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# Tumor necrosis factor type α stimulates human endothelial cells to produce granulocyte/macrophage colony-stimulating factor

(granulopoiesis/erythropoiesis/megakaryopoiesis)

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**ABSTRACT** **Tumor necrosis factor type α (TNF-α) is produced by monocytes and has been purified, sequenced, and cloned from the HL-60 cell line. Soluble products of monocytes stimulate endothelial cells to release multilineage hematopoietic colony-stimulating activity. To determine whether TNF-α could stimulate endothelial cells to produce these activities, we added recombinant human TNF-α to cultured human umbilical vein endothelial cells. Untreated endothelial cell conditioned medium and TNF-α-stimulated endothelial cell conditioned medium were tested for hematopoietic colony stimulating activity in colony-forming assays in methylcellulose. TNF-α stimulated growth factor production by endothelial cells. Fifth-passage human endothelial cells and multiply-passaged bovine aortic endothelial cells responded similarly to first-passage endothelial cells, indicating that the action of TNF-α on endothelial cells is direct and not due to contaminating lymphocytes or monocytes present in the first-passage cultures. To investigate the molecular basis for these findings, polyadenylylated RNA was prepared from the TNF-α-stimulated endothelial cells and probed for granulocyte–macrophage colony-stimulating factor and granulocyte colony-stimulating factor mRNA. Granulocyte–macrophage colony-stimulating factor, but not granulocyte colony-stimulating factor, message was detected. This finding suggests that at least some of the hematopoietic colony-stimulating activity released by the TNF-α-stimulated endothelial cells is granulocyte–macrophage colony-stimulating factor. These results demonstrate that a purified monocyte product can stimulate endothelial cells to produce the multilineage growth factor granulocyte–macrophage colony-stimulating factor and extend the role of this immunoregulatory protein to the regulation of hematopoiesis *in vitro*.**

Tumor necrosis factor (TNF) was first described as a factor present in infected, endotoxin-injected mice that caused hemorrhagic necrosis of transplanted sarcomas (1). Subsequent investigations revealed that the effects of TNF on cell growth are not limited to inhibition of tumors, but include a multitude of actions on normal cells. TNF may be identical to cachectin, which suppresses lipoprotein lipase activity in cultured adipocytes and may contribute to the catabolic state characteristic of chronic infections and malignancy (2, 3). TNF type α (TNF-α), a human monocyte product, stimulates the growth of normal human fibroblasts *in vitro* (4), activates neutrophils (5), increases neutrophil adherence to endothelial cells (6), and augments production of procoagulant activities by endothelial cells (7). Thus, TNF-α has been linked to aspects of normal cell growth, to the inflammatory response, and to the control of hemostasis. We report here that TNF-α stimulates human endothelial cells to produce at least one multilineage hematopoietic growth factor, granulocyte–macrophage colony-stimulating factor (GM-CSF), thus implicating TNF-α in the modulation of hematopoiesis.

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## MATERIALS AND METHODS

**Human TNF-α.** Purified, recombinant human TNF-α was a gift of Genentech (South San Francisco, CA). It was cloned from HL-60 cells and expressed in *Escherichia coli* (8, 9). The preparation, assayed (by the supplier) by its cytolytic activity on actinomycin D-treated L929 mouse fibroblasts, contained $3.6 \times 10^7$ units/mg.

**Endothelial Cells.** Endothelial cells were obtained by collagenase treatment of human umbilical cord veins and were cultured in RPMI 1640 (M. A. Bioproducts, Walkersville, MD) supplemented with 20% (vol/vol) heat-inactivated fetal calf serum (HyClone, Logan, UT) as described (10). The cells were maintained in 24-well microtiter plates (Costar, Cambridge, MA) at 37°C in a humidified atmosphere of 95% air/5% $CO_2$. First-passage endothelial cells were used for the majority of the experiments; fifth-passage endothelial cells were used where indicated. Endothelial cells were subcultured by a 2-min incubation in Hanks' balanced salt solution containing 0.05% trypsin and 0.02% EDTA (GIBCO), washed, divided into two cultures, and resuspended in RPMI 1640 supplemented with 20% (vol/vol) fetal calf serum/endothelial cell growth factor at 50 μg/ml (11) (gift of Russell Ross, University of Washington, Seattle)/heparin at 90 μg/ml (12). For one experiment, multiply-passaged bovine aortic endothelial cells were used (13).

**Endothelial Cell Conditioned Media.** Confluent endothelial cell monolayers were used for all experiments. The culture medium was removed and replaced with RPMI 1640 supplemented with 5% (vol/vol) fetal calf serum containing 0, 1, 10, 100, or 1000 units of TNF-α per ml. Twenty-four hours later unstimulated endothelial cell conditioned medium (ECM) and the TNF-α-stimulated endothelial cell conditioned medium ($ECM_\alpha$) were harvested and tested for hematopoietic growth factor content. To evaluate the time course of growth factor production, endothelial cells were incubated with and without TNF-α for 2, 4, 8, and 24 hr.

**Colony Assays.** The hematopoietic growth factor activity of each conditioned medium was assayed by its ability to support clonal growth of granulocyte–macrophage colony-forming units (CFU-GM), erythroid burst-forming units (BFU-E), and megakaryocytic colony-forming units (CFU-

Abbreviations: TNF, tumor necrosis factor; TNF-α, tumor necrosis factor type α; ECM, endothelial cell conditioned medium; $ECM_\alpha$, TNF-α-stimulated endothelial cell conditioned medium; GM-CSF, granulocyte–macrophage colony-stimulating factor; G-CSF, granulocyte colony-stimulating factor; LPS, lipopolysaccharide; CFU-GM, granulocyte–macrophage colony-forming unit(s); BFU-E, erythroid burst-forming unit(s); CFU-Meg, megakaryocytic colony-forming unit(s); PHA-LCM, phytohemagglutinin-stimulated lymphocyte conditioned medium.

Meg) from human bone marrow cells cultured in methylcellulose. Marrow cells were obtained from the posterior iliac crest of healthy adult volunteers after informed consent and approval of the University of Washington Human Subjects Review Committee. Mononuclear cells (<1.077 g/ml) were isolated by density centrifugation on Ficoll/diatrizoate (Litton Bionetics) and were depleted of adherent cells by overnight incubation in plastic tissue culture flasks. The nonadherent low-density marrow mononuclear cells were T-lymphocyte depleted by the E-rosette technique using neuraminidase-treated sheep erythrocytes. The nonadherent T-cell-depleted marrow cells were cultured at a concentration of $5 \times 10^4$ cells per ml of $\alpha$ medium (Flow Laboratories) containing 1% methylcellulose, 20% (vol/vol) fetal calf serum [or 25% (vol/vol) human plasma for megakaryocyte colony assays], 1% deionized bovine serum albumin (not used in megakaryocyte colony assay) (Reheis, Phoenix, AZ), $10^{-4}$ M 2-mercaptoethanol, and penicillin/streptomycin (14, 15). Human urinary erythropoietin, partially purified in our laboratory to a specific activity of 200 units/mg, was added at a concentration of 1 unit/ml on day 4 of culture (16). Samples being tested for growth factor activity were added to the cultures at a final concentration of 2%, 5%, or 10% (vol/vol). Phytohemagglutinin-stimulated lymphocyte conditioned medium (PHA-LCM) (15) served as a positive control. Colonies were counted on day 13 (14, 15).

**Monoclonal Antibodies.** Murine monoclonal antibodies 60.3 and 60.5 (both IgG2a, $\kappa$) were a gift of Patrick Beatty (Fred Hutchinson Cancer Research Center, Seattle).

Monoclonal antibody 60.3 recognizes a cell-surface antigen complex present on all peripheral blood leukocytes (17). Monoclonal antibody 60.5 identifies an HLA class I framework antigen present on endothelial cells and peripheral blood leukocytes.

**Cycloheximide.** Endothelial cells were incubated with cycloheximide (Sigma) at 1 $\mu$g/ml for 24 hr with or without TNF-$\alpha$ at 100 units/ml. The cycloheximide-containing ECM and $ECM_\alpha$ were dialyzed for 24 hr against phosphate-buffered saline (0.15 M sodium chloride/0.01 M sodium phosphate, pH 7.4) containing 0.02% Tween 20 to remove the cycloheximide, sterilized by passage through a 0.22-$\mu$m filter (Millipore), and tested for colony-stimulating activity.

**Trypsin Digestion of the Growth Factor.** One milliliter of ECM or $ECM_\alpha$ was digested with 50 units of agarose-bound trypsin (Sigma) for 20 min at 30°C. Peptide bond hydrolysis was stopped by centrifuging the mixture at $1500 \times g$ for 10 min to sediment the agarose-bound trypsin. The trypsin-digested ECM and $ECM_\alpha$ were tested for colony-stimulating activity.

**RNA Gel Blotting.** Second-passage human umbilical vein endothelial cells were incubated with TNF-$\alpha$ at 100 units/ml for 6 hr. Cellular RNA was extracted using guanidium thiocyanate (18) and poly(A)$^+$ RNA was selected by chromatography on an oligo(dT)-cellulose column (19). The RNA was denatured (20), size-fractionated through a 1.4% agarose gel, and transferred to nitrocellulose (21). RNA from lymphocytes cultured for 24 hr with phytohemagglutinin and RNA from the bladder carcinoma line 5637 were prepared in a similar fashion and included as positive controls. Synthetic oligonucleotide probes were designed based on the published sequence of human GM-CSF (22, 23) and human granulocyte colony-stimulating factor (G-CSF) (24, 25). Forty-base antisense oligonucleotides were synthesized by the phosphotriester method (Applied Biosystems model 380A) and end-labeled with [$\gamma$-$^{32}$P]ATP using T4 polynucleotide kinase (26) (specific activity $1–5 \times 10^8$ cpm/$\mu$g). The filter was hybridized (27) with $2 \times 10^6$ cpm/ml of probe, washed in 2× SSC (1× SSC = 0.15 M sodium chloride/0.015 M sodium citrate, pH 7.0.) with 0.1% $NaDodSO_4$ at 65°C, air dried, and autoradiographed.

## RESULTS

**TNF-$\alpha$ Stimulates Production of Colony-Stimulating Activity by Endothelial Cells.** Purified recombinant TNF-$\alpha$ was added to first-passage human umbilical vein endothelial cells at a final concentration of 100 units/ml. Twenty-four hours later the endothelial cell conditioned medium was harvested and tested for colony-stimulating activity (Table 1). Unstimulated endothelial cells did not constitutively produce significant levels of the growth factor(s), but the TNF-$\alpha$-stimulated endothelial cells were a potent source of colony-stimulating activity. TNF-$\alpha$ did not stimulate the growth of hematopoietic progenitors when added directly to the cultured marrow cells (Table 1) and at higher concentrations inhibited BFU-E and CFU-Meg growth. To evaluate for the presence of an inhibitor of colony growth, TNF-$\alpha$ at 100 or 1000 units/ml was added at 10% final concentration to nonadherent T-lymphocyte-depleted marrow cells cultured in methylcellulose containing 1–3% PHA-LCM. No inhibition of CFU-GM was detected, but TNF-$\alpha$ at 1000 units/ml inhibited BFU-E by 50% and CFU-Meg by 98% in two experiments (data not shown). This confirms a preliminary report that BFU-E and CFU-Meg are more sensitive than CFU-GM to the inhibitory effects of TNF-$\alpha$ (28). When TNF-$\alpha$ at 100 units/ml was added directly to endothelial cell conditioned medium and incubated at 37°C for 24 hr, no increment in growth factor production was found. This indicates that the TNF-$\alpha$ augments endothelial cell growth factor production rather than modifies a previously secreted protein. Dose–response experiments were done in which endothelial cells were incubated with TNF-$\alpha$ at 1, 10, 100, or 1000 units/ml for 24 hr. As little as 10 units of TNF-$\alpha$ per ml stimulated the endothelial cells to release colony-stimulating activity (Fig. 1). One unit of TNF-$\alpha$ per ml did not stimulate growth factor production by endothelial cells (data not shown). Endothelial cells were cultured with TNF-$\alpha$ at 100 units/ml for 2, 4, 8, and 24 hr to study the time course of growth factor production. TNF-$\alpha$-stimulated growth factor production was detected at 8 hr and was maximal at 8–24 hr (Table 2).

The recombinant TNF-$\alpha$ used for these experiments contained 0.8 ng of lipopolysaccharide (LPS, measured by the limulus amoebocyte lysate assay) per $2 \times 10^7$ units of TNF-$\alpha$, or 4 fg of LPS per 100 units of TNF-$\alpha$. To determine whether this quantity of LPS could stimulate growth factor production, first-passage endothelial cells were cultured with LPS (*E. coli* strain 055:B5, Sigma) at 1.5 pg/ml, 1.5 ng/ml, or 1.5 $\mu$g/ml for 24 hr. No increase in growth factor production was seen until a concentration of 1.5 $\mu$g of LPS per ml was reached. Boiling the TNF-$\alpha$ for 5 min destroyed its ability to augment growth factor production by endothelial cells; TNF-$\alpha$ is heat labile (6) and LPS is heat stable at 100°C. These results demonstrate that the increased growth factor produc-

Table 1. Hematopoietic growth factor production by TNF-$\alpha$-stimulated endothelial cells

| Addition | Colonies, no. | | |
|---|---|---|---|
| | CFU-GM | BFU-E | CFU-Meg |
| None | 5 ± 2 | 8 ± 1 | 0 |
| PHA-LCM | 107 ± 10 | 73 ± 4 | 29.3 ± 4.1 |
| TNF-$\alpha$ | 7 ± 6 | 4 ± 0 | 0 |
| ECM | 5 ± 1 | 7 ± 2 | 0 |
| $ECM_\alpha$ | 58 ± 10 | 31 ± 2 | 6.7 ± 1.8 |

Endothelial cells were incubated with TNF-$\alpha$ at 100 units/ml for 24 hr. TNF-$\alpha$, ECM, or $ECM_\alpha$ was added at a final concentration of 10% to $5 \times 10^4$ nonadherent T-cell-depleted marrow mononuclear cells cultured in methylcellulose. Ten experiments were done. Results of a typical experiment are shown and expressed as the mean ± SEM of triplicate plates.

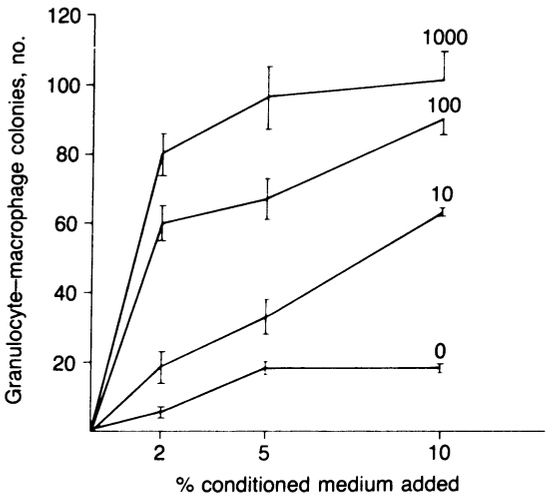


FIG. 1. Response of endothelial cells to increasing amounts of TNF-α. First-passage human umbilical vein endothelial cells were cultured with TNF-α at 0, 10, 100, or 1000 units/ml for 24 hr. ECM or $ECM_\alpha$ was added at a final concentration of 2, 5, or 10% to $5 \times 10^4$ nonadherent T-cell-depleted marrow cells cultured in methylcellulose. Two experiments were done. One experiment is shown, and the results are expressed as mean ± SEM of triplicate plates.

tion by TNF-α-stimulated endothelial cells is not due to LPS present in the TNF-α.

**Effect of TNF-α on Multiply Passaged Endothelial Cells.** To determine the extent to which cord blood mononuclear cells contaminated the endothelial cell preparation, first-passage endothelial cells were incubated with the murine monoclonal antibody 60.3, which recognizes an antigen present on all human peripheral blood leukocytes (17), followed by a fluoresceinated goat anti-mouse antibody. Monoclonal antibody 60.5, which identifies an HLA class I antigen present on both endothelial cells and leukocytes, was used as a positive control. Cytofluorographic analysis revealed that <0.5% of the cells in first-passage endothelial cell cultures were mononuclear cells. To exclude the possibility that this small fraction of mononuclear cells was contributing to growth factor production, we investigated the response of fifth-passage human umbilical vein endothelial cells and a bovine aortic endothelial cell line to TNF-α. Fifth-passage human umbilical vein endothelial cell cultures, stained with monoclonal antibody 60.3, were shown to contain no mononuclear leukocytes. After a 24-hr incubation with TNF-α at 100 units/ml, increased colony-stimulating activity was seen (two experiments). Endothelial cells from a bovine aortic endothelial cell line (13) also responded to TNF-α at 100 units/ml with a 2- to 5-fold increase in growth factor production (data not shown). The data from the fifth-passage human umbilical vein endothelial cell cultures and the bovine aortic endothelial cell line showed that the effect of TNF-α on endothelial cells is direct and not due to contaminating T lymphocytes or monocytes.

**Cycloheximide Inhibits TNF-α-Stimulated Growth Factor Production by Endothelial Cells.** The detection of colony-stimulating activity 8–24 hr after stimulation of endothelial cells with TNF-α is consistent with a requirement for new protein synthesis. However, murine TNF can induce human dermal fibroblasts to produce prostaglandin E (29), and prostaglandins of the E series enhance growth of BFU-E *in vitro* (30). To determine whether the production of growth factor(s) by the endothelial cells requires new protein synthesis, endothelial cells were cultured with TNF-α at 100 units/ml in the presence of cycloheximide at 1 μg/ml for 24 hr. The cycloheximide-treated ECM and $ECM_\alpha$ were dialyzed for 24 hr to remove the cycloheximide, then tested for growth factor activity. Cycloheximide treatment completely abrogated growth factor production by the endothelial cells (Table 3). Continued viability of the endothelial cells after cycloheximide treatment was demonstrated by replacing the cycloheximide-containing media with fresh culture media containing TNF-α at 100 units/ml. The endothelial cells retained their ability to respond to TNF-α, thus the decrement in growth factor production was not due to a nonspecific toxic effect of the cycloheximide. An inhibitor assay showed that the dialyzed cycloheximide-treated ECM and $ECM_\alpha$ did not inhibit colony growth (data not shown), confirming that the cycloheximide had been effectively removed by dialysis.

The hematopoietic growth factor produced by the TNF-α-stimulated endothelial cells was destroyed by digestion with trypsin. Sham-digested $ECM_\alpha$ ($ECM_\alpha$ mixed with agarose-bound trypsin and immediately centrifuged) was unaffected (data not shown). These results confirm that the growth factor is a protein.

**RNA Gel Blotting with Probes for GM-CSF and G-CSF.** Human GM-CSF has multilineage colony-stimulating activity (23, 31). The GM-CSF gene has been cloned from a human genomic library and expressed in COS cells (22, 23, 32). Human G-CSF, also known as pluripoietin, also supports the growth and differentiation of multiple hematopoietic progenitor cell types (24, 25). To investigate the molecular basis of growth factor production by the TNF-α-stimulated endothelial cells, RNA gel blot analysis of whole cellular and poly(A)-enriched RNA was performed using 40-base oligonucleotide probes designed to hybridize with GM-CSF- or G-CSF-specific mRNA. Endothelial cells cultured with TNF-α at 100 units/ml contain significant levels of GM-CSF-specific message (Fig. 2*B*). At this level of analysis, G-CSF-specific mRNA could not be detected (Fig. 2*B*). The results

Table 2. Time course of TNF-α-stimulated growth factor production by endothelial cells

| Addition* | Incubation, hr† | Colonies, no. CFU-GM | BFU-E | CFU-Meg |
|---|---|---|---|---|
| None | | 0 | 5 ± 0 | 0 |
| PHA-LCM | | 37 ± 4 | 46 ± 2 | 16.7 ± 2.4 |
| $ECM_\alpha$ | 2 | 9 ± 1 | 12 ± 1 | 0 |
| $ECM_\alpha$ | 4 | 12 ± 1 | 13 ± 3 | 3.3 ± 0.7 |
| $ECM_\alpha$ | 8 | 63 ± 6 | 27 ± 5 | 4.7 ± 0.7 |
| $ECM_\alpha$ | 24 | 90 ± 5 | 23 ± 3 | 2.0 ± 1.4 |
| ECM | 24 | 18 ± 1 | 17 ± 1 | 0.7 ± 0.7 |

Four experiments were done. Results of one experiment are given and expressed as the mean ± SEM of triplicate plates.
*ECM or $ECM_\alpha$ was added at a final concentration of 10% to $5 \times 10^4$ nonadherent T-cell-depleted marrow mononuclear cells cultured in methylcellulose.
†Endothelial cells were incubated for 2, 4, 8, or 24 hr with TNF-α at 100 units/ml.

Table 3. Effect of cycloheximide on growth factor production by TNF-α-stimulated endothelial cells

| Addition* | Cycloheximide | Colonies, no. CFU-GM | BFU-E |
|---|---|---|---|
| None | | 6 ± 4 | 19 ± 1 |
| PHA-LCM | | 32 ± 1 | 87 ± 4 |
| ECM | − | 3 ± 1 | 13 ± 2 |
| $ECM_\alpha$ | − | 33 ± 3 | 55 ± 4 |
| ECM | + | 4 ± 3 | 7 ± 1 |
| $ECM_\alpha$ | + | 6 ± 1 | 18 ± 5 |

+, Endothelial cells were incubated with cycloheximide at 1 μg/ml for 24 hr; −, endothelial cells were incubated without cycloheximide.
*$ECM_{TNF-\alpha}$ contained TNF-α 100 units/ml. ECM or $ECM_{TNF-\alpha}$ was tested for growth factor content at a final concentration of 10%.

A

GM-CSF: 5′ ttcaggagacgccgggcctcctggatggcattcacatgct 3′
794
G-CSF : 5′ tcttcctcacttgctctaagcacttgagcaggaagctctg 3′
191

B

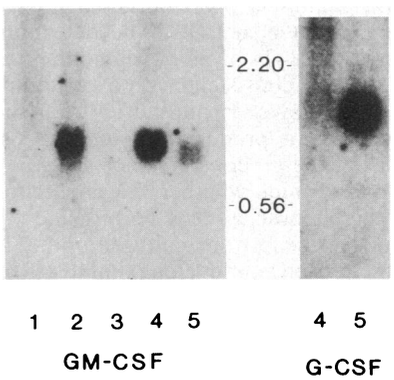


FIG. 2. RNA gel blot analysis of TNF-α-stimulated endothelial cell RNA. (*A*) Anti-sense oligonucleotides were used to probe the RNA gel blots. The base number refers to the base location within the gene for GM-CSF (23) and within the cDNA for G-CSF (24). (*B*) Ten micrograms of RNA were probed for GM-CSF and G-CSF specific mRNA. GM-CSF controls include whole cellular RNA from lymphocytes (lane 1), from lymphocytes stimulated with 1% phytohemagglutinin for 24 hr (lane 2), and poly(A)-enriched RNA from the bladder carcinoma line 5637 (lane 5). The G-CSF control is poly(A)-enriched RNA from the bladder carcinoma line 5637 (lane 5). Whole cellular RNA (lane 3) and poly(A)-enriched RNA (lane 4) were extracted from endothelial cells cultured with TNF-α at 100 units/ml. The size markers are 2.2- and 0.56-kilobase fragments of phage λ DNA.

for GM-CSF were confirmed with a full-length GM-CSF cDNA probe (data not shown). The bladder carcinoma line 5637, included as a positive control for G-CSF (25), was found to contain both G-CSF and GM-CSF transcripts (Fig. 2*B*). Pluripoietin α, which shares many of the characteristics of GM-CSF, has been partially purified from this bladder carcinoma line (33).

## DISCUSSION

TNF-α is one of a family of proteins that possesses tumor necrosis activity. Lymphotoxin, now known as TNF type β, is a lymphocyte-derived glycoprotein that has 47% amino acid sequence homology with TNF-α (9) and is also cytotoxic for tumors *in vitro* and *in vivo* (8, 34). The primary structure of TNF is highly conserved across species lines: murine TNF is 80% homologous to human TNF-α (35).

The full repertoire of activities of this family of TNFs remains to be defined. TNF may play a role in diverse aspects of cell metabolism including mobilization of triglycerides from adipose tissue (2, 3) and prostaglandin production (29). TNF enhances neutrophil adherence to endothelium and increases phagocytic and cytotoxic activities of neutrophils (5, 6) and thus is a potent inflammatory protein.

Monocytes constitutively release one or more soluble factors that stimulate endothelial cells to produce multilineage colony-stimulating activity (36–39). The identity of these monokines has remained elusive. We report here that TNF-α, a purified recombinant monocyte-derived protein, can stimulate growth factor production by human endothelial cells.

Growth factor activity was detected 8–24 hr after stimulation of endothelial cells with TNF-α (Table 2). Treatment of the endothelial cells with cycloheximide abolished TNF-α-stimulated growth factor production (Table 3), indicating that new protein synthesis is required for this effect. RNA gel blot analysis of endothelial cell RNA with probes for the multilineage growth factors GM-CSF and G-CSF revealed that the multilineage colony-stimulating activity released in response to TNF-α is due, at least in part, to GM-CSF production. Whether endothelial cells release colony-stimulating factors in addition to GM-CSF is unknown.

TNF-α is a candidate chemotherapeutic agent. The hematologic effects of TNF-α administered systemically for antitumor purposes may depend on the interplay of its direct marrow suppressive properties and its ability to stimulate growth factor production by endothelial cells, a component of the bone marrow microenvironment.

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