Transforming growth factor-β (TGF-β) is a multifunctional polypeptide that stimulates extracellular matrix deposition and fibroblast proliferation. Because both these features characterize Dupuytren’s contracture, we investigated a possible role for TGF-β in the etiology of this disorder. We studied receptor expression for TGF-β, effects of TGF-β1 on DNA-synthesis, and in vitro production of TGF-β1 and TGF-β2 in both normal and Dupuytren-derived fibroblasts. We also studied the effects of epidermal growth factor (EGF) on growth of the different cell types. TGF-β1 receptor profiles were different between the two cell types, as were TGF-β1 and EGF-induced stimulation of cell growth. Both cell types secreted both active and latent TGF-β. Our results suggest that growth factors such as TGF-β and EGF may play a role in Dupuytren’s contracture. (J Hand Surg 1995; 20A:101-108.)

Since its description over 150 years ago, the etiology and pathogenesis of Dupuytren’s contracture remain controversial. The pathology seen in Dupuytren’s disease consists of changes produced in the dermis and palmar fascia; there is uncontrolled tissue proliferation, with augmentation of myofibroblasts and enhanced synthesis of extracellular matrix (ECM). The error that initiates and perpetuates this tissue proliferation is not known, although many studies have reported clinical, biochemical, epidemiologic, and morphologic changes.1-6 With new developments in molecular biology and more insight into factors controlling cellular behaviour we might be able to uncover some of the regulating factors in this disease. Recently, a possible relationship has been proposed between two members of a large polypeptide growth factor family (platelet derived growth factor [PDGF] and basic fibroblast growth factor [bFGF]) and Dupuytren’s disease.7,8 Each of these growth factors might be involved in the proliferation of myofibroblasts. Another member of this polypeptide growth factor family is transforming growth factor β (TGF-β), originally detected in tumor extracts and termed “transforming” since it was thought to induce or maintain the neoplastic or transformed cell phenotype.9,10 TGF-β is ubiquitous in both normal and neoplastic tissues, affecting growth and differentiation in species ranging from Xenopus to humans. It has multifunctional and sometimes confusing effects, but in general TGF-β stimulates growth of mesenchymal cells, such as fibroblasts, and inhibits growth of ectodermal cells, such as keratinocytes and lymphocytes.9-12 A well-known effect of TGF-β is increased deposition of ECM, both through increasing its synthesis and decreasing its breakdown.8-11 This biologic effect, shown both in vivo and in vitro, has been linked to diseases such as pulmonary fibrosis and to keloids.12-14

TGF-β’s mechanism of action is still unknown, but it is most probably mediated through three different TGF-β cell surface receptors.15-17 All cells investigated have receptors for TGF-β, except retinoblastoma cell lines.18 The so-called type I and the
type II TGF-β receptors are proteoglycans and have weights of 53 kDa and 70–100 kDa, respectively. The type III TGF-β receptor is a betaglycan and has the highest molecular weight (280–330 kDa).

Since TGF-β stimulates fibroblast growth, fibroblast migration, and ECM production—characteristics of Dupuytren’s contracture—we explored a possible role of TGF-β in this disease. More specifically we looked at the presence of TGF-β receptors on these cells, effects of TGF-β and epidermal growth factor (EGF) on DNA-synthesis, and production of TGF-β1 and TGF-β2 by these cells. Comparisons were made with normal skin-derived fibroblasts.

**Material and Methods**

**Growth Factors**

Porcine TGF-β1 and porcine 125I-TGF-β1 were provided by M. B. Sporn, National Institute of Health, Bethesda, MD; EGF was purchased from Collaborative Research Inc., Bedford, MA; neutralizing antibodies to TGF-β1 and TGF-β2 and control IgG were purchased from R&D Systems, Minneapolis, MN.

**Cell Culture**

Dupuytren tissue was obtained at the time of surgery from 12 patients (11 male, 1 female, mean age 55 years, range 42–72 years). All patients had established Dupuytren’s disease with 30° or greater metacarpophalangeal joint contractures, or 20° or greater proximal interphalangeal joint contractures, or both. As a control, uninvolved skin tissue from the same patient was used whenever feasible, and uninvolved skin was used from an age-matched group of nine patients undergoing other, nonneoplastic orthopaedic procedures. Tissue was minced into pieces smaller than 1 mm and plated in 25 cm² culture flasks in Dulbecco’s Modified Eagle Medium (DMEM) (Gibco BRL, Grand Island, NY) containing 10% fetal calf serum (FCS), penicillin (100 U/ml), and streptomycin (100 μg/ml) (Mediatech, Washington, DC). Cultures were kept in 37°C humidified air containing 5% CO₂. Cells were subcultured and used for experiments between the third and ninth generation. Representative results are shown on 3–4 Dupuytren-derived cultures (Dup 1, 2, 3, and 4), and two normal tissue-derived cultures (Skin Fibroblast [SF] 1 and 2), each of these representing a different individual.

**TGF-β1 Receptor Assay**

The receptors for TGF-β were examined using a cross-linking method described by Massague.19 In short, cells were plated in 6-well plates and grown to confluence in DMEM plus 10% FCS plus antibiotics. At confluence the cell monolayer was washed with bicarbonate-free DMEM, 25 mM Hepes (pH = 7.4), and 1 mg/ml Bovine Serum Albumin (BSA). Then 100 pM 125I-TGF-β1 was added, and the plate was put on a rotating platform for 2.5 hours at 4°C. To show specificity of the binding we incubated a control with both iodinated and 100-fold excess unlabeled TGF-β1. Cells were washed and a cross-linking agent (disuccinimidyl suberate) was added for 1 hour. Finally the cells were solubilized using a mixture of Triton X-100, Tris, and EDTA. The supernatant was sampled and stored at −70°C until used. The solubilized cell proteins were separated using SDS-polyacrylamide gel electrophoresis under reduced conditions on a precast 4%–15% linear gradient gel (Bio-Rad, Richmond, CA). Gels were transferred to filterpaper and exposed for 14 days to x-rays for autoradiography at −70°C. Expression of TGF-β receptor types was measured using laser densitometry.

**Cellular Proliferation Studies**

Cells were routinely subcultured in 25 cm² flasks. For proliferation studies, the cells were plated in 96-well plates at an initial density of 4 × 10³ cells/well in DMEM plus 10% FCS plus antibiotics for 24 hours to allow attachment of the cells. Afterwards the cells were brought to a quiescent state by serum starvation for 24 hours. Different amounts of TGF-β1 (1 or 5 ng/ml), or EGF (8 ng/ml), or both were then added in DMEM plus 0.2% FCS plus antibiotics. Since TGF-β1-induced mitogenesis involves an indirect loop involving the production of PDGF,20 there is a delay of at least 24 hours.20 The maximum peak stimulus after EGF addition occurs after 18 hours or more; therefore, we added 1.6 uCi/well [3H]thymidine (NEN, Boston, MA) 20 hours after the addition of the respective growth factors. To measure the production of DNA, cells were washed with PBS and precipitated with trichloroacetic acid. DNA was then extracted with 3M NaOH, and aliquots were measured in a liquid scintillation counter.

**Preparation of Conditioned Medium**

Cells were plated at 1.5 × 10⁵ plus antibiotic cells/well in 6-well plates in DMEM plus 10% FCS. After 24 hours cells were washed extensively and concurrently incubated with serum-free DMEM/25 mM Hepes/0.5% BSA. After 48 hours conditioned medium was sampled and stored at −20°C until use. Cell numbers, counted after sampling the conditioned medium, were equal among the cell types.
Mink Lung Cell (Mv1Lu) Inhibition Assay

Mv1Lu cells (American Tissue Culture Collection, Rockville, MD) were plated in 96-well plates at 1.5 x 10^4 cells/well and left to attach for 12 hours in DMEM plus 10% FCS. After extensive washing with serum-free medium, diluted samples of conditioned medium were added into a total of 100 μl serum-free medium. After 48 hours the number of cells was estimated with the colorimetric Tetrazolium Salt (MTT) assay. This assay is based on reduction of MTT (Sigma Chemical, St. Louis, MO) by a mitochondrial succinyl dehydrogenase in viable cells to the purple insoluble formazan precipitate. Relative cell number can be estimated using a microplate colorimetric reader (570 nm wavelength). This assay was very accurate and reproducible (SEM less than 3%). Because TGF-β is usually produced in a latent inactive form that can be activated by acidification or alkalization, we tested each sample before and after acidification. To specify the isoform of TGF-β that was produced by the cells, we used antibodies to either TGF-β1 or TGF-β2 at a final concentration of 30 μg/ml. Samples were pre-incubated with antibodies for 1 hour at 37°C before adding to the Mv1Lu cells. The neutralizing antibodies at this concentration completely blocked the Mv1Lu inhibitory effect of .3 ng/ml TGF-β1.

Results

TGF-β Receptor Assay

On all primary cultures of Dupuytren cells and normal fibroblasts tested we found presence of three TGF-β receptor types. The type I receptor was expressed significantly less (Student t-test; p < 0.05), or had a lower affinity for TGF-β1 than the type II receptor in Dupuytren cultures. There was virtually no difference between binding of TGF-β1 to these two receptor types in normal fibroblasts. Binding was specific as shown by cross-linking in the presence of excess unlabeled TGF-β1. Representative results are shown in Figure 1.

Cellular Proliferation

Normal fibroblasts exhibited a greater mitogenic response to EGF than Dupuytren cells (Fig. 2). Addition of TGF-β1 alone (1 and 5 ng/ml) and in combination with EGF (TGF-β1 [5 ng/ml]/EGF [8 ng/ml]) stimulated more DNA synthesis in Dupuytren cells than in their normal counterparts (Fig. 2). DNA-synthesis in some Dupuytren cultures was stimulated up to eightfold by addition of the combination TGF-β1 (5 ng/ml) and EGF (8 ng/ml). TGF-β1 (5 ng/ml) alone stimulated mitogenesis up to fivefold in some Dupuytren cultures. These effects were less impressive in normal fibroblasts. An interesting finding was that the simultaneous addition of TGF-β1 and EGF resulted in synergistic stimulation of DNA-synthesis in Dupuytren cultures whereas this effect was only additive in normal fibroblasts.

Presence of TGF-β

TGF-β is a very specific inhibitor of Mv1Lu mink lung cells. Using a bioassay based on this feature we found presence of TGF-β in the conditioned medium of both cell types (Fig. 3). Acidification of the samples increased Mv1Lu inhibitory (= TGF-β) activity, which shows that both active and latent TGF-β is present (Fig. 3). Using specific antibodies neutralizing TGF-β1 or TGF-β2 activity, we found that Dupuytren cells made mostly TGF-β1 whereas we found no consistent difference between production of the two isoforms in normal cells (Fig. 4). Unexpectedly, normal fibroblasts produced more TGF-β than Dupuytren cells (Table 1).

Discussion

The concept of cells producing and secreting polypeptide growth factors that subsequently bind to their own receptors, resulting in a biologic response, was introduced as autocrine growth control in the early 1980s. Since then it has become common knowledge that cells exist in a balanced system of various growth factors, including PDGF, FGF, EGF, insulin-like growth factor (IGF), and TGF-β. If tissue-homeostasis is disturbed (e.g., following injury), adjustments in expression of growth factors or their receptors are part of the repair mechanisms needed to restore the balance. Assuming that tumorigenesis somewhat resembles a repair mechanism gone awry, it is not surprising that many tumors have been shown to express an increased synthesis of certain growth factors, or their receptors, or both. TGF-β’s potential role in tumorigenesis, however, is complicated by its bi-directional effects on growth (stimulatory for mesenchymal cells and inhibitory for epithelial cells), its complex receptor system, and the fact that it is produced in a latent (inactive) form. Loss of growth-inhibitory control by TGF-β has been linked to epithelial tumorigenesis, whereas increased TGF-β expression could theoretically lead to mesenchymal tumorigenesis. These mechanisms can be explained by alterations in production, secretion, and subsequent activation of latent TGF-β, by changes in TGF-β receptor expression, and by alterations in post-receptor cell signalling. Although the exact roles of TGF-β in tumorigenesis are still unclear, increased synthesis of TGF-β has been
Figure 1. Autoradiogram of representative affinity-labelling experiments. Two examples of normal skin fibroblasts are shown (SF1, SF2) and three Dupuytren derived fibroblasts (Dup 1, Dup 2, Dup 3). Lanes indicated with – represent cross-linking with $^{125}$I-TGF-$\beta$1 alone, whereas lanes indicated with + represent cross-linking with $^{125}$I-TGF-$\beta$1 in the presence of excess unlabeled TGF-$\beta$1. Molecular weights are indicated on the right. TGF-$\beta$ receptor types I, II, and III are marked on the left.

Figure 2. TGF-$\beta$1 and/or EGF stimulate DNA synthesis in normal skin-derived fibroblasts (n = 9) and Dupuytren-derived fibroblasts (n = 12). The mean and SEM of triplicate determinations is shown. ■, normal skin-derived fibroblasts; ■■, Dupuytren-derived fibroblasts.
Figure 3. Presence of active and latent TGF-β in conditioned medium. Level of TGF-β was determined using the Mv1Lu Mink lung cell inhibitory assay. Inhibition of cell growth was measured using the colorimetric MTT-assay. The higher the optical density, the more Mv1Lu cells (i.e., the less TGF-β) in the conditioned medium. The mean of triplicate determinations is shown; SEM was always lower than 5%. As control we assayed the effects of purified TGF-β1 on Mv1Lu cells (left side). SF, normal skin-derived fibroblasts; Dup, Dupuytren-derived fibroblasts; [ ], acidified samples; ■, non-acidified samples.

shown in diseases such as pulmonary fibrosis,\textsuperscript{13} keloids,\textsuperscript{14} meningiomas,\textsuperscript{25} and gliomas,\textsuperscript{26} suggesting that high levels of TGF-β somehow put these tumor cells at a growth advantage.

It is surprising that so far no report has suggested an autocrine mechanism involving TGF-β in Dupuytren’s disease, since this disease seems to exemplify classic features of TGF-β overexpression, including hyperproliferation of fibroblasts and excessive production of ECM. Although in our study the three

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Nanograms/ml (Active + Latent)</th>
<th>% Active TGF-β</th>
</tr>
</thead>
<tbody>
<tr>
<td>Skin fibroblast 1</td>
<td>9.5</td>
<td>21</td>
</tr>
<tr>
<td>Skin fibroblast 2</td>
<td>13.3</td>
<td>6</td>
</tr>
<tr>
<td>Dupuytren 1</td>
<td>1.1</td>
<td>5</td>
</tr>
<tr>
<td>Dupuytren 2</td>
<td>5.2</td>
<td>3</td>
</tr>
<tr>
<td>Dupuytren 3</td>
<td>5.7</td>
<td>12</td>
</tr>
</tbody>
</table>

Values are averages of triplicate measurements with an SEM smaller than 5%.

ingredients for an autocrine loop (i.e., expression of growth factor, presence of receptors, and responsiveness to exogenous growth factor) were shown both in Dupuytren and normal cultures, there were some interesting differences. First, Dupuytren cultures reacted consistently with a stronger mitogenic response to exogenous TGF-β1 (up to fivefold), than normal cultures. Second, the combination of exogenous TGF-β1 and EGF resulted in a stronger (up to eightfold) mitogenic response in Dupuytren cultures than in normal cultures. Moreover this effect was synergistic in Dupuytren cultures, whereas only additive in the normal cultures. Although these differences were consistent, mitogenic responses varied among the 12 Dupuytren cultures, which might reflect a specific stage of the disease process, since it is known that Dupuytren’s disease evolves from a high-proliferative towards a low-proliferative stage.\textsuperscript{27} There have been reports on the effects of TGF-β and EGF on keloid\textsuperscript{28} and neurofibroma-derived cells,\textsuperscript{29} diseases with characteristics common to Dupuytren’s contracture. They agree partly with our findings in that TGF-β1 stimulated the growth
of these tumor cells *in vitro* \(^{28,29}\) consistently higher than in their normal counterparts and that the effects of TGF-\(\beta\)1 and EGF were synergistic in tumor cells, as opposed to additive in normal cells. \(^{28}\) The recently reported findings of increased presence of PDGF protein in Dupuytren's contracture as compared with normal skin \(^{7}\) could be explained by increased TGF-\(\beta\) expression, since it is well known that mitogenic effects of TGF-\(\beta\) on connective tissue cells are induced via a complex control of an autocrine PDGF loop. \(^{20}\) Therefore, increased PDGF expression could be a normal response to an abnormal TGF-\(\beta\) expression. Third, normal cells seemed to produce more TGF-\(\beta\) than Dupuytren cells, which would not directly fit with our model of increased TGF-\(\beta\) expression in Dupuytren's contracture. However, growth factors do not always accumulate in the conditioned medium, \(^{28}\) and activation of latent TGF-\(\beta\) *in vivo* might be different between the two cell types. Fourth, receptor-binding studies demonstrate that both Dupuytren and normal cultures have three TGF-\(\beta\)1 receptor types, I, II, and III. An interesting difference was that the type II receptor on Dupuytren cells bound more TGF-\(\beta\)1 than the type I receptor. This can be caused either by a higher number of receptors or by a higher receptor affinity for the ligand. Lately it has become clear that the type I and the type II receptors are responsible for the biologic actions of TGF-\(\beta\), whereas the type III receptor functions mainly as an extracellular binding domain. \(^{30}\) Recent reports showed that a change in the expression of the type II and to a lesser extent the type I receptor resulted in significantly different effects of TGF-\(\beta\) on growth control. \(^{31-37}\) Analogously, our finding of a higher expression or affinity of the type II versus the type I TGF-\(\beta\) receptor on Dupuytren fibroblasts than on normal fibroblasts might lead to a qualitative altered growth pattern in favor of Dupuytren cells. Moreover, it might lead to a different transcriptional regulation for other features such as ECM production.

### References


34. Linask KK, D’Angelo M, Gehris AM, Greene RM.

