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Expression of types I, III and IV collagen genes in fibrotic skin and nerve lesions of toxic oil syndrome patients

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SUMMARY

We have studied the skin and nerve fibrosis in toxic oil syndrome by *in situ* hybridization using specific cDNA probes for types I, III, and IV collagens. Fibroblasts with high levels of type I and III collagen mRNA were observed in biopsies from fibrotic skin areas. Similarly, type IV collagen mRNA was abundant in cells within the fibrotic process of the nerves. These results suggest that the excessive accumulation of collagen in toxic oil syndrome results from transcriptional activation of collagen genes in a subpopulation of fibroblasts.

Keywords collagen gene expression fibrosis scleroderma-like diseases

INTRODUCTION

Toxic oil syndrome (TOS) is considered to be a scleroderma-like disease associated with the consumption of adulterated rapeseed oil. After an acute phase, 10–15% of affected individuals developed a chronic disease characterized by symptoms overlapping with those in scleroderma, sicca syndrome, Raynaud's phenomenon and pulmonary hypertension, in addition to a sensory and motor peripheral neuropathy. Histopathological examination of tissue samples disclosed a non-necrotizing vasculitis and an increase in fibrous tissue in various organs [1].

Immunohistochemical studies of scleroderma skin have shown increased amounts of type I and III collagens [2]. By *in vitro* and *in situ* hybridization studies, it has been found that the excessive accumulation of collagen in scleroderma involves activation of procollagen type I and type III gene expression in fibroblasts [3,4]. In other scleroderma-like diseases, an elevated expression of type I collagen mRNA has also been described [5].

To address the question whether similar activation of collagen gene expression occurs in TOS, we have studied skin biopsies of these patients by *in situ* hybridization with type I and type III collagen cDNA probes. In addition, we have investigated the neural fibrosis using a cDNA probe for type IV collagen to evaluate the contribution of this basement membrane collagen to the fibrotic process.

MATERIALS AND METHODS

The paraffin blocks used in this study had been processed 7 years earlier for histopathological studies of TOS. Skin biopsies had been obtained from affected shoulder regions of five patients and nerve biopsies from sural nerve of two patients with severe peripheral neuropathy. Preparation of tissue slices onto silanated slides, and *in situ* hybridization were performed as described previously in detail [6].

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Preparation of cDNA probes

Clones pHCAL1U, pHFS3 [6] and HT-21 [7] containing pro-alpha 1 (I), pro-alpha 1 (III) and pro-alpha 1 (IV) collagens cDNA were used for generating specific cDNA probes. Inserts and lambda phage DNA fragments were labelled with ^{35}S-deoxyadenosinetriphosphate (dATP) by a multiprime DNA labelling system (Amersham International, Amersham, UK). Specific activity of the probes was of approximately 1×10^8 ct/min per microgram.

Hybridization

Sections of 5 μm were deparaffinized by washing in xylene and ethanol. The sections were pretreated by immersion in 0·2 N HCl for 15 min, and by acetylation with 0·25% acetic anhydride in 0·1 M triethanolamine, pH 8·0 for 10 min. Slides were rinsed in distilled H_2O, sequentially dehydrated in ethanol and air dried. The hybridization mixture contained 0·1 μg/ml ^{35}S-labelled probe, 10 mM dithiothreitol (DTT), 1 mg/ml bovine serum albumin (BSA), 0·6 M NaCl, 50% formamide, 10% (w/v) dextran sulphate, 0·2 mg/ml sonicated calf thymus DNA, 0·02% (w/v) Ficoll, 0·02% (w/v) polyvinyl pyrrolidone, 10 mM Tris (pH 7·4), 0·5 mM EDTA. The mixture was heated to 100°C for 1 min, and cooled on ice. Aliquots of 20–60 μl (depending on sample size) were applied onto each slide, covered with siliconized coverslips, and sealed with rubber cement. The slides were hybridized at 42°C for 50 h.

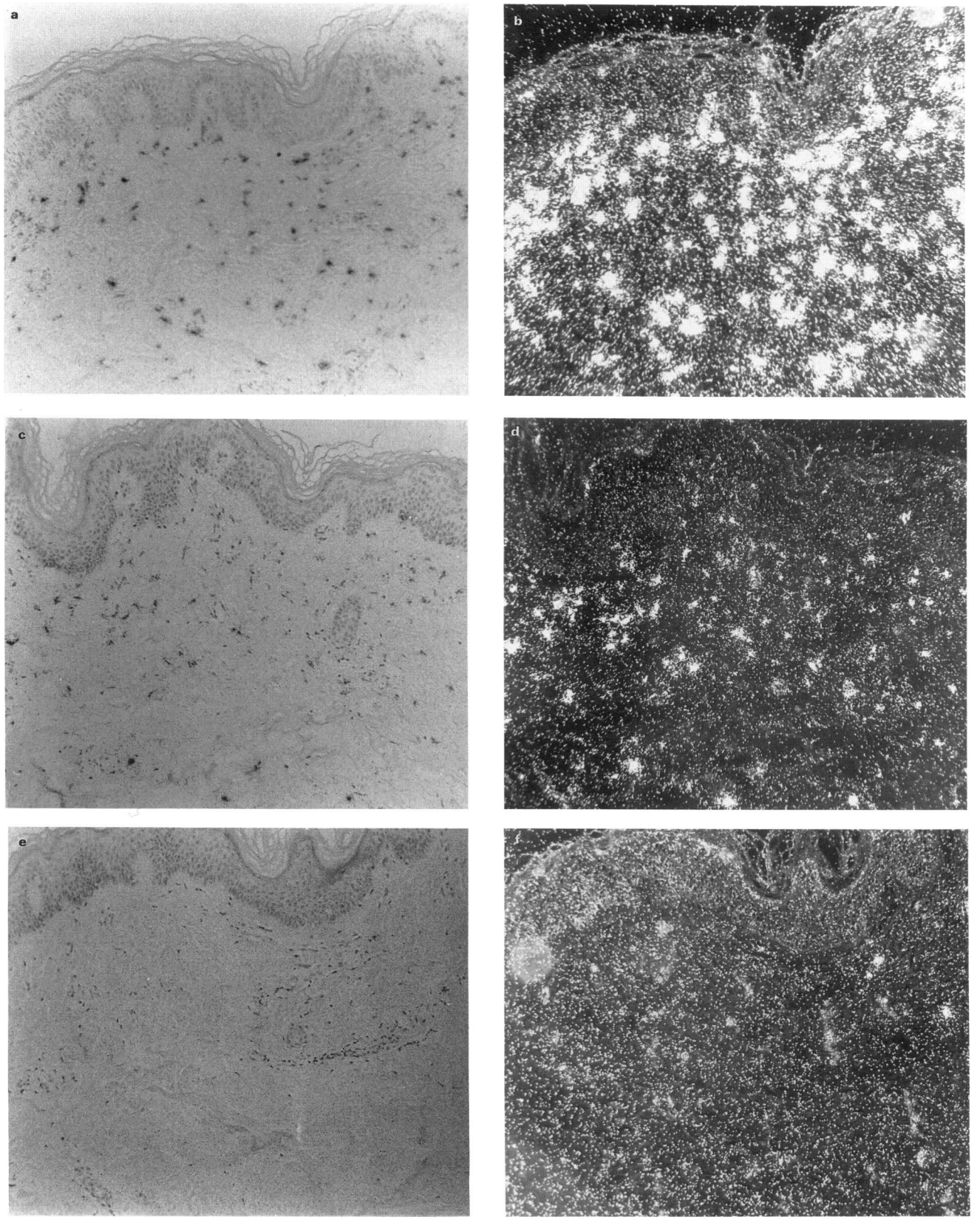

Fig. 1. *In situ* hybridization of skin sample from one toxic oil syndrome (TOS) patient with type I and type III procollagens ^{35}S-labelled cDNA. Hybridization was visualized by autoradiography and the sections counterstained with Gill's haematoxylin. (a) and (b) show abundant autoradiographic grains in upper dermis corresponding to type I procollagen mRNA. (c) and (d) show milder expression of grains, which are representative of type III procollagen mRNA. (e) and (f) show the dermis hybridized with ^{35}S-labelled lambda-DNA, devoid of grains. (b), (d) and (f) on the right show dark-field images of the tissue sections of the left (a), (c) and (e). (Original magnification × 10.)

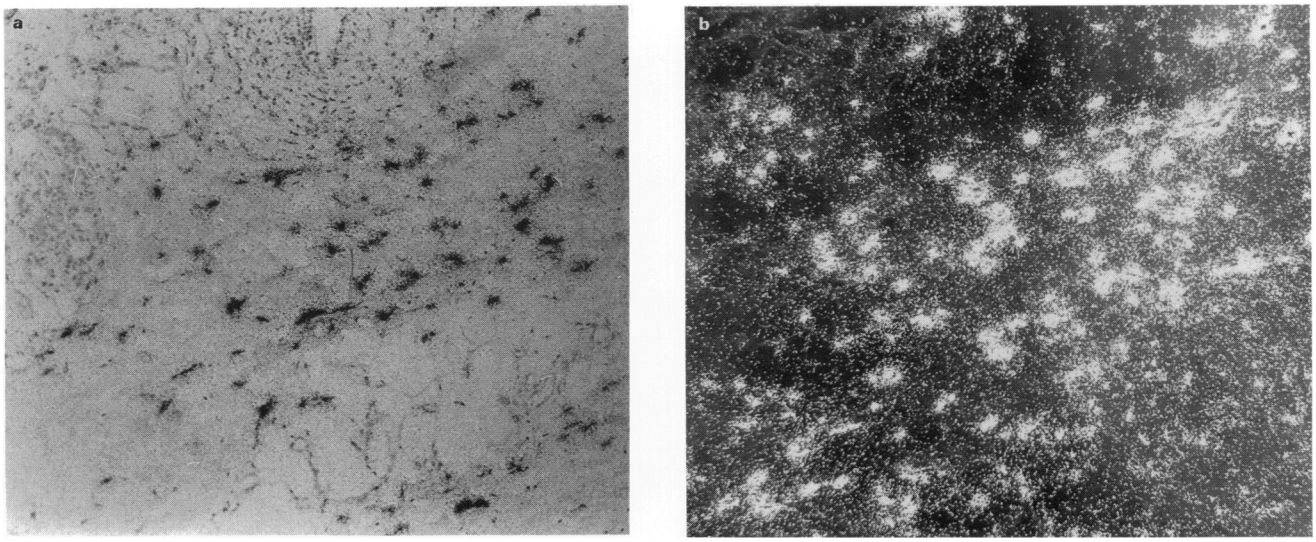

Fig. 2. *In situ* hybridization of skin sample from one toxic oil syndrome (TOS) patient with type I procollagen ^{35}S-labelled cDNA. (a) and (b) show abundant autoradiographic grains in hypodermis. There is a variability in individual expression of grains. (b) shows dark-field image of the tissue section of (a). (Original magnification × 20.)

Detection of bound probe

Autoradiography was performed by dipping the slides into NTB-3 nuclear track emulsion (Eastman Kodak, Rochester, NY) melted at 40°C and diluted 1:1 with 0·6 M ammonium acetate. After drying horizontally at room temperature for 5 h, the slides were exposed in desiccant-containing boxes at 4°C for 7–10 days. The exposed slides were developed in Kodak D-19 developer for 2–5 min at 15°C, rinsed, fixed for 5 min, stained with Gill's haematoxylin No 1, dehydrated and mounted with Permount (Fisher Scientific, Fair Lawn, NJ).

RESULTS

Histological examination of skin and nerve samples showed a marked increase in fibrous tissue with coarse bundles of collagen fibrils. Some inflammatory cells were also present. In skin, the fibrous tissue extended from papillary dermis to hypodermis. In the nerve, the fibrous process had both an epineural and perineural distribution.

In situ hybridization of all TOS skin samples showed several collagen-producing cells containing high levels of procollagen type I mRNA located in the dermis (Fig. 1) and subcutis (Fig. 2). Although some of these cells were in close proximity to blood vessels, others were located in apparently avascular areas. The distribution of fibroblasts containing high levels of type III collagen signal was similar, but the signals were weaker (Fig. 1). These high-collagen-producing fibroblasts were located among fibroblasts containing low levels of autoradiographic signal, thus demonstrating the existence of two distinct subpopulations of fibroblasts in fibrotic areas. Control biopsies incubated with lambda phage under identical conditions showed random distribution of radiographic grains (Fig. 1).

In the nerve, *in situ* hybridization disclosed collagen-producing cells with high levels of type IV collagen mRNA located in the epineurium and perineurium. Cells containing type IV autoradiographic grains had a uniform distribution (Fig. 3). No signal was detected with probes for type I and type III collagen, or for lambda phage.

DISCUSSION

In the acute phase of TOS, skin biopsies show moderate lymphohistiocytic infiltrates and the typical vascular lesion. In the chronic phase, thick bundles of collagen along with scanty cellular infiltrates occupy the dermis, the subcutis and the subcutaneous tissue [1]. The biopsies used in the present study correspond to the chronic phase of TOS. In scleroderma, cellular infiltration and fibrosis are somewhat more superficial than in TOS. In the newly described eosinophilia-myalgia syndrome (EMS), and in eosinophilic fasciitis these features occur more in the deep dermis, subcutis, subcutaneous tissue and fascia of muscle [5,8].

Fibrosis could result from recruitment and proliferation of fibroblasts with or without concomitant stimulation of collagen synthesis, decreased secretion of collagenase or increased secretion of its inhibitors. In scleroderma skin, an increase in type I and type III collagen synthesis produced by an accelerated rate of collagen gene transcription has been reported [9]. *In situ* hybridization experiments have shown elevated expression of type I and type III collagen mRNA [3]. Similarly, activation of type I collagen mRNA expression has been reported in eosinophilic fasciitis and EMS [4,5]. Here, we report an elevated expression of type I and type III collagen mRNA in the skin of TOS patients. There are several common findings in the reports involving scleroderma, eosinophilic fasciitis, EMS and TOS patients. First, although many activated cells are close to capillaries and inflammatory cells, many others are located away from blood vessels. Second, dual populations of fibroblasts are found: one with an elevated and the other with normal level of collagen gene expression, corresponding to the so-called high and low collagen-producer fibroblasts. Third, in scleroderma skin and in TOS skin, type III collagen mRNA expression is also activated parallel to type I collagen expression.

Neuromuscular lesions are among the most characteristic features of TOS. Epi- and perineural cellular infiltration followed by fibrosis are common in nerves of the patients. Nerve

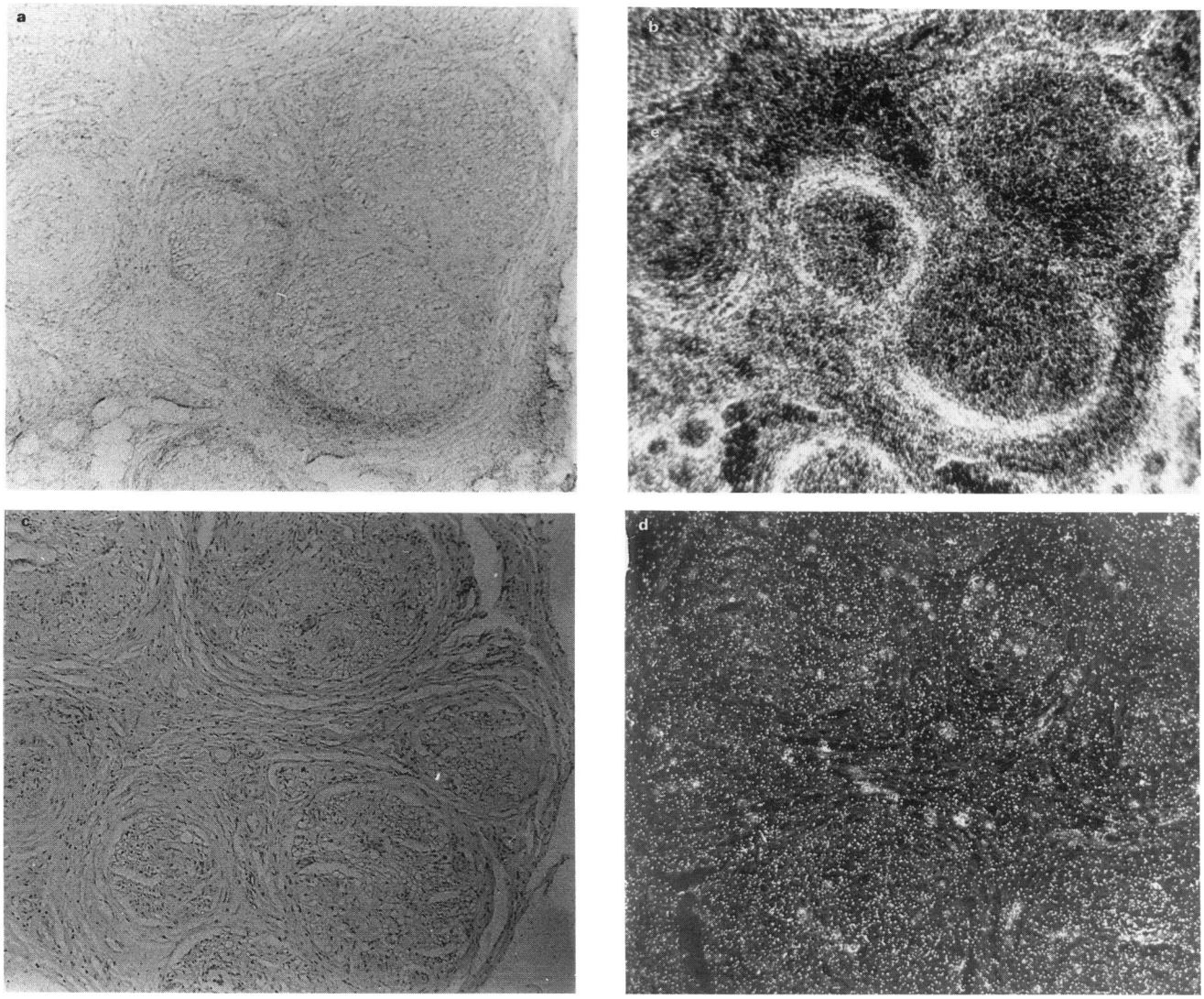

Fig. 3. *In situ* hybridization of sural nerve sample from one toxic oil syndrome (TOS) patient with type IV procollagen ^{35}S-labelled cDNA. (a) and (b) show an epi- and perineural distribution of autoradiographic grains, which are representative of type IV collagen mRNA. (c) and (d) show the nerve hybridized with ^{35}S-labelled lambda-DNA, devoid of grains. Panels on the right (b) and (d) show dark-field images of tissue section of the left (a) and (c). (Original magnification × 20.)

biopsies of the two patients studied showed extensive fibrosis. Perineural fibrosis in systemic sclerosis, in sarcoidosis and in the sensory perineuritis occurs only exceptionally [1]. Recently, an epineural infiltrate of mononuclear cells was reported in EMS [8]. *In situ* hybridization studies of TOS nerves revealed a large number of cells with positive type IV collagen hybridization signal in areas of fibrosis. Collagen type IV is the structural collagen of epithelial and endothelial basement membranes. One explanation of our findings is that persistent damage to blood vessels might have triggered collagen deposition in the nerve. Nevertheless, the contribution of perivascular cell infiltrates and/or factors produced in or around blood vessels cannot be excluded.

Several intracellular pathways can activate transcription of collagen genes. Cytokines and growth factors regulate those pathways [10]. Interaction with extracellular matrix may also control fibroblast functions [11]. Elevated blood levels of several cytokines have been reported in fibrotic conditions. Platelet-derived growth factor (PDGF), transforming growth factor-beta (TGF-β), tumour necrosis factor-alpha (TNF-α) and IL-1, IL-2, IL-4 and IL-6 levels are elevated in scleroderma and other fibrosing disorders [12,13]. PDGF and TGF-β stimulate the proliferation of fibroblasts and the production of collagen types I, III and V, as well as fibronectin and proteoglycans [12]. TGF-β mitogenic effect involves the induction of PDGF receptors in fibroblasts [14]. TGF-β and PDGF have been found in the periappendageal dermis in EMS patients, but not in TOS patients, suggesting that the genesis of tissue fibrosis in these two diseases may be dependent upon different peptide activation [15]. Comparison of scleroderma with scleroderma-like conditions should help to understand the pathogenic mechanisms of excessive accumulation of collagen.

EMS is associated with the consumption of L-tryptophan [16]. Elevated levels of serotonin in the carcinoid syndrome are also related to skin fibrosis. High blood levels of kynurenine have been reported in scleroderma, EMS [17], and recently, in

TOS [18]. Whether L-tryptophan or its metabolites directly or through the activation of cells producing fibrogenic cytokines, are involved in the fibrosis remains to be proved. Also, high levels of antibodies to native and denatured type I, type III and type IV collagens are present in TOS patients [19], as well as in scleroderma patients [20]. Anticollagen antibodies are characteristic of many inflammatory diseases of the connective tissues, but their role in the etiopathogenesis of the fibrotic process remains unknown. The destruction of endothelium seen in early lesions of TOS patients [21] could have damaged the underlying collagen and induced humoral immunity. The deposition of antibodies could then initiate a self-perpetuating cycle, with complement activation, immune-complex formation and antibody-dependent cell-mediated cytotoxicity. Arrival of mononuclear cells into the lesion and liberation of soluble factors could have led to fibrosis throughout fibroblast recruitment and proliferation. Search for similarities between TOS, scleroderma, eosinophilic fasciitis and EMS in connective tissue activation is one way towards understanding the molecular mechanisms leading to these fibrotic conditions. The current investigation has demonstrated by *in situ* hybridization analysis a remarkable similarity of the skin fibrosis in TOS and scleroderma. It remains to be shown whether this points towards a common activation mechanism in affected fibroblasts, or suggests that many different processes can result in a fibrotic response.

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