TGF-β Receptor Expression and Smad2 Localization Are Cell Density Dependent in Fibroblasts

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PURPOSE. In nonconfluent cultures, TGF-β induces differentiation of corneal fibroblasts to myofi-
broblasts. However, in confluent cultures, few fibroblasts differentiate to myofibroblasts after TGF-β1 addition. This study investigated the hypothesis that functional TGF-β receptor expression is greater in low-density cultures and is decreased in confluent cultures.

METHODS. Northern and western blot analyses were used to detect smooth muscle (SM) α-actin message and protein. 125I-labeled TGF-β1 was used in a radioreceptor-binding assay as an index of functional receptors on the cell surface of rabbit corneal fibroblast cultures prepared either at high density (cell–cell contact) or low density (absence of contact). Cell lysates were analyzed by SDS-PAGE and autoradiography. Total TGF-β receptor expression was evaluated in western blot analysis. Smad2, a downstream effector of TGF-β receptor activation, was immunodetected.

RESULTS. Low-density cultures expressed more SM α-actin mRNA and protein than high-density cultures, indicating that the low-density cells were differentiating into myofibroblasts. When 125I-TGF-β1 was added to low- and high-density fibroblasts, fibroblasts plated at low density bound more than fibroblasts in high density, confluent cultures. Furthermore, after the cells differentiated into myofibroblasts, they continued to bind 125I-TGF-β1. Specificity of 125I-TGF-β1 binding was demonstrated by complete inhibition by excess nonradioactive TGF-β1. Localization of Smad2 was correlated with SM α-actin induction: Smad was nuclear in low-density cells and cytoplasmic in high-density cells. After TGF-β1 treatment, Smad2 remained cytoplasmic in high-density cells but was localized to nuclei in cells that were nonconfluent.

CONCLUSIONS. Low cell density is correlated with increased functional expression of TGF-β receptors and promotion of signal transmission from these receptors. Thus, conditions that decrease cell density such as wounding favor myofibroblast differentiation in response to TGF-β. (Invest Ophthal mol Vis Sci. 2000;41:89–95)

A
fter corneal wounding, the normally quiescent kerato-
cyes are activated as fibroblasts.1,2 Myofibroblasts
arise, often immediately adjacent to a stromal incision,
and play critical roles in wound contraction and healing.3,4
Myofibroblasts are characterized by the novel expression of
the smooth muscle (SM) isoform of α-actin in their stress fibers and
the enhanced expression of cell–cell and cell–matrix adhesion
molecules that colocalize with the termination of SM α-actin
microfibrils.5–10

TGF-β has been shown to induce the myofibroblast
phenotype in situ and in culture.11,12 Modulation of the
response to TGF-β involves regulation at several levels: the
synthesis and functional expression of one or both of two
cell surface receptors that form the heterodimeric TGF-β

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receptor complex and regulation of the signaling molecules
downstream from the activated receptors. In a current
model, TGF-β binds to TGF-β type II receptors, which then
associate with type I receptors to form a heteromeric TGF-β
receptor complex. This initiates a serine–threonine kinase-
dependent signaling pathway.13 Molecular dissection of
events downstream of the TGF-β receptors in epithelial cells
has implicated nuclear translocation of Smad proteins as the
basis for TGF-β-dependent gene transcription.14 There have
been no reports of Smads in fibroblasts or of Smads playing a
role in myofibroblast differentiation.

The present study was undertaken to resolve an apparent
contradiction: although addition of TGF-β to cultures of sub-
confluent corneal fibroblasts induces myofibroblast differenti-
ation, if TGF-β1 is added to confluent cultures of corneal
fibroblasts, the majority of fibroblasts do not differentiate
into myofibroblasts.15 We have reported previously that fibroblasts
plated at low density secrete more, active TGF-β and give rise
to a myofibroblast culture without the addition of exogenous
TGF-β.15

We hypothesized that the absence of myofibroblast differ-
entiation in confluent cultures treated with TGF-β1 was asso-
ciated with a differential binding by TGF-β receptors. Thus,
one possibility was that fibroblasts in the low cell density
cultures had more functional TGF-β receptors, and the fibro-
blasts in high-density cultures had fewer functional TGF-β
receptors. Previous studies report that the number of several
growth factor receptors expressed on the cell surface is affected by cell density, but not in a predictable direction.\textsuperscript{16,17}

To evaluate the basis for the density-dependent induction of SM $\alpha$-actin and myofibroblast differentiation in response to TGF-$\beta_1$, we have examined the relative functional expression of TGF-$\beta$ receptors on corneal fibroblasts plated at high or low density. We found that cells plated at lower density exhibit greater binding of TGF-$\beta$ by TGF-$\beta$ receptors. Furthermore, the step after TGF-$\beta$ receptor activation, Smad translocation into the nucleus, is also density dependent.

**MATERIALS AND METHODS**

**Preparation of Corneal Fibroblasts and Myofibroblasts**

Corneas were dissected from New Zealand albino rabbits immediately after death. All procedures were performed according the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Human fibroblasts were cultured from discarded rims of corneas used for transplantation or from corneas not suitable for transplantation obtained from the Long Island Lions and New York City eye banks. Corneal fibroblasts emerged from stroma explants, cultured after the removal of epithelium and endothelium (two corneas/60-mm dish).\textsuperscript{18} Alternatively, the corneal stromas were treated with collagenase (Worthington, Lakewood, NJ) for 5 hours at 37°C to release keratocytes. The stromas were treated with collagenase (Worthington, Lakewood, NJ) for 5 hours at 37°C to release keratocytes. The cells were rinsed by multiple centrifugations (1000g/10min), resuspended, and placed in culture.\textsuperscript{19} Culture medium was Dulbecco’s modified Eagle’s medium supplied with Ham’s nutrient mixture F-12 (DMEM-F12; Sigma, St. Louis, MO), 10% fetal bovine serum (FBS; Gibco, Grand Island, NY), and antibiotic-antimycotic mix (10,000 units penicillin, 10 mg streptomycin, and 25 mg amphotericin B per milliliter in 0.9% sodium chloride), and gentamicin solution (10 mg/ml; Sigma).

We passaged fibroblasts by trypsinization (Trypsin-EDTA, Gibco) and used them in passages 2 through 6. Corneal fibroblasts resulted from the routine high-density plating (500–600 cells/mm\textsuperscript{2}), a 1:3 split of confluent fibroblasts.\textsuperscript{20} Myofibroblast cultures, identified and distinguished from fibroblasts by immunodetectable expression of SM $\alpha$-actin, were produced by passaging fibroblasts at very low density (6 cells/mm\textsuperscript{2}).\textsuperscript{15}

To understand the roles of cell density and TGF-$\beta$ in generating the myofibroblast phenotype, we wanted to evaluate cells early in the induction process. The earliest time at which this was feasible was 3 days after plating, when low-density cultures provided sufficient protein and RNA for these studies. In one series of experiments, TGF-$\beta_1$ (1 ng/ml, Collaborative Biomedical Products, Becton Dickinson Labware, Bedford, MA) was added to nonconfluent cells (intermediate density, 200 cells/mm\textsuperscript{2}) to induce myofibroblasts for comparison with myofibroblasts induced by low-density plating at 3 days. After 18 hours, the medium was changed to DMEM-F12 and 1% FBS with 1 ng/ml TGF-$\beta_1$ for an additional 2 days. All experiments were repeated at least three times.

**Northern Blot Analysis of SM $\alpha$-Actin mRNA**

Total RNA was prepared from cultures using TriZol Reagent following the manufacturer’s instructions (Molecular Research Center, Cincinnati, OH). Samples of 20 $\mu$g RNA, quantified by measuring UV absorption at an optic density of 260 nm, were separated on a 1% agarose-formaldehyde gel and transferred to a Nytran nylon membrane (Schleicher & Schuell, Keene NH). The membrane was hybridized with a $^{32}$P-labeled cDNA probe corresponding to a unique 111-nucleotide sequence that begins within the 3’ untranslated region of human SM $\alpha$-actin (1114-1335, EMBL accession no. X13839\textsuperscript{21}) at 37°C for 16 to 18 hours. A 28S RNA signal also detected with this probe was indicative of the amount of RNA loaded in each lane.\textsuperscript{22} The membrane was washed and exposed to film (Biomax MS; Kodak, Rochester, NY). Northern blot analyses were repeated on two independent preparations. The relative amount of each mRNA transcript was determined by scanning its autoradiogram with a flatbed scanner and an image analysis program (ImageQuant; Molecular Dynamics, Sunnyvale, CA). The intensity of the each mRNA band was normalized to a ratio of the 28S signal.\textsuperscript{22}

**TGF-$\beta$ Receptor Assay**

Plates (100-mm) were washed once with cold binding buffer (DMEM with 0.1% bovine serum albumin [BSA] and 25 mM HEPES) and then incubated at 37°C for two washes, 1 hour each, allowing dissociation of bound endogenous TGF-$\beta$ from the cells and from the tissue culture plastic.\textsuperscript{23,24} Cells were incubated with 80 to 400 pM $^{125}$I-TGF-$\beta_1$ (NEN Life Sciences, Boston, MA). Of five independent experiments performed, incubation with 150 pM $^{125}$I-TGF-$\beta_1$ was repeated three times. The dishes were placed in the 5% CO\textsubscript{2} incubator for 5 minutes and then sealed with parafilm and incubated with shaking for 2 to 3 hours at 0°C to 4°C. To terminate the incubation, we collected the medium and washed the cells twice with Hanks’ balanced salt solution (HBSS) buffered with 40 mM HEPES, pH 7.4 (Sigma), followed by incubation for 30 minutes at 4°C with the covalent cross-linking reagent, disuccinimidyl suberate (Pierce, Rockford, IL) in dimethyl sulfoxide at a final concentration of 0.3 mM in HBSS. The cross-linking reaction was quenched by washing three times with cold 250 mM sucrose, 10 mM Tris (pH 7.4), and 1 mM EDTA. The cells were lysed in 4× sodium dodecyl sulfate (SDS) sample buffer at a dilution to yield 1× SDS sample buffer. Protein was assayed by a highly sensitive quantitative colorimetric determination (Micro BCA, Pierce), and 20 $\mu$g per lane was electrophoresed in 10% SDS-polyacrylamide gel electrophoresis (PAGE) under reducing conditions along with prestained molecular size markers. After drying, the gel was exposed to film (Biomax MS; Kodak) with an intensifying screen for 1 to 3 days and developed. The relative amount of $^{125}$I-TGF-$\beta_1$ bound to receptors I and II (RI and RI) was determined by scanning the autoradiogram with a flatbed scanner and analyzing signal over background using the image analysis program.

To determine the specificity of TGF-$\beta_1$ binding, cells were incubated as above with 150 pM $^{125}$I-TGF-$\beta_1$ plus a 10-fold excess of unlabeled TGF-$\beta_1$, 1.5 nM.\textsuperscript{23}

**Smad Translocation in Response to TGF-$\beta$**

Fibroblasts were plated on coverslips in 15-mm diameter wells at low, intermediate, and high density (10\textsuperscript{3}, 5 × 10\textsuperscript{4}, and 5 × 10\textsuperscript{5} cells/ml, respectively) in DMEM-F12 with 10% FBS. The medium was replaced with TGF-$\beta_1$ (1 ng/ml) in DMEM-F12 containing 1% or 10% FBS, 6 or 24 hours after
plating. After a 1-hour incubation at 37°C in 5% CO₂, the coverslips were fixed and immunodetected as described later. Mink lung epithelial cells (American Type Culture Collection, Rockville, MD) plated at 5 × 10⁴ cells/ml provided a positive control for Smad antibody specificity and TGF-β-induced translocation. Twenty-four hours after plating at this density, mink lung epithelial cells were not confluent. Immunolocalization experiments were repeated three times.

Immunodetection in Cells and Western Blot Analysis

The culture phenotype was determined using antibody detection of SM α-actin in cells cultured on a coverslip that was removed from the 100-mm dish before lysis. Cells were fixed with 3% p-formaldehyde (Fisher Scientific, Fair Lawn, NJ) in phosphate-buffered saline (PBS; pH 7.4) for 15 minutes at room temperature or in absolute methanol at −20°C for 10 to 15 minutes. After blocking nonspecific binding with 3% normal serum, we incubated the cells with mouse monoclonal antibodies against SM α-actin conjugated to cy3 (Sigma) diluted 1:400 in PBS with 0.1% BSA (PBSA). Nuclei were labeled with bisbenzimide (Hoechst dye 33258; Sigma).

For localization of Smad2 in fixed cells, p-formaldehyde-fixed cells were incubated with rabbit polyclonal anti-human MADR2/Smad2 diluted 1:50 in PBSA (Upstate Biotechnology, Lake Placid, NY) for 1 hour at room temperature, rinsed three times with PBS, and blocked with normal serum followed by anti-rabbit fluorescein isothiocyanate. Cells were viewed with an epifluorescence microscope (Axiophot; Carl Zeiss, Thornwood, NY) and photographed (TMAX 400 film; Kodak) or imaged with a confocal microscope (CLSM; Leica, Deerfield, IL).

For western blot detection, cells were scraped up in 1% nonidet P-40 (NP-40), in 150 mM NaCl, 10 mM Tris-acetate buffer (pH 8.0) with protease inhibitors, as described previously. After 15 to 30 minutes on ice, the lysate was centrifuged for 2 minutes at 15,000 rpm in a microfuge (Eppendorf, Westbury, NY), and the pellet containing the nuclei was discarded. Samples of 20 μg protein of cell lysates were separated by electrophoresis in 10% SDS-PAGE gels and transferred to nitrocellulose. After blocking with 5% BSA, the blots were incubated with primary antibodies diluted in Tris-buffered saline with 0.1% Triton X-100 (TBS-T) with 5% BSA overnight at 4°C. Anti-SM α-actin (mouse monoclonal antibody, Sigma), anti-human TGF-β RI, and anti-TGF-β RI (V-22 and C-16, respectively, rabbit polyclonal antibodies, Santa Cruz Biotechnology, Santa Cruz, CA) were used to detect specific receptors or anti-MADR2/Smad2 (rabbit polyclonal antibodies, Upstate Biotechnology). After rinsing in TBS-T, we incubated the nitrocellulose blot with anti-mouse IgG- or anti-rabbit IgG-horseradish peroxidase, 30 to 60 minutes at room temperature and detected by enhanced chemiluminescence (ECL; Pierce) detected by reagents prepared according to the manufacturer’s instructions. Western blot analysis was repeated three times, and the relative amounts were determined by scanning the film with a flatbed scanner and analyzing signal over background using the image analysis program.

RESULTS

SM α-Actin mRNA and Protein Increase in Low-Density Cultures

When low-density cultures reach confluence (7–10 days), approximately 90% of the cells are myofibroblasts. Corneal fibroblasts result from routine high-density plating of fibroblasts. These cells are approximately 80% confluent 1 day after plating. At confluence (2–3 days), the corneal fibroblast culture has few, if any, myofibroblasts. The signature of myofibroblasts is the expression of SM α-actin in microfibrils.

In the current studies we used a cDNA probe to identify SM α-actin transcription in fibroblast cultures. Three days after plating at low density, a strong RNA signal was detected at 1.3 kb, whereas a weaker RNA signal was detected in high-density cultures (Fig. 1A). When normalized for amount of RNA loaded (see next section), the low-density 1.3-kb signal was 72 times more intense than the high-density signal. This is consistent with the conclusion that the myofibroblasts in the low-density cultures were significantly enriched compared to the high-density cultures.
detected a slightly larger RNA as well (Fig. 1A, lanes 1 and 3), bound 8.6 times more 125I-TGF-

protein in western blot analysis of lysates was very similar in cells cultured at high or low density (low density to high density ratio = 1.17; Fig. 2C). Thus, the increased binding of 125I-TGF-1 by low-density cells indicates that the receptor availability at the cell surface of fibroblasts is regulated by cell density. To show that the binding of 125I-TGF-1 was specific for TGF-1 receptors, we incubated cells in 150 pM 125I-TGF-1 and 1.5 nM unlabeled TGF-1, a 10-fold excess over the labeled form. The unlabeled TGF-1 effectively blocked the binding of 125I-labeled TGF-1, indicating that we were detecting specific TGF-1 receptors (Fig. 3). The Mr of the labeled bands in Figures 2 and 3 was as expected for TGF-1 RI and RII complexes, 70 and 89 kDa, respectively.24

We have previously demonstrated that the myofibroblast phenotype generated by low-density plating could be reversed. Replating those myofibroblasts at high density restores the fibroblast phenotype. However, when the myofibroblast phenotype is induced by 1 ng/ml exogenous TGF-1, replating at high density does not restore the fibroblast phenotype; addition of both FGF and heparin is required to restore the fibroblast phenotype to those replated myofibroblasts.27 We used the fact that myofibroblasts induced by exogenous TGF-1 retain their myofibroblast phenotype after replating at high density to evaluate whether the high level of functional receptor expression is maintained in differentiated myofibroblasts (Fig. 4). These myofibroblast cultures replated at high or low density exhibited high binding by TGF-1 receptors as well as SM a-actin expression similar to low-density cultures, which are differentiating into myofibroblasts (Fig. 2).

Smad2 Translocation to the Nucleus Depends on Cell Density and TGF-1 Treatment

Antibody made to a recombinant form of Smad2 (MADR2) recognized a protein of 55 to 60 kDa in lysates of the cytoplasm

with immunodetection of SM a-actin protein expression in the 3-day cultures of low-density cells (Fig. 1B). In addition to the SM a-actin mRNA, in the lanes that did not have strong signal for SM a-actin message, we confirmed that SM a-actin probes detected a slightly larger RNA as well (Fig. 1A, lanes 1 and 3), which may be 18S RNA or a-7 actin mRNA (Fig. 1A in Taubman et al.25). Furthermore, the probe detects a band at 28S ribosomal RNA (4.4 kb), and we used the strength of this signal to normalize loading of RNA.22

In one series of experiments, TGF-1 (1 ng/ml) added to nonconfluent cells plated at intermediate density (200 cells/mm2) at 3 days showed SM a-actin message comparable to the low-density plating (Fig. 1A, lane 2).

TGF-1 Binding Is Increased in Low-Density Cultures and Is Specific

As a functional assay of expression of TGF-1 receptors, we evaluated binding of 125I-TGF-1 to fibroblasts 3 days after plating at high or low density. Functional TGF-1 RI (arrowhead) and TGF-1 RII (two arrowheads) are expressed more highly on corneal fibroblasts plated at low density than those plated at high density (Fig. 2A). In the low-density culture TGF-1 RI bound 2.55 times and TGF-1 RII bound 8.6 times more 125I-TGF-1 than did cells plated at high density. This pattern was seen in cultures treated with either 150 or 400 pM 125I-TGF-1. In all cases, before autoradiography we verified by Coomassie blue staining of the gel that equal amount of proteins were loaded in each lane (Fig. 2B).

However, this differential binding indicating greater receptor expression in low-density cells did not parallel the total amount of receptor in the cells. In contrast to the greater receptor binding in the low-density cells, TGF-1 RI and RII protein detected in western blot analysis of lysates was very similar in cells cultured at high or low density (low density to high density ratio = 1.17; Fig. 2C). Thus, the increased binding of 125I-TGF-1 by low-density cells indicates that the receptor availability at the cell surface of fibroblasts is regulated by cell density.
of rabbit fibroblasts. After 24 hours of TGF-β1 treatment of nonconfluent cells (intermediate density), the immunodetectable Smad2 was greatly decreased in lysates of cytoplasm (Fig. 5A). Because the lysates contain cytosolic proteins but not nuclei, the decrease in detectable cytoplasmic Smad2 could indicate translocation of the molecule out of the cytoplasm into the nucleus.

In immunocytochemical studies we found that both cell density and TGF-β1 treatment determined whether Smad2 was localized primarily to the cytoplasm or the nucleus. To determine the early effects of TGF-β1, we used intermediate, nonconfluent cells and localized Smad2 in cells 1 hour after addition of TGF-β1. After 1 hour of TGF-β1 treatment, Smad2 was detected in nuclei of the majority of cells rather than being confined to the cytoplasm in cells not treated with TGF-β1. This was most obvious in regions of lower density (arrows, Fig. 5D) but was also seen in some cells in areas of higher density (arrows, Fig. 5C) consistent with decrease of immunodetectable Smad2 in cytoplasmic lysates, after longer treatment with TGF-β1 (24 hours; Fig. 5A). In contrast, in the control intermediate-density cells, Smad2 was still primarily localized to the cytoplasm after a 1-hour TGF-β1 treatment of high-density fibroblasts. Bar, 40 μm.

Finally, we compared Smad2 localization in cells plated at low density and high density and grown for 3 days. In the high-density cells, Smad2 was primarily in the cytoplasm (arrow, Fig. 6A) even after a 1-hour TGF-β1 treatment (Fig. 6C). In contrast, in low-density cultures Smad2 was highly expressed in the nuclei (arrows, Fig. 6B), presumably in response to autocrine TGF-β secreted by low-density cells.
DISCUSSION

In the present study, we found that fibroblasts plated at low density bind more TGF-β than fibroblasts plated at high density, indicating that the low-density cells have more functional TGF-β receptors. Furthermore, we found that nuclear translocation of Smad2 is cell-density dependent.

Fibroblasts can undergo phenotypic conversion to myofibroblasts. This process is induced in granulation tissue by delivery of TGF-β by macrophages and platelets. Administration of TGF-β in situ or in vitro reproduces the myofibroblast differentiation. In addition, most cells have the capacity to synthesize and release TGF-β after wounding and in culture on plastic supports. It was therefore not surprising that in culture, fibroblasts in response to autocrine TGF-β differentiated into myofibroblasts before they became confluent. It was surprising, however, that addition of TGF-β to high cell density cultures did not induce myofibroblast differentiation. The present study indicates differential functional expression of TGF-β receptors: binding of TGF-β is increased in cells growing at low density and probably plays a critical role in the TGF-β response. That low-density cells secrete more TGF-β and also express more functional receptors suggests that the cytokine up-regulates the cell surface expression of its own receptor. Additionally, increased numbers of functional receptors may be related to larger surface area of the low-density fibroblast and the resultant myofibroblast.

Wounding produces several changes in the stromal cell types similar to the phenotypes and densities in our model system. Fibroblasts replace the formerly quiescent keratocytes. Functions between keratocytes are disrupted by apoptosis and cell rounding. In our model system, the cultured fibroblast activated by serum represents the activated stromal fibroblast found in vivo after wounding. Similar to the myofibroblasts that arose in our low-density culture, we predict that these in situ low-density fibroblasts that appear after wounding have a higher expression of TGF-β1 receptors than the keratocytes.

What is downstream of TGF-β activation in myofibroblast differentiation? The recent discovery of the Smad proteins has provided one mechanism by which signals from TGF-β receptors in the plasma membrane are conveyed into the nucleus. Smad2 and Smad3 have been shown to be direct substrates for the type I TGF-β receptor serine–threonine kinase domain. In unstimulated cells, Smad2 is present throughout the cytoplasm. After phosphorylation and activation in epithelial cells, heterodimeric Smad2 localizes to the nucleus where it acts as a transcriptional regulator. In mink lung epithelial cells, Smad translocation to the nucleus was correlated with TGF-β-induced inhibition of cell proliferation.

The present study presents evidence for a TGF-β-induced Smad nuclear translocation in nonconfluent fibroblasts. These are the same conditions under which the cells become myofibroblasts. In contrast, high-density cultures seem to favor retention of Smad in the cytoplasm. Future studies will explore whether nuclear translocation of Smad2 is directly responsible for myofibroblast differentiation and what mechanisms prevent its nuclear translocation in high-density cells.

The ability to regulate the proportion of fibroblasts and myofibroblasts in the cornea could be helpful in the healing process, because phenotypic differences in secretion of matrix, matrix-degrading enzymes and adhesion characteristics suggest they may play different roles. Our present study provides direct evidence that the fibroblasts' ability to respond to TGF-β treatment is influenced by cell density and suggests that increasing fibroblast density at a wound site may be a mechanism to prevent myofibroblast differentiation.

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References


