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Role of the Mac-1 and VLA-4 integrins, and concomitant Th2-cytokine production, in nitric oxide modulated eosinophil migration from bone marrow to lungs in allergic mice

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ABSTRACT

Although numerous studies demonstrate the participation of nitric oxide (NO) in various inflammatory diseases, the precise function of NO in allergic asthma remains unclear. We investigated whether iNOS inhibition could interfere with the kinetics of VLA-4 and Mac-1 expression and adhesion properties of bone marrow and peripheral blood eosinophils of sensitized mice after antigen exposure. Treatment of allergic mice with 1400W (iNOS inhibitor) increased the adhesion of bone marrow eosinophils to ICAM-1, but not blood eosinophils, at 24 h and 48 h after OVA-challenge. Conversely, adhesion of blood eosinophils from 1400W-treated mice to VCAM-1 diminished at 24 h and was almost completely blocked at 48 h. 1400W did not induce any change in the adhesion of bone marrow eosinophils to VCAM-1, at 24 h, but cells collected 48 h after challenge showed significantly lower adherence. Flow cytometry demonstrated that 1400W resulted in a significantly increased Mac-1 expression on bone marrow eosinophils at 24 h, as compared to control mice. However, at 24 h, 1400W significantly decreased Mac-1 and VLA-4 expressions on blood eosinophils. At 48 h, the expressions of both Mac-1 and VLA-4 returned to previous levels. Results show a temporal effect of iNOS upon Mac-1 expression and function, the chief adhesion molecule involved in the eosinophil efflux from the bone marrow at 24 h. In contrast, Mac-1 and VLA-4 were involved in eosinophil mobilization from blood to lungs at 48 h after antigen challenge. Data suggest an important role of the Mac-1 and VLA-4 in the iNOS-modulated migration of eosinophils to the lungs of allergic mice.

1. Introduction

Asthma is a chronic inflammatory disease of the respiratory tract, characterized by an accumulation of eosinophils, mast cells, and activated lymphocytes in lung tissues [1]. In allergic diseases, it has been suggested that T helper (Th) cells, through the release of specific cytokines, orchestrate the inflammatory response leading to pulmonary eosinophil accumulation. It is now recognized that allergic asthma is driven by a Th1/Th2-cytokine imbalance that favors Th2 activation [2]. Th2 cytokines, such as IL-4 and IL-13, promote immunoglobulin isotype class switching to IgE and are also involved in mucus hypersecretion and allergen-induced airway hyper-responsiveness [2]. Additionally, IL-5 is involved in the maturation of eosinophils and basophils, the main effector cells that secrete mediators of the allergic response [1]. Regulatory T cells have been implicated in the suppression of Th2-cell responses involving the inhibitory cytokines, IL-10 and TGFβ [3]. There is strong evidence that the CC chemokines, eotaxin and RANTES, act in synergy with Th2 cytokines to recruit eosinophils to asthmatic airways [4]. Among the cytokines implicated in leukocyte recruitment, only IL-5 and eotaxin selectively regulate eosinophil trafficking [5].

Inflammatory diseases of the airways are commonly associated with elevated production of nitric oxide (NO) and increased indices of NO-dependent oxidative stress. Asthma is often characterized by an increased expression of the inducible isoform of nitric oxide synthase (iNOS) within respiratory epithelial and inflammatory immune cells, and a markedly elevated local production of NO [6]. Recent data indicate that the measurement of exhaled nitric oxide in exhaled air may be a useful biomarker of asthma [7].

Several studies have revealed that the NO produced during the allergic inflammatory responses is involved in the up-regulation of eosinophil migration to airways [8–13], although the mechanism by which NO promotes allergic eosinophil influx in allergic inflammation

Abbreviations: BAL, bronchoalveolar lavage; iNOS, inducible nitric oxide synthase; ICAM-1, intercellular adhesion molecule-1; LFA-1, leukocyte function-associated molecule-1; Mac-1, membrane-activated complex-1; 1400W, N-3-aminomethyl-benzyl-acetamidine; NO, nitric oxide; OVA, ovalbumin; VCAM-1, vascular adhesion molecule 1; VLA-4, very late antigen-4.

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is incompletely understood. It has been suggested that NO could regulate cytokine production and adhesion molecule expression and function, where the VLA-4 (CD49d/CD29) and Mac-1 (CD11b/CD18) integrins are thought to be key adhesion molecules involved in eosinophil accumulation in several allergic disorders. In turn, VCAM-1 and ICAM-1, expressed on endothelial cells, act as receptors for VLA-4 and Mac-1, respectively. [14].

Our previous study suggests that inhibition of NO synthesis could delay efflux of eosinophils from bone marrow to peripheral blood that could result in reduced eosinophil infiltration to the lungs [13], however the NOS isoform involved in this mechanism remains undefined. While some studies using iNOS-deficient animals or iNOS inhibitors, such as the highly selective iNOS inhibitor 1400W, indicate participation of iNOS in eosinophil migration [11,15,16], others have not found any association between the iNOS isoform and eosinophil recruitment to the lungs [8,12,17]. The purpose of this study was to investigate whether iNOS inhibition could interfere with the kinetics of VLA-4 and Mac-1 expression on the eosinophils of the bone marrow and peripheral blood after antigen exposure. Additionally, we evaluated the adhesion of eosinophils to immobilized VCAM-1 and ICAM-1 and the levels of the C-C chemokine, eotaxin, and cytokines secreted by Th2 lymphocytes (IL-4, IL-5 and IL-13) in lung tissue.

2. Material and methods

2.1. Drugs

The following materials and reagents were used: Ovalbumin (OVA grade V), Eagle's minimum essential medium (MEM) and Protease Inhibitor Cocktail (Sigma Chem. Co., St. Louis, MO, USA); N-3-aminomethyl-benzyl-acetamidine-dihydrochloride (1400W; Calbiochem, San Diego, CA); neutralizing antibodies to VLA-4 and Mac-1, PE-conjugated anti-CD49d and PeCy7-conjugated anti-CD11a (BD Bioscience, San Jose, CA, USA); recombinant mouse VCAM-1 and ICAM-1, IL-4, IL-5, IL-13, eotaxin immunoassay kit and FITC-conjugated anti-CCR3 (R&D System, Minneapolis, USA); APC-conjugated anti-CD11b (eBioscience, San Diego, CA, USA).

2.2. Animals

This study was approved by the Animal Ethics Committee of San Francisco University, Brazil. Female BALB/c mice, 5–8 weeks old, were obtained from the Multiinstitutional Centre for Biological Investigation (CEMIB), State University of Campinas (UNICAMP), Brazil. The mice were maintained under conventional animal housing conditions with food and water ad libitum.

2.3. Sensitization, airway challenge and treatment

Mice were sensitized subcutaneously at day 0 and day 7 with 400 μl of a suspension of 100 mg ovalbumin (OVA grade V) bound to 4 mg of aluminum hydroxide, prepared in PBS. Seven days after the second sensitization, the animals were briefly anesthetized with halothane and challenged intranasally with either 10 μg of OVA in 50 μl of sterile saline or with saline alone. These OVA or saline exposures were performed twice a day for 4 consecutive days. Groups of animals were treated with the iNOS-specific inhibitor, N-3-aminomethyl-benzyl-acetamidine-dihydrochloride (1400W; 1.0 mg/kg in 300 μl of sterile saline). The inhibitor was administered intraperitoneally (i.p.) at 2 h before and 4 h and 12 h after OVA-challenge. Control groups received saline i.p. alone. Thus, for each time-point after challenge, our protocols resulted in four experimental groups: (1) control non-challenged; (2) control OVA-challenged; (3) 1400W non-challenged; (4) 1400W OVA-challenged. All groups were sensitized with OVA.

2.4. Cell collection and sample processing

To obtain the bronchoalveolar lavage (BAL), mice were anesthetized with halothane, peripheral blood was collected, and lungs were washed four times with 500 μl of saline. The samples were immediately centrifuged (20 °C, 300×*g*, 10 min); the pellet was resuspended in Eagle's minimum essential medium (MEM; pH 7.2) and the supernatant was collected and frozen at −80 °C. After the BAL procedure, lungs were removed from the mice, flash frozen in liquid nitrogen and stored at −80 °C. Femurs were isolated from the mice to obtain the bone marrow. The epiphyses were cut transversely and bone marrow cells were flushed out with PBS containing heparin (20 UI/ml). The red blood cells of peripheral blood and bone marrow were then lysed with lysis buffer (155 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA) for 15 min at 4 °C. The white blood cells were resuspended in Eagle's minimum essential medium (MEM), pH 7.2, with 0.01% bovine serum albumin. The total cell numbers in bone marrow, blood and BAL were determined using standard hematological procedures. Differential leukocyte counts were carried out on a minimum of 400 cells using cytospin preparation of the cell suspension, stained with Diff-Quick (Scientific Products). Both bone marrow and blood eosinophils were evaluated as a mixture of mature and immature forms, as recognized by the intensely eosinophilic granules. For bone marrow and BAL cell analysis, as well as cytokine measurements, the mice were killed at 24 h, 48 h, 72 h and 96 h after the first challenge. *In vitro* eosinophil adhesions and flow cytometric analyses were carried out at 24 h and 48 h after the first challenge.

2.5. Eosinophil cell adhesion assays

The assay was carried out employing a previously described method. In brief, bone marrow and peripheral blood cells (1.0×10^6 cells/ml) were pre-incubated with or without neutralizing antibodies to VLA-4 and Mac-1 (10 μg/ml) for 20 min at 4 °C. Subsequently, the cells were subjected to an adhesion assay in 96-well plates pre-coated with recombinant mouse VCAM-1 (2.5 μg/ml) or with recombinant mouse ICAM-1 (5.0 μg/ml) for 15 min at 37 °C, 5% CO_2. After incubation, non-adhered cells were removed and the remaining cells were washed twice with PBS. Fifty microliters of varying concentrations of the original cell suspension in minimum essential media (MEM) were added to empty wells to form a standard curve. Eosinophil adhesion was calculated by measuring residual eosinophil peroxidase activity of adherent cells. Fifty microliters of eosinophil peroxidase substrate (1 mM H_2O_2, 1 mM o-phenylenediamine and 0.1% Triton X-100 in Tris buffer pH 8.0) were added to each well. After 30 min of incubation at room temperature, 25 ml of 4M H_2SO_4 were added to each well to stop the reaction and absorbance was measured at 490 nM with a microplate reader (Versamax, Molecular Devices, Sunnyvale, CA, U.S.A.). Adherence was calculated by comparing absorbance of unknowns to that of the standard curve.

2.6. Measurement of cytokine concentrations in lungs

After accurately weighing, 50 mg of airway tissues was homogenized in 1.0 ml of buffer (10 mM potassium phosphate buffer, 0.1 mM EDTA, pH 7.4) containing protease inhibitor cocktails. These homogenates were centrifuged at 800×*g* for 10 min at 4 °C. The supernatant fraction was then removed and stored at −80 °C. IL-4, IL-5, IL-10, IL-13, eotaxin, TNF-α and IFN-γ levels in the lung tissue supernatant were determined using a commercial immunoassay kit obtained from R&D Systems (Minneapolis, USA). The minimum detectable levels of mouse IL-4, IL-5, IL-13, eotaxin were 2.0, 7.0, 1.5 and 3.0 pg/ml, respectively.

2.7. Flow cytometric analysis

Bone marrow and peripheral blood cells, collected at 24 h and 48 h after OVA-challenge, were stained with FITC-conjugated anti-CCR3,