Original Article

STAT6 Mediates Apoptosis of Human Coronary Arterial Endothelial Cells by Interleukin-13

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Interleukin (IL)-13 is a cytokine produced by type 2 helper T cells that has pathophysiological roles in allergic inflammation and fibrosis formation. IL-13 shares many functional properties with IL-4, which promotes apoptosis of endothelial cells (ECs). We here investigated the effects of IL-13 on apoptosis using human coronary artery endothelial cells (HCAECs). Assessment by WST-1 assay demonstrated that IL-13 as well as IL-4 significantly inhibited cell growth. IL-13 significantly attenuated the cell viability and induced apoptosis of HCAECs as well. Expression of mRNA for vascular endothelial cell growth factor, which maintains survival of ECs, was significantly diminished by IL-13. The effects of IL-13 and IL-4 were abolished by depletion of STAT6 using RNA interference. These results suggest that IL-13 attenuates EC viability by inducing apoptosis, and that STAT6 plays pivotal roles on IL-13– and IL-4–induced apoptosis in ECs. (*Hypertens Res* 2008; 31: 535–541)

***Key Words*: apoptosis, cell growth, endothelial cell, interleukin-13, STAT6**

Introduction

T helper 2 type cytokine interleukin (IL)-13 is a pleiotropic immunomodulatory cytokine sharing many functional properties with IL-4 (*1*). The mechanism of its actions is explained by a receptor subunit consisting of IL-4Rα and IL-13Rα1, and a common signaling pathway through phosphorylation of Janus kinases (JAKs) and STAT6 (signal transducer and activator of transcription 6) (*1*, *2*). STAT6$^{-/-}$ mice are deficient in their ability to mount a Th2 response (*3*, *4*). Binding of IL-13 and IL-4 to the α chains of the receptors of IL-13 and IL-4, respectively, triggers phosphorylation of JAKs, leading to activation of STAT6 in human umbilical vein endothelial cells (ECs) (*2*, *5*).

It has been demonstrated that IL-4 stimulation inhibits cell growth and induces apoptosis in human cancer cells (*6–9*) and normal hepatocytes (*10*) by STAT6 activation (*6*, *10*) and by augmentation of caspase 3, 8 and 9 activity (*10*). In ECs, IL-4 inhibits proliferation (*11–13*) and induces apoptosis by activating caspase 3 (*12*). In addition, the JAK/STAT signaling pathway has been shown to play a critical role in ischemia-induced apoptosis in cardiomyocytes (*14*). However, little is known about the effects of IL-13 on proliferation and apoptosis in ECs.

We have recently observed that plasma IL-13 levels were increased in patients with ischemic heart disease (*15*). IL-13 has been reported to induce atherosclerosis in apolipoprotein E–deficient (apoE$^{-/-}$) mice due to EC damage (*16*), suggesting that IL-13 is directly involved in the exacerbation of ischemic heart diseases. In this study, to explore how the increased IL-13 affects the pathogenesis of ischemic heart diseases, we investigated the effects of IL-13 and IL-4 on the cell viability, cell growth and apoptosis of human coronary artery endothelial cells (HCAECs). We also demonstrated the roles of STAT6 activation on IL-13- and IL-4–induced EC apoptosis and suppression of vascular endothelial growth factor (VEGF) mRNA expression in HCAECs.

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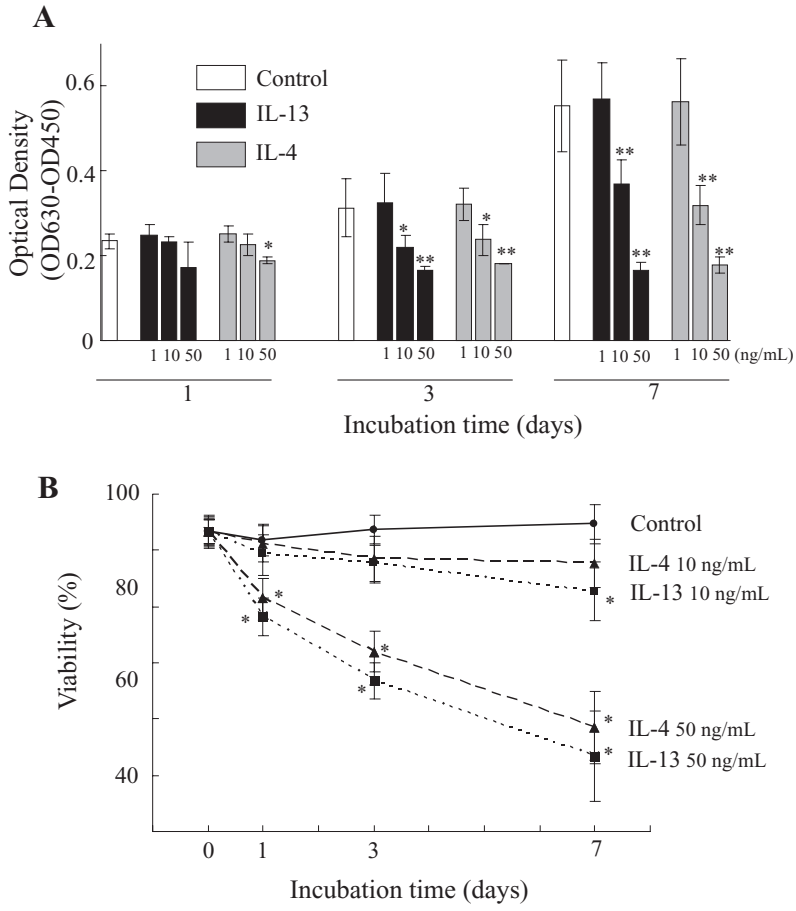

Fig. 1. *Inhibition of HCAEC growth and viability by IL-13 and IL-4. A: Cell growth. HCAECs were incubated for the periods indicated in control medium (control), or medium containing 1, 10 or 50 ng/mL IL-13 (IL-13) or IL-4 (IL-4). Medium containing IL-13 or IL-4 was changed every 2 days. Cell growth was determined by WST-1 assay. Data are shown as the means ± SEM from three experiments. *p < 0.05, **p < 0.01 vs. the control. B: Viability. HCAECs were incubated for the periods indicated in control medium (control), or medium containing 10 ng/mL or 50 ng/mL IL-13 (IL-13) or IL-4 (IL-4). Medium containing IL-13 or IL-4 was changed every 2 days. The cell number was counted after staining with trypan blue. Data are shown as the means ± SEM from three independent experiments. *p < 0.05 vs. the control.*

Methods

Cell Culture and Stimulation

HCAECs (Cell Applications Inc., San Diego, USA) were cultured in Dulbecco's modified Eagle medium (D'MEM) F-12 (Lonza Walkersville, Basel, Switzerland) containing 5% fetal bovine serum, 100 U/mL penicillin, 100 μg/mL streptomycin, and the appropriate growth hormones, according to the manufacturer's instructions. The cells were stimulated with recombinant human IL-13 or IL-4 (Peprotech, London, UK). Medium containing IL-13 or IL-4 was changed every 2 days.

Transfection of Small Interfering RNA

In order to deprive the cells of STAT6, small interfering RNA (siRNA) for STAT6 (ID#4501; Ambion, Austin, USA) was used. The cells were seeded at 1×10^5 cells/well in 24-well plates and then transfected with 20 pmol/μL siRNAs in combination with Lipofectamine 2000 (Invitrogen, Carlsbad, USA) in OPTI-MEM (Invitrogen), according the manufacturer's instructions.

Immunoblotting Analysis

Cells (1×10^6 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, 1 mmol/L Na_3VO_4, pH 7.4) and then centrifuged at 13,000 × *g* for 15 min at 4°C.

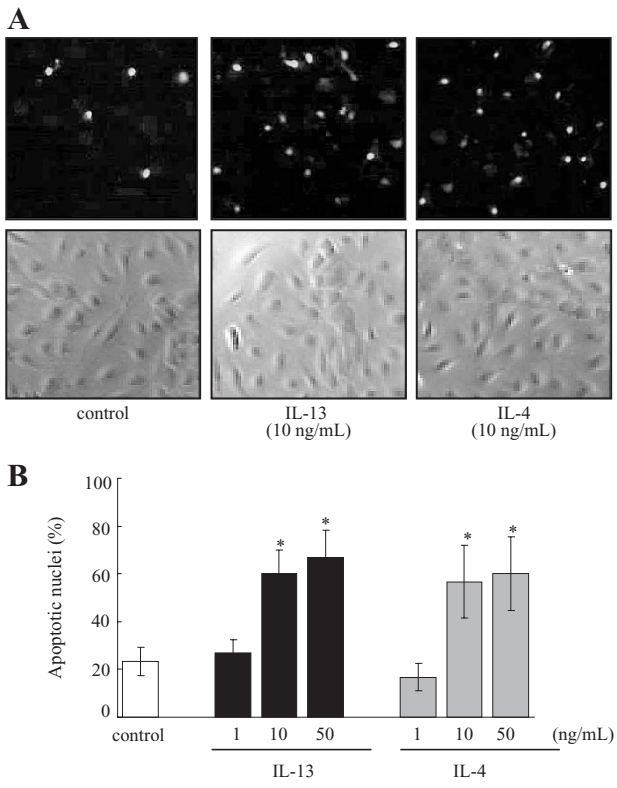

Fig. 2. *Induction of apoptosis by IL-13 and IL-4 in HCAECs. A: HCAECs were treated with 1, 10 or 50 ng/mL IL-13 or IL-4 for 24 h. The apoptotic cells were detected by TUNEL staining (upper panels). Total cells of the phase contrast images are shown in the lower panels. B: Apoptotic nuclei were counted in 200 random nuclei and the proportion of apoptotic nuclei was calculated. Data are shown as the means±SEM from four independent experiments. *$p < 0.01$ vs. the control.*

The supernatants were collected, and the protein concentrations were determined by a Bio-Rad protein assay (Bio-Rad, Hercules, USA). An aliquot of the cell lysates was mixed with an equal volume of 2 × Laemmli's sample buffer and boiled at 100°C for 5 min. Equal amounts of the proteins in each sample were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis gels and transferred onto polyvinylidene difluoride membranes. The blots were then probed with antibodies against STAT6 (Santa Cruz Biochemistry, Santa Cruz, USA), or against tyrosine-phosphorylated STAT6 (Cell Signaling Technology, Danvers, USA).

Reverse Transcription–Polymerase Chain Reaction

The level of VEGF mRNA was determined by reverse transcription–polymerase chain reaction (RT-PCR). Total RNA was prepared from HCAECs using ISOGEN (NIPPON GENE, Tokyo, Japan) according to the manufacturer's instructions. RT-PCR was performed using a RETROscript kit (Ambion) and a primer set of which sequences for VEGF mRNA are 5′-CCATGAACTTTCTGCTGTCTT-3′ and 5′-TCGATCGTTCTGTATCAGTCT-3′. A set of primers for β-actin (Ambion) was used as an internal control. PCR was performed for 26 cycles of 95°C for 30 s, 65°C for 30 s and 72°C for 60 s.

Cell Viability Assay

The viability of HCAECs treated with or without IL-13 and IL-4 was evaluated by counting the cells using a hemocytometer in phosphate-buffered saline (pH 7.4) containing 0.4% trypan blue.

WST-1 Assay

We employed a viability assay based on reduction of tetrazolium salt to formazan by mitochondrial dehydrogenase activity (*17*) using WST-1 reagent (Roche Applied Science, Mannheim, Germany). The assay was performed in 96-well microtiter plates following the manufacturer's protocol, and absorbance at 450 nm was measured. At least eight wells/group were analyzed.

Evaluation of Cells Undergoing Apoptosis

Apoptotic cells were evaluated using an *In Situ* Cell Death Detection Kit (Roche Applied Science).

Statistical Analysis

The values are presented as the means±SEM. Differences between single pairs of treatments were tested by Student's *t*-test (two tailed), and differences between groups were tested by one-way analysis of variance with Fisher's test for pairwise multiple comparisons. A p value <0.05 was considered statistically significant.

Results

IL-13 and IL-4 Impaired Cell Growth and Viability in HCAECs

Growth of HCAECs was evaluated by a WST-1 assay. As shown in Fig. 1A, both IL-13 and IL-4 significantly reduced cell growth compared to the control in a concentration-dependent manner at all time points examined. Cell viability gradually decreased when subconfluent HCAECs were cultured in 50 ng/mL IL-13 and IL-4, whereas the viability of the cells in control media did not change (Fig. 1B), suggesting that IL-13 and IL-4 suppressed the viability of HCAECs. IL-13 and IL-4 at 10 ng/mL also significantly suppressed HCAEC viability (Fig. 1B).

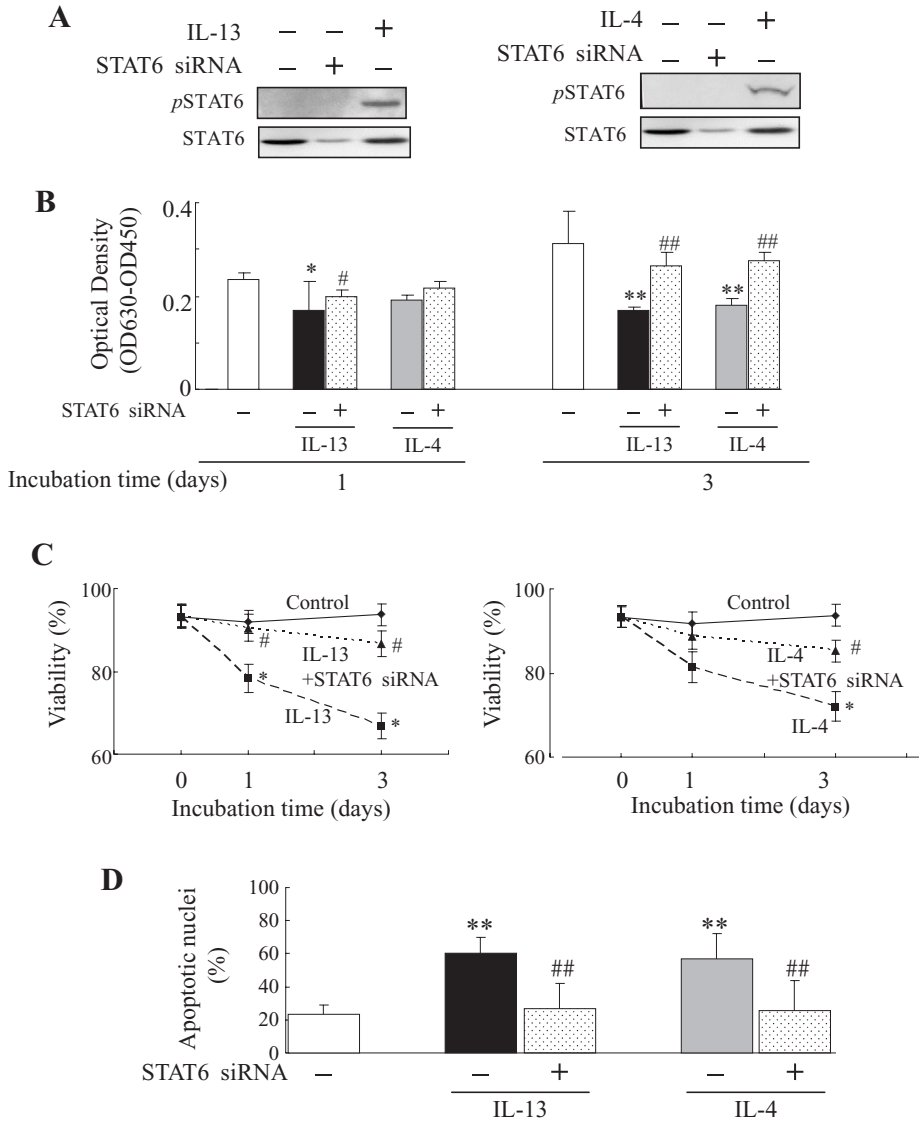

Fig. 3. *Effects of abrogation of STAT6 on IL-13– or IL-4–induced HCAEC apoptosis. A: HCAECs were incubated with 20 pmol/μL siRNA for STAT6 for 24 h followed by stimulation with 10 ng/mL IL-13 or IL-4. Medium containing IL-13 or IL-4 was changed every 2 days. The cell lysates prepared were subject to immunoblotting with the anti-phospho-STAT6 (top) or with anti-STAT6 (bottom). B, C, D: HCAECs were preincubated with 20 pmol/μL siRNA for STAT6 for 24 h prior to the stimulation with 50 ng/mL IL-13 or IL-4 for the durations indicated. Medium containing IL-13 or IL-4 was changed every 2 days. Cell growth was determined by WST-1 assay (B). Cell numbers were counted after trypan blue staining (C). Apoptotic nuclei were counted in 200 random nuclei after TUNEL staining, and the proportion of apoptotic nuclei was calculated (D). Data are shown as the means±SEM from three independent experiments. *$p<0.05$, **$p<0.01$ vs. the control. $^{\#}p<0.05$, $^{\#\#}p<0.01$ vs. IL-13– or IL-4–treated cells.*

IL-13 and IL-4 Induced Apoptosis in HCAECs

We performed a TUNEL assay to examine whether IL-13 and IL-4 induce apoptosis of HCAECs. As shown in Fig. 2A, treatment with either IL-13 or IL-4 at 10 ng/mL for 24 h significantly increased the number of TUNEL-positive nuclei compared to the control, suggesting that IL-13 and IL-4 induced apoptosis in HCAECs. IL-13 and IL-4 induced HCAEC apoptosis to a similar extent at 10 and 50 ng/mL (Fig. 2B).

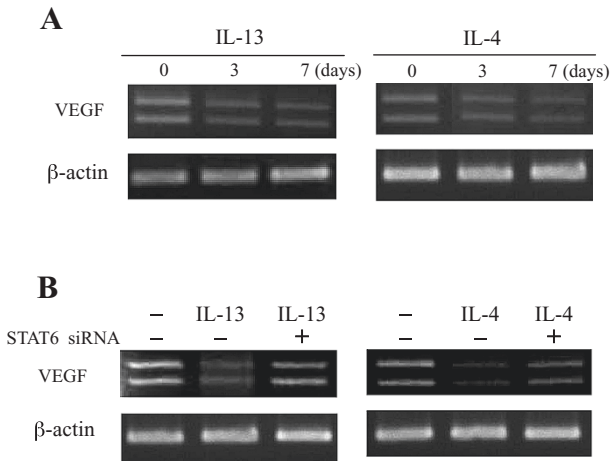

Fig. 4. *Involvement of STAT6 in suppression of VEGF mRNA expression by IL-13 and IL-4. A: HCAECs were treated with 10 ng/mL IL-13 or IL-4 for the durations indicated. Total RNA was extracted and VEGF and β-actin mRNA levels were determined by RT-PCR. B: HCAECs were preincubated with 20 pmol/μL siRNA for STAT6 for 24 h prior to the stimulation with 50 ng/mL IL-13 or IL-4.*

STAT6 Activation Was Involved in HCAEC Apoptosis Induced by IL-13 and IL-4

We next investigated the signaling pathway in response to IL-13 and IL-4 stimuli. As shown in Fig. 3A, STAT6 was markedly tyrosine-phosphorylated 15 min after stimulation with IL-13 or IL-4 at 10 ng/mL in HCAECs. To more directly examine the roles of STAT6 activation, we depleted STAT6 by RNA interference. The effective rate of protein depletion was more than 75%, which was confirmed by immunoblotting (Fig. 3A). Transfection of STAT6 siRNA recovered the growth that was suppressed by either 50 ng/mL IL-13 or IL-4. (Fig. 3B). Transfection of STAT6 siRNA also abolished the inhibitory effect of IL-13 and IL-4 on HCAEC viability (Fig. 3C) and the number of TUNEL-positive nuclei (Fig. 3D). These results suggest that STAT6 mediated the HCAEC apoptosis induced by IL-13 and IL-4.

IL-13 and IL-4 Down-Regulated VEGF mRNA Expression *via* STAT6 in HCAECs

Because VEGF induces proliferation and maintains the survival of ECs (*18*), and ECs are capable of producing VEGF by themselves (*19*, *20*), we hypothesized that IL-13 and IL-4 may alter VEGF production by HCAECs. As shown in Fig. 4A, VEGF mRNA expression was indeed reduced in response to 50 ng/mL IL-13 and IL-4. In addition, down-regulation of VEGF mRNA expression by IL-13 and IL-4 was reversed by transfection with STAT6 siRNA (Fig. 4B).

Discussion

Our results show that IL-13 attenuates cell viability and cell growth, and induces apoptosis. Similar effects have been reported for IL-4 (*12*), a cytokine sharing a receptor and signaling systems with IL-13. In general, IL-4 and IL-13 are thought to exhibit similar biological activities, although there are also some biological activities specific to each cytokine. For instance, IL-4 but not IL-13 has an activity to induce immunoglobulin class switching (*1*). On the other hand, IL-13 can promote fibrosis formation, which IL-4 fails to induce (*1*). Our present results indicate that there is no difference between the abilities of IL-4 and IL-13 to induce apoptosis in ECs. In other results of the present study, the WST-1 assay (Fig. 1) indicated that IL-13 and IL-4 had a stronger effect when administered at a concentration of 50 ng/mL than at 10 ng/mL, whereas the TUNEL assay (Fig. 2) showed that these cytokines had similar effects at either 10 ng/mL or 50 ng/mL. We consider that this discrepancy might have been due to the greater sensitivity of the TUNEL assay for detecting pre-apoptotic cells compared to WST-1 assay. We have recently shown that IL-13 and IL-4 impair angiogenesis by suppressing tube formation and cell migration, and that inhibition of STAT6 abrogates IL-13– and IL-4–mediated anti-angiogenic effects (*21*). Since angiogenesis consists of several steps, including proliferation, migration, formation of new capillary tubes and maintenance of their survival, we consider that the induction of apoptosis is one of the mechanisms by which IL-13 and IL-4 inhibit vascular tube formation.

There are conflicting reports on the effects of IL-13 and IL-4 on EC apoptosis (*12*, *13*, *22*, *23*). Our results support the findings of Volpert *et al*. (*13*), who reported that IL-13 and IL-4 inhibited angiogenesis in the rat cornea, and in human and bovine microvascular ECs. In addition, induction of the IL-4 gene significantly suppressed angiogenesis in a rat model of adjuvant-induced arthritis (*24*). On the other hand, there are reports (*22*, *23*) that apoptosis induced by TNF-α in combination with cycloheximide was inhibited by IL-4 and IL-13. The discrepancy between the previous reports and the present report might be due to the difference in experimental conditions. In the present study, ECs were treated with IL-13 in the presence of growth factors but without agents promoting apoptosis. In the previous studies, ECs were preincubated without growth factors, and then treated with IL-13. We presume that IL-13 has dual effects on apoptosis; *i.e.*, it shows proapoptotic activity in the presence of growth factors, and it shows anti-apoptotic activity in the presence of proapoptotic factors and/or in the absence of factors supporting survival. It is also likely that considerable numbers of ECs were already dead of apoptosis under the conditions of the previous study (*22*, *23*), because the cells were pretreated with IL-4 and IL-13 for 72 h before the treatment with TNF-α in combination with cycloheximide. Nonetheless, the precise causes of these discrepant findings need to be clarified in a later study.

Our results suggest that the proapoptotic effects of IL-13 and IL-4 are mediated by STAT6, because STAT6 siRNA abrogated all the suppressive effects of IL-13 and IL-4 on cell growth, cell viability, apoptosis, and VEGF mRNA expression. In this series of experiments, we changed the medium containing IL-13 and IL-4 every 2 days, thereby exposing the ECs to fresh cytokines. Therefore, it is quite likely that repetitive stimulation rather than one-time stimulation by IL-13 and IL-4 caused sustained activation of STAT6 and subsequent suppression of VEGF mRNA. Nuclear factor-κB (NF-κB) is likely to be a key candidate for a target molecule of STAT6, since STAT6 activation has been reported to suppress NF-κB activation (*25*). NF-κB is activated by adhesion through αvβ3 (*26*) and by VEGF (*27*), and its activation is thought to suppress EC apoptosis by inducing several "survival genes," such as A20 (*28*, *29*), Bcl-2 (*27*), and survivin (*27*). It is also possible that STAT6 suppresses EC proliferation by modulating the expression of the genes related to the cell cycle because STAT6 alters the levels of proteins such as $p21^{waf1}$, p53, cyclin D1, cyclin E (*11*), and $p27^{Kip1}$ (*30*). p53 is reported to play an important role in the apoptosis of mouse bone marrow mast cells by stimulation with IL-4 and IL-10 (*31*). STAT6 activation following IL-4 stimulation causes mitochondrial damage (*32*) and caspase activation (*10*), which might be involved in the process of HCAEC apoptosis by IL-13 as well. Further studies are required to clarify the mechanisms for HCAEC apoptosis induced by IL-13.

We demonstrated that IL-13 decreased VEGF mRNA expression. VEGF acts as an endothelial growth factor (*18*) and a vascular survival factor (*33*) and plays an important role in promoting all steps of angiogenesis. Vascular ECs are reported to produce VEGF in response to hypoxia (*19*) and FGF-2 stimulation (*20*). In our system, HCAECs constitutively express VEGF mRNA. This may be due to the presence of growth factors in the culture medium for ECs. It has been reported that VEGF production is suppressed by IL-4 in rat C6 glioma cells (*34*) and peripheral blood mononuclear cells from patients with inflammatory bowel diseases (*35*) or minimal-change nephritic syndrome (*36*). Our results are the first to show that IL-13 and IL-4 suppress VEGF production in ECs. Interestingly, the suppression of VEGF expression by IL-13 and IL-4 was completely blocked by the treatment with STAT6 siRNA, suggesting that STAT6 activation following IL-13 and IL-4 stimulation might be involved in the suppression of VEGF expression. Further studies will be needed to clarify the precise mechanisms involved in the suppression by STAT6.

Recently, we demonstrated that the plasma IL-13 level was significantly increased in patients with ischemic heart failure (*15*). In the present study, we demonstrated that IL-13 has a negative effect on EC survival. Taken together, our results suggest that IL-13 exacerbates ischemic heart diseases due to induction of apoptosis in HCAECs, which finally leads to inhibition of angiogenesis. The finding that injection of IL-13 into $apoE^{-/-}$ mice resulted in EC damage and development of atherosclerosis (*16*) supports this idea.

In conclusion, we demonstrated that IL-13 suppresses the survival and induces apoptosis of HCAECs. STAT6 was suggested to be involved in this phenomenon, since both IL-13– and IL-4–induced apoptosis was completely reversed by STAT6 siRNA. Further studies on the mechanisms of the apoptosis by IL-13 and IL-4 and on the source of these cytokines *in vivo* will clarify the role of IL-13 and IL-4 in ischemic heart diseases.

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