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Expression of adenosine receptors in monocytes from patients with bronchial asthma

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ABSTRACT

Adenosine is generated from adenosine triphosphate, which is released by stressed and damaged cells. Adenosine levels are significantly increased in patients with bronchial asthma (BA) and mediate mast cell degranulation and bronchoconstriction. Over the last decade, increasing evidence has shown that adenosine can modulate the innate immune response during monocytes differentiation towards mature myeloid cells. These adenosine-differentiated myeloid cells, characterized by co-expression of monocytes/macrophages and dendritic cell markers such as CD14 and CD209, produce high levels of pro-inflammatory cytokines, thus contributing to the pathogenesis of BA and chronic obstructive pulmonary disease. We found that expression of *ADORA2A* and *ADORA2B* are increased in monocytes obtained from patients with BA, and are associated with the generation of $CD14^{pos}CD209^{pos}$ pro-inflammatory cells. A positive correlation between expression of *ADORA2B* and *IL-6* was identified in human monocytes and may explain the increased expression of *IL-6* mRNA in asthmatics. Taken together, our results suggest that monocyte-specific expression of A2 adenosine receptors plays an important role in pro-inflammatory activation of human monocytes, thus contributing to the progression of asthma.

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1. Introduction

Monocytes rapidly infiltrate lung tissue after injury [1] and contribute to initiation and amplification of inflammation via mechanisms, including acceleration of neutrophil migration to the lungs and generation of reactive oxygen/nitrogen species [2,3]. It has been also shown that monocytes promote inflammation through differentiation into inflammatory macrophages and dendritic cells (DCs) [4–6]. DCs play a crucial role in primary and secondary immune responses in lung inflammation and development of BA [7]. Under inflammatory conditions, monocytes contribute to the DCs pool and promote Th2-mediated immune response in asthma [8–11]. Using animal models of lung inflammation, it has been established that the local microenvironment plays an essential role in the regulation of monocytes differentiation towards mature myeloid cells (macrophages and DC) with different properties.

Adenosine is an endogenous purine nucleoside with a broad spectrum of immunomodulatory activities. Adenosine production is significantly increased during lung inflammation, resulting in enhanced extracellular levels of adenosine in patients with BA [12,13]. Adenosine mediates its action via signaling through adenosine receptors (ADORA), namely A_1, A_{2A}, A_{2B} and A_3. We have shown previously that adenosine, acting via A_{2B} receptors, directs differentiation of monocytes into inflammatory cells characterized by co-expression of monocytes/macrophages and DC markers [14]. We demonstrated a pro-inflammatory role of A_{2B} during chronic pulmonary inflammation using a mouse model of allergen-induced chronic pulmonary inflammation [15]. The role of A_{2B} signaling in pro-inflammatory activation of monocytes has been further demonstrated in an allergic-airway inflammation with myeloid cell specific deletion of Adora2b in mouse models [16]. However, little is known about the expression of ADORA in monocytes and their

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functional properties in patients with BA. In the current study, we performed analysis of ADORA expression in peripheral blood monocytes obtained from patients with BA and healthy subjects. We used an established model of adenosine-driven monocyte differentiation *in vitro* [14] to demonstrate the functional significance of ADORA expression for differentiation towards cells promoting inflammation. We hypothesized that the expression levels of adenosine receptors in circulating monocytes may predict their differentiation towards pro-inflammatory mature myeloid cells in asthmatics. We found that the expression of *ADORA2* mRNA is increased in BA patients and adenosine induces their differentiation towards pro-inflammatory $CD14^{pos}CD209^{pos}$ cells. We documented a positive correlation between expression of *ADORA2B* and *IL-6* in human monocytes. These findings may have implications for the rational employment of ADORA antagonists to target adenosine-induced inflammation in patients with BA. Our results support the hypothesis that the expression of A_{2B} receptors in circulating monocytes can play a significant role in the pathogenesis of chronic airway inflammation in asthmatics.

2. Methods

2.1. Subjects

We recruited mild-moderate allergic asthmatic and control subjects (Table 1). Individuals were defined as mild-moderate asthmatics according to “The Global Initiative for Asthma (GINA, 2010)” as previously described [17]. The protocol was approved by The Ethical Committee of the Siberian State Medical University, and written informed consent was obtained from all patients included in the study. All subjects met the following inclusion criteria: males and females aged 18 years or older and 65 years or younger. Asthmatic subjects had no history of other cardiopulmonary diseases. Healthy subjects were negative for allergies and respiratory diseases. The relatively small number of included patients was attributed to the invasive procedure used in the study.

2.2. Purification of peripheral blood monocytes

Human peripheral blood monocytes purification was performed by two-step gradient centrifugation procedure as described [18] with modifications. Briefly, peripheral blood was diluted with Hank's balanced salt solution (HBSS) (1:1), loaded on Ficoll–Hypaque gradient (Sigma, Moscow, Russia) and centrifuged for 30 min at 600 × *g* at room temperature. Peripheral blood mononuclear cells (PBMC) were collected, washed twice in HBSS (pH 7.4), resuspended in serum-free RPMI and mixed with 1.5X volume of isotonic Percoll solution (IPS) (percoll:PBS, 9:1 v/v, p = 1123 g/ml). Then cells was carefully overlaid with Percoll-RPMI solution 1 (IPS:RPMI, p = 1,064 g/ml) and Percoll-RPMI solution 2 (IPS:RPMI, p = 1,032 g/ml). Monocytes were collected from RPMI/percoll interface after centrifugation at 2000× *g* for 50 min at 20 °C. The purity of monocytes was 75–85% as determined after analysis of $CD14^{pos}$ cells using a FACSCalibur flow cytometer.

2.3. In vitro culture and stimulation of human peripheral blood monocytes

Isolated monocytes were resuspended in 10% FBS RPMI medium containing 20 mM HEPES, 50 μM β-mercaptoethanol, 1X antibiotic antimycotic mix (Sigma, Moscow, Russia) and supplemented with 10 ng/ml of human GM-CSF and human IL-4 (both from ProSpec-Tany technoGene, Ness Ziona, Israel). Monocytes were seeded in 24 well plates at concentration of 2×10^5 cell/well and stimulated in the absence (DMSO) or presence of 30 μM stable adenosine analog NECA for 3 days at 37 °C in CO_2-incubator.

2.4. Real-time reverse transcription-polymerase chain reaction

Total RNA was isolated from purified monocytes using RNeasy Mini kit (Qiagen, Valencia, CA). Real-time RT-PCR was performed on a DT-96 Sequence Detection System (Dna-technology, Moscow, Russia). For human *ADORA1* forward primer was: 5′-CTACTTCCA-CACCTGCCTC-3′, and the reverse primer was – 5′-GTCACCAC-CATCTTGTAC-3′; human *ADORA2A*: 5′-GAGCTCCATCTTCAGTCTCC-3′ (forward), 5′-GCATGGGAGTCAGGCCGATG-3 (reverse); human *ADORA2B*: 5′-GTCGACAGATACCTGGCCATC-3′ (forward), 5′-CAGT TGTTGGTGGCACTGTC-3′ (reverse); human *ADORA3* primers: 5′-GTTGTCCGCAAGGCTGACC-3′ (forward), 5′-CAAATGACTGATTACA-GAG-3′ (reverse). The human *VEGFA* forward primer was 5′-GGG CAGAATCATCACGAAGTG-3′, and the reverse primer was 5′-ATTG-GATGGCAGTAGCTGCG-3′; for human *IL-8*, the forward primer was 5′-TGCCAAGGAGTGCTAAAG-3′ and the reverse primer was 5′-TCC ACAACCCTCTGCAC-3′, human *IL-6* forward: 5′-CACAGACAGCCAC TCACCTC-3′, reverse – 5′-TTTTCTGCCAGTGCCTCTTT-3′. For human *ACTB* the forward primer was 5′-CGCCCCAGGCACCAGGGC-3′, and the reverse primer was 5′-GGCTGGGGTGTTGAAGGT-3′.

The relative mRNA quantity for a given gene measured from a single reverse transcription reaction was divided by the value obtained for β-actin to correct for fluctuations in input RNA levels and varying efficiencies of reverse transcription reactions.

2.5. Flow cytometry

After treatment with FcR Blocking Reagent (Miltenyi Biotec Inc., Auburn, CA), monocytes (10^6 cells/ml) were labeled using relevant antibodies for 20 min on ice. All antibodies were obtained from BD Bioscience Pharmingen (San Jose, CA). Data acquisition was performed on a FACScalibur flow cytometer, and the data were analyzed with WinList 5.0 software. Non-viable cells were excluded by using 7-amino actinomycin D. Antigen negativity was defined as having the same fluorescent intensity as the isotype control.

2.6. Statistical analysis

Normally distributed variables are expressed as mean ± SEM.

Table 1
Patient demographics.

Criteria	Bronchial asthma patients		Control subjects
	Mild (n = 14)	Moderate (n = 5)	(n = 20)
Age (years)	37.3 (30.7–43.8)	37.6 (25.6–49.6)	32.2 (28.7–35.7)
Gender (M/F)	(5/9)	(2/3)	(6/14)
Duration of disease (years)	10.4 (6.1–14.6)	16.8 (0.6–31.1)	N/A
FEV1, % of predicted	87.8 (83.4–92.1)	68.4 (51.8–85.0)	ND

Values reported as mean with mean with 95% confidence intervals. Definition of abbreviations: N/A – not applicable; ND – not determined; F = females, FEV1 = forced expiratory volume in 1 s, M = males.

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