# Connective Tissue Growth Factor Gene Expression in Tissue Sections From Localized Scleroderma, Keloid, and Other Fibrotic Skin Disorders

Atsuyuki Igarashi, Kiyoko Nashiro,* Kanako Kikuchi,* Shinichi Sato,† Hironobu Ihn,† Manabu Fujimoto,* Gary R. Grotendorst,§ and Kazuhiko Takehara‡

Division of Dermatology, Kanto Teishin Hospital, Tokyo; *Department of Dermatology, Tokyo University Branch Hospital, Tokyo; †Department of Dermatology, Faculty of Medicine, Tokyo University, Tokyo; §Department of Cell Biology and Anatomy, University of Miami School of Medicine, Miami, Florida; and ‡Department of Dermatology, Faculty of Medicine, Kanazawa University, Ishikawa, Japan

**Connective tissue growth factor (CTGF) is a novel peptide that exhibits platelet-derived growth factor–like activities and is produced by skin fibroblasts after activation with transforming growth factor–β. Coordinate expression of transforming growth factor–β followed by CTGF during wound repair suggests a cascade process for control of tissue regeneration. We recently reported a significant correlation between CTGF mRNA expression and histologic sclerosis in systemic sclerosis. To confirm the relation between CTGF and skin fibrosis, we investigated CTGF gene expression in tissue sections from patients with localized scleroderma, keloid, and other sclerotic skin disorders using nonradioactive *in situ* hybridization. In localized scleroderma, the fibroblasts with positive signals for CTGF mRNA were scattered throughout the sclerotic lesions with no preferential distribution around the inflammatory cells or perivascular regions, whereas the adjacent nonaffected dermis was negative for CTGF mRNA. In keloid tissue, the fibroblasts positive for CTGF mRNA were diffusely distributed, especially in the peripheral expanding lesions. In scar tissue, however, the fibroblasts in the fibrotic lesions showed partially positive signals for CTGF mRNA. In eosinophilic fasciitis, nodular fasciitis, and Dupuytren's contracture, CTGF mRNA was also expressed partially in the fibroblasts of the fibrotic lesions. Our findings reinforce a correlation between CTGF gene expression and skin sclerosis and support the hypothesis that transforming growth factor–β plays an important role in the pathogenesis of fibrosis, as it is the only inducer for CTGF identified to date. *Key words: fibroblasts/TGF-β/fibrosis/*in situ *hybridization. J Invest Dermatol 106:729–733, 1996***

Previous investigations have revealed increased production of collagen and other matrix proteins in cultured fibroblasts from the affected skin of systemic or localized scleroderma (LeRoy, 1974; Uitto *et al*, 1979; Backingham *et al*, 1980; Fleischmajer *et al*, 1981; Krieg *et al*, 1983; Kähäri *et al*, 1984; Vuorio *et al*, 1985). Recently, factors derived from inflammatory cells have been suggested to play an important role in the initiation of scleroderma. Among these, transforming growth factor-β (TGF-β) has been focused on because it was revealed to stimulate collagen and fibronectin production by fibroblasts *in vitro* (Raghow *et al*, 1987; Varga *et al*, 1987). *In vivo*, overexpression of TGF-β has been demonstrated in systemic or localized scleroderma as well as in eosinophilic fasciitis (Gruschwitz *et al*, 1990; Kulozik *et al*, 1990; Peltonen *et al*, 1990; Peltonen *et al*, 1991b; Gabrielli *et al*, 1993; Sfikakis *et al*, 1993; Higley *et al*, 1994).

Overproduction of extracellular matrix is also prominent in keloid tissue, which has a genetic predisposition and is known to be caused by trauma. Keloid differs from scars in that the regeneration process does not cease after recovery of the damaged tissue, because of the abnormal regulatory pathway of wound healing. Enhanced biosynthesis of collagen and fibronectin in cultured fibroblasts from keloid tissue has been reported (Diegelmann *et al*, 1979; Abergel *et al*, 1985; Babu *et al*, 1989), and TGF-β expression was also observed in the tissue (Peltonen *et al*, 1991a). Thus, it is generally agreed that TGF-β plays a key role in the development of these fibrotic skin diseases.

Connective tissue growth factor (CTGF) is a cysteine-rich peptide, originally identified from human umbilical endothelial cells, that exhibits platelet-derived growth factor-like activities and appears to be antigenically related to platelet-derived growth factor (Bradham *et al*, 1991). Our recent study showed that human foreskin fibroblasts produce high levels of CTGF mRNA and protein after activation with TGF-β (Igarashi *et al*, 1993). In the wound chamber model, coordinate TGF-β expression followed by CTGF in regenerating tissue has been observed, suggesting a cascade process for the control of tissue regeneration and repair (Igarashi *et al*, 1993). More recently, we reported that CTGF mRNA is strongly expressed in the fibroblasts located in sclerotic

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Reprint requests to: Dr. Atsuyuki Igarashi, Division of Dermatology, Kanto Teishin Hospital, 5-9-22 Higashigotanda, Shinagawa-ku, Tokyo, 141, Japan.

Abbreviation: CTGF, connective tissue growth factor.

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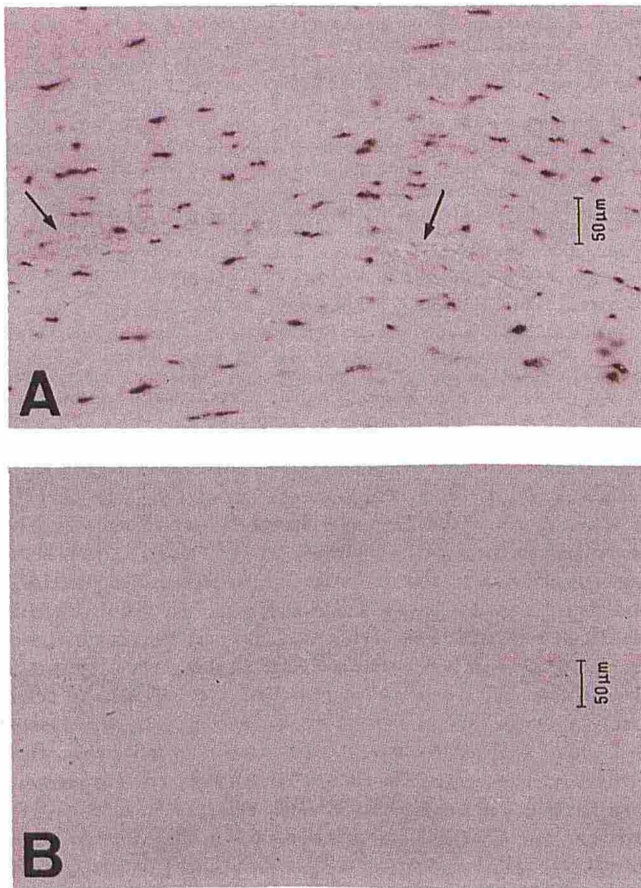


**Figure 1. CTGF mRNA is present in fibroblasts from patients with localized scleroderma.** The tissue from patients with localized scleroderma was hybridized with CTGF antisense strand probe (*A*) and sense strand probe (*B*), as described in the text. *Arrows* in *A* point to vascular endothelial cells negative for CTGF mRNA.

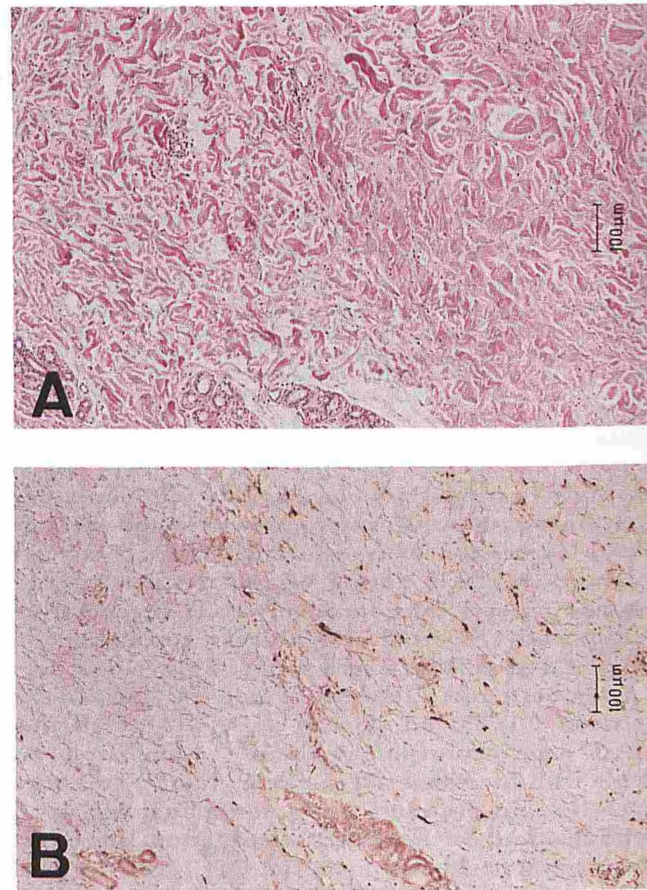


**Figure 2. CTGF mRNA is expressed in the sclerotic lesion, but not in the adjacent unaffected skin region of localized scleroderma.** *A*, same lesion as in *B*; hematoxylin and eosin–stained section of localized scleroderma biopsy specimen taken from the edge of the lesion. The section was hybridized with CTGF antisense strand probe (*B*).

lesions from patients with systemic sclerosis, but not in those from normal controls, indicating a significant correlation between CTGF gene expression and skin sclerosis (Igarashi *et al*, 1995). We speculate that CTGF is a candidate autocrine stimulator released in response to TGF-$\beta$ in skin fibroblasts and participates in the process of skin fibrosis.

To confirm this hypothesis, we examined CTGF gene expression in other sclerotic diseases including localized scleroderma, keloid, scars, nodular fasciitis, and eosinophilic fasciitis by nonradioactive *in situ* hybridization. CTGF mRNA-positive fibroblasts were demonstrated in these lesions.

## MATERIALS AND METHODS

**Tissue Preparation** We evaluated 22 formalin-fixed, paraffin-embedded skin tissues for CTGF mRNA expression. These consisted of 12 localized scleroderma (seven morphea, three generalized morphea, two linear scleroderma; mean age 30 y, range 9–56 y; mean disease duration 3 y, range 1 mo to 15 y), three keloid, three scar, one nodular fasciitis, one eosinophilic fasciitis, and two Dupuytren's contracture. Hematoxylin and eosin-stained sections of all lesions were reviewed, and the respective diagnoses were confirmed.

***In Situ* Hybridization** Formalin-fixed, paraffin-embedded skin tissues were investigated using a slight modification of the nonradioactive *in situ* hybridization procedure using digoxigenin-labeled RNA probes. Briefly, paraffin-embedded sections were cut at a thickness of 4 $\mu$m, mounted on silane-coated slides, deparaffinized, and treated with 0.2 M HCl for 15 min, then digested with 1.5 mg/ml proteinase K for 15 min at 37°C. The sections were post-fixed with 4% paraformaldehyde in phosphate-buffered saline and soaked in phosphate-buffered saline containing 2 mg/ml glycine. After rinsing with phosphate-buffered saline, the samples were immersed in 2× standard saline citrate buffer (SSC) containing 50% formamide and hybridized.

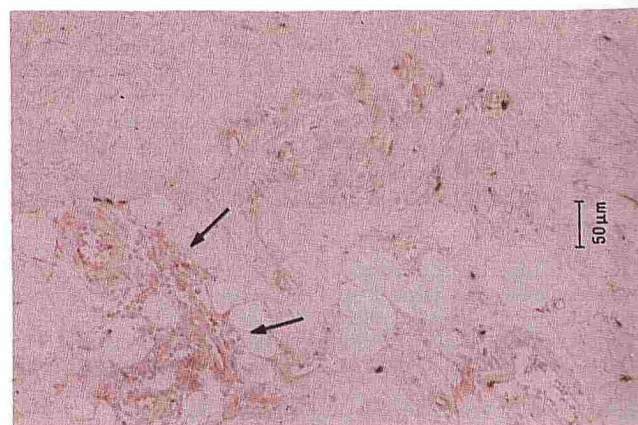


**Figure 3. No preferential distribution of fibroblasts positive for CTGF mRNA in the inflammatory lesion or around the blood vessels in the tissue from localized scleroderma.** The section was hybridized with antisense strand probe. *Arrows* indicate the perivascular inflammatory cell infiltration.

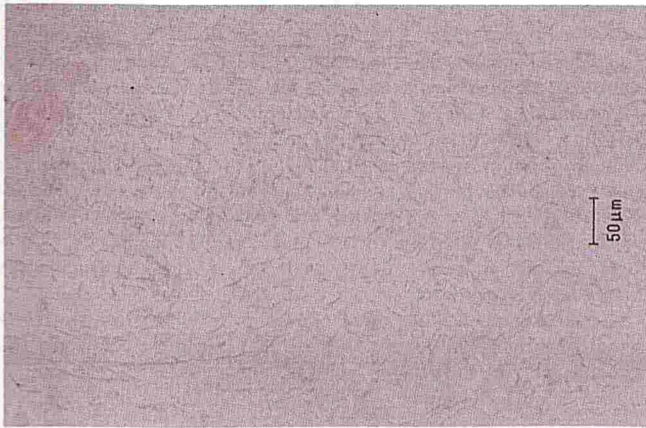


**Figure 4. No CTGF gene is expressed in this case of localized scleroderma, although histologic sclerosis is apparent.** The section was hybridized with CTGF antisense strand probe.

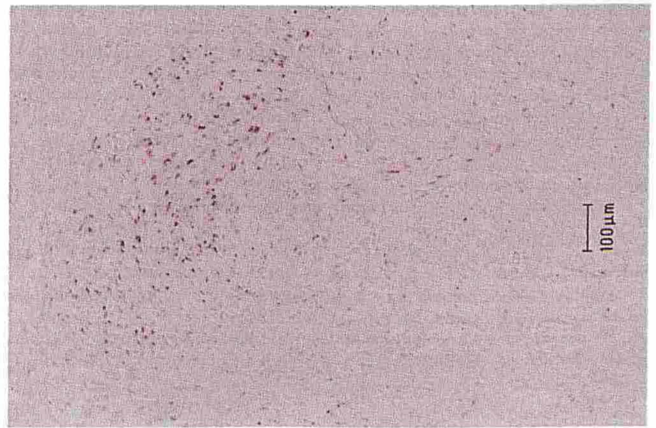


**Figure 6. Fibroblasts expressing CTGF mRNA are partially located in the sclerotic lesion of the scar tissue.** The section was hybridized with CTGF antisense strand probe.

A 2,100-bp fragment from CTGF cDNA was subcloned into the *Eco*RI site of the Bluescript phagemid (Bradham *et al*, 1991) and used for making probes. After linearizing with *Xba*I, antisense RNA was generated using T3 RNA polymerase. T7 RNA polymerase allowed the production of a sense RNA after linearizing with *Xho*I. The probes were labeled with digoxigenin-11-UTP using a DIG RNA-labeling kit (Boehringer Mannheim Biochemica, Germany). The amount of nonradioactive CTGF RNA probes was quantified on a 1% agarose gel and in a dot blot with the labeled control RNA using a DIG nucleic acid detection kit (Boehringer Mannheim Biochemica). These riboprobes were tested by Northern blotting; the antisense probe recognized the correct mRNA, whereas the sense probe did not give any signals.

The labeled RNA probe (final 1 $\mu$g/ml), in a mixture containing 50% formamide, 10% dextran sulfate, 1× Denhardt's solution, 100 $\mu$g/ml tRNA, 5× SSC, 0.25% sodium dodecyl sulfate, 1 mM ethylenediamine tetraacetic acid, and 50 mM $NaH_2PO_4$, was placed on the slides and covered with a coverslip. Hybridization was performed in a humidified chamber for 18 h at 45°C, after which the specimens were washed in 2× SSC with 50% formamide at 50°C. Unhybridized probes were digested in 2.5 $\mu$g/ml RNase A, 500 mM NaCl, 1 mM ethylenediamine tetraacetic acid, and 10 mM Tris HCl, pH 8.0, for 15 min at 37°C. The slides were then washed for 15 min in 2× SSC and twice in 0.2× SSC at 50°C. After post-hybridization washing, the digoxigenin-labeled probes were visualized as described in the DIG nucleic acid detection kit protocol. The sections were counterstained with nuclear fast red.

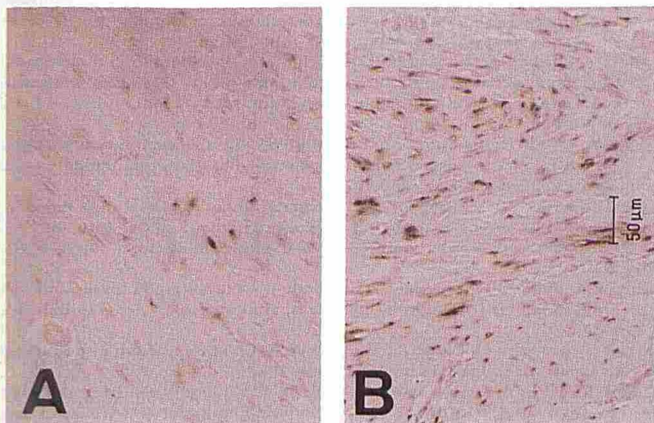


**Figure 5. Fibroblasts in the peripheral lesion of keloid tissue strongly express CTGF mRNA.** The section was hybridized with CTGF antisense strand probe. *A*, central lesion; *B*, peripheral lesion of the keloid tissue.

## RESULTS

**CTGF mRNA Expression in Fibroblasts of Sclerotic Lesions From Localized Scleroderma Specimens** When tissues from 12 patients with localized scleroderma were examined by *in situ* hybridization with the CTGF antisense strand probe, dermal fibroblasts with positive signals were demonstrated in ten cases. Fibroblasts displaying CTGF mRNA were scattered throughout the sclerotic dermis, and no preferential accumulation in the perivascular regions was observed **(Fig 1*A*)**. Dermal vascular endothelial cells were negative for CTGF mRNA. To confirm the specificity of the hybridization with CTGF mRNA, we performed *in situ* hybridization with the CTGF sense strand probe, and no positive signals were obtained **(Fig 1*B*)**. **Figure 2*A*** shows a hematoxylin and eosin-stained section taken from the edge of the morphea, which shows sclerotic changes in the right upper portion and a normal region in the left lower portion. As shown in **Fig 2*B***, the fibroblasts with positive signals were distributed in the sclerotic tissue, whereas no hybridization was observed in the adjacent unaffected region. Nonspecific background staining was observed in the eccrine sweat glands. In one case that showed pronounced inflammatory cell infiltration, CTGF mRNA expression was moderate and no preferential distribution was observed around the inflammatory foci **(Fig 3)**. In two cases, virtually no CTGF gene expression was detected, although histologic sclerosis was apparent **(Fig 4)**.

**Differential Expression of CTGF mRNA in Keloid and Other Sclerotic Skin Diseases** Next, we investigated CTGF gene expression in keloid, scars, and other fibrotic skin disorders. In keloid tissue, the fibroblasts with positive hybridization signals were distributed throughout the lesions, especially in the peripheral areas **(Fig 5)**. In scar tissue, CTGF mRNA was partially expressed in the fibroblasts of the lesion, although CTGF mRNA-negative lesions were also observed in the fibrotic tissue **(Fig 6)**, as seen in nodular fasciitis. In eosinophilic fasciitis, fibroblasts located in the thickened fascia also expressed CTGF mRNA (data not shown). Of the two cases of Dupuytren's contracture, one specimen gave positive signals for CTGF mRNA in the fibroblasts of the fibrotic lesions, whereas the other sample revealed negative hybridization (data not shown).

The results of the above experiments are summarized in **Table I**.

## DISCUSSION

CTGF has platelet-derived growth factor–like biologic activity and is secreted from human umbilical endothelial cells (Bradham *et al*, 1991), as well as from human foreskin fibroblasts after activation with TGF-$\beta$ *in vitro* (Igarashi *et al*, 1993). CTGF is a member of a

**Table I. CTGF mRNA Expression in Sclerotic Disorders**[a]

| Disease | CTGF(+)/No. of Cases[b] |
|---|---|
| Morphea | 6/7 |
| Generalized morphea | 3/3 |
| Linear scleroderma | 1/2 |
| Keloid | 3/3 |
| Scar | 2/3 |
| Nodular fasciitis | 1/1 |
| Eosinophilic fasciitis | 1/1 |
| Dupuytren's contracture | 1/2 |

[a] Paraffin-embedded sections were hybridized with CTGF antisense strand probe, and the signals were visualized as mentioned in the text.

[b] Positive/number of cases examined. The case that had apparent purple-stained particles was defined as positive.

family of peptides that includes the v-*src*–induced CEF-10 peptide, identified in *src*-transformed chicken embryo fibroblasts (Simmons *et al*, 1989), *cyr61* cloned from mouse BALB/c 3T3 fibroblasts after serum induction (O'Brien *et al*, 1990), and the serum-induced *fisp12* (Ryseck *et al*, 1991) or TGF-$\beta$-induced $\beta$*IGM2* transcript identified in 3T3 cells (Brunner *et al*, 1991). CTGF is also related to the *nov* gene product, which is overexpressed in nephroblastomas induced by myeloblastosis-associated virus (Joliot *et al*, 1992). Among CTGF-related genes, expression of *cyr61* was reported to be correlated with chondrogenesis during embryonic development (O'Brien and Lau, 1992), yet the biologic roles of these CTGF-related gene products are poorly understood.

We have recently reported that dermal fibroblasts of systemic sclerosis were positive for CTGF mRNA in all cases that showed histologic sclerosis. Fibroblasts with positive hybridization signals were more abundant in the tissue from the sclerotic stage than in that from the inflammatory stage, indicating a close correlation between CTGF gene expression and fibrosis (Igarashi *et al*, 1995).

In localized scleroderma, the fibroblasts with positive hybridization for CTGF mRNA expression were also scattered in the sclerotic lesions of the tissue. This observation is basically consistent with that in systemic sclerosis, although the level of gene expression differed in each case examined and was not correlated closely with the histologic sclerosis, as observed in systemic sclerosis. In systemic sclerosis, it is well known that the sclerotic lesions diminish spontaneously during the disease process when they reach the atrophic stage. It is likely that extracellular matrix synthesis and degradation proceed simultaneously in systemic sclerosis; enhanced biosynthesis exceeds the rate of degradation at the sclerotic stage, but when the disease reaches the atrophic stage, the fibroblasts lose their activities and degradation predominates, resulting in the disappearance of skin fibrosis. These aspects can explain the close correlation between CTGF mRNA expression and histologic sclerosis in systemic sclerosis. In localized scleroderma, however, the extracellular matrix degradation process seems to be different from that in systemic sclerosis. This is why histologic sclerosis was still obvious although the CTGF gene was no longer expressed in some cases of localized scleroderma observed in this study. Thus, we speculate that the level of CTGF mRNA expression is correlated with the activity of fibroblasts. Our examination of keloid tissue and scars supports this speculation. CTGF expression was pronounced in keloid, especially in the expanding border of the lesions. In scars, however, CTGF gene expression was partially positive; there was one fibrotic lesion in which no CTGF mRNA was expressed, indicating that disease activity was already diminished. These observations also support the hypothesis that CTGF is a potential autocrine stimulator released in response to TGF-$\beta$ in skin fibroblasts, and participates in the process of tissue regeneration and skin fibrosis.

It remains unclear, however, why the CTGF gene is expressed continuously in the lesions, despite the proposed role of TGF-$\beta$ in initiation of the disease process. Two possibilities can be postulated: (i) Unknown factor(s) *in vivo* may function to maintain CTGF gene expression; or (ii) the fibroblasts of the affected lesions already elude the normal TGF-$\beta$ regulatory program in the very earliest stage of the disease and maintain CTGF production. Altered responses to TGF-$\beta$ in fibroblasts derived from keloid or localized scleroderma have been reported (Babu *et al*, 1992). The latter possibility is thus the more likely explanation.

The regulation processes of CTGF, collagen, and fibronectin gene expression are similar because these genes are induced by TGF-$\beta$ and expressed in fibrotic skin tissue. It remains unknown, and should be targeted for further investigation, whether CTGF is merely expressed simultaneously or interacts with the metabolism of these matrix proteins.

In conclusion, CTGF gene expression was demonstrated in several fibrotic skin diseases, suggesting a pathogenic role for this molecule in skin fibrosis.

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## REFERENCES


Abergel RP, Pizzurro D, Meeker CA, Lask G, Matsuoka LY, Minor RR, Chu M-L, Uitto J: Biochemical composition of the connective tissue in keloid and analysis of collagen metabolism in keloid fibroblast cultures. *J Invest Dermatol* 84:384–390, 1985

Babu M, Diegelmann R, Oliver N: Fibronectin is overproduced by keloid fibroblasts during abnormal wound healing. *Mol Cell Biol* 9:1642–1650, 1989

Babu M, Diegelmann R, Oliver N: Keloid fibroblasts exhibit an altered response to TGF$\beta$. *J Invest Dermatol* 99:650–655, 1992

Backingham RB, Prince RK, Rodnan GP, Barnes EL: Collagen accumulation by dermal fibroblast cultures of patients with linear localized scleroderma. *J Rheumatol* 7:130–142, 1980

Bradham DM, Igarashi A, Potter RL, Grotendorst GR: Connective tissue growth factor: a cysteine-rich mitogen secreted by human vascular endothelial cells is related to the SRC induced immediate early gene product CEF-10. *J Cell Biol* 114:1285–1294, 1991

Brunner A, Chinn J, Neubauer M, Purchio AF: Identification of a gene family regulated by transforming growth factor $\beta$. *DNA Cell Biol* 10:293–300, 1991

Diegelmann RF, Cohen IK, McCoy BJ: Growth kinetics and collagen synthesis of normal skin, normal scar and keloid fibroblasts in vitro. *J Cell Physiol* 98:341–346, 1979

Fleischmajer R, Perlish JS, Krieg T, Timpl R: Variability in collagen and fibronectin synthesis by scleroderma fibroblasts in primary culture. *J Invest Dermatol* 76:400–403, 1981

Gabrielli A, Di Loreto C, Taborro R, Candela M, Sambo P, Nitti C, Danieli MG, DeLustro F, Dasch JR, Danieli G: Immunohistochemical localization of intracellular and extracellular associated TGF$\beta$ in the skin of patients with systemic sclerosis (scleroderma) and primary Raynaud's phenomenon. *Clin Immunol Immunopathol* 68:340–349, 1993

Gruschwitz M, Müller P, Sepp N, Hofer E, Fontana A, Wick G: Transcription and expression of transforming growth factor type beta in the skin of progressive systemic sclerosis: a mediator of fibrosis? *J Invest Dermatol* 94:197–203, 1990

Higley H, Persichitte K, Chu S, Waegell W, Vancheeswaran R, Black C: Immunocytochemical localization and serologic detection of transforming growth factor $\beta$1. *Arthritis Rheum* 37:278–288, 1994

Igarashi A, Nashiro K, Kikuchi K, Sato S, Ihn H, Grotendorst GR, Takehara K: Significant correlation between connective tissue growth factor gene expression and skin sclerosis in tissue sections from patients with systemic sclerosis. *J Invest Dermatol* 105:280–284, 1995

Igarashi A, Okochi H, Bradham DM, Grotendorst GR: Regulation of connective tissue growth factor gene expression in human skin fibroblasts and during wound repair. *Mol Cell Biol* 4:637–645, 1993

Joliot V, Martinerie C, Dambrine G, Plassiart G, Brisac M, Crochet J, Perbal B: Proviral rearrangements and overexpression of a new cellular gene (nov) in myeloblastosis-associated virus type 1-induced nephroblastomas. *Mol Cell Biol* 12:10–21, 1992

Kähäri VM, Vuorio T, Näntö-Salonen K, Vuorio E: Increased type I collagen mRNA levels in cultured scleroderma fibroblasts. *Biochim Biophys Acta* 781:183–186, 1984

Krieg T, Braun-Falco O, Perlish JS, Fleischmajer R: Collagen synthesis in generalized morphea. *Arch Dermatol Res* 275:393–396, 1983

Kulozik M, Hogg A, Lankat-Buttgereit B, Krieg T: Co-localization of transforming growth factor $\beta$2 with $\alpha$1(I) procollagen mRNA in tissue sections of patients with systemic sclerosis. *J Clin Invest* 86:917–922, 1990

LeRoy EC: Increased collagen synthesis by scleroderma skin fibroblasts in vitro. *J Clin Invest* 54:880–889, 1974

O'Brien TP, Lau LF: Expression of the growth factor-inducible immediate early gene cyr61 correlates with chondrogenesis during mouse embryonic development. *Cell Growth Differ* 3:645–654, 1992

O'Brien TP, Yang GP, Sanders L, Lau LF: Expression of cyr61, a growth factor-inducible immediate early gene. *Mol Cell Biol* 10:3569–3577, 1990

Peltonen J, Hsiao LL, Jaakkola S, Sollberg S, Aumailley M, Timpl R, Chu M-L, Uitto J: Activation of collagen gene expression in keloid: co-localization of type I and VI collagen transforming growth factor-β1 mRNA. *J Invest Dermatol* 97:240–248, 1991a

Peltonen J, Kähäri L, Jaakkola S, Kähäri V-M, Varga J, Uitto J, Jimenez SA: Evaluation of transforming growth factor β and type I procollagen gene expression in fibrotic skin diseases by in situ hybridization. *J Invest Dermatol* 94:365–371, 1990

Peltonen J, Varga J, Sollberg S, Uitto J, Jimenez SA: Elevated expression of the genes for transforming growth factor-β1 and type VI collagen in diffuse fasciitis associated with the eosinophilia-myalgia syndrome. *J Invest Dermatol* 96:20–25, 1991b

Raghow R, Postlethwaite AE, Keski-Oja J, Moses HL, Kang AH: Transforming growth factor-beta increases steady state levels of type I procollagen and fibronectin messenger RNAs posttranslationally in cultured human dermal fibroblasts. *J Clin Invest* 79:1285–1288, 1987

Ryseck R-P, MacDonald-Bravo H, Mattei M-G, Bravo R: Structure, mapping and expression of fisp-12, a growth factor-inducible gene encoding a cysteine-rich protein. *Cell Growth Differ* 2:225–233, 1991

Sfikakis PP, McCune BK, Tsokos M, Aroni K, Vayiopoulos G, Tsokos G: Immunohistological demonstration of transforming growth factor-β isoforms in the skin of patients with systemic sclerosis. *Clin Immunol Immunopathol* 69:199–204, 1993

Simmons DL, Levy DB, Yannoni Y, Erikson RL: Identification of a phorbol ester-repressible v-src-inducible gene. *Proc Natl Acad Sci USA* 86:1178–1182, 1989

Uitto J, Bauer EA, Eisen AZ: Scleroderma. Increased biosynthesis of triple-helical type I and type III procollagens associated with unaltered expression of collagenase by skin fibroblasts. *J Clin Invest* 64:921–930, 1979

Varga J, Rosenbloom J, Jimenez SA: Transforming growth factor β (TGFβ) causes a persistent increase in steady-state amounts for type I and type III collagen and fibronectin mRNA's in normal human dermal fibroblasts. *Biochem J* 297:597–604, 1987

Vuorio T, Mäkelä JK, Vuorio E: Activation of type I collagen genes in cultured scleroderma fibroblasts. *J Cell Biochem* 28:105–113, 1985