IL-13 Attenuates Vascular Tube Formation Via JAK2-STAT6 Pathway

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Background  Interleukin (IL)-13, which is a cytokine produced by type 2 helper T cells, has pathophysiological roles in allergic inflammation and fibrosis formation. IL-13 shares many functional properties with IL-4, which is known to inhibit angiogenesis.

Methods and Results  The effects of IL-13 on angiogenesis were examined using human coronary artery endothelial cells (HCAECs), in addition to investigating the mechanism(s) of this action. Using an in vitro assay of angiogenesis it was demonstrated that IL-13, as well as IL-4, significantly inhibited capillary-like tube formation. Migration of HCAECs, considered to be a process of new capillary tube formation, was also significantly inhibited by IL-13. IL-13 activated signal transduction and transcription 6 (STAT6) as a result of the activation of Janus kinase 2 (JAK2). The inhibitory effect of IL-13 on angiogenesis was abolished by depletion of JAK2 and STAT6 by RNA interference.

Conclusion  IL-13 has anti-angiogenic activity as a result of activation of JAK2 and subsequent activation of STAT6. (Circ J 2008; 72: 469–475)

Key Words: Angiogenesis; Endothelial cell; Interleukin-13; JAK2; STAT6

The T helper 2 (Th2) type cytokine interleukin (IL)-13 has been shown to play an important role in allergic asthma, inflammatory bowel disease and fibrosis. IL-4–/– mice have an impaired Th2 response, but not completely, whereas IL-13–/– mice, and IL-13–/–IL4–/– mice have a completely impaired Th2 response, suggesting that IL-13 is required for the full Th2 response. Both IL-13–/– and IL-4–/– mice have compromised allergic reactions and protection against helminthes because of impaired Th2 reactions.

IL-13 exerts its actions by binding to the IL-13 receptor (IL-13Rα1 and IL-13 receptor (IL-13Rα2) as a signaling-competent IL-13R, together with the IL-4-receptor [chain (IL-4Rα)]. IL-13Rα1 forms a signaling-competent IL-13R, together with the IL-4 receptor β–chain (IL-4Rβ). IL-13Rα2 has no intracellular domain and can bind to IL-13 without mediating signal transduction, suggesting that it works as a decoy receptor.

Binding of IL-13 to IL-13Rα1, as well as to IL-4Rα, triggers phosphorylation of the Janus kinases (JAKs), leading to activation of signal transduction and transcription 6 (STAT6). STAT6 regulates inflammatory injury, with STAT6–/– mice shown to be unable to mount Th2 responses as with IL-4–/–IL-13–/– mice. In addition, the STAT6 signaling pathway plays a critical role in ischemia-induced apoptosis in cardiomyocytes and also accelerates cardiac dysfunction during ischemia and reperfusion. In endothelial cells (ECs), STAT6 activation induced by IL-4 stimulation upregulates expression of P-selectin, an action which is likely to contribute to a number of chronic inflammatory diseases, including atherosclerosis. However, little is known concerning the signal transduction and biological activities of IL-13 in ECs, despite the receptors for IL-13 having been identified.

The process of angiogenesis consists of several steps, including migration of ECs and the formation of new capillary tubes. Dysregulation of angiogenesis occurs in pathophysiological conditions such as ischemic heart disease, cancer, diabetes and chronic inflammation, including atherosclerosis. It has been demonstrated that IL-4 has the potential to inhibit angiogenesis by reducing EC proliferation and migration; however, the effect of IL-13 on angiogenesis and the mechanism of its action remain unclear, despite IL-4 and IL-13 sharing many biological activities. In this study, we investigated the effects of IL-13 on coronary artery angiogenesis using human coronary artery ECs (HCAECs). We also assessed the relationship between the biological activity of IL-13 and its signaling pathway.

Methods

Cell Culture and Stimulation
HCAECs (Cambrex Bioscience, Walkersville, MD, USA) were cultured in DMEM F-12 medium containing 5% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin and appropriate growth hormones, according to the manufacturer’s instructions. The cells were stimulated with recombinant human IL-13 or IL-4 (Peprotech, London, UK). In order to neutralize the effects of the cytokines, the ECs were pre-incubated for 3 h prior to stimulation with 30 μg/ml of either anti-IL-13 or anti-IL-4 subunit blocking antibodies (R&D Systems, Inc, Minneapolis, MN, USA).

Transfection of siRNA
In order to deprive the cells of JAK2 and STAT6, the siRNAs for JAK2 (ID#607) and STAT6 (ID#4501, Ambion,
Austin, TX, USA) were used. The cells were seeded when subconfluent and then transfected with 20 pmol/l of the siRNAs with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) in OPTI-MEM (Invitrogen), according to the manufacturer’s instruction.

**Immunoblotting Analysis**

Cells (1x10^6 cells) were lysed by a RIPA buffer (10 mmol/L Tris, 1% NP-40, 0.1% deoxycholate, 0.1% SDS, 0.15 mol/L NaCl, 1 mmol/L EDTA, 10 mmol/L NaF, 0.1 mol/L Na3VO4, pH 7.4) and then centrifuged at 15,000 g for 15 min at 4°C. The supernatants were collected and the protein concentrations determined by the Bio-Rad protein assay (Hercules, CA, USA). An aliquot of the cell lysate was mixed with 2 volumes of Laemmli’s sample buffer and boiled at 100°C for 5 min. Equal amounts of the proteins in each sample were resolved by SDS-PAGE gels and transferred onto PVDF membranes. The blots were then probed with antibodies against JAK1, JAK2 and STAT6, TYK2 and tyrosine-phosphorylated-JAK1, JAK2, TYK2 and STAT6 (Cell Signaling Technology, Danvers, MA, USA), followed by rabbit anti-mouse immunoglobulin conjugating horseradish peroxidase (GE Healthcare Life Sciences, Uppsala, Sweden). Kodak BioMax film (Rochester, NY, USA) was exposed to the membrane, which had been treated using the ECL Western Blotting Detection System (GE Healthcare Bio Sciences), and developed. The developed bands were quantified using software (ImageJ; PC version of NIH image software, downloaded from http://rsb.info.nih.gov/ij/download.html).
Scrape Wound-Induced Migration Assay
Confluent cells were wounded by a razor, rinsed and then incubated in medium for 24 h.23 Phase-contrast photographs of the migrated cells were taken using an AXIOVERT 40CFL microscope (Zeiss, Oberkochen, Germany), and counted using NIH imaging software.

In Vitro Angiogenesis Assay
In vitro angiogenesis was assessed in 3-dimensional fibrin matrices using an angiogenesis assay kit (Chemicon, Temecula, CA, USA), as described previously.24 Diluted ECMatrix™ solution was transferred to each well of a 96-well tissue culture plate and incubated at 37°C for 1 h to allow the matrix solution to solidify. The HCAECs were harvested and resuspended in appropriate media and then seeded at a density of 1×10^4 cells per well in 24-well plates onto the surface of the polymerized ECMatrix™, followed by incubation at 37°C for 8 h. Tube formation was observed with an inverted light microscope at magnifications between ×40 and ×200. The images of tube length were converted to black-scale and subjected to image processing using NIH imaging software in order to calculate total tube length. At least 8 wells were analyzed for each sample.

Statistical Analysis
The values are presented as mean±SEM. Differences between single pairs of treatments were tested by Student’s t-test (2-tailed) and differences between groups by 1-way analysis of variance with Fisher’s test for pair-wise multiple comparisons. A p-value <0.05 was considered statistically significant.

Results
IL-13 and IL-4 Impair Angiogenesis Through IL-13Rα1 and IL-4Rα in HCAECs
We first performed an in vitro angiogenesis assay to assess the effects of IL-13 and IL-4 on new capillary-like tube formation (Fig 1). HCAECs were harvested and resuspended in medium with 10ng/ml IL-13 or IL-4 or control medium that contained endothelial growth factors, and then seeded onto the extracellular matrix proteins. After 8 h, tube structures were detected when HCAECs were incubated in control media, but tube formation was completely blocked by IL-13 and IL-4. The inhibitory effect of each cytokine was abolished by anti-IL-13Rα1 and anti-IL-4Rα antibody, respectively. An inhibitory effect on tube structure was more evident with IL-13 than with IL-4.

IL-13 and IL-4 Inhibit HCAEC Migration Via JAK2-STAT6 Pathway Through IL-13Rα1 and IL-4Rα
We assessed whether IL-13 and IL-4 affected cell mobility using a scrape wound-induced migration assay because proliferation of ECs does not start until 24 h after scraping.
so the scrape wound-induced migration assay reflects the migration process of ECs exclusively. In the presence of 10 ng/ml IL-13 or IL-4, the migration of HCAECs was inhibited significantly compared with controls (Fig 2). However, pretreatment with anti-IL-13R\(\alpha\)1 and anti-IL-4R\(\alpha\) antibodies significantly reversed the decrease in migration caused by IL-13 and IL-4, respectively (Fig 2).

**IL-13 Induces Phosphorylation of STAT6 Via Activation of JAK2 in HCAECs**

We investigated the signaling pathway in response to stimulation with IL-13. As shown in Figs 3A,B, treatment of HCAECs with 10 ng/ml of IL-13 caused tyrosine phosphorylation of JAK2 within 5 min, which peaked at 15 min and then declined to baseline by 30 min. In contrast, IL-13-induced phosphorylation of STAT6 peaked at 15 min and then gradually declined. No phosphorylation of JAK1 and TYK2 in response to IL-13 was observed at the time points examined. This finding was confirmed by immunoblotting with anti-phospho-JAK1 and anti-phospho-TYK2 (Figs 3A,B). Immunoblotting by an anti-phosphotyrosine antibody with immunoprecipitates of HCAEC lysate by anti-JAK1 and TYK2 showed consistent results (data not shown).

When HCAECs were pretreated with an anti-IL-13R\(\alpha\)1 antibody and then stimulated with IL-13, tyrosine-phosphorylation of both JAK2 and STAT6 was blocked by anti-IL-13R\(\alpha\)1 (Figs 3C,D) and anti-IL-4R\(\alpha\) antibodies (data not shown).

**Depletion of JAK2 and STAT6 Impairs Inhibitory Effects of IL-13 on Angiogenesis**

To more directly examine the roles of JAK2 and STAT6, we depleted each molecule using specific siRNAs for each signaling molecule. Treatment with siRNA successfully depleted JAK2 and STAT6 proteins by more than 75% compared with the level of these proteins in untreated cells (Fig 4A). This finding was confirmed by immunoblotting. Treatment with JAK2 siRNA did not affect the level of STAT6 protein (Fig 4A Left, Bottom). Tyrosine phosphorylation of STAT6 in response to IL-13 was clearly blocked by depletion of JAK2, suggesting that JAK2 phosphorylation leads to STAT6 phosphorylation in HCAECs.

We next investigated the contribution of the JAK2/STAT6 pathway to capillary-like tube formation in response to IL-13, using JAK2- and STAT6-depleted HCAECs (Figs 4B,C). IL-13-induced suppression of tube formation was reversed completely by treatment with JAK2 or STAT6 (Figs 4B,C). Furthermore, depletion of JAK2 and STAT6 with their siRNAs reversed cell migration significantly, with these effects being attenuated by IL-13 (Fig 5).

**Discussion**

The major finding of this study is that the Th2 cytokine IL-13 is a potent inhibitor of coronary artery tube formation in vitro, similar to the action of IL-4. Reports on the effects of IL-4 and IL-13 on angiogenesis have been con-
Our results support those of Volpert et al, who showed that IL-4 and IL-13 inhibited angiogenesis in rat cornea and in human and bovine microvascular ECs. We observed enhancement of vascular tube formation at a low concentration (ie, 1 ng/ml) of IL-13 and IL-4 (data not shown), similar to their observations, but recently, it was reported that introduction of the IL-4 gene suppressed angiogenesis significantly in adjuvant-induced arthritis in rats. The discrepancy in results might be attributed to experimental conditions. We incubated HCAECs in a medium containing endothelial mitogens so as to augment tube formation. Fukushi et al did not seem to add growth factors, whereas Volpert et al and we examined the effects of IL-4 and IL-13 under the presence of endothelial mitogens. Therefore, the presence of endothelial mitogens (ie, growth factors) appears to be the greatest difference between the experimental conditions. Taken together, our results suggest that IL-13 and IL-4 inhibit tube formation and the migration of ECs, because they antagonize the effects of growth factors. In addition, we have already demonstrated that IL-13 and IL-4 suppress growth and induce apoptosis in HCAECs. IL-13 and IL-4 also suppress expression of vascular endothelial growth factor, an important growth factor for ECs. Because angiogenesis consists of several steps (ie, proliferation, migration, the formation of new capillary tubes and maintenance of their survival), we consider that growth suppression and the induction of apoptosis is the mechanism by which IL-13 and IL-4 inhibit vascular tube formation.

We also investigated the role of the signaling pathway mediated by the IL-13 receptors on inhibition of tube formation in HCAECs. In HUVECs, JAK2 is activated by tyrosine-phosphorylation following stimulation by IL-4 and IL-133 rather than by JAK1 and JAK3, which are commonly activated in hematopoietic cells. We demonstrated that IL-13 induced tyrosine phosphorylation of JAK2, whereas it had no effect on either JAK1 or TYK2 through IL-13R. Because angiogenesis consists of several steps (ie, proliferation, migration, the formation of new capillary tubes and maintenance of their survival), we consider that growth suppression and the induction of apoptosis is the mechanism by which IL-13 and IL-4 inhibit vascular tube formation.

In this study, we used a scrape wound-induced migration assay to evaluate cell migration, because it is used frequently to study the effects of physical injury involved in vascular repair and maintenance of stringently controlled monolayers. The scrape wound-induced migration assay has several advantages for the assessment of whether the many compounds that modulate the repair process have effects on cell mobility and motility. As proliferation of ECs does not start until 24 h after scraping, the scrape wound-induced migration assay reflects the migration process of ECs exclusively. Therefore, IL-13 presumably suppressed wound healing as it decreased mobility and/or motility, thereby reducing directional migration into open space. The finding that IL-13-mediated suppression of EC mobilization was abrogated by the siRNAs for JAK2 and STAT6 strongly suggests that STAT6 activation following JAK2 activation is actively involved in the migration process of ECs. Our study is the first to demonstrate a relationship between STAT6 activation and EC motility and we are currently carrying out an investigation on how STAT6 affects cell mobility in ECs, which includes determining whether transcriptional activation is involved in this process.

Depletion of JAK2 and STAT6 resulted in abrogation of inhibition of tube formation by IL-13, which suggests that activation of STAT6 mediates suppression of angiogenesis by IL-13. Our experimental system for assaying angiogenesis, ECMatrix™ (Chemicon), contains laminin, type 4 collagen, the heparin sulfate proteoglycans, entactin and nidogen as components of extracellular matrix, basic FGF and TGF-β as angiogenic factors and proteolytic enzymes such as metalloproteases, tissue-type plasminogen activator and plasminogen that enable ECs to readily form vascular tubes. Recently, several inhibitors of these proangiogenic factors have been reported. Tumstatin and angiostatin, which are fragments of collagen type IV and plasminogen and thrombostatin-1 have been shown to inhibit angiogenesis and EC migration. Tissue inhibitors of metalloproteases (TIMPs) are internal inhibitors of the protease activity of MMPs. TIMP2 is capable of inhibiting angiogenesis independent of blocking MMP activity. We pre-
sume that IL-13 stimulation and subsequent activation of STAT6 inhibits angiogenesis by production of these inhibitors, although the precise mechanisms remain to be elucidated.

This study is the first to show that both IL-13 and IL-4 have potent inhibitory effects on angiogenesis in ECs derived from coronary arteries as a result of STAT6 activation. We have found that the plasma IL-13 level is increased significantly in patients with ischemic heart failure, suggesting that IL-13 is directly involved in exacerbation of ischemic heart diseases. Furthermore, there is evidence that STAT6 signaling plays an important role in cardiac dysfunction during ischemia and reperfusion. Taken together, these findings suggest that locally produced IL-13 has a pivotal role in ischemic heart diseases. On the basis of our current results we consider that inhibition of angiogenesis by IL-4 and IL-13 may be also involved. Therefore, inhibition of either IL-13 production or STAT6 activation may be beneficial for healing ischemic tissues after myocardial infarction. Examination of IL-13 and IL-4 levels in blood, and of JAK2 and STAT6 activation in the coronary artery, using an animal model such as the acute myocardial infarction or hindlimb ischemia model will provide confirmatory information on this in vitro result.

In conclusion, this study demonstrated that IL-13 inhibits coronary artery angiogenesis by activating JAK2 and STAT6. Suppression of IL-13 production and the JAK2–STAT6 pathway may therefore have significant therapeutic implications in ischemic heart disease.

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