Tumor Necrosis Factor Employs a Protein-tyrosine Phosphatase to Inhibit Activation of KDR and Vascular Endothelial Cell Growth Factor-induced Endothelial Cell Proliferation*

(Received for publication, November 24, 1999)

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Vascular endothelial cell growth factor (VEGF) binds to and promotes the activation of one of its receptors, KDR. Once activated, KDR induces the tyrosine phosphorylation of cytoplasmic signaling proteins that are important to endothelial cell proliferation. In human umbilical vein endothelial cells (HUVECs), tumor necrosis factor (TNF) inhibits the phosphorylation and activation of KDR. The ability of TNF to diminish VEGF-stimulated KDR activity was impaired by sodium orthovanadate, suggesting that the inhibitory activity of TNF was mediated by a protein-tyrosine phosphatase. KDR-initiated responses specifically associated with endothelial cell proliferation, mitogen-activated protein kinase activation and DNA synthesis, were also inhibited by TNF, and this was reversed by sodium orthovanadate. Stimulation of HUVECs with TNF induced association of the SHP-1 protein-tyrosine phosphatase with KDR, identifying this phosphatase as a candidate negative regulator of VEGF signal transduction. Heterologous receptor inactivation mediated by a protein-tyrosine phosphatase provides insight into how TNF may inhibit endothelial cell proliferative responses and modulate angiogenesis in pathological settings.

Angiogenesis, the sprouting of new blood vessels from pre-existing endothelium, is a significant component of embryonic vascular development and differentiation, wound healing, and organ regeneration, but it also contributes to the progression of pathologies that depend on neovascularization, including tumor growth, diabetes mellitus, ischemic ocular diseases, and rheumatoid arthritis (1, 2). Two important steps in blood vessel development, which are induced and regulated by a network of mitogens and cytokines, are degradation and remodeling of the extracellular matrix and the proliferation and migration of capillary endothelial cells. Tumor necrosis factor (TNF),1 transforming growth factor, angiogenin, and prostaglandin E₂, are believed to indirectly affect angiogenesis, whereas other factors that can affect neovascularization, such as the acidic and basic fibroblast growth factors and platelet-derived growth factor, are mitogens for many cell types (1, 2). Perhaps the most important mediator of angiogenesis is vascular endothelial cell growth factor (VEGF) (3–8). VEGF is an endothelial cell-specific mitogen and chemotactic agent that plays a significant role in the physiology of normal vasculature. VEGF is so crucial to the development of the cardiovascular system that the loss of even a single allele is embryo lethal (9).

The first step in VEGF action is binding to either of two cell surface receptor tyrosine kinases, KDR (the human homolog of Flk1) or Flt1 (10–14). Mouse embryos null for Flk1 or Flt1 die in utero, showing that each receptor is essential for development and is biologically active (15, 16). Experiments employing VEGF mutants that selectively bind KDR or Flt1 (17) associate KDR activity with endothelial cell proliferation and survival. The ability of VEGF and placental growth factor (PIGF), a VEGF homolog that binds Flt1 but not KDR, to induce chemotaxis and procagoulant activity suggests that these responses are induced, at least in part, by Flt1 (18–20).

VEGF is involved in wound repair, inflammation, and tumor growth, processes in which cytokines, such as TNF, are also elaborated (21, 22). TNF modulates VEGF action by promoting VEGF production (23) and by affecting the expression of the VEGF receptors (24, 25). Additionally, TNF and VEGF synergistically induce endothelial cell tissue factor (26, 27). The effects of tissue factor are complex as it can inhibit tumor growth through its clot-promoting procoagulant activity but promote angiogenesis by inducing the production of growth regulatory molecules, including VEGF (28, 29). The effects of TNF on new vessel growth and its interplay with VEGF led us to define its effects on VEGF receptor function. The present study shows that TNF acutely inhibits the ability of VEGF to activate KDR and endothelial cell proliferative responses dependent on KDR, among which are MAPK activation and DNA synthesis. This function of TNF does not require down-regulation of KDR expression but rather is mediated by activation of a protein-tyrosine phosphatase and therefore was blocked by sodium orthovanadate. SHP-1 associates with KDR on treat-

1 The abbreviations used are: TNF, tumor necrosis factor; VEGF, vascular endothelial cell growth factor; PIGF, placental growth factor; HUVEC, human umbilical vein endothelial cell; MAPK, mitogen-activated protein kinase.
ment of HUVECs with TNF, identifying this tyrosine phosphatase as a potential mediator of the negative-regulatory effects of TNF on VEGF-initiated proliferative responses. Heterologous receptor inactivation mediated by protein-tyrosine phosphatase activity provides a mechanistic basis for understanding the ability of TNF to modulate VEGF function and angiogenesis.

EXPERIMENTAL PROCEDURES

MATERIALS—Recombinant human TNF and VEGF were gifts from Genentech (South San Francisco, CA). SU5416 was a gift from Sugen Inc. (South San Francisco, CA). PI3’G’F was purchased from R&D Systems (Minneapolis, MN). Horseradish peroxidase-conjugated monoclonal antiphosphotyrosine antibody (BC20) and agarose-conjugated monoclonal antiphosphotyrosine antibody (PY20) were from Transduction Laboratories (Lexington, KY). Polyconal antiserum to Flk-1/KDR and anti-SHP-1 were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Antibodies to phospho-MAPK and MAPK were from New England Biolabs (Beverly, MA).

Cell Culture—HUVECs were grown on 0.2% gelatin-coated tissue culture plates in endothelial cell growth medium (Clonetics, San Diego, CA) in a humidified incubator under 5% CO2 at 37 °C. Prior to acute incubations with TNF, HUVECs were starved in endothelial cell basal medium (Clonetics) containing 1% bovine serum albumin for 5 h and then treated as described.

Immunoprecipitation and Immunoblotting—After treatments, HUVECs were washed twice with ice-cold phosphate buffered saline and lysed by incubation in 50 mM Hepes, pH 7.0, 150 mM NaCl, 10% glycerol, 1.2% Triton X-100, 1.5 mM MgCl2, 1 mM EGTA, 10 mM sodium pyrophosphate, 100 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 0.15 mg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml pepstatin A, and 1 mM sodium orthovanadate for 30 min at 4 °C. Immunoprecipitations using 1–1.5 mg of protein, SDS-polyacrylamide gel electrophoresis, and Western blotting were then conducted as described previously (30).

In Vitro Kinase Assays—Immunoprecipitated KDR was washed twice with lysis buffer and then three times with kinase assay buffer (10 mM Hepes, pH 7.4, 50 mM NaCl, 5 mM MgCl2, 5 mM MnCl2, 0.1 mM sodium orthovanadate). The agarose beads used for precipitations were suspended in kinase assay buffer containing 0.25 μCi/ml [γ-32P]ATP and then incubated at room temperature for 30 min. The beads were washed three times with kinase assay buffer, suspended in Laemmli sample buffer, and heated for 5 min at 100 °C. After SDS-polyacrylamide gel electrophoresis, 32P-labeled KDR was detected by autoradiography.

Binding of 125I-VEGF to HUVECs—125I was coupled to VEGF using IODO-GEN. 125I-VEGF (specific activity, ~60 Ci/g) was separated from NaI by chromatography on Dowex 1X2-200. Monolayers of subconfluent HUVECs grown in 12-well plates were incubated for 16 h at 4 °C in Dulbecco’s modified Eagle’s medium, 1% bovine serum albumin with 125I-VEGF in the absence or presence of a 100-fold excess of unlabeled VEGF. The monolayers were washed twice with ice-cold Dulbecco’s modified Eagle’s medium, solubilized into 1% SDS, and assayed in a gamma counter for specific binding (the difference in radioactive uptake between the absence and presence of excess unlabeled VEGF).

Thymidine Incorporation into DNA—HUVECs in endothelial cell growth medium were seeded into 96-well tissue culture plates at a density of 1 × 103 cells/well. The next day, the growth medium was changed to endothelial cell basal medium (10% dialyzed fetal bovine serum) and growth medium were seeded into 96-well tissue culture plates at a density of 1 × 103 cells/well. The next day, the growth medium was changed to endothelial cell basal medium (10% dialyzed fetal bovine serum) and

RESULTS

To characterize how TNF might affect VEGF signal transduction, we tested its effect on the ability of VEGF to induce tyrosine phosphorylations in HUVECs. As shown in Fig. 1, VEGF induced the tyrosine phosphorylation of numerous proteins in HUVECs. TNF acutely inhibited the ability of VEGF to induce these phosphorylations; thus, inhibition was detected within 30 min, and the magnitude of the blockade increased over 3 h, at which time, the ability of VEGF to induce the tyrosine phosphorylation of many cellular proteins was completely blocked. The inhibition of so many tyrosine phosphorylations led us to postulate that TNF might act on an early event in the VEGF signaling cascade. Because we previously found that such tyrosine phosphorylations and HUVEC proliferation are mediated by KDR (31, 32), we tested whether TNF might impair KDR activation by VEGF. To test this, HUVECs were incubated with 1 nM TNF for various times before being stimulated with 1 nM VEGF for 5 min. TNF was able to inhibit VEGF-induced tyrosine phosphorylation of KDR (Fig. 2A). In vitro kinase assays revealed that the ability of TNF to inhibit the tyrosine phosphorylation of KDR was accompanied by a parallel decrease of KDR activity (Fig. 2B).

Because down-regulation of endothelial cells with TNF has been reported to decrease the mRNA levels for KDR and Flt1 (24). This led us to test whether the acute ability of TNF to inhibit KDR activation could be explained by diminished expression of VEGF receptors on the surface of HUVECs. As shown in Fig. 3, acute treatment of HUVECs with TNF (1–3 h) did not alter the capacity of the cells to specifically bind 125I-VEGF. Thus, the inhibitory effect of TNF on KDR activity occurred in the absence of and/or prior to any change in the amount of KDR available to bind VEGF.

Because down-regulation of receptor expression did not account for diminished KDR activity in TNF-treated HUVECs, we tested for the induction of tyrosine phosphatase activity by TNF. As illustrated by Fig. 4, sodium orthovanadate (a tyrosine phosphatase inhibitor) increased the basal level of tyrosine phosphorylation in HUVECs and impaired the ability of TNF to block tyrosine phosphorylation reactions induced by VEGF. The data in Fig. 5 show that sodium orthovanadate reversed the capacity of TNF to affect VEGF-stimulated tyrosine phosphorylation of KDR. These results suggest that a TNF-activated protein-tyrosine phosphatase impairs the ability of VEGF to activate KDR.

An experiment using an antibody that exclusively recognizes dually phosphorylated (activated) MAPKs evaluated the following points: 1) whether VEGF mediates activation of this enzyme system by acting through KDR or Flt1; 2) whether TNF would affect such activation; and, 3) if so, whether the TNF effect was mediated by a tyrosine phosphatase. To determine which VEGF receptor transduces the signal necessary for MAPK activity, HUVECs were treated with either VEGF, which activates KDR and Flt1, or PI3’G’F, which only activates Flt1 (18–20). As shown in Fig. 6A, VEGF, but not PI3’G’F, induced MAPK (ERK1 and ERK2) phosphorylation. This result supports the conclusion that MAPK is activated by signaling through KDR. Treatment of cells with TNF impaired the ability of VEGF to activate MAPK, and this effect was blocked by sodium orthovanadate (Fig. 6B). These observations show that
TNF blocks the induction of the MAPK signaling cascade that initiates at KDR by activating a protein-tyrosine phosphatase.

Finally, we evaluated a functional consequence of the TNF inhibition of KDR activation by assaying de novo DNA synthesis, an index of cell proliferation. VEGF, but not PlGF, induced thymidine incorporation by HUVECs, and this response was abrogated by SU5416, a specific KDR antagonist (33) (Fig. 7A). These observations show that KDR is the mediator of the endothelial cell proliferative response to VEGF. This observation prompted experiments to test whether TNF would inhibit VEGF-promoted endothelial cell proliferation and, if so, whether it would utilize a protein-tyrosine phosphatase. As shown in Fig. 7B, VEGF augmented the incorporation of thymidine into DNA about 2.5-fold, and TNF antagonized this response. Sodium orthovanadate increased thymidine incorporation into DNA and antagonized the ability of TNF to impair DNA synthesis stimulated by VEGF. These observations show that TNF and VEGF are antagonists, insofar as endothelial cell proliferation is concerned, and that TNF mediates its growth inhibitory activity through activation of a protein-tyrosine phosphatase.

Two protein-tyrosine phosphatases, SHP-1 and SHP-2, have been reported to associate with KDR (34). Although SHP-1 and SHP-2 have similar domains, and considerable sequence identity, the former functions as a negative regulator and the latter as a positive regulator of proliferative signals emanating from...
were treated with vehicle, 0.1 nM VEGF, or 1 nM TNF for 16 h or were
incorporation into DNA was then assayed. A, VEGF.
incorporation into DNA was then assayed. 1 h at 37 °C and then with 1 n M VEGF or PlGF for 16 h. Thymidine
treated with sodium orthovanadate in the absence or presence of 0.1 nM
of TNF on KDR signal transduction. SHP-1 as a candidate mediator for the negative-regulatory role
inhibition of a protein-tyrosine phosphatase with KDR and identify
a substantial increase in the amount of SHP-1 that bound to
HUVECs with TNF for 3 h prior to VEGF treatment resulted in
each induced association of SHP-1 with KDR. Pretreatment of
exposure to VEGF. As illustrated in Fig. 8, VEGF and TNF
cubated with VEGF or TNF or pretreated with TNF prior to
immunoprecipitated from untreated HUVECs or HUVECs in-
might act, at least in part, through SHP-1. Thus, KDR was
expression of major histocompatibility class I antigens and
receptor protein-tyrosine kinases. Our observations implicat-
ing a protein-tyrosine phosphatase in the negative regulatory
effect of TNF on KDR activity led us to determine whether it
might act, at least in part, through SHP-1. Thus, KDR was
immunoprecipitated from untreated HUVECs or HUVECs in-
cubated with VEGF or TNF or pretreated with TNF prior to
exposure to VEGF. As illustrated in Fig. 8, VEGF and TNF
each induced association of SHP-1 with KDR. Pretreatment of
HUVECs with TNF for 3 h prior to VEGF treatment resulted in
a substantial increase in the amount of SHP-1 that bound to
KDR. These observations show that TNF potentiates association
of a protein-tyrosine phosphatase with KDR and identify
SHP-1 as a candidate mediator for the negative-regulatory role of
TNF on KDR signal transduction.

DISCUSSION

TNF, a potent multifunctional hormone produced predomi-
nantly by activated macrophages, is cytotoxic to transformed
cells and induces the hemorrhagic necrosis and regression of
tumors in animals (21). In the endothelium, TNF increases the
expression of major histocompatibility class I antigens and
stimulates the production of chemotactic factors and other cy-
tokines to promote immunity (35, 36). Additionally, TNF in-
duces expression of cell adhesion molecules, such as ICAM-1 and E-selectin, on endothelial cells. The acquisition of surface
adhesive properties is important for inflammatory processes
dependent on transendothelial cell migration of neutrophils
and lymphocytes. TNF also alters the barrier function of the
endothelium and increases the permeability of endothelial cell
monolayers to macromolecules.

TNF can promote or inhibit angiogenesis. In the rabbit cor-
nnea (37) and the chick chorioallantoic membrane (38), TNF
induces new vessel growth, possibly by stimulating stromal or
inflammatory cells to produce proangiogenic factors. TNF in-
duces production of B61 (39), the ligand for the Eck receptor
tyrosine kinase, fibroblast growth factor (40), VEGF (23) and
platelet activating factor (41), which are all angiogenic. By
inducing the expression of urokinase-type plasminogen activa-
tor, TNF cooperates with basic fibroblast growth factor and
VEGF to form capillary-like tubular structures in three dimen-
sional fibrin matrices (42). TNF is cytotoxic to endothelial cells,
an activity antagonized by VEGF (43), and impairs tumor
growth by inducing procoagulant activity in synergy with
VEGF, thereby blocking access of the tumor to a blood supply
(26–29).

The phenotypes of the VEGF receptor knockout animals and
studies with endothelial cell cultures suggest that both VEGF
receptors are functional, but they mediate responses through
distinct mechanisms (15–20). In KDR/Flk1 null mutant mice,endothelial and hematopoietic cell development is impaired
(15), whereas in Flt1 null mutant mice, endothelial cells over-
grow and blood vessels are disorganized (16). Flt1 tyrosine
kinase-deficient homozygous mice develop normal blood vessels
and survive, indicating that Flt1 lacking the tyrosine kinase
domain is sufficient for normal angiogenesis and development
(44). In contrast to the unique, tyrosine kinase-independent
mechanism through which Flt1 may function, we previously
showed that the kinase activity of KDR is essential to the
tyrosine phosphorylation and activation of cytoplasmic signal-
proteins that contain Src homology 2 domains and associ-
ated this process with endothelial cell proliferation. Further-
more, activation of the Akt serine threonine kinase, a cell
survival factor, and MAPK, which plays an obligate role in
endothelial cell proliferation, requires activation of KDR, as
does DNA synthesis (31, 45).

The present study shows that TNF inhibits signal transduc-
tion through KDR and consequently affects all of the signaling
events and cellular responses elicited by this receptor. TNF ac-
cutely inhibits the ability of VEGF to activate KDR and tyro-
sine phosphorylations induced by KDR, which are important to
VEGF-induced HUVEC proliferation (30). TNF also blocks
VEGF-induced MAPK activation and DNA synthesis. Thus,
TNF negatively regulates the ability of VEGF to induce signal-
ing events that originate at KDR and culminate in HUVEC
proliferative responses.

Two recent studies show that TNF may modulate VEGF
receptor expression. It has been reported that TNF down-reg-

![Fig. 7. TNF inhibits endothelial cell proliferation induced by VEGF. A. HUVECs were treated with vehicle or 1 μM SU5416 (SU) for 1 h at 37 °C and then with 1 nM VEGF or PlGF for 16 h. Thymidine incorporation into DNA was then assayed. Con, control. B. HUVECs were treated with vehicle, 0.1 nM VEGF, or 1 nM TNF for 16 h or were treated with sodium orthovanadate in the absence or presence of 0.1 nM VEGF, 1 nM TNF, or 0.1 nM VEGF and 1 nM TNF for 16 h. Thymidine incorporation into DNA was then assayed.](http://www.jbc.org/Downloaded from http://www.jbc.org/)
TNF Inhibits Endothelial Cell Proliferation

TNF activates the expression of the mRNAs for both VEGF receptors through a transcriptional mechanism; maximal (70%) inhibition of mRNA expression occurred within 24 h of exposure of HUVECs to TNF (24). However, a second study concludes that TNF up-regulates the expression of KDR and its co-receptor neuropilin-1 through a transcriptional mechanism (25). The former study suggests that TNF exerts antiangiogenic activity through down-regulation of VEGF receptor expression, whereas the latter investigation concludes that TNF exerts proangiogenic activity through up-regulation of VEGF receptor expression.

We have assayed the effects of TNF on the capacity of HUVECs to specifically bind 

$^{125}$I-VEGF and observed a modest decrease in VEGF receptor expression, but only after days of cellular exposure to TNF. This effect did not occur rapidly enough to account for the ability of TNF to impair acute events induced by VEGF, such as KDR activation, tyrosine phosphorylation of KDR substrates, or MAPK activation. Even after 2 days of TNF treatment, the diminished binding capacity of HUVECs was not of sufficient magnitude to account for the capacity of TNF to abrogate VEGF-promoted DNA synthesis. Our observations suggest that although VEGF receptor mRNA levels may be changed by chronic cellular exposure to TNF, such changes are not necessarily reflected at the protein level. We also found that the effect of TNF on VEGF receptor expression varied with HUVECs from different umbilical cords. Some cell populations were more refractory to TNF-induced down-regulation of VEGF receptor expression than others, leading us to propose that the way in which such primary cells are cultured, the individual source of the cells, and other experimental variables may account for the different effects of TNF on VEGF receptor expression.

Tyrosine phosphorylations induced by receptor tyrosine kinases promote cellular proliferative responses (46). Protein-tyrosine phosphatases counterbalance the activities of tyrosine kinase receptors (46). The present study provides evidence that TNF impairs VEGF activity by activating a protein-tyrosine phosphatase. Thus, the ability of TNF to block KDR activation and VEGF-induced tyrosine phosphorylations, MAPK activation, and DNA synthesis was reversed by sodium orthovanadate.

SHP-1 (also called PTP1C/HCP/SH-PTP1), a cytoplasmic protein-tyrosine phosphatase that contains two Src homology 2 domains in its N-terminal region, is predominantly expressed in hematopoietic cells (47–51). SHP-1 interacts with many activated cytokine, growth factor, and antigen receptors and appears to play a negative regulatory role in signal transduction pathways by dephosphorylating receptors or receptor substrates to which it binds (52–57). The role of SHP-1 as a negative regulator of hematopoietic signal transduction is consistent with the multiple defects found in a naturally occurring mouse mutant, motheaten, which expresses inactive SHP-1 and is characterized by overgrowth of hematopoietic cells (58). SHP-1 (47–50) and KDR/Flik1 are expressed by hematopoietic (59) as well as endothelial cells, cell types that share a common lineage (60). Experiments were conducted to determine whether SHP-1 might be used by TNF to affect the VEGF/KDR signaling system. The demonstration that VEGF and TNF promote association of SHP-1 with KDR identifies a putative mechanism through which KDR/Flik1 action can be attenuated by homologous and heterologous factors. Further experimentation will be required to determine whether SHP-1 is unique or is one of a number of phosphatases that may affect angiogenic signaling initiated by KDR.

The ability of one receptor system to communicate with and impair the activity of a second, a process of heterologous receptor inactivation, identifies a mechanism through which TNF can negatively regulate mitogen induced cell proliferation and thereby modulate angiogenesis. As the capacity of TNF to impair insulin-induced glucose utilization and induce a state of insulin resistance is inhibited by sodium orthovanadate (61), protein-tyrosine phosphatases and the process of heterologous receptor inactivation are likely to be of generalized importance to TNF action.

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*J. Biol. Chem.* 2000, 275:11216-11221. 
doi: 10.1074/jbc.275.15.11216

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