Signal Transducer and Activator of Transcription 3 Is Required for Glycoprotein 130-mediated Induction of Vascular Endothelial Growth Factor in Cardiac Myocytes*

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Activation of glycoprotein (gp) 130 transduces hypertrophic and cytoprotective signals in cardiac myocytes. In the present study, we have demonstrated that signals through gp130 increase the expression of vascular endothelial growth factor (VEGF) in cardiac myocytes via the signal transducer and activator of transcription (STAT) 3 pathway. After activation of gp130 with leukemia inhibitory factor (LIF), expression of VEGF mRNA rapidly increased with a peak at 3 h in cultured cardiac myocytes. Cardiotrophin-1 also enhanced VEGF mRNA expression in a dose-dependent manner. VEGF protein production and secretion to the medium were also enhanced by LIF and cardiotrophin-1 but not by interleukin-6. Adenovirus transfer of the dominant-negative form of STAT3 to cultured cardiac myocytes inhibited induction of VEGF expression induced by LIF, but neither PD98059 nor wortmannin was affected. In murine hearts, intravenous administration of LIF augmented expression of VEGF mRNA; however, the hearts of transgenic mice overexpressing dominant-negative STAT3 showed reduced expression of VEGF mRNA that was not induced after LIF stimulation. These data provide the first evidence that a STAT family protein functions as a regulator of angiogenic growth factors and suggest that gp130/STAT signaling in cardiac myocytes can control vessel growth during cardiac remodeling.

Glycoprotein (gp) 130 is a signal transducer and a common receptor subunit of interleukin (IL)-6-related cytokines (1). Stimulation of gp130 leads to the activation of the Janus kinase (JAK)/signal transducer and activator of transcription (STAT) pathway. Following the activation of JAK, STAT proteins are phosphorylated on tyrosine residues, translocate to nuclei, and activate the transcription of target genes (2). STAT proteins are reported to be involved in regulating cell survival, proliferation, and differentiation (3). Signals through gp130 also play an important role in the regeneration or remodeling of various tissues in vivo. For example, hepatocyte regeneration was found to be impaired in IL-6-deficient mice (4), and coexpression of IL-6 and its receptor causes regenerative hyperplasia in the liver (5).

In cardiac myocytes, gp130 stimulation results in the activation of downstream signaling pathways, including the JAK/STAT, mitogen-activated kinase (MAPK) (6), and phosphatidylinositol 3-kinase (PI3-K) pathways (7). Pathophysiologically, gp130/STAT is activated through the autocrine/paracrine system of IL-6 related cytokines, including cardiotrophin-1 (CT-1), in response to mechano-stretch, hypoxia, and other cytokines such as IL-1β and tumor necrosis factor-α (8, 9). It is also reported that the activation of STAT transduces hypertrophic signals coupled with the positive regulation of cell survival in vitro (10, 11). Taken together, these data suggest that the gp130/STAT system plays an important role in cardiac remodeling, such as hypertrophy triggered by extracellular stress.

Recently it has been shown that cardiac-specific disruption of gp130 results in cardiomyopathy in response to mechano-stress with an increase in apoptosis (12). Thus the inactivation of STAT3 resulting from the loss of gp130 may be a key event in the transition from cardiac hypertrophy to heart failure. Although the molecular mechanisms for the onset of dilated cardiomyopathy in cardiac-specific gp130 knockout mice remains unidentified, perturbed vascular function may contribute to the cardiomyopathy. This hypothesis arises from the observations that impaired vasculature caused by disruption of VEGF (13) or the sarcoglycan complex in vascular smooth muscle cells (14) leads to cardiac dysfunction, possibly because of ischemic stress-induced reduction in cell viability. In other words, proper vascular growth is essential for normal cardiac development and remodeling.

Based on this reasoning, we explored the possibility that signals through gp130 regulate vascular growth factors in heart via STAT pathways. In the present study, we demonstrate that only gp130 up-regulates VEGF expression both in vitro and in vivo and that STAT3 is required for gp130-mediated VEGF induction. These data suggest that activation of gp130 can play a pivotal role in regulating interactions between myocytes and the endothelium through a VEGF sig-
naling mechanism. This mechanism may function to coordinate vessel growth with cardiac remodeling.

**EXPERIMENTAL PROCEDURES**

**Reagents**—Murine LIF, medium-199 (M-199), fetal calf serum, and moloney murine leukemia virus reverse transcriptase were purchased from Life Technologies, Inc. Oligo(dT) (d(T)$_{12-18}$, 5'-OH, Na$^+$ salt) and protein A-Sepharose were obtained from Amersham Pharmacia Biotech. Taq polymerase was from Takara. PRIME IT II for labeling cDNA was from Stratagene. Polyvinylindene difluoride membrane was from Millipore Co. (ωP1DCTP and ECL detection system were purchased from New England BioLabs, Inc. Anti-STAT3 antibody was purchased from Santa Cruz Biotechnology Inc. VEGF ELISA Kit (Quantikine M) was obtained from R & D Systems. Human cardiotoxin-1 was purchased from alomone labs. All other chemicals were reagents of molecular biology grade and were obtained from standard commercial sources.

**Animal Preparation**—Male DDY mice (8 weeks old) obtained from Kiwa Dobutsu (Wakayama, Japan) were injected intravenously with either LIF (1 × 10$^5$ units/0.1 ml of PBS) or vehicle. The care of all animals in the present study was in accordance with Osaka University Animal Care guidelines.

**Cell Cultures**—Primary cultures of cardiac myocytes were prepared from the ventricles of 1–2-day-old Wister rats (Kiwa Dobutsu, Wakayama, Japan) as described previously (6). In brief, hearts were treated with trypsin and collagenase for 30 min. Isolated cells were collected by centrifugation and resuspended in M-199 containing 10% fetal calf serum. Cultures were enriched with myocytes by preplating for 40 min to deplete the population of nonmyocytes. Nonattached cells were plated onto plastic culture dishes at an appropriate cell density (data not shown).

**Generation of Recombinant Adenovirus**—The recombinant replication-defective adenovirus expressing dominant-negative form of STAT3 (dnSTAT3) was prepared as described previously (11). The recombinant viruses were purified and concentrated as described previously (15).

**Protocol for Adenovirus Infection**—36 h after plating, cardiac myocytes were transfected with adenovirus vectors in M-199 with 10% fetal calf serum at a multiplicity of infection of 20 and incubated for 12 h. After removal of viral suspension, cardiac myocytes were serum-starved for 8 h and stimulated with reagents. Transfection efficiency was analyzed by Lac-Z gene expression, as described above. Adenovirus vector expressing β-galactosidase was used as a control.

**Generation of Transgenic Mice with Cardiac-specific Overexpression of the dnSTAT3 and Wild Type STAT3**—A 5.5-kilobase (kb) fragment of murine α-musino heavy chain (α-MHC) gene promoter (generously provided by Dr. Jeffrey Robbins, Children’s Hospital Research Foundation, Cincinnati, OH) and a 2.2-kb murine dnSTAT3 cDNA (generously provided from Dr. S. Akira, Osaka University, Osaka, Japan) were subcloned into pBluescriptIISK(+) plasmid. The plasmids were digested with BamHI to generate 8.4-kb DNA fragment consistent with α-MHC gene promoter, dnSTAT3 cDNA, and poly(A) of the human growth hormone. Transgenic mice (BCF1 strain) were generated as described elsewhere (16). To identify exogenous gene, genomic DNA was extracted from tail of 3-month-old mice. Southern blot analysis using probes of transgenic mice carrying cardiac-specific overexpression of dnSTAT3 (dnSTAT3 TG) was performed by polymerase chain reaction analysis using a set of primers, 5'-CAGGACCATCCTCGGTTGTTTCA-3' and 5'-GACATCGGACAACTCCCTTGTTGTTTCA-3', which correspond to the sequences obtained from exon 19 and exon 23 of the murine STAT3 gene (17). Polymerase chain reaction conditions were 96 °C for 4 min, followed by 30 cycles of 96 °C for 1 min, 58 °C for 1 min, and 72 °C for 2 min. The length of amplified DNA was 201 base pairs. Transgenic mice carrying cardiac-specific overexpression of wild type STAT3 (wtSTAT3 TG) were generated as described previously (18).

**Northern Blot Analysis**—Total RNA was prepared with the acid guanidinium isothiocyanate-phenol-chloroform method (19). Rat VEGF cDNA was obtained by reverse transcription-polymerase chain reaction methods using specific oligonucleotides primers (sense, 5'-AAGCGAGC

CAGTATGGAGGATGAG-3' and antisense, 5'-TACCCGGCTTGGCTGTCACATCT-3'). The 201 base pairs DNA fragment was cloned into pCR2.1 and the nucleotide sequence was determined.

**Primer Extension Analysis**—Polymerase chain reaction fragment was subcloned into pCR2.1, and the nucleotide sequence was determined. Reverse transcription was performed with primer 5'-AGGGTCCCGGATCCCTTCC-3' with PRIME IT II labeling kit. Total cellular RNA (8 μg) was separated on 1.5% formaldehyde agarose gel and transferred to a nylon membrane in the presence of 20× SSC (300 mM sodium chloride and 300 mM sodium citrate, pH 7.0). Prehybridization was performed at 42 °C for 1 h in 650 mM sodium chloride, 100 mM sodium phosphates, pH 6.8, 5× Denhardt’s solution, 0.1% SDS, 10 mg/ml of denatured salmon sperm DNA, and formamide at a final concentration of 50%. After 12 h of hybridization, the membrane was washed twice with 2× SSC and 0.1% SDS at 60 °C. Autoradiography was performed at 80 °C. The intensity was analyzed by densitometry (Immuno Reader NJ-2000) (InterMed Inc.).

**Western Blot Analysis**—10 μg of proteins were separated in 7.5% SDS-polyacrylamide gel and electrophoretically transferred onto a polyvinylindene difluoride membrane with transfer buffer (25 mM Tris, 190 mM glycine, and 20% methanol). Membrane was blocked with 5% skim milk and probed with anti-STAT3 antibody at a 1:1000 dilution for 1 h.

**RESULTS**

**Effect of Leukemia Inhibitory Factor on the Expression of VEGF in Vitro**—To investigate whether IL-6-related cytokines regulate VEGF mRNA expression, we performed Northern blot analyses of LIF-stimulated cardiac myocytes. After 8 h of culture in serum-depleted medium, cardiac myocytes were stimulated with 10$^8$ units/ml LIF for the time indicated (Fig. 1).

Enhancement of VEGF mRNA expression was detected within 1 h with a maximum level at 3 h (140% increase) followed by a decrease within 24 h in cardiac myocytes. On the other hands, no enhancement of VEGF mRNA expression was detected in response to LIF stimulation in nonmyocytes (data not shown). As shown in Fig. 2, LIF also induced VEGF mRNA expression in a dose-dependent manner.

To investigate whether the increase in VEGF mRNA expression is accompanied by an increase in protein level, secreted VEGF protein was quantified in the medium using a VEGF ELISA system. Cardiac myocytes were starved from serum for 8 h and stimulated with fresh medium containing various concentrations of LIF for 24 h. As shown in Fig. 2C, LIF augmented VEGF protein production in cardiac myocytes in a dose-dependent manner. Consistent with the VEGF mRNA expression, no enhanced VEGF protein expression was detected in the culture medium from LIF-treated nonmyocytes (data not shown).

**VEGF Expression Was Enhanced by CT-1 but Not by IL-6**
Because CT-1 activates the LIF receptor (20) followed by gp130 activation, we analyzed the effects of CT-1 on the expression of VEGF. As shown in Fig. 3A, CT-1 also induced VEGF mRNA expression in a dose-dependent manner in cardiac myocytes. Moreover, as in the case with LIF, enhancement of VEGF mRNA accompanied an increase in protein (Fig. 3B). On the other hand, IL-6 showed no effect on VEGF expression in either mRNA (data not shown) or protein levels, which is consistent with the finding that the IL-6 receptor is expressed at low levels in cultured cardiac myocytes (21).

**Dominant-negative STAT3 (dnSTAT3) Inhibits LIF-induced Increase in VEGF Expression**—Activation of gp130 is known to result in the activation of downstream signaling pathways, including JAK/STAT, MAPK, and PI3-K (6, 7). To identify the downstream effector for gp130-mediated VEGF induction, the effects of dnSTAT3, PD98059, and wortmannin on VEGF induction were examined. First, cultured cardiac myocytes were transfected with adenovirus vectors expressing dnSTAT3 or β-galactosidase and stimulated with LIF. As shown in Fig. 4A, transduction of β-galactosidase had no effect on the expression level of VEGF mRNA, whereas transduction of dnSTAT3 inhibited LIF-induced VEGF mRNA expression.

To examine the involvement of other pathways in VEGF gene regulation, PD98059, a specific MEK-1 inhibitor, was used. As shown in Fig. 4B, pretreatment with PD98059 had no effect on LIF-mediated VEGF induction, suggesting that the MAPK pathway is not a major regulatory pathway under these conditions. Wortmannin, a PI3-K inhibitor, also did not affect LIF-mediated VEGF mRNA induction (data not shown). Finally, transfection of dnSTAT3 inhibited LIF-induced VEGF protein induction (Fig. 4C).

**LIF Augments VEGF Expression in Murine Hearts**—To evaluate the importance of STAT3 in VEGF induction in vivo, we examined the effect of LIF on VEGF expression in adult murine heart. 8-week-old mice were treated intravenously either with LIF (1 × 10³ units/0.1 ml) or with an equal volume of PBS, and the hearts were excised at various time points. As shown in Fig. 5, enhancement of VEGF mRNA expression was detected within 1 h of LIF administration and reached it maximum level at 3 h.

To clarify the involvement of STAT3 in VEGF regulation in vivo, transgenic mice with cardiac-specific overexpressing dnSTAT3 under control of the α-MHC promoter (Fig. 6A) were examined. Protein extracts were prepared from the hearts of 8-week-old control littermates (WT) or transgenic mice carrying dnSTAT3 (dnSTAT3 TG) and immunoblotted with anti-STAT3 antibody. As shown in Fig. 6B, a 10-fold increase in STAT3 protein was observed in dnSTAT3 TG hearts compared with that in WT. LIF (1 × 10³ units) was then injected into both animals, and VEGF mRNA expression was examined in the hearts after 1 h. As shown in Fig. 6C, little or no remarkable induction of VEGF mRNA expression was observed in dnSTAT3 TG after LIF treatment. Similar results were obtained using a second line of dnSTAT3 transgenic animals (data not shown).

Furthermore, transgenic mice with cardiac-specific overex-
pressing wild type STAT3 (wtSTAT3 TG) were generated and analyzed the concentration of VEGF protein in the heart. As shown in Fig. 7, 20% increase of VEGF protein was observed in wtSTAT3 TG hearts compared with that in WT at basal level, and enhancement of VEGF induction was observed after LIF stimulation in wtSTAT3 TG hearts.

**DISCUSSION**

IL-6 related cytokines bind to their specific receptors, followed by the activation of gp130 (1). Signals through gp130 are known to activate STATs, MAPK, and PI3-K (6, 7) and known to transduce hypertrophic and anti-apoptotic signals in cardiac myocytes (10, 11, 22). Recently, it was reported that transgenic mice with cardiac-specific overexpression of STAT3, a downstream effector of gp130, manifested mild cardiac hypertrophy at the age of 12 weeks and recovered from doxorubicin-induced cardiomyopathy (18). Here we propose a new function for gp130 signaling, the regulation of angiogenic growth factors.

In the present study, we demonstrated that signals through gp130 rapidly up-regulate VEGF expression in cardiac myocytes both in vitro and in vivo. Using an adenovirus vector expressing dnSTAT3, we showed that STAT3 is essential for VEGF induction in vitro. To address whether the STAT3-dependent signaling pathway is essential for VEGF induction in the heart, we generated transgenic mice expressing dominant-negative STAT3 from a myocardial-specific promoter. dnSTAT3 TG hearts showed reduced basal VEGF mRNA expression and little or no remarkable increase after LIF stimulation, indicating that STAT3 plays an important role in the transcriptional regulation of VEGF gene in cardiac myocytes in vivo. Because the transgene is driven by the α-MHC promoter, reduction of VEGF mRNA expression in hearts is likely to be derived from cardiac myocytes not from cardiac fibroblasts. Furthermore, it should be noted that VEGF mRNA is down-regulated in naive dnSTAT3 TG hearts, suggesting physiological significance of the STAT3 pathway in adult hearts. The biological relevance of this signaling pathway was also supported by the experiments using wtSTAT3 TG hearts, which showed higher levels of VEGF protein with enhanced expression in response to LIF (Fig. 7), implying that STAT3 mediates VEGF induction in vivo.

VEGF is a potent angiogenic growth factor, and heterozygous deletion of the VEGF gene results in embryonic lethality (23). Recently, disruption of VEGF isoform has been reported to induce heart failure because of ischemia, which is in turn induced by impaired vascular structure. This suggests that...
STAT3 Mediates VEGF Expression

A. α-MHC promoter
dnSTAT3 cDNA
hGH polyA

B. WT
dnSTAT3 TG

C. LIF
WT
dnSTAT3 TG

Fig. 6. VEGF mRNA expression is not observed in dnSTAT3 transgenic mice hearts. A, schematic representation of the transgene, a 2.3-kb murine dominant-negative STAT3 (dnSTAT3) cDNA was ligated downstream of a 5.5-kb fragment of the murine α-MHC gene promoter and upstream of a 0.6-kb human growth hormone (hGH) polyA). B, the lysates were obtained from the hearts of 8-week-old control littermates (WT) or dnSTAT3 TG mice. Crude protein extracts (10 μg) from the heart were separated by SDS-PAGE and immunoblotted with anti-STAT3 antibody as described under “Experimental Procedures.” C, 8-week-old dnSTAT3 TG or WT were treated either with 0.1 ml of vehicle or with LIF (1 × 10^5 units/0.1 ml of PBS). Total RNA was purified from the hearts 1 h after the stimulation. VEGF mRNA expression was analyzed by Northern blot analysis. Experiments were repeated using a second line of dnSTAT3 TG, and similar results were obtained.

VEGF/protein (pg/μg)  

LIF
WT
dnSTAT3 TG

Fig. 7. VEGF protein is highly expressed in wtSTAT3 transgenic mice hearts. 8-week-old WT and wtSTAT3 TG were injected with LIF (1 × 10^5 units/0.1 ml of PBS) or vehicle intravenously. 3 h later, crude extracts were prepared, and VEGF concentrations were measured as described under “Experimental Procedures.” VEGF contents were normalized with protein concentration and expressed as the ratio of VEGF protein over total protein. Values are the means ± S.E. from three samples. *p < 0.01 versus LIF (--), †p < 0.05 versus WT LIF (--).

VEGF expression is essential for postnatal heart development (13). However, despite the reduction in VEGF expression, dnSTAT3 TG did not show any abnormality in the hearts (data not shown). The difference in cardiac phenotype between the VEGF isoform knockout mice and dnSTAT3 TG may be explained by the difference between the two experimental systems. In our transgenic strategy, dominant-negative STAT3 is driven by the α-MHC promoter and activated after birth (24), whereas the VEGF deletion occurs before birth. Another explanation for the difference is that STAT3-mediated VEGF induction is not required for the heart development but essential under stress conditions. This speculation is supported by a recent report that cardiac-specific gene targeting of gp130 resulted in little cardiac abnormality during development but generated dilated cardiomyopathy in response to mechanical stress (12). To evaluate the pathological significance of STAT3 in VEGF regulation in vivo, it would be helpful to determine whether a reduction of VEGF contributes to the onset of heart failure in gp130 conditional knockout mice.

Our observations would also give a new insight into the role of STAT3 in tumorgenesis. Recently it was shown that STAT3 is involved in the transformation by v-Src (25, 26) and that the constitutively active form of STAT3 functions as an oncogene (27). Importantly, cells expressing an active form of STAT3 form solid tumor in vitro. Because the formation of solid tumor larger than 2 mm^3 requires tumor angiogenesis for continuous growth and maintenance (28), it is possible that activation of STAT3 leads to the tumor anglogiogenesis, at least in some cases.

In summary, signals though gp130 induce VEGF via STAT3 in cardiac myocytes both in vitro and in vivo. This represents the first identification of STAT family proteins as regulators of an angiogenic growth factor.

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