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CD40 Expression by Peripheral Blood Eosinophils from Asthmatics*

CD40在支气管哮喘患者外周血嗜酸性粒细胞的表达

Liang Guorong Shi Huanzhong Liang Xiangdong Deng Jingmin Xie Zhengfu
梁国容 施焕中 梁象东 邓静敏 谢正福

(Department of Internal Medicine, First Affiliated Hospital, Guangxi Medical University, 6 Binhulu, Nanning, Guangxi, 530021, China)
(广西医科大学第一附属医院呼吸内科 南宁市滨湖路 6号 530021)

Abstract To identify the differences in CD40 expression on the eosinophils from patients with allergic asthma and healthy people, the purified eosinophils were isolated from 10 allergic asthmatics and 8 non-allergic normal subjects. Flow cytometry was used to detect the surface expression of CD40 on eosinophils and immunocytochemical techniques were used to determine the intracellular CD40 expression in eosinophils. Intracellular CD40 expression was observed in the eosinophils from both allergic asthmatics and normal donors. The eosinophils from normal donors lacked CD40 surface expression; in contrast, the eosinophils from allergic asthmatics were all positive for CD40 surface expression. It could be concluded that human eosinophils appear uniformly to contain CD40, but vary in the levels of CD40 expressed on the outer plasma membrane with expression enhanced on the eosinophils from allergic asthmatics.

Key words asthma, eosinophil, CD40

摘要 探讨过敏性哮喘患者和正常人外周血嗜酸性粒细胞 (EOS) 表达 CD40的差异。采集 10例过敏性哮喘患者和 8例正常对照者静脉血用于纯化 EOS, 以流式细胞义检测 CD40在 EOS表面的表达, EOS胞浆中的 CD40蛋白则以铭疫细胞化学方法显示。结果表明, 哮喘患者 EOS表面存在 CD40表达, 但 CD40并不表达于正常人 EOS的表面。无论是哮喘患者还是正常人, 其 EOS的胞浆均可检测到 CD40蛋白。人 EOS均能合成和储存 CD40, CD40分子可以移行到哮喘患者 EOS的表面而不能移行到正常人 EOS的表面。揭示 CD40在哮喘的发病机理中具有某种作用。

关键词 哮喘 嗜酸细胞 CD40
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CD40 is a 50-kDa glycoprotein that is a member of the tumor necrosis factor (TNF) receptor family, which also includes the TNF receptor, nerve growth factor receptor and Fas. CD40 was initially identified on the surface of B cells, where it binds to a surface-expressed ligand (CD40L) on the activated CD4 T lymphocytes and plays a critical role in B cells activation and isotype switching[1]. CD40 signaling can be initiated by cross-linking CD40 with anti-CD40 monoclonal antibody (mAb) or by interaction with CD40L, now referred to as gp39, which is expressed by CD4 T lymphocytes[2, 3]. The role of CD40-CD40L costimulation in mediating T cell responses *in vivo* was first shown in the studies of using CD40L-deficient ($CD40L^{-/-}$) mice. In these studies, T cell activation and production of cytokines such as interferon-γ were markedly impaired in response to protein antigens *in vivo*[4]. Ohkawara et al. in 1996 showed that the eosinophils from allergic subjects express human CD40 mRNA by both reverse transcriptase-PCR and Northern blot analysis. And the constitutive CD40 mRNA expression in eosinophils could be upregulated by being exposured to IgA immune complexes and downregulated by interleukin (IL) -10[5]. However, there has been a lack of direct evidence to reveal the difference of CD40 expression on the eosinophils from allergic asthmatics and normal healthy subjects. The primary objective of the present study was to determine whether CD40 expression on the eosinophils from patients with allergic asthma was upregulated when compared with healthy people.

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1 Materials and Methods

1.1 Subjects

Ten nonsmoking patients (6 males, 4 females; 18 to 54 years of age), who met the criteria for a diagnosis of asthma as defined by Chinese Medical Association[6], were enrolled in this study. All patients had mild atopic asthma, with baseline forced expiratory volume in one second (FEV_1) greater than 70% of predicted value, and intermitting use of inhaled β_2-agonists when necessary only. All patients had a provocative concentration of methacholine, which was less than < 8 g/L at a 20% fall in FEV_1. Each patient had one or more documented positive skin prick test responses to aeroallergens, but none of them was being received immunotherapy or corticosteroid therapy in a month before enrolled in this study. Eight nonsmoking non-allergic normal healthy subjects (4 males, 4 females; 20 to 47 years of age) served as controls. Each one had a negative history for lung, heart, liver, or kidney disease as well as a normal chest radiograph and pulmonary function tests. No acute respiratory illness had occurred in the preceding 6 weeks.

1.2 Eosinophil Isolation

80 ml peripheral blood was obtained from each subject, and mixed with 20 ml citrate-anticoagulate. Each 50 ml of citrate-anticoagulated peripheral blood was added with 10 ml 6% dextran (McGaw, Irvine, CA), and was allowed to sediment for 45 min at room temperature. The leukocyte-rich supernatant was overlaid onto an equal volume of Ficoll-Pagque (Pharmacia, Uppsala, Sweden), and centrifuged at 400 g for 20 min at room temperature. Granulocytes were recovered from the pellet and washed in Ca^{2+} /Mg^{2+} free Hanks' balance salt solution (HBSS). Residual erythrocytes were lysed with hypotonic saline. Eosinophils were purified by negative immunomagnetic selection, using a magnetically activated cell separator system (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) according to the method reported by Weller et al[7]. To eliminate mononuclear cells, the anti-CD3-, anti-CD14-, and anti-CD19-coated micromagnetic beads were added to the anti-16/granulocyte mixture. By negative selection, we obtained the highly purified eosinophils (> 99.5%) and depleted of neutrophils ($CD16^+$ cells) and any contaminating mononuclear cells ($CD3^+$ /$CD14^+$ /$CD19^+$ cells).

1.3 Flow cytometry

Freshly isolated eosinophils were resuspended at 4℃ in HBSS containing 1% BSA and 0.1% sodium azide at 2×10^5 cells per 90μl. 90μl of cell suspension was incubated with 10μl of anti-CD40 mAb (5C3) directly conjugated FITC or isotype-matched control Ab (5C3; PharMingen) at 4℃ in the dark for 30 min. Cells were washed in the same medium, and fixed in 0.5% paraformaldehyde. Flow cytometry was performed with a Becton Dickinson FACScan flow cytometer within 24 hours.

1.4 Immunocytochemistry

Human eosinophils isolated from asthmatics and normal controls were washed twice in Ca^{2+} /Mg^{2+} free HBSS, cytospun onto slides, and fixed in 3% formaldehyde for 10 min at room temperature. Fixed cells were permeated with 0.05% saponin/HBSS solution for 20 min, and then blocked with 10% normal goat serum. After had been washed, cytospin preparations were incubated for 1 h at room temperature with mouse anti-CD40 mAb (5C3; PharMingen) as primary Ab. Nonimmune mouse serum or purified mouse IgG_1, at the same concentration as the primary Ab, were used as controls. After three washes of 5 min in 0.05% saponin/HBSS, the preparations were incubated with biotin-conjugated goat-anti-mouse IgG (Vector Laboratories, Burlingame, CA) for 1 h. The immunoreactive CD40 eosinophils were identified following the manufacturer's instructions in the ABC Vectastain glucose-oxidase kit (Vector Laboratories). The glucose-oxidase immunostaining was visualized under light microscopy.

2 Results

2.1 CD40 expression on eosinophil surface

The freshly isolated eosinophils from 8 normal donors were found to be uniformly negative for CD40 (Fig. 1A). However, Using directly fluorescent anti-CD40 mAb 5C3, the highly purified and freshly isolated eosinophils from 10 allergic asthmatics were all stained positively for CD40 (Fig. 1B). The mean fluorescence intensity of CD40 expression in allergic asthmatics were 14.2± 3.1 (from 9.9 to 23.5). This is confirmed by simultaneous flow cytometric studies using two other FITC-labeled anti-CD40 mAbs (EA-5 and BE-1) and an appropriated isotype matched IgG_1 as a negative control and a lymphoblastic B cell line (Daudi) that constitutively expresses CD40 as a positive control. We also tried to induce CD40 expression on the eosinophil surface from normal nonatopic donors by incubating highly purified eosinophils with cytokines and performe flow cytometry at various time points. The following cytokine combinations failed to induce cell surface CD40 expression: granulocyte-macrophage colony stimulating factor (GM-CSF), IL-5, GM-CSF+ interferon-γ, GM-CSF+ IL-4, GM-CSF+ IL-5, GM-CSF+ IL-10, GM-CSF+ TNF-α and IL-4+ IL-5 (data not shown).

2.2 Intracellular CD40 protein expression in eosinophils

Intracellular CD40 positive staining was observed in the eosinophils from all asthmatics. Surprisingly, the eosinophils from all 8 normal donors who lacked CD40 surface eosinophil expression of CD40 contained CD40 detectable on the permeated eosinophils, although the positive CD40 staining in normal donors were usually weaker than that in allergic asthmatics under the same experimental conditions.

3 Discussion

Recently, Lei et al[8] used $CD40^{-/-}$ mice to investigate the involvement of the CD40–CD40L interaction in a murine model of allergic airways inflammation. They found that $CD40^{-/-}$ mice and littermates developed a bone marrow eosinophile response which consisted with the increased levels of IL-5 in both the serum and bronchoalveolar lavage fluid. However, the inflammatory response of $CD40^{-/-}$ mice in both the airways and the lungs was markedly abrogated. In contrast to control littermates, $CD40^{-/-}$ mice had undetectable levels of ovalbumin-specific IgE and IgG_1 in the serum, and negligible levels of IL-4 and TNF-α. Local replacement of both TNF-α and IL-4 by means of an adenoviral-mediated gene transfer approach resulted in full reconstitution of the inflammatory response in the airways. These findings demonstrate the importance of CD40–CD40L interactions in the development of antigen-induced immune-inflammatory response in the airways. In the present study, we have showed that intracellular CD40 expression was observed in the eosinophils from both allergic asthmatics and normal donors. We also found that allergic asthmatics had much stronger intracellular positive staining signal for CD40 than normal subjects did. On the other hand, the eosinophils from normal donors lacked CD40 surface expression; in contrast, the eosinophils from allergic asthmatics were all positive for CD40 surface expression. It thus could be concluded in our data that human eosinophils appear uniformly to contain CD40, but vary in the levels of CD40 expressed on the outer plasma membrane with expression enhanced on the eosinophils from allergic asthmatics. We believed that understanding the regulation of eosinophil CD40 expression, especially how intracellular CD40 is stored and mobilized to the cell surface as well as what stimuli are active in eliciting both the formation of CD40 and its translocation on the cell surface, will make a contribution to further elucidation of the pathogenesis of bronchial asthma.

Our data suggested that CD40 might be involved in the pathogenesis of bronchial asthma in human, although the exact role of CD40 expression on eosinophils is still unknown. Allergen-induced recruitment of eosinophils into the airways is correlated with roles of $CD4^+$ T lymphocytes, presumably Th_2 cells, and cytokines released by such T cells[9, 10]. In humans, IL-5 not only induces eosinophil infiltration into the asthmatic airways, but also activates the infiltrating eosinophils, and thus promotes airway hyperreactivity[11, 12]. The accumulation of eosinophils in asthmatic airways correlates with measures of local T cell activation. For instance, increases in activated T lymphocytes, eosinophils, and cytokines mRNA for IL-5 and GM-CSF have been documented in bronchial biopsies after allergen inhalation challenge in allergic asthmatics[13]. Thus, there has been an increasing recognition that eosinophil accumulation and enhanced effector functions in the asthmatic airways may be intimately related to lymphocyte activation, especially by nominally Th_2-like cytokines such as IL-5 and GM-CSF.

There should exist collaborative interactions between lymphocytes and eosinophils in respiratory tract tissue environments. If eosinophils function to help regulating lymphocytes responses to aeroallergens encountered in the airways, such functions may be deleterious in contribution to sustaining or propagating allergic reactions within the airways. Our previous *in vitro* experiments have shown that eosinophils are able to present antigen to T cells and thus induce T cells to proliferate, and that CD80 and CD86 molecules, which are expressed on the surface of eosinophils, provide costimulatory signals to antigen presentation[14, 15]. CD40 has been reported to be a costimulatory molecule for eosinophils to present antigen to T cells, since T cell proliferation was inhibited by anti-CD40 mAb when T cells were cocultured with eosinophils in the presence of antigen[16]. We speculated that CD40 on eosinophils might be responsible for providing costimulation to the activated T cells in asthmatic airways.

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较明显,可作为种间类群鉴别的依据,但同类型的花粉外壁纹饰之间不同种的差别不太明显,如广东蜘蛛抱蛋、乐山蜘蛛抱蛋。由于取材、制片处理及电镜扫描拍照角度不同都对纹饰突起的形状、大小及分布密度等有一定的影响,因此,同类型不同种的花粉纹饰之间的细微差别是否是物种本身的反映,能否成为种的鉴别依据,有待进一步研究。

铃兰族内花粉外壁纹饰演化主要通过两条途径,一方面通过小穿孔扩大演化出大穿孔纹饰,大穿孔进一步扩大,产生网状纹饰;另一方面通过穿孔退化,演化出皱波状纹饰和各式瘤状纹饰[3]。Walker[7](1974)对一些原始科属的研究表明花粉表面纹饰的演化趋势为:表面光滑→表面网状、条纹状→表面疣状、刺状。根据铃兰族外类群对比及花粉演化的规律,本文认为这10种蜘蛛抱蛋的外壁纹饰演化趋势为:皱波状纹饰→小芽孢状纹饰,即由表面具孔的、突起较少的皱波状纹饰演变为表面无孔的、突起较多的小芽状纹饰。

10种蜘蛛抱蛋植物的花粉外壁纹饰与其花部式样的变化有一定的相关性。花粉外壁纹饰原始的种,如棒蕊蜘蛛抱蛋,它的花被钟状,裂片内侧无隆起,雄蕊着生位置高于柱头,柱头较小,为棒状,不明显膨大,表面结构及纹饰简单,这些花部式样在蜘蛛抱蛋属中属原始性状;花粉外壁纹饰进化的种,如罗甸蜘蛛抱蛋,它的花被坛状,花被裂片内弯,裂片内侧有隆起,雄蕊着生位置低于柱头,柱头较大,其膨大明显,表面结构及纹饰复杂、特化。这些花部式样在蜘蛛抱蛋属中属较进化的性状。以上表明,蜘蛛抱蛋属的花粉外壁纹饰演化趋势与其花部式样演化趋势是一致的。

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