TUMOR NECROSIS FACTOR–MEDIATED RELEASE OF PLATELET-DERIVED GROWTH FACTOR FROM CULTURED ENDOTHELIAL CELLS

BY KATHERINE A. HAJJAR,*‡ DAVID P. HAJJAR,§ ROY L. SILVERSTEIN,‡ AND RALPH L. NACHMAN‡

*From the Divisions of Hematology-Oncology, Departments of *Pediatrics and ‡Medicine, the Departments of §Pathology and Biochemistry, and the Specialized Center of Research in Thrombosis, Cornell University Medical College, New York, 10021*

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Tumor necrosis factor (TNF)[1] is a 17,500-M_r polypeptide produced by macrophages and related monocyte-like cell lines that induces hemorrhagic necrosis of murine sarcomas (1). In vitro, TNF also binds with high affinity (K_d 200–610 pM; number of binding cites per cell [B_{max}] 2,200–7,500) to a variety of cell lines (2–4), affects proliferation of normal and transformed cells (5), and modulates the hemostatic properties of cultured endothelial cells (EC) (6, 7). TNF also stimulates production of IL-1 in both resting macrophages (8) and EC (9, 10).

IL-1 promotes growth of fibroblasts and mitogen-stimulated lymphocytes, but not vascular smooth muscle cells (SMC) grown under low-serum conditions (11). Recent evidence, however, suggests that IL-1 promotes EC production of a factor that stimulates growth of bovine aortic SMC (12). EC constitutively produce both platelet-derived growth factor (PDGF) (13–16), and EC-derived growth factor (17). Release of PDGF, in particular, is significantly enhanced by agents that stimulate production of IL-1 such as phorbol esters and endotoxin (18), and also by coagulation factor X_a (19) and thrombin (20).

The present study was designed to examine the effect of TNF on EC production of SMC mitogenic activity. EC challenged with TNF released SMC mitogenic activity in a saturable, concentration- and time-dependent fashion. Release of SMC mitogenic activity was totally inhibited by antibody to platelet PDGF. Similar mitogenic activity was also released upon stimulation of EC with IL-1. The stimulatory effect of TNF was largely abrogated by pretreatment of cells with actinomycin D, and Northern blot analysis revealed a 2.5-fold increase in c-*sis* hybridizable RNA after stimulation with TNF. Thus, we have demonstrated

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Address correspondence to Dr. Katherine A. Hajjar, Div. of Hematology-Oncology, Dept. of Medicine, Cornell University Medical College, 1300 York Ave., New York, NY 10021.

[1] *Abbreviations used in this paper:* B_{max}, number of binding sites per cell; EC, endothelial cells; EC_{50}, agonist concentration resulting in half-maximal response; LDH, lactate dehydrogenase; PDGF, platelet-derived growth factor; $PDGF_c$, platelet-derived growth factor–like molecules; SMC, smooth muscle cells; TNF, tumor necrosis factor.

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that TNF stimulates release from EC of PDGF-like molecules ($PDGF_c$). This effect may be partially mediated by EC release of autocrine IL-1, or may involve other mediators.

Materials and Methods

Endothelial Cell Culture. Human umbilical vein EC were cultured according to published methods (21–24). Briefly, early passage (P_2–P_5) EC were grown to confluency on gelatin-coated 24-well tissue culture plates (Falcon Labware, Oxnard, CA). The cells were plated at a density of 4×10^4 cells per well, reached confluence by 48–72 h, and were used within 3–5 d of plating. Culture medium consisted of Medium 199 (Grand Island Biological Co., Grand Island, NY) containing 50 U/ml penicillin, 50 μg/ml streptomycin, 2.5 μg/ml fungizone, 2.0 mM L-glutamine, 20 μg/ml endothelial cell growth factor (Meloy Laboratories, Inc., Springfield, VA), 90 μg/ml porcine intestinal mucosa heparin (170 USP units/mg), and 20% pooled human serum. For cell passage, monolayers were washed with Hepes-buffered saline, and incubated for 5 min in the same containing 0.05% Type I collagenase, 0.01% EDTA, and 0.25% BSA at 37°C. After addition of an equal volume of serum-containing medium, the cells were washed once, resuspended, and plated. Cells were grown at 37°C in a humidified atmosphere with flowing 5% CO_2/95% air.

SMC Culture. SMC were cultured from bovine arteries by the procedures of Ross (25) and Hajjar et al. (26). Briefly, early passage (P_2–P_6) SMC were grown from explants on Falconware in MEM (Gibco Laboratories, Grand Island, NY) containing 50 U/ml penicillin, 50 μg/ml gentamicin, 2.5 μg/ml fungizone, 2.0 mM L-glutamine, and 20% FCS (heat-inactivated). For routine cell passage, cultures were washed, removed from the plates, and replated (25, 26). Cells were propagated in a humidified atmosphere containing 5% CO_2/95% air at 37°C.

Treatment of Endothelial Cells with TNF and IL-1. Confluent EC were washed twice with PC-1 medium (Ventrex Laboratories Inc., Portland, ME), and treated for various periods of time (2–24 h) with PC-1 containing various concentrations (0–3.0 nM) of recombinant human TNF (Genentech, South San Francisco, CA) or recombinant IL-1β. IL-1β was a gift from Dr. Charles Dinarello, Tufts University School of Medicine, Boston, MA. The resulting postculture medium was harvested and added immediately to semiconfluent bovine SMC previously washed twice with PBS. In some experiments, polymyxin B (10 μg/ml; Sigma Chemical Co., St. Louis, MO) was added to exclude possible endotoxin effects (27).

Treatment of Endothelial Cells with Actinomycin D. Cells were preincubated with 0.16 μM actinomycin D (Sigma Chemical Co.) for 30 or 120 min (28), and then washed three times with PC-1 before treatment with TNF as described above.

Mitogenesis Assay. Mitogenic activity of arterial SMC was measured as described extensively by DiCorleto and Bowen-Pope (29). All samples were assayed in quadruplicate; standard deviations averaged 10% of the mean.

Lactate Dehydrogenase (LDH)-release Assay. LDH activity in postculture medium was measured using a commercially available kit (Sigma LD-L; Sigma Chemical Co.), according to the method of Wacker et al. (30). Release of LDH from EC was determined for postculture medium samples after treatment of cells with PC-1 alone, PC-1 plus TNF, or sonication at 4°C (Heat Systems-Ultrasonics, Inc., Farmingdale, NY; power 4, duty cycle 40%, 10-s continuous pulse). Linearity of the assay was verified using normal and high LDH standards provided by the manufacturer.

Immunoinhibition Studies. Postculture medium from TNF-stimulated cells was preincubated with rabbit anti–human platelet PDGF. This antibody, a gift from Dr. Thomas Deuel, Washington University, St. Louis, MO, was obtained from rabbits immunized with purified human PDGF and judged monospecific in immunodiffusion analyses against platelet lysates, serum, and plasma (31). Additional samples of postculture medium were preincubated with anti-EC antibody (24), or nonimmune goat serum (CooperBiomedical, Inc., Malvern, PA) as described in the figure legends. In some experiments, EC were stimulated with TNF in the presence of various antibodies, including rabbit anti–recom-

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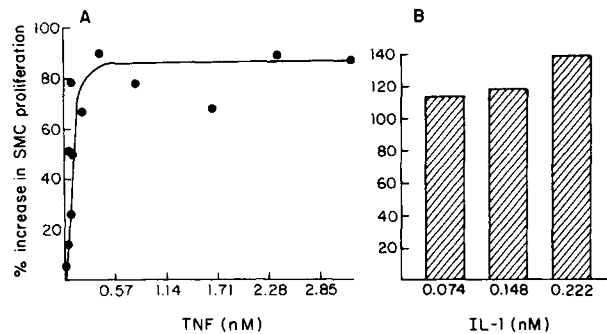

FIGURE 1. Effect of various doses of TNF and IL-1 on the release of mitogenic activity by EC. EC, grown to confluency in 24-well plates, were washed twice in PC-1 and incubated in PC-1 containing increasing amounts of rTNF or IL-1 for 18 h at 37°C. EC postculture medium was then harvested and applied directly (0.8 ml/well) to washed semiconfluent SMC. SMC were incubated for 18 h (37°C) in the presence of [^{3}H]thymidine (sp act, 18.2 Ci/mM; 1 μCi/10,000 cells; 50,000 cells/well). The SMC were then washed, trypsinized, and the TCA-precipitable material was counted as described in Materials and Methods. (*A*) Effect of TNF. Each point represents the mean of five samples stimulated with various concentrations of TNF (0.013–3.18 nM). All samples were normalized for cell number. Control cells represented incorporation of 65,921 ± 6,738 cpm ± SE of [^{3}H]thymidine per well. (*B*) Effect of IL-1. Each bar represents the mean of five samples stimulated with three different concentrations of IL-1 (0.074, 0.148, and 0.222 nM). Results are expressed as percent increase over control (0% or 30,241 ± 9,450 cpm/well ± SE).

binant IL-1β, a gift from Dr. Charles Dinarello, Tufts University School of Medicine, Boston, MA. This antibody contained a gamma globulin concentration of 16 mg/ml and neutralized IL-1β at a concentration of 1:100 to 1:1,000. It was inactive against IL-1α (Dinarello, C., personal communication).

Northern Blot Analysis. Human umbilical vein EC (P_4) were grown to confluency in 75-cm^2 flasks (Falcon Labware). The cells were washed twice with PC-1, and then treated with either TNF (25 ng/ml in PC-1) or PC-1 alone and incubated for 18 h at 37°C in a 5% CO_2 humidified incubator. The cell monolayers were then washed three times with Hepes-buffered saline and detached with 0.05% Type I collagenase, 0.01% EDTA, and 0.25% BSA (5 min, 37°C). Cells in the resulting single-cell suspension (total number of EC ~22.5 × 10^6, treated and control) were washed three times with PBS. Total cellular RNA was prepared from these pellets by extraction in 4 M guanidium isothiocyanate, 5 mM sodium citrate, 0.1 M 2-ME, and 0.5% sarkosyl followed by centrifugation through CsCl (32, 33). Total cellular RNA (14 μg) from TNF-treated or control cells was first denatured with formaldehyde, electrophoresed in a 1% agarose gel, and then transferred to nitrocellulose filters (33). In addition, 30 μg of total cellular RNA isolated from the human melanoma cell line A875 was electrophoresed and transferred. Hybridization was carried out at 42°C for 15 h in 50% formamide, 1× Denhart's solution, 100 μg/ml salmon sperm DNA, 10% Dextran sulfate, 0.75 M NaCl, 0.075 M sodium citrate, 0.05 M NaH_2PO_4 H_2O, 5 mM EDTA, pH 7.4, using a ^{32}P-labeled c-*sis* DNA probe nick-translated to a sp act of 3.9 × 10^7 dpm/μg. The c-*sis* probe used was a 1.6-kb Bam HI human genomic fragment designated P1 335 and was generously provided by F. Wong-Staal and R. Gallo, National Institutes of Health, Bethesda, MD (34). The filter was then washed in 0.3 M NaCl, 0.03 M sodium citrate, 0.1% SDS, pH 7.0, at 68°C and autoradiograms were obtained after 5, 14, and 24 h of exposure. The relative intensity of the *sis* probe hybridizing band was estimated by laser densitometric scanning of the autoradiograms.

Results

Incubation of SMC with postculture medium from EC stimulated with various doses of TNF for 18 h led to a dose-dependent and saturable increase in incorporation of [^{3}H]thymidine into SMC (Fig. 1*A*). This stimulatory effect became maximal at a TNF dose of ~10 ng/ml (570 pM) where the rate of SMC proliferation was increased 90% over controls. Half-maximal stimulation was reached at a TNF dose of ~2 ng/ml, resulting in an EC_{50} for this interaction of

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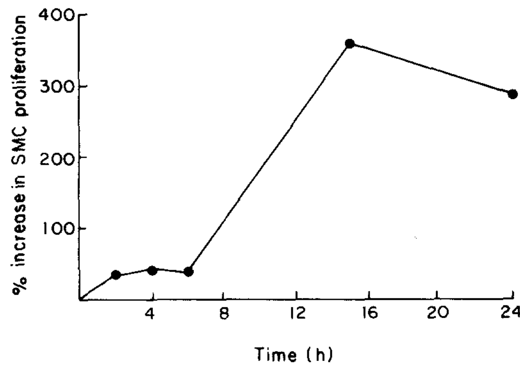

FIGURE 2. Time course of appearance of mitogenic activity in postculture medium from TNF-stimulated EC. EC were stimulated with TNF (0.08 nM) for various periods of time as indicated on the abscissa. EC postculture medium was assayed for mitogenic activity as described in the legend to Fig. 1. Each point represents the mean of quadruplicate samples. Results are expressed as percent increase over control (0% or 82,451 ± 7,601 cpm/well ± SE).

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~114 pM. This increase in [^{3}H]thymidine incorporation was paralleled by cellular proliferation as cell counts rose from a mean of 52,000 ± 4,004 (SE, n = 4) to a mean of 147,500 ± 14,362 (SE, n = 4) after 18 h of treatment with TNF (10 ng/ml). A similar effect was achieved by stimulating EC with IL-1 (60–250 pM), which resulted in a 100% increase in the SMC proliferation rate (Fig. 1*B*). These results suggest a high affinity interaction between these monokines and the endothelial cell involving one or more specific binding sites.

The time course for the production of mitogenic activity by TNF stimulation of EC is shown in Fig. 2. A slight (34.7–43.5%) increase in mitogenic activity was detectable after 2–6 h of TNF stimulation. This was followed by a major peak of activity at 15 h, which represented a tripling of baseline activity and persisted for up to 24 h. Thus, the initial small increase in mitogenic activity might reflect constitutive release of $PDGF_c$ while it appears that an initial lag period may be required for full expression of the stimulatory effect of TNF.

To determine whether the mitogenic effect of EC postculture medium could be attributed to passive transfer of TNF, the direct effect of TNF on SMC mitogenesis was studied. Only a slight increase in mitogenesis (17%) was observed at concentrations of 53.2 ng/ml (3.0 nM). This dose was ~10 times that used routinely to stimulate EC that responded with a 100–300% increase in SMC proliferation. Postculture medium from nonstimulated EC showed minimal enhancement of [^{3}H]thymidine incorporation into dividing SMC (32 ± 6%, SE), even when added to SMC in the presence of TNF (10 ng/ml) (34.4 ± 4%, SE). IL-1 alone had no mitogenic effect when added directly to SMC. In addition, polymyxin B had no effect on SMC mitogenesis in response to postculture medium from TNF-stimulated EC.

Immunoinhibition studies were carried out to examine the mechanism by which postculture medium from TNF-stimulated EC promoted SMC proliferation (Fig. 3). Postculture medium from nonstimulated EC enhanced SMC mitogenesis by 29.4%, while postculture medium from TNF-stimulated cells produced a 96.8% increase in mitogenic activity. When TNF-stimulated postculture medium was preincubated with anti-platelet PDGF, its mitogenic activity was completely abrogated (0%) and cellular proliferation returned to the baseline level. Antibody to intact EC and nonimmune goat serum failed to significantly inhibit mitogenic activity (94.9 and 123.3% of control, respectively) produced

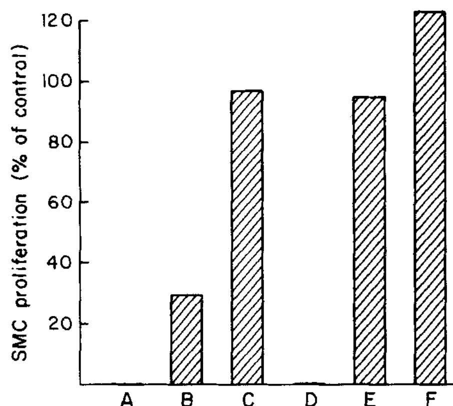

FIGURE 3. Immunoinhibition of mitogenic activity by anti-$PDGF_c$. Semiconfluent SMC were incubated with preculture medium (40,040 ± 1,965 cpm/well ± SE) (*A*) or postculture medium from nonstimulated EC (*B*) or from EC stimulated with TNF (1.43 nM) (*C–F*). Postculture medium from stimulated EC was pretreated with either anti-platelet PDGF (*D*), anti-endothelial cell antiserum (*E*), or nonimmune goat serum (*F*) at dilutions of 1:200 for 1 h at 37°C. SMC mitogenesis was measured by uptake of [^{3}H]thymidine as described in the legend to Fig. 1. Each bar represents the mean of four samples normalized for cell number.

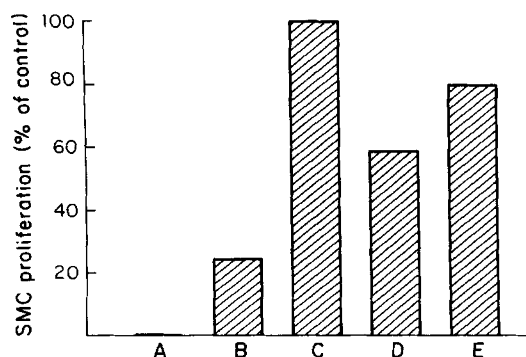

FIGURE 4. Effect of anti-IL-1 on TNF induction of mitogenic activity. Semiconfluent SMC were incubated (37°C, 18 h) with preculture medium (231,212 ± 27,638 cpm/well ± SE) (*A*), postculture medium from nonstimulated EC (*B*), postculture medium from EC stimulated with TNF (0.40 nM) alone (*C*) or TNF in the presence of either anti-IL-1 (*D*) or anti-EC (*E*) antibody. All antisera were diluted 1:200 (vol/vol) in serum-free medium and used immediately. SMC mitogenesis was measured as described in the legend to Fig. 1. Each bar represents the mean of four samples normalized for cell number.

by TNF stimulation. In similar experiments, mitogenic activity in postculture medium from nontreated EC was partially (68.8%) inhibited by treatment with anti-PDGF at a dilution of 1:200 (49,108 ± 17,518 cpm/well ± SE, $n = 4$) as compared with treatment without antibody (157,465 ± 11,919 cpm/well ± SE, $n = 4$) or with control (anti-EC) antibody (103,530 ± 23,660 cpm/well ± SE, $n = 4$). Anti-PDGF also blocked the mitogenic activity induced by IL-1-stimulated EC (data not shown). Thus, PDGF-like molecules appeared to play a major role in stimulating SMC proliferation.

Treatment of EC with TNF in the presence of anti-IL-1 enhanced SMC mitogenic activity to 58.9% (± 2.2%, SE, $n = 4$) over control as compared with a 97.4% (± 4.1%, SE, $n = 4$) increase over control for TNF alone (Fig. 4). When EC were treated with TNF in the presence of an irrelevant antibody (anti-EC), the SMC proliferative effect in the resulting postculture medium represented an 80.9% (± 7.6%, SE, $n = 3$) increase over control. These results suggested that elaboration of SMC proliferative activity by TNF-stimulated EC may have been partially mediated by TNF-induced secretion of autocrine IL-1 by the endothelial cell.

The effect of inhibition of RNA synthesis on production of mitogenic activity by TNF-stimulated EC was also examined. Preincubation of EC with 0.16 μM actinomycin D for 120 min blocked all but 26.3% of mitogenic activity released by TNF-treated EC, while after 30 min of actinomycin D blockade, release of

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TABLE I

Effect of Actinomycin D on TNF-induced $PDGF_c$ Release by EC

Culture condition	Mitogenic activity	Amount above control
		%
ECCM	30,264 ± 5,719	0
ECCM (TNF)	58,974 ± 9,803	94.9
ECCM (TNF, Act D, 30′)	49,797 ± 3,659	64.6
ECCM (TNF, Act D, 120′)	37,824 ± 3,818	25.0

EC were prepared as described in the legend to Fig. 1. After two washes with PC-1, EC monolayers were preincubated with actinomycin D (0.16 μM in PC-1) for 30 min or 120 min as indicated. Culture medium was then removed, and the cells were washed twice with PC-1, and incubated with TNF (0.08 nM) in PC-1 or PC-1 alone for 18 h. The resulting EC postculture medium (ECCM) was then added to proliferating SMC and its mitogenic activity was assessed. Activity is expressed as [^{3}H]thymidine incorporation (dpm ± SE, $n = 4$), and as percent above control (ECCM).

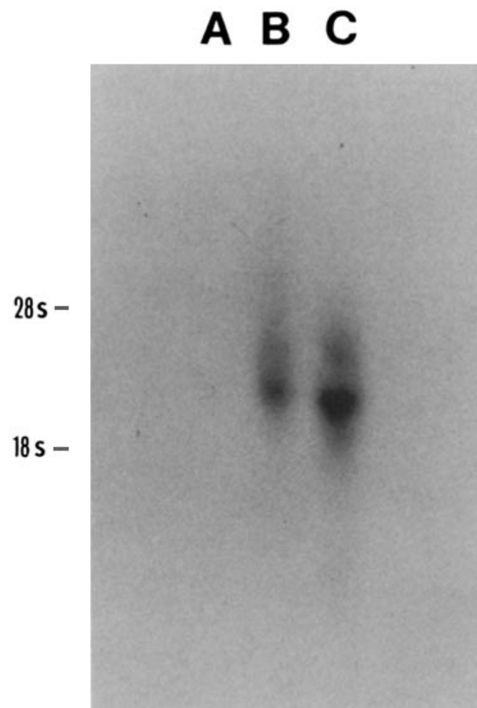

FIGURE 5. Level of c-*sis* mRNA in TNF-treated EC. RNA samples were electrophoresed in 1% agarose in the presence of formaldehyde, transferred to nitrocellulose, and then hybridized with a ^{32}P-labeled c-*sis* DNA probe. Lanes are (*A*) 30 μg total cellular RNA extracted from human melanoma A935 cells, (*B*) 14 μg total cellular RNA from control EC, and (*C*) 14 μg total cellular RNA from TNF-treated EC. Molecular weight markers are indicated on the left as the position of migration of 18S (1.9 kb) and 28S (5.1 kb) species.

mitogenic activity by TNF was 68.0% of control (Table I). This inhibitory effect did not reflect cell death or injury since neither exclusion of trypan blue nor release of LDH in actinomycin D–treated samples was elevated over untreated controls (data not shown). In addition, Northern blot analysis was carried out. Both TNF and control EC showed a strong band at ~3.5–3.7 kb hybridizing with the specific *sis* probe (Fig. 5). Melanoma cells did not express the *sis* gene. Densitometric analysis revealed a 2.5-fold increased expression of the *sis* gene

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by the TNF-treated cells compared with control. In addition, a minor band at ~4.3 kb was also noted in the TNF-treated samples; the significance of this band is currently unknown.

Discussion

The present data indicate for the first time that recombinant human TNF stimulates release of an EC factor that is mitogenic for bovine SMCs and that is completely neutralized by antibody to platelet PDGF. Elaboration of $PDGF_c$ appears to be dose related and saturable, with half-maximal stimulation occurring at ~2 ng/ml (~114 pM) TNF, suggesting the presence of discrete, high-affinity binding sites (Fig. 1*A*) for this peptide. In agreement with the literature (12), a similar effect was achieved by stimulation with rIL-1 at 1–4 ng/ml (60–240 pM) (Fig. 1*B*).

The mitogenic activity in postculture medium from TNF-stimulated EC can be completely neutralized by antibody to platelet PDGF (Fig. 3). Thus, $PDGF_c$ are apparently released by EC in response to TNF challenge. Although freshly isolated adult rat SMC produce $PDGF_c$ during their first few days in culture (35, 36), the rate of production of $PDGF_c$ declines upon further subculture, suggesting that the mitogenic effect in these passaged cells is not due to autocrine stimulation by endogenous $PDGF_c$ from SMC. TNF alone at doses up to 53 ng/ml had no significant mitogenic effect on bovine SMC. Therefore, the observed proliferation cannot be attributed to passive transfer of TNF in the postculture medium.

Time-course studies (Fig. 2) suggested that the major release of mitogenic activity occurs only after 6 h of TNF stimulation. Earlier time points showed only a slight (35–45%) increase in activity, which probably reflects constitutive production of $PDGF_c$ (16, 29). This finding raises the question of whether a series of intermediate events might be required before release of $PDGF_c$ in response to TNF occurs. Upon stimulation with thrombin (20), human EC released $PDGF_c$ in an approximately linear fashion for up to 24 h; significant $PDGF_c$ release was first detected at 1.5 h. Bovine EC elaborate $PDGF_c$ after challenge with factor X_a in an apparently biphasic fashion over 16 h, with first appearance of $PDGF_c$ within as early as 2 min (19). These various release patterns may reflect differential modes of agonist processing by the endothelial cell, and suggest that these agents may act through different mechanisms.

Release of $PDGF_c$ from human umbilical vein EC was associated with PDGF-specific RNA synthesis since c-*sis* hybridizable RNA was increased 2.5-fold after TNF stimulation of EC (Fig. 5), and since release of proliferative activity was substantially inhibited by preincubating cells with actinomycin D (Table I). In cultured adipocytes, TNF is known to downmodulate the activity of differentiation-specific enzymes such as lipoprotein lipase by acting at the level of transcription (37–40). Similarly, in dermal fibroblasts and synovial cells, TNF enhances production of prostaglandin E_2 and collagenase (41). In addition, the recently reported effects of TNF on the endothelial cell, such as induction of IL-1 secretion (9, 10), increase in tissue factor activity (6, 7), and promotion of neutrophil adhesion (42) also appear to reflect new synthesis of mRNA and hence protein. It now appears that the observed release of $PDGF_c$ from EC may

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represent an additional example of gene expression that is controlled by this inflammatory mediator.

TNF is known to bind to human umbilical vein EC (apparent K_d ~125 pM) and to augment release of IL-1 through a protein synthesis–dependent mechanism (9, 10). Immunoinhibition studies reported herein (Fig. 4) indicate that TNF-induced release of $PDGF_c$ by EC was partially blunted by antibody to IL-1, suggesting a role for this monokine. On the other hand, the release of mitogenic activity was also clearly associated with a several-fold increase in synthesis of mRNA specific for $PDGF_c$. Therefore, it appears that $PDGF_c$ release may reflect a dual mechanism, involving both secretion of autocrine IL-1 and *de novo* synthesis of $PDGF_c$.

In the endothelial cell, TNF is an important inducer of IL-1 secretion (9, 10). IL-1, in turn, appears to feed back upon the cell, thereby modulating its role in hemostasis by promoting its procoagulant properties (43, 44), and by inducing synthesis of plasminogen activator–inhibitor (45–47). In addition, both monokines promote leukocyte adhesion to endothelial monolayers (42, 48). Our data suggest an additional role for TNF, namely release of EC-derived $PDGF_c$. Thus, through a series of cell–cell interactions, the release of key monokines may play an important role in vessel wall biology, not only by favoring the maintenance of fibrin, but also by modulating vascular tone (49) and SMC proliferation. In this way, TNF and IL-1 may play a role in both normal and abnormal responses to vascular inflammation, wound healing, and atherogenesis.

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Summary

Platelet-derived growth factor (PDGF) is a 30,000-M_r glycoprotein that is chemotactic and mitogenic for vascular smooth muscle cells (SMC). It is also a potent vasoconstrictor. In the present study, we found that the macrophage-derived polypeptide, tumor necrosis factor (TNF), releases a factor from human umbilical vein endothelial cells (EC) that is mitogenic for SMC. Postculture medium from TNF-stimulated EC induced a 90% increase in mitogenesis as compared with controls. This effect was half-maximal at a TNF dose of 114 pM, reflected a 2.5-fold increase in PDGF-specific mRNA synthesis, and peaked at 15 h of TNF stimulation. Mitogenic activity was completely abrogated by preincubation of postculture medium with antibody to platelet PDGF. Stimulation of EC with IL-1 (60–240 pM) led to the release of similar mitogenic activity. Thus, in addition to its effects on the hemostatic and adhesive properties of EC, TNF also promotes release of PDGF, which may serve to modulate proliferation of vascular SMC during wound healing, inflammation, and atherogenesis.

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