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Transforming Growth Factor-β1 Expression in Cultured Corneal Fibroblasts in Response to Injury

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Abstract The mechanisms underlying TGF-β regulation in response to injury are not fully understood. We have developed an in vitro wound model to evaluate the expression and localization of transforming growth factor-β1 in rabbit corneal fibroblasts in response to injury. Experiments were conducted in the presence or absence of serum so that the effect of the injury could be distinguished from exogenous wound mediators. Cultures were wounded and evaluations conducted over a number of time points. Expression of TGF-β1 RNA was determined using Northern blot analysis and in situ hybridization, while the TGF-β receptors were identified by affinity cross-linking. Injury increased the expression of TGF-β1 mRNA in cells at the wound edge after 30 min; this response was amplified by the addition of serum. TGF-β1 mRNA expression was observed in a number of cells distal from the wound. After wound closure, TGF-β1 mRNA was negligible and resembled unwounded cultures. The half-life of TGF-β1 mRNA was two times greater in the wounded cultures, indicating that the injury itself maintained the expression, while cell migration was present. Analogous to these findings, we found that binding of TGF-β to its receptors was maximal at the wound edge, decreasing with time and distance from the wound. These results indicate that injury increases the level of expression of TGF-β1 mRNA and maintains a higher level of receptor binding during events in wound repair and that these might facilitate the migratory and synthetic response of stromal fibroblasts. J. Cell. Biochem. 77:186–199, 2000.

Key words: corneal fibroblasts; wound healing; TGF-β1 expression; growth factor

Wound repair is a complex process involving inflammation, cell proliferation, matrix deposition and tissue remodeling [Clark, 1997]. The extracellular matrix (ECM) of the corneal stroma supports and mediates signal transduction events that regulate cell function. Stromal keratocytes are surrounded by a well-organized ECM composed mainly of collagen types I, V, and VI and of proteoglycans possessing either keratan sulfate side chains or chondroitin/dermatan sulfate side chains. Unlike other tissues, heparan sulfate is not detected in the normal uninjured cornea [Brown et al., 1994]. The collagen molecules are organized in highly ordered lamellae, and the interrelationship of collagens and proteoglycans is thought to play a role in maintaining corneal transparency [Trinkaus-Randall, 1997]. Thus, injury has been demonstrated to induce rapid changes in cell-cell and cell-ECM interactions, often resulting in a loss of transparency.

Our goal was to characterize cellular changes that occur in response to injury. An injury to the corneal stroma causes changes in collagen fibril organization and glycosaminoglycan synthesis. The injury-induced glycosaminoglycan side chains are more highly sulfated and contain increased amounts of iduronic acid. The ratio of chondroitin sulfate (CS)/dermatan sulfate (DS) to keratan sulfate (KS) increases after injury, and heparan sulfate proteoglycans (HSPGs) are detected at the edge of the injury where cells are migrating [Hassell et al., 1983]. There is also an associated change in cellular phenotype with injury [Jester et al., 1998]. Furthermore, matrix-

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associated growth factors such as transforming growth factor-β (TGF-β) and basic fibroblast growth factor (bFGF, FGF-2) have been detected transiently after injury and delineate the wound margin in vivo [Trinkaus-Randall and Nugent, 1998]. The presence of these growth factors was correlated with the appearance of heparan sulfate [Brown et al., 1995]. The expression of TGF-β1 has also been localized to epidermal wounds in rabbit, porcine, and human [Assoian et al., 1983, 1987; Shipley et al., 1986; Gailit et al., 1994]. The transient appearance of growth factors in vivo suggests that they play a role in regulating the synthesis of matrix molecules.

The TGF-β family includes several structurally homologous proteins that affect cell growth and differentiation [Massague, 1990; Cross et al., 1991; Attisano et al., 1994]. The effects of TGF-β depend on cell type and the characteristics of the ECM. While TGF-β inhibits epithelial and leukocyte proliferation, it stimulates the proliferation of smooth muscle cells, skin fibroblasts, and stromal fibroblasts [Assoian et al., 1987; Grotendorst et al., 1989; Massague, 1990; Cross et al., 1991; Kay et al., 1998; Brown et al., 1999]. We have recently demonstrated that TGF-β modulates the interaction of stromal cells with their ECM by inducing the synthesis of specific proteoglycan core proteins and their respective glycosaminoglycans [Brown et al., 1999].

TGF-β are typically secreted in a biologically latent form, and activation is induced in vivo through a complex process of proteolytic activation and dissociation of latency protein subunits [Massague, 1998]. Active TGF-β is a 25-kDa disulfide-linked homodimer. TGF-β Receptors I and II are transmembrane glycoproteins of 55 and 70 kDa. Betaglycan (TGF-β receptor III) is a cell surface proteoglycan that has both heparan and chondroitin sulfate chains on its extracellular domain. Although much is known about the distribution of TGF-β and its receptors in various tissues, little is known about the time course and localization of TGF-β1 expression during the repair process.

Previous data have demonstrated that the major proteoglycan species secreted by stromal fibroblast cultures are decorin and perlecan and that their synthesis is mediated by TGF-β1 [Brown et al., 1999]. We have used this culture system to evaluate the relationship between growth factor expression and injury. When cells were wounded in serum-free medium, we found that the injury alone increased the expression of TGF-β1 mRNA along the wound margins. When cells were cultured in 1% serum, there was an increase in TGF- β1 mRNA along the wound margin and the presence of serum accelerated the rate of the response. Injury resulted in an increase in the half-life of the TGF-β1 mRNA. An increase in the amount of TGF-β1 protein associated with the wounded cells compared with unwounded control was observed. TGF-β receptors I, II, and III were present under both wounded and unwounded conditions, yet receptor occupancy was maximal at the leading edge of the wound. These findings may have implications in the regulation of wound repair.

MATERIALS AND METHODS

Cell Culture

Rabbit eyes were obtained from Pel Freeze (Rogers, AK), shipped on ice, and used within 24 h of enucleation. Briefly, epithelium and endothelium were removed, and minced stromas were digested in 1 mg/ml collagenase A (Sigma, St. Louis, MO) [Brown et al., 1999]. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 4% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin, 1% nonessential amino acids, and antifungal agents (Gibco, Grand Island, NY). Cells were subcultured and, at confluence, cells were serum starved for 24 h. Medium containing 0% or 1% FCS was added, and cells were incubated further for 24 h. The conditioned medium was removed before injury and linear wounds were made every 2 mm with a 25-gauge 7/8 needle. The conditioned medium was then added back to the cells, and the cells were evaluated at several time points over 24 h.

Probes

The plasmid: pGEM3zf (−) human TGF-β1 (host strain: DH5α) was a gift from Dr. R. Derynck. TGF-β1 cDNA restriction fragments 322 bp (*Sac*I/*Bst*E II) were generated for the nonisotopic in situ hybridization experiments, while 523 bp (*Sac*I/*Bam*HI) was used for Northern blot analysis. A 545-bp (*Xba*I/*Hin*dIII) restriction fragment of human cDNA glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used.

RNA Isolation and Northern Blot Analysis

Total cellular RNA was isolated from stromal fibroblasts using TRIzol Reagent (GibcoBRL). A total of 15 μg of total RNA was denatured and separated by electrophoresis, using a 1% agarose gel containing 1.9% formaldehyde. Equal gel loading and integrity of 18S and 28S RNA was verified using ethidium bromide. RNA was transferred to a Duralose membrane (Stratagene, La Jolla, CA) and ultraviolet (UV) cross-linked [Grushkin-Lerner et al., 1997].

Blots were hybridized with TGF-β1 and GAPDH cDNA probes that were labeled by random priming with [α^{32}P] dCTP (New Life Science Products, Boston, MA), using the Random prime DNA labeling kit (Pharmacia, Piscataway, NJ). Prehybridization and hybridization at 68°C were performed in Rapid-Hybridization buffer (Stratagene, La Jolla, CA). Blots were washed twice at room temperature in 2× SSC buffer (2× saline-sodium citrate [SSC]: 0.15 M NaCl, 15 mM sodium citrate) containing 0.1% sodium dodecyl sulfate (SDS), once in 1× SSC buffer containing 0.1% SDS at 68°C, and once in 0.1× SSC containing 0.1% SDS at 60°C. Membranes were exposed to Amersham Hyperfilm for 24–48 h, and mRNA was quantified using NIH Imaging Software (National Institutes of Health [NIH]). Statistical analysis was performed using Student's *t*-test to compare wounded and unwounded groups. Differences were considered significant when $P < 0.05$.

Stability of TGF-β1 mRNA

To investigate whether changes in the levels of TGF-β1 mRNA were related to changes in mRNA turnover, mRNA stability in control and wounded cultures was evaluated. Quiescent cultures were established and incubated in medium containing 1% serum and wounded with a 25-gauge 7/8 needle. 5,6-Dichlorobenzimidazole riboside (DRB; Sigma Chemical Co.) [Zandomeni et al., 1986], 30 μg/ml, was added 3 h after injury to the cultures, to inhibit new transcription. Parallel unwounded cultures were incubated in DRB. Cells were evaluated at 30, 60, 90, 120, and 180 min after the addition of DRB. Total RNA was extracted and subjected to Northern blot analysis for TGF-β message levels.

Nonisotopic In Situ Hybridization

Replicate experiments were performed to evaluate the localization of cells expressing TGF-β1 in response to injury. Nonisotopic in situ hybridization for TGF-β1 mRNA was conducted [Singer et al., 1986; Grushkin-Lerner et al., 1997]. Cells were fixed in 4% paraformaldehyde containing 5.0 mM $MgCl_2$ pH 7.4 for 15 min, washed, and stored in PBS at 4°C overnight. Before hybridization, cells were incubated in Tris-HCI, pH 7.4, 0.1 M glycine. The cDNA restriction fragment of TGF-β1 (322 bp *Sac*I/*Bst*EII) was prepared and labeled using the nick-translational kit (Boehringer Mannheim, IN) and digoxigenin-11-UTP. Southern blot analysis of the labeled probe was conducted to ensure that the probe was labeled specifically. The cDNA probe melted in 100% formamide at 90°C was combined with an equivalent volume of hybridization buffer containing 20× SSC, 2% BSA, 50% dextran sulfate, and vanadyl ribonucleoside complex (VRC; 1:1:2:1) and added to cells and incubated in a humidified chamber at 37°C for 15 min After hybridization and extensive washing with 2× SSC, the cells were incubated in anti-digoxigenin-FITC for 1 h at 37°C. Cells were washed and coverslipped, and images were recorded using confocal laser scanning microscopy. The pixel intensities of cells at the leading edge and one cell back were measured. Simultaneously transmitted light images were recorded to calculate the number of cells expressing TGF-β1. A minimum of 50 cells was counted for each parameter.

TGF-β Binding Studies

To evaluate the presence of receptors in response to injury, cells were radiolabeled with ^{125}I-TGF-β1 as described [Chen et al., 1998; Massague, 1998]. Wounded and unwounded cultures were established as previously described. Cultures were incubated in DMEM containing 1% serum and binding of ^{125}I-TGF-β1 (1 ng/ml) was performed at 4°C for 2.5 h Cells were washed with binding buffer containing DMEM, 0.05% gelatin, and 25 mM HEPES and extracted with Triton X-100. Samples were counted in an Auto-5650 gamma counter (Packard Instruments, Downers Grove, IL). Nonspecific binding was determined with a 200-fold excess of unlabeled

TGF-β1 and the counts subtracted from the experimental data.

TGF-β1 receptor proteins were identified by chemically cross-linking ^{125}I-TGF-β1 to its receptor using disuccinimidyl suberate [Chen et al., 1998; Massague, 1998]. ^{125}I-TGF-β1 receptor complexes were solubilized in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer and electrophoresed on a 4–20% SDS-polyacrylamide gradient gel. The radiolabeled proteins were visualized by autoradiography.

Quantification of TGF-β1 Protein

To evaluate the total TGF-β1 protein in wounded and parallel unwounded cell cultures at various time points in serum-free medium, a quantitative sandwich enzyme-linked immunosorbent assay (ELISA) specific to TGF-β1 (R&D Systems) was used. Cell cultures were wounded as described previously. At each time point, the media was collected and frozen at −20°C until all time points were obtained. The cells were extracted using a Tris-NaCl buffer containing 1% Triton X-100, 150 mM NaCl, 10 mM Tris pH 7.4, 1 mM EDTA, 0.2 mM Na vanadate, and 0.2 mM phenylmethylsulfonyl fluoride (PMSF). To activate any latent TGF-β1, 1 N HCl diluted in PBS was added for 10 min and neutralized with PBS containing 1.2 N NaOH and 0.5 M Hepes. The ELISAs were conducted according to the manufacturer's directions. The immunoassay plate was read on an OptiMax microplate reader (Molecular Devices) at 450 nm with background correction set at 540 nm. All values were normalized to cell number as determined by relative acid phosphatase activity [Brown et al., 1999].

Localization of TGF-β Receptors

To localize TGF-β1 binding sites at various times after injury (0, 3, 6, and 24 h), wounded and unwounded cultures were incubated in DMEM with 1% FBS and TGF-β (1.5 nM) at 4°C for 2.5 h in binding buffer (DMEM, 0.05% gelatin and 25 mM HEPES). Cells were rinsed twice with binding buffer and PBS and fixed for 10 min with formaldehyde (3.7% in PBS, pH 7.2). Cells were rinsed again in PBS and blocked with PBS-BSA (3%) for 1 h at room temperature. Anti-TGF-β antibody (150 μg/ml) in PBS-BSA (2%) was hybridized for 1 h at 37°C. After three washes with PBS-BSA (2%) and two washes with PBS, the secondary anti-IgG-FITC (1:100) in PBS-BSA (2%) was hybridized for 1 h at 37°C. Cells were washed and anti-fade (Molecular Probes, Eugene, OR) was added to the coverslip.

Confocal Microscopy

Cells were imaged on a Zeiss inverted LSM 510 confocal laser scanning microscope (CLSM) equipped with Ar and He lasers. All experimental cultures were examined under the same conditions [Trinkaus-Randall et al., 1993; Wu et al., 1995; Grushkin-Lerner et al., 1997]. Images were stored on Zip disks and transferred to a Macintosh workstation, where images were configured in Adobe Photoshop.

RESULTS

Expression of TGF-β1 Induced by Injury

Previous work has described the synthesis of glycosaminoglycan and core proteins by stromal fibroblasts in response to TGF-β and serum [Brown et al., 1999]. We have also demonstrated that linear wounds to stromal cultures close by 24 h and that a fissure line can be detected by 48 h [Haq et al., 1998]. The goal of the present study was to investigate the potential that an injury could modulate TGF-β expression. The response was analyzed both in the presence and in the absence of serum to evaluate whether additional soluble wound mediators might participate in regulating TGF-β expression. Cells were serum-starved for 24 h and kept in serum-free or switched to 1% FBS for 24 h. Immediately before injury, the media were removed, the cultures were injured and washed in PBS, and the conditioned media (0% or 1% FBS) were returned to the respective cultures and incubated for 0, 0.25, 0.5, 3, 6, and 24 h. Expression was evaluated using Northern blot analysis and in situ hybridization, and the latter was visualized using confocal microscopy.

Four representative confocal images of wounded cells cultured in 1% serum were taken 15 min after injury (Fig. 1A–D). In all four images, TGF-β1 mRNA was localized along the wound margins of both sides of the injury. There was some wound-to-wound variation, but there was a strong consistency in the localization of message to the wound margin and the lack of signal in cells a distance from the wound. Fluorescence was negligible in

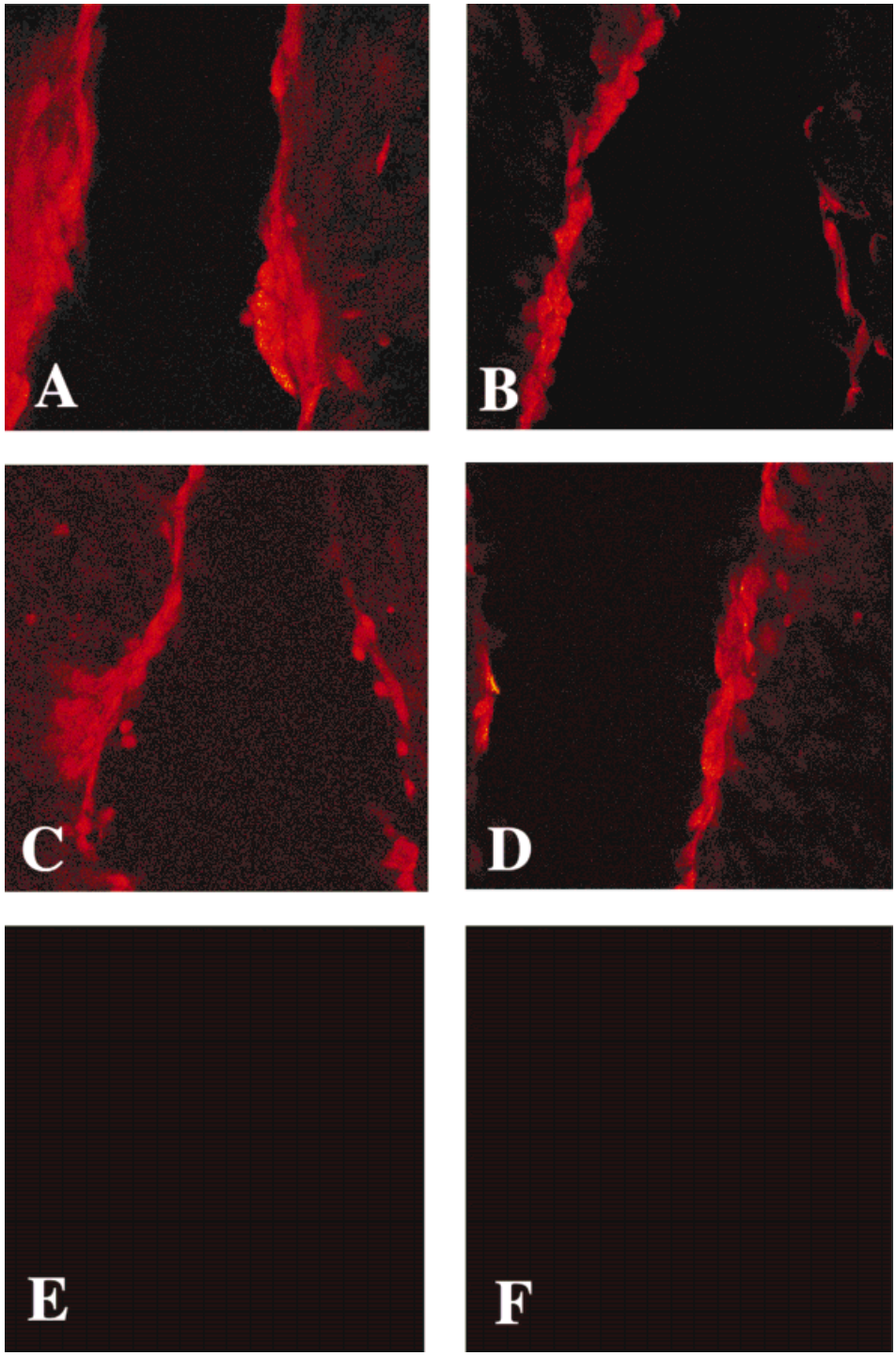

Fig. 1. Localization of transforming growth factor-β1 (TGF-β1) mRNA of cells injured in 1% serum using nonisotopic in situ hybridization and imaged with confocal microscopy. There are four representative images of cultures 15 min after injury. There is an increase in the expression of TGF-β mRNA at the wound edge. Only a small number of cells distal to the wound express TGF-β1 (**A–D**). Fluorescence was negligible in RNase control samples (**E, F**) (each frame is 125 μm).

RNase control samples (Fig. 1E,F). To follow changes in localization over time, cultures incubated in the presence and absence of serum were evaluated (Fig. 2). When cells were cultured in 1% serum, TGF-β1 mRNA was localized to cells at the leading edge within 15 min

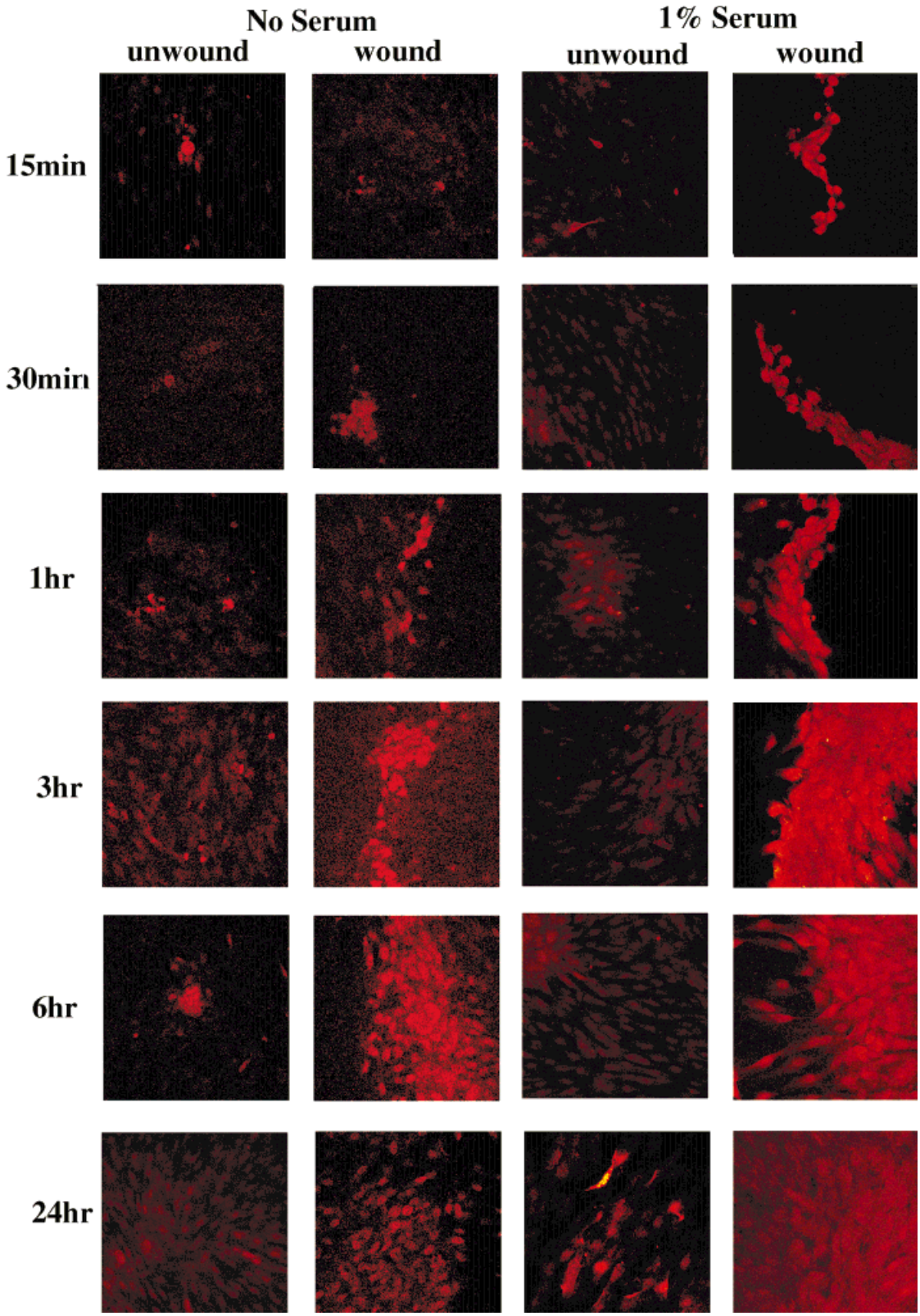

Fig. 2. Localization of transforming growth factor-β (TGF-β) mRNA in wounded and control cultures, using nonisotopic in situ hybridization and imaged with confocal microscopy. Cultures were serum starved, incubated in the presence and absence of serum for 24 h, injured, and evaluated for an additional 24 h. Control cultures were not wounded and are represented at the same time points. TGF-β1 mRNA was negligible in unwounded cultures and back from the migrating edge of the wound. TGF-β1 was expressed in 1% serum at the leading edge of the wound by 15 min and became intense by 30 min. Expression was detected in cells distal to the wound edge at 1, 3, and 6 h in 1% serum. In serum-free medium, the response was detected at the leading edge by 30 min, and the response was delayed compared with cells incubated in serum (each frame is 125 μm). The images are representative of three individual experiments.

after injury (Fig. 2). However, the change in localization was not detected at 15 min when cells were injured in the absence of serum. With increasing incubation time, the number of cells that expressed TGF-β mRNA distal to the wound margin increased. At 30 min, a population of cells 3–5 cells distal to the injury expressed TGF-β mRNA in the presence of 1% serum, while only a few cells expressed message under serum-free conditions. By 3 h, more than 10 "rows" of cells distal to the leading edge expressed TGF-β1 in 1%, while an increasing number of cells distal to the leading edge expressed TGF-β1 in serum free conditions. At 6 h after injury, the number of cells expressing TGF-β1 mRNA in cells cultured in 1% serum decreased, while the cells cultured in serum-free conditions exhibited an increased expression. These results indicate that the presence of the serum does not change the overall response but does seem to enhance the rate of response to the injury. Fluorescence was negligible in the unwounded controls in both serum-free cultures and in cultures containing 1% serum, demonstrating that the effect is dependent on the wound (Fig. 2).

While in situ analysis provides information regarding the position of the expressing cells with respect to injury, Northern blot analysis was also conducted on the entire cell population to evaluate the effects on the overall TGF-β1 mRNA levels. When cells cultured in the absence of serum were injured, increased expression of TGF-β1 mRNA was detected 3 h after injury and remained elevated for 24 h (Fig. 3A). When the Northern blots were subjected to densitometric analysis and normalized to unwounded cultures, there was a 1.5-fold increase at 3 h, and the change in message was maximal at 12 h (2.2-fold increase). These data correlated well with the data from the in situ analysis, demonstrating a large increase at 3 h (Fig. 2). The integrity of RNA was monitored using ethidium bromide staining of 28S and 18S. In addition, there was no significant change in GAPDH over time or condition. The absence of serum delayed the response, but elevated expression was manifested for a longer period of time. These data indicate that injury alone induced an overall increase in TGF-β1 mRNA, even averaged over the entire culture.

We also evaluated steady-state TGF-β1 mRNA in the presence of 1% serum. When cells were cultured in 1% serum, expression was maximal at 3 h and by that time had increased more than 3.5 times relative to unwounded cultures (Fig. 3B). The integrity of RNA was monitored using ethidium bromide staining of 28S and 18S. Since Northern blot analyses represent an average of the entire culture which includes both wounded and unwounded cells, we hypothesize that the time course in the Northern blot is delayed because the increase in expression induced by the injury (15 min; see Figs. 1 and 2) was masked by the unwounded cells in the culture.

Injury-Stabilized TGF-β1 mRNA

The injury-induced increase in TGF-β1 mRNA levels observed may reflect an increase in the transcriptional activity of this gene, an increase in message stability, or a combination of both. To evaluate whether injury altered the stability of TGF-β1 mRNA, cells were incubated in the presence and absence of DRB, an inhibitor of new gene transcription. At 3 h after injury, cells were incubated both with and without DRB, and RNA was extracted every 30 min for 3 h. Replicate experiments were conducted on unwounded cells. Under these conditions, no significant change in GAPDH mRNA was observed over time (Fig. 4A). The rate of TGF-β1 mRNA degradation was markedly reduced in the wounded cultures compared with unwounded cultures (Fig. 4A,B). The half-life of the message in the unwounded cultures was 45 min, while that in wounded cultures was 90 min. These data suggest that injury and the respective changes in cell-cell and cell-matrix communication may increase the stability of TGF-β1 mRNA.

Differential Association of TGF-β1 Protein

To determine whether the changes in TGF-β1 mRNA observed in the injured cultures translated into an increase in TGF-β1 protein production, TGF-β1 protein levels in wounded and unwounded cultures were quantitated by ELISA. The amount of TGF-β1 in unwounded cultures increased with time in culture in both the cell associated and media fractions, indicating that corneal fibroblasts constitutively express TGF-β1 in serum-free conditions (Fig. 5A,B). There was a 60% increase in cellular-associated TGF-β1 at 4–8 h in wounded cells, compared with a smaller increase (25%) in unwounded cultures. At the 8-h

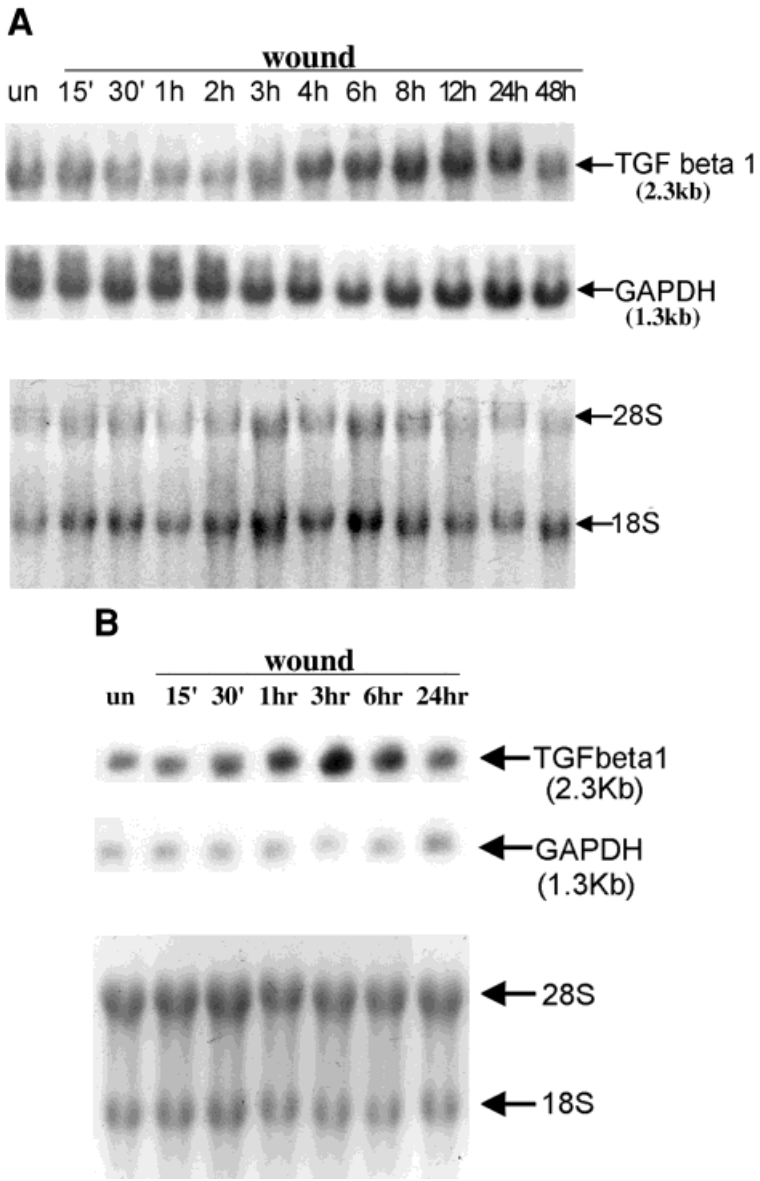

Fig. 3. **A:** Injury enhances transforming growth factor-β1 (TGF-β1) mRNA expression in the absence of serum. Northern blots were probed with TGF-β1 and GAPDH cDNA probes. Multiple wounds were made throughout the culture and RNA extractions represent the entire culture. Expression was elevated 4 h after injury and remained high for 24 h. Expression dropped to unwounded control levels 48 h after injury. Ethidium bromide staining of 18 and 28S indicates integrity of RNA. Data are representative of three experiments. **B:** Injury increases TGF-β1 mRNA along the margin of the wound in 1% serum. TGF-β1 mRNA levels in unwounded cultures did not change over the time course. Northern blots were probed with TGF-β1 and GAPDH cDNA probes. Multiple wounds were made throughout the culture and RNA extractions represent the entire culture. Change in GAPDH mRNA was negligible over time. When TGF-β1 mRNA was normalized to GAPDH mRNA, expression was maximal at 3 h. Ethidium bromide staining of 28 and 18S RNA is shown. Data are representative of at least three experiments.

time point the level of TGF-β in the media decreased while the amount TGF-β in the media of unwounded cultures remained elevated.

TGF-β Binding to Stromal Fibroblasts

To determine whether there were local injury-induced changes in binding of TGF-β1 to stromal fibroblasts, experiments were designed to localize the binding of exogenous TGF-β after injury. Binding was detected at the leading edge minutes after injury indicating that the injury changed the availability of receptors (Fig. 6B). Staining became more intense along the cells at the leading edge of the wound at 3 h

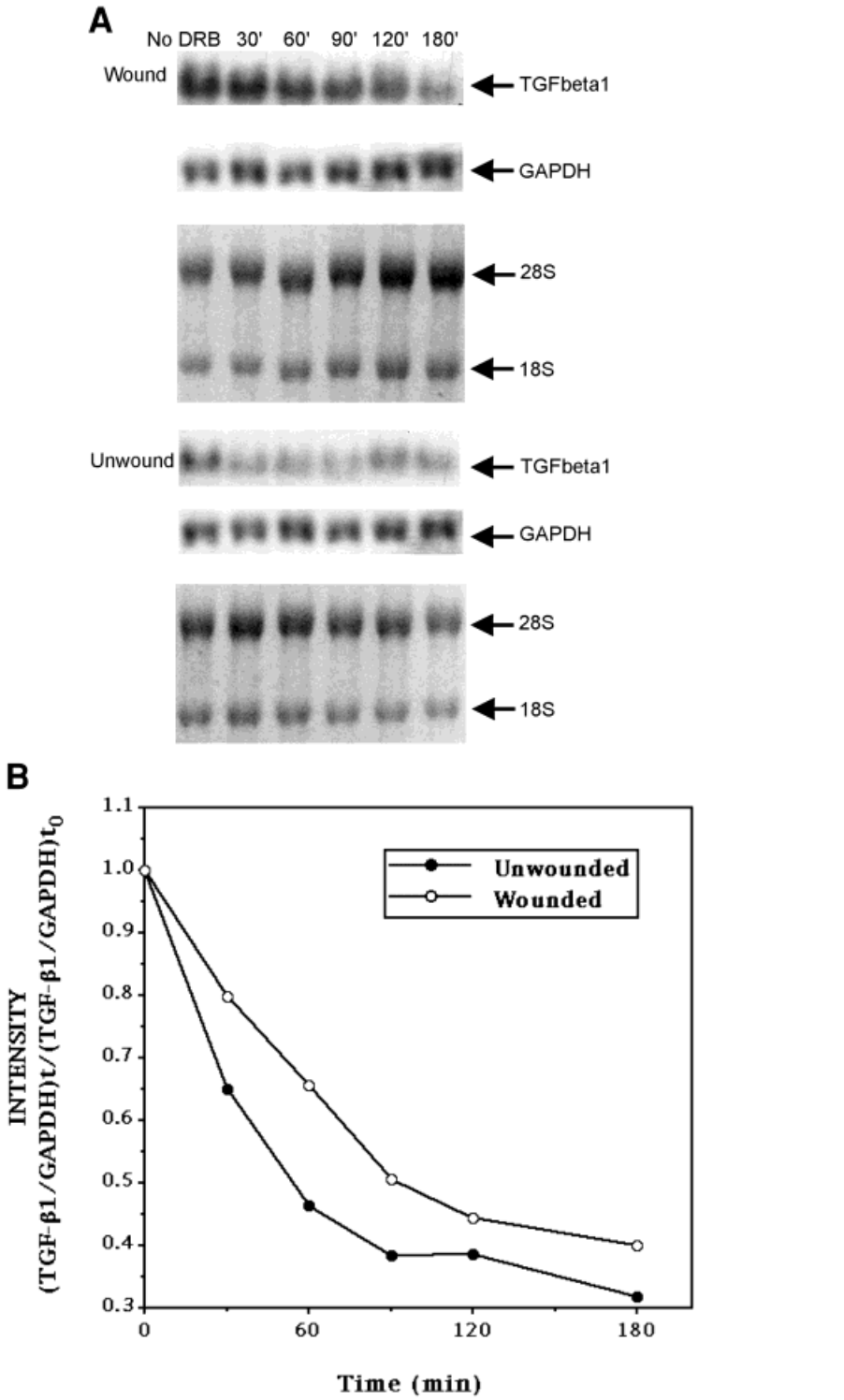

Fig. 4. **A:** Injury enhances stability of transforming growth factor-β1 (TGF-β1) mRNA. Quiescent cultures were injured, and 5,6-dichlorobenzimidazole riboside was added 3 h after injury. Parallel-unwounded cultures were incubated in inhibitor. Ethidium bromide staining of 18 and 28S shows integrity of RNA. GAPDH mRNA did not change over time. **B:** The half-life of the unwounded cultures was 45 min, while that of the wounded cultures was 90 min. Data are representative of three experiments.

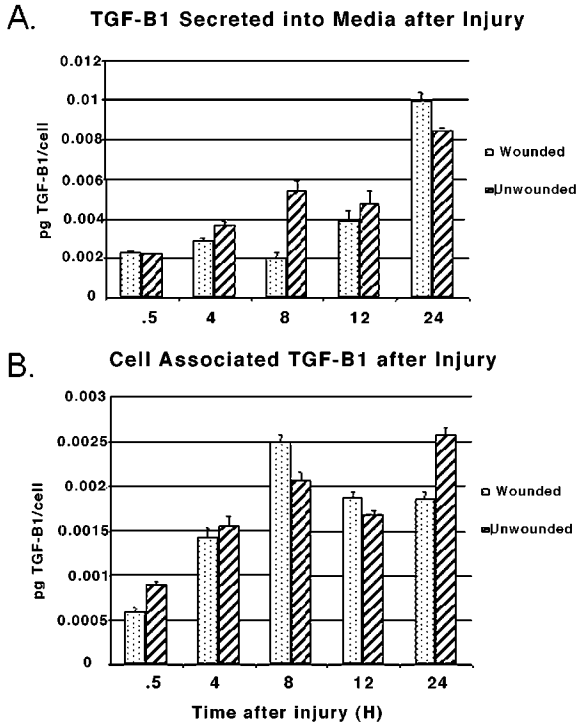

Fig. 5. Injury mediated the transforming growth factor-β (TGF-β) protein secreted into serum free media and that which was cell associated. TGF-β was quantified using an enzyme-linked immunosorbent assay (ELISA). Cell extracts and media fractions were normalized to cell number and latent TGF-β1 was acid activated. Error bars depict SE, and all experiments were repeated a minimum of 3 times. **A:** Media fractions were analyzed. TGF-β1 was present at significantly lower levels 8 h after injury compared with unwounded cultures. **B:** Cell extracts were analyzed. TGF-β1 was present at significantly higher levels at 8 h after injury compared with unwounded controls.

(Fig. 6C), while cells distal to the wound showed significantly less binding (Fig. 6A). At 6 h after injury, fluorescence decreased, and after wound closure (24 h) staining was negligible (Fig. 6D,E, respectively). When exogenous TGF-β was added to the cultures, staining was negligible (data not shown) as the antibody bound to the exogenous TGF-β. In unwounded cultures, fluorescence was negligible (Fig. 6F). Fluorescence was not detected when the primary antibody was omitted (data not shown).

The presence of receptors in wounded and unwounded cultures was evaluated using affinity-cross-linking experiments. Cultures labeled with ^{125}I-TGF-β were subjected to cross-linking and were extracted and separated electrophoretically on 4–20% SDS-PAGE gels. TGF-β receptors I, II, and III were present in both control and wounded cultures. When experiments were conducted at 3 and 24 h, no significant differences were detected between wounded and unwounded cultures (Fig. 7). As the wounded cultures contain a significant number of unwounded cells, this analysis represented an average of the total cellular population; therefore, significant localized alterations in TGF-β receptor expression or availability could have been masked. Controls with an excess of unlabeled TGF-β demonstrated specificity (data not shown).

DISCUSSION

In this study, we evaluated the expression of TGF-β1 in response to injury in a defined culture model [Haq et al., 1998; Brown et al., 1999]. We hypothesized that injury alters the cell-cell and cell-matrix environment and modulates growth factor availability resulting in a change in receptor occupancy. We found that injury increased the expression of TGF-β1 and that the increased expression was restricted to cells at, or adjacent to, the leading edge. The presence of serum resulted in a more rapid response accompanied by a more rapid decline occurring before wound closure. Thus, changes in the extracellular environment might trigger the intensity of the cellular response.

The role of growth factors and of their receptors in wound repair has been widely studied [Clark, 1997], yielding insight into the mechanisms of scarring. It is established that TGF-β is found in high concentrations in platelets [Assoian et al., 1983] and is produced by other cells present at the wound site including activated macrophages, neutrophils, fibroblasts, and keratinocytes [Shipley et al., 1986; Assoian et al., 1987; Thompson et al., 1989; Pittelkow et al., 1991; Levine et al., 1993; Gailit et al., 1994; Grotendorst et al., 1998]. Several review studies have discussed the role of growth factors in wound healing [Schultz et al., 1992; Bennett et al., 1993a,b]. These observations suggest that growth factors act in a coordinated manner and must be under strict control to regulate normal tissue repair.

In our wound system, the expression of TGF-β was enhanced rapidly after injury and the addition of serum to the entire culture in the absence of injury did not replicate these events. In preliminary experiments, we have shown that expression is specific to injury and that FGF-2 mRNA was not increased with injury (data not shown). These results indicate

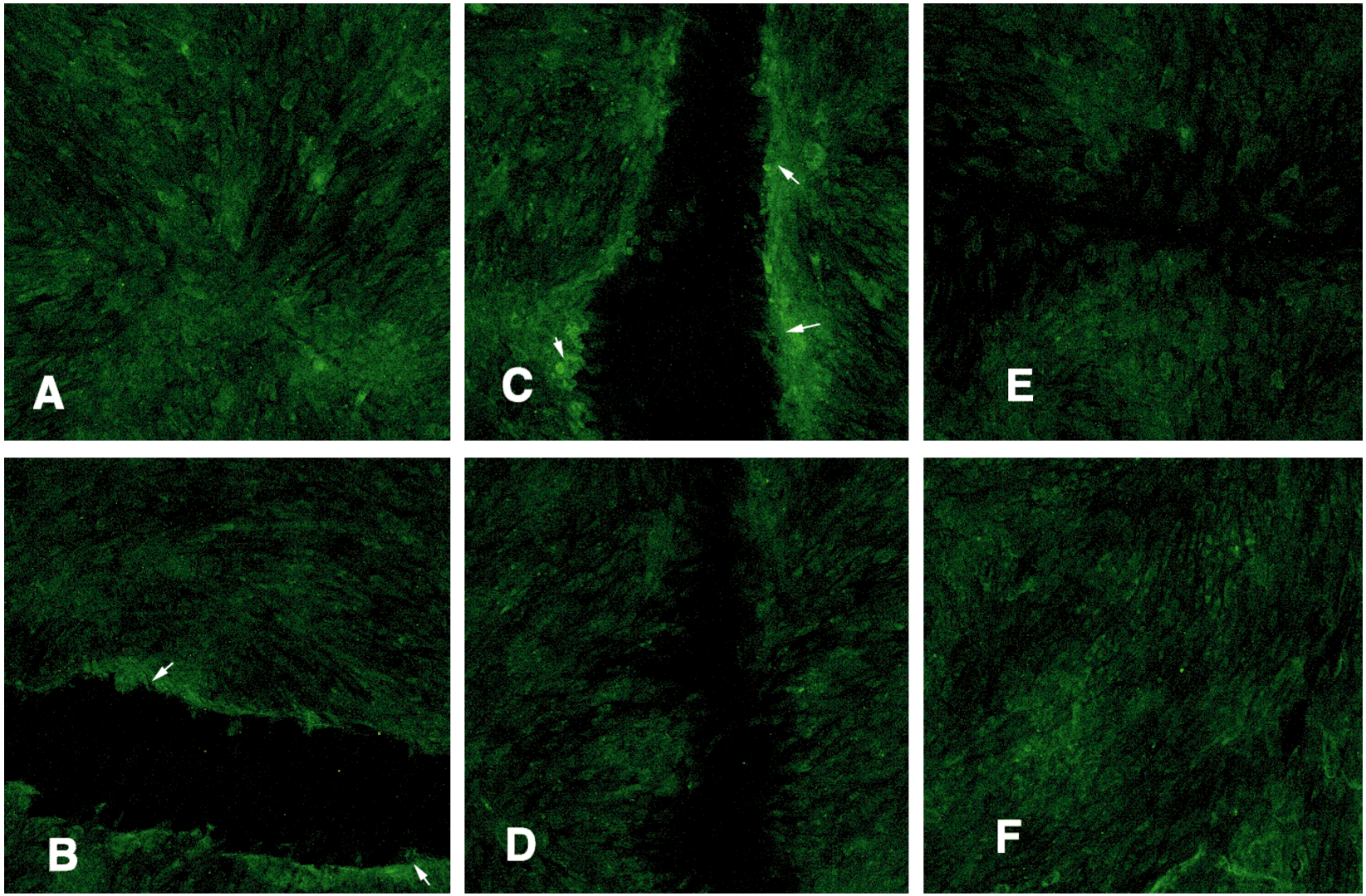

Fig. 6. To determine whether there were local injury-induced changes in binding of transforming growth factor-β1 (TGF-β1) to stromal fibroblasts, experiments were designed to localize the binding of exogenous TGF-β binding after injury. Serum-starved fibroblasts were wounded and incubated for the times indicated. TGF-β1 (1.5 nM) was bound for 2.5 h at 4°C, fixed and immunolocalized by confocal laser scanning microscopy. **A:** Distal to wound edge. **B:** At 2 min after injury. **C:** At 3 h after injury. **D:** At 6 h after injury. **E:** At 24 h after injury. **F:** At unwounded cell cultures (1302.7 μm per frame). Experiments are representative of three independent experiments.

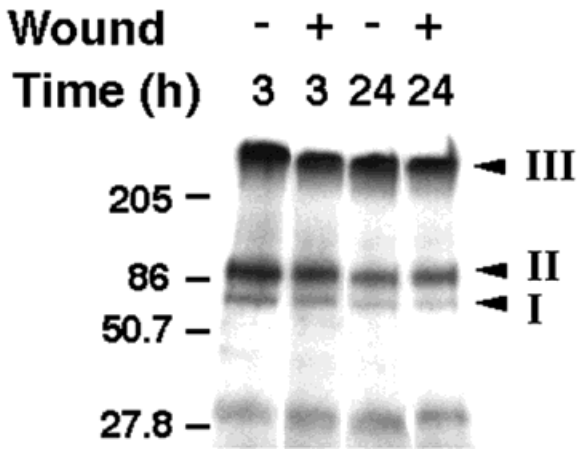

Fig. 7. Transforming growth factor-β1 (TGF-β1) receptors were identified in wounded and control cultures by chemical cross-linking. Wounded and control cultures were washed and chilled to 4°C either 3 or 24 h after injury and incubated with ^{125}I-TGF-β1 (40 pM) for 3 h at 4°C. Unbound ^{125}I-TGF-β1 was washed from the cells and the bound label cross-linked to its receptors using disuccinimidyl suberate. Cells were solubilized in boiling sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer and subjected to electrophoresis (4–20% gradient) and the radiolabeled proteins visualized by autoradiography. Equal amounts of total protein were applied to each lane. The gel is representative of three experiments.

that the initial stages of injury might be mediated by TGF-β1 expression, which alters the synthesis of additional growth factors such as FGF-2. Initially, the increase in expression indicates that injury alters the level of TGF-β binding. In this system, we predict that there exists a level of negative feedback that would shutdown TGF-β expression of cells as they move through the various stages of wound repair. These results indicate that the timing of the administration of growth factors in wound healing is critical. Difficulties in mediating the wound repair cascade may also be attributed to observations that growth factor bioavailability can change dramatically when the microenvironment of the tissue changes [Logan and Hill, 1992].

Three TGF-β isoforms—TGF-β1, TGF-β2, and TGF-β3—are present in mammals during wound repair and share a 64–85% amino acid sequence homology [Massague, 1990]. Each member of the TGF-β family is stored as a latent multimeric complex [Massague, 1990; Piez et al., 1990]; activation of these complexes is one aspect of cellular control. Since TGF-β interacts with extracellular matrix molecules the sulfated proteoglycans present in the stroma may modulate receptor activation and binding. TGF-β are known to have different functions in specific cell types. While TGF-β is mitogenic for fibroblasts, it is known to inhibit the proliferation of epithelial and endothelial cells. All TGF-β isoforms are known to enhance the expression of matrix and adhesion proteins [Massague, 1990; Piez et al., 1990; Brown et al., 1999]. Thus, TGF-β has the potential to modulate cell function at the site of injury in a coordinated manner, with the response dependent on the characteristics of the cell type and the surrounding extracellular matrix.

All three mammalian TGF-β isoforms are expressed during wound repair and development in vivo and have a unique distribution [Levine et al., 1993] suggesting a differential regulation of the three genes and their products. The studies of Shah et al. [1995] suggest that TGF-β1 and TGF-β2 induce cutaneous scarring, whereas TGF-β3 does not. Therefore, enhanced TGF-β expression might stimulate scarring, yet a decrease might delay repair. We found that injury enhanced the stability of the TGF-β1 mRNA. In addition, TGF-β1 enhances the expression of perlecan and other heparan sulfate proteoglycans in a dose-dependent manner and perlecan has also been shown to be present at wound margins in vivo [Nugent et al., 1995; Brown et al., 1999]. We have also demonstrated that TGF-β1 is secreted into the media. Andresen et al. [1997] demonstrated that concentrations of 0.1–1 ng/ml were necessary to induce cellular migration. However, concentrations greater than 1 ng/ml were inhibitory. These data suggest that TGF-β might enhance the synthesis of specific matrix proteins that facilitate cellular migration.

Studies on keratinocyte growth factor provide additional evidence that growth factors enhance the rate of wound healing. Keratinocyte growth factor is expressed at reduced levels during wound repair in glucocorticoid-treated mice [Brauchle et al., 1995], and the inhibition can be reversed by the topical application of exogenous growth factors, TGF-β or bFGF [Pierce et al., 1989; Beck et al., 1991, 1993; Klingbeil et al., 1991]. In these studies, the injury enhanced expression of TGF-β1 mRNA and protein along with altered availability of binding sites might contribute to localized changes in the cellular wound response. Our results suggest that TGF-β may be a local mediator of wound response, providing posi-

tional and temporal control in cell-cell communication, migration, and matrix synthesis that occur after injury.

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