The Deletion of Transforming Growth Factor- β -Induced Myofibroblasts Depends on Growth Conditions and Actin Organization

Pamela D. Arora and Christopher A. G. McCulloch

From the MRC Group In Periodontal Physiology, Faculty of Dentistry, University of Toronto, Toronto, Ontario, Canada

Myofibroblasts are important but transient mediators of normal wound contraction and are characterized phenotypically by their high levels of α -smooth-muscle actin (SMA). During wound maturation, these cells disappear. We have examined the mechanisms that lead to myofibroblast deletion in a fibroblast culture model. Transforming growth factor- β (TGF- β) was used to increase SMA content in gingival fibroblasts (three- to sixfold). After replating TGF- β -induced cells at low density with serum, there was a fivefold decrease in SMA protein content, SMA protein synthesis, and SMA mRNA as cells proliferated. These reductions were due to reduced SMA mRNA stability. For TGF- β -induced cells plated at high density without serum (ie, quiescent conditions), protein content was reduced by only 20% over 12 days. TGF- β protected SMA-positive cells against apoptosis in serum-free cultures. Those cells that were protected against apoptosis exhibited well-developed stress fibers enriched in SMA. We conclude that, in quiescent myofibroblasts, SMA protein turnover is slow, and cells are long-lived. In proliferative conditions SMA protein and mRNA turn over quickly, and the myofibroblast phenotype dissipates. The reduced apoptosis of myofibroblasts in quiescent conditions is due in part to the organization of SMA into stress fibers. (*Am J Pathol* 1999, 155:2087-2099)

The fibroblast is the most abundant cell type in normal connective tissues and plays a central role in synthesis, degradation, and remodeling of the extracellular matrix in health and disease. Despite their somewhat uniform appearance in normal tissues *in vivo*, fibroblasts can exhibit considerable heterogeneity of morphology and function in pathological or remodeling states. *In vitro*, they demonstrate widespread variations of proliferative status, matrix protein synthetic profiles, and phenotypic markers.^{1,2} Currently the fate of fibroblasts and the mechanisms of their deletion in remodeling or wound-healing sites are poorly understood, in part because of the lack of reliable markers for fibroblast subpopulations. Considerable effort has been devoted to the characterization of fibroblast

subpopulations.^{1,2,3} α -Smooth-muscle actin (SMA) is an actin isoform that is strongly expressed in myofibroblasts^{3,4,5} and that may mark discrete subpopulations of fibroblasts with constitutively high levels of contractile capacity.⁶ SMA-containing myofibroblasts are found in fibrocontractive lesions but are present only transiently during healing of dermal wounds.⁷ In culture, fibroblasts may acquire several phenotypic features of myofibroblasts including stress fibers.⁸ These force-generating elements are often enriched with SMA⁶ and may have *in vivo* counterparts that are involved in wound contraction.⁹

In vivo, wound myofibroblasts are thought to arise locally from quiescent fibroblasts without SMA.³ These SMA-null cells proliferate and form myofibroblasts, and then, after wound maturation, many of these cells disappear by apoptosis.^{7,10} Although little is known about the factors that regulate the differentiation and subsequently the deletion of myofibroblasts, their transient development from local fibroblastic cells suggests that, at least in certain cases, cell differentiation in fibroblasts is not controlled by genetically determined steps but instead may be a short-lived response to locally available cytokines, such as transforming growth factor (TGF)- β , interferon (IFN)- γ , platelet-derived growth factor (PDGF), or heparin.¹¹ Thus, although smooth-muscle cells constitutively express high levels of SMA,¹² fibroblasts can be induced to express SMA by TGF- β *in vitro* when the cells are grown on rigid matrices.^{13,14} Further, the level of SMA expression in cultured fibroblasts is modulated by their proliferative status,¹⁴ substrate flexibility,¹⁵ and cell density.¹⁶ Although a great deal is known about the synthesis and stability of SMA in quiescent and proliferating smooth-muscle cells,^{12,17} in which SMA is a highly abundant and constitutively synthesized protein, there is much less information on SMA metabolism in myofibroblasts, which may be of central importance in phenotypic stability.

Little is known about the mechanisms that are involved in the formation and deletion of SMA-containing fibroblasts. In one possible regulatory system, the apparent

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Address reprint requests to C. A. G. McCulloch, Room 244, Fitzgerald Building, University of Toronto, 150 College St., Toronto, Ontario, Canada M5S 3E8. E-mail: christopher.mcculloch@utoronto.ca.

lifetime of myofibroblasts may be regulated by the turnover rates of SMA mRNA and protein. For example, there is an apparently long-lived pool of SMA protein in gingival fibroblasts when grown on rigid but not compliant substrates.¹⁵ It is noteworthy that, on rigid substrates, incorporation of SMA into stress fibers is maximal, and the cells exhibit apparent high levels of intracellular tension. When cultured on certain types of matrix proteins, SMA expression may modulate cell shape¹⁵ and the migratory capacity of cells,¹⁸ suggesting that the SMA content of cells is influenced by intracellular tension and the substrate to which the cells are attached. In addition, the shape of cells and their relative degree of intracellular tension and spreading appear to be critical factors in determining whether cells *in vitro* live or die.¹⁹ In view of these reports, we have examined the importance of substrate-dependent SMA incorporation into stress fibers and the relative apoptotic susceptibility of cells on different substrates. We have also characterized the stability of SMA protein and mRNA in gingival fibroblasts to obtain insights into the plasticity of the myofibroblastic phenotype after TGF induction.

Materials and Methods

Cell Culture and Experimental Conditions

Primary cultures of human gingival fibroblasts were obtained from biopsies of normal gingiva in patients aged 10 to 16 years, as previously described.⁶ Cells at passages 3 to 12 were used for all experiments. The cells were grown as monolayer cultures in α -minimal essential medium (MEM) plus 15% v/v fetal bovine serum on plastic tissue culture dishes, fibronectin-coated plastic (10 μ g/ml of coating solution), or collagen-coated plastic as described.¹⁵ Confluent cultures were serum-starved for 48 hours and treated with TGF- β_1 (R&D Systems, Minneapolis, MN) or phosphate-buffered saline (PBS) vehicle for 72 hours in serum-free medium. In preliminary experiments to optimize myofibroblast formation, dose-response studies using 1, 5, 10, and 20 ng/ml TGF- β_1 were conducted. Immunoblotting (see below) showed that the optimal SMA response was 10 ng/ml TGF- β , and all subsequent experiments were performed at this dose. Others have used doses of TGF- β for SMA gene expression that are equivalent to levels of TGF- β_1 found in wound fluid.²⁰

After TGF- β or vehicle treatment, the cells were replated at high density in serum-free conditions or at low density in the presence of serum (Figure 1). For our experiments, cells were collected at 0, 3, 7, and 12 days. The initial time point (ie, 0 day) indicates samples obtained after TGF- β or vehicle treatment but before replating, and time points 3, 7, and 12 days indicate samples obtained after replating.

Fluorescence Microscopy

The phenotype of fibroblastic cells was studied after plating in 8-well multichamber slides (7.5×10^4 cells/

Figure 1. Flow chart illustrating the overall experimental design. α -SMA, α -smooth-muscle actin.

cm^2) coated with either 10 μ g/ml fibronectin (Sigma Chemical Co., St. Louis, MO) or 0.5 mg/ml collagen (Vitrogen, Celltrix, Palo Alto, CA) coated as thin films or as a thick gel (~ 1 mm). These different thicknesses of gels were used because previous data¹⁵ and preliminary experiments had shown that the actin organization was influenced by the thickness of the collagen gel; in thick gels, the cells burrowed into the gels, and their morphology was not as well spread as in thin gels. Improved imaging of the cells burrowing into collagen gels was obtained by confocal microscopy (see below). Cells were washed with PBS, fixed with 1.5% paraformaldehyde, permeabilized with 0.3% v/v Triton-X 100 in PBS, and stained for SMA with a mouse anti-human SMA monoclonal antibody (1:50 dilution, clone 1A4; Sigma) for 1 hour at 37°C followed by either a fluorescein isothiocyanate (FITC)-conjugated or rhodamine-conjugated goat anti-mouse antibody (1:100, Sigma) for 1 hour at 37°C. Pre-immune mouse and goat sera were used to block non-specific staining before antibody incubation. For study of focal adhesion formation in cells on different substrates, cells were stained for talin (1:30 dilution, mouse monoclonal, clone 8d4; Sigma). Cells were also stained for desmin (1:50 dilution, clone DE-U-10; Sigma) and for vimentin (1:50 dilution, clone vim-13.2; Sigma). The cells were examined in a Leitz Orthoplan microscope, equipped with a specific filter for fluorescein or rhodamine, and were photographed. In some experiments quantitative immunofluorescence for SMA or for rhodamine phalloidin staining of stress fibers was performed with a confocal microscope (LCSM; Leica) on single cells. In this assay, single cells were imaged, and the fluorescence due to phalloidin binding of stress fibers within an area of known dimensions was measured. Data

were normalized to the area and expressed as fluorescence per cell.

Western Blotting

We quantified TGF- β_1 -induced modulation of SMA content by immunoblot analysis. Cell extracts were prepared in sodium dodecyl sulfate (SDS) sample buffer and boiled for 3 minutes at 95°C. Equal amounts of protein as determined by the BioRad assay were resolved by electrophoresis on 10% SDS gels, transferred to nitrocellulose filters, probed with the mouse monoclonal antibody for SMA followed by an HRP-conjugated second antibody, and developed with ECL reagents (Amersham, Oakville, Ontario, Canada). Subsequently, X-OMAT Kodak films were exposed to the blots, and the density of the bands was estimated by Scan Analysis (Biosoft, Cambridge, U.K.). Blots were stripped and probed with a monoclonal antibody for β -actin (clone AC-15; Sigma) for comparison. The ratio of the density of SMA to β -actin was calculated for each sample. In some experiments we prepared cytoskeletons that were enriched in stress fibers. Briefly, cells were permeabilized in 500 μ l of buffer (1% Triton-X 100, 50 mmol/L KCl, 2 mmol/L $MgCl_2$, 0.2 mmol/L adenosine 5'-triphosphate, 10 mmol/L Tris, pH 7.4, 2 mmol/L ethylene glycol bis(β -aminoethyl ether)-N,N,N',N'-tetraacetic acid [ethylenebis(oxyethylene)trilo]tetraacetic acid, 0.5 mmol/L dithiothreitol) containing 1 μ mol/L phalloidin (Sigma) to stabilize actin filaments. To obtain cytoskeletal preparations containing stress fibers, cell lysates were centrifuged at 100,000 $\times g$ for 1 hour. The pellet consisted of actin filaments and was dissolved in 50 μ l of SDS sample buffer and analyzed by immunoblotting.

Flow Cytometry and Cell Counting

Single-cell suspensions were prepared (0.01% trypsin), fixed with 3.7% formaldehyde, permeabilized in 0.02% Triton with PBS, and stained for SMA as described.⁶ First (anti-SMA) and second (FITC-conjugated goat anti-mouse) antibody dilutions were 1:25 and 1:50, respectively. Cells were washed and resuspended in Mg^{2+} and Ca^{2+} -free PBS. Samples were analyzed on a FACSTAR Plus flow cytometer (Becton-Dickinson, Mississauga, Ontario, Canada) with 488-nm excitation and 530/30-nm band pass filter for FITC. For all flow cytometry analyses, at least 1×10^4 cells were assessed in each sample, and only cells with forward and orthogonal light scatter characteristics similar to whole, intact fibroblasts were included in the analysis by electronic gates previously established for fibroblasts. For radioautography experiments of [³H]thymidine-labeled cells, flow cytometry was used to sort cells by fluorescence intensity due to SMA staining, and cytopsin preparations were made. To estimate the fraction of S-phase cells in the whole population, cell suspensions were stained with 4',6-diamidino-2-phenylindole (1 μ g/ml in 0.1% Nonidet P-40). LYSIS software (Becton Dickinson) was used for estimation of the S-phase population. For estimation of cell counts in cul-

tures, 10- μ l aliquots of cell suspensions were counted electronically (Coulter, Hialeah, FL).

[³H]Thymidine Labeling

For estimation of the fraction of S-phase cells, cultures were labeled with [³H]thymidine (20 Ci/mmol, 1 μ Ci/ml; Amersham) for 3 hours before fixation, and radioautographs of flow-sorted cells in cytopsin preparations were prepared using liquid emulsion (Kodak, Rochester, NY). The percentage of labeled cells was estimated after light-microscopic examination.

Apoptosis Assay

To measure the proportion of apoptotic cells, breaks in DNA strands were detected by the terminal deoxynucleotidyltransferase-mediated UTP end-labeling (TUNEL) reaction. Cells were treated with vehicle or TGF- β (72 hours, 10 ng/ml). Cells were replated and grown in serum-free or serum-containing conditions. Samples were collected at various time points after TGF- β treatment, and cytopsin preparations were prepared. These samples were assayed for apoptotic cells with a TUNEL detection kit (Boehringer Mannheim, Montreal, Quebec, Canada). In this kit, terminal deoxynucleotidyl transferase catalyzes polymerization of biotinylated nucleotides to free 3'-OH DNA ends in a template-independent manner. Streptavidin-FITC was used for detection of biotinylated nucleotides, and the percentage of labeled cells was quantified by fluorescence microscopy.

Northern Analysis

Northern blotting was performed on RNA isolated from monolayer cultures grown on plastic tissue culture dishes after 3 days of treatment with TGF- β_1 or vehicle, followed by replating and incubation with or without serum as described above. RNA was isolated by the guanidium hydrochloride method. Total RNA was separated in denaturing 1.3% formaldehyde-agarose gels, transferred to a nylon membrane (Bio-Rad), and cross-linked by UV light. The McMolly Tetra program (SoftGene) was used to design 32-mer oligonucleotides (5'-TCCACAGGACATTCACAGTTGTGTGCTAGAGA-3' and 5'-CCATGCCAATCTCATCTTGTCTTCTGCGCAAG-3') complementary to specific sequences of the SMA and β -actin mRNA 3'-untranslated regions, respectively. The oligonucleotides were labeled with [³²P]ATP (Dupont, NEN, Markham, Ontario, Canada), using 3'-end labeling. Hybridization conditions, film exposure, and reprobing for glyceraldehyde phosphate dehydrogenase were performed as described previously.¹⁵

mRNA Stability

Northern analyses of SMA mRNA were conducted on cells that had been previously incubated with actinomycin D at 1 μ g/ml for 24 hours. The concentration and

Figure 2. Photomicrographs showing TGF- β -induced gingival fibroblasts. **A:** 4',6-diamidino-Z-phenylindole-stained nuclei; **B:** Same cells stained for desmin show no staining reaction; **C** and **D:** Separate cell preparations are positively stained for vimentin (**C**) and SMA (**D**).

duration of actinomycin D treatment were optimized in preliminary experiments in which it was found that 1 μ g/ml actinomycin D inhibited 98% of [3 H]uridine incorporation into RNA without affecting cell attachment and cell morphology over a 24-hour interval.

[35 S]Methionine Labeling and Immunoprecipitation

Cells were treated with TGF- β_1 or vehicle, replated in the presence or absence of serum, and harvested on days 3, 7, and 12 as described above (Figure 1). Before harvest, the cells were metabolically labeled for 4 hours with [35 S]methionine (100 μ Ci/ml, ICN Biochemicals) in methionine-free MEM. Cells were solubilized in 200 μ l of lysis buffer (10 mmol/L Tris-HCl, pH 7.0, 50 mmol/L NaCl, 0.5% Triton X-100, 30 mmol/L $\text{Na}_4\text{P}_2\text{O}_7$, 50 mmol/L NaF, 100 μ mol/L Na_3VO_4 , 0.1% bovine serum albumin, 4 mmol/L MgCl_2 , 2 mmol/L phenylmethyl sulfonyl fluoride, 1% aprotinin, 1 mmol/L benzamide). Insoluble material was removed by centrifugation at $10,000 \times g$ for 5 minutes at 4°C. The radioactivity in cell lysates was counted, and equal amounts of radioactivity were used in immunoprecipitation assays. Supernatants were immunoprecipitated with SMA antibody (4 μ g/ml) overnight at 4°C. Immunocomplexes were recovered by binding to protein

A-sepharose (Zymed) and were washed four times with 25 mmol/L Tris-buffered saline (pH 7.4), containing 0.5% Triton X-100 and 1 mg/ml bovine serum albumin, and twice with 0.5 mmol/L NaCl and 25 mmol/L Tris-HCl (pH 7.4). The immunocomplexes were analyzed by electrophoresis on 10% polyacrylamide gels followed by fluorography and were scanned to quantify the density of the band.

Statistical Analysis

When appropriate, quantifications of blot or fluorograph densities and cell counts were collated, and the mean $\pm$ SEM was calculated. When direct comparisons between groups were made, an unpaired *t*-test was conducted, and statistical significance was computed. For all experiments, $n = 3$ per sample group or greater, and statistical significance was accepted at $P < 0.05$.

Results

Phenotypic Characteristics and TGF- β Induction

In culture, gingival fibroblasts constitutively express low but detectable levels of SMA and are negative for keratin

Figure 3. Flow cytometry analysis of three representative cell populations grown from three different individual explants. Cells were treated with vehicle (a) or TGF- β (b) for 3 days, plated, grown in the presence of serum for 7 days, and analyzed by flow cytometry for SMA and β -actin. Note that the x-axis for fluorescence intensity on the flow cytographs is a log plot. For each flow cytometry analysis, $n = 10,000$ cells.

and factor VIII,⁶ indicating that they are indeed fibroblastic and are not epithelial or endothelial cells. Because proliferation of cultured vascular smooth-muscle cells causes loss of SMA expression and confluency augments SMA levels,²¹ we immunostained for desmin to confirm that the gingival fibroblast cultures were not contaminated by smooth-muscle cells. We found that these cells stained positively for vimentin but were negative for desmin (Figure 2), which corresponds to the V⁺A⁺D⁻ fibroblasts described by Skalli et al.²²

Because previous work has shown heterogeneity of SMA expression within untreated fibroblast populations,⁶ we first examined the effect of TGF- β (10 ng/ml, for 3 days) on SMA protein. Flow cytometry analysis of very early-passage cultures (passages 1–3 after explantation; $n = 5$ different explant cultures) showed relatively low levels of SMA in unstimulated cells from different explants and increased SMA levels in response to TGF- β between cultures from different explants (Figure 3). TGF- β -treated cells from all explants exhibited an upward, unimodal

Figure 4. Histograms of SMA (α -SMA) to β -actin protein content ratios. **A:** Cells were grown in the presence (proliferating cultures) or absence (non-proliferating; confluent, density-arrested cultures) of serum. Cells were incubated with TGF- β_1 (10 ng/ml) or vehicle for 3 days. Immunoblots of SMA and β -actin were scanned, and the densities plotted (mean ratio $\pm$ SEM; $n = 3$ per group). **B:** Cells were pretreated with TGF- β or vehicle for 3 days, replated at different densities (low, 1.28×10^4 cells/cm²; moderate, 1.28×10^5 cells/cm²; high, 1.28×10^6 cells/cm²) and grown for 5 days and immunoblotted. Data are expressed as ratios of the blot densities of SMA to β -actin (mean ratio $\pm$ SEM).

shift of staining for SMA, indicating that all cells were responsive to TGF- β and that the SMA-positive populations, even after 7 days without TGF- β , were relatively uniform in their SMA content as indicated by the relatively narrow distributions of SMA staining (Figure 3). Western blotting for SMA and β -actin showed increases of SMA compared with β -actin and total protein, which corresponded to the increases observed by flow cytometric analyses (not shown). Because endogenous TGF- β production by cells from the different explant cultures could account for some of the observed increase of SMA, we measured TGF- β levels by an enzyme-linked immunosorbent assay (ELISA) (R&D Systems). In serum-containing and serum-free cultures, the ELISA showed that endogenous levels of active TGF- β in both types of culture were always less than 5 pg/ml, which is 1×10^{-3} of the dosage required to obtain induction of SMA by TGF- β for these cells.

Because culture conditions can affect SMA content in fibroblasts,¹⁶ we conducted a series of experiments to study the effect of cell culture conditions on SMA levels. Depending on the growth phase of cells in culture, gingival fibroblasts exhibited different levels of SMA when induced with TGF- β . At the log phase of growth in proliferating low-density cultures, the relative TGF- β induction of SMA was less than in cells that were induced under

Figure 5. TGF- β -pretreated cells (TGF+) or vehicle-treated cells (TGF-) were replated at low density with serum or at high density with no serum. Cells were electronically counted at days 0, 3, 7, and 12. The initial time point (ie, 0 days) reflects counts obtained after TGF- β pretreatment at the time of replating; time points 3, 7, and 12 days indicate counts obtained after replating the treated cells ($n = 3$ per time point).

density-arrested conditions (ie, nonproliferating) in confluent cultures (Figure 4A). Consequently we next examined whether SMA expression depended on the density at which the cells were plated (Figure 4B). Cells were treated with TGF- β or vehicle for 3 days in serum-free medium at a range of cell densities from low (1.28×10^1 cells/cm²) to moderate (1.28×10^3 cells/cm²) to high (1.28×10^4 cells/cm²) and were analyzed at 5 days after replating. When normalized for β -actin content, the SMA levels of TGF- β -treated samples were severalfold higher for cells plated at the higher densities (2.8-fold higher for cells plated at 1.28×10^4 cells/cm², compared with 1.28×10^3 cells/cm² and 40-fold higher for cells plated at 1.28×10^4 cells/cm², compared with cells plated at 1.28×10^1 cells/cm²; Figure 4B). Consequently, to optimize the inductive effect of TGF- β on SMA expression but also to allow cell proliferation, cells were replated at 1.28×10^1 cells/cm² for experiments performed in the presence of serum.

In preliminary experiments we found that, when TGF- β -treated cells were replated in serum-free conditions, SMA content was stable for up to 7 days, after which it declined ~20% (see below). In serum-containing cultures, SMA was reduced three- to sixfold by 12 days. Although the reductions of SMA were modest at days 1–2, large-scale reductions of SMA were marked towards the end of the culture period. Thus by day 12 in serum-containing conditions, SMA content returned to the baseline levels originally exhibited by these cells before TGF- β treatment. Before performing detailed experiments on SMA metabolism and phenotypic stability of myofibroblasts, we first examined the behavior of cells in serum-

containing and serum-free media over a 12-day period after TGF- β treatment.

Behavior of Replated Cells in Serum-Containing and Serum-Free Conditions

In rat dermal wounds, the relative abundance of myofibroblastic cells expressing SMA varies at different stages of wound healing⁷; up to 70% of fibroblastic cells express SMA at day 6, but they regress thereafter so that by day 30 myofibroblasts are no longer detectable. To better understand the mechanisms involved in the formation and deletion of these cells, we studied the fate of SMA cells in expanding or static cell culture populations. We measured changes in SMA content under serum-containing and serum-free conditions for 12 days and assessed cell loss and cell growth in both conditions. TGF- β -induced and vehicle-treated controls were replated and grown in the presence or absence of 10% serum to provide proliferating and quiescent conditions that model wound healing or resting connective tissues, respectively. In serum-free conditions when cells were plated at high densities (1.28×10^4 cells/cm²), the difference in cell numbers between day 0 (replated cells) and day 12 showed a $64 \pm 4\%$ and $57 \pm 5\%$ decrease in TGF- β -treated and vehicle-treated controls, respectively, which was not significantly different between treatments (Figure 5; $P > 0.2$). In contrast, when cells were plated at low densities (1.28×10^1 cells/cm²) in serum-containing conditions, cell numbers were increased 69-fold and 78-fold compared with initial cell input in TGF- β -treated and ve-

With serum

No Serum

With serum

No Serum

A

B

Figure 6. A: Experiment showing ratios of SMA (α -SMA) to β -actin protein content in the presence or absence of serum after pretreatment with TGF- β or vehicle for 72 hours. Cells in serum were replated at low density, and cells without serum were replated at high density. Day 0 indicates the time point after 72 hours of TGF- β or vehicle treatment; 3, 7, and 12 days are the time points after the same pretreatments and at various time periods after replating. Note that the low-density plating in serum reduces the ratios of SMA to β -actin content compared with high-density plating without serum. However, the difference in ratios between TGF- β and vehicle treatments is equivalent at 0 days. $n = 3$ per group. **B:** Representative experiment showing the differences in rates of SMA (α -SMA) protein synthesis in serum-containing and serum-free conditions. Cells were metabolically labeled with [35 S]methionine (100 μ Ci/ml; for 4 hours) before harvest at each time point. Incorporation of [35 S]methionine into nascent SMA was determined by immunoprecipitation followed by fluorography. Data are mean $\pm$ SEM of fluorographic density ratios. $n = 3$ per group.

hicle-treated samples, respectively ($P < 0.05$). When cells were plated at higher densities in the presence of serum, cell growth was also robust although the increases were not as marked, because, at higher densities, the cells became confluent after 12 days of growth. There were no detectable S-phase cells in the serum-free cultures over the 12-day time course. For serum-containing cultures plated at low densities with or without previous TGF- β treatment, the percentage of S-phase cells was 19% at 3 days and 21% at 7 days. At most, the difference in the percentage of S-phase cells between TGF- β pretreatment or vehicle was $\sim 1\%$ ($P > 0.2$). By day 12, 5.6% of cells were in S-phase in vehicle-treated controls, and 4.4% of cells were in S-phase after TGF- β treatment. These data indicated that, at the plating densities used here and in the presence of serum, the cells proliferated, whereas in serum-depletion conditions, proliferation was nil. Further, the effect of TGF- β pretreatment on cell proliferation appeared to be only small, a finding

that is consistent with earlier data showing cell type-dependent variation of TGF- β -induced proliferation.²³

SMA Metabolism

To better understand the mechanisms regulating the variability of SMA expression in wound healing and in resting connective tissues, cellular protein and mRNA were examined over the same 12-day time course in serum-containing and serum-free conditions with vehicle or TGF- β -pretreated cells plated at 1.28×10^1 cells/cm² for serum-containing conditions and 1.28×10^4 cells/cm² for serum-free conditions. Western analysis showed that, in serum-containing conditions, the relative SMA protein content decreased over time, particularly between days 0 and 3 of the time course; by day 12, SMA content had returned to baseline levels. In serum-free conditions the SMA content was much longer-lived and was reduced

Figure 7. Representative experiment showing the effect of serum on SMA (α -SMA) and β -actin content of cells in high-density (confluent) and low-density (proliferating) conditions. Confluent and proliferating samples were treated with vehicle (TGF-) or TGF- β (TGF+) for 72 hours, then plated at high density (confluent) or low density (proliferating) in the presence of serum for 5 days. Control cells were replated at high density in serum-free conditions. SMA and β -actin protein contents were analyzed by immunoblotting. Data are mean $\pm$ SEM of blot density ratios. $n = 3$ per group.

only 20% by day 12 ($P < 0.01$; Figure 6A). These results were also confirmed by flow cytometric analysis at 0 and 12 days in serum-containing cultures, which showed a loss of SMA staining intensity in cells over time (vehicle-treated cells at 0 and 12 days, respectively, 81 ± 1.9 and 73 ± 1.5 fluorescence channel; TGF-treated cells, 283 ± 4.2 and 89 ± 1.7 at days 0 and 12, respectively). It is noteworthy that comparisons of SMA content by flow cytometry showed no large reductions of SMA content between days 0 and 1. The observed changes in the SMA content could be due to differences in the rates of SMA synthesis as shown earlier for smooth-muscle cells.²¹ We measured the incorporation of [³⁵S]methionine into nascent SMA by immunoprecipitation with SMA antibody. The decrease of SMA content in serum-containing conditions was parallel with the corresponding reduction in the fractional synthesis of nascent SMA. Similarly, in serum-free conditions, the SMA synthesis corresponded closely with the SMA content (Figure 6B).

Because factors in serum such as platelet-derived growth factor may inhibit SMA expression,³ we determined whether serum factors contributed to the decrease in SMA content in serum-containing conditions, independently of proliferation. Cultures treated with TGF- β for 3 days or vehicle-treated controls were replated at high density with serum (confluent cultures), low density with serum (proliferating cultures), or high density without serum (controls) and were cultured for an additional 5 days. In proliferating cultures there was a reduction of SMA content to that of untreated controls, whereas the SMA content of confluent cultures with serum was similar to that of confluent cultures without serum (Figure 7). These results suggested that cessation of proliferation and not the absence of serum factors was responsible for the maintenance of SMA content in high-SMA-containing cells.

We examined steady-state levels of SMA mRNA over the same time course in serum-containing and serum-free conditions. As shown in Figure 8A, the ratio of SMA mRNA to β -actin mRNA content showed a >twofold decrease between 0 and 3 days in cells that were pre-treated with TGF- β and then replated in serum-containing medium ($P < 0.01$). By day 12, there was no detectable difference between controls and TGF- β -treated samples ($P > 0.2$). In serum-free conditions mRNA levels were stable up to 7 days and showed a marked decrease of SMA mRNA only by day 12.

We explored the mechanisms involved in the reduced mRNA levels in the serum-containing samples. Turnover of SMA mRNA after 1 day of culture was studied by blocking mRNA transcription with actinomycin D over a 24-hour time course followed by Northern blotting. There were pronounced decreases in SMA mRNA levels for TGF- β -treated cultures grown in serum, indicating that increased turnover of mRNA might account for the large drop of steady-state mRNA levels early in serum-containing cultures (Figure 8B). Untreated controls and serum-free cultures showed no significant changes in mRNA turnover over the actinomycin D time course. We examined whether the more rapid turnover of SMA mRNA could be explained by a dilutional effect because of more rapid proliferation of SMA-negative cells. Flow cytometry showed that, over the experimental time course (0–1 days), there was no significant downward shift of SMA content in the whole-cell population; as a result, a large proportion of SMA-negative cells did not appear in this time period. Further, we studied cell proliferation in the SMA-positive and SMA-negative cell populations. [³H]Thymidine labeled SMA-immunostained cells were sorted by flow cytometry into high- and low-SMA-expressing cells based on thresholds set for above background staining. Autoradiography of cytopspins showed no significant differences between the percents labeled cells in either the high- or low-SMA-expressing cells and between controls and TGF- β -treated cultures under proliferating conditions. For example on day 1, the labeling index (mean $\pm$ SEM) for high- and low-SMA-expressing cells differed by only 1.7% (high, $16.9 \pm 1.3\%$; low, $15.2 \pm 0.5\%$; $P > 0.2$), and by day 5 the difference was only 0.5% ($P > 0.2$). Thus the observed reduction of SMA mRNA stability was not due to selective and more rapid overgrowth of SMA-negative cells during the time period studied here.

Effect of Substrate on Actin Organization

We examined whether extracellular matrix proteins such as collagen and fibronectin may influence the stability of the SMA protein, perhaps by regulating cell adhesion and consequently the supramolecular organization of SMA into the actin cytoskeleton. We prepared cytoskeletal proteins that were enriched with stress fibers, using a Triton X-100 buffer and phalloidin stabilization to preserve actin filaments and stress fibers. Immunoblotting of these preparations showed that TGF- β -pretreated cells on fibronectin-coated plates after 12 days exhibited ra-

With serum

No serum

A

With serum

actinomycin-D

No serum

B

Figure 8. A: Steady-state levels of SMA (α -SMA) and β -actin mRNA were determined at indicated time points in serum-containing and serum-free cultures. mRNA levels were measured by Northern hybridization using oligonucleotide probes specific for either human SMA or β -actin. Note the pronounced decrease between 0 and 3 days in the proliferating serum-containing cultures. Data are mean $\pm$ SEM of Northern blot density ratios. $n = 3$ per group. **B:** Representative experiment showing turnover of SMA and β -actin mRNA to determine the cause of the large decrease in steady-state SMA mRNA between 0 and 3 days as shown in **A**. TGF- β -induced and/or vehicle-treated cells were replated and incubated with actinomycin D ($1 \mu\text{g/ml}$) after 1 day for an additional 24-hour time course. Total RNA was isolated from cells at intervals after actinomycin D addition and subjected to Northern analysis. 0 hours reflects samples untreated with actinomycin D. Data are mean $\pm$ SEM of Northern blot density ratios. $n = 3$ per group.

tios of SMA to β -actin that were similar to those of cells on tissue culture plastic (Figure 9). Cells on collagen-coated plates showed $\sim 50\%$ reduction of the SMA-to- β -actin ratio after 12 days ($P < 0.05$). For control cells on fibronectin or collagen that were not treated with TGF- β , after 3 days of culture there was a twofold increase in the ratio of SMA to β -actin, reflecting an increase of SMA incorporation into stress fibers. *In situ* analysis of SMA and stress fiber organization by quantitative confocal microscopy showed that TGF- β treatment increased SMA levels for cells on all types of substrates (Table 1). As shown by quantitative confocal microscopy of rhodamine phalloidin-stained preparations, TGF- β -treated cells on fibronectin-coated substrates exhibited stable levels of rhodamine phalloidin fluorescence throughout the culture period of 12 days, whereas vehicle-treated cells and all cells on collagen-coated substrates showed marked loss of staining over the time course.

Apoptosis

Apoptotic cells appear more frequently during the later stages of wound healing,^{7,10} suggesting that apoptosis is an important mechanism for the deletion of myofibroblasts. To monitor the proportion of apoptotic cells in serum-containing or serum-free conditions, TGF- β -treated and untreated samples were immunostained for SMA and sorted by flow cytometry into high- and low-SMA cells at 3, 7, and 12 days after replating on plastic. High- and low-SMA-expressing populations were stained for TUNEL to determine the percentage of apoptotic cells (Figure 10). The sampling period of day 12 was chosen as the last sampling period because, from preliminary experiments, it was coincident with a large reduction in cell numbers that occurred in serum-free cultures after this time (not shown). Consistent with this result, at day 12 in serum-free cultures, there were, respectively, 67% and

Figure 9. Cells were pretreated with TGF- β , plated on collagen-coated plastic or fibronectin-coated plastic, and incubated for 0, 3, or 12 days in MEM without serum. Cytoskeletal preparations enriched for stress fibers were prepared using Triton X-100 and phalloidin to stabilize stress fiber complexes. Lysates were immunoblotted for SMA or β -actin and quantified as mean ratios $\pm$ SEM.

66% apoptotic cells in the high- and low-SMA populations treated with vehicle ($P > 0.4$), whereas in TGF- β -treated samples there were 4% and 60% apoptotic cells in high- and low-SMA populations, respectively ($P < 0.01$). In cultures in which serum was added after TGF- β pretreatment, there were 10% and 1% TUNEL +ve cells

Table 1. Quantification of Stress Fibers and SMA on Different Substrates

Substrate type	Day 0 intensity units*	Day 3 intensity units	Day 12 intensity units
Collagen gels			
TGF- F-actin	43 $\pm$ 8	40 $\pm$ 9	17 $\pm$ 4 [†]
TGF+ F-actin	49 $\pm$ 7	40 $\pm$ 8	18 $\pm$ 8 [†]
TGF- SMA	33 $\pm$ 2	21 $\pm$ 4	17 $\pm$ 7 [†]
TGF+ SMA	43 $\pm$ 5	41 $\pm$ 7	28 $\pm$ 9
Collagen-coated plates			
TGF- F-actin	44 $\pm$ 6	34 $\pm$ 5	22 $\pm$ 7 [†]
TGF+ F-actin	66 $\pm$ 3	33 $\pm$ 4 [†]	24 $\pm$ 3 [†]
TGF- SMA	40 $\pm$ 3	27 $\pm$ 8	10 $\pm$ 1 [†]
TGF+ SMA	53 $\pm$ 7	38 $\pm$ 8	28 $\pm$ 2 [†]
Fibronectin-coated plates			
TGF- F-actin	55 $\pm$ 10	54 $\pm$ 11	28 $\pm$ 5 [†]
TGF+ F-actin	67 $\pm$ 12	56 $\pm$ 12	54 $\pm$ 8
TGF- SMA	23 $\pm$ 6	33 $\pm$ 2	22 $\pm$ 3
TGF+ SMA	53 $\pm$ 9	44 $\pm$ 7	40 $\pm$ 10

Cells were pretreated with TGF- β (TGF+) or with vehicle (TGF-) and then plated on collagen gels, or collagen-coated plastic (collagen-coated plates) or fibronectin-coated plates without serum for 3 or 12 days as indicated. Cells were fixed and stained with rhodamine phalloidin for F-actin or with antibody to SMA for SMA immunofluorescence.

*Whole-cell fluorescence intensity due to rhodamine-phalloidin staining of F-actin and to immunofluorescence staining for α -SMA was measured by confocal microscopy on a single-cell basis. For each condition, 10 cells were measured, and data from three replicated experiments were analyzed to compute means $\pm$ standard errors.

[†]Indicates significant difference ($p < 0.05$) compared to day 0 and day 3.

[‡]Indicates significant difference compared to day 0.

for the high- and low-SMA cells ($P < 0.01$), and in vehicle-treated controls there were 4% and 13% for high- and low-SMA cells ($P < 0.05$). These data show that, in proliferating cultures, TGF- β pretreatment may have contributed to the selective deletion of the SMA-expressing cells. In contrast, TGF- β pretreatment in static cultures may protect high-SMA-expressing cells from apoptosis.

Because the expression of SMA appeared to provide protection against apoptosis in static cultures, we examined in more depth the relationship of apoptosis to the supramolecular organization of SMA in the cytoskeletons and also the effect of extracellular-matrix ligands on apoptosis (Table 2). First, cells were stained with TUNEL, talin, or rhodamine phalloidin to examine the relationship between apoptosis, focal adhesions, and stress fiber formation. Second, cells were stained for SMA and TUNEL to determine whether SMA expression and the supramolecular organization of actin protect cells from apoptosis. Cells were dichotomized into groups of either poorly developed or well-developed stress fibers and examined *in situ* (Figure 11). Cells plated on thick collagen gels,¹⁵ collagen-coated plates, or fibronectin-coated plates showed significant protection from apoptosis if the cells were preincubated with TGF- β . For all substrates, cells with well-developed stress fibers were more resistant to apoptosis at 12 days. It is noteworthy that cells on fibronectin more frequently showed prominent stress fibers and abundant focal adhesions, whereas cells on collagen were more likely to show poor stress fiber development and fewer focal adhesions. Consistent with the data of cells plated on tissue culture plastic, TGF- β reduced the percentage of apoptotic cells on fibronectin or collagen by up to tenfold. Cells that were not preincubated with TGF- β showed very high percentages of apoptosis when there were poorly developed stress fibers or when the staining levels of SMA were low (Table 2). Indeed for cells on fibronectin-coated substrates, there was a 35-fold-higher rate of apoptosis in cells with poorly developed stress fibers than cells with well-developed stress fibers.

Discussion

In chronic wounds, fibrocontractive lesions, or fibromatosis, myofibroblasts may persist³ and contribute to pathological scarring and contracture. The reason for their persistence in pathological states is poorly understood, as is the physiological deletion of these cells in normal wound-healing processes. The main findings of this study are that, first, TGF- β -induced myofibroblasts with high-SMA expression have long-lived SMA protein and mRNA under quiescent conditions, whereas in proliferating cells SMA protein and mRNA are relatively short-lived. Second, the supramolecular organization of SMA into the actin cytoskeleton protects against apoptotic deletion of myofibroblasts. Third, the organization of SMA into stress fibers and the resultant protection against apoptosis are very dependent on the type of extracellular-matrix proteins. These *in vitro* findings may provide insight into *in vivo* phenomena in which wound cytokine-induced myo-

With serum

No serum

Figure 10. TGF- β -treated cells or vehicle-treated controls were replated and grown with serum or without serum. Cells were flow sorted into high SMA content (H) or low SMA content (L) and stained for TUNEL at days 0, 3, 7, and 12 to detect DNA strand breaks. TUNEL-positive cells were counted by fluorescence microscopy. TGF+ indicates pretreatment with TGF- β . TGF- indicates pretreatment with vehicle. $n = 3$ samples per group; each group included >100 cells counted.

fibroblasts with abundant SMA appear shortly after the wounding stimulus *in vivo*⁴; these cells gradually disappear in open-wound-healing models.^{7,10} In the myofibroblast model system examined here, our data point to the importance of actin organization and substrate for cell deletion by apoptosis.

Model Systems

The mechanisms that are involved in the expansion of specific fibroblast subpopulations during early wound-healing conditions are poorly understood. In these situations high levels of serum-derived proteins, cytokines, and provisional matrix proteins are present that pro-

foundly affect fibroblast function.²⁴ TGF- β peaks at day 7 in rat dermal wounds and falls off dramatically thereafter.²⁵ Our results are consistent with earlier findings showing that TGF- β treatment at 10 ng/ml for 3 days induces high levels of SMA in fibroblasts^{14,26} and show that TGF- β -induced cells replated on solid substrates demonstrate high-SMA expression over prolonged periods of time,¹⁵ long after the withdrawal of the inductive stimulus. This system generated high levels of SMA and a high proportion of SMA-expressing cells and is analogous to the types of conditions that would be anticipated in the first 4 to 8 days of wound healing when TGF- β levels²⁵ are those used in the present *in vitro* study (ie, ~10 ng/ml).

Table 2. Percent Apoptotic Cells on Different Substrates

	Collagen gels		Collagen plates		Fibronectin plates	
	Day 3	Day 12	Day 3	Day 12	Day 3	Day 12
TGF+						
Poor S/F	0	8 $\pm$ 0.8	2 $\pm$ 0.1	10 $\pm$ 1	0.1 $\pm$ 0.1	4 $\pm$ 0.3
Developed S/F	0	1 $\pm$ 0.01	1 $\pm$ 0.1	6 $\pm$ 0.9	0	2 $\pm$ 0.5
Low SMA	0	7 $\pm$ 0.7	2 $\pm$ 0.8	8 $\pm$ 0.7	1 $\pm$ 0.3	5 $\pm$ 0.2
High SMA	0	2 $\pm$ 0.5	1 $\pm$ 0.1	2 $\pm$ 0.1	0	1 $\pm$ 0.2
TGF-						
Poor S/F	1 $\pm$ 0.4	90 $\pm$ 10	40 $\pm$ 8	95 $\pm$ 14	0	35 $\pm$ 5
Developed S/F	0	2 $\pm$ 0.7	3 $\pm$ 0.9	5 $\pm$ 0.8	0	1 $\pm$ 0.1
Low SMA	0	85 $\pm$ 11	25 $\pm$ 5	90 $\pm$ 12	1 $\pm$ 0.2	30 $\pm$ 4
High SMA	0	0	2 $\pm$ 0.7	4 $\pm$ 0.8	0	1 $\pm$ 0.2

Cells were pretreated with TGF- β (TGF+) or with vehicle (TGF-) and then plated on collagen gels, or collagen-coated plastic (collagen plates) or fibronectin-coated plates (fibronectin plates) without serum for 3 or 12 days as indicated. Cells were fixed and stained with rhodamine phalloidin. Poorly developed stress fibers (Poor S/F) or well-developed stress fibers (Developed S/F) were dichotomized in the whole-cell population by visual examination as described in Figure 11. Cells in separate experiments were immunostained for SMA and dichotomized into low-SMA (<20 intensity units) or high-SMA (>20 intensity units) groups by quantitative confocal microscopy. The percentage of apoptotic cells was determined by TUNEL staining.

Figure 11. Fluorescence micrographs of rhodamine phalloidin-stained fibroblasts exhibiting either well-developed stress fibers (**A**) and abundant focal adhesions (**C**) or poorly developed stress fibers (**B**) and sparse focal adhesions (**D**) on collagen. This qualitative method for dichotomizing stress fiber organization was used to study the effect of supramolecular organization of actin on apoptosis (see Table 1).

SMA Metabolism

In proliferating conditions SMA content decreased very rapidly, owing to reduction in the synthesis of nascent SMA as shown by [^{35}S]methionine incorporation into the newly synthesized protein. This reduced SMA protein synthesis was evidently related to the pronounced decrease in SMA mRNA levels of TGF-induced myofibroblasts, which in turn indicated a rapid rate of turnover of the SMA mRNA, a similar finding to that reported earlier for smooth-muscle cells.^{12,17} The rapid turnover of SMA mRNA but not β -actin mRNA was confirmed by blocking mRNA transcription with actinomycin D over 1 day of culture. It is noteworthy that the rapid turnover rate of SMA mRNA was not observed in untreated controls. This effect appeared selective for SMA in that there was no evidence of a similar change in β -actin mRNA stability. Further, the more rapid loss of SMA mRNA was not due to a dilutional effect caused potentially by more rapid growth of SMA-negative cells. We conclude that the precipitous loss of SMA in proliferating cells is due to a rapid expansion in cell number that is simultaneous with an increase of SMA mRNA turnover and a concomitant loss of SMA mRNA.

Apoptosis

TGF- β -induced myofibroblasts grown in serum-free medium exhibited a low but steady decrease in cell numbers over time. There was also a progressive increase in the percentages of cells staining positive for TUNEL, which, by day 12, presumably resulted in the observed large-scale loss of cells from the culture. Indeed, by day 12,

$\approx 65\%$ of the cells grown on plastic were apoptotic, which was consistent with the decrease in SMA protein content that was seen in immunoblots. Thus in contrast to the proliferating-cell model, it appears that the main reasons for the loss of SMA protein content in the nonproliferating model were related to reduced numbers of myofibroblasts and increased cell death.

Different substrates conferred variable levels of protection against apoptosis in serum-free cultures. In particular, cells on fibronectin showed much lower incidence of apoptosis compared with cells on collagen. Because cells on fibronectin also exhibited more well-developed stress fiber systems enriched with SMA and abundant focal adhesions, our data indicate a measure of protection against apoptosis that is attributable to the supramolecular organization of actin and to the relative adhesive strength of the cells to the substrate. Previous work on endothelial cells has shown that well-spread, tightly adherent cells are more resistant to apoptosis than poorly adherent cells.¹⁹ Because high levels of SMA content may also inhibit motility in fibroblasts,¹⁸ we surmise that the organization of SMA into the stress fibers of well-spread, adherent cells confers protection against apoptosis for those cells that are more likely to contract the matrix and to remain *in situ* compared with cells that migrate. This effect appears to be mediated in part by TGF- β , because this cytokine not only enhances SMA expression but does so more efficiently in cells that are attached to rigid substrates and with high levels of intracellular tension.¹⁵ Indeed if TGF- β was present during myofibroblast induction, then TUNEL staining was greatly reduced. Because TGF- β can be present at very high levels in chronic inflammatory lesions,²⁷ we suggest that the persistence of myofibroblasts in nonproliferating conditions may be related to the persistence of high TGF- β levels and the organization of SMA in the actin cytoskeletons. This finding has important implications for wound healing and scar tissue formation, because it suggests a mechanism by which the very cells that are most likely involved in scar formation (ie, the SMA-rich myofibroblast) are also those that are most protected against apoptosis and are strongly induced by TGF- β . Perhaps by therapeutically altering the composition of the extracellular matrix or by affecting the physical properties of the matrix,¹⁵ significant blockage of this positive feedback loop could inhibit myofibroblast formation, because TGF- β induction of myofibroblasts is very dependent on the compliance of the matrix. Notably, the apoptosis data that we provide here were determined from TUNEL assays that in addition to estimating apoptotic processes, can also reflect DNA repair²⁸ and RNA synthesis.²⁹ In view of our previous use of DNA-laddering methods,³⁰ electron microscopy,³⁰ and TUNEL³¹ to measure apoptosis in gingival fibroblasts, we think that nonapoptotic phenomena have probably not contributed significantly to errors in estimation of apoptosis.

Actin Organization

Previous data have indicated that the state of actin assembly may regulate actin expression by a feedback

loop³²; thus, cells with incorporation of actin into heavily cross-linked filaments would be expected to show a slower rate of actin turnover. Because this prediction applies to the myofibroblasts described here, we speculate that the myofibroblasts with well-developed stress fibers and stable pools of SMA are more phenotypically stable because the actin filaments are more stable in these cells and less likely to convert to monomer. Exactly how the putative feedback system in cultured fibroblasts can convert the sensing of intracellular tension and actin organization into more stable mRNAs for SMA is unclear, but the data reported here are remarkably consistent with the concept proposed earlier.³² A particularly novel finding in this study is that the supramolecular organization of the actin cytoskeleton appears not only to stabilize SMA mRNA and protein but also to protect myofibroblasts from deletion by apoptosis. Thus the expression of SMA may not only be linked to wound contraction and retardation of motility in fibroblasts¹⁸ but may also enhance the lifetime of the cells in which it is most strongly expressed.

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