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IL-4 alters expression patterns of storage components of vascular endothelial cell-specific granules through STAT6- and SOCS-1-dependent mechanisms

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ABSTRACT

IL-4 develops Th2-biased immunity or allergic inflammation through activation of STAT6-dependent signaling. In vascular endothelial cells (ECs), IL-4 elicits regulatory effects on chemokine production and adhesion molecule expression to recruit T cells and eosinophils. In this study, we examined how IL-4 affects Weibel–Palade bodies (WPBs), EC-specific storage granules capable to store multiple protein components, including von Willebrand factor (vWF), P-selectin, eotaxin-3, IL-8 and angiopoietin-2 (Ang-2). Among 11 WPB component genes that we examined, IL-4 potently upregulated the expression levels of P-selectin and eotaxin-3, whereas it downregulated the expression levels of IL-8 and Ang-2. Both regulatory effects were dependent on STAT6. In addition, the IL-4-induced downregulatory effect on WPB component genes depended on the negative feedback regulation by SOCS-1 induced by STAT6 signaling. Furthermore, IL-4-regulated gene expression through STAT6 and SOCS-1 was consistent with WPB compositional changes in cultivated ECs and capillary-like tube networks. Since WPBs enable ECs to rapidly regulate multiple critical functions of vasculatures, IL-4-induced alteration of expression patterns of WPB storage components may convert the physiological functions of WPBs into Th2-biased immune functions or allergic functions.

1. Introduction

The pleiotropic cytokine IL-4 is produced by Th2 cells, eosinophils, basophils and mast cells and is associated with immune protection against helminthic parasites. Dysregulation of IL-4 expression often leads to the development of allergic reactions and chronic inflammatory diseases (Lee and Hirani, 2006; Li-Weber and Krammer, 2003; Tepper et al., 1990; Wills-Karp, 1999). IL-4 exerts its activities via binding to cell surface IL-4 receptor complexes, IL-4Rα with the common γ chain (type I IL-4 receptor) or with IL-13Rα1 (type II IL-4 receptor) (Chatila, 2004; Nelms et al., 1999). IL-4 ligation triggers activation of tyrosine kinases JAK1 and JAK3, which phosphorylate tyrosine residues in the cytoplasmic domain of IL-4Rα and common γ chain, respectively. This event then activates signal transducer and activator of transcription 6 (STAT6), resulting in its homodimerization and subsequent translocation into the nucleus, where it regulates gene transcription (Chatila, 2004; Takeda et al., 1996). So far, more than 35 different STAT6 target genes have been identified, and it has been shown that many of them are involved in Th2-associated immune processes (Hebenstreit et al., 2006).

In vascular endothelial cells (ECs), IL-4 regulates expression of adhesion molecules, with an upregulatory effect on VCAM-1 and a downregulatory effect on E-selectin, and regulates production of CC chemokines, including MCP-1 and eotaxins (Lampinen et al., 2004; Lukacs, 2001; Øynebråten et al., 2004; Schleimer et al., 1992; Shinkai et al., 1999; Yao et al., 1996). This shift in the balance of expression of adhesion molecules and chemokine production by IL-4 is thought to favor the recruitment of T cells and eosinophils, rather than neutrophils and basophils, from the bloodstream to the surface of the endothelium, leading to their infiltration into a site of allergic inflammation (Schleimer et al., 1992; Shinkai et al., 1999; Thornhill et al., 1990). In addition, IL-4-induced selective recruitment of such cells is partly mediated by STAT6-dependent expression of P-selectin/CD62P and eotaxin-3/CCL26 in ECs (Lampinen et al., 2004). However, it is not fully understood how IL-4 alters normal functions of ECs into Th2-biased or allergic functions.

Abbreviations: Ang-2, angiopoietin-2; EC, endothelial cell; LAMP-3, lysosomal-associated membrane protein 3; OPG, osteoprotegerin; qRT-PCR, quantitative real-time reverse transcription-polymerase chain reaction; SOCS, suppressor of cytokine signaling; STAT6, signal transducer and activator of transcription 6; t-PA, tissue plasminogen activator; vWF, von Willebrand factor; WPB, Weibel–Palade body.

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ECs have specific storage granules, designated Weibel–Palade bodies (WPBs) (Weibel and Palade, 1964). WPBs were originally identified as an intracellular storage vehicle for von Willebrand factor (vWF), a plasma protein that mediates platelet adhesion, but an increasing number of other components, including P-selectin, eotaxin-3, IL-8/CXCL8, angiopoietin-2 (Ang-2), lysosomal-associated membrane protein 3 (LAMP-3)/CD63, endothelin-1, tissue plasminogen activator (t-PA) and osteoprotegerin (OPG), has been revealed to be present within WPBs (Metcalf et al., 2008; Rondaij et al., 2006). The features of WPB components indicates a crucial role for degranulation of WPBs, which provides the endothelium with the ability to rapidly respond to changes in the micro-environment and to regulate multiple biological functions of vasculatures, such as inflammation, hemostasis, vascular tone regulation and angiogenesis. Regulated degranulation of WPBs can be initiated through an increase in intracellular calcium or cyclic adenosine monophosphate level following stimulation of ECs with thrombin, histamine, TNF, bacterial products or extracellular adenosine triphosphate (Inomata et al., 2007; Into et al., 2007; Metcalf et al., 2008; Rondaij et al., 2006).

Several studies have demonstrated that inflammatory cytokines, such as TNF-α and IL-1β, induce proinflammatory IL-8 production in ECs, enabling storage of IL-8 within WPBs as "memory" of past EC reactions (Utgaard et al., 1998; Wolff et al., 1998). Such a change in WPB components may lead to alteration of normal physiological features of WPBs into proinflammatory features. We therefore hypothesized that IL-4 alters the normal expression pattern of WPB components into patterns associated with Th2-biased immunity or allergic diseases. In this study, we first examined whether IL-4 alters expression levels of WPB component genes in ECs. We here report that IL-4 regulates expression levels of several WPB component genes through a STAT6-dependent mechanism. We also examined whether such changes in gene expression are indeed consistent with WPB compositional changes.

2. Materials and methods

2.1. Reagents, plasmids and cell culture

Human rIL-4 was obtained from R&D Systems. Histamine was obtained from Sigma–Aldrich. An expression plasmid encoding myc-SOCS-1 was a kind gift from Akihiko Yoshimura (Division of Molecular and Cellular Immunology, Medical Institute of Bioregulation, Kyushu University). Human umbilical vein endothelial cells (HUVECs) were purchased from Cambrex (at least 3 different lots isolated from different donors), grown in EBM-2 complete endothelial growth medium (Cambrex) under 5% CO_2 at 37 °C and used for experiments from passages 4 to 8. HUVECs were stimulated with IL-4 after reaching confluence. IL-4 did not influence cell survival and proliferation in the confluent HUVEC culture (data not shown).

2.2. Gene expression analysis by quantitative real-time reverse transcription-polymerase chain reaction

Total RNA was prepared from HUVECs using an RNeasy extraction kit (Qiagen). One μg of total RNA was reverse-transcribed using ReverTraAce reverse transcriptase (TOYOBO) with both an oligo21dT primer and a random 6 primer set. qRT-PCR was performed using Power SYBR Green PCR Master Mix (Applied Biosystems) on a 7300 Fast Real-Time PCR System (Applied Biosystems) according to the manufacturer's instructions. All primer sequences are listed in Supporting Table 1. We confirmed that there was no critical difference between the values normalized to the levels of three different house-keeping genes, *Gapdh*, *Ppia1* and *Hprt1*. Results shown in this study were normalized to the level of *Gapdh*.

2.3. DNA transfection and RNA interference

DNA transfection in HUVECs was performed using Opti-MEM I (Invitrogen) and Lipofectin reagent (Invitrogen) as described previously (Into et al., 2007). 'ON-TARGET *plus*' siRNAs for human *Stat6* (006690) and *Socs1* (011511) and a non-targeting control RNA (D-001810-01) were purchased from Dharmacon. siRNA sequences were provided by the manufacturer. Transfection of siRNA in HUVECs was performed as described previously (Inomata et al., 2007). After 24 h of incubation, the transfection media were changed and cells were used for experiments.

2.4. Capillary-like tube formation

For formation of capillary-like tube networks in vitro, HUVECs were harvested and resuspended in 200 μl EBM-2 and then seeded at a density of 1×10^5 cells in 24-well plates onto the surface of 150 μl of polymerized Matrigel (Becton Dickinson). After 12 h of incubation at 37 °C, Matrigel containing EC tubes was pervaded with EBM-2 containing 10 ng/ml IL-4 and then incubated for an additional 48 h. IL-4 did not affect the number of capillary-like tubules when experiments were performed after tubule formation (data not shown). To fix the tubes in gels, incubation media were carefully removed and 1 ml of methanol was added to the culture, followed by incubation at room temperature for 30 min.

2.5. Immunofluorescent staining of WPBs

Immunofluorescent staining of WPBs was performed by reference to our protocols described previously (Into et al., 2008; Into et al., 2007). Briefly, HUVECs were fixed at −20 °C with methanol for 30 min. Then cells or capillary-like cells in Matrigel were treated with an anti-vWF rabbit polyclonal antibody (sc-14014, Santa Cruz Biotechnology) and an anti-P-selectin mouse monoclonal antibody (sc-19672, Santa Cruz Biotechnology) at room temperature for 1 h. The former primary antibody was detected with Alexa Fluor 488-conjugated anti-mouse IgG antibody (Invitrogen) and the latter antibody was detected with Alexa Fluor 568-conjugated anti-rabbit IgG antibody (Invitrogen). To avoid background staining of Matrigel by antibodies, potassium chloride at a final concentration of 200 mM was added to the antibody solution while staining. The detection of Ang-2 was performed using an anti-Ang-2 goat polyclonal antibody (AF623, R&D Systems) and Alexa Fluor 568-conjugated anti-goat IgG antibody (Invitrogen). Images were obtained by a fluorescent microscope IX71 with DP70 image capture (Olympus) in the presence of Prolong Gold antifade reagent (Invitrogen) and then processed using Adobe Photoshop, version 7.0.

2.6. Immunoblotting

Confluent HUVEC seeded on 60 mm plates were stimulated or unstimulated with IL-4 for 48 h. The cytoplasmic cell lysates were obtained by incubating cells with a buffer consisting of 20 mM Tris–hydrochloride (pH 7.2), 150 mM sodium chloride, 5 mM EDTA and 1% Triton X-100 and protease inhibitor cocktails (Roche) at 4 °C for 15 min, followed by clarification by centrifugation at $12{,}000 \times g$ for 10 min. These cell lysates were diluted by an equal volume of SDS sample buffer consisting of 0.5 M Tris–hydrochloride (pH 7.2), 10% glycerol, 2% SDS, 5% 2-mercaptoethanol and 0.05% bromphenol blue. Samples were boiled for 5 min and separated under reducing conditions on 12% SDS–PAGE gels and then transferred onto polyvinylidene fluoride membranes. Membranes were blocked at room temperature for 1 h with 5% non-fat skim milk solved in PBS and then reacted with primary antibodies to IL-8 (sc-7922), eotaxin-3 (sc-19353), Ang-2 (sc-20718) and GAPDH (sc-25778) (all