Transforming growth factor type β: Rapid induction of fibrosis and angiogenesis in vivo and stimulation of collagen formation in vitro

ANITA B. ROBERTS*, MICHAEL B. SPORN*, RICHARD K. ASSOIAN†, JOSEPH M. SMITH‡, NANEET S. ROCHE*, LALAGE M. WAKEFIELD*, URSULA I. HEINE*, LANCE A. LIOTTA*, VINCENT FALANGA†, JOHN H. KEHRL‡, AND ANTHONY S. FAUCI‡

*National Cancer Institute, Bethesda, MD 20892; †University of Pittsburgh School of Medicine, Pittsburgh, PA 15213; and ‡National Institute of Allergy and Infectious Diseases, Bethesda, MD 20892

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ABSTRACT Transforming growth factor type β (TGF-β), when injected subcutaneously in newborn mice, causes formation of granulation tissue (induction of angiogenesis and activation of fibroblasts to produce collagen) at the site of injection. These effects occur within 2–3 days at dose levels of <1 μg. Parallel in vitro studies show that TGF-β causes marked increase of either proline or leucine incorporation into collagen in either an NRK rat fibroblast cell line or early passage human dermal fibroblasts. Epidermal growth factor (EGF) and platelet-derived growth factor (PDGF) do not cause these same in vivo and in vitro effects; in both rat and human fibroblast cultures, EGF antagonizes the effects of TGF-β on collagen formation. We have obtained further data to support a role for TGF-β as an intrinsic mediator of collagen formation: conditioned media obtained from activated human tonsillar T lymphocytes contain greatly elevated levels of TGF-β compared to media obtained from unactivated lymphocytes. These activated media markedly stimulate proline incorporation into collagen in NRK cells; this effect is blocked by a specific antibody to TGF-β. The data are all compatible with the hypothesis that TGF-β is an important mediator of tissue repair.

Transforming growth factor type β (TGF-β) was originally identified in neoplastic cells (1–4) and then found in a wide variety of non-neoplastic tissues (1–5); the first total purification of this 25-kDa peptide was from three non-neoplastic sources—namely, human blood platelets, human placenta, and bovine kidney (6–8). Recently, the human gene for TGF-β has been cloned (9), and mRNA transcripts have been found in both neoplastic and non-neoplastic cells. There is a marked induction of TGF-β mRNA when peripheral blood lymphocytes are activated (9). The presence of TGF-β in cells or cell fragments of hematopoietic origin suggests that it may play some intrinsic role in inflammation and tissue repair. It had been shown previously that extrinsic TGF-β could stimulate the formation of connective tissue and collagen within wire-mesh wound-healing chambers implanted in rats (10, 11). We now report that subcutaneous injection of <1 μg of TGF-β in newborn mice induces angiogenesis and causes rapid activation of fibroblasts to produce collagen; the new tissue formed after injection of TGF-β is essentially granulation tissue, resembling that found during physiological wound repair. Furthermore, we show that TGF-β in vitro has marked stimulatory effects on the formation of collagen by a variety of rodent and human fibroblasts and that these effects are different from those of two other peptide growth factors that have been implicated in tissue repair—namely, epidermal growth factor (EGF) and platelet-derived growth factor (PDGF).

Activated lymphocytes and macrophages have long been implicated in the production of growth factors that stimulate fibroblast proliferation and collagen synthesis, although the chemical nature of many of the specific peptide growth factors that are involved has not been well-defined (12–16). Here, we report that T lymphocytes secrete greatly elevated levels of TGF-β into their culture medium when they are activated in vitro (17) and that the conditioned media from such activated lymphocytes can stimulate proline incorporation into collagen in rodent fibroblasts. Moreover, we show that specific antibodies to TGF-β will partially abolish this stimulatory effect. The present data thus indicate that TGF-β is a functionally significant component of the set of lymphokines produced by activated lymphocytes.

MATERIALS AND METHODS

Growth Factors. TGF-β, homogeneous by analysis on sodium dodecyl sulfate/polyacrylamide gels, was purified from human platelets (6). EGF was purified from male mouse salivary glands (1). Pure human PDGF was the generous gift of Russell Ross and Elaine Raines and contained <0.1% TGF-β by radioreceptor assay (18).

In Vitro Studies. Carrier-free sterile TGF-β (40 μg) was dissolved in 200 μl of 2 mM HCl to which 600 μl of phosphate-buffered saline (PBS) and 200 μl of PBS containing NaCl at 9 mg/ml was added. Swiss mice (1 day old) were injected subcutaneously each day in the nape of the neck with a 27-gauge needle, with 20 μl of TGF-β or with saline control, and tissue samples were obtained as described in Results. Sterile murine EGF was also injected in the same vehicle in the same manner. At the end of each experiment, the firm nodular tissue at the site of injection of TGF-β (or the corresponding area at the site of injection of saline or EGF in the control animals) was removed and fixed in either neutral formalin or glutaraldehyde. Paraffin sections of the samples fixed in formalin were then stained with either hematoxylin and eosin or with Masson trichrome (for collagen). Samples of tissue fixed in glutaraldehyde were examined by electron microscopy.

Cell Culture. NRK cells, clone 49F, were maintained in Dulbecco’s modified Eagle’s medium (DMEM) (GIBCO) containing 10% calf serum (GIBCO) supplemented with penicillin (50 units/ml) and streptomycin (50 μg/ml) in humidified 5% CO2/95% air at 37°C. Normal human dermal fibroblasts, cultured from biopsies taken from dorsal forearm skin of donors, were grown under identical conditions except that fetal bovine serum was used.

Assays for Collagen Formation in Vitro. NRK 49F cells were seeded in 1 ml of DMEM/10% calf serum, in 24-well multi-dishes (1 × 105 cells per 16-mm well). After the cells

Abbreviations: TGF-β, transforming growth factor type β; EGF, epidermal growth factor; PDGF, platelet-derived growth factor.
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reached confluence, the medium was changed to minimal essential medium (MEM) (containing glutamine, 20 mM Hepes buffer, and 2% plasma-derived serum), and growth factors were added in 20 μl of 4 mM HCl containing bovine serum albumin at 1 mg/ml. After 16 hr, the medium was changed to 300 μl per well (containing 0.25 mM ascorbate and other supplements as described above, but without plasma); growth factors were re-added. After 15 min, 6 μCi of L-[2,3-3H]proline (29.1 Ci/mmol; 1 Ci = 37 GBq) was added and the incubation continued for 3 hr at 37°C. Collagen was determined on the pooled medium from triplicate wells using bacterial collagenase (Advance Biofactures, Lynbrook, NY) by a modification (15) of the method of Peterkofsky (19).

Measurement of Amino Acid Uptake. NRK cells (3 × 10^6) were seeded in 3 ml of medium in six-well (35 mm) multi-dishes and treated with growth factors as described above for collagen determinations. For the uptake experiments, 1 μCi of [1-14C]methylaminobutyric acid (48.4 mCi/mmol; New England Nuclear) was added to each well (1 ml of MEM supplemented as for collagen determinations) and incubated for 6 min at 37°C, followed by four rapid washes with PBS at 0°C. The washed cells were dissolved in 0.5 M NaOH/1% NaDodSO₄, and the radioactivity was determined in a scintillation counter (20). Nonspecific uptake, measured in the presence of 10 mM L-proline, was subtracted from all values. Collagen determinations were made on duplicate wells to facilitate direct comparison of the two measurements. All results were normalized to cell number.

Antibodies to TGF-β. Human platelet TGF-β of >98% purity (6) was coupled to keyhole limpet hemocyanin (KLH). Rabbits were immunized with three doses of KLH-TGF-β (100 μg of TGF-β per dose per rabbit) in Freund’s adjuvant at 3-week intervals. The IgG fraction was purified from the resulting antiserum by affinity chromatography on protein A-Sepharose (21). Anti-TGF-β antibodies prepared in this way inhibited the binding of TGF-β to its specific receptors on NRK cells and also suppressed the growth of NRK cells in soft agar in response to exogenous TGF-β (L.M.W., unpublished observations). The IgG fraction of normal rabbit preimmune serum prepared in the same way served as a control. Both IgG preparations were extensively dialyzed against PBS and contained 4–5 mg of protein per ml. For determination of the effect of antibodies on collagen formation, growth factor solutions in MEM plus 2% plasma-derived serum were preincubated overnight at 4°C in the presence of immune or preimmune IgG at 40 μg/ml, then added directly to the cells and incubated for an additional 16 hr at 37°C prior to determination of proline incorporation.

Determination of TGF-β in Media Conditioned by Lymphocytes. Tonsillar T lymphocytes were prepared and cultured as described (17). Serum-free HB-102 medium, conditioned by 10⁶ cells per ml, was dialyzed against 1 M acetic acid, lyophilized, and reconstituted (20 to 50-fold more concentrated) in 4 mM HCl containing bovine serum albumin (1 mg/ml). Concentrations of TGF-β were determined both by a competitive radioimmunoassay assay measuring binding of [125I]-labeled TGF-β to A549 human lung carcinoma cells (18) and by comparison of dilution curves of colonies of NRK cells (assayed in the presence of 0.8 mM EGF) in soft agar induced either by standard human platelet TGF-β or by the lymphocyte conditioned medium (1).
chase experiments (data not shown) have shown that the antagonistic effects of these two growth factors do not result from activation of an extracellular protease or collagenase activity by EGF. Incorporation of \[^{14}C\]proline into secreted collagen following a 2-hr pulse of cells treated with either 50 pM TGF-\(\beta\), or 1 nM EGF (or 300 pM PDGF) remains stable for up to 4 hr after addition of unlabeled proline to a final concentration of 10 mM (chase).

Since some of the effects of growth factors on formation of new protein may be mediated by their ability to stimulate uptake of free amino acids into cells (20, 25–28), we have compared the ability of TGF-\(\beta\), EGF, and PDGF to stimulate uptake of \[^{14}C\]methylaminoisobutyric acid into NRK cells, as well as the ability of these three growth factors to stimulate \[^{14}C\]proline incorporation into collagen, as shown in Fig. 3. Methylaminoisobutyric acid, an amino acid that is not metabolized, was chosen because it is taken up by cells by system A transport (26), the same system that accounts for proline and glycine uptake. Although all three growth factors stimulated methylaminoisobutyric acid uptake, TGF-\(\beta\) alone increased incorporation of proline into collagen; thus, the differential effects of these growth factors on proline incorporation into collagen apparently are not the result of effects on amino acid uptake. Confirmation of these results is seen in Fig. 4, which compares the effects of TGF-\(\beta\), PDGF, and EGF on incorporation of \[^{14}C\]leucine (which is taken up by

![Image A](image1.png)

![Image B](image2.png)

![Image C](image3.png)

![Image D](image4.png)

**Fig. 1.** Histologic effects of TGF-\(\beta\) on induction of a fibrotic and angiogenic response after subcutaneous injection. Mice (1 day old) were injected each day in the nape of the neck with 20 \(\mu\)l of a solution of saline alone or a saline solution of TGF-\(\beta\) (800 ng); tissue samples were obtained as described in Materials and Methods. (A) Control injection (72 hr). This section shows the interface below the reticular dermis, between the subcutaneous adipose tissue (containing hair follicles, top), and the underlying skeletal muscle (bottom). Only a small number of fibroblasts are normally found at this interface, as indicated by arrows. (hematoxylin and eosin; \(\times 430\).) (B) TGF-\(\beta\) injection (48 hr). The subcutaneous interface is expanded by fibroblasts, macrophages, granulocytes, and newly formed collagen bundles (blue). (Masson trichrome; \(\times 260\).) (C) TGF-\(\beta\) injection (72 hr). The subcutaneous space is now further expanded by sheets of fibroblasts, endothelial cells, and macrophages, surrounded by a collagenous network. (hematoxylin and eosin; \(\times 430\).) (D) TGF-\(\beta\) injection (72 hr). This section shows pronounced neovascularization, with newly formed capillary loops, surrounded by fibroblasts and occasional macrophages. Extravasated erythrocytes are also present. (hematoxylin and eosin; \(\times 430\).)

Table 1. Stimulation of proline incorporation into collagen by human dermal fibroblasts

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Incorporation of [^{14}C]proline into collagen, cpm per cell</th>
<th>Relative collagen biosynthesis, %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Donor 1</td>
<td>Donor 2</td>
</tr>
<tr>
<td>Control</td>
<td>1.5 ± 0.3</td>
<td>1.5 ± 0.7</td>
</tr>
<tr>
<td>TGF-(\beta) (0.2 nM)</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>TGF-(\beta) (0.4 nM)</td>
<td>12.0 ± 1.8</td>
<td>9.0 ± 1.6</td>
</tr>
<tr>
<td>EGF (0.8 nM)</td>
<td>1.4 ± 0.5</td>
<td>1.4 ± 0.5</td>
</tr>
<tr>
<td>EGF (0.8 nM) plus</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>TGF-(\beta) (0.4 nM)</td>
<td>2.3 ± 0.8</td>
<td>1.7 ± 0.8</td>
</tr>
</tbody>
</table>

Normal human dermal fibroblasts of four different donors were used in their third in vitro passage. Cells were treated with growth factors for 40 hr prior to labeling with \[^{14}C\]proline (2 \(\mu\)Ci) for 20 hr in the presence of ascorbate (25 \(\mu\)g/ml) and \(\beta\)-aminopropionitrile (20 \(\mu\)g/ml). Determinations of secreted collagen were as described in Materials and Methods. Results are the mean ± SD of triplicate determinations. ND, not determined.
cells by system L transport; ref. 26) into collagen, as well as on proline incorporation. It is clear that the use of leucine as a tracer gives the same relative results as seen with proline.

**TGF-β in Media Conditioned by Lymphocytes.** Recently, activated lymphocytes have been shown to produce mRNA for TGF-β (9) and to secrete TGF-β itself (17); there is a 10- to 30-fold increase in TGF-β in conditioned media of activated T lymphocytes compared to media obtained from unactivated lymphocytes (Fig. 5; ref. 17). To test whether TGF-β secreted by lymphocytes might play an intrinsic role in formation of granulation tissue in vivo, we assayed the media conditioned by activated T lymphocytes for its ability to stimulate incorporation of proline into collagen. Fig. 5 shows that when lymphocytes are stimulated by phytohemagglutinin, there is a marked increase in both TGF-β secretion and the ability of their conditioned medium to enhance the incorporation of [3H]proline into collagen. Although activated lymphocytes produce many growth factors other than TGF-β (12–16), experiments using rabbit anti-TGF-β antibodies demonstrate that this peptide may be a significant mediator of the effects of lymphocytes on collagen formation in fibroblasts; preincubation in 2% plasma of anti-TGF-β IgG (40 μg per ml) with either standard platelet TGF-β or tonsillar T-lymphocyte conditioned media (at final concentrations up to 50 pM TGF-β) blocked 89% of the activity of platelet TGF-β and 59% of the activity of the lymphocyte conditioned media, when compared to the stimulation obtained from samples treated with equal amounts of preimmune IgG.

**DISCUSSION**

We have shown that TGF-β has marked effects on the formation of collagen, both in vivo and in vitro, and that TGF-β can also cause a striking angiogenic response in the newborn mouse. Furthermore, activation of lymphocytes causes them to produce amounts of TGF-β that cause a marked stimulation of proline incorporation into collagen in model human and rodent in vitro systems. All of the above data, obtained from both in vivo and in vitro experiments, are compatible with the hypothesis that TGF-β is a significant mediator of tissue repair.

The mechanism of action of TGF-β in promoting angiogenesis is unknown and will involve an entire cascade of cellular and molecular events (30–34), including the possibilities that TGF-β may be chemotactic for cells involved in angiogenesis, or even less directly that TGF-β may induce cells to secrete other peptides with angiogenic activity. It has not yet been demonstrated that TGF-β itself has any direct effect on proliferation of capillary endothelial cells.
The mechanism of action of TGF-β in promoting collagen formation is similarly unknown. Certainly the increased numbers of fibroblasts at the site of injection (which might result from direct or indirect chemotactic actions of TGF-β rather than direct proliferative actions) might be sufficient to explain the increased collagen deposition. However, in vitro experiments suggest that TGF-β also increases formation of other matrix substances, such as glycosaminoglycans (V.F., unpublished results). In addition, although its functional implications are unknown, it may be noted that the TGF-β precursor contains the minimal cellular recognition sequence, Arg-Gly-Asp-X, identified in the adhesion glycoprotein, fibronectin (35).

Finally, the known presence of TGF-β in both platelets and activated lymphocytes suggests that this peptide may be an important intrinsic physiological paracrine mediator of repair. Although in this report we have emphasized the action of TGF-β by itself, it is clear that in vivo TGF-β acts in combination with other peptide growth regulators, such as EGF or its homolog, TGF-α (36), PDGF (36, 37), and basic fibroblastic growth factor (32), and that better understanding of its true role will require further study of these interactions.

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