HIV infection is associated with increased NTPDase activity that correlates with CD39-positive lymphocytes

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Abstract

Infection with the human immunodeficiency virus (HIV) results in alterations in immune cells such as an increase or decrease of cytokine secretion and immunodeficiency. HIV causes a state of chronic cellular activation that can induce apoptosis in lymphocyte T-helpers, making the patient susceptible to opportunistic infections. The biochemical mechanisms involved in this immune response to HIV have been researched. Here, we have shown for the first time that ATP and ADP hydrolysis are essential for the immune response to HIV. Our results clearly indicate an increase of NTPDase-1 (EC 3.6.1.5) activity in lymphocytes of HIV-positive patients, confirmed by an enhanced CD39 expression on its surface. These results suggest that NTPDase-1 may be important to keep an adequate balance between the generation and consumption of ATP and to preserve cellular integrity and immune response to the HIV infection.

Keywords: NTPDase; CD39; Human lymphocyte; HIV-1

1. Introduction

NTPDase-1 (EC 3.6.1.5) is an enzyme that hydrolyzes extracellular nucleoside tri- and/or diphosphates forming AMP that can serve as a substrate for an ecto-5′-nucleotidase (EC 3.1.3.5) with release of adenosine a modulator of vascular tone and inhibitor of platelet aggregation. This class of enzyme has been described in mammals [1–4] and NTPDase-1 was identified as a lymphoid cell antigen CD39 protein [5]. Extracellular nucleotides and nucleosides play an important role in signaling cascades that include several cellular events such as neurotransmission, platelet aggregation and inflammatory and immune responses [6]. Nucleotides, after exerting their function through purinoreceptors, are hydrolyzed in the extracellular medium [7]. Thus, the duration and magnitude of purinergic signaling is governed by nucleotide-degrading ectoenzymes such as NTPDase-1 [8].

NTPDase-1 (CD39) activity occurs in lymphocytes from human peripheral blood, thymocytes and mouse spleen lymphocytes and its activity is higher in human B cells than in T cells [9]. The increased expression of CD39 is associated with an enhancement in ATPase and ADPase activities in these cells besides demonstrating a state of cellular activation in lymphocytes [10]. The fact that CD39 is expressed only on subsets of activated T lymphocytes suggests a possible role of CD39 in cell–cell adhesion and thus CD39+ cells may facilitate more efficiently the interaction among lymphocytes with other cells of the immune system [11].

NTPDase-1 activity was recently characterized by Leal et al. [4] in human peripheral lymphocytes and its presence was confirmed by a low density of fluorescence detected by cytometric analysis of CD39. The enzyme characterized showed cation dependency, optimum activity in pH 8.0,
preference for ATP and ADP as substrates and enzymatic associations were eliminated by the use of selective inhibitors. However, its possible association with immune diseases has not yet been evaluated. Here, for the first time, we have investigated the association of NTPDase activity with the expression of CD39 in the lymphocytes of HIV-patients. Most importantly, here we have shown that these patients present an increase of NTPDase activity followed by an enhanced CD39 expression.

2. Materials and methods

2.1. Chemicals

Nucleotides and Trizma base were purchased from Sigma (St. Louis, MO, USA). Ficol-Hypaque (Lymphoprep™) was purchased from Nycomed Pharma (Oslo, Norway). Antibodies for flow cytometry analysis (R-Phycocerythrin-[R-PE]-conjugated mouse anti-human monoclonal antibody against CD39, fluorescein isothiocyanate (FITC)-conjugated mouse anti-human monoclonal antibody against CD25, peridinin chlorophyll protein (PerCP)-conjugated mouse anti-human monoclonal antibody against CD45 and fluorescein isothiocyanate (FITC)-conjugated mouse anti-human monoclonal antibody against CD45) were purchased from BD PharMingen Technical Data Sheet (San Jose, CA, USA). All other reagents used in the experiments were of analytical grade and of the highest purity.

2.2. Patients

The sample consisted of 50 healthy volunteers and 50 HIV-positive patients from the Hospital of the Federal University of Santa Maria (Santa Maria, RS, Brazil). All subjects gave written informed consent to participate in the study. The Human Ethics Committee of the Health Science Center from the Federal University of Santa Maria approved the protocol under the number 13690. The control group was carefully selected by clinical evaluation consisting on 50 individuals aged 20–40 years, 50% males and 50% females who did not undergo any pharmacological therapy during the last month. The 50 patients who did not undergo any pharmacological therapy during the last month. The 50 patients infected by HIV were individuals aged 25–42 years, 50% males and 50% females, both being treated and not by antiretroviral treatment.

2.3. Isolation of mononuclear cells from human blood

Mononuclear leukocytes were isolated from human blood collected with EDTA and separated on Ficol–Hypaque density gradients as described by Boyum [12].

2.4. Enzyme assays

After the isolation of mononuclear cells, the NTPDase activity was determined as described by Leal et al. [4] where the reaction medium contained 0.5 mM CaCl₂, 120 mM NaCl, 5 mM KCl, 60 mM glucose, and 50 mM Tris–HCl buffer, pH 8.0, at a final volume of 200 µL, and inhibitors of other ATPases was added to confirm NTPDase activity. Twenty microliters of intact mononuclear cells suspended on a saline solution were added to the reaction medium (2–4 µg protein) and preincubated for 10 min at 37 °C. The reaction was started by the addition of substrate (ATP or ADP) at a final concentration of 2 mM and stopped with 200 µL 10% trichloracetic acid (TCA) to provide a final concentration of 5%. The enzyme incubation times and protein concentrations were chosen in order to ensure the linearity of the reactions. The samples were chilled on ice for 10 min before assaying for the release of inorganic phosphate (Pi) as described for Chan et al. [13], using malachite green as a colorimetric reagent and KH₂PO₄ as standard. Controls with the addition of enzyme preparation after mixing TCA were used to correct for non-enzymatic hydrolysis of substrate. All samples were run in duplicate or triplicate and the specific activity is reported as nmol Pi released/min/mg protein.

2.5. Protein determination

Protein was measured by the Coomassie Blue Method using bovine serum albumin as standard as described for Bradford [14].

2.6. Flow cytometry analysis for CD39 and CD25

Peripheral blood cells were incubated with anti-CD39, anti-CD25 and anti-CD45 (20 µL/10⁶ cells) for 25 min, lysed with reagent FACS (Fluorescence Activated Cell Sorter) lysis and incubated again for 15 min in the dark. Cells were washed twice in PBS buffer (pH 7.4) containing 0.02% (w/v) sodium azide and 0.2% (w/v) BSA. The cells were then resuspended in PBS buffer (pH 7.4) and immediately analyzed by FACScalibur flow cytometer using Cellquest software (Becton Dickinson, San Jose, CA, USA), without fixation.

2.7. Flow cytometry analysis for CD3, CD4, CD8 and CD4/CD8 ratio

Fifty microliters of peripheral blood cells (with K₃EDTA as anticoagulant) of HIV-positive patients and controls were incubated with 20 µL of BD TriTEST™ for 15 min in tubs with TruCOUNT™ controls (Becton Dickinson). The red cells were lysed with 450 µL of BD FACSLysing™ solution and the sample incubated again for 15 min in the dark. After incubation, the tub was immediately analyzed by FACScalibur™ flow cytometer using MultiSET™ software (Becton Dickinson, San Jose, CA, USA), without fixation.

2.8. Viral load determination

The viral load was performed using the VERSANT® HIV-1 RNA 3.0 assay (Bayer Corporation, Berkeley, CA, USA), a signal amplification nucleic acid probe assay for the direct quantification of human immunodeficiency virus type 1 (HIV-1) RNA in plasma, using the Bayer® system 340 b-DNA Analyzer. 1 mL of plasma with K₃EDTA as anticoagulant was used and the minimal limit detection was 50 copies/mL [15].

2.9. Hematological parameters determination

Quantitative determinations of leucocytes were performed by hematology analyzer Coulter STKS (Diamond Diagnostics, Holliston, MA, USA) in peripheral blood. The parameters analyzed were the total number of leucocytes and the absolute count of lymphocytes as for the control group as the HIV-positive group.

2.10. Statistical analysis

Data were analyzed by the non-parametric U test (Mann–Whitney test), considering a level of significance of 5% to determine the differences between the study group and controls.

3. Results

3.1. Hematological parameters and viral load

Other cellular markers, besides CD39, were determined in the peripheral blood by flow cytometry, such as a count of CD4⁺, CD8⁺ and CD3⁺ cells in the control group and the HIV-positive group. Moreover, it was verified the absolute number of lymphocytes and total leukocytes in the human peripheral blood in both groups. In the HIV-positive patients group, the quantification of viral load by HIV-1 was performed. The results showed comparing HIV group with controls, an increase of CD8⁺ cells (948 ± 54 and 615 ± 54 cells/µL), a decrease of CD4⁺ cells (418 ± 39 and 1091 ± 136 cells/µL) and CD3⁺ cells (1434 ± 82 and 1794 ± 157 cells/µL) together to
lower CD4/CD8 ratio (0.47±0.045 and 1.80±0.21) and absolute count of leukocytes (5954±284 and 7796±296 cells/μL) and lymphocytes (1844±86 and 2290±84 cells/μL). Results are expressed as the mean±SE (n = 50 for each group). These data are according to immune response to HIV infection and have been established by many laboratories. The statistical analysis comparing CD39 expression and NTPDase-1 activity with CD4, CD8 and CD3 expression, leukocytes and lymphocytes number and viral load did not show a significant correlation between these variables. A tendency to increase CD39 expression together with CD8 enhancement was observed and the same behavior was observed for NTPDase-1 activity (data not shown). The viral load was determined to confirm the infection in HIV group, showing as result a mean of 9552±3248 copies/mL (S.E.M.; n = 50).

3.2. NTPDase activity

Lymphocytes from HIV-infected patients had a significant increase of about 40% in the specific activity of ATP and ADP hydrolysis confirming Mann–Whitney U test (P<0.0001) when compared to controls (Fig. 1).

3.3. Cytometric analysis of the CD39 and CD25 expression

By statistical analysis the difference was significant (P<0.0001) between the control group and the HIV-positive group according to the Mann–Whitney U test, when observed the percentage of CD39-positive cells. In line with this, the flow cytometry analysis indicated that the percentage of CD39-positive lymphocytes in HIV-patients was higher than in controls (8.04±0.42/5.25±0.17) (Fig. 2). The research of positive cells for CD25 indicated higher percentages in HIV group when comparing with controls (3.80±0.58 and 2.32±0.56, respectively; P<0.05) and the difference between the groups in relation to CD39+/CD25+ cells (0.85±0.17 and 0.51±0.17, respectively) it was not significant. Fig. 3 illustrates histograms of the two groups, obtained by flow cytometry.

4. Discussion

This study evaluated NTPDase-1 activity and the percentage of lymphocytes that express CD39 on their surface in HIV-infected patients. It is known that CD39 is expressed in activated lymphocytes and presents NTPDase activity [5,16]. Our aim was to verify its expression and activity in lymphocytes of HIV-positive patients, where T cells show an increased turnover, demonstrating a chronic activation state [17]. The activation of these cells is ATP-dependent as an energy source, which is catered by mitochondria through the electron transport chain and oxidative phosphorylation [18].

It was observed that ATP suppress the proliferation of peripheral lymphocytes due to adenosine production [19] and incites the DNA synthesis in lymphoid cells of timo, spleen, lymph nodes and peripheral blood [20]. Lymphocytes express P2Z receptors, which make with that ATP and other nucleotides can induce alterations in intracellular calcium store. The bind of ATP to P2X7 receptor forms a transmembrane pore, which modifies the permeability to mono and divalents ions and increases the intracellular calcium such as P2Z receptor [21]. Moreover, to make believe that P2X receptor role in lymphocytes proliferation control is more complex than only the effectors actions that take to cellular died [22].

CD39 was identify as a lymphoid activation marker expressed in B lymphocytes, cytotoxic T lymphocytes, NK cells and endothelial cells, been too recognized in platelets and megakaryocytes [11,23–25]. It is able to do signs that increase cell–cell interaction [26,27]. NTPDase1 executes important role at control of lymphocytes function [28], including antigen recognition and/or activation of effectors activities of cytotoxic T cells.

Ecto-ATPase activity expression in T cells, but not in naive T cells, suggests that its expression is activated by antigen recognition, showing its involvement in activation regulation of T cells or effectors functions. The adhesion of T cells to antigen introduced by APC (antigen presentation cell) is sufficient to start the T cell activation. CD39 enzymatic activity (NTPDase) may be responsible for the hydrolysis of
extracellular ATP, which appears to be necessary for Th1 cytokine secretion, essential for an immune response to viral diseases [29]. Its enzymatic action involves the extracellular ATP hydrolysis process and it was observed that the depletion of this nucleotide inhibits the type Th1 cytokine secretion. The main Th1 cytokine is the interferon-γ (IFN-γ) that, once liberated, activates other subsets of lymphocytes such as TCD8 cells and B cells or macrophages. Th1-cytokine secretion (IFN-γ and TNF-α) is a form of cellular response maintenance, through an inflammatory process, activating granulocytes and macrophages, besides TCD8 and B lymphocytes, being that these go on to produce opsonizant antibodies to help the phagocyte actions.

TCD8 lymphocytes are the main cells able to respond to the presence of virus, as well as NK cells, and their interaction with extracellular ATP seems to be essential to raise the degranulation of these cells and their cytotoxic effect [30]. The degranulation of these cells enables the release of ATP in the extracellular medium. Besides that, there is a release of perforin and granzyme that induces the apoptosis and consequently lyses cells with more ATP release in the medium [31]. ATP may be toxic to cells by activating P2X7 purinoreceptors, also causing a lysis of the target cell by increasing of calcium influx and caspase activation [22]. NTPDase hydrolysis ability is related to essential events in lymphocyte activation in immune responses and the main role does not seem to be that of providing nucleotides to purine salvation pathway [8,29].

In the HIV infection, the immune response suffers severe disorders such as CD4 cell depletion, as a consequence of an apoptotic process triggered by chronic cell activation. TCD4 lymphocytes of its chronic activation state express Fas and FasL (Fas ligand), leading to the activation of caspase-8 and causing apoptosis with subsequent massive ATP release in the extracellular medium. CD4 lymphocyte depletion makes the organism immunodeficient, as it is more apt to opportunistic infections. Extracellular ATP is also able to induce cell destruction through the P2X7 receptor [22] and thus it would be appropriate that cells use mechanisms to protect themselves from these effects. In that case, the increase of NTPDase activity enhances ATP hydrolysis, saving cells from apoptotic effects. Furthermore, TCD8 cells that are responsible for defense against virus can be exhausted by continual activation.
and fail to develop immunological memory. In this context, ATP hydrolysis from NTPDase is very important, as it inactivates both the ATP released by the cytotoxic lymphocytes and that which is released by the destroyed cells (cells lysed by cytotoxic lymphocytes), which could be a compensatory response. Our results show that HIV-positive patients have an NTPDase activity increased with an higher number of CD39-positive lymphocytes, that is consistent with the cellular activation. The same can be confirmed by higher CD25 expression, other activation marker (IL-2 receptor).

ATP generation and consumption are important to maintain its concentration in narrow ranges, controlled in part by NTPDases. It has already been related that lymphoid cells possess two enzymes that could produce ATP from the AMP released in the NTPDase hydrolysis. These enzymes could be adenylate kinase (EC 2.7.4.3) and ecto-NTP kinase (EC 2.7.4.6) that may restore the ATP pool in the extracellular medium [8]. This restoration may be enhanced by a low 5'-nucleotidase activity in human lymphocytes. Peripheral lymphocytes seem to have low 5'-nucleotidase activity and according to the literature, patients with AIDS present a decrease in this activity compared to healthy subjects, which could indicate cellular immaturity, as well [32]. What then would be the destination of the ATP formed? We can suggest that the AMP formed by NTPDase could be rephosphorylated by adenylate kinase and NDP kinase, restoring the extracellular ATP pool. In this case, there could be a constant balance between ATP that must be hydrolyzed to avoid cellular apoptosis and the ATP that must be formed, activating important immune functions. We suggest that NTPDase participates in the maintenance of this balance. Furthermore, we can speculate that 5'-nucleotidase activity is low, producing low adenosine levels, which could be beneficial to the cell. It is known that adenosine could incite apoptosis, contributing to the increase of the immunodeficiency state [33]. Adenosine would have various undesirable actions in this type of infection such as a decrease of interleukin-12, an increase of interleukin-10 and an inversion of the Th1 cellular response to the Th2 humoral response, contributing to the pathogenesis of the infection [29]. Besides, adenosine has anti-inflammatory and lympho toxic effects, decreasing Class II MHC expression in macrophages and dendritic cells and lowering leukocyte adhesion [34].

In this scenario, our results suggest that extracellular nucleotides and their metabolism may be involved in the immune response to the HIV infection. Our data propose that during chronic HIV infection there is an increase of ATP released in the extracellular medium by apoptotic process and NTPDase-1 hydrolysis of these nucleotides become important. This hydrolysis could decrease the levels of ATP and its toxic effects on the cells and save them from nucleotide cytolytic events. In an injured immune system, it is necessary to save cells that are essential to response against viral infection and in this case, NTPDase-1 performs this role, maintaining the ATP balance. Thus, not only are low ATP concentrations important to maintain the immune functions, but also toxic ATP concentrations are avoided. These findings may help to understand the mechanisms involved in immune response against HIV-1 and perhaps NTPDase-1 or CD39 expression may become useful as a prognostic marker of the HIV infection.

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