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Down-regulation of adenosine A_1 and A_{2A} receptors in peripheral cells from idiopathic normal-pressure hydrocephalus patients

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ABSTRACT

Idiopathic normal-pressure hydrocephalus (iNPH) is a neurological disease that usually develops in the elderly. Natural history of iNPH is still unknown. It has been hypothesized that cerebrovascular diseases could have a role in etiology of chronic hydrocephalus and studies show an increased prevalence of cardiovascular diseases in iNPH patients. Moreover, evidences show a possible alteration of immune system in iNPH patients.

Adenosine (Ado) is a metabolite produced in response to metabolic stress and injury. Adenosine and its receptors play an important role in vascular protection and in the modulation of inflammatory reactions and neuroinflammation.

Our aim is to evaluate gene and protein expression of A_1R and $A_{2A}R$ in the peripheral blood mononuclear cells (PBMCs) from iNPH patients compared to control subjects. We investigate if Ado system, that plays an important role in central nervous system, in vascular system, and also in inflammation, is involved in pathophysiology of iNPH disease.

Our analysis showed that A_1R mRNA levels and A_1R density in PBMCs from iNPH patients were significantly lower than CT subjects (0.84 ± 0.12 and 2.42 ± 0.42, $p < 0.001$ and 0.31 ± 0.02 and 0.42 ± 0.04, $p = 0.043$; respectively). About $A_{2A}R$, the gene expression in PBMCs was significantly lower in iNPH than CT (0.65 ± 0.09 and 1.5 ± 0.14, $p < 0.001$) as well as there was a trend in protein expression: iNPH and CT (0.51 ± 0.05 and 0.62 ± 0.03; $p = 0.172$).

This preliminary study underlines the involvement of Ado system in iNPH disease whose pathophysiology is still unclear.

1. Introduction

Idiopathic normal-pressure hydrocephalus (iNPH) is a neurological disease that usually develops in the elderly with symptoms of impaired gait and mobility, urinary urgency and incontinence, and mild cognitive impairment or dementia (Hakim triad) in presence of ventriculomegaly and normal intracranial pressure [1]. The pathophysiology and natural history of this disease are still unknown [1].

It has been hypothesized that cerebrovascular diseases could have a role in etiology of chronic hydrocephalus [2]. Moreover, many studies show a significantly increased prevalence of cardiovascular diseases and risk factors for vascular diseases in iNPH compared to healthy subjects [3]. So far contrasting data have been reported on inflammatory involvement in iNPH patients; some studies suggest an alteration of immune system in this pathology [4], but other authors deny it [5].

The purine ribonucleoside adenosine (Ado) is a naturally occurring metabolite that is ubiquitously distributed throughout the body as a metabolic intermediary. Intra- and extracellular Ado levels rise in response to physiological stimuli and with metabolic/energetic perturbations, inflammatory challenges, and tissue injury. The physiological effects of adenosine are transduced through the link between the Ado receptors and different subtypes of G-protein, that regulate, in opposite directions, the second messenger cAMP: A_1 receptor (A_1R) linked to inhibitory G_i-protein and A_{2A} receptor ($A_{2A}R$) linked to excitatory G_s-protein, thereby decreasing and increasing cAMP levels, respectively. Adenosine receptors are ubiquitously distributed throughout brain and spinal cord, heart, inflammatory cells, liver, adipose tissue, kidney and in many other tissues [6].

There are very robust studies showing that Ado receptors control the dynamics of the brain vasculature, they have multiple effects on cerebral arteries and arterioles, resulting in a complex influence on brain perfusion both under resting conditions and during states of increased synaptic activity [7]. In the central nervous system (CNS) A_1R slows metabolic activity and has an important role on brain tissue protection

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against multiple types of brain insults including ischemia, hypoxia, excitotoxicity, trauma and neurodegenerative diseases [8]. Instead, contrasting data have been reported on the beneficial/detrimental effects of $A_{2A}R$ on brain cells [9]. In the cardiovascular system, A_1R plays a cardioprotective role. Stimulation of A_1R has a myocardial depressant effect, slows heart rate and conduction, and reduces atrialventricular contractility and activity of pacemaking cells [6]. $A_{2A}R$ enhances heart contractility, plays a primary role in coronary and systemic vasoregulation, contributes to endothelial dependent and independent dilatation [6]. It also plays a role in vasculogenesis, angiogenesis and in vascular protection [6].

Moreover, increasing evidences support the notion that Ado system is implicated in the regulation of inflammation [10]. A_1R activation serves as a chemoattractant for immature dendritic cells as well as a maturation signal to up-regulate T cells proliferation. $A_{2A}R$ blocks the synthesis of pro-inflammatory cytokines, down-regulates the expansion of effector T cells and induces immunosuppression mediated by T regulatory cells and a recent study has showed that A_1R and $A_{2A}R$ cross-talk is implicated in the down-regulation of inflammation [10]. Ado and its receptors play an important role also in the control of brain inflammation and microglia reactivity. A_1R activation inhibits the proinflammatory microglial phenotype [11], while the blockade of $A_{2A}R$ confers robust neuroprotection and controls microglia reactivity in the brain [12]. Therefore, Ado receptors play a critical role in the modulation of neuroinflammatory reactions, influencing functional outcome in a broad spectrum of pathologies including neurodegeneration [13].

The aim of this preliminary study is to investigate A_1R and $A_{2A}R$ gene expressions and protein levels in peripheral blood mononuclear cells (PBMCs) as these cells seem to participate to processes until now thought to be confined in CNS [14]. We compared Ado receptors expression in iNPH patients and healthy controls (CT) to investigate if Ado system, that plays an important role in CNS, in vascular system, and also in inflammation, is involved in pathophysiology of iNPH disease.

2. Materials and methods

2.1. Study design

The study involved 22 iNPH (mean age ± standard error: 84.3 ± 1.0 years) and 50 non-demented CT subjects (80.2 ± 0.7 years) matched for gender and for the major risk factors for vascular disease (hypertension, high cholesterol, obesity, diabetes, tobacco exposure).

Subjects were recruited from outpatients attending the Geriatric Unit of the Fondazione IRCCS Ca' Granda of Milan, Italy. Diagnosis of iNPH was formulated according to International Guidelines published in 2005 [15]. All patients were older than 75 years and had insidious onset and progression of symptoms (gait disturbance, impairment in cognition and incontinence) for at least 3 months. Brain computed tomography (CT) or magnetic resonance imaging (MRI) scans of all participants showed ventricular enlargement (Evans ratio > 0.3) and no macroscopic obstruction to cerebrospinal fluid (CSF) flow. The vascular damage was evaluated by Fazekas scale after brain MRI [16]. All patients had normal CSF opening pressure at lumbar puncture. All iNPH patients underwent the tap test (TT) as CSF drainage procedure (30–50 cc) performed by lumbar puncture. Only the patients that showed an improvement of at least one of the symptoms of the Hakim triad were included in the study. On CSF samples we evaluated amyloid-β (Aβ), tau, and phosphorylated tau (p-tau) by ELISA (Innogenetics, Ghent, Belgium). We analyzed these biomarkers in order to exclude patients that could be affected by Alzheimer's disease (AD).

Blood samples from all subjects were collected between 8 and 9 a.m, after a 6-hour fast. For the iNPH patients, blood was collected both before and one week after TT. None of the selected subjects showed any clinical signs of inflammation with normal plasmatic albumin, transferring, and protein C-reactive levels and caffeine consumption was about 80 mg/die (one cup of coffee) or less for all subjects.

All participants gave their informed consent to the study, which had been previously approved by the local ethics committee.

2.2. PBMCs isolation

PBMCs were collected from whole blood by using a density gradient centrifugation procedure (Lympholyte-H kit Cedarlane Laboratories Limited, Burlington, ON) and stored at −80 °C pending analysis.

2.3. A_1R and $A_{2A}R$ mRNA expressions

Total RNA was extracted from PBMCs using Chomczynski's and Sacchi's modified method [17]. Quantitative relative PCR (qPCR) was performed on all samples using a Chromo4 instrument and analyzed using Opticon Monitor2 (Celbio, Italy).

A_1R and $A_{2A}R$ reactions were conducted with thermal cycling conditions of 10 min at 95 °C followed by 40 cycles at 95 °C for 15 s, 64 °C (for A_1R) or 56 °C (for $A_{2A}R$) for 40 s and 72 °C for 30 s, with a ramp of 5 °C/s. qPCR for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was performed simultaneously. The mix contained iQ SYBER Green Supermix (Bio-Rad, Italy) and the primers were Forward 5′-GCCCGCTCCACCGCACTC-3′ and Reverse 5′-CCCCCTCGCCATCCTCATCA-3′ for A_1R, Forward 5′-GGCTGCCCCTACACATCATC-3′ and Reverse 5′-GCCAGGTACATGAGCCAGAGA-3′ for $A_{2A}R$ and Forward 5′-ATTCCACCCATGGCAAATTC-3′ and Reverse 5′-TGGGATTTCCATTGATGACAAG-3′ for GAPDH. The quantification of A_1R and $A_{2A}R$ mRNA was carried out by using the comparative cycle threshold (Ct) method and the formula: normalization ratio $= 2^{-\Delta\Delta Ct}$.

2.4. A_1R and $A_{2A}R$ densities

Proteins of 22 iNPH and 30 CT subjects were extracted from PBMCs [18] and A_1R and $A_{2A}R$ levels were measured by Western blot [18]. A_1R bands were visualized with polyclonal rabbit anti-human A_1R (1:250) (Santa Cruz, USA) and goat anti-rabbit IgG-HRP (1:2000) (Santa Cruz, USA). $A_{2A}R$ bands were visualized with polyclonal mouse anti-human $A_{2A}R$ (1:100) (Santa Cruz, USA) and goat anti-mouse IgM-HRP (1:2000) (Santa Cruz, USA). The membranes were stripped and then re-probed with monoclonal anti-human GAPDH (1:8000) (Chemicon International, USA) and rabbit anti-mouse IgG-HRP (1:8000) (Sigma, Italy). The density of the protein bands was estimated by means of IM1D software (Bio-Sciences, Italy) and was expressed as Arbitrary Units (A_1R/GAPDH or $A_{2A}R$/GAPDH ratio).

2.5. Statistical analysis

Statistical analysis was performed using the SPSS statistical package (SPSS version 22, Chicago, IL). The differences in mRNA and protein levels, expressed as mean ± standard error, were calculated using the Student's t-test. The correlation between the vascular damage and the receptors expressions was performed by linear regression analysis. A p value < 0.05 was considered statistically significant.

3. Results

Our analysis showed that A_1R mRNA levels in PBMCs from iNPH were significantly lower than CT subjects (0.84 ± 0.12 and 2.42 ± 0.42, respectively; $p < 0.001$) (Fig. 1A). Along this line, A_1R density was significantly lower in iNPH than CT (0.31 ± 0.02 and 0.42 ± 0.04; $p = 0.043$) (Fig. 1C).

About $A_{2A}R$, the gene expression in PBMCs was significantly lower in iNPH than CT (0.65 ± 0.09 and 1.5 ± 0.14; $p < 0.001$) (Fig. 1B) as well as there is a trend in protein expression: iNPH and CT (0.51 ± 0.05 and 0.62 ± 0.03; $p = 0.172$) (Fig. 1D).

Although iNPH patients treated with TT showed an improvement of at least one of the symptoms of the Hakim triad, we did not find