Co-Localization of Transforming Growth Factor β2 with α1(I) Procollagen mRNA in Tissue Sections of Patients with Systemic Sclerosis

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Abstract

The role of transforming growth factor β2 (TGF-β2) in the pathogenesis of systemic sclerosis (SSc) was investigated by in situ hybridization of skin biopsies from six patients with SSc. Two patients with acute systemic lupus erythematosus (SLE), one with acute dermatomyositis (DM), and three healthy individuals were used as controls. TGF-β2 mRNA was found to be co-localized with proα1(I) collagen expression around dermal blood vessels in all patients with the inflammatory stage of SSc, whereas there was no expression of either gene in the dermis of patients in the fibrotic stage, the SLE patients or the normal controls. These findings provide evidence that TGF-β2 released by inflammatory cells around blood vessels may play a role in mediating the collagen gene disregulation in fibrosis. (*J. Clin. Invest.* 1990. 86:917–922.) Key words: TTS-beta • collgen synthesis • fibrosis • systemic sclerosis

Introduction

Collagen synthesis in fibroblasts is physiologically induced during wound healing or after inflammatory damage of tissue. However, inadequate collagen production can lead to fibrosis and severe impairment of organ function. Systemic sclerosis (SSc)[1] is a disease characterized by excessive deposition of connective tissue proteins (1). In the initial stages of SSc a perivascular mononuclear cell infiltrate is found in the dermis and other involved organs and is associated with increased collagen synthesis in the surrounding fibroblasts (2–4). The cause for this increased collagen synthesis is unknown.

Growth factors released from inflammatory cells have long been implicated in the pathogenesis of fibrosis (1, 3–6). Due to the recent availability of purified preparations of lymphokines, a clear picture has emerged as to how these components can influence collagen synthesis in cultured fibroblasts. In vitro, transforming growth factor-β (TGF-β) was found to increase the gene expression of collagen type I and type III (6–8), whereas interferon-gamma showed the opposite effect (9). TGF-β is known to be present in two highly homologous forms, TGF-β1 and TGF-β2 (10). It is secreted by activated macrophages (10) and lymphocytes (11, 12) and is found in large amounts in platelets (10). In vivo, TGF-β produces rapid fibrosis and angiogenesis when injected subcutaneously into newborn mice (5). Recently, TGF-β2 rather than TGF-β1 was found in high levels in intraocular fluid aspirates from patients with intraocular fibrosis (13). Furthermore, TGF-β1 could not be detected in tissue sections from patients with the inflammatory stage of SSc (14). To investigate the role of TGF-β2 in the pathogenesis of fibrosis in SSc, we have cellularly localized TGF-β mRNA in biopsies obtained from patients with different stages of SSc.

In this paper we report the co-localization of collagen type I and TGF-β2 gene expression around dermal blood vessels in inflammatory stages of SSc and not in the inflammatory infiltrate of SLE, DM, and controls, providing further evidence that TGF-β2 may be one of the factors playing an important role in the pathogenesis of fibrosis.

Methods

Biopsies were taken from affected sites of six patients with SSc, two patients with acute SLE, one with acute DM, and from three healthy individuals after obtaining informed consent. The diagnosis of SSc was confirmed by histological examination of paraffin embedded biopsies and the criteria of the American Rheumatism Association (15). Clinical data on the patients are shown in Table I. Diagnosis of SLE and DM was made by histological examination as well as immunological investigations. The SLE biopsies were taken from the forearm of a 21- and a 53-yr-old female patient, respectively. The DM biopsy was taken from the thigh of an 11-yr-old girl. The patients with SLE and DM had acute skin lesions and were on no treatment at the time of the biopsies. Control sections were taken from one male and two female healthy volunteers aged 48, 34, and 39 yr. The biopsy sites were upper arm, thigh, and forearm, respectively.

RNA isolation. Human adult keratinocytes were isolated from a healthy individual according to standard procedures (16), total RNA was isolated by the guanidinium isothiocyanate-cesium chloride method (17), and poly (A)+ RNA was prepared from the keratinocytes using oligo-dT-cellulose (Collaborative Research Inc., Bedford, MA) following standard procedures (18).

Preparation of RNA probes. The TGF-β2 clone was kindly donated by Sandoz (Basel, Switzerland). A 859-bp fragment from the 3′ end of the original G-TsF clone enclosing the coding sequence for the mature peptide (19) was subcloned into the Hind III/Eco RI site of the Gemini 3 vector (Promega Biotec, Madison, WI). The subcloning of proα1(I) collagen has been described previously (3). A differentiation associated keratin probe (K10) was used as a control in hybridization to demonstrate the integrity of mRNA (3). Following linearization with an appropriate restriction enzyme, in vitro transcription was carried out as published previously using $[^{35}S]\alpha$-UTP (New England Nuclear, DuPont, Dreieich, FRG) (3) for in situ and $[^{32}P]\alpha$-ATP (Amersham International, Amersham, UK) for Northern blot hybridization.

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[1] *Abbreviations used in this paper:* DM, dermatomyositis; HE, hematoxylin-eosin; PDGF, platelet-derived growth factor; poly(A)+RNA, polyadenylated enriched RNA; SSc, systemic sclerosis; TGF-β, transforming growth factor beta.

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Table I. Patients Characteristics

Patient	Sex	Age	Duration	Biopsy site	Stage of disease	Lymphocytic infiltrate
—			*yr*			
B.W.	F	45	5	Lower leg	Inflammatory	+++
Y.K.	F	44	0.5	Forearm	Inflammatory	+++
H.S.	M	40	1	Neck	Inflammatory	+++
W.H.	M	54	5	Chest	Inflammatory sclerotic	+
E.B.	F	45	5	Forearm	Sclerotic	+/−
F.E.	F	71	0.5	Forearm	Sclerotic	−

Northern blot analysis. Poly(A)+ RNA was separated in a 1% agarose gel under denaturing conditions using formaldehyde. The RNA was transferred onto Hybond-N membrane (Amersham International) and ultraviolet crosslinked to the filter according to the suppliers' protocol. The filter was then hybridized at 42°C overnight with antisense or sense TGF-β2 riboprobes. After washing the filters following the suppliers' protocol, they were incubated with RNase A (2 μg/ml; Sigma Chemical Co., St. Louis, MO) and RNase T1 (0.02 μg/ml; Boehringer, Mannheim, FRG) in 2× SSC (20× SSC is 3 M NaCl/0.3 M Na3 citrate) for 30 min at 37°C. Filters were then washed again as above and autoradiographed for 14 d.

In situ hybridization. In situ hybridization was carried out on cryosections of the same biopsies as used for histological examination. The detailed methods are described elsewhere (3). Briefly, 5-μm frozen sections mounted on Poly-*l*-lysine-coated slides (100 μg/ml, Sigma Chemical Co.) were hybridized with riboprobes for TGF-β2, proα1(I) collagen and keratin. After hybridization the slides were washed and treated with RNase to remove nonspecific binding. The slides were washed again and dried by passage through ethanol series. Subsequently, slides were dipped into Kodak NTB2 emulsion (Eastman Kodak Co., Rochester, NY) and exposed for 3 d at 4°C. Slides were developed in D19 (Kodak) and sections were stained with hematoxylin-eosin (HE).

Immunohistochemistry. 5-μm cryosections were cut and air dried for 30 min. Immunologic reactions were carried out using vimentin and von Willebrand's factor as well as BMA 120 as described elsewhere (20).

Results

The histology of all patients with SSc revealed closely packed, thick collagen bundles in the dermis. Three patients (B.W., H.S., and Y.K.) showed clinical and histological signs of the inflammatory stage of SSc with a marked lymphocytic infiltrate around deep dermal blood vessels (Table I). In two cases (H.S. and Y.K.) the lymphocytic infiltrate was also evident around superficial blood vessels. Patient W.H. showed an intermediate stage of SSc in that there were few but distinct mononuclear cells around dermal blood vessels. Patient E.B. and F.E. represented a sclerotic stage of SSc. The dermal collagen was thickened and closely packed, and in some areas appeared amorphous. A mild mononuclear infiltrate was seen in patient E.B. but was absent in patient F.E. In particular, the blood vessels in patient E.B. were unaffected.

The SLE and DM biopsies showed a typical lymphocytic infiltrate along the dermo-epidermal junction and of the superficial and deep blood vessels as well as hydropic degeneration of the basal cell layer.

In patients B.W., Y.K., and H.S. the TGF-β2 expression was seen around inflamed dermal blood vessels (Fig. 1, *a* and *b*). Hybridization with the proα1(I) collagen chain probe revealed the same pattern of distribution (Fig. 2, *a* and *b*). In patient W.H. there was less TGF-β2 and proα1(I) gene expression compared to patients in the active stages of SSc. Again the

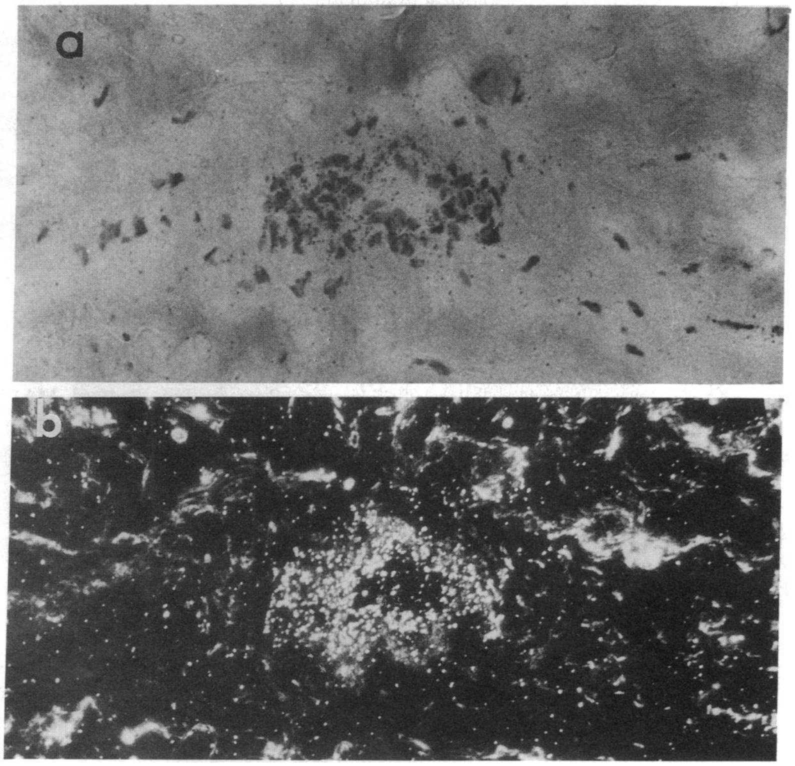

Figure 1. In situ hybridization of SSc skin with the TGF-β2 antisense probe. (*a* and *b*) Bright and dark field micrograph of frozen section of a biopsy from a patient with inflammatory stage of SS showing intense labeling of the blood vessel indicating high levels of TGF-β2 mRNA. ×216. 5-μm frozen sections from biopsies were hybridized with [^{35}S]UTP labeled riboprobes, washed under stringent conditions and treated with RNase to remove nonspecific hybridization. Sections were subjected to autoradiography and stained with HE.

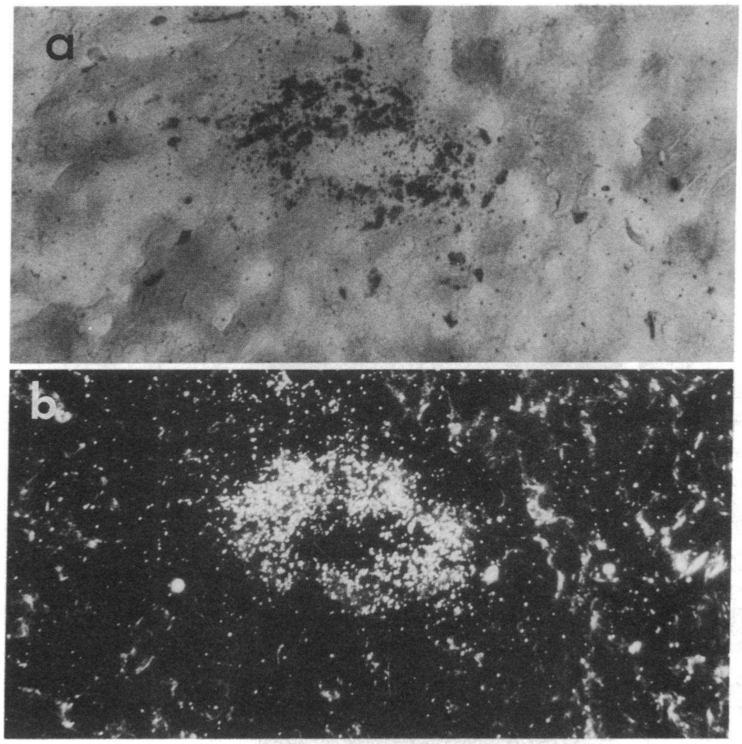

Figure 2. In situ hybridization with proα1(I)collagen antisense probe of a parallel section using the same biopsy as for Fig. 1 showing intense labeling around a deep dermal blood vessel. Bright field and dark field photograph. ×216. Same procedure as under Fig. 1.

distribution of signals generated by both probes were similar and mainly located around distinct dermal blood vessels.

In patient E.B. the TGF-β2 probe labeled cells around very few deep and superficial dermal blood vessels and was less intense than in patient W.H.

In cryosections from patient F.E., from the SLE patients, and from the normal controls neither expression of TGF-β2 nor proα1(I) collagen was present in inflammatory cells or around dermal blood vessels (Fig. 3). In cryosections from the young girl with DM no TGF-β2 mRNA was found in the dermal infiltrate.

The integrity of the RNA in all biopsies was demonstrated by specific hybridization of the keratin probe to cells in the supra-basal layers of the epidermis (Fig. 4 *a*). Furthermore, there was specific labeling of basal cells with the TGF-β2 probe in all specimens from SSc patients and from the normal individuals, thus serving as an internal control for the integrity of the probe as well as the mRNA within the section (Fig. 4, *b* and *c*).

The specificity of the TGF-β2 riboprobes was demonstrated by Northern blot analysis of poly(A)+ RNA from cultured keratinocytes. The antisense probe hybridized to mRNA of 4,000 and 6,000 bp in length, which is in agreement with the published data (19). The sense probe did not give any autoradiographic signal (data not shown).

Immunohistochemistry showed an abundance of fibroblasts throughout the dermis, and in particular around blood vessels, while very few endothelial cells were identified in the very center of the blood vessels.

Discussion

The pathophysiological events of fibrosis in SSc are not yet fully understood. There is evidence that the excessive accumulation of type I and type III collagen in the affected organs is due to increased collagen gene expression (1, 3, 21). This appears to be induced by external factors (22). In situ hybridization of skin biopsies from patients with SSc has demonstrated specific enhancement of collagen mRNA in dermal fibroblasts (3, 4). In the early edematous phase of the disease the activated cells are found around small blood vessels in the deep dermis and the fatty tissue near the inflammatory infiltrates.

In vitro studies have shown that mononuclear cells as well as platelets synthesize growth factors such as platelet-derived growth factor or TGF-β, both of which are known to induce collagen synthesis and fibroblast proliferation (6–8, 22). TGF-β is present in two highly homologous forms, termed TGF-β1 and TGF-β2, and the effects of TGF-β observed in vitro and in vivo may be attributed to a combination of the two forms (10). Both TGF-β1 and TGF-β2 have been shown to similarly increase the activity of the collagen α2(I) gene promoter (23). However, TGF-β2 rather than TGF-β1 has been found in vitreous aspirates from patients with intraocular fibrosis (13), and TGF-β1 could not be detected in tissue sections from patients with SSc (14). Therefore, our working hypothesis has been that TGF-β2 may also be involved in the pathogenesis of fibrosis in SSc. In our study we found a co-localization of TGF-β2 mRNA with proα1(I) mRNA around dermal blood vessels surrounded by a mononuclear cell infiltrate.

Since ^{35}S-labeled riboprobes penetrate beyond a single cell border and stringent washing procedures alter histological characteristics of cells, the exact identification of the cell type expressing TGF-β2 mRNA is difficult. The cells that are present in and around these inflamed blood vessels are endothelial cells, platelets, monocytes, and fibroblasts. Using immunohistochemical staining in SSc the close proximity of these cells

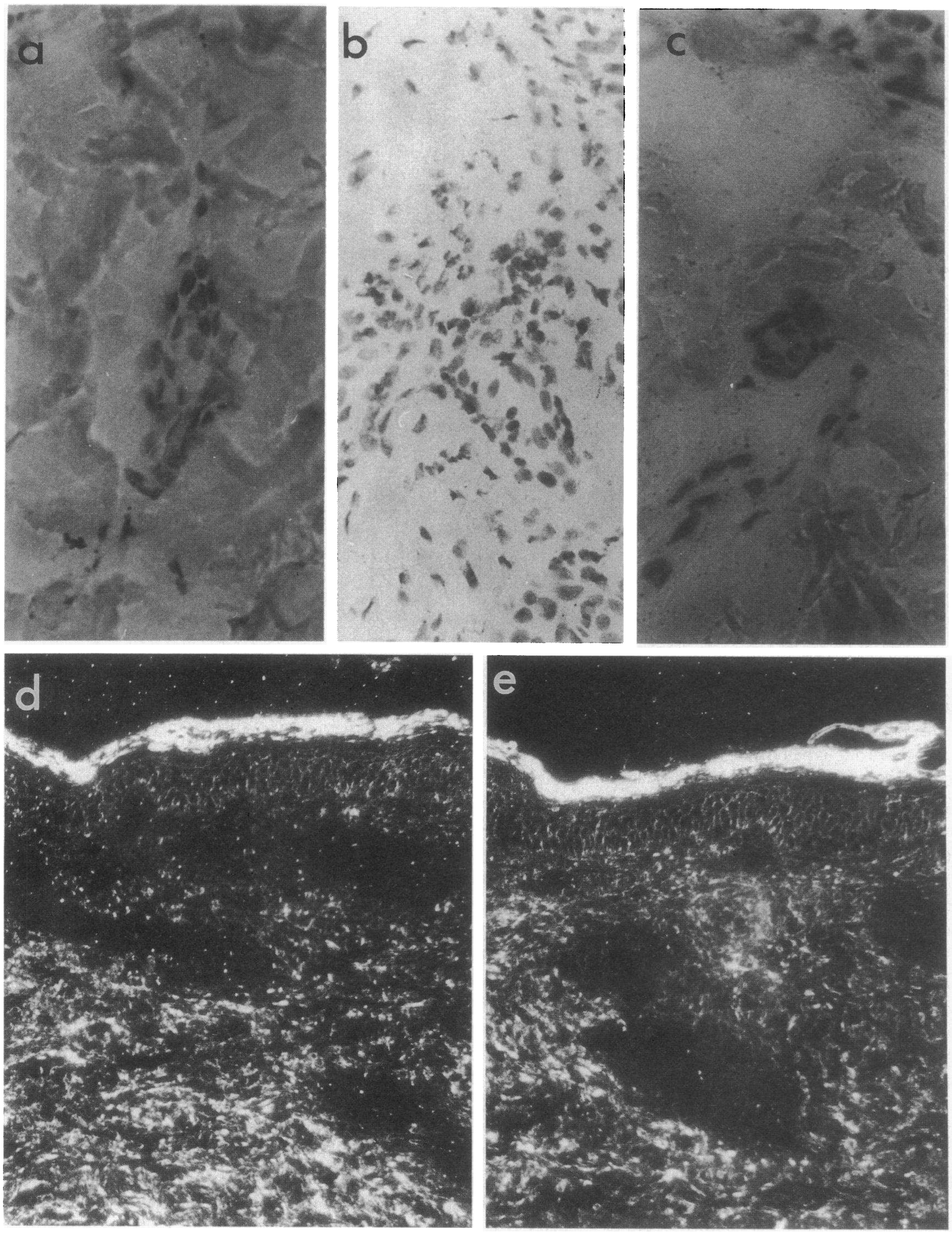

Figure 3. In situ hybridization of control (*a, c*) and SLE (*b, d, e*) with TGF-β2 and proα(I) collagen probes. Same procedure as under Fig. 1. (*a*) No expression of the TGF-β2 probe is seen around a blood vessel in the control section. ×324. (*b*) No expression of the TGF-β2 probe is found in the inflammatory infiltrate in a patient with acute SLE. ×270. (*c*) Blood vessel without grains in a normal control section indicating that there are no detectable levels of proα(I) mRNA. ×270. (*d*) Dark field photograph of a section from a patient with SLE labeled with the TGF-β2 probe showing only background labeling, which indicates no expression of TGF-β2 mRNA in SLE. ×144. (*e*) Dark field photograph of a parallel section labeled with the proα1(I) collagen gene expression in SLE showing no more than background labeling. ×144.

around blood vessels is apparent and very few cells stain with the endothelial cell markers. Since human platelets only contain TGF-β1 they are not the source of TGF-β2. Alterations of endothelial cells in SSc are well known features indicating that these cells may release mediators, which either directly act on fibroblasts or activate mononuclear cells. However, endothelial cells seem unlikely to be the major source of TGF-β2 since there are very few in the sections and a large number of cells far away from the vessels express TGF-β2. Fibroblasts in vitro do not express TGF-β2 (Kulozik, M., unpublished results). All

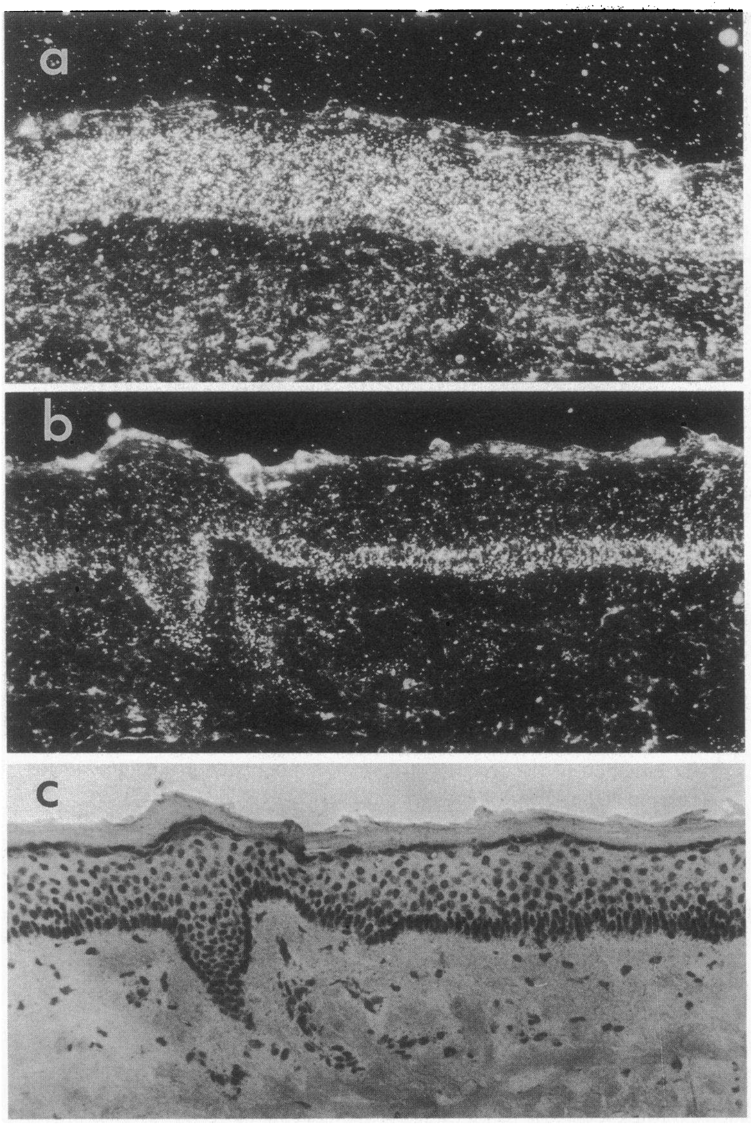

Figure 4. In situ hybridization of SSc skin with the keratin (*a*) and TGF-β2 antisense probe (*b, c*). Same procedure as under Fig. 1. (*a*) Dark field micrograph of hybridization with keratin riboprobe showing uniform labeling of the epidermis. ×144. (*b, c*) Dark field and bright field photograph of a parallel section with the TGF-β2 antisense probe demonstrating intense expression of TGF-β2 mRNA in basal cells. ×144.

these data indicate that inflammatory cells are the major source of TGF-β2 expression. However, since a coexpression of TGF-β2 and proα1(I) collagen mRNA was present in the same sites, our observation may allow an alternative interpretation. We cannot totally exclude that activated fibroblasts also express TGF-β2 in vivo acting in an autocrine fashion. Our controls consisted of normal skin, SLE, and DM with inflammatory infiltrates. In none of these biopsies was TGF-β2 mRNA found. These controls indicate that TGF-β2 is specifically transcribed in the inflammatory infiltrate of SSc and not in SLE and DM. Although the prime event in SSc as well as the events leading to a transcriptional activation of TGF-β2 remain unknown, we presume from our data that induction of TGF-β2 leads to an increased transcription of proα1(I) in fibroblastic cells surrounding the inflamed blood vessels.

Since a transcriptional regulation of TGF-β2 has been shown in activated T lymphocytes (12), it appears likely that the increased mRNA levels seen here reflect enhanced TGF-β2 protein synthesis. However, TGF-β2 is secreted in a latent form and can be activated by transient acidification in vitro (24). It is interesting to note that Connor et al. found that 87% of TGF-β in vitreous aspirates of eyes with intraocular fibrosis was latent TGF-β but that the degree of fibrosis correlated well with the levels of TGF-β in the aspirates (10). This probably indicates that most of TGF-β becomes activated but that the activated form is shortlived and thus can only be detected in small quantities.

TGF-β2 induces collagen type I gene expression in vitro (6–8). It is the major form of TGF-β in intraocular fibrosis (13). However, it should also be noted that TGF-β has a variety of other activities that may also contribute to the pathogenesis of fibrotic processes. It is a potent chemotactic ligand for fibroblasts (10) and monocytes and induces the gene expression of interleukin 1 in the latter (25). This factor is known to stimulate fibroblast proliferation (25). In addition, TGF-β may inhibit the degradation of the extracellular matrix by decreasing the secretion of proteases and increasing the production of protease inhibitors (10).

An unexpected finding was the expression of TGF-β2 mRNA in basal epidermal cells of normal as well as SSc skin (Fig. 4, *b* and *c*), which is supported by the identification of

TGF-$\beta 2$ mRNA in a Northern blot analysis using RNA prepared from primary human keratinocytes. The exact role of TGF-$\beta 2$ in the epidermis is not yet clear, but TGF-$\beta 1$ has been reported to be synthesized in an epidermal cell line (11) and has been shown to have antiproliferative effects on a variety of epithelial cells (10). It has therefore been implicated in the regulation of epidermal homeostasis. There is no induction of proα1(I) collagen in fibroblasts situated just below the epidermis. It is conceivable that TGF-$\beta 2$ is not activated or simply cannot penetrate the basement membrane and acts locally.

In summary, although the initial activation of TGF-$\beta 2$ transcription remains unknown, this study provides direct evidence that in SSc TGF-$\beta 2$ mRNA expression is localized around inflamed blood vessels in close proximation of fibroblasts characterized by a high expression of proα1(I) collagen mRNA. Since TGF-$\beta 2$ is also involved in intraocular fibrosis (13) the data suggest that TGF-$\beta 2$ might therefore play a general role in the pathogenesis of fibrotic processes.

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