Clinica Chimica Acta 433 (2014) 105–110

Contents lists available at ScienceDirect

Clinica Chimica Acta

journal homepage: www.elsevier.com/locate/clinchim

Alterations of ectonucleotidases and acetylcholinesterase activities in lymphocytes of Down syndrome subjects: Relation with inflammatory parameters

Rodrigo Rodrigues [a], Gabriela Debom [a], Fabiano Soares [a], Caroline Machado [a], Jéssica Pureza [a], William Peres [a], Gilberto de Lima Garcias [c], Marta Frescura Duarte [b], Maria Rosa Chitolina Schetinger [b], Francieli Stefanello [a], Elizandra Braganhol [a], Roselia Spanevello [a,*]

[a] Programa de Pós-Graduação em Bioquímica e Bioprospecção, Centro de Ciências Químicas, Farmacêuticas e de Alimentos, Universidade Federal de Pelotas, Campus Universitário S/N, Pelotas, RS, Brazil
[b] Programa de Pós-Graduação em Bioquímica Toxicológica, Centro de Ciências Naturais e Exatas, Universidade Federal de Santa Maria, Av. Roraima, 97105-900 Santa Maria, RS, Brazil
[c] Faculdade de Medicina, Universidade Federal de Pelotas, Pelotas, RS, Brazil

ARTICLE INFO

Article history:
Received 16 August 2013
Received in revised form 22 February 2014
Accepted 3 March 2014
Available online 12 March 2014

Keywords:
Down syndrome
Lymphocytes
NTPDase
Adenosine deaminase
Acetylcholinesterase
Cytokines

ABSTRACT

Background: Subjects with Down syndrome (DS) have an increased susceptibility to infections and autoimmune disorders. ATP, adenosine, and acetylcholine contribute to the immune response regulation, and NTPDase, adenosine deaminase (ADA) and acetylcholinesterase (AChE) are important enzymes in the control of the extracellular levels of these molecules. We evaluated the activities of these enzymes and the cytokine levels in samples of DS individuals.
Methods: The population consisted of 23 subjects with DS and 23 healthy subjects. Twelve milliliters of blood was obtained from each subject and used for lymphocyte and serum preparation. Lymphocytes were separated on Ficoll density gradients. After isolation, NTPDase and AChE activities were determined.
Results: The NTPDase activity using ADP as substrate was increased in lymphocytes of DS patients compared to control ($P < 0.05$); however, no alterations were observed in the ATP hydrolysis. An increase was observed in the AChE activity in lymphocytes and in ADA activity in serum of DS patients when compared to healthy subjects ($P < 0.05$). In DS subjects, an increase in the levels of IL-1β, IL-6, TNF-α and IFN-γ and a decrease in the IL-10 levels were also observed ($P < 0.05$).
Conclusions: Alterations in the NTPDase, ADA and AChE activities as well changes in the cytokine levels may contribute to immunological alterations observed in DS.

1. Introduction

Down syndrome (DS), also known as trisomy of 21, is a chromosomal alteration characterized by the presence of an extra copy of the chromosome 21 [1,2]. It is the most common genetic disorder affecting 1 in 700 newborns [3]. Data from literature have shown that subjects with DS have an increased susceptibility to infections and autoimmune disorders, which are the main causes of mortality and morbidity observed in this genetic alteration [4].

The immune system dysfunction in DS has been associated to B lymphocyte decreased number, T-cell subset modifications as well as anti and pro-inflammatory cytokine level alterations [5–8]. However, the molecular mechanisms leading to immune defects and the contribution of these alterations to the increased risk of infections have not been fully elucidated.

Extracellular adenine nucleotides and nucleosides such as ATP and adenosine have been recognized as key components of immune and inflammatory processes [9]. ATP, acting through specific cell surface purinergic receptors, is involved in pro-inflammatory actions such as lymphocyte stimulation and proliferation as well as cytokine release, including IL-2, IFN-γ, IL-1β and TNF-α [9,10]. In opposite, adenosine, a product of ATP hydrolysis, exhibits potent anti-inflammatory and immunosuppressive actions by inhibiting both proliferation of T cells and secretion of pro-inflammatory cytokines, such as TNF-α and IFN-γ [11,12].

Extracellular ATP and adenosine levels are regulated by cell surface ectoenzymes, such as ectonucleoside triphosphate diphosphohydrolase (NTPDase) and adenosine deaminase (ADA). NTPDase1 (CD39) is involved in the breakdown of ATP and ADP to AMP which is hydrolyzed by ecto-5′-nucleotidase to adenosine [13,14]. ADA is considered a key enzyme in purine metabolism, catalyzing the irreversible deamination

* Corresponding author at: Programa de Pós-Graduação em Bioquímica e Bioprospecção, Centro de Ciências Químicas, Farmacêuticas e de Alimentos, Universidade Federal de Pelotas, Campus Universitário Capão do Leão, 96010-900 Pelotas, RS, Brazil. Tel.: +55 53 39217233.
E-mail address: rspanevello@gmail.com (R. Spanevello).

http://dx.doi.org/10.1016/j.cca.2014.03.002
0009-8981/

of adenosine to inosine, thus regulating extracellular adenosine availability [15]. Since NTPDase1 and ADA activities potentially modulate extracellular levels of pro-inflammatory ATP and anti-inflammatory adenosine, the role of these enzymes has been evaluated in the pathogenesis of immune and inflammatory diseases [16,17]. Indeed, the activities of these enzymes have been altered in such conditions, indicating the crucial role of NTPDase and ADA in the regulation of immunologic responses.

Acetylcholinesterase (AChE) is another enzyme involved in immune functions. This enzyme is expressed in both T and B lymphocytes and promotes the hydrolysis of the acetylcholine (ACh) to choline and acetate [18]. ACh is known to promote anti-inflammatory actions by suppressing the production of pro-inflammatory cytokines [19,20]. In this line, AChE emerges as a potential contributor in the pathways controlling inflammatory and immune responses mediated by muscarinic and nicotinic receptors [21]. Because of its key physiological role, AChE activity has been studied in different pathological and experimental conditions [22].

Despite the importance of NTPDase1, AChE and ADA in modulating inflammatory and immune responses, to the best of our knowledge, there are few reports evaluating the activity of these enzymes in lymphocytes from DS subjects. Considering the alterations in lymphocyte functions observed in DS, the aim of this study was to evaluate NTPDase, AChE and ADA activities in lymphocytes as well as the serum cytokine levels of DS subjects.

2. Material and methods

2.1. Chemicals

Nucleotides, Trizma base, acetylthiocholine iodide (ASCh), 5,5′-dithiobis-2-nitrobenzoic acid (DTNB), Ficoll-Histopaque (Lymphoprep™) and Malachite Green Carbinol Base were purchased from Sigma Chem. Co. All other reagents used in the experiments were of analytical grade and the highest purity.

2.2. Study population

The sample consisted of 23 DS and 23 healthy individuals (control group). All the subjects included in this study were residents of the Pelotas City, located in the south region of the state of the Rio Grande do Sul (Brazil). The general characteristics of the individuals are shown in Table 1. All subjects gave written informed consent to participate in this study. Written informed consents were obtained from parents/guardians of DS subjects. The Human Ethics Committee of the Health Science Center from Universidade Federal de Pelotas approved the study protocol. Twelve milliliters of blood was obtained from each subject by venous puncture and used for lymphocyte preparation and other biochemical determinations described as follows.

2.3. Hematological determination

A complete hemogram was performed in the blood samples collected in tubes containing 7.2 mg dipotassium EDTA as anticoagulant. The hematological parameters were carried out with the aid of the automatic counter Horiba ABX Pentra C+60 (Horiba ® ABX), which is an association of the principles of electrical impedance, flow cytometry and spectrophotometry for blood analysis. Hematological parameters of the DS group and control are shown in Table 2. All the individuals showed a hemogram compatible with their age and gender in accordance with reference values.

Table 1
Characteristics of Down syndrome (DS) and control individuals applied in this study.

	Control	DS
Number	23	23
Women	14	12
Men	9	11
Age of women	29.66 ± 9.91	29.33 ± 9.78
Age of men	26.15 ± 7.36	26.00 ± 7.1

2.4. Isolation of lymphocytes from human blood

Lymphocytes were isolated from human blood collected with EDTA and separated on Ficoll-Histopaque density gradients as described by [23]. Protein was measured by the Coomassie blue method according to [24] using serum albumin as standard.

2.5. NTPDase enzyme assays in lymphocytes

After lymphocyte isolation, the NTPDase activity was determined as described by [16] in a reaction medium containing 0.5 mM $CaCl_2$, 120 mM NaCl, 5 mM KCl, 6 mM glucose and 50 mM Tris–HCl buffer (pH 8.0), at a final volume of 200 μl. Twenty microliters of intact mononuclear cells suspended in saline solution was added to a reaction medium (2–4 μg of protein) and pre-incubated for 10 min at 37 °C. The enzyme reaction was initiated by the addition of substrate (ATP or ADP) at a final concentration of 2 mmol/l and it was stopped following 70 min of incubation by adding 200 μl of 10% trichloroacetic acid (TCA). The released inorganic phosphate (Pi) was assayed by the method of [11] using Malachite Green as colorimetric reagent and KH_2PO_4 as standard. Controls were carried out by adding the enzyme preparation after TCA addition to correct for non-enzymatic nucleotide hydrolysis. All samples were run in triplicate and the specific activity is reported as nmol Pi released/min/mg of protein.

2.6. AChE enzyme assays in lymphocytes

After lymphocyte isolation, the AChE activity was determined by colorimetric assay [25] modified [26]. The reaction mixture was composed of 1 mmol/l acetylthiocholine, 0.1 mmol/l 5,5′-dithio-bis-2-nitrobenzoic acid (DTNB), and 0.1 mol/l phosphate buffer (pH 8.0) and 100 μl of the intact mononuclear cells suspended in saline solution was added to the reaction. The proteins of all samples were adjusted to 0.1–0.2 mg/ml. The absorbance was read on a spectrophotometer at 412 nm before and after incubation for 30 min at 27 °C. All samples were run in triplicate and the specific activity of lymphocyte AChE was calculated from the quotient between lymphocyte AChE activity and protein content, and results are expressed as μmol of AcSch/h/mg of protein.

2.7. ADA enzyme assay in blood serum

ADA activity in serum was determined according to previous studies [27]. Briefly, 50 μl of serum reacted with 21 nmol/l of adenosine (pH 6.5) and the incubation was carried out at 37 °C for 60 min. This method is based on the direct production of ammonia when ADA acts in excess of adenosine. Results were expressed in units per liter (U/l). One unit (1 U) of ADA is defined as the amount of enzyme required to release 1 mmol of ammonia from adenosine per minute at standard assay conditions.

2.8. Quantification of cytokines

Cytokine quantification was assessed by ELISA using commercial kits for human IFN-γ, TNF-α, IL-1β, IL-6 and IL-10 (eBio-Science), according to the manufacturer's instructions. The cytokine presence and concentration were determined by the intensity of the color measured by spectrometry in a micro ELISA reader. Standard curves for the cytokines ranged from 1 to 50 pg/ml for IL-1β, 2 to 200 pg/ml for IL-6, 4 to