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EFFECT OF TGF-BETA1 ON PDGF RECEPTORS EXPRESSION IN HUMAN SCAR FIBROBLASTS

Messadi DV[1,2] , Le A[1], Berg S[1], Huang G[1], Zhuang W[1] and Bertolami CN[3]

[1] School of Dentistry, [2] Dental Research Institute, University of California, Los Angeles, California, [3] School of Dentistry,University of California, San Francisco, California, USA,

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1. ABSTRACT

This study examined the effect of exogenous TGF –beta1 on platelet derived growth factor alpha and beta (PDGF-alpha, beta) receptor expression in human dermal fibroblasts derived from both normal cutaneous tissues (normal skin [NSk]) and (normal scar [NSc]) and abnormal scar (keloid). TGF-beta and PDGF are present in the early phases of wound healing and are implicated in tissue fibrosis. In this study, replicate samples of NSk, NSc and keloid fibroblasts were grown to subconfluency in DMEM/10% FBS followed by replacement of media with DMEM/0.1%FBS for 24 hrs. One group of cells (NSk, NSc and keloid) were exposed to 10 ng/mL of exogenous TGF-beta1 for 24 hours, while the other group was used as control with no exposure to exogenous TGF-beta1. RadioImmunoBinding assays, Western and Northern blot analysis were performed to examine both PDGF-alpha and PDGF-beta receptor expression at the transcriptional and post-trancriptional levels. cDNA receptor probes were synthesized using polymerase chain reaction (PCR) with selected primer sets derived from published sequences. Beta–actin probe was used as a control to confirm that the same quantity of RNA was used for each experimental condition. TGF-beta1 was found to upregulate the expression of PDGF-alpha receptor for keloid fibroblasts but not for NSk or NSc fibroblasts. No effect was observed for TGF-beta1 on PDGF-beta receptor expression for any of the cell lines examined.

2. INTRODUCTION

Abnormal healing after traumatic injury is often manifested as excessive fibrosis and is recognized clinically as hypertrophic scars or keloids. These conditions represent debilitating derangement in remodeling and are associated with impaired function and significant dysmorphea. They are defined largely by an abnormal accumulation of extracellular matrix (1). Previous studies have independently suggested a role for transforming growth factor beta1 (TGF-beta1) (2), and platelet derived growth factors (PDGF) in tissue repair (3); however their involvement in abnormal healing has not been adequately explored nor has the specific relationship between TGF-beta1and PDGF receptors, in orofacial tissue repair been studied.

TGF-beta1 is a well known and potent biological response modifier implicated in a wide array of biologic processes, including tissue repair and modulation of extracellular matrix accumulation (4).

PDGF isoforms are potent mitogens and chemoattractants for fibroblasts. They have been suggested to play an important role in wound healing and have been implicated in several fibroproliferative disorders such as systemic sclerosis, pulmonary fibrosis and atherosclerosis (5). Three different isoforms of PDGF have been identified (AA, AB, BB). These isoforms bind and dimerize two PDGF receptor subtypes (alpha and beta)

(6). PDGF-AA binds only PDGF alpha–receptor subtype (PDGF-alpha R), whereas PDGF-AB and PDGF-BB bind both PDGF-receptors subtypes (alpha and beta) Matsui and coworkers (7), also Seppa and coworkers (8) have reported that both PDGF receptors -(alpha and beta) transduce the signals for both mitogenesis and chemotaxis. It has been previously demonstrated that TGF-beta1 can upregulate or downregulate the PDGF-alpha R fibroblasts depending on the cell type (9). TGF-beta1 has been reported to downregulate the PDGF- alpha R on human smooth muscle cells and human foreskin fibroblasts (10) and also on human periodontal ligament fibroblasts (11). It upregulates PDGF- alpha R on fibroblasts derived from scleroderma patients with no effect on PDGF-beta receptor (12) . The reason for these contradictory results is not clear, but may be due to TGF-beta modulating PDGF receptors in both cell and species specific manner.

We have previously shown (13) that fibroblasts derived from scar tissues (normal scar and keloid) secrete TGF-beta1 in active form, while normal skin fibroblasts (NSk) secrete fibroblasts in latent form. Moreover, we have shown that scar fibroblasts exhibit greater autocrine responsiveness to TGF-beta1 than do skin fibroblasts. Such findings suggest that TGF-beta1 functions differently in normal skin than it does in scar tissues. The aim of this study is to clarify the roles of TGF-beta1 and PDGF in abnormal scar formation after traumatic injury. To do so, we examined the effect of TGF-beta1 on fibroblasts derived from normal skin (NSk) and scar tissues (both normal scar [NSc] and abnormal scar [keloid]), with regard to PDGF alpha and beta receptor cell surface production and gene expression.

3. MATERIALS AND METHODS

The effect of TGF- beta 1 treatment on PDGF alpha and beta receptor cell surface expression for NSk, NSc and keloid fibroblasts was analyzed using RadioImmunoBinding assay. All cells were serum starved for 24 hours; one half of the cultures were then preincubated for 24 hours with 10 ng/mL of TGF- beta 1. Using monoclonal antibodies specific for PDGF alpha and beta receptors, the TGF- beta 1 stimulatory effect on the different cell lines was examined.

3.1 Cell cultures

Three replicate cultures each of human NSk, NSc and keloid fibroblasts were cultured as previously described (14). Patients ages ranged from 18 to 50 years. Normal skin tissue samples were matched for age, race and sex. In brief, freshly biopsied tissues, were obtained from the operating room of the University of California Medical Center, Los Angeles (UCLA) and Martin Luther King Hospital, Los Angeles as part of therapeutic procedures. Tissues were enzyme digested, the cells being propagated and maintained in Dulbecco's Modified Eagle's Medium (D-MEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin streptomycin. The cells were studied at passages 4-8.

3.2 Addition of exogenous TGF- beta 1

Twenty-four hours before treating cultures with TGF-beta1, regular medium was replaced with serum-free medium. Half of the preconfluent cultures received TGF-beta1 (Sigma Company, St. Louis, MO) at a concentration of 10 ng/mL in serum-free medium for 24 hours. The TGF-beta1-containing medium was replaced with serum-free medium for another 24 hours before harvesting. This dosage and exposure regimen is based on our previous work (13) and the work of Westergren-Thorsson and coworkers (15) showing that TGF-beta1-induced effects on human fibroblast proliferation and activation lasts up to 48 hours after initial exposure.

3.3 Radioimmunobinding assay

Quantitative analysis was performed to determine whether statistically significant differences exist in the expression of PDGF alpha and beta receptors in response to the addition of exogenous TGF-beta1 for the different cell lines. A Dot Microfold apparatus from V&P Scientific, Inc. (San Diego, CA) was used. The different cell lines were fixed with 2% glutaraldehyde and seeded at 5×10^4 cells in the manifold. The manifold has the configuration of a 96 well plate whose bottom surface consists of glass microfilter paper (Whatman, Clifton, NJ). Each well was incubated with 100 µl of the various primary antibodies—either anti-PDGF alpha receptor antibody, PDGF-beta receptor antibody (R&D System Inc., Minneapolis, MN) or mouse IgG as a negative control. The wells were washed several times with PBS + 2% FBS. Radiolabeled secondary antibody [^{125}I]-conjugated mouse antibody was used at 0.1 µCi/well (ICN, Irvine CA), vacuum applied, and vigorous washing performed to eliminate any nonspecific binding. Wells were cut separately and each was counted for its immune reactivity using a liquid scintillation counter (14). Results were expressed as disintegration per minute (dpm) for each cellular condition and each antibody tested.

3.4 RNA extraction

To determine mRNA levels for PDGF-alpha and beta receptors, fibroblasts from the various tissues were cultured in 75-cm^2 tissue culture flasks in the presence or absence of TGF-beta1 as described above. At the end of the incubation period, total cellular RNA was extracted from cell cultures using an acid guanidium thiocyanate-phenol-chloroform described by Chomczymski and Saachi (16). Briefly, cells were washed twice with PBS, lysed by adding 10 mL of 4M guanidium thiocyanate buffer, and collected in 50 ml conical tubes. The following solutions were added in succession with vortexing 2-5 minutes each time: 2M sodium acetate pH 4, followed by water saturated phenol, followed by chloroform-isoamyl alcohol. The mixture was centrifuged at 4oC, RNA was ethanol precipitated, centrifuged and resuspended in lysing buffer, centrifuged and the RNA pellet resuspended in 75% ethanol at room temperature. The RNA mixture was then centrifuged, vacuum dried, and the RNA pellet dissolved in 0.5% SDS. Absorbance was checked at OD 260.

3.5 Polymerase chain reaction amplification

Total RNA was isolated from AG 1518 human foreskin fibroblasts (Human Genetic Mutant Cell Repository, Camden, New Jersey) by the method of Chirgwin and coworkers (17). This was used for first strand synthesis in accordance with the work of Claesson-Welch and coworkers (18) who have shown abundant amplification product for PDGF-alpha receptor by this method. RT-PCR was performed using the GeneAmp RNA PCR kit (Perkin Elmer Cetus, Norwalk, CT) according to the manufacturer's instructions. For cDNA amplification, PCR was performed for 30 cycles. Throughout successive stages of re-amplification and purification, 1.6% Agarose Gels (1XTBE, ethidium bromide) was used to monitor the integrity of the product. The presence of a PDGF-alpha receptor transcript was indicated by the PCR amplification of appropriately sized product generated by the deoxyoligonucleotide primers derived from the published PDGF-alpha cDNA sequences (18) shown to yield a PCR product of 989 bp. PDGF-beta receptor and beta–Actin cDNA probes, the latter used as positive control, were purchased from American Type Culture Collection (Rockville, MD).

3.6 Northern blot analysis

Ten µg of total RNA was separated by electrophoresis in a 0.66 M formaldehyde/1 % agarose gel according to the method of Sambrook and coworkers (19) using a 0.24 to 9.5 kb RNA ladder (BRL, Life Technologies, Inc., Gaithersburg, MD) as size markers. Gels were stained for 10 minutes in ethidium bromide and destained in water. Bands were visible under UV light and then photographed. The agarose gels were transferred to Zeta probe blotting membranes (Bio-Rad Laboratories, Richmond, CA). The membranes were cross- linked by ultraviolet irradiation using a Stratalinker (Stratagene, La Jolla, CA), then prehybridized in the prehybridization buffer containing 50% deionized formamide, 5X Denhardt's solution, 1% sodium dodecyl sulfate (SDS), 5X SSPE and 100 µg/ml herring sperm DNA. Membranes were prehybridized for 6 hours at 42^{o}C, then hybridized overnight at 42^{o}C with the different probes which were labeled using a random primed labeling kit (Boehringer Mannheim Biochemicals, Indianapolis, IN) according to the manufacturer's protocol with [alpha-^{32}P] dCTP (-3000 Ci/mmol, Amersham Corporation, Arlington Heights, IL.) in the same prehybridization buffer but without the herring sperm DNA. Membranes were washed twice in 2X SSPE for 15 minutes at room temperature, twice in 2 X SSPE/2% SDS for 45 minutes at 55^{o}C and twice in 0.1 x SSPE for 15 minutes at room temperature. Then membranes were exposed to Kodak X Omat AR film at –70^{o}C overnight.

3.7 Western blot analysis

Confluent fibroblast cultures derived from NSk, NSc and keloid in the presence or absence of TGF-beta1 as described above, were solubilized in cold lysis buffer containing 10mM Tris-HCl, 150 mM NaCl, (TSA) 5 mM EDTA, 1.2% Triton X-100, 2 mM phenyl methylsulfonyl fluoride (PMSF), 2 mM iodoacetamide, 0.15 U/ml aprotinin, and 10 ug/ml leupeptin. Cell suspensions were sheared using a syringe and centrifuged at 2000 rpm for 5 minutes at 4^{o}C. Lysate pellets were stored at –70^{o} C. The protein concentration of the different cell lysates were measured using the BCA protein assay kit (Pierce, Rockford, IL) and analyzed by a spectrophotometer.

Immunoprecipitation of PDGF alpha and beta receptors was conducted using 1:25 dilution of anti-PDGF alpha and beta receptors monoclonal antibodies respectively followed by incubation in 100 µl of protein A/G Agarose beads for 2 hours at 4^{o}C. The protein was then washed with TSA buffer and denatured by boiling for 10 minutes in 2X SDS. Equal amounts of immunoprecipitates were run on a 10% acrylamide SDS gels and transferred onto a Hybond nitrocellulose membranes (Amersham, Life Sciences Inc., Arlington Heights, IL). The membranes were blocked with 5% milk in 0.05% Tween in TBS (T-TBS) washed, and incubated with 1:1000 dilution of anti-PDGF alpha and beta receptor antibodies overnight. The membranes were then incubated with 1:2000 dilution of horseradish peroxidase-conjugated secondary antibody in 1% milk in T-TBS for 2 hours. Protein bands were visualized using Amersham's enhanced chemiluminesence substrate (ECL) and exposure to X-ray film.

4. RESULTS

4.1. Effect of TGF- beta 1 on PDGF receptors subunits: RadioImmunoBinding assay

By RadioImmunoBinding assay all cell lines expressed both PDGF-alpha and beta receptors before TGF- beta 1 treatment but only at low levels. Scar cells showed higher PDGF-beta receptor levels than NSk. TGF- beta 1 treatment induced no significant increase in PDGF-beta receptors expression for any of the cell lines i.e: NSk, NSc and Keloid "figure 1". In case of PDGF-alpha receptor , TGF- beta 1 treatment upregulated the expression of the alpha receptor subunit on keloid fibroblasts only ($p<0.01$) with no effect on NSk ($p> 0.05$) and NSc fibroblasts ($p>0.05$), "figure 2". All testing of differences between groups of data utilized the Student t test; significance was established at a p value of < 0.05.

4.2 TGF- beta 1 Modulation of PDGF-alpha and beta receptor Protein Synthesis

Protein synthesis as determined by Western analysis is shown in "figure 3, and relative optical densities shown in table 1". The amount of PDGF-alpha receptor subunit in keloid fibroblasts was shown to be increased after TGF- beta 1 stimulation for 24 hours, whereas no changes was observed with NSk or NSc fibroblasts "figure 3A, table 1". In the case of PDGF-beta there was no significant increase in PDGF-beta receptor expression for any of the cell line tested whether with or without TGF-beta 1 stimulation "figure 3B, table 1".

Table 1. Western blot analysis

LANES	RELATIVE OPTICAL DENSITY	
	PDGF-alpha receptor	PDGF-beta receptor
1. Kel (+)	22149	22461
2. Kel (-)	15122	19051
3. NSc (+)	13617	18915
4. NSc (-)	13348	18685
5. Kel II (+)	17934	17990
6. Kel II (-)	11048	16394
7. NSk (+)	12931	21051
8. NSk (-)	12338	25749

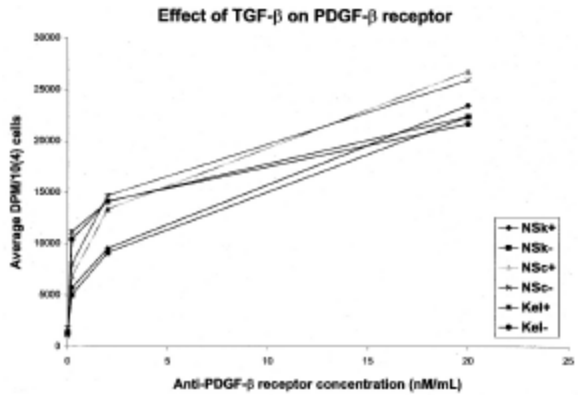

Figure 1. Effect of TGF- beta 1 on PDGF-beta receptor cell surface expression. Using a RadioImmunoBinding assay, normal skin (NSk), normal scar (NSc) and Keloid (kel) fibroblasts were examined. Half of the subconfluent cultures were preincubated with TGF- beta 1 (10ng/mL) for 24 hours. Monoclonal mouse anti-human PDGF-beta receptor antibody was added at a concentration range of (0-20 nM/mL). Data are expressed as average disintegrations per minute (dpm) of [^{125}I]-conjugated mouse secondary antibody per 10^4 cells per well. Each data point represents the average of values for triplicate wells for each cell line.

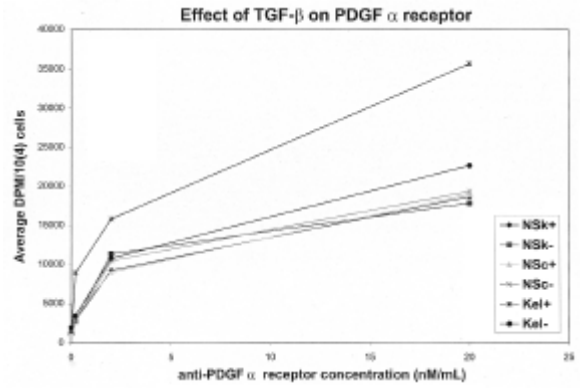

Figure 2: Comparison of PDGF-alpha receptor cell surface expression after TGF- beta 1 treatment. In normal skin (NSk), normal scar (NSc) and Keloid (kel) fibroblasts. Half of the subconfluent cultures were preincubated with TGF- beta 1 (10ng/mL) for 24 hours. Monoclonal mouse anti-human PDGF-alpha receptor antibody was added at a concentration range of (0-20 nM/mL). Data are expressed as average disintegrations per minute (dpm) of [^{125}I]-conjugated mouse secondary antibody per 10^4 cells per well. Each data point represents the average of values for triplicate wells for each cell line.

4.3 TGF- beta 1 Effect on PDGF-alpha and beta receptor mRNA Expression

Northern analysis showed that 24-hr treatment with 10 ng/mL of TGF- beta 1 upregulated the mRNA expression of the 6.5- kb PDGF-alpha receptor transcript for keloid scar tissues. There was also a slight upregulation for NSc, but there was no effect on NSk "figure 4A and relative optical densities shown in table 2". In contrast to the results of the alpha receptor; the PDGF-beta–receptor transcript (5.6 kb) mRNA levels were not altered after TGF-beta 1 treatment for any of the cell lines tested figure 4B, table 2". Levels of PDGF- alpha and PDGF-beta receptor mRNA are presented relative to beta-Actin mRNA levels.

5. DISCUSSION

TGF- beta 1 is a multifunctional regulatory factor exerting a diversity of different effects on cell and tissue differentiation, growth, and wound healing (20). Both PDGF and TGF-beta are released from the alpha granules of human platelets when these platelets aggregate and degranulate at the site of injury. Several investigators have shown that both PDGF and TGF-beta promote connective tissue deposition *in vivo* (21, 22), and can enhance the healing of dermal wounds in certain animal models (23, 24,25).

In this study, the effects of TGF- beta 1 on PDGF receptors (alpha and beta) in human dermal fibroblasts were studied both at the transcriptional and posttranscriptional levels. The present data demonstrate that TGF- beta 1 upregulates the expression of PDGF alpha receptor for keloid fibroblasts only, with no discernible effect on normal skin fibroblasts. The differences in TGF-beta 1 modulation of PDGF alpha receptor membrane expression in the cell lines tested also corresponds to the effect of TGF- beta 1 on PDGF alpha receptor subunit mRNA levels. Recent studies indicate that selected growth factors have the capacity to modulate proliferating responses to PDGF isoforms by affecting the expression of PDGF receptor subunits. Investigators have shown that scleroderma fibroblasts experience a distinct upregulation of PDGF alpha receptor after treatment with TGF-beta as well as increased responsiveness to PDGF-AA (26). This upregulation was not seen in normal human adult and foreskin fibroblasts treated under the same conditions (27). The authors speculate that this characteristic of scleroderma fibroblasts may be important for the in vivo expansion of this cell population, the same maybe true for the expansion of keloid fibroblasts. In studying the regulation of TGF-beta 1 on PDGF beta receptors for normal skin, normal scar and keloid fibroblasts, both at the protein and mRNA levels, no modulatory effect for any of the cell lines was found. One possibility is that PDGF-beta receptor levels are already maximal, or that more than one growth factor is needed for the upregulation of the PDGF-beta receptors in these cells. Other studies of PDGF receptor expression in wounds, have found that the PDGF-beta receptor is elevated in both chronic wounds and

Table 1. Northern blot analysis

LANES	RELATIVE OPTICAL DENSITY	
	PDGF-alpha receptor	PDGF-beta receptor
1. Kel I (-)	21200	20677
2. Kel I (+)	28591	18052
3. NSc (-)	13438	16960
4. NSc (+)	15624	17880
5. NSk (-)	10825	19257
6. NSk (+)	12672	20232
7. Kel II (-)	22974	20285
8. Kel II (+)	27929	19178

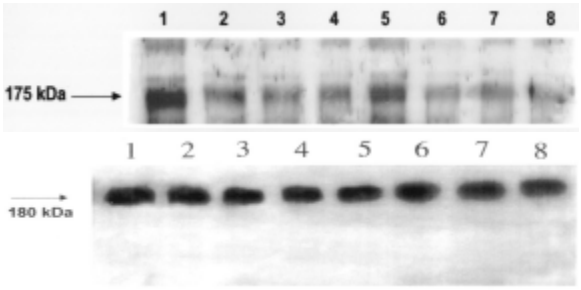

Figure 3: Effects of TGF- beta 1 on PDGF alpha and beta receptor protein levels. Immunoprecipitates prepared from equivalent numbers of cells were analyzed by Western blot. Half of the cells were pre-incubated with (+) or without (-) TGF- beta 1 (10 ng/mL) for 24 hours. Lanes 1 keloid I (+), lanes 2 keloid I (-), lanes 3 NSc (+), lanes 4 NSc(-), lanes 5 keloid II (+), lane 6 keloid II (-), lanes 7 NSk (+) and lanes 8 NSk (-). (A:top) Analyzed with anti-PDGF-alpha receptor antibody. (B:bottom) Analyzed with anti-PDGF-beta receptor antibody.

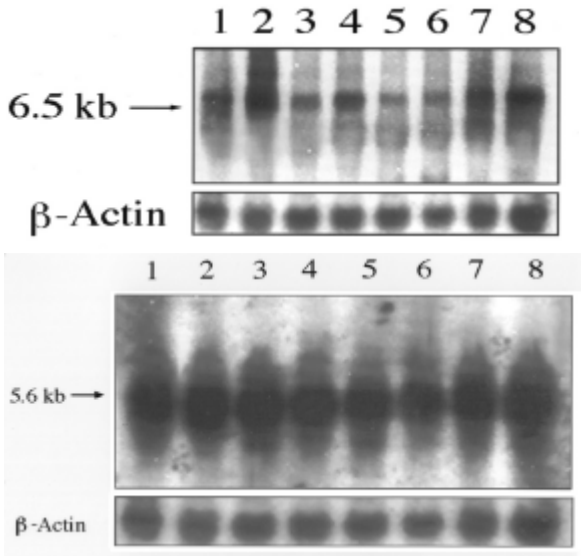

Figure 4: Northern Blot analysis. Total cellular RNAs were isolated from the different cell lines after stimulation with (+) or without (-) TGF- beta 1 (10 ng/mL) for 24 hours. Lanes 1 keloid I (-), lanes 2 keloid I (+), lanes 3 NSc (-), lanes 4 NSc (+), lanes 5 NSk (-), lanes 6 NSk (+), lanes 7 keloid II (-), lanes 8 keloid II (+). Equal amounts of RNAs were loaded in each lane, electrophoresed, transferred to nitrocellulose membrane and hybridized with (A:top) 32P-labeled PDGF-alpha receptor cDNA probe. (B; bottom) 32P-labeled PDGF-β receptor cDNA probe. beta -Actin was used as a control probe.

psoriasis, with no increase in PDGF- alpha receptors (28), the same is true for normal healing wounds (29), and in systemic sclerosis (30). Elevation of PDGF-alpha receptor expression in keloid fibroblasts only and not in normal skin was also demonstrated by Haisa and coworkers (31), who speculate that elevated levels of PDGF-alpha receptor in the keloid fibroblasts present in the keloid-derived cells would make them more responsive to PDGF AA as a chemoattractant and mitogen. The presence of PDGF AA in the tissue could selectively recruit cells with elevated PDGF alpha receptors forming a site with distinct subpopulation of fibroblasts. The reason for this differential expression of the PDGF-alpha receptor expression after TGF- beta 1 treatment in keloid fibroblasts only is still unknown. It may be due to differences in TGF-beta 1 signal-transduction pathways in keloid and normal skin fibroblasts, or it may reflect differences between the two cell lines in the levels or activities of transcription factors implicated in the regulation of the PDGF- alpha receptor gene.

At present, mechanisms of fibrosis in keloid scars and other fibroproliferative diseases such as scleroderma and pulmonary fibrosis are not fully understood. Factors such as PDGF and TGF-beta which are consistently found in fibrotic lesions, may stimulate fibroblasts to proliferate and synthesize extracellular matrix (31). Distinctive properties of abnormal scar fibroblasts such as increased extracellular matrix synthesis and abnormal phenotypic proliferation may play an essential role in the development of fibrosis. At present, the origin of abnormal fibroblasts residing in these lesions is unknown. One can speculate that, proliferation of a subpopulation of fibroblasts occurs—a subset expressing genes promoting increased production of, for instance, matrix collagen and fibronectin. The selective amplification of such a substrain of fibroblasts maybe associated with increased responsiveness of growth factors present in the lesion and, correspondingly, continued propagation of the particular strain(s).

These results indicate that an intertwining network of growth factors present in the early phases of the wound healing process may play a role in the pathogenesis of fibrotic disorders such as keloid scarring. This work accentuates the critical role of TGF-beta in this process.

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__Send correspondence to: Dr. Diana V. Messadi, Associate Adjunct Professor, UCLA School of Dentistry, 10833 LeConte Ave., CHS 20-118, Los Angeles, CA 90095-1668, Tel: (310) 206-7399, Fax: (310) 825-7232, E-mail: dmessadi@dent.ucla.edu