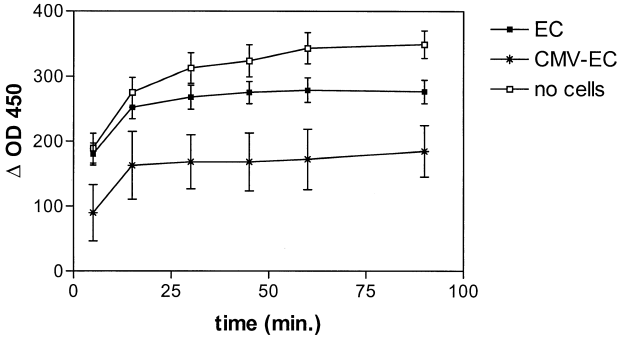

Fig. 3. Activity of fMLP stimulated PMN in the presence of CMV infected or uninfected endothelial cells. Superoxide production of PMN is shown in the absence (□) or presence of CMV infected endothelial cells (*) or uninfected endothelial cells (■). Data represent mean ± S.E.M. of four independent experiments performed in quadruple.

3.3. Anti-inflammatory effect of CMV infected HUVEC upon PMN in vitro

Coculture of monolayers of CMV infected endothelial cells and PMN inhibited the oxygen radical production by PMN after stimulation with fMLP ($P < 0.001$), whereas monolayers of uninfected EC did not inhibit the oxygen radical production. The incubation of PMN with endothelial cells without fMLP had no stimulatory effects on oxygen radical production (Fig. 3). Incubation of PMN stimulated with fMLP and conditioned medium derived from CMV infected or uninfected endothelial cells did not alter the production of oxygen radicals (data not shown).

4. Discussion

In the present study we describe that CMV infection induced the increased expression and activity of ecto-ATPase (CD39) and ecto-5′nucleotidase (CD73) in endothelial cells. These enzymes are involved in the extracellular adenine nucleotide metabolism. The increased expression of functional ecto-5′nucleotidase after CMV infection was demonstrated in supernatants of infected cells by the higher turnover of exogenously added AMP. However, we were unable to demonstrate the increased activity of ecto-ATPase by differences in extracellular nucleotide levels. Stimulated PMN produced lower amounts of oxygen radicals in the presence of CMV infected endothelial cells than with uninfected endothelial cells. The amount of oxygen radical production by PMN was not influenced by the presence of conditioned medium containing a lot of viral particles and multiple other CMV induced factors, indicating the requirement of a cell surface associated enzyme such as ecto-5′nucleotidase. It would seem likely that CD73 catalyzes the formation of adenosine from AMP, upon which oxygen radicals are scavenged by adenosine.

The metabolism of extracellular nucleotides is an important regulatory tool to maintain a coagulant state as well as a 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 [19], and is well studied in rat models [14]. Recently, the importance of ecto-ATPase was confirmed in CD39-deficient mice, which had prolonged

bleeding times and platelet hypofunction [20]. 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. Moreover, ecto-5′nucleotidase may enhance the role of ecto-ATPase by converting AMP to adenosine, a known inhibitor of platelet aggregation. After contact with an inflammatory plasma factor as shown in the renal perfusion model ex vivo, the expression and activity of ecto-ATPase is severely reduced [12]. The reduction of ecto-ATPase expression during inflammation is due to the sensitivity of this exoenzyme for oxygen radicals, and was observed in rats [21] as well as in activated human endothelial cells [22]. The expression of ecto-5′nucleotidase is influenced by ischemic conditions, reflected by upregulation of this enzyme [6]. 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 [23]. A delayed onset of graft function after transplantation resulted in a decreased expression of ecto-ATPase as well [24]. Obviously, these patients had suffered from considerable reperfusion damage with release of oxygen radicals.

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 by proinflammatory cytokines such as TNF-α; otherwise ecto-ATPase would probably be downregulated [25]. 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 [6]. We did not measure the adenosine concentration in the cytochrome *c* 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 [26,27]. Therefore to maintain the anti-aggregatory and anti-inflammatory effects of adenosine in vitro 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 [7], including CMV [8], 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 hemostasis.

Furthermore, adenosine has a modulatory role in the inflammatory response. Binding of ATP to purine receptors on the PMN has a proinflammatory 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 modulate 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 endothelial cells 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, affecting the regulation of coagulation, inflammation and integrity of the endothelial layer.

Acknowledgements: We thank Juul Baller and Patrick Lockhorst for technical assistance, C. Sinzger for HCMV clinical isolate TB42 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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