Transforming Growth Factor-β1 Induces α-Smooth Muscle Actin Expression in Granulation Tissue Myofibroblasts and in Quiescent and Growing Cultured Fibroblasts

Alexis Desmoulière, Antoine Geinoz, Françoise Gabbiani, and Giulio Gabbiani

University of Geneva, Department of Pathology, CMU, 1211 Geneva 4, Switzerland

Abstract. Granulation tissue fibroblasts (myofibroblasts) develop several ultrastructural and biochemical features of smooth muscle (SM) cells, including the presence of microfilament bundles and the expression of α-SM actin, the actin isoform typical of vascular SM cells. Myofibroblasts have been proposed to play a role in wound contraction and in retractile phenomena observed during fibrotic diseases. We show here that the subcutaneous administration of transforming growth factor-β1 (TGFβ1) to rats results in the formation of a granulation tissue in which α-SM actin expressing myofibroblasts are particularly abundant. Other cytokines and growth factors, such as platelet-derived growth factor and tumor necrosis factor-α, despite their profibrotic activity, do not induce α-SM actin in myofibroblasts. In situ hybridization with an α-SM actin probe shows a high level of α-SM actin mRNA expression in myofibroblasts of TGFβ1-induced granulation tissue. Moreover, TGFβ1 induces α-SM actin protein and mRNA expression in growing and quiescent cultured fibroblasts and preincubation of culture medium containing whole blood serum with neutralizing antibodies to TGFβ1 results in a decrease of α-SM actin expression by fibroblasts in replicative and non-replicative conditions. These results suggest that TGFβ1 plays an important role in myofibroblast differentiation during wound healing and fibrocontractive diseases by regulating the expression of α-SM actin in these cells.

G RANULATION tissue formation and contraction are critical steps for the healing of an open wound (for review see Skalli and Gabbiani, 1988). These processes are characterized by the involvement of a specialized mesenchymal cell with morphologic and biochemical features between those of the fibroblast and of the smooth muscle (SM)[1] cell, called myofibroblast (Gabbiani et al., 1971; and for review see Schürch et al., 1992). Myofibroblasts disappear in normal scars, but persist in hypertrophic scars and in fibrotic lesions of many organs, including stroma reaction to epithelial tumors (Dvorak, 1986; Skalli et al., 1989b; Sappino et al., 1990b; Kuhn and McDonald, 1991; Gressner and Meyer, 1992), where they are allegedly involved in retractile phenomena as well as in extracellular matrix accumulation (Gabbiani et al., 1976; Bazin et al., 1980; Gressner and Meyer, 1992; and for review see Schürch et al., 1992). The hallmark of the myofibroblastic phenotype is the expression of α-SM actin (Skalli et al., 1989b; Darby et al., 1990), the actin isoform present in SM cells and myoepithe-

lial cells, and particularly abundant in vascular SM cells (Rubenstein, 1990; McHugh et al., 1991). Many aspects of myofibroblast biology are still mysterious, such as the factors regulating the expression of α-SM actin by these cells. Previous work from our laboratory has shown that growth factors known to elicit fibroblast replication in vivo and in vitro — e.g., PDGF or tumor necrosis factor-α (TNFα) — do not induce α-SM actin expression by these cells (Rubbia-Brandt et al., 1991; Desmoulière et al., 1992b). Here we report that the local administration of transforming growth factor-β1 (TGFβ1) in vivo results in the formation of a granulation tissue in which α-SM actin expressing myofibroblasts are particularly abundant. Moreover, TGFβ1 induces α-SM actin expression in growing and quiescent cultured fibroblasts and neutralizing antibodies to TGFβ1 inhibit partly the expression of α-SM actin in fibroblasts cultured in the presence of whole blood serum (WBS) but not of plasma-derived serum (PDS). These results suggest that TGFβ1 plays an important role in myofibroblast differentiation during wound healing and fibrosis.

1. *Abbreviations used in this paper:* DTAF, dichlorotriazinyl amino fluoresceine; IL-1, interleukin-1; PDS, plasma-derived serum; SM, smooth muscle; TNFα, tumor necrosis factor-α; WBS, whole blood serum.

Materials and Methods

In Vivo Experimental Procedures

All experiments were performed on 6-wk-old female Wistar rats. Seven

animals per group were used. Osmotic minipumps (model 2001; Alza Corp., Palo Alto, CA) were filled under sterile conditions with TGFβ1 isolated from human platelets (Sigma Immunochemicals, St. Louis, MO; and a gift of Dr. A. B. Roberts, Laboratory of Chemoprevention, National Cancer Institute, Bethesda, MD), or recombinant-human TGFβ1 (gift of Dr. A. B. Roberts). Recombinant murine TNFα (gift of Dr. G. Grau, Department of Pathology, University of Geneva, Geneva, Switzerland, with an endotoxin contamination <1 ng/ml, Grau et al., 1987; Piguet et al., 1990) was used. Minipumps were implanted subcutaneously into the back of the animals, through a channel (~3-cm long) prepared immediately before with blunt sterile scissors. Incisions were always performed over the sacral region. In controls, the pumps were filled with carrier solution. Pump outlets were oriented cranially. After 7 d of continuous delivery, the pumps were removed and their content verified. Animals in which the pumps were not empty were discarded. Newly formed granulation tissues surrounding perfusion pumps were collected systematically in the region close to the outlet and in the middle of the pump; they were either frozen in liquid isopentane immersed in liquid nitrogen and stored at −80°C for indirect immunofluorescence and for biochemical studies or fixed for light and EM.

Drugs were delivered at the following doses per day: TGFβ1, 140 ng; TNFα, 1 μg (Desmoulière et al., 1992b). The substances were brought to final concentration in sterile 150 mM NaCl just before implantation.

Cells and Culture Conditions

Cells were cultured in MEM (Gibco AG, Basel, Switzerland) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine, and containing 10% FCS (Gibco AG). Rat and human PDS and WBS were prepared according to Vogel et al. (1978). Cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2/95% air with medium changes twice a week.

Rat fibroblasts were obtained from explants of subcutaneous tissue of the back. Human fibroblasts were obtained from explants of normal subcutaneous tissue of breast skin from women undergoing reduction mammoplasty for cosmetic reasons. We have also used the HFL-1 cell line derived from human embryo lung (American Type Culture Collection, Rockville, MD) and bovine aortic endothelial cells (Gabbiani et al., 1984).

Passage 5 cells were treated with TGFβ1 in different culture conditions. In most cases, doses between 5 and 10 ng/ml which has been shown to induce α-SM actin in human arterial SM cells (Björkerud, 1991) were used. Medium was removed 24 h after plating and fibroblasts were incubated for 7 d with MEM plus serum alone or supplemented with TGFβ1. To study TGFβ1 effects on growth-arrested cells, the cytokine was added for 7 d to cultures where FCS had been substituted with 10% PDS and in which no growth was observed (Desmoulière et al., 1992b), or to a confluent monolayer of fibroblasts. The cell number was determined by performing haemocytometer cell count. A total of seven experiments were done using cell populations from different donors.

To neutralize the activity of TGFβ1, chicken (IgY; Research & Diagnostic Systems, Inc., Minneapolis, MN) or turkey (gift of Dr. A. B. Roberts; Flanders et al., 1988) neutralizing antibodies to TGFβ1 (1–15 μg/ml) were added to the culture medium and incubated for 1 h at room temperature. As a control, the same protein concentration of immunoglobulins from non-immunized chickens or turkeys was used.

Immunofluorescence Staining

Double immunofluorescence was performed using anti-αSM-1, a mouse mAb specific for α-SM actin (Skalli et al., 1986) and either a rabbit polyclonal anti-actin antibody recognizing all actin isoforms (Skalli et al., 1986), an affinity-purified rabbit polyclonal anti-SM myosin antibody (Benzonana et al., 1988) or an affinity-purified rabbit polyclonal anti-desmin antibody (Kocher et al., 1984). TRITC-labeled goat anti–mouse Ig (Nordic Immunological Laboratories, Tilburg, The Netherlands) and dichlorotriazinyl amino fluorescein (DTAF)-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Labs, Inc., West Grove, PA) were used for the second step. To minimize cross reactions, second antibodies were passed on solid immunoabsorbants of rat serum cross-linked with glutaraldehyde. Control staining was performed using non-immune IgGs at the same dilution used for the primary antibody. After washing, preparations were mounted and observed on a Zeiss Axiophot microscope (Carl Zeiss, Oberkochen, FRG) equipped with epiillumination and specific filters for rhodamine and fluorescein (Carl Zeiss). Photographs were taken, using Plan Neofluar 40×/0.90 objective, on T-Max Kodak black and white film (Eastman Kodak, Rochester, NY).

Cells were fixed in methanol for 5 min at −20°C. Immunofluorescence was performed in culture dishes or on cells detached by trypsinization and cytocentrifuged at 125 g for 5 min (Shandon Scientific-Co., London, UK). Cell counts were performed using the interactive image analysis system VIDAS (Kontron Elektronik GmbH, Eching, FRG) connected to an Intensified CCD camera type FMC-4005 (AVT-Horn, Aalen, FRG).

Cryostat sections (3–4-μm thick) of tissue samples were fixed in acetone at −20°C for 5 min and air dried at room temperature for 2 h. Adjacent frozen sections were stained with hematoxylin and eosin in order to correlate histologic with immunohistochemical evaluations.

In Situ Hybridization

A 33-mer synthetic oligonucleotide (dTAGCACACAACTGTGAATGTTTT-GTGGATCAGC-OH) derived from the rat α-SM actin 3'-untranslated region was used as single-stranded template (Bochaton-Piallat et al., 1992). An 11-mer synthetic oligonucleotide (dATCCACAAAAC-OH) was used as primer and extended by means of Klenov fragment of DNA polymerase I (sp act >3,000 U/mg; Boehringer Mannheim GbmH, Mannheim, FRG) with deoxyribonucleotide triphosphates (except dCTP) and ^{32}P-labeled dCTP (sp act >3,000 Ci/mM; Amersham Corp., Arlington Heights, IL) according to Sambrook et al. (1989). The labeled oligonucleotide (29 mer) was run on a 12% polyacrylamide sequencing gel with 7 M urea (Bio-Rad Laboratories AG, Glattbrugg, Switzerland). After gel autoradiography, the band was located, cut out, and eluted overnight in sterile distilled water with shaking.

Cryostat sections (3–4-μm thick) of tissue samples were deposited on slides pretreated with 3-aminopropyl-triethoxysilane (Fluka Chemie AG, Buchs, Switzerland), fixed in 4% paraformaldehyde (Merck, Darmstadt, FRG) at 4°C for 20 min, dehydrated, and stored at −80°C until use. The sections were incubated in 0.25% acetic anhydride (Fluka Chemie AG) in 0.1 M triethanolamine-HCl, pH 8.0 (Fluka Chemie AG), for 10 min, and then prehybridized in 50% deionized formamide (Fluka Chemia AG), 30 mM phosphate buffer, 0.02% Denhardt's solution (Sambrook et al., 1989), 1 mg/ml denatured salmon sperm DNA (Sigma Chemical Co.), 0.6 M NaCl, and 5 mM EDTA (Fluka Chemie AG) at 30°C for 4 h. Hybridization was performed in the same solution with the labeled oligonucleotide at 30°C overnight. Slides were washed in 2× SSC (SSC is 0.15 M NaCl, 0.015 M sodium citrate) for 1 min at room temperature, and then in 1× SSC at 40°C with shaking for 1 h and rinsed in ethanol. Slides were dipped into NTB2 autoradiographic emulsion (Eastman Kodak) and stored at 4°C. Preparations were observed on a Zeiss Axiophot microscope. Photographs were taken, using Plan Neofluar 40×/0.90 objective, on Ektachrome 64T color film. Densitometric evaluation of labeling was performed using the interactive image analysis system VIDAS (Kontron Elektronik GmbH) connected to an Intensified CCD camera type FMC-4005 (AVT-Horn).

Light and Electron Microscopy

For light microscopy, tissue samples were fixed in ethanol and embedded in paraffin wax. Sections (5-μm thick) were cut and stained with hematoxylin and eosin, or with Van Gieson (for collagen)-elastin.

For EM, tissues were cut in small cubes, fixed 4 h at room temperature in 2% glutaraldehyde (Merck, Darmstadt, FRG) in 0.1 M cacodylate (Merck) buffer and stored in buffer overnight at 4°C. They were postfixed in 2% OsO_4 (Merck), dehydrated in graded ethanols, and embedded in Epon 812 (Fluka Chemie AG, Buchs, Switzerland). Semithin sections were cut and stained with methylene blue. Representative areas were selected for thin sections. These were collected on copper grids, double stained with uranyl acetate (Merck) and lead citrate (Merck), and examined with a Philips 400 electron microscope.

For immunoelectron microscopy, specimens were cut in small cubes, fixed for 5 h at room temperature in 2% paraformaldehyde (Merck) and 0.5% glutaraldehyde (Merck) in 0.1 M phosphate buffer, and then rinsed overnight in the same buffer. The next day, tissues were dehydrated through graded ethanols at decreasing temperatures, up to −35°C in a LTE 020 Balzers apparatus (Balzers Union, Vaduz, Liechtenstein) and maintained at this temperature all through the subsequent infiltrations in Lowicryl K4M resin (Chemische Werke Lowi, Waldkraiburg, FRG). Polymerization was carried out in UV light first at −35°C for 24 h, and then at room temperature for additional 48 h. Representative regions of the tissues were selected on semithin sections and cut in thin sections which were collected on Formvarcoated nickel grids covered by a carbon film. The grids were first incubated for 30 min at room temperature in PBS (150 mM NaCl in 10 mM phosphate buffer, pH 7.2) containing 5% normal goat serum (Aurion, Wageningen,

The Netherlands), 0.8% BSA (Fluka Chemie AG) and 0.1% gelatin (Cold Water Fish Skin gelatin 40%; Aurion), and then for 2 h on drops of anti–α-SM-1 diluted at 1:10 in PBS containing 1% normal goat serum, 0.8% BSA, and 0.1% gelatin, and then rinsed three times for 10 min in PBS containing BSA and gelatin. The second incubation was performed with goat anti–mouse IgG conjugated to 0.8-nm gold particles (GP-Ultra Small; Aurion) diluted at 1:100 in PBS containing normal goat serum, BSA, and gelatin for 2 h at room temperature. They were rinsed three times for 15 min in PBS containing BSA and gelatin, three times for 5 min in PBS, and finally rinsed in sterile distilled water. Amplification of the gold staining was performed by reaction with colloidal silver (Aurion) for 15 min at room temperature according to Danscher (1981). Grids were counterstained with uranyl acetate and lead citrate and examined in a Philips 400 electron microscope.

SDS-PAGE and Immunoblotting

For SDS-PAGE, cells were trypsinized and, after haemocytometer cell counts, were mixed in buffer containing 1% SDS (Bio-Rad Laboratories AG), 1% DTT (Fluka Chemie AG), 1 mM PMSF (Merck), 1 mM Nα-P-Tosyl-L-arginine methylester (Sigma Immunochemicals) in 0.4 M Tris HCl, pH 6.8 (Laemmli, 1970). The samples were immediately sonicated and boiled for 3 min. Protein content was determined according to Bradford (1976). 40 μg protein was electrophoresed on a 5–20% gradient gel and stained with Coomassie blue. For quantification of total actin, gels were scanned with a computerized laser beam densitometer (Genofit SA, Geneva, Switzerland) as previously described (Kocher et al., 1985; Desmoulière et al., 1991).

Western blotting with anti–αSM-1, a mouse mAb-specific for α-SM actin, was performed according to Skalli et al. (1986). Briefly, 5 to 20 μg protein were electrophoresed on a 5–20% gradient gel. Separated proteins were transferred to nitrocellulose filters (Towbin et al., 1979) which were incubated with anti–αSM-1. A second incubation was done with a rabbit anti–mouse IgG conjugated with HRP (Nordic Immunological Laboratories). Enhanced chemiluminescence (Amersham International plc, Buckinghamshire, England) was used for detection.

RNA Extraction and Northern Blot Hybridization

Cultured cells were scraped from Petri dishes using a rubber policeman and homogenized in a sterile solution, pH 7.4, containing 4.5 M guanidine isothiocyanate (Fluka Chemie AG), 50 mM EDTA (Fluka Chemie AG), 0.1 M β-mercaptoethanol (Fluka Chemie AG), 25 mM sodium citrate (Merck) and 2% N-lauroylsarcosine (Sigma Immunochemicals) with a syringe through a 23-gauge needle as previously described (Kocher and Gabbiani, 1987). RNA was purified by ultracentrifugation through a cushion of 5.7 CsCl (Chirgwin et al., 1979). The RNA pellets were resuspended in 10 mM Tris HCl, pH 7.4, 0.5% SDS (Bio-Rad Laboratories AG) and 1 mM EDTA (Fluka Chemie AG), extracted twice with saturated phenolchloroform, and then once with chloroform-isoamylalcohol (24:1, vol/vol). The RNA was ethanol precipitated, resuspended in sterile water, and stored at –70°C.

Samples (5–20 μg) of total RNA were denatured with glyoxal, separated by electrophoresis in a 1% agarose gel, and then transferred overnight on a Hybond-N filter (Amersham Corp.). Next day, the membrane was baked for 2 h at 80°C under vacuum and stained with 0.04% methylene blue in 0.5 M Na-acetate to verify correct loading and transfer. Hybridization was performed with a riboprobe derived from the coding region of the rat α-SM actin mRNA which recognizes all actin mRNAs (total actin probe pRAoαA-C; Kocher and Gabbiani, 1987) or with synthetic oligonucleotides specific either for rat α-SM actin (Bochaton-Piallat et al., 1992) or for human α-SM actin (Barret and Benditt, 1987) mRNAs respectively. For the riboprobe, ^{32}P-labeling was carried out according to Melton et al. (1984) and Northern blot hybridization was performed as previously described (Kocher and Gabbiani, 1987) with minor modifications. The Northern blots were washed twice for 20 min at 58°C in 3× SSC and 2× Denhardt's solution (Sambrook et al., 1989), and subsequently three times in 0.2× SSC, 0.1% SDS (Bio-Rad Laboratories AG), and 0.1% Na-pyrophosphate, pH 7.0, for 20 min at 70°C. ^{32}P-labeling of the oligonucleotides and hybridization were performed according to Sambrook et al. (1989). Filters were exposed to Kodak X-Omat SO-282 film at –70°C between intensifying screens and developed films were analyzed by means of computerized densitometric scanning (Desmoulière et al., 1991; Bochaton-Piallat et al., 1992).

Results

Effect of TGFβ1 In Vivo

7 d after implantation, the reactive connective tissue around all pumps appeared macroscopically as a capsule, and was always distinct from the non-reactive subcutaneous tissue (Desmoulière et al., 1992b). In controls, the pumps remained mobile and the capsule was very thin. In animals treated with TNFα, the capsules were adherent to the subcutaneous tissue and clearly thicker than the control capsule. In animals treated with TGFβ1, the capsules were adherent to the subcutaneous tissue and less thick than those induced by TNFα.

At light microscopic examination, the newly formed capsules had different features according to the treatment. Tissue surrounding control pumps consisted of fibroblasts, new blood vessels, and extracellular matrix accompanied by a very weak inflammatory reaction. After TNFα treatment, a typical granulation tissue was observed; in addition, mononuclear cells and few polymorphonuclear cells were present, particularly close to the surface facing the osmotic pump (Desmoulière et al., 1992b). With TGFβ1, pronounced neovascularization and extensive collagen deposits surrounding fibroblastic cells were seen; leucocyte infiltration was mild.

After immunofluorescence staining, the walls of arterioles, venules and capillaries were positive for α-SM actin and desmin (Fig. 1), as well as for SM myosin. In fibroblastic cells of capsules surrounding pumps delivering NaCl and TNFα (Fig. 1, a and b), α-SM actin and desmin stainings were never detected. In the presence of TGFβ1 (Fig. 1 c), a diffuse positive staining for α-SM actin was observed in each animal; the cellular staining was often clearly distributed in bundles and most pronounced in the tissue portion close to the pump. A weak but definite staining for desmin was also observed (Fig. 1 d).

By in situ hybridization with the α-SM actin probe a high labeling was localized on the vessel walls, particularly in arterioles, of subcutaneous tissue (Fig. 2 a). In granulation tissue, autoradiographic silver grains were diffusely distributed at a high density compared to the adjacent unaffected subcutaneous tissue (Fig. 2 b). Densitometric analysis showed that in α-SM actin expressing granulation tissue and in vessel walls, OD values were increased to $172 \pm 3\%$ ($P < 0.001$) and $217 \pm 3\%$ ($P < 0.001$), respectively, compared to the levels of the subcutaneous tissue.

EM showed that fibroblasts of control capsules were characterized by a smooth nuclear outline and a cytoplasm rich in RER, Golgi apparatus, and mitochondria. In TNFα-treated tissues, in addition to such fibroblasts, several myofibroblastic cells were present showing typical cytoplasmic microfilament bundles or stress fibers (Desmoulière et al., 1992b). After TGFβ1 treatment, stress fibers, often located beneath the plasmalemma and parallel to the main axis of the cell, were present in a large proportion of fibroblastic cells. Dense bodies were scattered within these bundles. Microfilament bundles were also located along cytoplasmic extensions, frequently observed in these myofibroblasts. These cells showed also a dilated RER suggesting their involvement in an active synthetic activity.

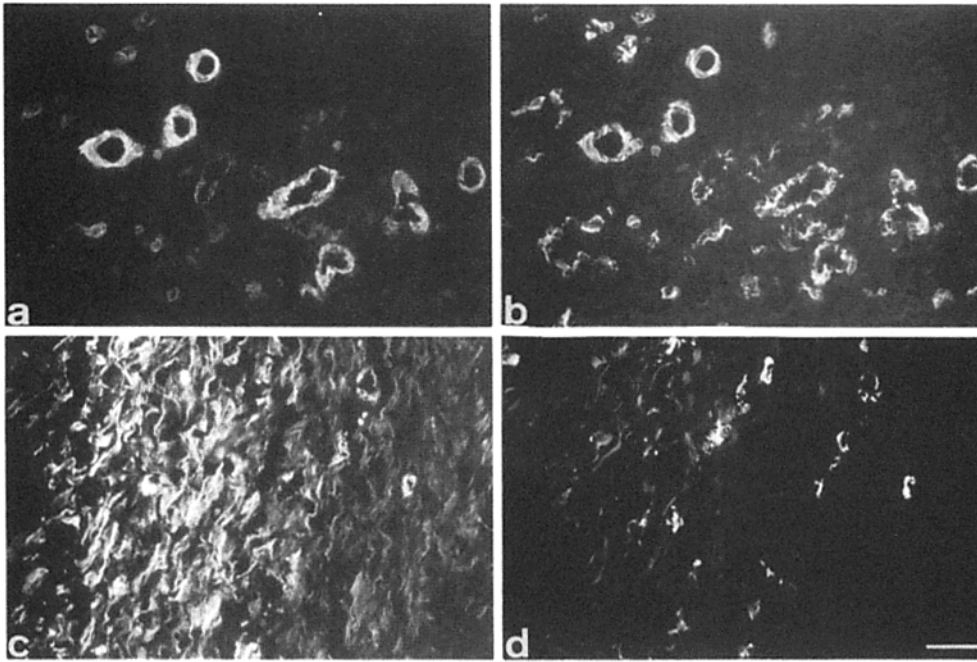Immunogold EM with anti–αSM-1 was performed on TNFα- and TGFβ1-induced capsules. As described previ-

Figure 1. Double immunofluorescence stainings for α-SM actin (*a* and *c*) and desmin (*b* and *d*) of capsules from animals treated with TNFα (*a* and *b*) or with TGFβ1 (*c* and *d*). Vessels are labeled for both α-SM actin and desmin. Numerous fibroblasts positive for α-SM actin (*c*) and few positive also for desmin (*d*) are recognized in TGFβ1-treated tissue. Bar, 10 μm.

ously (Skalli et al., 1989*a*; Darby et al., 1990), pericytes were always labeled. TNFα-induced granulation tissue showed fibroblastic cells containing microfilament bundles not decorated (Fig. 3 *a*) or decorated by few gold granules (Desmoulière et al., 1992*b*). In granulation tissue induced by TGFβ1, practically all myofibroblasts were stained with anti-αSM-1 with many colloidal silver particles localized over extensive stress fibers (Fig. 3 *b*).

Effect of TGFβ1 on Cultured Fibroblasts

In 10% FCS and 10% WBS, we observed an antiproliferative effect of TGFβ1 (10 ng/ml, 65.1 ± 1.3% and 40.1 ± 2.2% of control conditions).

Immunoblot analysis showed that the expression of α-SM actin was increased in the presence of TGFβ1 when human subcutaneous fibroblasts were cultured in medium plus 10% FCS (Fig. 4, *a–d*) or 10% WBS. This increase was higher when TGFβ1 was added to sparse subconfluent cells compared to confluent growth-arrested cells (data not shown). Moreover, an increase of α-SM actin expression by TGFβ1

treatment was observed in fibroblasts cultured in PDS (Fig. 4, *e–h*). A dose–response study with a confluent culture of human subcutaneous fibroblasts (∼3 × 10⁶ cells per 100 mm Petri dish) showed that TGFβ1 was able to induce α-SM actin expression at a dose of 1 ng/ml (8 ml per Petri dish). By densitometric scanning of Western blots, values expressed as percentage of the 10% PDS value were 380 for 10 ng/ml, 430 for 5 ng/ml, 330 for 2 ng/ml, and 270 for 1 ng/ml (standard errors of means were always <5% of the values).

Quantitative changes of total actin content were examined by densitometric analysis of SDS-PAGE (Fig. 4, *a*, *b*, *e*, and *f*). Total actin contents were similar in 10% FCS and 10% PDS cultured fibroblasts. TGFβ1 treatment did not modify significantly the total actin content which was 13.8 ± 0.4% of total cellular protein in subcutaneous adult rat fibroblasts, and 12.7 ± 0.3% in human subcutaneous fibroblasts.

Immunofluorescence studies (Fig. 5) showed that the increase of α-SM actin expression by TGFβ1 treatment in human subcutaneous fibroblasts cultured in PDS was at least in part due to a significant increase in the percentage of

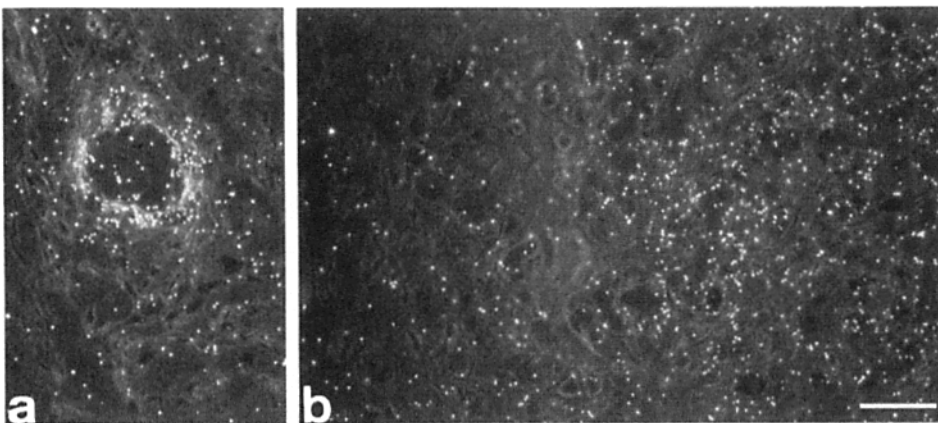*Figure 2.* α-SM actin mRNA expression in TGFβ1-treated connective tissue revealed by in situ hybridization. As internal control, a significant labeling is present in the vessel wall of a subcutaneous arteriole (*a*). *b* in α-SM actin expressing granulation tissue (*right*), a density of silver grains comparable to that present in the arteriole is observed, compared to the underlying subcutaneous tissue (*left*) where the labeling is lower. Bar, 10 μm.
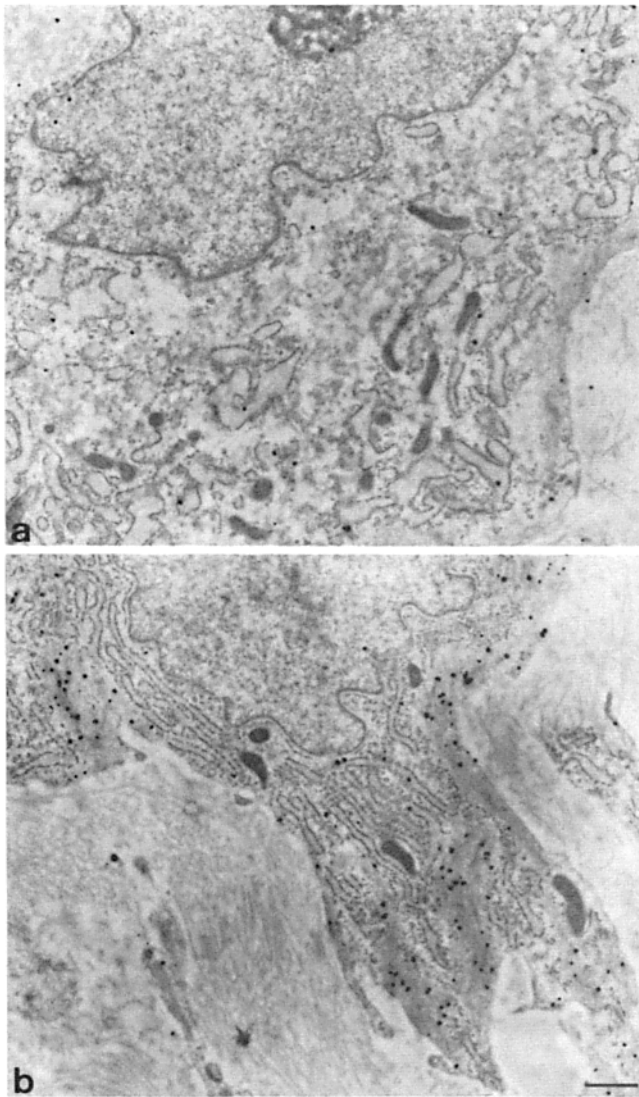
Figure 3. Immunoelectron microscopic details of myofibroblasts from TNFα (*a*)- and TGFβ1 (*b*)-treated capsules after labeling for α-SM actin. Some myofibroblasts of TNFα induced capsule show no or very weak labeling (*a*) of stress fibers. Silver particles are abundant over stress fibers of myofibroblasts in granulation tissue produced by TGFβ1 treatment (*b*). Bar, 1 μm.

Figure 4. Effect of TGFβ1 on α-SM actin expression in human sub-cutaneous fibroblasts measured by Western blot analysis. Cells were cultured in the presence of 10% FCS (*a–d*) or 10% PDS (*e–h*) without (*a, c, e,* and *g*) or with (*b, d, f,* and *h*; 5 ng/ml) TGFβ1. Densitometric scanning of Coomassie blue–stained gels (*a, b, e,* and *f*) shows that the percentage of actin per total protein is not significantly modified in the presence of 10% FCS or 10% PDS and by TGFβ1 treatment (for values see Results). Immunoblotting after SDS-PAGE (*c, d, g,* and *h*) shows that TGFβ1 increases the α-SM actin content of growing and quiescent human fibroblasts. *A,* actin.

α-SM actin positive cells (45.3 ± 2.1%) compared to control cells (7.5 ± 0.8%, *P* < 0.001). No significant modifications concerning the number of desmin-positive cells (<10%) or the number of SM myosin positive cells (<15%) were observed. When fibroblasts were treated by TGFβ1 (10 ng/ml or less) in the presence of 10% PDS, we never observed cell death as previously described for cultured SM cells (Björkerud, 1991).

We examined also the influence of FCS, WBS, and PDS on the expression of α-SM actin in cultured human fibroblasts. An increase of α-SM actin expression was observed in the presence of 1% serum (FCS or WBS) compared to 10% serum. Densitometric scanning of Western blots showed that in fibroblasts cultured in the presence of 1% WBS, the level of α-SM actin expression was clearly increased compared to the value obtained for fibroblasts cultured in 10% WBS (Fig.
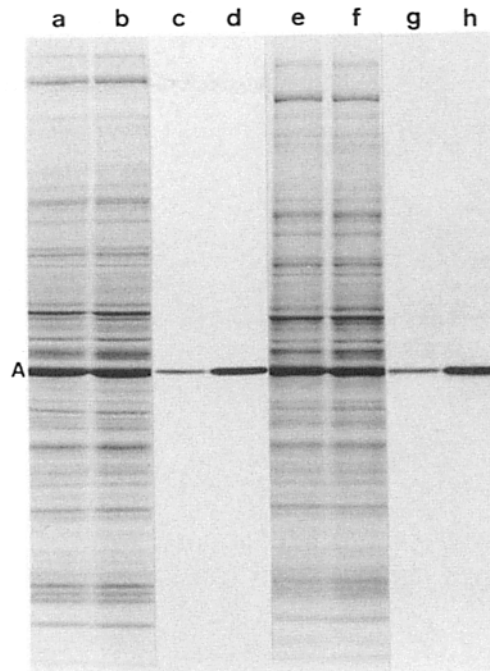
6). Neutralizing antibodies to TGFβ1 abolished the increase of α-SM actin expression observed in 10% PDS plus 1 ng/ml TGFβ1. Moreover, preincubation of medium containing 10% WBS (inducing cell replication) or 1% WBS (maintaining fibroblasts quiescent with high levels of α-SM actin) with neutralizing antibodies to TGFβ1 resulted in a decrease of α-SM actin expression in both 10% WBS (*P* < 0.01) and 1% WBS (*P* < 0.001, Fig. 6). When neutralizing antibodies to TGFβ1 were incubated with medium containing PDS, no changes in the expression of α-SM actin were observed compared to controls.

A marked increase of α-SM actin expression by TGFβ1 was also observed in HFL-1 cells. By densitometric scanning of Western blots, values expressed as percentage of the 10% FCS value were 3,070 for 5 ng/ml, 2,740 for 2 ng/ml, and 2,070 for 1 ng/ml. In these culture conditions, no effect was observed with a dose of 0.1 ng/ml. Cultured bovine aortic endothelial cells did not express α-SM actin normally and upon treatment with TGFβ1.

Hybridization with the total actin probe pRAoαA-C of total RNA preparations from human subcutaneous fibroblasts resulted in two bands corresponding to β- + γ- (2.1 kb) and α- (1.7 kb) actin mRNAs (Kocher and Gabbiani, 1987). In cells cultured in 10% PDS and treated with TGFβ1 (5 ng/ml) for 7 d, the 1.7-kb band was markedly increased compared to control cells. An induction of the 2.1-kb band was also observed (Leof et al., 1986). Densitometric scanning of North-
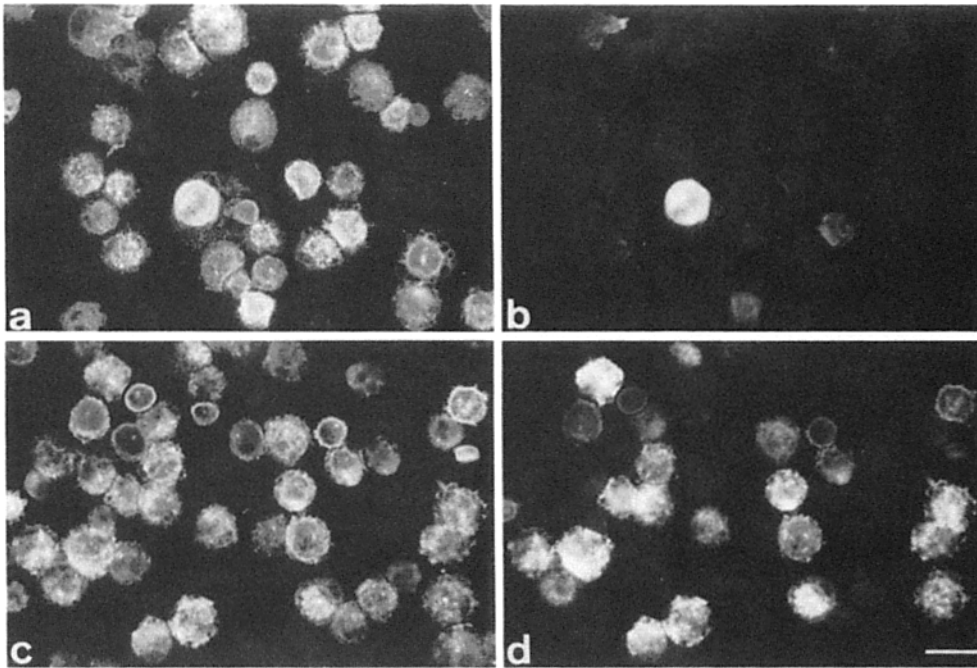
Figure 5. Effect of TGFβ1 on α-SM actin expression in human subcutaneous fibroblasts fixed after cytocentrifugation and stained by means of double immunofluorescence for total actin (*a* and *c*) and α-SM actin (*b* and *d*). Cells were cultured without (*a* and *b*) or with (*c* and *d*; 5 ng/ml) TGFβ1 in the presence of 10% PDS. TGFβ1 increases significantly the proportion of α-SM actin containing cells. Bar, 10 μm.

ern blots hybridized with the total actin probe showed that after TGFβ1 treatment, total actin mRNA was increased to 215 ± 3% of the level of controls. Densitometric scanning of Northern blots hybridized with our specific oligonucleotide showed that after TGFβ1 treatment, α-SM actin mRNA was increased to 380 ± 2% of the level of controls (Fig. 7). Similar results showing an important action of TGFβ1 on α-SM actin mRNA expression were also obtained in rat fibroblasts (data not shown).

Discussion

Many morphological and functional features of the myofibroblast have been recently defined (for review see Schürch

et al., 1992). Thus, it is established that during the modulation from fibroblast to myofibroblast, this cell acquires the expression of α-SM actin and, albeit more rarely, of desmin (Skalli et al., 1989*b*), but almost never of SM myosin heavy chains (Benzonana et al., 1988; Eddy et al., 1988). There is a correlation between the expression of these SM cell markers by myofibroblastic cells and the presence of an active retractile fibrosis in the affected tissues (Darby et al., 1990; Schmitt-Gräff et al., 1991). Myofibroblasts are also considered to be responsible, at least in part, for the production of connective tissue matrix components which characterizes fibrotic situations (Ramadori et al., 1987; Schäfer et al., 1987; Skalli and Gabbiani, 1988) or stroma reaction to epithelial tumors (Schürch et al., 1981; El-Torky et al.,
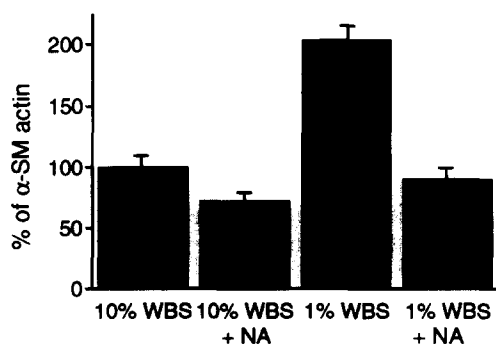

Figure 6. Effect of neutralizing antibodies to TGFβ1 on α-SM actin expression in human fibroblasts. Cells were cultured in the presence of 10% WBS or 1% WBS without or with neutralizing antibodies (*NA*). Densitometric scanning of Western blots shows that preincubation of medium containing WBS with NA to TGFβ1 results in a decrease of α-SM actin expression in both replicating (*10% WBS*) and non-replicating (*1% WBS*) fibroblasts. The effect is more important in non-replicating fibroblasts. Values are expressed as mean percentage (±SEM) of the 10% WBS value.

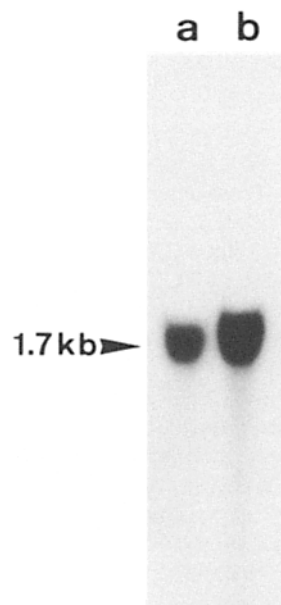*Figure 7.* Effect of TGFβ1 on actin mRNA expression in human subcutaneous fibroblasts. Hybridization of Northern blots with the oligonucleotide specific for human α-SM actin mRNA shows that α-SM actin mRNA expression is increased in TGFβ1-treated cells (*b*; 5 ng/ml) compared to control cells (*a*).

108

1985). The problem of the origin of myofibroblasts has not been yet fully clarified. It has been suggested that myofibroblasts of wounds arise locally from quiescent fibroblasts (Darby et al., 1990) but they may also originate from more specialized cells such as perisinusoidal cells in the liver (Ballardini et al., 1988; Ramadori, 1991; Schmitt-Gräff et al., 1991), mesangial cells in the glomerulus (Johnson et al., 1991), interstitial cells in the lung septa (Mitchell et al., 1989; Kapanci et al., 1990; Kuhn and McDonald, 1991), and perivascular SM cells (Shum and McFarlane, 1988; for a detailed review on this subject see Schürch et al., 1992). All these observations support the view that, whatever the origin, myofibroblastic modulation is an important step in wound healing and in pathological phenomena characterized by tissue retraction and fibrosis. However, little is known about the factors regulating on one hand the differentiation and the disappearance of myofibroblasts and on the other hand their functional activities. We show here that TGFβ1 stimulates in vivo the modulation of fibroblasts into α-SM actin expressing myofibroblasts. It has been recently reported that wounds in adult rats treated with a neutralizing antibody to TGFβ presented a reduced inflammatory response and restitution of the dermal architecture without scarring, thus suggesting that TGFβ plays a role in wound retraction and scar formation in vivo (Shah et al., 1992). Moreover, inhibition of TGFβ by decorin significantly reduced scarring in an experimental model of glomerulonephritis (Border et al., 1992).

When several growth factors were tested in vivo for their capacity to generate α-SM actin rich myofibroblasts, it was observed that interleukin-1 (IL-1), PDGF, and TNFα did not possess the capacity of inducing α-SM actin expression, which however was exerted by granulocyte macrophage-colony-stimulating factor (GM-CSF). GM-CSF activity is allegedly indirect since this cytokine, differently from TGFβ1, had no effect on α-SM actin expression on cultured fibroblasts (Rubbia-Brandt et al., 1991). After GM-CSF local application, the appearance of α-SM actin-rich myofibroblasts is preceded by an accumulation of macrophage clusters (Vyalov et al., 1993) which conceivably could produce one or more α-SM actin expression inducing factors, among which TGFβ1 is a likely candidate. In the same work (Rubbia-Brandt et al., 1991), we had described that TGFβ has no action of α-SM actin expression in vivo: since our present results have been repeatedly obtained with TGFβ1 isolated from human platelets supplied by two sources (see Materials and Methods) and with recombinant TGFβ1, we assume that TGFβ1 used for the previous experiment was contaminated by a substance inhibiting α-SM actin expression. Work is in progress in our laboratory to identify this contaminant.

We have also recently reported (Desmoulière et al., 1992b) that heparin stimulates the expression of α-SM actin in fibroblastic cells, both in vivo and in vitro. However, heparin acts only when fibroblasts undergo replication, suggesting that it produces a selection of α-SM actin–positive cells rather than an activation of the α-SM actin gene. It has been shown that heparin potentiates the biological action of TGFβ (McCaffrey et al., 1989, 1992). Indeed many similarities exist between the action of heparin or heparin-like molecules and TGFβ, particularly on cell proliferation and extracellular matrix synthesis (McCaffrey et al., 1992).

In addition to its action in vivo, our results show that TGFβ1 stimulates de novo the synthesis of α-SM actin in cultured fibroblasts growing in the presence of 10% FCS as well as in quiescent fibroblasts after confluence in the presence of 10% FCS or subconfluent in the presence of PDS. In this last case, the percentage of fibroblasts expressing α-SM actin was increased about six times compared to controls. We do not know whether TGFβ1 induces the transcription of α-SM actin mRNA or modifies the degradation of such mRNA in a cell which already expresses it. Further studies are necessary to clarify this point. In any event it appears that TGFβ1 is up to now the only substance known to be capable of stimulating α-SM actin synthesis in cultured quiescent fibroblastic cells. TGFβ increases also the expression of α-SM actin in cultured SM cells (Björkerud, 1991). We have previously shown that a population of cultured fibroblasts always contains a subpopulation of α-SM actin positive cells, even after cloning, suggesting that the expression of α-SM actin is a constitutive feature of a fibroblast population in vitro, where serum and hence TGFβ are present (Desmoulière et al., 1992a). TGFβ could represent one of the main regulators of this fibroblastic feature. This is supported by the observation that incubation of medium containing exogenous TGFβ1 in addition to PDS as well as of medium containing WBS, but not of medium containing only PDS with neutralizing antibodies to TGFβ1 reduces significantly the expression of α-SM actin in both replicating and quiescent fibroblasts. It remains to be established if and how TGFβ modulates the fibroblastic phenotype in vivo during wound healing. We suggest that during this phenomenon, fibroblast replication and movement as well as stress fiber formation (resulting in myofibroblasts without α-SM actin [for a discussion of the heterogeneity of myofibroblastic phenotype see Sappino et al., 1990b]) are under the control of factors other than TGFβ. This cytokine could play a role subsequently by stimulating collagen and α-SM actin synthesis and thus allowing the formation of a typical granulation tissue with α-SM actin-rich myofibroblasts, such as seen in rats 8 to 15 d after an experimental wound (Darby et al., 1990).

The study of growth factor and cytokine activities has allowed an important progress in the understanding of wound healing mechanisms (Deuel et al., 1991; Kovacs, 1991; Robson, 1991; Pierce et al., 1991, 1992). We know that many growth factors and cytokines are capable of stimulating fibroblast replication in vitro (Chen and Rabinovitch, 1990; Thornton et al., 1990; Kumar et al., 1991) and in vivo (Rubbia-Brandt et al., 1991; Desmoulière et al., 1992b), collagen synthesis in vitro (Roberts et al., 1986) and fibrosis formation in vivo (Krummel et al., 1988; Kovacs, 1991). However, with the exception of TGFβ, these growth factors do not affect directly α-SM actin expression by fibroblasts (Rubbia-Brandt et al., 1991), hence they presumably are not responsible for the differentiation of typical myofibroblastic cells. Extracellular matrix components are also capable of modifying the phenotype and the functional features of fibroblasts (Gospodarowicz et al., 1980; Kao et al., 1984; Desmoulière et al., 1992b). However the only extracellular matrix component capable of influencing α-SM actin expression in these cells, i.e., heparin, acts by selecting α-SM actin-positive cells among replicating fibroblasts (Desmoulière et al., 1992b).

It is well established that TGFβ plays a role in the differen-

tiation of several cells and tissues (Barnard et al., 1990; Sporn and Roberts, 1991; Border and Ruoslahti, 1992; Massagué et al., 1992). TGFβ has also been shown to stimulate collagen, fibronectin (Ignotz and Massagué, 1986; Roberts et al., 1986), and protease inhibitors (Laiho et al., 1986) synthesis and to inhibit collagenase production (Chiang and Nilsen-Hamilton, 1986; Matrisian et al., 1986; for review see Sporn et al., 1987). The fibrosis stimulating activity of TGFβ is generally interpreted on the light of its action on extracellular matrix compounds (Border et al., 1992; Shah et al., 1992; Sporn and Roberts, 1992). The demonstration that TGFβ1 promotes the synthesis of a structural protein such as α-SM actin, which is a well accepted differentiation marker (Sappino et al., 1990a; McHugh et al., 1991), confirms and extends the notion of the role of TGFβ in both differentiation and fibrosis. Taken together with our results, all these properties of TGFβ1 support the assumption (Sporn et al., 1987) that this cytokine plays a crucial role in granulation tissue evolution and particularly in myofibroblast formation, possibly after that other cytokines or growth factors (e.g., PDGF, IL-1, and TNFα) have directly or indirectly stimulated fibroblast proliferation.

Our results may help in the understanding of the mechanisms involved in the production and control of granulation tissue and are in accordance with the possibility that, at least in certain cases, cell differentiation is not controlled by genetically irreversible steps, as it has been assumed for a long time, but is modulated locally by microenvironmental influences exerted by cytokines, growth factors and extracellular matrix components (Blau and Baltimore, 1991).

References

Ballardini, G., M. Fallani, G. Biagini, F. B. Bianchi, and E. Pisi. 1988. Desmin and actin in the identification of Ito cells and in monitoring their evolution to myofibroblasts in experimental liver fibrosis. *Virchows Arch. [B] Cell Pathol.* 56:45–49.

Barnard, J. A., R. M. Lyons, and H. L. Moses. 1990. The cell biology of transforming growth factor β. *Biochim. Biophys. Acta.* 1032:79–87.

Barrett, T. B., and E. P. Benditt. 1987. Sis (platelet-derived growth factor B chain) gene transcript levels are elevated in human atherosclerotic lesions compared to normal artery. *Proc. Natl. Acad. Sci. USA.* 84:1099–1103.

Bazin, S., M. Le Lous, V. C. Duance, T. J. Sims, A. J. Bailey, G. Gabbiani, G. d'Andiran, G. Pizzolato, A. Browski, C. Nicoletis, and A. Delaunay. 1980. Biochemistry and histology of the connective tissue of Dupuytren's disease lesions. *Eur. J. Clin. Invest.* 10:9–16.

Benzonana, G., O. Skalli, and G. Gabbiani. 1988. Correlation between the distribution of smooth muscle or non muscle myosins and α-smooth muscle actin in normal and pathological soft tissues. *Cell Motil. Cytoskeleton.* 11:260–274.

Björkerud, S. 1991. Effects of transforming growth factor-β1 on human arterial smooth muscle cells in vitro. *Arteriosclerosis Thromb.* 11:892–902.

Blau, H. M., and D. Baltimore. 1991. Differentiation requires continuous regulation. *J. Cell Biol.* 112:781–783.

Bochaton-Piallat, M. L., F. Gabbiani, P. Ropraz, and G. Gabbiani. 1992. Cultured aortic smooth muscle cells from newborn and adult rats show distinct cytoskeletal features. *Differentiation.* 49:175–185.

Border, W. A., and E. Ruoslahti. 1992. Transforming growth factor-β in disease: the dark side of tissue repair. *J. Clin. Invest.* 90:1–7.

Border, W. A., N. A. Noble, T. Yamamoto, J. R. Harper, Y. Yamaguchi, M. D. Pierschbacher, and E. Ruoslahti. 1992. Natural inhibitor of transforming growth factor-β protects against scarring in experimental kidney disease. *Nature (Lond.).* 360:361–364.

Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72:248–254.

Chen, Y., and P. S. Rabinovitch. 1990. Mitogen response and cell cycle kinetics of Swiss 3T3 cells in defined medium: differences from human fibroblasts and effects of cell density. *Exp. Cell Res.* 190:145–150.

Chiang, C. P., and M. Nilsen-Hamilton. 1986. Opposite and selective effects of epidermal growth factor and human platelet transforming growth factor-beta on the production of secreted proteins by murine 3T3 cells and human fibroblasts. *J. Biol. Chem.* 261:10478–10481.

Chirgwin, J. M., A. E. Przybyla, R. J. MacDonald, and W. J. Rutter. 1979. Isolation and biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry.* 18:5294–5299.

Danscher, G. 1981. Localization of gold in biological tissue. A photochemical method for both light and electronmicroscopy. *Histochemistry.* 71:81–88.

Darby, I., O. Skalli, and G. Gabbiani. 1990. α-Smooth muscle actin is transiently expressed by myofibroblasts during experimental wound healing. *Lab. Invest.* 63:21–29.

Desmoulière A., L. Rubbia-Brandt, and G. Gabbiani. 1991. Modulation of actin isoform expression in cultured arterial smooth muscle cells by heparin and culture conditions. *Arteriosclerosis Thromb.* 11:244–253.

Desmoulière, A., L. Rubbia-Brandt, A. Abdiu, T. Walz, A. Macieira-Coelho, and G. Gabbiani. 1992a. α-Smooth muscle actin is expressed in a subpopulation of cultured and cloned fibroblasts and is modulated by γ-interferon. *Exp. Cell Res.* 201:64–73.

Desmoulière, A., L. Rubbia-Brandt, G. Grau, and G. Gabbiani. 1992b. Heparin induces α-smooth muscle actin expression in cultured fibroblasts and in granulation tissue myofibroblasts. *Lab. Invest.* 67:716–726.

Deuel, T. F., R. S. Kawahara, T. A. Mustoe, and G. F. Pierce. 1991. Growth factors and wound healing: platelet-derived growth factor as a model cytokine. *Annu. Rev. Med.* 42:567–584.

Dvorak, H. F. 1986. Tumors: wounds that do not heal. Similarities between tumor stroma generation and wound healing. *N. Engl. J. Med.* 315:1650–1659.

Eddy, R. J., J. A. Petro, and J. J. Tomasek. 1988. Evidence for the nonmuscle nature of the "myofibroblast" of granulation tissue and hypertrophic scar. An immunofluorescence study. *Am. J. Pathol.* 130:252–260.

El-Torky, M., L. I. Giltman, and M. Dabbous. 1985. Collagens in scar carcinoma of the lung. *Am. J. Pathol.* 121:322–326.

Flanders, K. C., A. B. Roberts, N. Ling, B. E. Fleurdelys, and M. B. Sporn. 1988. Antibodies to peptide determinants in transforming growth factor β and their applications. *Biochemistry.* 27:739–746.

Gabbiani, G., G. B. Ryan, and G. Majno. 1971. Presence of modified fibroblasts in granulation tissue and their possible role in wound contraction. *Experientia.* 27:549–550.

Gabbiani, G., M. Le Lous, A. J. Bailey, S. Bazin, and A. Delaunay. 1976. Collagen and myofibroblasts of granulation tissue. A chemical, ultrastructural and immunologic study. *Virchows Arch. [B] Cell Pathol.* 21:133–145.

Gabbiani, G., F. Gabbiani, R. L. Heimark, and S. M. Schwartz. 1984. Organization of actin cytoskeleton during early endothelial regeneration in vitro. *J Cell Sci.* 66:39–50.

Gospodarowicz, D., D. Delgado, and I. Vlodavsky. 1980. Permissive effect of the extracellular matrix on cell proliferation in vitro. *Proc. Natl. Acad. Sci. USA.* 77:4094–4098.

Grau, G. E., L. F. Fajardo, P. F. Piguet, B. Allet, P. H. Lambert, and P. Vassalli. 1987. Tumor necrosis factor (cachectin) as an essential mediator in murine cerebral malaria. *Science (Wash. DC).* 237:1210–1212.

Gressner, A. M., and D. Meyer. 1992. The role of proteoglycans in liver fibrosis. *In* Molecular and Cell Biology of Liver Fibrosis. A. M. Gressner, and G. Ramadori, editors. Kluwer Academic Publishers, Dordrecht, The Netherlands. 115–136.

Ignotz, R. A., and J. Massagué. 1986. Transforming growth factor-β stimulates the expression of fibronectin and collagen and their incorporation into the extracellular matrix. *J. Biol. Chem.* 261:4337–4345.

Johnson, R. J., H. Iida, C. E. Alpers, M. W. Majesky, S. M. Schwartz, P. Pritzl, K. Gordon, and A. M. Gown. 1991. Expression of smooth muscle cell phenotype by rat mesangial cells in immune complex nephritis. α-Smooth muscle actin is a marker of mesangial cell proliferation. *J. Clin. Invest.* 87:847–858.

Kao, R. T., J. Hall, L. Engel, and R. Stern. 1984. The matrix of human breast tumor cells is mitogenic for fibroblasts. *Am. J. Pathol.* 115:109–116.

Kapanci, Y., S. Burgan, G. G. Pietra, B. Conne, and G. Gabbiani. 1990. Modulation of actin isoform expression in alveolar myofibroblasts (contractile interstitial cells) during pulmonary hypertension. *Am. J. Pathol.* 136:881–889.

Kocher, O., and G. Gabbiani. 1987. Analysis of α-smooth muscle actin mRNA expression in rat aortic smooth-muscle cells using a specific cDNA probe. *Differentiation.* 34:201–209.

Kocher, O., O. Skalli, W. S. Bloom, and G. Gabbiani. 1984. Cytoskeleton of rat aortic smooth muscle cells: normal conditions and experimental intimal thickening. *Lab. Invest.* 50:645–652.

Kocher, O., O. Skalli, D. Cerutti, F. Gabbiani, and G. Gabbiani. 1985. Cytoskeletal features of rat aortic cells during development: an electron microscopic, immunohistochemical, and biochemical study. *Circ. Res.* 56: 829–838.

Kovacs, E. J. 1991. Fibrogenic cytokines: the role of immune mediators in the development of scar tissue. *Immunol. Today.* 12:17–23.

Krummel, T. M., B. A. Michna, B. L. Thomas, M. B. Sporn, J. M. Nelson, A. M. Salzberg, I. K. Cohen, and R. F. Diegelmann. 1988. Transforming growth factor beta (TGF-β) induces fibrosis in a fetal wound model. *J. Pediat. Surg.* 23:647–652.

Kuhn, C., and J. A. McDonald. 1991. The roles of the myofibroblast in idiopathic pulmonary fibrosis. Ultrastructural and immunohistochemical features of sites of active extracellular matrix synthesis. *Am. J. Pathol.* 138:1257–1265.

Kumar, R. K., R. O'Grady, W. Li, L. W. Smith, and G. C. Rhodes. 1991. Primary culture of adult mouse lung fibroblasts in serum-free medium: responses to growth factors. *Exp. Cell Res.* 193:398–404.

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.).* 227:680–685.

Laiho, M., O. Sakesela, P. A. Andreasen, and J. Keski-Oja. 1986. Enhanced production and extracellular deposition of the endothelial-type plasminogen activator inhibitor in cultured human lung fibroblasts by transforming growth factor-beta. *J. Cell Biol.* 103:2403–2410.

Leof, E. B., J. A. Proper, M. J. Getz, and H. L. Moses. 1986. Transforming growth factor type β regulation of actin mRNA. *J. Cell. Physiol.* 127:83–88.

Massagué, J., S. Cheifetz, M. Laiho, D. A. Ralph, F. M. B. Weis, and A. Zentella. 1992. Transforming growth factor-β. *Cancer Surv.* 12:81–103.

Matrisian, L. M., P. Leroy, C. Ruhlmann, M. C. Gesnel, and R. Breathnach. 1986. Isolation of the oncogene and epidermal growth factor-induced transin gene: complex control in rat fibroblasts. *Mol. Cell Biol.* 6:1679–1686.

McCaffrey, T. A., D. J. Falcone, C. F. Brayton, L. A. Agarwal, F. G. P. Welt, and B. B. Weksler. 1989. Transforming growth factor-β activity is potentiated by heparin via dissociation of the transforming growth factor-β/α_2-macroglobulin inactive complex. *J. Cell Biol.* 109:441–448.

McCaffrey, T. A., D. J. Falcone, and B. Du. 1992. Transforming growth factor-$\beta1$ is a heparin-binding protein: identification of putative heparin-binding regions and isolation of heparins with varying affinity for TGF-$\beta1$. *J. Cell. Physiol.* 152:430–440.

McHugh, K. M,. K. Crawford, and J. L. Lessard. 1991. A comprehensive analysis of the developmental and tissue-specific expression of the isoactin multigene family in the rat. *Dev. Biol.* 148:442–458.

Melton, D. A., P. A. Krieg, M. R. Rebagliati, T. Maniatis, K. Zinn, and M. R. Green. 1984. Efficient in vitro synthesis of biologically active RNA and RNA hybridization probes from plasmids containing a bacteriophage SP6 promoter. *Nucleic Acids Res.* 12:7035–7056.

Mitchell, J., J. Woodcock-Mitchell, S. Reynolds, R. Low, K. Leslie, K. Adler, G. Gabbiani, and O. Skalli. 1989. α-Smooth muscle actin in parenchymal cells of bleomycin-injured rat lung. *Lab. Invest.* 60:643–650.

Pierce, G. F., J. Vande Berg, R. Rudolph, J. Tarpley, and T. A. Mustoe. 1991. Platelet-derived growth factor-BB and transforming growth factor beta1 selectively modulate glycosaminoglycans, collagen, and myofibroblasts in excisional wounds. *Am. J. Pathol.* 138:629–646.

Pierce, G. F., J. E. Tarpley, D. Yanagihara, T. A. Mustoe, G. M. Fox, and A. Thomason. 1992. Platelet-derived growth factor (BB homodimer), transforming growth factor-$\beta1$, and basic fibroblast growth factor in dermal wound healing. Neovessel and matrix formation and cessation or repair. *Am. J. Pathol.* 140:1375–1388.

Piguet, P. F., G. E. Grau, and P. Vassalli. 1990. Subcutaneous perfusion of tumor necrosis factor induces local proliferation of fibroblasts, capillaries, and epidermal cells, or massive tissue necrosis. *Am. J. Pathol.* 136:103–110.

Ramadori, G. 1991. The stellate cell (Ito-cell, fat-storing cell, lipocyte, perisinusoidal cell) of the liver. New insights into pathophysiology of an intriguing cell. *Virchows Arch. [B] Cell Pathol.* 61:147–158.

Ramadori, G., H. Rieder, T. Knittel, H. P. Dienes, and K. H. Meyer zum Büschenfelde. 1987. Fat storing cells of rat liver synthesize and secrete fibronectin. Comparison with hepatocytes. *J. Hepatol.* 4:190–197.

Roberts, A. B., M. B. Sporn, R. K. Assoian, J. M. Smith, N. S. Roche, L. M. Wakefield, U. I. Heine, L. A. Liotta, V. Falanga, J. H. Kehrl, and A. S. Fauci. 1986. Transforming growth factor type-beta: rapide induction of fibrosis and angiogenesis in vivo and stimulation of collagen formation in vitro. *Proc. Natl. Acad. Sci. USA.* 83:4167–4171.

Robson, M. C. 1991. Growth factors as wound healing agents. *Curr. Opin. Biotechnology.* 2:863–867.

Rubbia-Brandt, L., A. P. Sappino, and G. Gabbiani. 1991. Locally applied GM-CSF induces the accumulation of α-smooth muscle actin containing myofibroblasts. *Virchows Arch. [B] Cell Pathol.* 60:73–82.

Rubenstein, P.A. 1990. The functional importance of multiple actin isoforms. *BioEssays.* 12:309–315.

Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular cloning. A Laboratory Manual (second edition). Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Sappino, A. P., I. Masouyé, J. H. Saurat, and G. Gabbiani. 1990a. Smooth muscle differentiation in scleroderma fibroblastic cells. *Am. J. Pathol.* 137:585–591.

Sappino, A. P., W. Schürch, and G. Gabbiani. 1990b. Differentiation repertoire of fibroblastic cells: expression of cytoskeletal proteins as marker of phenotypic modulations. *Lab. Invest.* 63:144–161.

Schäfer, S., O. Zerbe, and A. M. Gressner. 1987. The synthesis of proteoglycans in fat-storing cells of rat liver. *Hepatology.* 7:680–687.

Schmitt-Gräff, A., S. Krüger, F. Bochard, G. Gabbiani, and H. Denk. 1991. Modulation of alpha smooth muscle actin and desmin expression in perisinusoidal cells of normal and diseased human livers. *Am. J. Pathol.* 138: 1233–1242.

Schürch, W., T. A. Seemayer, and R. Lagacé. 1981. Stromal myofibroblasts in primary invasive and metastatic carcinomas. A combined immunological, light electron microscopy study. *Virchows Arch. [A] Pathol. Anat.* 391: 125–139.

Schürch, W., T. A. Seemayer, and G. Gabbiani. 1992. Myofibroblast. *In* Histology for Pathologists. S. S. Sternberg, editor. Raven Press, NY. 109–144.

Shah, M., D. M. Foreman, and M. W. J. Ferguson. 1992. Control of scarring in adult wounds by neutralising antibody to transforming growth factor β. *Lancet.* 339:213–214.

Shum, D. T., and R. M. McFarlane. 1988. Histogenesis of Dupuytren's disease: an immunohistochemical study of 30 cases. *J. Hand Surg. [Am].* 13A:61–67.

Skalli, O., and G. Gabbiani. 1988. The biology of the myofibroblast. Relationship to wound contraction and fibroconnective diseases. *In* The Molecular and Cellular Biology of Wound Repair. R. A. F. Clark, and P. M. Henson, editors. Plenum Publishing Corporation, NY. 373–401.

Skalli, O., P. Ropraz, A. Trzeciak, G. Benzonana, D. Gillessen, and G. Gabbiani. 1986. A monoclonal antibody against α-smooth muscle actin: a new probe for smooth muscle differentiation. *J. Cell Biol.* 103:2787–2796.

Skalli, O., M. F. Pelte, M. C. Peclet, G. Gabbiani, P. Gugliotta, G. Bussolati, M. Ravazzola, and L. Orci. 1989a. α-Smooth muscle actin, a differentiation marker of smooth muscle cells, is present in microfilamentous bundles of pericytes. *J. Histochem. Cytochem.* 37:315–321.

Skalli, O., W. Schürch, T. Seemayer, R. Lagacé, D. Montandon, B. Pittet, and G. Gabbiani. 1989b. Myofibroblasts from diverse pathological settings are heterogeneous in their content of actin isoforms and intermediate filament proteins. *Lab. Invest.* 60:275–285.

Sporn, M. B., and A. B. Roberts. 1991. Interactions of retinoids and transforming growth factor-β in regulation of cell differentiation and proliferation. *Mol. Endocrinol.* 5:3–7.

Sporn, M. B., and A. B. Roberts. 1992. Transforming growth factor-β: recent progress and new challenges. *J. Cell Biol.* 119:1017–1021.

Sporn, M. B., A. B. Roberts, L. M. Wakefield, and B. de Crombrugghe. 1987. Some recent advances in the chemistry and biology of transforming growth factor-beta. *J. Cell Biol.* 105:1039–1045.

Thornton, S. C., S. B. Por, B. J. Walsh, R. Penny, and S. N. Breit. 1990. Interaction of immune and connective tissue cells: I. The effect of lymphokines and monokines on fibroblast growth. *J. Leukocyte Biol.* 47:312–320.

Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA.* 76:4350–4354.

Vogel, A., E. Raines, B. Kariya, M. J. Rivest, and R. Ross. 1978. Coordinate control of 3T3 cell proliferation by platelet-derived growth factor and plasma components. *Proc. Natl. Acad. Sci. USA.* 75:2810–2814.

Vyalov, S., A. Desmoulière, and G. Gabbiani. 1993. GM-CSF induced granulation tissue formation: relationships between macrophage and myofibroblast accumulation. *Virchows Arch. [B] Cell Pathol.* In press.