ORIGINAL ARTICLE

TGF-β1 regulates TGF-β1 and FGF-2 mRNA expression during fibroblast wound healing

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Aims: To evaluate the expression of transforming growth factor β1 (TGF-β1) and fibroblast growth factor 2 (FGF-2) mRNA in stromal cells in response to injury in the presence of either TGF-β1 or FGF-2. It has been shown previously that heparan sulfate proteoglycans and FGF-2 are present transiently during wound repair in vivo and that an increase in TGF-β1 mRNA is detected rapidly after injury.
Methods: Primary corneal fibroblasts were cultured to confluency, serum starved, and linear wound(s) were made in medium containing TGF-β1 or FGF-2. TGF-β1 and FGF-2 mRNA expression were evaluated using both northern blot analysis and in situ hybridisation. Both dose dependent and time course experiments were performed. Whole eye organ culture experiments were also carried out and growth factor expression was assessed.
Results: Injury and exogenous TGF-β1 increased TGF-β1 mRNA values. The increase in expression of FGF-2 mRNA was not detected until wound closure. In contrast, FGF-2 inhibited the expression of TGF-β1. TGF-β1 increased TGF-β1 mRNA stability but did not alter that of FGF-2. Migration assay data demonstrated that unstimulated stromal cells could be activated to migrate to specific growth factors.
Conclusions: TGF-β1 specifically enhances cellular responsiveness, as shown by increased stability after injury and the acquisition of a migratory phenotype. These data suggest that there is an integral relation during wound repair between TGF-β1 and FGF-2.

Wound repair is a complex process beginning with the rapid disruption of cell–cell and cell–matrix interactions and the activation of signalling mechanisms. This is followed by a repair phase that encompasses inflammation, cell proliferation, matrix degradation and deposition, and tissue remodelling. The corneal stroma is a logical tissue to use for the evaluation of signalling mechanisms in wound repair because it is an avascular tissue with a highly organised architecture, where cells make contact via gap junctions between collagen lamellae. The stroma is composed mainly of collagen types I, V, and VI and proteoglycans possessing either keratan sulfate side chains or chondroitin/dermatan sulfate side chains. It is believed that the highly ordered lamellae and the inter-relation of collagens and proteoglycans maintain corneal transparency.[1 2] Unlike other tissues, heparan sulfate is not detected in the unwounded cornea, but is present in the injured stroma and cultured cells.[3–5]

Our goal was to characterise the cellular changes that occur in response to injury and the addition of exogenous growth factors (transforming growth factor β1 (TGF-β1) and fibroblast growth factor 2 (FGF-2)). Previously, it was shown that injury to the corneal stroma causes glycosaminoglycan side chains to become more highly sulfated, with increased amounts of iduronic acid.[6] In addition, the ratio of chondroitin sulfate to keratan sulfate increases and heparan sulfate proteoglycans are detected at the edge of the injury, where cells are migrating.[3 6–8] We showed previously that TGF-β1 and FGF-2 were present after injury in vivo.[9] Growth factors were also localised to the region in the stroma where migrating cells moved into an implanted porous polymer.[3 9] TGF-β1 has also been localised to epidermal wounds in rabbit, porcine, and human models.[10–13] The transient appearance of these growth factors in vivo suggests that they play a role in regulating the synthesis of matrix molecules and are crucial to the early and delayed phases in wound repair.

> "The corneal stroma is a logical tissue to use for the evaluation of signalling mechanisms in wound repair because it is an avascular tissue with a highly organised architecture, where cells make contact via gap junctions between collagen lamellae"

The TGF-β family includes several structurally homologous proteins and their effects depend on cell type and the characteristics of the extracellular matrix.[14–16] Although TGF-β inhibits epithelial and leucocyte proliferation, it can stimulate the proliferation of smooth muscle cells, skin fibroblasts, and stromal fibroblasts.[4 11 15 17 18] TGF-β molecules are typically secreted in a biologically latent form and activation is induced in vivo through a complex process of proteolytic activation and the dissociation of latency protein subunits.[19] Active TGF-β is a 25 kDa disulfide linked homodimer. TGF-β receptors I and II are transmembrane glycoproteins of 55 and 70 kDa, whereas TGF-β receptor III (betaglycan) is a large cell surface proteoglycan that can have both heparan and chondroitin sulfate chains on its extracellular domain. Intracellular signalling occurs as a result of TGF-β binding to cell surface receptors, primarily through the type I and type II receptors.[20] Betaglycan may have several roles, one being to bind TGF-β and present it to the type II receptor.[21]

FGF-2 is a member of a large family of growth factors implicated in numerous biological processes, including cell proliferation, differentiation, migration, angiogenesis, and wound healing.[5 22] FGF-2 is an 18 kDa protein, and three high molecular mass forms have been described (22, 22.5, and 24 kDa).[23–25] FGF-2 belongs to a family containing more than 20 heparin binding proteins and its activity is mediated by binding to heparan sulfate proteoglycans and to high affinity cell surface

Abbreviations: BSA, bovine serum albumin; DRB, 5,6-dichlorobenzmidazole riboside; FBS, fetal bovine serum; FGF-2, fibroblast growth factor 2; FISH, fluorescent in situ hybridisation; FITC, fluorescein isothiocyanate; PBS, phosphate buffered saline; RT-PCR, reverse transcriptase polymerase chain reaction; SDS, sodium dodecyl sulfate; SSC, saline sodium citrate; TGF-β1, transforming growth factor β1

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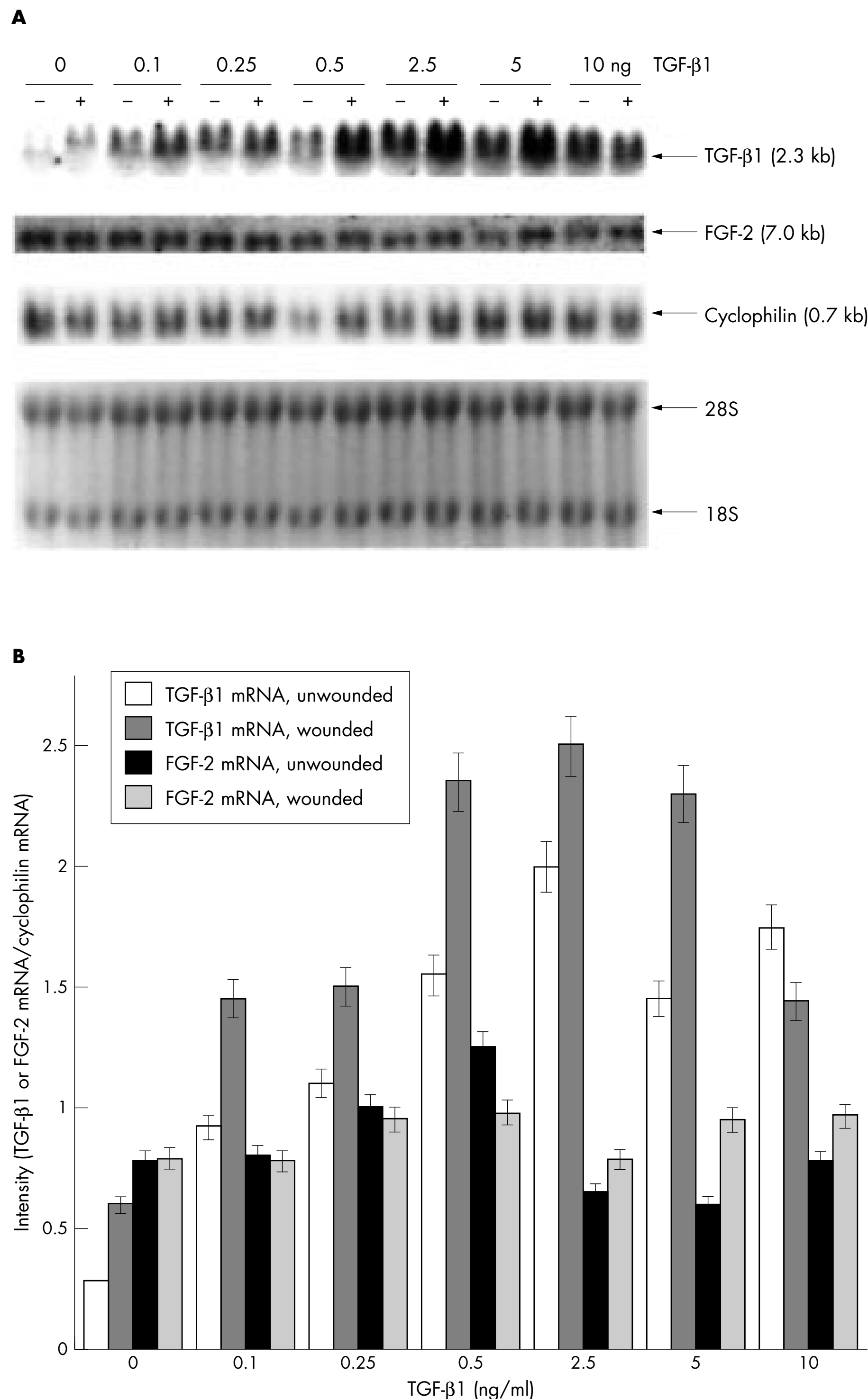

Figure 1 Transforming growth factor β1 (TGF-β1) and injury mediate expression of TGF-β 1 mRNA. (A) TGF-β1 mRNA and fibroblast growth factor 2 (FGF-2) mRNA expression in response to TGF-β1 and injury. Cultures were serum starved, multiple wounds were made throughout the culture (+), and cells were incubated in serum free DMEM containing a series of concentrations of TGF-β1 for six hours. Parallel unwounded cultures (–) were also harvested. RNA extractions represent the entire culture and ethidium bromide staining of 18S and 28S rRNA indicates the integrity of RNA. Northern blots were probed with TGF-β1, FGF-2, and cyclophilin cDNA probes; n = 3. (B) Densitometric analysis of northern blots. TGF-β1 and FGF-2 mRNA were normalised to cyclophilin mRNA.

receptor tyrosine kinases.[22] The transient localisation of FGF-2 six weeks after injury in vivo and the coincident expression of heparan sulfate suggest that changes in growth factors in an avascular tissue may be used to mediate the crucial regulation of proteoglycans.[3 9]

Previously, we showed that TGF-β modulates the interaction of stromal cells with their extracellular matrix by inducing the synthesis of specific proteoglycan core proteins and their respective glycosaminoglycans.[4] More recently, Song and colleagues[26] demonstrated that injury rapidly increases TGF-β1 mRNA expression along the wound margin. In our present study, we aimed to evaluate the relation between injury and growth factors in mediating the expression of TGF-β1 and FGF-2. The addition of exogenous TGF-β1 rapidly

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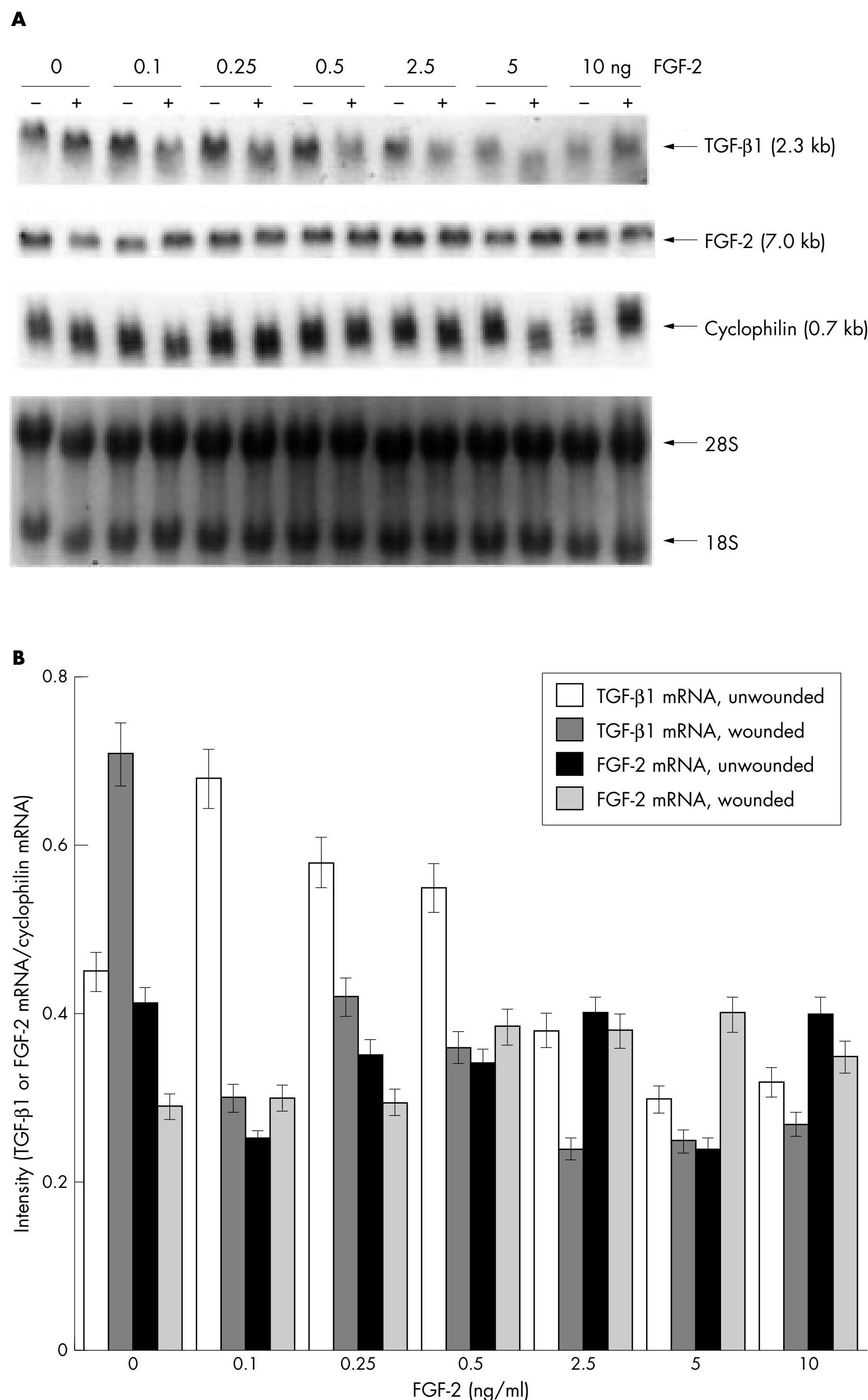

Figure 2 Fibroblast growth factor 2 (FGF-2) mediates the expression of transforming growth factor β1 (TGF-β1) mRNA in injured cultures. (A) TGF-β1 mRNA and FGF-2 mRNA expression in response to FGF-2 and injury. Multiple wounds were made throughout the culture (+) and cells were incubated in serum free DMEM containing a series of concentrations of FGF-2 for six hours. Parallel unwounded cultures (–) were also harvested. RNA extractions represent the entire culture and ethidium bromide staining of 18S and 28S rRNA indicates the integrity of RNA. Northern blots were probed with TGF-β1, FGF-2, and cyclophilin cDNA probes; n = 3. (B) Densitometric analysis of northern blots. TGF-β1 and FGF-2 mRNA were normalised to cyclophilin mRNA.

increased the level of its own mRNA. TGF-β1 also played a role in the increase in expression of FGF-2 following wound closure. In contrast, exogenous FGF-2 did not alter the expression of itself and inhibited that of TGF-β1. These results suggest a process whereby injury activates TGF-β and then primes cells to respond to other growth factors. These events may have implications in the regulation of wound repair.

MATERIALS AND METHODS

Cell culture

Rabbit eyes were obtained from Pel-Freeze (Rogers, Arkansas, USA), shipped on ice, and used within 24 hours of enucleation. Briefly, epithelium and endothelium were removed and stromas were cut into small pieces and digested in 2 mg/ml collagenase A (Sigma, St Louis, Missouri).[4] Cells were

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cultured in DMEM containing 4% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin, 1% non-essential amino acids, and antifungal agents (Life Technologies, Grand Island, New York, USA) and passed once. At confluency, cells were serum starved for 24 hours and linear wounds were made every 2 mm with a 25G7/8 needle, as described previously.[26 27] To evaluate the role of exogenous growth factors on the injury response, wounds were made, growth factors (TGF-β1 or FGF-2) were added (at concentrations of 0, 0.1, 0.25, 0.5, 2.5, 5.0, and 10 ng/ml) to serum free medium, and the response was assessed over a period of 48 hours. Morphological and biochemical assays were performed to evaluate the expression and localisation of mRNA.

Organ culture

Eyes were stabilised on customised paraffin wax posts in tissue culture wells. Full thickness wounds were made using a 25 gauge needle and injured whole eyes were cultured in DMEM containing TGF-β1 (5 ng/ml), 100 U/ml penicillin, 100 μg/ml streptomycin, 1% non-essential amino acids, and antifungal agents for 15 minutes, one, three, six, and 24 hours. During the incubation period, the wells were rotated on a nutator and viability was assessed at the end of each time period. Eyes were washed using phosphate buffered saline (PBS) containing 5.0 mM $MgCl_2$, pH 7.4, and fixed in 4% paraformaldehyde at 4°C overnight. Corneas were removed and maintained in PBS containing 0.1% azide until the completion of the experiment. They were then evaluated using immunohistochemical and/or fluorescent in situ hybridisation (FISH).

Antibodies

The following antibodies were used: polyclonal anti-TGF-β receptor I (human TGF-β RI rabbit IgG) from Research Diagnostics (Flanders, New Jersey, USA), anti-TGF-β1 receptor II (human TGF-β RII goat IgG), and anti-TGF-β receptor III (human TGF-β RIII goat IgG) from R & D Systems (Minneapolis, Minnesota, USA).

Probes

The plasmid pGEM3zf (−) human TGF-β1 (host strain, DH5α) was a gift from Dr R Derynck, NIH NCI, Bethesda, USA. TGF-β1 cDNA restriction fragments 322 bp (Sac I/BstE II) were generated for the non-isotopic in situ hybridisation experiments, whereas a 523 bp restriction fragment (SacI/BamHI) was used for northern blot analysis. A 1400 bp FGF-2 cDNA was cut by EcoR-I from the plasmid pGEM3zf (−) bovine FGF-2 (host strain, DH5α) for northern blot analysis. Reverse transcriptase polymerase chain reaction (RT-PCR) was used to amplify the 407 bp cDNA for in situ hybridisation experiments. The sequence of the upstream FGF-2 primer was 5′-CCG CCC TGC CGG AGG ATG GAG GCA-3′ and the downstream FGF-2 primer was 5′-GCC TTC TGC CCA GGT CCT GT-3′. The primers were complementary to the sequence of the rabbit FGF-2 cDNA (L12034).[28] The 216 bp cDNA of cyclophilin was also amplified by RT-PCR. The forward PCR primer for cyclophilin was 5′-CCA TCG TGT CAT CAA GGA CTT CAT-3′ and the reverse PCR primer was 5′-TTG CCA TCC AGC CAG GAG GTC T-3′ (Ambion, Austin, Texas, USA). Cyclophilin was used for normalisation in northern blot analysis.

RNA isolation and northern blot analysis

Total cellular RNA was isolated from stromal fibroblasts using TRIzol reagent (Gibco/BRL, Gaithersburg, Massachusetts, USA). A 15 μg aliquot of total RNA was denatured and separated by electrophoresis using a 1% agarose gel containing 1.9% formaldehyde. Equal gel loading and the integrity of the 18S and 28S RNAs were verified using ethidium bromide. RNA was transferred to a Duralose membrane (Stratagene, La Jolla, California, USA) and UV crosslinked. Blots were hybridised with TGF-β1, FGF-2, and cyclophilin cDNA probes that were labelled by random priming with [α-^{32}P] dCTP (New Life Science Products, Boston, Massachusetts, USA) using the random prime DNA labelling kit (Pharmacia, Piscataway, New Jersey, USA). Prehybridisation and hybridisation were performed in Rapid-Hybridisation buffer (Stratagene) at 68°C. Blots were washed twice at room temperature in 2× saline sodium citrate (SSC) buffer (1× 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 to 48 hours and mRNA was quantified using NIH Imaging Software (NIH).

Stability of TGF-β1 and FGF-2 mRNA

To investigate whether the increases in TGF-β1 and FGF-2 mRNA were related to a change in mRNA turnover, quiescent unwounded cultures were preincubated with TGF-β1 (5 ng/ml) for three hours. 5,6-Dichlorobenzmidazole riboside (DRB; Sigma) (30 μg/ml) was added to the cultures to inhibit new transcription. Parallel cultures were incubated in DRB alone. Cells were evaluated at 30, 60, 90, 120, and 180 minutes after the addition of DRB. Total RNA was isolated at each time point and northern blots were probed with TGF-β1 and FGF-2 cDNA probes.

Non-isotopic in situ hybridisation

Experiments were performed to evaluate the localisation of cells expressing TGF-β1 and FGF-2 mRNA in response to TGF-β1 or FGF-2 during the wound healing process. Non-isotopic in situ hybridisation for TGF-β1 and FGF-2 mRNA was performed.[26 29 30] Cells or organs were fixed in 4% paraformaldehyde containing 5.0 mM $MgCl_2$, pH 7.4, for 15 minutes, washed, and stored in PBS at 4°C overnight. Before hybridisation, cells were incubated in a Tris/HCl buffer, pH 7.4, containing 0.1 M glycine. The cDNA restriction fragments of TGF-β1 (322 bp SacI/BstEII) and FGF-2 (407 bp; see probes section) were prepared and labelled using the nick translational kit (Roche Molecular Biochemicals, Indianapolis, Indiana, USA) and digoxigenin-11-UTP. Southern blot analysis of the labelled probe was carried out each time to ensure that the probe was labelled specifically. The cDNA probe, melted in 100% formamide at 90°C, was combined with an equivalent volume of hybridisation buffer containing 20× SSC, 2% bovine serum albumin (BSA), 50% dextran sulfate, and vanadyl ribonucleoside complex (1/1/2/1), added to cells, and incubated in a humidified chamber at 37°C for 15 minutes. After hybridisation and extensive washing with 2× SSC, cells were incubated in antidigoxigenin–fluorescein isothiocyanate (FITC) for one hour at 37°C. Cells were washed and coverslipped, images were recorded using a Zeiss LSM 510 confocal laser scanning microscope, and image analysis was performed using Zeiss LSM 510 software 2.5. Control cells (those lacking cDNA probe) were analysed first and the gain and per cent laser transmission were set at a value where the fluorescence of the control was negligible. The background staining for the secondary antibody was set at zero mean pixel intensity for each unit area; this parameter was measured for each field. All experimental images were scanned using these parameters. Simultaneous transmitted light images were recorded in the second channel and the total number of cells in the field determined. The proportion of cells expressing mRNA was determined (cells expressing mRNA/total number of cells). To calculate the average fluorescence intensity of an image, the wound region was not included and only cells exceeding a minimum threshold pixel unit of 25 were counted. A minimum of 50 cells was counted for each parameter.

Localisation of TGF-β receptors

To localise TGF-β1 binding sites at various times after injury (zero, three, six, and 24 hours), wounded and unwounded cultures were incubated in binding buffer (serum free DMEM with

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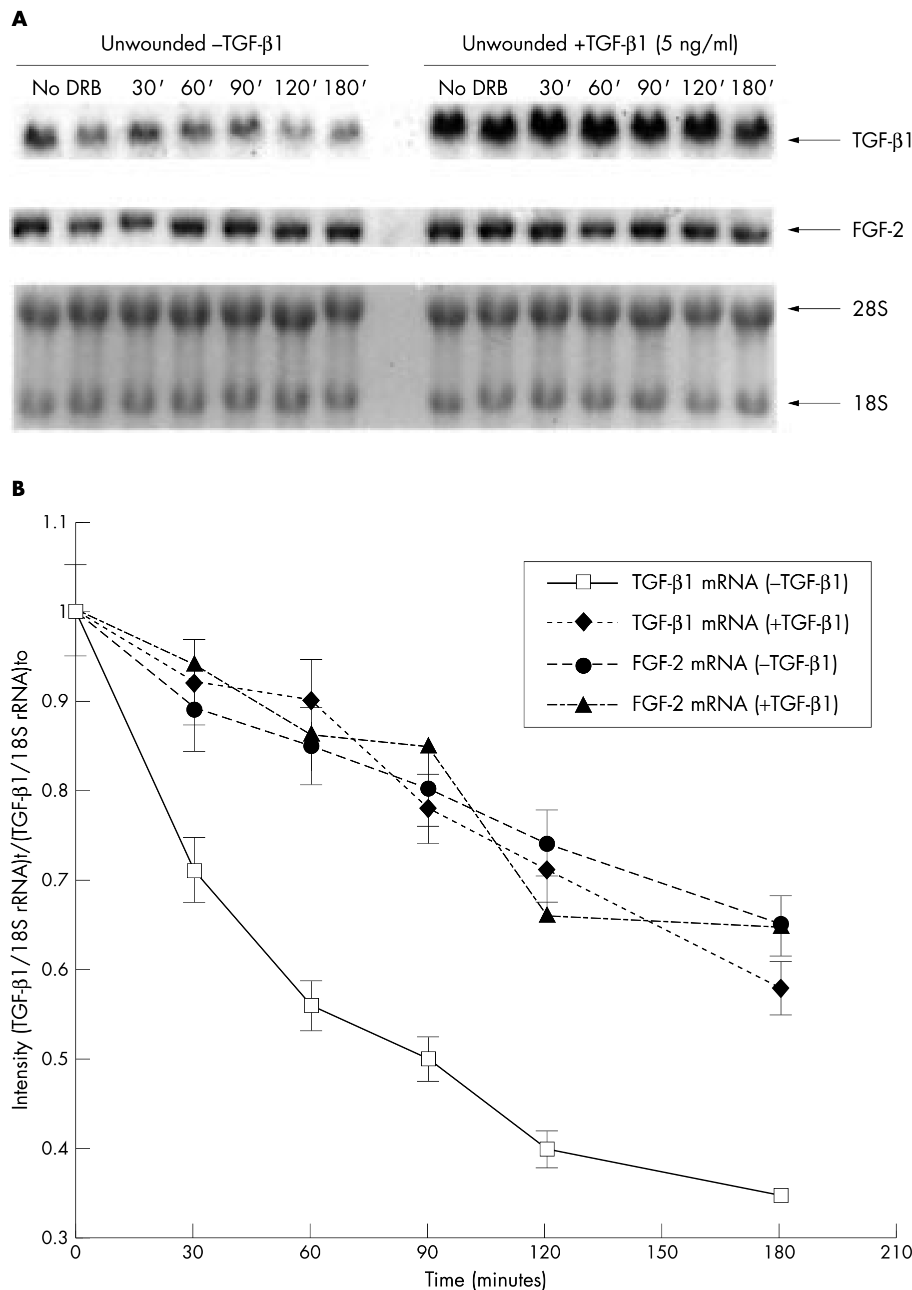

Figure 3 Exogenous transforming growth factor β1 (TGF-β1) enhances the stability of TGF-β1 mRNA. (A) Quiescent unwounded cultures were incubated in the presence or absence of TGF-β1 (5 ng/ml) for three hours, and 5,6-dichlorobenzimidazole riboside (DRB), an inhibitor of transcription, was added. Total RNA was extracted every 30 minutes for three hours. Ethidium bromide staining of 18 and 28S rRNA. (B) The half life of TGF-β1 treated cultures was 90 minutes compared with a shorter half life in untreated cultures. Fibroblast growth factor 2 (FGF-2) mRNA stability was not altered by TGF-β1. Data are representative of at least three experiments.

0.05% gelatin and 25 mM HEPES) containing TGF-β (1.5 nM) at 4°C for 2.5 hours. Cells were rinsed twice with binding buffer, once with PBS, and fixed for 10 minutes with formaldehyde (3.7% in PBS, pH 7.2). Cells were rinsed again in PBS and blocked with PBS/BSA (3%) for one hour at room temperature. Anti-TGF-β antibody (150 μg/ml) in PBS/BSA (2%) was hybridised for one hour at 37°C. After three washes with PBS/BSA (2%) and two washes with PBS, the secondary anti-IgG–FITC (1/100 dilution) in PBS/BSA (2%) was hybridised for one hour at 37°C. Cells were washed and antifade (Molecular Probes, Eugene, Oregon, USA) was added to the cover slip. To localise TGF-β receptors (I, II, and III) at various times in response to wounding and/or TGF-β1, wounded and unwounded cultures were incubated in DMEM and TGF-β1 (5 ng/ml) for 15 minutes, 30 minutes, one hour, and three hours. Cells were washed with PBS and fixed in 3.7% formaldehyde for 15 minutes at room temperature. Cells were washed with PBS and blocked in a solution containing 3% BSA. Cells were incubated with one of the three antibodies to receptors I, II, and III (150 μg/ml) in a solution containing 1% BSA on a rocker at 4°C. After extensive washes with PBS, cells were incubated in PBS/BSA (1%) containing the appropriate secondary antibody (anti-IgG–FITC; 1/100 dilution) for one hour at 37°C. Cells were washed and antifade was added to the coverslip.

Confocal microscopy

Cells were imaged using a Zeiss inverted LSM 510 confocal laser scanning microscope (CLSM) equipped with one argon

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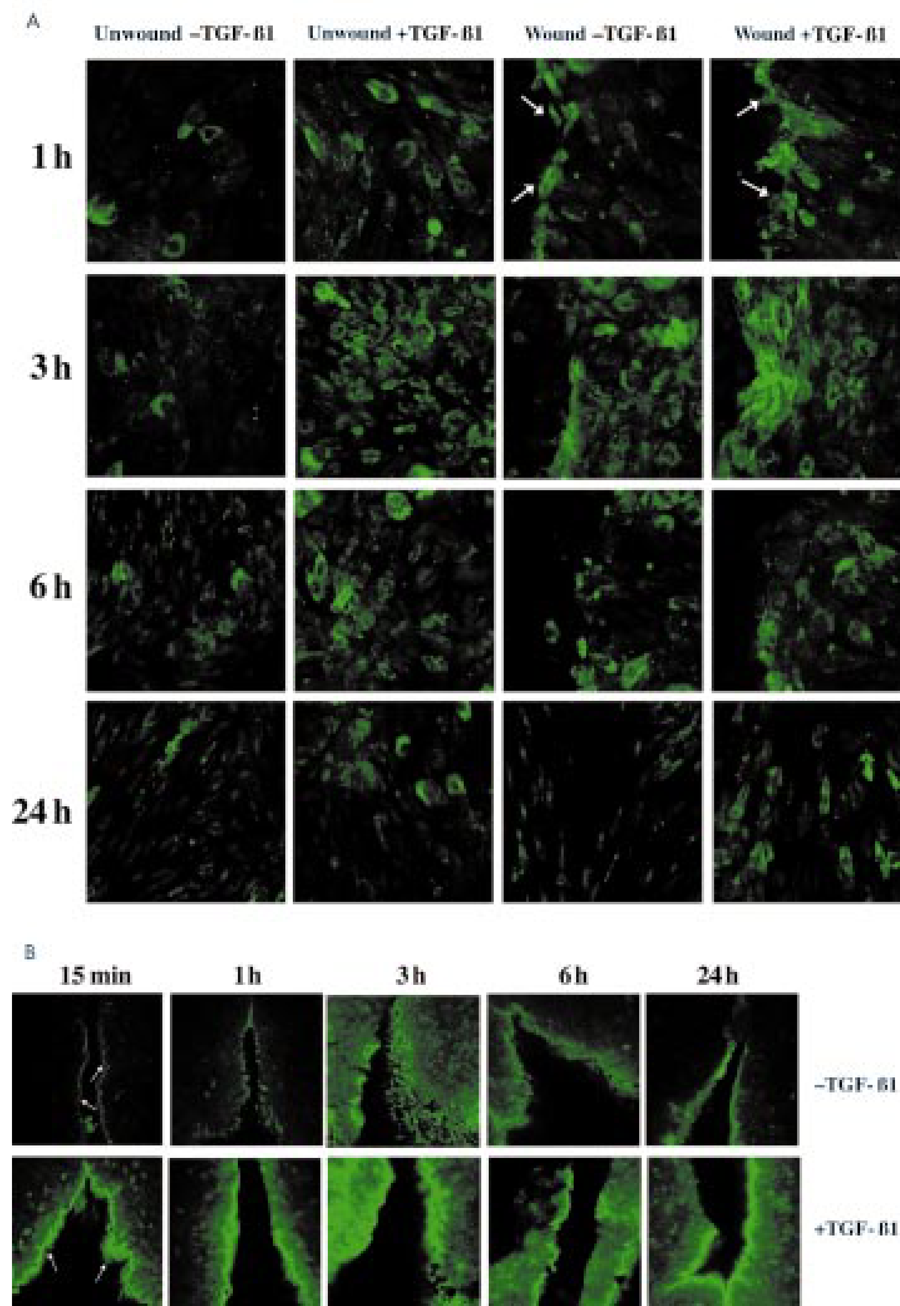

Figure 4 Localisation of transforming growth factor β1 (TGF-β1) mRNA in response to injury and exogenous growth factor. (A) Wounded and unwounded cultures were incubated in the presence or absence of TGF-β1 and localisation of TGF-β1 mRNA was determined using non-isotopic in situ hybridisation and imaged by confocal microscopy. Cultures were serum starved, injured, and incubated in the presence or absence of TGF-β1 (5 ng/ml) for one to 24 hours. TGF-β1 mRNA was negligible in unwounded cultures and in cells distal to the wound edge. TGF-β1 was expressed at the leading edge of the wound by one hour and intensity was maximal at three hours. (B) Parallel in situ hybridisation experiments were performed on whole eye organ cultures in the presence or absence of TGF-β1 (5 ng/ml). The response was evaluated at 15 minutes to 24 hours after injury. Expression was again localised along the leading edge of the wound (arrows). Images are representative of three experiments (dimensions of each frame: 125 × 125 μm).

and two helium lasers, as described previously.[26 29 31–33] Control cultures (cells lacking either primary antibody or cDNA probe) were imaged at settings where the fluorescence was negligible. All experimental images were then captured at these control settings. All images were acquired using an optical slice of 1.5 μm. Image analysis was performed using Zeiss LSM 510 software 2.5.

Migration assay

To evaluate the functional role of TGF-β, the migration of stromal cells was measured using Transwell migration chambers (24 mm diameter polycarbonate membrane; 5 μm pore size). Binding buffer (0.05% gelatin and 25 mM HEPES in DMEM) was used for both diluting growth factors and resuspending the cells. Stromal cells were isolated under serum free conditions and cultured in P-100s for 48 hours.[34] Cells were incubated in the presence or absence of 5 ng/ml TGF-β1 for 72 hours. After inducing quiescence by incubating the cells for 24 hours in serum and factor free medium, cultures were suspended with trypsin and trypsin was inhibited with an equal volume of soybean trypsin inhibitor (1 mg/ml in DMEM). The viability of the cells was monitored using dye

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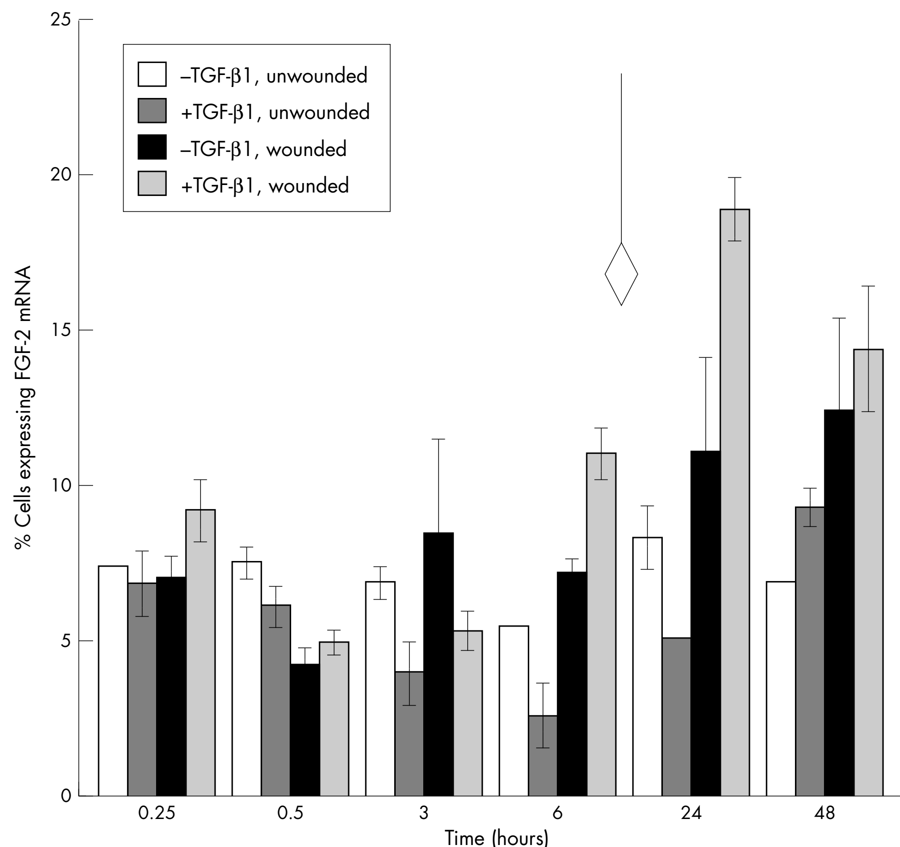

Figure 5 Percentage of cells expressing fibroblast growth factor 2 (FGF-2) mRNA. The response of wounded and control cultures in the presence or absence of growth factor was evaluated. Cultures were serum starved, injured, and incubated in the presence or absence of transforming growth factor β1 (TGF-β1; 5 ng/ml) for one to 24 hours. Confocal microscopy was used to quantitate the in situ hybridisation experiments by calculating the background pixel intensity for three images collected at each time point. The total number of cells in each field was counted, as was the number of cells with an intensity above background. The data are expressed as the percentage of cells expressing FGF-2 mRNA. Time of wound closure is noted (arrow). Injured cultures treated with exogenous TGF-β1 display an increase in the expression of FGF-2 coincident with wound closure. Data are representative of three experiments.

exclusion assays and only cultures with a minimum of 90% viability were used. Cells were centrifuged and resuspended in binding buffer to a final cell density of 30 000 cells/100 µl. A 100 µl sample of the cell suspension was added to the top of each Transwell chamber and binding buffer (0.600 µl) with varying concentrations of TGF-β1 (0.1, 1, 10, and 100 ng/ml) was added to the bottom chambers (binding buffer alone was used as a negative control for migration, whereas 10% FBS in binding buffer was used as a positive control). The migration assay was conducted at 37°C for 12 hours, after which time the cells were rinsed and fixed with methanol for 10 minutes at room temperature. The non-migrated cells were removed and the migrated cells were permeabilised with 0.1% Triton X-100 in PBS for one minute and stained with propidium iodide for 10 minutes at room temperature (1/200 dilution). This was done to assess migratory cells, and only those cells with detectable nuclei were counted as positive. The polycarbonate membranes were removed, mounted on to glass slides, antifade was added to each membrane, and coverslips were applied. For each membrane, the total number of cells was counted in each of six random fields (1.5 mm^2/field). The migration experiments were repeated four times and the average number of cells migrating in each field for each condition was calculated (± SE of the mean).

RESULTS

Previous work has shown that linear wounds made in primary cells cultured in serum free medium close by 24 hours.[27] We have also shown that injury elicits a highly defined response that includes a rapid upregulation in the expression of TGF-β1 mRNA at the leading edge of the wound, along with a minimal increase in signal a distance from the wound.[26] The aim of our present study was to determine the response of cells to exogenous TGF-β1 and/or FGF-2 and to evaluate the regulatory role of both growth factors upon injury. In all experiments, cells were maintained in serum free medium to evaluate the response to specific growth factors. Confirmatory experiments were performed using whole eye organ culture and expression was evaluated with FISH.

Expression of TGF-β1 and FGF-2 mRNA in response to injury and growth factors

To characterise the response of wounded cultures to growth factors, both dose dependent and time course experiments were performed. In the dose dependent experiments, cells were injured mechanically in serum free medium, incubated in several concentrations of either FGF-2 or TGF-β1 (0, 0.1, 0.25, 0.5, 2.5, 5.0, and 10 ng/ml), and evaluated at a single time point using northern blot analysis. Northern blots were subjected to densitometric analysis and the expression of growth factor was normalised to cyclophilin. The response represents an average of the entire culture, including wounded and unwounded cells.

The expression of TGF-β1 and FGF-2 mRNA was evaluated six hours after cells were injured and cultured in serum free medium containing several concentrations of TGF-β1. The integrity of the RNA was monitored using ethidium bromide staining of 18S and 28S RNA and expression was normalised to cyclophilin. There was an increase in the expression of TGF-β1 mRNA in response to injury and this was used to

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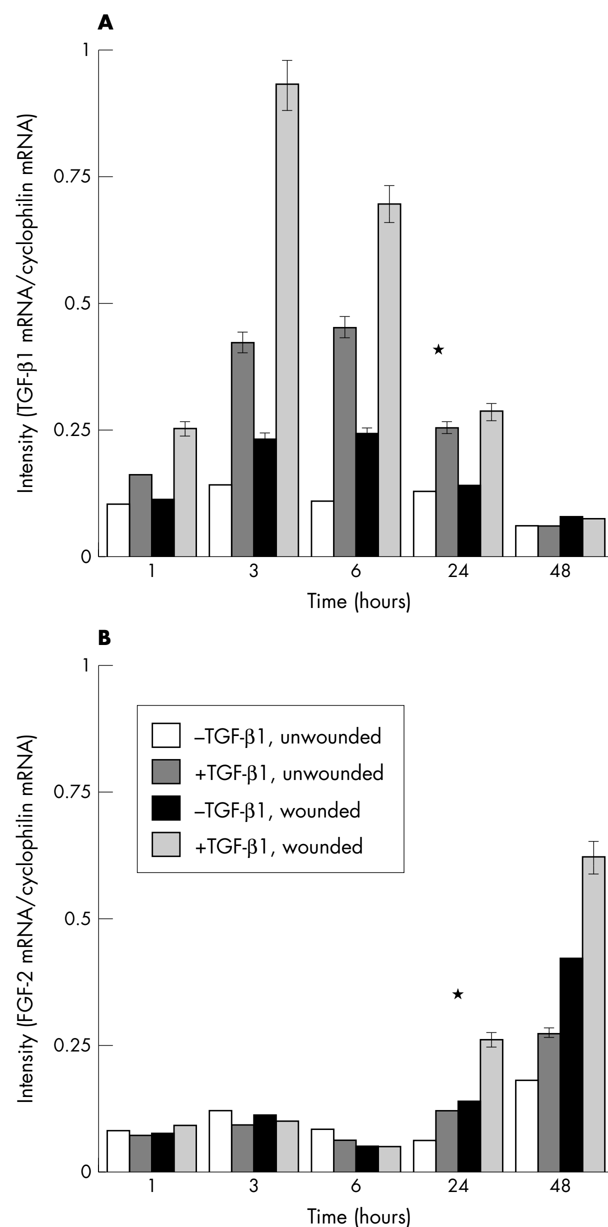

Figure 6 Expression of transforming growth factor β1 (TGF-β1) and fibroblast growth factor 2 (FGF-2) mRNA after injury. Cultures (+/− injury) were incubated in the presence or absence of TGF-β1. Extracts were collected and northern blots were probed with TGF-β1, FGF-2, and cyclophilin cDNA probes. Densitometric analysis was performed and data normalised to cyclophilin. Time of wound closure is noted with an asterisk. (A) TGF-β1 enhanced TGF-β1 mRNA expression in control and wounded cultures was maximal at three hours and remained raised for six hours after injury. (B) Injury and TGF-β1 enhanced FGF-2 mRNA expression after wound closure. Data are representative of three experiments.

establish a baseline to monitor changes in response to growth factors and injury. Exogenous TGF-β1 further increased TGF-β1 mRNA values in both wounded and unwounded cultures (on average fourfold increase over the wounded control (no addition of growth factor)) (fig 1A,B). The increase was detected at concentrations as low as 0.1 ng/ml, and the response was maximal between 0.5 and 5.0 ng/ml (fig 1A,B). In contrast, FGF-2 was expressed constitutively and neither injury nor TGF-β1 significantly altered its expression at six hours (fig 1A,B). These data indicate that TGF-β1 mediates the expression of its own mRNA, but does not alter that of FGF-2 mRNA when cells are actively migrating.

Reciprocal experiments were performed and the expression of TGF-β1 and FGF-2 was evaluated in response to exogenous FGF-2 after six hours of incubation. In the control lacking growth factor, there was a similar increase in the TGF-β1 mRNA ratio of wounded to unwounded cultures when expression was normalised to cyclophilin (figs 1B, 2A,B). The addition of FGF-2 to the wounded cultures inhibited the expression of TGF-β1 mRNA in a dose dependent manner (fig 2A,B). Interestingly, low concentrations of exogenous FGF-2 (0–0.5 ng/ml) enhanced the expression of TGF-β1 over control in unwounded cultures. In contrast, exogenous FGF-2 did not significantly alter its own regulation of FGF-2 mRNA.

The increase in expression of TGF-β1 may reflect an increase in transcriptional activity, an increase in message stability, or potentially a combination of both. Previously, we demonstrated that injury alone induced an increase in stability.[26] Here, we evaluated whether the addition of exogenous TGF-β1 altered the stability of TGF-β1 and/or FGF-2 mRNA. Cells were incubated in the presence or absence of DRB (an inhibitor of new gene transcription) and TGF-β1 (5 ng/ml). RNA was extracted every 30 minutes for three hours. Replicate control experiments were conducted in the absence of exogenous TGF-β1. The half life of TGF-β1 mRNA in TGF-β1 treated cultures increased from 45 to 90 minutes compared with untreated cultures (fig 3A,B). The addition of TGF-β1 did not alter the stability of FGF-2 mRNA.

Expression and localisation of TGF-β1 and FGF-2 mRNA in response to TGF-β1 and injury

To assess changes in localisation of FGF-2 and TGF-β1 mRNA over time, cultures were incubated as described and evaluated using FISH. Figure 4A shows representative confocal images of unwounded and wounded cells (arrows indicate wound) cultured in the presence or absence of TGF-β1 (5 ng/ml) at one, three, six, and 24 hours. As described previously, wound closure occurs by 24 hours.[27] In the control unwounded cultures, there was minimal expression of TGF-β1 mRNA. Between 3% and 5% of the cells in each field exceeded the threshold pixel intensity unit of 25. When the unwounded cultures were incubated in the presence of TGF-β1, the proportion of cells expressing over the threshold increased to 7% (fig 4A).

In the wounded cultures TGF-β1 was localised along the wound margins at one hour and expression was negligible distal to the wound edge. By three hours there was an increase in cells that expressed TGF-β1 mRNA at a distance from the wound (8–9%) (fig 4A). When exogenous TGF-β was present in the medium of injured cultures, maximal expression was detected at three hours, with 10–12% of cells a distance from the wound expressing TGF-β1 mRNA.

Parallel experiments were performed on whole eye organ cultures in serum free medium in the presence or absence of growth factor and the intact cornea was evaluated using confocal microscopy. A response was detected and localisation was similar to that detected in the in vitro experiments—wounded corneas showed an increase in the number of cells expressing TGF-β1 mRNA along the wound edge. Expression was enhanced further when the corneas were wounded and incubated in serum free medium containing 5 ng/ml TGF-β1 at 15 minutes and one hour (fig 4B; arrows). By three hours, there was a significant increase in the number of cells expressing TGF-β1. At each time point, phase images were taken simultaneously to evaluate the integrity of the tissue (data not shown). In the organ culture experiments, the wound was not sutured and because each side of the wound is at a distance from the other, we predict that the prolonged increased response correlated with the open wound.

Parallel experiments were conducted to evaluate whether injury and TGF-β1 affected FGF-2 mRNA values in our cell culture system. The cells were found to express FGF-2 mRNA constitutively, with minimal change in expression of FGF-2

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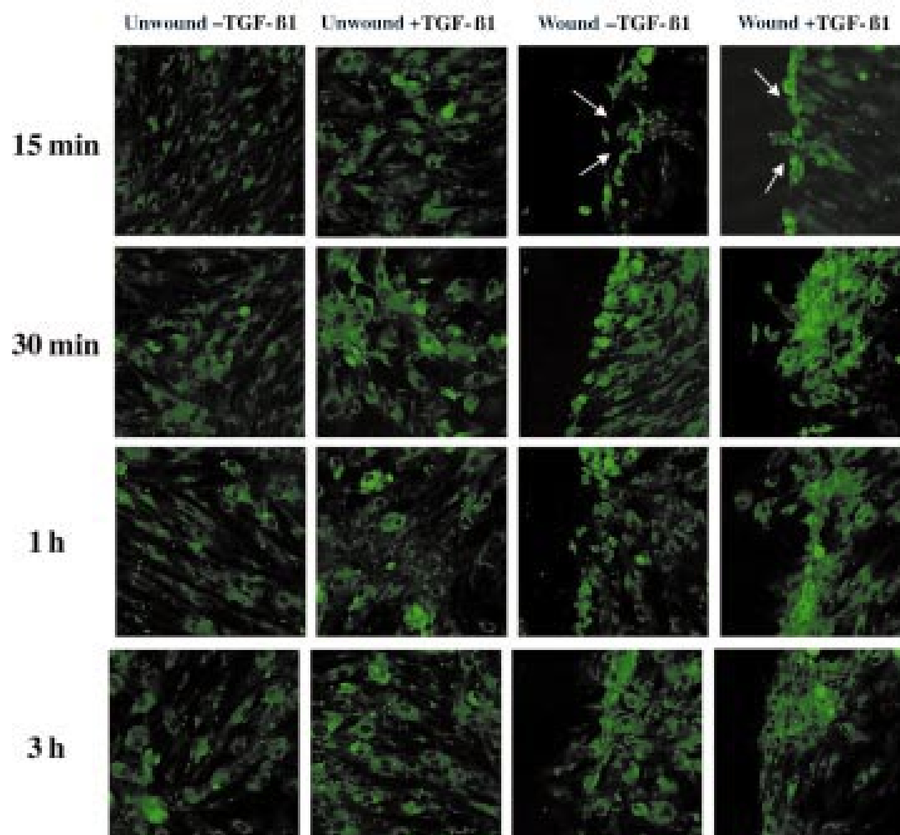

Figure 7 Expression of transforming growth factor β1 (TGF-β1) receptors in response to injury. Cultures were wounded and incubated in the presence or absence of TGF-β1 (5 ng/ml). Wounded cultures were compared with control cultures. Arrows indicate the leading edge of the wound and the orientation is kept the same throughout the time course. TGF-β receptor I is detected in control cultures and was found predominantly along the margins of the wound. By three hours, the localisation of receptors was diffuse and resembled unwounded controls. Images are representative of three experiments (dimensions of each frame: 125 × 125 μm).

mRNA for the first six hours after injury (fig 5). To evaluate whether there was a delayed response, cultures were incubated over a period of 48 hours. In the presence of TGF-β, there was a slight increase in the proportion of cells expressing FGF-2 mRNA (fig 5). In the wounded cultures, there was no significant increase in expression until 24 hours (wound closure), and this was only detected in the cultures containing exogenous TGF-β1. By 48 hours, there was a similar increase in both wounded cultures (fig 5). In addition, experiments were conducted to determine whether TGF-β1 altered the rate of wound closure, and micrographs were taken at specific times after injury and morphometry was carried out.[18] We found that the rate of repair was accelerated at the six hour time point in cultures containing growth factor, but at later time points there was no detectable difference, and in both wounds closure occurred by 24 hours.

Northern blot analysis was carried out on the entire cell population to evaluate the effects of injury and growth factors on the overall degree of expression over a 48 hour period. Densitometric analysis was performed at each time point and changes in either TGF-β1 or FGF-2 were normalised to cyclophilin. When cells were incubated under control conditions there was negligible change over time. When TGF-β1 was added there was an increase in TGF-β1 mRNA that was detected at one hour, became maximal at three hours (threefold increase in the unwounded and a 3.9-fold increase in wounded cultures), and remained raised for six hours after injury (fig 6A). There was no detectable change in the expression of FGF-2 mRNA for the first 24 hours in the control wounded cultures. In cultures containing TGF-β1 there was an increase in FGF-2 mRNA at 24 hours. At 48 hours, there was a 1.4-fold increase in wounded controls and a sixfold increase in the wounded cultures containing TGF-β1. The increase occurred at a time when the expression of TGF-β1 was negligible (fig 6A,B). These results indicate that a crucial balance in growth factor regulation is required, and that TGF-β1 may play a role in mediating cellular events that elicit an increase in FGF-2 mRNA. To maintain the balance, an increase in FGF-2 (at a concentration greater than 0.5 ng/ml) decreases the expression of TGF-β1, suggesting a regulatory feedback mechanism.

Changes in the localisation of TGF-β receptors after injury

In immunocytochemical studies, we found that both injury and TGF-β1 alter the localisation of TGF-β receptors I and III. In the absence of exogenous growth factors, TGF-β receptors I and III were detected along the margins of the wound (figs 7,8; arrows), and by 30 minutes there was an increase in the number of cells that expressed TGF-β receptors I and III at a distance from the wound (figs 7,8). The change in localisation of receptors along the wound edge (in the absence of TGF-β1) agreed with the increase in binding localised to the wound edge and detected within minutes after injury.[26] The addition of exogenous TGF-β1 caused an enhanced response in TGF-β receptors I and III between 30 minutes and one hour (figs 7,8). After three hours, the enhanced expression returned to unwounded control values. Interestingly, the addition of TGF-β1 to the unwounded cultures did not result in the enhancement that was detected with injury. Under these conditions, binding was detected rapidly after injury, was present for three hours, and then decreased to background values (data not shown). In contrast, the only noticeable change in TGF-β receptor II was present at the earliest timepoint with

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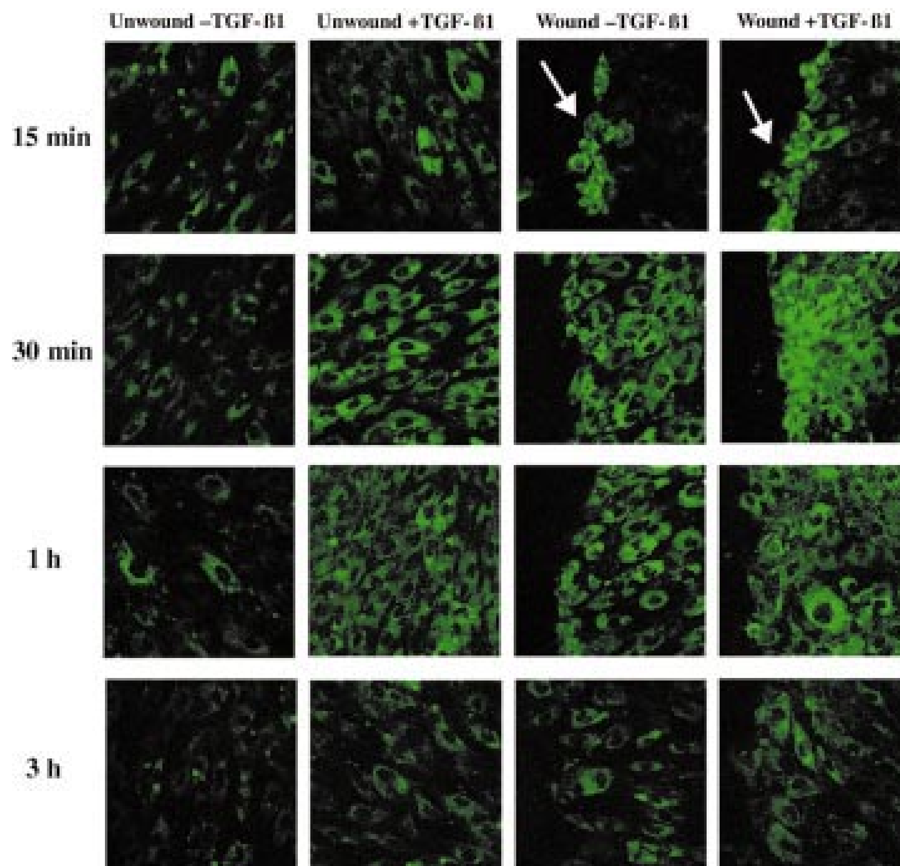

Figure 8 Expression of transforming growth factor β1 (TGF-β1) receptors in response to injury. Cultures were wounded and incubated in the presence or absence of TGF-β1 (5 ng/ml). Wounded cultures were compared with control cultures. Arrows indicate the leading edge of the wound and the orientation is kept the same throughout the time course. Low amounts of TGF-β receptor III (betaglycan) are present in control cultures and are increased in the presence of growth factor. There is a change in localisation in response to injury and/or exogenous TGF-β1 that is detected between 30 minutes and one hour. TGF-β receptor III returned to background values by three hours. Images are representative of three experiments (dimensions of each frame: 125 × 125 μm).

both injury and exogenous growth factor (fig 9). Therefore, the combination of exogenous TGF-β1 and injury elicit a maximal response, indicating that these factors play a role in mediating the availability of receptors.

TGF-β induces a migratory cellular phenotype

To isolate a specific biological process of wound repair, namely motility, a migration assay was used. Because TGF-β1 was detected after injury, and injury increased the stability of TGF-β1 mRNA, unstimulated stromal cells were primed with this growth factor to migrate in response to TGF-β1 or FGF-2. These cells were isolated and cultured under serum free conditions and possessed a dendritic-like phenotype (modification of Beales *et al*).[34] Their motility was compared with that of unstimulated cells that were cultured in serum free medium containing TGF-β1 (5 ng/ml) for three days. These cells underwent a transformation from the small dendritic-like cells to large myofibroblast-like cells ("TGF-β cells"). Both phenotypes were cultured in serum free medium for 24 hours before the migration assay and allowed to migrate for 12 hours in the presence of TGF-β1, FGF-2, binding buffer (negative control), or binding buffer containing 10% FBS (positive control).

Both cell phenotypes migrated in the presence of 10% FBS, indicating that the cells could be stimulated with a rich composite of factors (fig 10). However, the migrational ability was altered when cells were exposed to a single growth factor. When the unstimulated cells were migrated for 12 hours in the presence of TGF-β1 there was a twofold increase in the number of cells migrating when compared with the negative control. These cells also showed similar migration in the presence of FGF-2, indicating that over time quiescent cells can be stimulated by exposure to specific growth factors (fig 10). The migration of TGF-β cells in the presence of TGF-β1 or FGF-2 was enhanced in comparison with the unstimulated cells. However, it should be noted that the TGF-β cells achieved the same high degree of motility even when migrated in the absence of any growth factor. These results indicate that the initial stimulation required to induce the myofibroblast-like phenotype caused a highly migratory state that was not enhanced by additional exposure to either growth factor alone. Cells were also evaluated for proliferative ability in response to either growth factor alone and none was detected (data not shown).

DISCUSSION

Many investigators have studied growth factors and their receptors to evaluate the mechanisms of scarring. Injury induces the recruitment of cells to the wound site, including activated macrophages, neutrophils, and platelets, which play a role in secreting such factors (see Clark Richard for a review).[35] In the cornea, an additional source of mediators is the lacrimal gland.[36–38] Our study evaluated the expression of TGF-β1 and FGF-2 in response to injury and exogenous growth factors using both a primary cell line derived from stromal cells and an organ culture system. We found that TGF-β1 mediates the early phases of wound repair, whereas FGF-2 plays a role in modulating later responses. In addition, the combination of injury and exogenous TGF-β1 induces a synergistic response with respect to TGF-β mRNA expression

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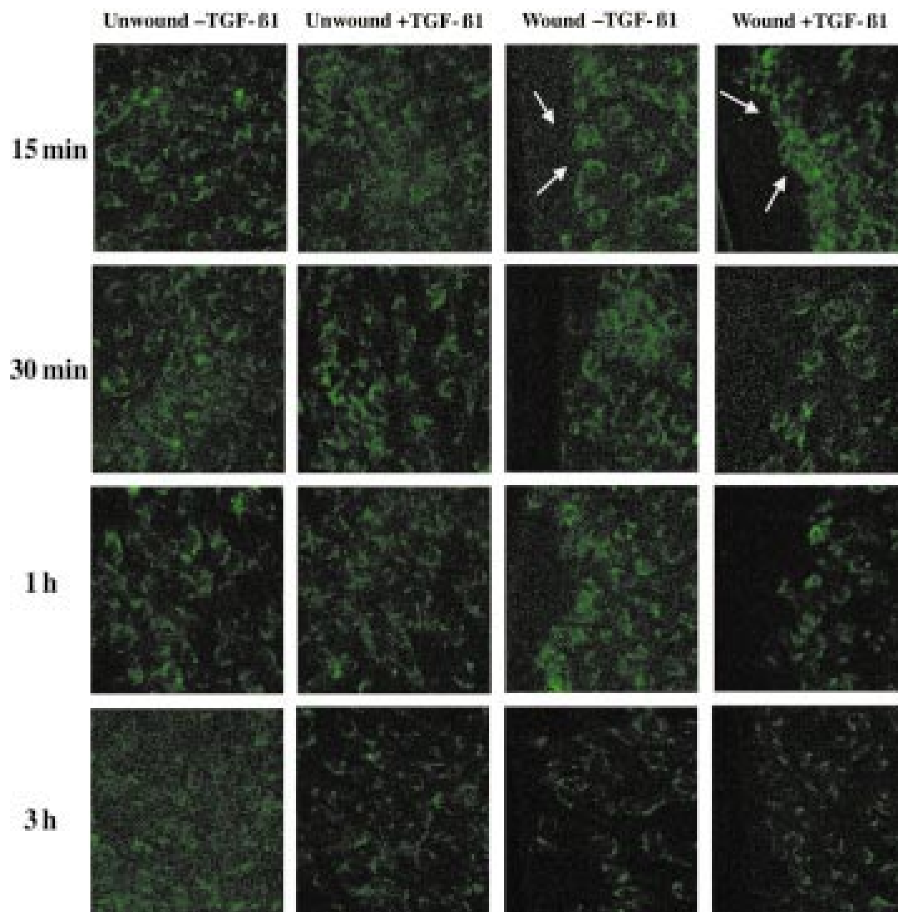

Figure 9 Expression of transforming growth factor β1 (TGF-β1) receptors in response to injury. Cultures were wounded and incubated in the presence or absence of TGF-β1 (5 ng/ml). Wounded cultures were compared with control cultures. Arrows indicate the leading edge of the wound and the orientation is kept the same throughout the time course.(C) The localisation and expression of TGF-β receptor II did not change in the presence of exogenous growth factor or injury over three hours. Images are representative of three experiments (dimensions of each frame: 125 × 125 μm).

during the phase of wound repair associated with cell migration. These data support the observation of others that growth factors act in a coordinated manner.[39 40]

Our current in vitro studies were developed from a large body of work where polymers or synthetic devices were placed into corneas and wound repair was monitored over a period of months and years (reviews by Trinkaus-Randall).[1 2] In those studies the synthesis, deposition, and localisation of collagen(s), glycosaminoglycans, and growth factors (TGF-β1 and FGF-2) were monitored and the localisation of the growth factors was found to correlate with the appearance of specific glycosaminoglycans. These results suggested that there was an interaction between changes that occur in injury and the bioavailability of growth factors. Initial studies performed in vitro showed that cells respond to injury in serum free medium with an increase in tyrosine phosphorylation of FAK and paxillin,[27] and an increase in TGF-β1 mRNA that is localised initially along the wound edge.[26]

Our results indicate that injury itself may regulate the availability and expression of receptors and that this response is enhanced by TGF-β1. Furthermore, preliminary results have shown that connective tissue growth factor is also affected by injury in our wound model, and mediates the expression of TGF-β1 and is stimulated by TGF-β1 in a dose dependent manner, indicating the presence of a potential feedback loop.[41] We also found that if exogenous FGF-2 is added during wound repair, there is an inhibition in the expression of TGF-β1 during the active phase of repair. This suggests that TGF-β1 expression is regulated by the status of the wound repair. The increase in the expression of FGF-2 mRNA at a later time in culture correlates well with the delayed and transient appearance of FGF-2 seen in the tissue in vivo.[9] The multiplicity of roles that FGF-2 plays has been shown by other investigators who have demonstrated that FGF-2 plays a crucial role in mediating cell proliferation, differentiation, migration, angiogenesis, and wound healing.[22]

"Our results indicate that injury itself may regulate the availability and expression of receptors and that this response is enhanced by transforming growth factor β1"

The maximal response in FGF-2 mRNA occurred when the system was altered by both injury and exogenous TGF-β1, suggesting that injury activates TGF-β, which primes cells to respond to other growth factors. Previously, Nugent and Edelman[42] showed that TGF-β1 synergistically regulated FGF-2 activity by altering FGF-2–proteoglycan interactions. In those experiments, TGF-β1 increased [^{125}I] FGF-2 binding to the extracellular matrix of BALB/c3T3 cells by increasing the number of FGF-2 binding sites. However, it is likely that there are several potential pathways, because the addition of TGF-β1 to primary fibroblasts did not result in increased FGF-2 binding.[5]

In other similar cell culture systems it has been shown that TGF-β1 can enhance the expression of perlecan and other heparan sulfate proteoglycans in a dose dependent manner.[4] Perlecan has been shown to be present at wound margins in vivo.[43] Because TGF-β interacts with extracellular matrix molecules, it is also probable that changes in the sulfated

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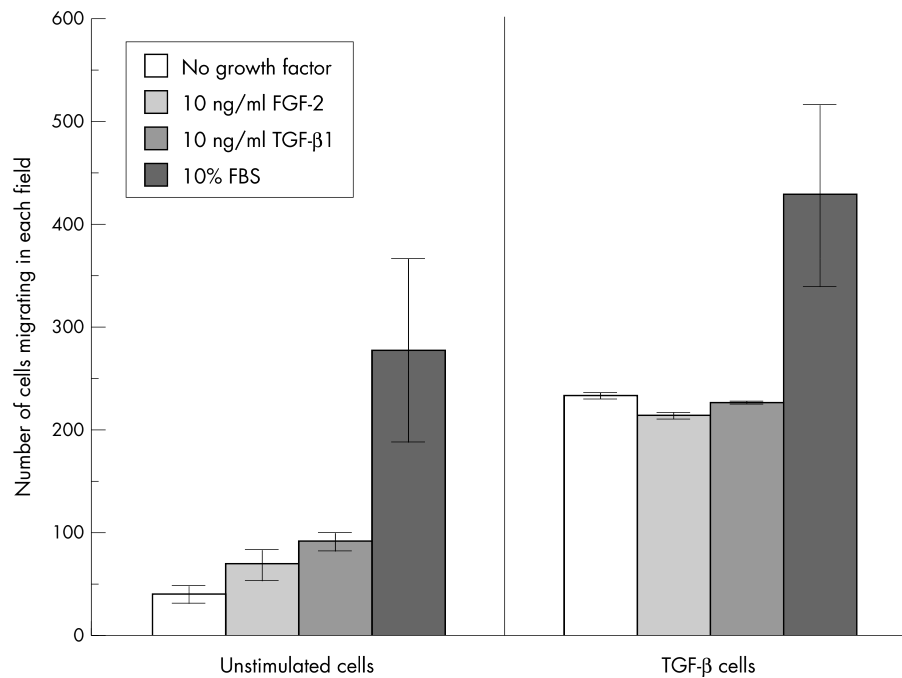

Figure 10 Comparison of migratory ability of stimulated and unstimulated cells. Cells were isolated under serum free conditions and incubated for three days in either serum free medium (unstimulated cells) or 5 ng/ml transforming growth factor β1 (TGF-β1) (myofibroblast-like cells). Before the migration assay, cells were quiescent for 24 hours and then allowed to migrate for 12 hours in the presence of 10 ng/ml TGF-β1, 10 ng/ml fibroblast growth factor 2 (FGF-2), binding buffer (negative control), or 10% fetal bovine serum (FBS; positive control) using the Costar Transwell system. The average number of cells migrating in a 1.5 mm^2 area was calculated for each condition (average of six areas). Both cell phenotypes migrated in the presence of 10% FBS. Unstimulated cells showed a twofold increase in migration in the presence of TGF-β1 over control. Myofibroblast-like cells were highly migratory in the presence or absence of either growth factor. The experiment was repeated three times and the mean and SEM are shown.

proteoglycans that occur with injury may modulate TGF-β receptor activation and binding.[3 4] Thus, alterations in the environment surrounding the injury, such as cell–cell and cell–substrate disruption, can trigger a cellular response that is further mediated by changes in the bioavailability of growth factors. In our system, there was an increase in the localisation of TGF-β receptors I and III detected at the wound margin, which decreased with time and correlated with a transient increase in growth factor binding.[26] The specific increase in the type I receptor could suggest a role in regulating or limiting activity by mediating a single receptor in the entire complex. These variables ultimately add a level of complexity to the wound repair cascade.

TGF-β molecules are known to have different functions in specific cell types. Although TGF-β1 is mitogenic for fibroblasts, it inhibits the proliferation of epithelial and endothelial cells. TGF-β has been shown to cause varied responses in migration and these again correlate with cell type. Andresen and colleagues[44] showed that concentrations between 0.1 and 1 ng/ml were necessary to induce cellular migration of fibroblasts. However, concentrations greater than 1 ng/ml were inhibitory. Our migration studies using a Costar Transwell system demonstrated that quiescent cells that have never been exposed to serum or growth factors can be stimulated to acquire a migratory phenotype when treated with higher concentrations of TGF-β1. In our assay, quiescent cells were activated after 12 hours of exposure to either FGF-2 or TGF-β1. In addition, when the quiescent cells were cultured in the presence of TGF-β1 for three days, they acquired a myofibroblast phenotype (TGF-β cells), which was conserved even after removing the growth factor for 24 hours before migration. Other laboratories have also shown that TGF-β or cell density can alter cell phenotype and expression of stromal cells.[45 46] These TGF-β cells were highly motile and did not lose the ability to migrate when growth factor was removed. We predict that migration was not enhanced further with either growth factor because the receptors were saturated. When similar migration experiments were performed using cells cultured with serum rather than TGF-β1, the cells failed to migrate in the presence of TGF-β1 (data not shown). These results indicate that a factor(s) present in the serum inhibits the stimulatory action of TGF-β and may potentially function to mediate expression of cells after wound closure when migration is no longer necessary.

Several potential therapeutic roles for TGF-β1 have been explored. The administration of TGF-β1 enhances the repair of injured tissue in several models. When TGF-β1 is applied topically, healing is improved in several wound models, including incisional and excisional wounds, punch wounds, and ulcers.[44] Others have hypothesised that TGF-β increases the rate of healing and the breaking strength of repairing tissue by stimulating the local secretion of other growth factors.[47 48] These findings may underlie why the stability of TGF-β1 mRNA is naturally enhanced with injury or exogenous TGF-β1—because it promotes healing. In addition, investigators have shown that in mice overexpressing TGF-β there was no scarring in linear wounds, but there was a compensatory change in the regulation of other isoforms of TGF-β.[49]

Our data suggest that there is an integral relation during wound repair between TGF-β1 and FGF-2. TGF-β1 primes cells and may cause a delayed increase in FGF-2 by first altering the synthesis and degradation of specific proteins. In addition, FGF-2 inhibits the expression of TGF-β1. Thus, the increase in FGF-2 values may prevent the continuous upregulation of TGF-β1 during the later phases of wound repair.

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Take home messages

- Transforming growth factor β1 (TGF-β1) appears to mediate the early phases of wound repair, whereas fibroblast growth factor 2 (FGF-2) seems to play a role in modulating later responses
- TGF-β1 and FGF-2 appear to act together during wound repair: TGF-β1 primes cells and may cause a delayed increase in FGF-2 and FGF-2 inhibits the expression of TGF-β1
- Thus, the increase in FGF-2 values may prevent the continuous upregulation of TGF-β1 during the later phases of wound repair
- The combination of injury and exogenous TGF-β1 induces a synergistic response with respect to TGF-β mRNA expression during the phase of wound repair associated with cell migration
- Injury and exogenous increased TGF-β1 and fibroblast growth factor 2 (FGF-2) mRNA values, although the increase in FGF-2 was not seen until wound closure
- FGF-2 inhibited the expression of TGF-β1
- TGF-β1 increased TGF-β1 mRNA stability but had no effect upon FGF-2
- Migration assays indicated that unstimulated stromal cells could be activated to migrate to specific growth factors. TGF-β1 specifically enhances cellular responsiveness, as shown by increased stability after injury and the acquisition of a migratory phenotype. These data suggest that there is an integral relation during wound repair between TGF-β1 and FGF-2.

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