Tumor Necrosis Factor-α Inhibits Growth Factor–Mediated Cell Proliferation Through SHP-1 Activation in Endothelial Cells

Hironori Nakagami, Tai-Xing Cui, Masaru Iwai, Tetsuya Shiuchi, Yuko Takeda-Matsubara, Lan Wu, Masatsugu Horiuchi

Abstract—Src homology 2–containing protein-tyrosine phosphatase 1 (SHP-1) is known to regulate signal transduction through the dephosphorylation of tyrosine kinases. In this study, we addressed the role of SHP-1 under tumor necrosis factor-α (TNF-α) stimulation in endothelial cells. The addition of recombinant vascular endothelial growth factor (50 ng/mL) or epidermal growth factor (50 ng/mL) significantly increased thymidine incorporation and c-fos promoter activity, whereas TNF-α (5 ng/mL) attenuated these effects in human or bovine aortic endothelial cells. In bovine aortic endothelial cells, we confirmed endogenous SHP-1 expression and that TNF-α activated SHP-1. Importantly, overexpression of dominant-negative SHP-1 attenuated the effect of TNF-α on thymidine incorporation and c-fos promoter activity. In addition, TNF-α attenuated vascular endothelial growth factor– and epidermal growth factor–induced extracellular signal–regulated kinase phosphorylation, whereas overexpression of dominant-negative SHP-1 prevented this inhibitory effect of TNF-α. Taken together, our results suggested that TNF-α inhibited growth factor–mediated cell proliferation through SHP-1 activation. (Arterioscler Thromb Vasc Biol. 2002;22:238-242.)

Key Words: SHP-1 ■ vascular endothelial growth factor ■ epidermal growth factor ■ tumor necrosis factor-α ■ endothelial cells

Because dysfunction of endothelial cells may be a trigger in atherosclerosis and arteriosclerosis, it is considered very important for endothelial cells to maintain a well-controlled condition. In numerous studies, the potential roles of growth factors, such as vascular endothelial growth factor (VEGF) and epidermal growth factor (EGF), have been examined, especially in angiogenesis, which depends on neovascularization, including tumor growth, diabetes mellitus, ischemic ocular diseases, and rheumatoid arthritis.1–3 Among these, VEGF is a potent mitogen that promotes the growth and maintenance of vascular endothelial cells and the development of new blood vessels through interactions with its endothelial cell–specific receptors, KDR/flk-1 and flt-1.5,6 These families of endothelium-specific receptor tyrosine kinases, the VEGF receptor family and the novel Tie family of receptors, have been shown to play important roles in pathological angiogenesis in adult tissues.7–9

To fully understand the roles of growth factors in the vasculature, it is necessary to understand the signaling pathways that drive the cellular responses downstream from these receptors in much more detail. Phosphorylation of tyrosine residues mediated by the action of protein-tyrosine kinases creates docking sites for the recruitment and activation of src homology 2 (SH2)-containing cytosolic molecules. One of these SH2-containing cytosolic molecules is SHP-1 (previously known as SH-PTP1, PTP1C, HCP, and SHP), which has 2 SH2 domains in the N-terminal half. The SH2 domains function not only to recruit the enzyme to tyrosine-phosphorylated molecules but also to regulate the enzymatic activity. SHP-1 is a cytoplasmic non–receptor-like protein-tyrosine phosphatase (PTP) that is primarily expressed in hematopoietic cells, ischemic ocular diseases, and rheumatoid arthritis. Among these, VEGF is a potent mitogen that promotes the growth and maintenance of vascular endothelial cells and the development of new blood vessels through interactions with its endothelial cell–specific receptors, KDR/flk-1 and flt-1.5,6 These families of endothelium-specific receptor tyrosine kinases, the VEGF receptor family and the novel Tie family of receptors, have been shown to play important roles in pathological angiogenesis in adult tissues.7–9

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From the Department of Medical Biochemistry, Ehime University Medical School, Ehime, Japan.

Correspondence to Masatsugu Horiuchi, MD, PhD, Department of Medical Biochemistry, Ehime University School of Medicine, Shitsukawa, Shigenobu, Onsen-gun, Ehime 791-0295, Japan. E-mail horiuchi@m.ehime-u.ac.jp

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in a dose- and time-dependent fashion. In the present study, we focused on the interaction between TNF-α and SHP-1 in endothelial cells and the possibility that TNF-α may be negatively regulated through SHP-1 activation in growth factor–mediated endothelial cell proliferation.

Methods

Cell Culture
Human aortic endothelial cells (HAECs) and bovine aortic endothelial cells (BAECs), at passage 3 and 1, respectively, were obtained from Clonetics Corp and cultured in modified MCDB131 medium supplemented with 5% FCS, 50 μg/mL gentamicin sulfate, 50 ng/mL amphotericin B, 10 ng/mL EGF, and 1 mmol/L hydrocortisone in the standard fashion. These cells showed the specific characteristics of endothelial cells by immunohistochemical examination and morphological observation and were used within passages 5 to 8.

Plasmid Constructs and Transfection
BAECs were used because of their higher transfection efficiency compared with that of HAECs, and they were transiently transfected with the plasmid pcDNA3 or a vector encoding a catalytically inactive mutant of SHP-1 in which Cys453 was changed to Ser. This mutation in the catalytic domain decreases enzymatic activity but still allows enzyme-substrate binding and, when overexpressed, has a negative-dominant effect in cells. Transfection was performed with LipofectAMINE2000 in combination with PLUS reagent ( Gibco-BRL) according to the manufacturer’s instructions.

Measurement of DNA Synthesis by Thymidine Incorporation
DNA synthesis was assayed by measuring [3H]thymidine incorporation as previously described. Briefly, endothelial cells in growth medium were equally seeded into 24-well culture plates. Next day, the growth medium was changed to medium supplemented with 0.5% FCS, and incubation was continued for 2 days. On day 3, the medium was changed to fresh serum-free medium containing TNF-α (5 ng/mL) and VEGF or EGF (50 ng/mL) and incubated for 24 hours. Twelve hours before harvest, [3H]thymidine (1 μCi/mL) was added to each well of the culture plate. DNA was precipitated with cold 10% trichloroacetic acid for 20 minutes, and the precipitated material was resuspended in 0.2 mL of 0.5 mol/L NaOH and assayed for [3H] in a liquid scintillation counter.

c-fos Promoter Assay
BAECs were seeded in 6-well plates and transfected with the c-fos-lucerase reporter gene (p2FTL) as previously described. The c-fos–lucerase reporter gene consists of 2 copies of the c-fos 5′–regulated enhancer element (−357 to −276), the herpes simplex virus thymidine kinase gene promoter (−200 to 70), and the lucerase gene. At 24 hours after transfection, transfected cells were incubated with serum-free medium for 24 hours. Quiescent cells were treated with 50 ng/mL VEGF or EGF with or without 5 ng/mL TNF-α for 4 hours, washed with PBS, and lysed for 15 minutes with 200 μL cell lysis buffer (Promega Corp) at room temperature. Then, 10 μL cell extract was mixed with 100 μL lucerase assay reagent (Promega Corp), and the light production was measured for 30 seconds by use of a luminometer.

Western Blotting
Western blotting was performed for analysis of extracellular signal–regulated kinase (ERK) by using a phospho-specific antibody as previously described. Briefly, the treated cells were extracted with lysis buffer (50 mmol/L Tris-HCl, 2.5 mmol/L EGTA, 1 mmol/L EDTA, 10 mmol/L NaF, 0.1% sodium deoxycholate, 1% Triton X-100, 1 mmol/L phenylmethylsulfonyl fluoride, and 1 mmol/L Na3VO4). Samples containing 20 μg proteins were separated on 10% SDS-polyacrylamide gels, transferred to nitrocellulose membranes (Hybond ECL, Amersham Life Science Inc.), and incubated with a polyclonal antibody to phospho-specific or total ERK (anti-human, -rat, -mouse, or -rabbit IgG, 1:1000, Cell Signaling Technology) at 4°C overnight. The membranes were then washed and incubated with a 1:2000 dilution of rabbit immunoglobulin horseradish peroxidase–conjugated antibody (Amersham). Bound antibodies were detected by enhanced chemiluminescence (ECL, Amersham) with Hyperfilm-MP (Amersham).

Immunoprecipitation of SHP-1 and PTP Assay
SHP-1 activity was measured by immunoprecipitation of SHP-1 and PTP assay (New England Biolabs), as previously described. Subconfluent endothelial cells were incubated in serum-free medium for 24 hours and treated with TNF-α (5 ng/mL). After treatment, the cells were rinsed with ice-cold PBS and solubilized in lysis buffer (25 mmol/L Tris-HCl, pH 7.4, 1.5% CHAPS, 10 μmol/L Na3VO4, 50 mmol/L NaF, 1 mmol/L phenylmethylsulfonyl fluoride, and 20 μg/mL aprotinin). The extracts were centrifuged, and total proteins (300 μg) were immunoprecipitated with an anti–SHP-1 antibody and protein A/G-agarose (Santa Cruz Biotechnology Inc) and incubated with rocking. After 2 hours, immunocomplexes were washed 3 times with ice-cold lysis buffer (lacking Na3VO4 and NaF) and once with phosphate assay buffer (50 mmol/L Tris-HCl, pH 7.0, 1 mmol/L EDTA, 5 mmol/L dithiothreitol, and 0.01% Brij 35 solution). Finally, tyrosine phosphatase activity was measured as the release of [32P]orthophosphate from radiolabeled myelin basic protein (MBP) as previously described.

Materials
Human recombinant VEGF was obtained from R&D, and human recombinant TNF-α and EGF were obtained from Peprotech. A PTP assay kit was obtained from New England Biolabs. Other materials were obtained from Sigma Chemical Co.

Statistical Analysis
All values are expressed as mean±SEM. ANOVA with a subsequent Bonferroni/Dunnett test was used to determine the significance of differences in multiple comparisons. Values of P<0.05 were considered to be statistically significant.

Results

Effect of TNF-α on Endothelial Cell Proliferation Induced by Growth Factors
To determine whether TNF-α blocks the proliferative effect of VEGF and EGF on human endothelial cells, we measured [3H]thymidine incorporation as a marker of DNA synthesis after stimulating HAECs with human recombinant VEGF. As shown in Figure 1A, VEGF treatment alone (50 ng/mL) enhanced [3H]thymidine incorporation 2.4-fold in HAECs compared with control cells, whereas treatment with TNF-α (5 ng/mL) attenuated the effect of VEGF on DNA synthesis in HAECs. Similarly, treatment with TNF-α inhibited EGF-induced [3H]thymidine incorporation (Figure 1B). Pretreatment with TNF-α also attenuated the effects of VEGF and EGF on DNA synthesis in BAECs (data not shown). Because VEGF stimulation is known to enhance the expression of a variety of genes, including the immediate early growth response genes such as the proto-oncogene c-fos, we next examined the effect of TNF-α on VEGF- and EGF-mediated promoter activation of c-fos by using lucerase activity. As shown in Figure 2A and 2B, VEGF or EGF treatment enhanced lucerase activity ≈3-fold, whereas treatment with TNF-α attenuated these increases in BAECs. These results demonstrate that TNF-α inhibited the proliferation of endothelial cells in response to growth factors.
Effect of TNF-α on SHP-1 Activity in BAECs

Next, we focused on the role of PTP, especially SHP-1. In this experiment, we used BAECs, which are reported to be transfected with high efficiency. In BAECs, we demonstrated that TNF-α activated SHP-1 and reached a peak after 3 minutes of stimulation (Figure 3A). To confirm the role of SHP-1 in the proliferation of endothelial cells, we transfected dominant-negative (dn) SHP-1. Endogenous SHP-1 protein was expressed in endothelial cells, and the transiently transfected dnSHP-1 gene was evaluated by the increase in SHP-1 protein by Western blotting (Figure 3B). SHP-1 activity was lower, and the further increase by TNF-α was attenuated in dnSHP-1–overexpressing cells compared with control vector–transfected cells (Figure 3C).

Effect of TNF-α and Growth Factors on ERK Phosphorylation

We focused on the signal pathway of growth factors, especially ERK activity, because the ERK pathway activated by growth factors is involved in mediating cellular proliferation, transformation, and differentiation. Stimulation with VEGF and EGF markedly increased ERK phosphorylation after 10 minutes of treatment in BAECs, whereas a pretreatment with TNF-α for 10 minutes attenuated this phosphorylation of ERK. Importantly, overexpression of dnSHP-1 prevented TNF-α–induced inhibition of ERK phosphorylation (Figure 4).

Effect of SHP-1 on Endothelial Cell Proliferation

We next examined the effect of SHP-1 on DNA synthesis. In control vector–transfected cells, treatment with TNF-α (5 ng/mL) attenuated the effect of VEGF on DNA synthesis, whereas the overexpression of dnSHP-1 attenuated this effect of TNF-α and enhanced VEGF-induced thymidine incorporation (Figure 5A). Similarly, the overexpression of dnSHP-1 enhanced EGF-induced thymidine incorporation and attenuated the inhibitory effect of TNF-α (Figure 5B). In addition, the overexpression of dnSHP-1 also attenuated the effect of TNF-α on c-fos promoter activity (Figure 5C).

Discussion

Growth factors, such as VEGF and EGF in endothelial cells, play an important role in angiogenesis, a process known to be
required for embryonic development and for the progression of many clinically important diseases. Moreover, dysfunction of endothelial cells may promote abnormal vascular growth, such as that in atherosclerosis and arteriosclerosis. From this viewpoint, we have focused on the regulation of growth factors and cytokine-mediated cellular effects.

As a central regulator of the local inflammatory response, TNF-α modulates the expression of many genes in endothelial cells. Consistent with recent reports,31 our present study demonstrated signaling cross talk; ie, TNF-α directly inhibits VEGF- and EGF-mediated cellular effects through SHP-1 activation. Although inhibition of endothelial cell growth and survival may serve as a brake on the inflammatory response during the early stages of endothelial activation, this growth inhibition may also contribute to pathophysiological responses in the later stages of cytokine-mediated diseases. The other discovery that TNF-α and VEGF induce angiogenesis through 2 distinct αv integrin–mediated pathways is of particular interest,32 because our findings propose the possibility that a factor from one such pathway with bifunctional angiogenic activity (TNF-α) may actually inhibit the growth factor–mediated effects by acting through another pathway.

SHP-1, a member of these nonreceptor PTP families, is predominantly a negative regulator of signal transduction; eg, B and T cells prepared from SHP-1–deficient me/me mice are hyperresponsive to immune receptor stimulation that is due to loss of the activity of SHP-1, which normally dephosphorylates signal transduction molecules.14–17 SHP-1 is primarily expressed in hemopoietic tissues but is also present in endothelial cells31 and is known to associate with multiple signaling molecules, such as growth factor receptor tyrosine kinases, activated cytokine receptors, and the erythropoietin receptor.14–17 We demonstrated that treatment with TNF-α significantly increases SHP-1 activity and, consequently, attenuates growth factor–induced ERK phosphorylation through PTP activation. Interestingly, dnSHP-1 overexpression increased the growth factor–induced cellular effect, such as ERK phosphorylation, thymidine incorporation, and c-fos promoter activity. From these results, we suggest that SHP-1 may play an important role in the basic regulation of signal transduction through possible binding to tyrosine kinase receptors of growth factors and that SHP-1 may be more active in negatively regulating the signal transduction of TNF-α. The present study has demonstrated that SHP-1 may play a role in the negative regulation of the growth factor–induced cellular effect and that SHP-1 may be a key molecule in the prevention of endothelial dysfunction in atherosclerosis and in the induction of angiogenesis in ischemic diseases.
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