Activation of Collagen Gene Expression in Keloids: Co-Localization of Type I and VI Collagen and Transforming Growth Factor-β1 mRNA

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Untreated, clinically active keloids were examined as a model system to study the spatial expression of extracellular matrix and transforming growth factor-β1 (TGF-β1) genes in fibrotic skin diseases. In situ hybridizations localized active expression of type I and VI collagen genes to the areas containing an abundance of fibroblasts and apparently representing the expanding border of the lesions. Within this zone, microvascular endothelial cells also expressed the type I collagen genes, as evaluated by simultaneous use of in situ hybridization for collagen gene expression and immunolocalization for factor VIII-related antigen, a marker for endothelial cell differentiation. Slot-blot hybridizations of RNA isolated from this zone suggested that the expression of type I and VI collagen genes was selectively enhanced, as compared to type III collagen gene expression. TGF-β1 protein and mRNA were also detected in areas active in type I and type VI collagen gene expression, indicating that TGF-β1 gene is transcribed and the corresponding protein is deposited in areas of elevated collagen gene expression, including microvascular endothelial cells. We conclude that the initial step in the development of fibrotic reaction in keloids involves the expression of the TGF-β1 gene by the neovascular endothelial cells, thus activating the adjacent fibroblasts to express markedly elevated levels of TGF-β1, as well as type I and VI collagen genes. *J Invest Dermatol 97:240–248, 1991*

Keloids are acquired cutaneous lesions with a strong genetic predisposition and they are characterized by an excessive accumulation of the extracellular connective tissue matrix. The lesions often develop as a result of trauma to the predilection sites, such as the ear lobes, upper chest and back [1–3].

Previous biochemical analyses have indicated that collagen is the major extracellular matrix component of keloids [4,5]. Several lines of evidence have suggested that collagen biosynthesis is enhanced in keloids, leading to an accumulation of collagen [1]. Specifically, the rate of collagen biosynthesis, as measured by the formation of radioactive hydroxyproline, is increased both in keloid tissue and in fibroblast cultures established from active lesions [1,6,7]. Accordingly, the activity of prolyl 4-hydroxylase, the critical enzyme in the biosynthetic pathway of collagen, is elevated in clinically active keloids, and in fibroblast cultures established from such lesions [4,8,9]. Thus, enhanced biosynthetic pathway apparently exceeds the rate of degradation, resulting in an accumulation of collagen.

Our previous studies have indicated that type I procollagen mRNA levels were selectively increased whereas the levels of type III procollagen mRNAs were unaltered, thus resulting in markedly elevated type I:III procollagen mRNA steady-state ratios in most keloid fibroblast cultures [10]. However, the expression of the genes encoding type VI collagen in keloids has not been examined either in vitro or in vivo.

Type VI collagen is a heterotrimer consisting of three different alpha chains: α1(VI), α2(VI), and α3(VI) [11–14]. Type VI collagen has been shown to be an abundant extracellular matrix component that forms microfibrils in the skin and other tissues [15–17]. Type VI collagen mRNA is also a relatively abundant gene product of cultured human skin fibroblasts [18,19]. Because type VI collagen may play a distinct role in the organization of the extracellular matrix architecture, possibly through its cell-adhesive properties, aberrations in its biology could possibly explain the altered collagen fibrillogenesis in pathologic situations. In this study, we have examined the expression of type I, III, and VI collagen genes in keloid tissue.

Previous studies have demonstrated that TGF-β1 markedly enhances the synthesis of type I collagen [20,21], and it has been postulated that this growth factor polypeptide may play a role in the development of fibrotic skin lesions in diseases, such as scleroderma and eosinophilic fasciitis [22,23]. In this study, the expression of

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Abbreviations:

BSA: bovine serum albumin
DTT: dithiothreitol
EDTA: ethylenediamine tetraacetic acid
PAP: peroxidase antiperoxidase
TBS: tris-buffered saline
TGF-β1: transforming growth factor-β1

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both the TGF-β1 gene and the presence of TGF-β1 protein were demonstrated in keloids by in situ hybridizations and peroxidase antiperoxidase (PAP) immunodetection.

MATERIALS AND METHODS

Tissue Specimens A total of 10 keloids, diagnosed on the basis of their clinical appearance, anatomic location, and lack of previous surgery in the area, were excised for cosmetic reasons at the Department of Dermatology, Jefferson Medical College, from Afro-American patients, 18–25 years of age. All keloids were removed from the ear lobes, and the lesions varied from approximately 5 mm to 4 cm in diameter. The lesions were clinically expanding and none had been previously treated, e.g., by intralesional steroids or excision. All lesions displayed histopathology diagnostic for keloids [24]. Tissues from six lesions were snap- frozen in liquid nitrogen for in situ hybridization and for immunohistochemistry.

In Situ Hybridizations Five-micrometer-thick cryosections were cut from snap-frozen tissue specimens and immediately post-fixed with fresh 4% paraformaldehyde in phosphate-buffered saline for 20 min. The samples were then pre-treated and pre-hybridized, as described previously [25–27]. The samples were hybridized for 16 h at 42°C in a solution containing 0.1 μg/ml of a ^{32}P-labeled cDNA probe, 50% formamide, 10 mM dithiothreitol (DTT), 1 mg/ml bovine serum albumin (BSA), 0.6 M NaCl, 10% (w/v) dextran sulfate, 200 μg/ml denatured and sheared salmon sperm DNA, 0.5 mM ethylenediamine tetraacetic acid (EDTA), 0.02% (w/v) ficoll, 0.02% (w/v) polyvinyl pyrrolidone and 10 mM Tris-HCl, pH 7.4 [26]. After hybridization, the samples were washed as described previously [26], the final stringency of washing being 0.2 × SSC (1 × SSC = 0.15 M NaCl, 0.015 M sodium citrate, pH 6.8), at 42°C. Some tissue sections were subjected to simultaneous detection of proα1(I) collagen mRNA and factor VIII-related antigen as a marker for endothelial cell differentiation [28].

In situ hybridization using RNA probes was carried out as previously described [29] with slight modifications. For pre-hybridization and hybridization (3 h each at 50°C), a solution containing 50% formamide, 100 mM DTT, 1 mg/ml salmon sperm DNA, 10 mg/ml tRNA (yeast), and 2 mg/ml BSA was used. The slides were then washed in 2 × SSC, incubated with RNase A (100 μg/ml)/RNase T_1 (1 μg/ml) in 2 × SSC, washed again, dehydrated and mounted.

The [^{32}P]cDNA-mRNA hybrids were detected by immersing the samples into Kodak NTB-3 autoradiography emulsion (Eastman Kodak, Rochester, NY) diluted with an equal volume of 0.6 M ammonium acetate, and exposing them in a desiccant-containing box for 5–21 d at 4°C. The samples were developed with Kodak D-19 developer (Eastman Kodak), stained with hematoxylin, dehydrated with ethanol, cleared in xylene, and mounted.

Quantitation of mRNA by Slot-Blot Analyses Total RNA was isolated from frozen tissue specimens by a single-step method [30]. Varying amounts of RNA (0.5–4.0 μg) were then dotted on nitrocellulose filters and immobilized by heating at 80°C under vacuum for 90 min [31]. The filters were first pre-hybridized and then hybridized with one of the human sequence-specific cDNA, and the radioactive cDNA-mRNA hybrids were detected by autoradiography. The relative quantities of mRNA were determined by scanning densitometry with a laser scanner (LKB, Bromma, Sweden). The values were corrected for differences in the specific activity and the length of the probes, as well as for the chain composition of type I and VI collagens, as described previously [32].

cDNA and cRNA Probes The human sequence-specific cDNA used in this study were: For type I collagen, a 1.8-kb proα1(I) (Hf677) cDNA [33], and a 4.5-kb proα2(I) (HP 2010) cDNA [34]; for type III collagen, a 1.3-kb proα1(III) (Hf 934) cDNA [35]; for type VI collagen, three different cDNA, 2.1 (p18), 1.4 (p8), and 1.5 (p24) kb in size, corresponding to α1(VI), α2(VI), and α3(VI) chains, respectively [36]. A 0.3-kb TGF-β1 cDNA was developed by polymerase chain reaction amplification. For this purpose, mRNA from cultured human fetal skin fibroblasts was used as a template for synthesis of cDNA, and a 308-bp region defined by synthetic oligonucleotide primers corresponding to published TGF-β1 sequences [37] was amplified by polymerase chain reaction [38]. The 0.3-kb cDNA was isolated by agarose gel electrophoresis, subcloned into Bluescript vector, and its identity was confirmed by nucleotide sequencing [39]. In vitro transcription of TGF-β1 was performed using the T_3 and T_7 (for control hybridizations) polymerase binding sites of the cloning vector and a RNA transcription kit (Stragene, La Jolla, CA). After transcription reaction, the radiolabeled RNA transcripts were purified on RNase free G-50 columns (Boehringer-Mannheim, Indianapolis, IN).

In control cDNA in situ hybridizations, a 525-bp proα1(II) collagen cDNA (pHCAR1) [40] was used, and in control cRNA hybridization, the sense cRNA was used. In all control hybridizations, only a faint uniform background binding signal was observed.

For slot-blot hybridizations, cDNA was labeled with [^{32}P]dCTP by nick translation [41] to a specific activity of at least 1×10^8 cpm/μg. For in situ hybridizations, the probes were double-labeled with [^{32}P]dCTP and [^{32}P]dGTP to a specific activity of approximately 1×10^9 cpm/μg.

Immunolocalization of Type VI Collagen, TGF-β1 and Factor VIII Related Antigen Epitopes For indirect immunofluorescence, 5-μm thick frozen sections were rinsed with tris-buffered saline (TBS, pH 7.6), and pre-incubated 15 min in TBS containing 1% bovine serum albumin. The samples were then exposed to affinity-purified rabbit antibodies to human type VI collagen [42] overnight at 4°C. The sections were washed in TBS for 60 min with five changes, and incubated with tetramethyl-rhodamine isothiocyanate-conjugated goat–anti-rabbit IgG secondary antibodies (Miles Laboratories, Inc., Elkhart, IN). After a 60-min incubation at room temperature, the sections were washed in TBS with five changes for 60 min, rinsed with distilled water, air dried, mounted with Fluoromount (Fisher Scientific) and examined with a fluorescence microscope (Optiphot, Nikon Inc., Garden City, NY), equipped with filters for detection of fluorescein isothiocyanate and tetramethyl-rhodamine isothiocyanate. Representative sections were photographed using Tri-X film (Eastman Kodak). In control reactions, the primary antibody was omitted or replaced with sera from non-immunized animals. Only faint uniform background was observed in all controls.

Peroxidase antiperoxidase (PAP) immunostaining was a slight modification of a method previously described in detail [26,43]. Briefly, 5-μm thick sections were incubated in 0.01 M HCl containing 10 U/ml pepsin (Sigma Chemical Co., St. Louis, MO). Endogenous peroxidase activity was blocked by incubating the sections in 0.3% hydrogen peroxide in methyl alcohol. To prevent non-specific antibody binding, the sections were pre-incubated 30 min in a solution containing 1% bovine serum albumin. For immunostaining, sections were first incubated with an affinity-purified rabbit antibodies to TGF-β1 (Lot No H905, R&D Systems Inc., Minneapolis, MN). Swine–anti-rabbit antiserum (Accurate Chemical & Scientific Corp., Westbury, NY) was then used as the linking antibody, and the sections were incubated with rabbit PAP (Accurate Chemical & Scientific Corp.). Peroxidase activity was detected by incubation with 3,3′-diaminobenzidine tetrahydrochloride in 0.01% H_2O_2. PAP-immunostaining with antibodies to factor VIII-related antigen (Dakopatts, Glostrup, Denmark) following in situ hybridizations was carried out with slight modifications, to a method previously described [44].

RESULTS

Localization of Collagen mRNA in Keloid Tissue A total of six, previously untreated keloids were subjected to in situ hybridizations. The lesions were clinically active, and the diagnosis was confirmed by characteristic histopathology [1,2,24].

Previous studies have suggested that type I procollagen gene expression is enhanced in cultured keloid fibroblasts [6,10]. To deter-

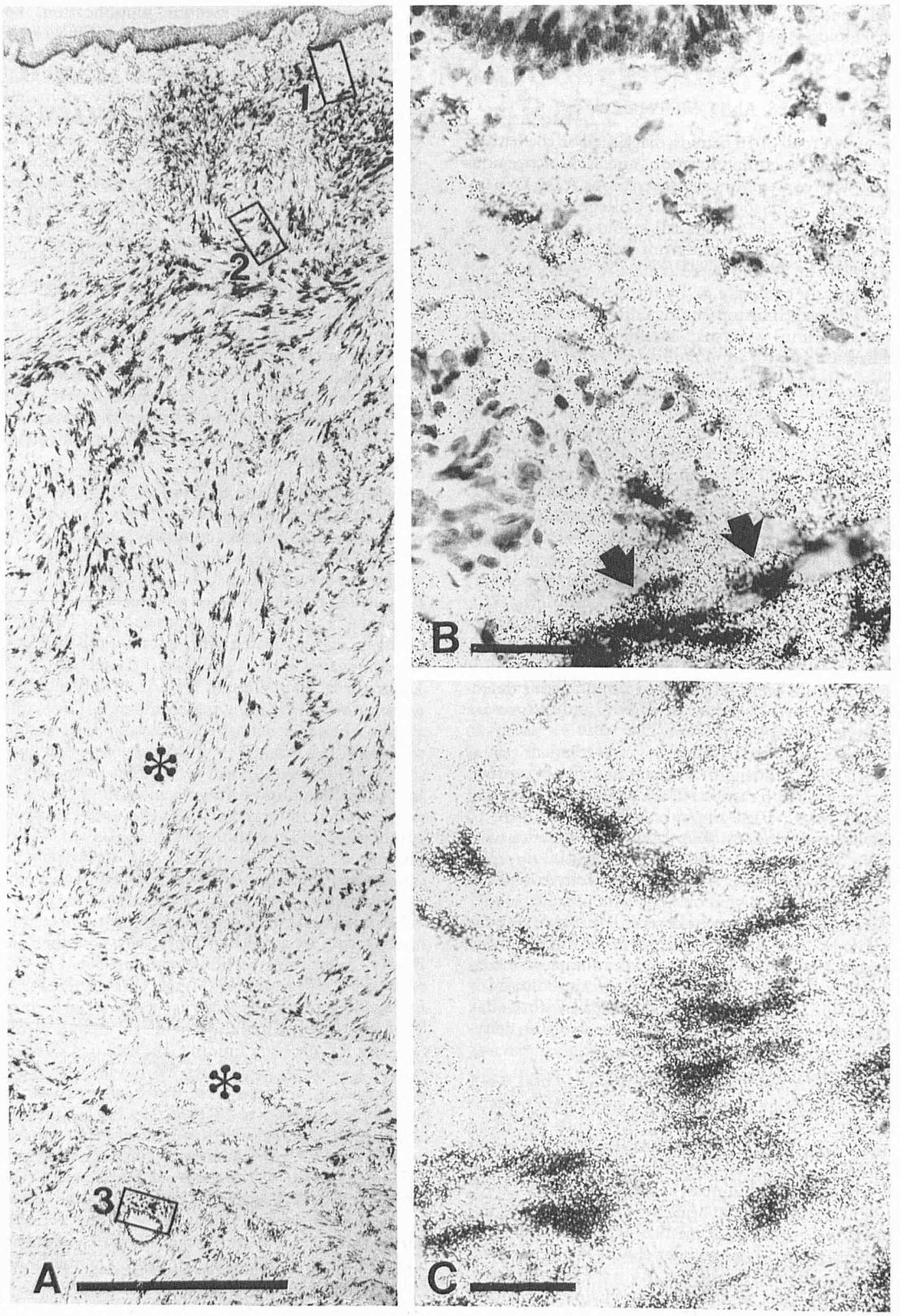
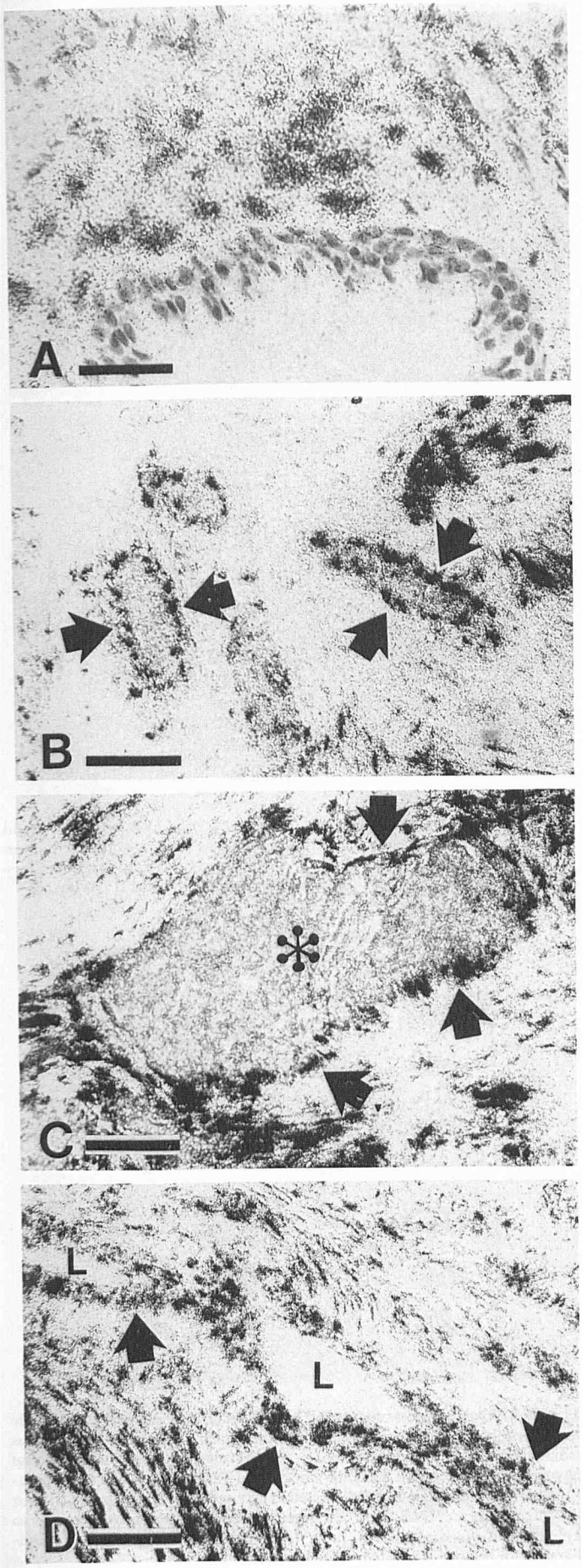


Figure 1. Demonstration of type I collagen gene expression in keloid tissue by in situ hybridization. *A,* Low magnification reveals fibroblastic cells actively expressing the proα1(I) collagen gene, as detected by the presence of [^{32}P]cDNA-mRNA hybrids in the upper reticular dermis, but not in the papillary dermis *(box 1)*. Active expression of the proα1(I) collagen gene is also noted in the periphery of a blood vessel within the keloid lesion *(box 3)*. Note the relatively low level of expression in the center of the lesion *(asterisks)*. *B,* Higher magnification of *box 1* outlined in *A*. Note that the cells in the papillary dermis are not active in type I collagen gene expression, whereas the majority of cells in the upper reticular dermis *(arrows)* display a large number of proα1(I) collagen mRNA. *C,* Higher magnification of *box 2* outlined in *A*. Note that most of these cells, which are located at the periphery of the keloid lesion, contain an abundance of autoradiographic grains. (*Bars: A,* 1 mm; *B* and *C,* 50 μm.)



mine if such activation is also detectable in situ, keloid tissues were first hybridized with a human proα1(I) collagen cDNA. The results indicated distinct regions within the keloid lesions that actively expressed the proα1(I) collagen gene (Figs 1 and 2). Specifically, grains representative of type I procollagen [^{32}P]cDNA-mRNA hybrids were detected in high abundance in regions apparently representative of the actively expanding border of the keloid lesion (Fig 1*A–C*). Deeper parts of the lesions contained a large number of cells, but only relatively few of them expressed the proα1(I) collagen gene (Fig 1*A*). Careful examination of the apparently normal papillary dermis overlying the keloid revealed that only a few cells were active in expressing the type I procollagen genes (Fig 1*B*). Similar spatial distribution was detected with a proα2(I) collagen cDNA, but as expected from the 2:1 stoichiometry of α1 and α2 chains of type I collagen, the signal was less prominent (not shown).

It was of interest to note that in keloids, the cells in the center of the lesion, which was characterized by accumulation of thick, coarse collagen bundles, did not demonstrate active type I procollagen gene expression. However, within the keloid lesions, there were numerous small blood vessels that were often surrounded by fibroblasts active in expressing the type I procollagen genes, as detected by the presence of perivascular grains representative of the proα1(I) collagen mRNAs (Fig 2*A*). In the case of capillaries in the expanding border, the autoradiographic grains were often intimately associated with the cells of the blood vessel wall (Fig 2*B*). The precise identification of these cells on the morphologic basis was not possible. However, they were identified as endothelial cells by simultaneous immunostaining with antibodies to factor VIII-related antigen [28,44] (Fig 3). Thus, certain keloid regions appear selectively to be active in type I procollagen gene expression.

Further attempts were made to examine the expression of the type III procollagen gene in keloid tissue by in situ hybridizations. In five of six cases, only a few scattered cells ($<$ 1% of the total population) contained detectable levels of type III procollagen mRNA. In one case, however, the presence of proα1(III) collagen mRNA could be detected in a large number of fibroblastic cells (not shown). These observations support the notion that, in most keloids, type III collagen gene expression is not activated to the same extent as that of type I procollagen genes. However, demonstration of active expression of type III procollagen genes in one of the keloids suggests heterogeneity within these lesions. It was of interest to note that the keloid actively expressing the type III procollagen gene was the smallest in size in our clinical material and possibly had the most recent onset. Besides suggesting individual variability, these observations could indicate that type III collagen gene expression is only transiently activated during the development of a keloid lesion. The enhanced type I collagen gene expression and relatively low level of type III collagen gene expression appears to correlate with previous analyses of genetically distinct collagens within keloids. For example, collagen analyses by us have demonstrated that type I collagen represents approximately 95% of the total collagen in keloids; the corresponding value in normal human skin was found to be about 75% [4]. The relative concentration of type III

Figure 2. Demonstration of type I and type VI collagen gene expression in keloid tissue. *A* and *B*, Type I collagen gene expression in the proximity of *(A)* a blood vessel or *(B)* in association with cells of the blood vessel wall, as detected by in situ hybridizations with a proα1(I) collagen cDNA. The resolution of frame *B* does not allow definitive identification of the cell type containing the autoradiographic grains, but their location is consistent with their identity as endothelial cells *(arrows)*. *C*, Type I procollagen gene is expressed in the periphery of an inflammatory cell infiltrate *(asterisk)*, as detected by the presence of autoradiographic grains representing proα1(I) collagen mRNA *(arrows)*. The morphology of the inflammatory cell infiltrate was partially lost during the processing of the specimen for in situ hybridization, but originally was similar to that noted in Fig 6*A* and *D*. *D*, Demonstration of type VI collagen gene expression in the proximity of a small blood vessel within the keloid tissue. The presence of α2(VI) collagen mRNA is detected by in situ hybridization. The section is longitudinal to the blood vessel lumen (L). *Bars: A*, 50 μm; *B–D*, 100 μm.

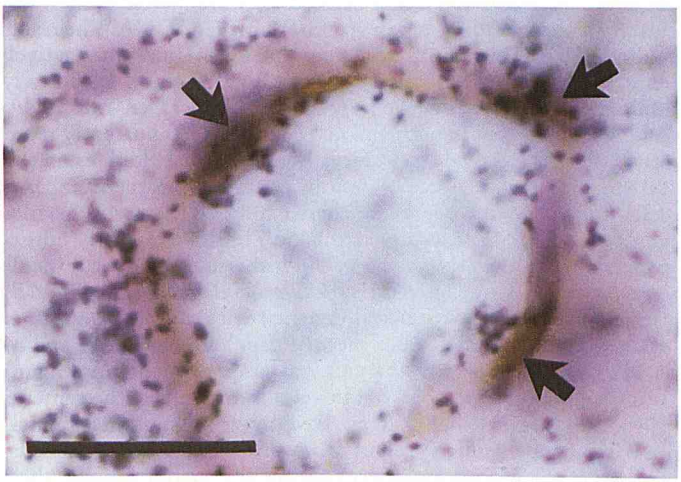


Figure 3. Simultaneous detection of proα1(I) collagen mRNA and factor VIII-related antigen epitopes in keloid tissue. Endothelial cells can be identified by a positive staining reaction for factor VIII-related antigen *(arrows)*, whereas autoradiographic grains represent ^{32}P-labeled cDNA/mRNA hybrids. Note the strong hybridization signal for type I procollagen within the endothelial cells. Hematoxylin counterstain. *Bar,* 20 μm.

collagen in keloids was ~4%, compared to ~21% in the control skin [4].

As indicated above, type VI collagen gene expression has not been previously studied in keloids. Thus, parallel sections from the keloid tissues were subjected to in situ hybridizations with a human α2(VI) collagen cDNA. The results indicated that cells in the mid or lower dermis, and within the keloid lesions themselves, contained a large number of grains representative of the α2(VI) collagen mRNA (Fig 4). Furthermore, areas immediately adjacent to the vessel walls contained α2(VI) collagen mRNA, in a manner similar to that observed with a proα1(I) collagen cDNA (Fig 2*D*). In contrast to the observations made with type I procollagen cDNA, most cells in the papillary dermis also actively expressed the α2(VI) collagen gene (Fig 4*A*). Thus, the active expression of type I and type VI collagen genes in general, had similar spatial distribution within the affected tissues. In addition, type VI collagen mRNA was abundantly present in a number of cells in the papillary dermis, an area that was largely devoid of type I collagen mRNA (Fig 1*B* and 4*A*). This disparity between type I and type VI collagen gene expression may relate to the differences between papillary and reticular fibroblasts [45,46].

Enhanced expression of type VI procollagen gene has been noted recently in different fibrotic lesions, including cutaneous neurofibromas, progressive systemic sclerosis, and eosinophilic fasciitis [16,47,48]. Type VI collagen has been shown to localize into distinct microfibrils within the dermal connective tissue, and it has been suggested that these fibers play a role in the organization of the extracellular matrix [15]. Thus, the presence of type VI collagen in keloids may contribute both to the physical properties of the extracellular matrix and to the clinical characteristics of keloids.

Quantitation of Collagen mRNA To quantitate the relative concentrations of type I, III, and VI procollagen mRNA, total RNA was extracted from keloid tissues, and the collagen mRNA steady-state levels were determined by slot-blot hybridizations as illustrated in Fig 5. When compared to normal human skin, samples of three keloids demonstrated enhanced levels of type I and VI collagen mRNA. On the average, proα1(I) collagen mRNA levels were two- to threefold higher, and α1(VI) collagen mRNA levels were four- to sixfold higher in keloids than in controls. The levels of type proα1(III) collagen mRNA were also increased in keloids about 1.4 times. When normalized by the levels for type III procollagen mRNA, the type I procollagen mRNA levels were increased by

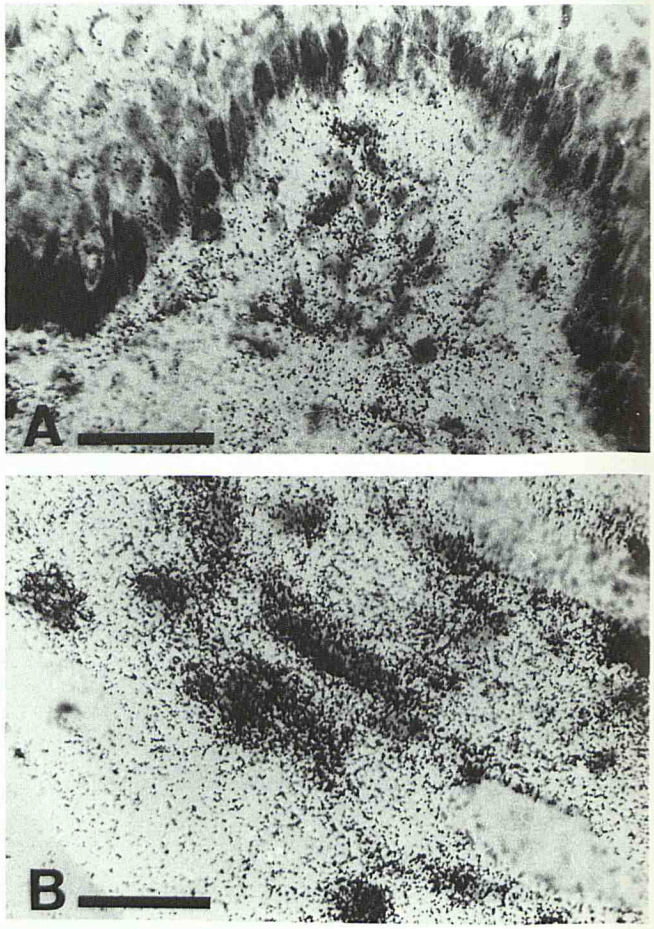


Figure 4. Demonstration of type VI collagen gene expression in keloid tissue and papillary dermis. In situ hybridizations with an α2(VI) collagen cDNA reveals an abundance of autoradiographic grains in the *(A)* papillary dermis and *(B)* deep within the keloid lesion. *Bars: A* and *B,* 50 μm.

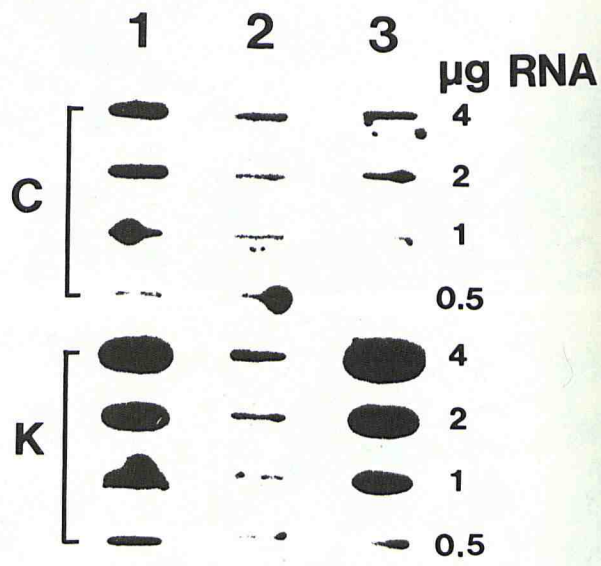


Figure 5. Quantitation of type I, III, and VI collagen mRNA steady-state levels in keloid tissue specimens by slot-blot hybridizations. Total RNA was isolated from (K) a keloid or from (C) a specimen of skin from a sex- and age-matched individual. Varying amounts of RNA (4.0–0.5 μg) were applied to nitrocellulose and hybridized either with proα1(I), proα1(III) or α1(VI) collagen cDNA. Note the significantly stronger signal detected in keloid RNA preparation with proα1(I) and α1(VI) collagen cDNA, compared with control RNA hybridized in parallel.

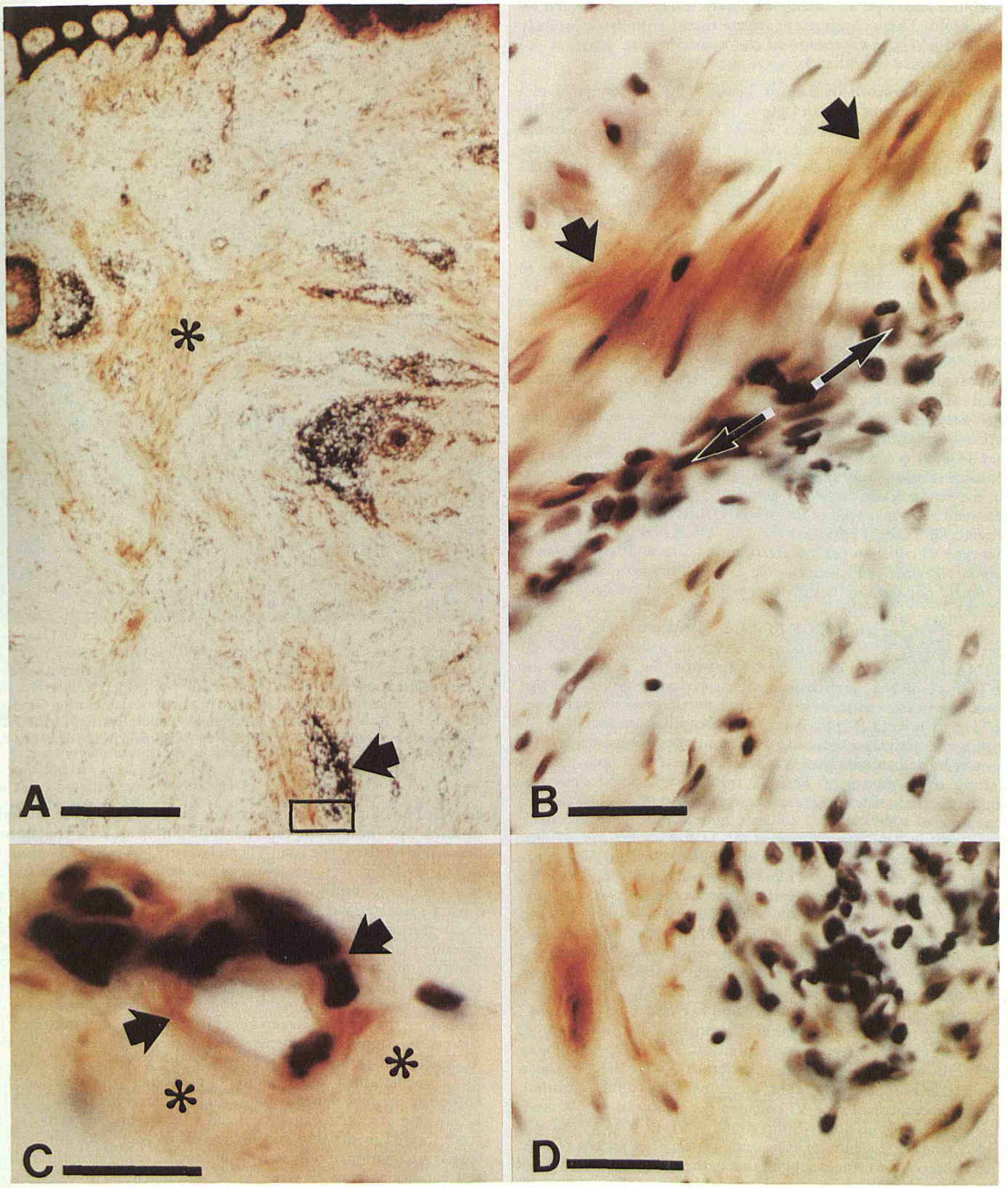


Figure 6. Demonstration of TGF-β1 epitopes within a keloid lesion by peroxidase antiperoxidase immunodetection. *A*, Note the presence of TGF-β1 epitopes, as detected by the brownish deposition of DAB precipitates, in the periphery of the keloid lesion *(asterisk)*, in a similar distribution noted for the activation of type I and type VI collagen genes (see Figs 1, 2, and 4). *B*, The presence of TGF-β1 epitopes in association with spindle-shaped fibroblastic cells *(large arrows)* in the proximity of a blood vessel cut in longitudinal orientation *(small arrows)*. *C*, TGF-β1 epitopes were also detected in association with a blood vessel either directly on the endothelial cells *(arrows)* or diffusely in the proximity of the vessel *(asterisks)*. *D*, Higher magnification of the area outlined in *A* reveals the presence of TGF-β1 epitopes in the proximity of an inflammatory cell infiltrate consisting predominantly of mononuclear cells. *Bars: A*, 500 μm; *B* and *D*, 50 μm; *C*, 25 μm.

~twofold and type VI collagen mRNA levels were elevated by ~threefold. These observations are consistent with the apparently increased levels of expression of the corresponding genes, as detected by in situ hybridizations (see above).

TGF-β1 Expression in Keloid Tissue TGF-β1, member of a family of pleiotropic growth factors, has been shown to up-regulate collagen gene expression in a variety of experimental situations [20,21,49]. To examine the possible role of TGF-β1 in the activation of collagen gene expression in keloids, the presence of TGF-β1 mRNA was examined by in situ hybridizations, and the corresponding protein epitopes were detected by peroxidase antiperoxidase method. The presence of TGF-β1 protein was demonstrated by immunodetection in the same general areas of the keloid lesions that displayed elevated levels of type I and type VI collagen mRNA (Fig 6). Specifically, relatively large areas containing the TGF-β1 epitopes could be demonstrated in the upper parts of the keloid lesions bordering the lower dermis, areas corresponding to active type I and VI collagen gene expression (Fig 6*A*). Also, TGF-β1 protein could be demonstrated in close proximity to blood vessels in the association of fibroblastic cells (Fig 6*B*) and of cells in the vessel wall of small capillaries (Fig 6*C*). The spatial distribution of TGF-β1 protein in these locations correlates well with type I procollagen gene expression in similar locations (see Figs 2*A, B, D*). It should be noted that TGF-β1 could not be detected in the adjacent normal skin by the same methodology and in the same tissue specimens. In particular, the papillary dermis, which actively expressed the α2(VI) collagen gene but was devoid of the proα1(I) mRNA, did not reveal the presence of TGF-β1 epitopes (Fig 6*A*). These observations suggest that type VI collagen gene expression is endogenously activated in papillary dermis and further attest to the differences between the fibroblast populations within the papillary and reticular dermis [45,46].

Hybridizations with a TGF-β1 cDNA, labeled with ^{32}P to a high specific activity ($> 10^9$ cpm/μg), revealed the presence of TGF-β1 mRNA in distinct regions of the keloid tissue (Fig 7). Also, the use of TGF-β1 cRNA, with enhanced sensitivity, demonstrated that fibroblasts in areas adjacent to the vascular wall showed the presence of TGF-β1 mRNA (Fig 8). Furthermore, capillary endothelial cells within the actively expanding border of the lesions displayed a positive hybridization signal (Fig 8). In summary, the areas demonstrating the presence of TGF-β1 mRNA and protein epitopes within the keloid lesion were in general the same that showed activation of type I and VI collagen gene expression (see above). Thus, cells in keloid tissue express the TGF-β1 gene, and the production of this growth factor may up-regulate the expression of the matrix genes encoding type I and VI collagens. In support of this suggestion are previous observations indicating that type I procollagen gene expression is markedly enhanced by TGF-β in a variety of in vivo and in vitro situations [20,21]. In particular, type I collagen gene expression has been shown to be up-regulated by TGF-β, and it has been suggested that this activation occurs primarily on the transcriptional level of gene expression [49]. In addition to enhanced transcriptional activity, TGF-β may lead to elevated levels of type I procollagen mRNA by stabilizing the corresponding mRNA [49]. In case of type VI collagen gene expression, TGF-β has been found specifically to increase the expression of the α3(VI) collagen gene in normal skin fibroblast cultures (T. Krieg, personal communication). The enhancement of type VI collagen gene expression in keloids was detected by in situ hybridizations with an α2(VI) collagen cDNA, and by slot-blot analysis with an α1(VI) collagen cDNA. These observations would suggest that in the case of keloids, TGF-β may up-regulate the gene expression for all three subunit polypeptides of type VI collagen.

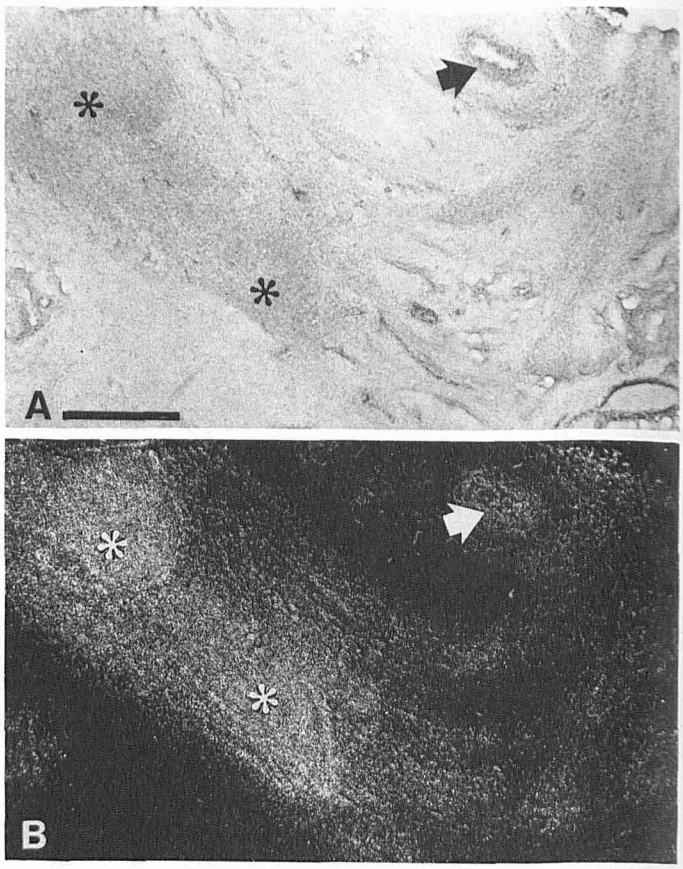


Figure 7. Expression of TGF-β1 gene in a keloid lesion, as detected by in situ hybridization with a human sequence-specific cDNA. Note the presence of autoradiographic grains, representative of [^{32}P]cDNA-mRNA hybrids, in a diffuse area *(asterisks)* and also in a perivascular location *(arrows)*. The picture corresponds to the approximate middle portion of the full-thickness tissue section shown in Fig 1*A*. *B*, A darkfield image of *A*. *Bars: A* and *B*, 500 μm.

DISCUSSION

Our study presented here indicates that type I and type VI collagen gene expression is activated in keloids. Similar enhancement of collagen gene expression has been previously noted in other fibrotic diseases of the skin. However, the spatial distribution of cells with activated phenotype in keloids is quite different from other fibrotic diseases. Specifically, the regional distribution of cells containing detectable levels of type I and VI collagen mRNA in keloid lesions is in a marked contrast to observations made on cutaneous neurofibromas, another example of fibrotic skin lesions [16,26]. Similar studies with a proα1(I) and α2(IV) collagen cDNA with neurofibroma tissue sections revealed scattered distribution of cells actively expressing these collagen genes throughout the lesions [16,26]. These observations suggested that factors responsible for the activation of type I procollagen gene expression in neurofibromas may be more diffusely distributed within the lesion. Regional distribution of cells expressing the type I collagen genes has also been demonstrated in cutaneous lesions from patients with progressive systemic sclerosis, morphea, and eosinophilic fasciitis [22,50]. In the case of eosinophilic fasciitis, such cells were found to reside in the lower dermis bordering the subcutaneous adipose tissue and in the fascia [22,51]. In the case of systemic and localized scleroderma, the active cells were located in the mid and lower dermis [22]. The differential spatial distribution of cells actively producing collagen in these clinical conditions may give rise to an accumulation of collagen in different compartments of the skin and adjacent structures, thus explaining variable clinical phenotypes of these cutaneous lesions.

An intriguing observation on keloids in our study was the activation of type I and VI collagen gene expression in close proximity of blood vessels and even on the endothelial cells of small capillaries. The enhanced collagen gene expression was accompanied by the presence of TGF-β1 mRNA and protein epitopes in the same distribution. These observations raise the possibility that vascular damage

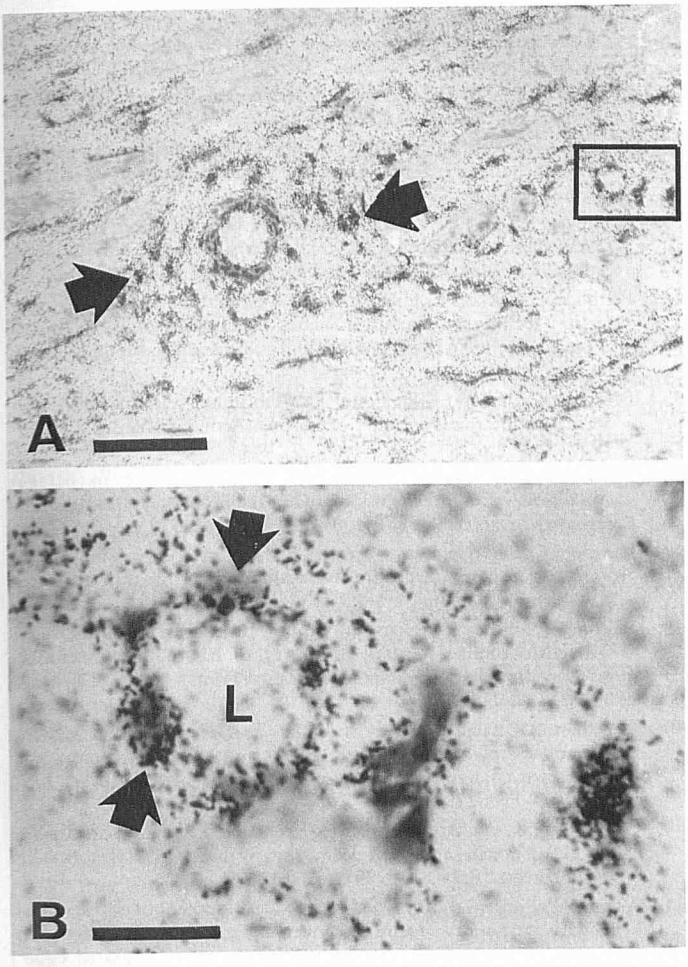


Figure 8. Demonstration of TGF-β1 gene expression in keloid tissue. In situ hybridizations were performed with a single-stranded cRNA probe. Note that the autoradiographic grains, representative of [^{32}P]cRNA-mRNA hybrids, are associated with *(A)* fibroblasts *(arrows)*, and with *(B)* endothelial cells of capillaries *(arrows)*. *L*, the lumen of the blood vessel. The area outlined by *rectangular box* in *A* is enlarged in *B*. *Bars: A*, 100 μm; *B*, 20 μm.

may be one of the early events leading to keloid formation, in a similar manner proposed for cases of progressive systemic sclerosis [52]. TGF-β would then serve as a mediator resulting in the fibrotic tissue reaction, and thus intimately participating in the pathogenesis of keloids. It should be noted that in addition to activating gene expression, TGF-β could enhance collagen deposition through an alternate, and potentially complementary, mechanism, *viz* inhibition of collagenase activity [53,54]. Furthermore, different members of the TGF-β family may be operative in different diseases. For example, in case of keloids, as shown in this study, the primary form of TGF-β appears to be TGF-β1, whereas in the case of progressive systemic sclerosis the predominant form is TGF-β2 [22,23,55]. Nevertheless, the initial TGF-β–mediated reaction in the endothelial cells could then be propagated by TGF-β gene expression in fibroblasts in the adjacent extracellular matrix. The latter suggestion is consistent with the demonstration that TGF-β1 can positively regulate its own expression in fibroblasts in an autocrine manner [56]. This cascade reaction would manifest clinically as an actively expanding keloid.

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