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 retractive 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.
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 Immunocouloratories, St. Louis, MO) and a gift of Dr. A. B. Roberts. Recombinant mouse 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 animal 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% CO2/95% air with medium changes in paraffin wax. Sections (5-µm thick) were cut and stained with hematoxylin and eosin, or with Van Gieson (for collagen)-elastin.

**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 (dTAGACACAAACTGTAAGTTGTTTT- GTGATAGC-G-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 (dATCCACAAAC-OH) was used as primer and extended by means of Klenov fragment of DNA polymerase I (sp act >3,000 U/mg; Boehringer Mannheim GmbH, Mannheim, FRG) with deoxyribonucleotide triphosphates (except dCTP and 32P-labeled dCTP (sp act >3,000 Ci/mmol, Amersham Corp., Airlington 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.

**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 fixed in small cubes, fixed 4 h at room temperature in 2% glutaraldehyde (Merck, Darmstadt, FRG) in 0.1 M cacodylate buffer, pH 7.4 for 1 h, and then prehybridized in 0.5% deionized formamide (Fluka Chemie AG), 30 mM phosphate buffer, 0.2% 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 4°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).
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-I diluted at 1:100 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 gold anti-mouse IgG conjugated to 0.8-nm gold particles (GP-Ultra Small; SM-1) diluted at 1:10 in PBS containing normal goat serum, 0.8% BSA, and 0.1% gelatin, and then rinsed 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), 0.1 M β-mercaptoethanol (Fluka Chemie AG), 25 mM sodium citrate buffer, 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 3-20% 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-α-SM-1, a mouse Ab-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-α-SM-1. A second incubation was done with a rabbit anti-mouse IgG conjugated with HRP (Nordic Immunological Laboratories) as previously described (Kocher et al., 1985; Desmoulière et al., 1991). 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 phenol-chloroform, and then once with chloroform-isoamylalcohol (24:1, vol/vol). The RNA was precipitated with ethanol, was 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 prA006-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, 32P-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 baked 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. 32P-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 ± 3% (P < 0.0001) and 217 ± 3% (P < 0.0001), 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-α-SM-I was performed on TNFα and TGFβ1-induced capsules. As described previ-
ous (Skalli et al., 1989a; 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., 1992b). In granulation tissue induced by TGFβ1, practically all myofibroblasts were stained with anti-α-SM-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 × 10^6 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 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.

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.
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 (Björkérud, 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 pRAactA-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-
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., 1989b), 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 retractive fibrosis in the affected tissues (Darby et al., 1990; Schmitt-Graff 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., 1991; El-Torky et al., 1988).
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., 1989; 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 (Björknerud, 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 TGFβ 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., 1990) 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; Robinson, 1991; Pierce et al., 1991, 1992). We know that many growth factors and cytokines are capable of stimulating fibroblast replication in vitro (Chen and Rabinovich, 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-
TGF/β1 promotes the synthesis of a structural protein such as collagen, fibronectin (Ignotz and Massagué, 1986; Roberts et al., 1986), and protease inhibitors (Laiho et al., 1986) 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 TGF/β 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 TGF/β 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 TGFβ 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 understanding 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).

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