IL-4 Upregulates Tenascin Synthesis in Scleroderma and Healthy Skin Fibroblasts

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Tenascin (TN), a large extracellular matrix glycoprotein, is transiently expressed during embryonic development, but is absent from most normal adult tissues. TN is reexpressed, however, in healing wounds, in the stroma of some tumors, and in fibrotic diseases such as systemic sclerosis (SSc) and rheumatoid arthritis. To clarify the mechanisms regulating TN expression, we studied the effects of selected cytokines (PDGF, bFGF, TGF-β, IL-1, IL-4, IL-6, IFN-γ, and TNF-α) found in fibrotic tissue on TN expression by dermal fibroblasts. IL-4 strongly induced TN protein levels (up to 10-fold over the basal level), whereas PDGF and bFGF were less potent inducers of TN than IL-4. All other cytokines tested, including TGF-β, did not stimulate TN synthesis. IL-4 also increased TN mRNA expression, and this effect was blocked by actinomycin D. Cycloheximide increased basal TN mRNA expression and induced TN mRNA in IL-4–treated fibroblasts, suggesting that repressor protein(s) may regulate transcription of the TN gene. Although no differences in constitutive TN expression or effects of cytokines on TN expression were observed between SSc and healthy fibroblasts, these data are consistent with the observations that high levels of both IL-4 and TN are present in the affected skin of patients with SSc. These results suggest that the high level of TN found in the affected tissue of patients with SSc results from the high level of IL-4 present. ***Key words: PDGF/bFGF/TGFβ. J Invest Dermatol 107:856–859, 1996***

Systemic sclerosis (SSc), an autoimmune disorder, is characterized by the excessive deposition of extracellular matrix components in the skin and internal organs (Fagundus and LeRoy, 1994). Tenascin is an extracellular matrix glycoprotein that may play a role in the pathogenesis of scleroderma. It is a large disulfide-linked hexameric glycoprotein with a subunit molecular mass ranging from 220 to 320 kDa (Erickson and Bourdon, 1989). In adult tissues, tenascin is present as a thin band in the papillary dermis (Lightner *et al*, 1989) and is absent from most other tissues. Tenascin is transiently reexpressed in wound healing at the stages preceding collagen deposition and cell migration (Whitby and Ferguson, 1991; Gailit and Clark, 1994). Tenascin reappears in some pathologic conditions, including cancer (Koukoulis *et al*, 1991) and fibrotic and immunologic diseases such as SSc and rheumatoid arthritis (Lacour *et al*, 1992; Salter, 1993).

Although immunohistochemistry has shown that tenascin is increased in the dermis of patients with scleroderma (LaCour *et al*, 1992), the production of tenascin by dermal fibroblasts has not been demonstrated. We therefore investigated the regulation of tenascin by cytokines believed to be involved in fibrosis, using cultured dermal fibroblasts obtained from healthy subjects and from patients with scleroderma. We tested the effects of platelet-derived growth factor (PDGF), basic fibroblast growth factor (bFGF), transforming growth factor–β (TGF-β), interleukin (IL)-1, IL-4, IL-6, interferon-γ (IFN-γ), and tumor necrosis factor–α (TNF-α) on tenascin expression at both the protein and mRNA levels. Our data indicate that IL-4 is a potent inducer of tenascin, whereas PDGF and bFGF are moderate inducers.

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Abbreviation: SSc, systemic sclerosis.

MATERIALS AND METHODS

Cell Cultures Fibroblasts were obtained by skin biopsy from the affected areas (dorsal forearm) of patients with diffuse cutaneous SSc with less than 2 y of skin thickening. Control fibroblasts were obtained by skin biopsy of healthy donors (within several days of SSc biopsy); these were matched with each SSc patient for age, race, gender, and biopsy site and were processed in parallel. Newborn foreskin fibroblasts were obtained from the delivery suites of affiliated hospitals. Primary explant cultures were established and propagated as described previously (Yamakage *et al*, 1992). Fibroblasts between the third and fifth subpassages were used for experiments.

Cytokines and Chemicals IL-4, IL-1, IFN-γ, actinomycin D, and cycloheximide were purchased from Sigma (St. Louis, MO); TNF-α was obtained from Promega (Madison, WI); bFGF, PDGF, and TGF-β were obtained from Upstate Biotechnology (Lake Placid, NY); and IL-6 was purchased from Pepro Tech (Rocky Hill, NJ).

RNA Preparation and Northern Blot Analysis Fibroblasts were grown to confluence in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, and then incubated for 48 h in serum-free medium (Dulbecco's modified Eagle's medium plus 0.1% bovine serum albumin). The cells were stimulated with various cytokines—IL-1, IL-4, IL-6, TNF-α, or IFN-γ—for 3, 6, or 24 h. Total RNA was extracted and analyzed by Northern blot as described previously (Yamakage *et al*, 1992). Tenascin cDNA probe was kindly provided by Dr. Mario Bourdon (La Jolla Institute for Experimental Medicine, La Jolla, CA). The filters were scanned with a phosphorimager scanner (Molecular Dynamics, Sunnyvale, CA). Filters were stripped by boiling for 30 min in 0.1% sodium dodecyl sulfate

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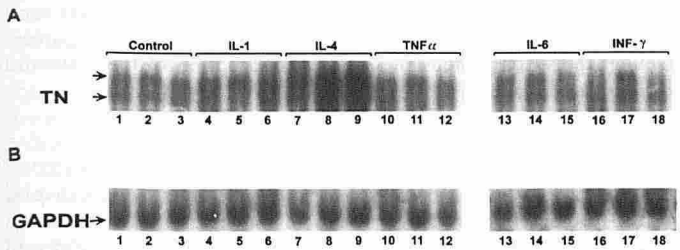

Figure 1. Effects of cytokines on tenascin (TN) mRNA levels in healthy fibroblasts. Cells were stimulated with the indicated cytokines for 3 h *(lanes 4,7,10,13,16)*, 6 h *(lanes 5,8,11,14,17)*, and 24 h *(lanes 6,9,12,15,18)*. Control cells received only serum-free medium for 3 h *(lane 1)*, 6 h *(lane 2)*, and 24 h *(lane 3)*. Membranes were hybridized with tenascin cDNA probe *(A)* and rehybridized with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe *(B)*.

and 0.1 × sodium citrate/chloride buffer and rehybridized with glyceraldehyde-3-phosphate dehydrogenase probe.

Immunoblotting Cells at 10^5 were plated in 1 ml of 10% fetal bovine serum in 12-well plates, allowed to adhere for 6–8 h, and then incubated in serum-free medium (1 ml) for 24 h. The indicated cytokines were added in serum-free medium. After 3 d of incubation, the medium was collected. Remaining cells and deposited extracellular matrix (denoted as cell/matrix layer) were lysed in lysis buffer (5 mM Tris-Cl, pH 8.0, 150 mM NaCl, 0.02% sodium azide [NaN_3], 0.1% sodium dodecyl sulfate, 1 μg aprotinin per ml, 1% Nonidet P-40, 0.5% sodium deoxycholate, 1 mM sodium orthovanadate, and 100 μg phenylmethylsulfonyl fluoride per ml). Aliquots of conditioned medium (35 μl; 3.5% of the medium from one well) or cell/matrix layers (20 μg; about 20% of the cell/matrix layer in one well) (protein content measured by BCA Pierce assay; Rockford, IL) were separated on 5% sodium dodecyl sulfate–polyacrylamide gels and transferred to nitrocellulose membranes. The membranes were stained with amido black before blocking to control for equal loading. The membranes were blocked with 1% bovine serum albumin and incubated overnight with an anti-human tenascin monoclonal antibody (NEC1b) (kindly provided by Dr. Wolfgang Rettig, Thomae Biberach, Germany) (Rettig and Garin-Chesa, 1989). The membranes were washed in Tris-buffered saline/Tween, incubated with anti-mouse antibody conjugated to horseradish peroxidase, and washed again. The tenascin bands were detected using the Enhanced Chemiluminescence Detection System (Amersham, Arlington Heights, IL).

RESULTS

IL-4 Upregulates Tenascin mRNA Levels in Dermal Fibroblasts To investigate the regulation of tenascin in dermal fibroblasts, we studied the effects of IL-1, IL-4, IL-6, TNF-α, and IFN-γ on tenascin mRNA levels in SSc and in healthy skin fibroblasts at concentrations ranging from 0.1 to 100 ng per ml. Based on these preliminary experiments, the following concentrations (which are the minimal concentrations providing maximal stimulation) were used in subsequent experiments: IL-1, 10 ng per ml; IL-4, 2 ng per ml; IL-6, 10 ng per ml; TNF-α, 10 ng per ml; and IFN-γ, 100 U per ml. Total RNA was extracted from cultured fibroblasts after 3, 6, or 24 h of stimulation and analyzed by Northern blot **(Fig 1)**. Two tenascin mRNA transcripts were detected. Increases in tenascin mRNA levels were induced by IL-4 at all time points, with maximum stimulation at 24 h. No other cytokines showed stimulation at 3 or 6 h, but after 24 h of treatment, IL-1 and TNF-α showed slight stimulatory effects. IL-6 had no effect and IFN-γ slightly decreased tenascin mRNA levels. Only IL-4 significantly induced tenascin mRNA expression in both SSc (fold increase: 3.4 ± 0.6, mean ± SEM, $p < 0.01$, data not shown) and healthy fibroblasts (3.3 ± 0.6, $p < 0.001$) **(Fig 2)**. Co-stimulation with IFN-γ slightly decreased the stimulatory effects of IL-4 in SSc and healthy fibroblasts **(Fig 2)**. No significant differences were detected in basal levels of tenascin mRNA between healthy and SSc fibroblasts. The pattern of responses to cytokines with regard to tenascin mRNA expression was similar in SSc and healthy fibroblasts.

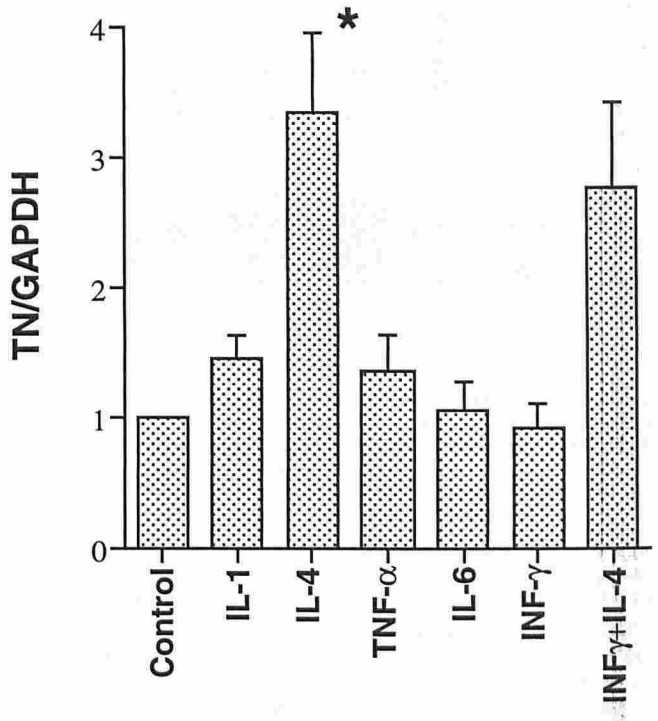

Figure 2. IL-4 increases tenascin mRNA levels in dermal fibroblasts. Tenascin/glyceraldehyde-3-phosphate dehydrogenase ratio (TN/GAPDH) in normal (n = 4) fibroblasts after stimulation with various cytokines for 24 h. *Statistically significant difference in ratio, $p < 0.001$. *Error bars*, mean ± SEM.

IL-4, bFGF, and PDGF Stimulate the Production of Tenascin in Dermal Fibroblasts Tenascin protein levels were measured in conditioned medium and in cell/matrix layers obtained from fibroblasts treated with cytokines for 72 h. Two polypeptides (320 and 220 kDa), corresponding to the two isoforms of tenascin, were detected **(Fig 3)**. The larger isoform was detected in both the cell/matrix layers and in conditioned medium, whereas the small isoform was detected only in conditioned medium. IL-4 both increased the deposition of tenascin in the cell/matrix layers **(Fig 3*A*)** and induced the secretion of tenascin into conditioned medium **(Fig 3*B*)**. To quantify the increase of tenascin secretion after IL-4 stimulation, aliquots of conditioned medium obtained from untreated fibroblasts were run adjacent to aliquots representing various amounts of conditioned medium obtained from IL-4–treated cells. The intensity of the major tenascin polypeptide from 35 μl of unstimulated fibroblast medium was slightly lower than the intensity from 3.5 μl of stimulated fibroblast medium, indicating stimulation of tenascin secretion of about 10-fold by IL-4 (data not shown). Treatment of fibroblasts with IL-1, IL-6, TNF-α, IFN-γ, or TGF-β did not change tenascin production significantly **(Fig 3)**.

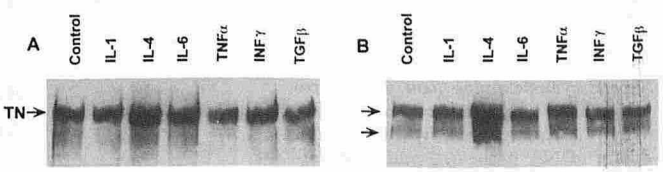

Figure 3. Induction of tenascin (TN) protein synthesis in healthy fibroblasts after IL-4 stimulation. Cultured fibroblasts were stimulated with the indicated cytokines for 72 h, the medium was collected, and the cells and extracellular matrix were lysed in lysis buffer, as described in *Materials and Methods*. Aliquots of cell/matrix layer *(A)* or conditioned media *(B)* were analyzed by western blot as described in *Materials and Methods*.

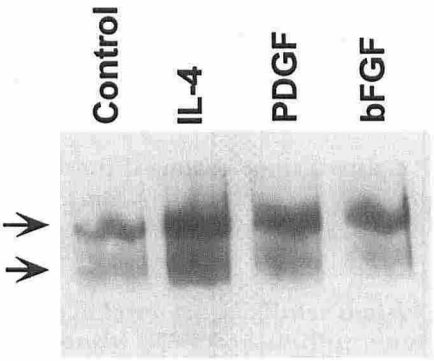

Figure 4. PDGF AB and bFGF induce tenascin protein synthesis in scleroderma fibroblasts. Cultured fibroblasts were stimulated with PDGF AB (20 ng per ml), bFGF (10 ng per ml), and IL-4 (2 ng per ml). Aliquots of conditioned medium, normalized for cell number, were analyzed by western blot as described in *Materials and Methods*.

Previous reports indicated that PDGF and bFGF induce tenascin synthesis in several types of cells (Sharifi *et al*, 1992; Rettig *et al*, 1994). We tested the effects of these growth factors on tenascin protein synthesis in SSc and healthy dermal fibroblasts. As shown in **Fig 4**, both PDGF and bFGF induced tenascin synthesis, but with a lesser potency than IL-4. It was interesting that PDGF did not induce tenascin mRNA levels (data not shown). The effects of bFGF on tenascin mRNA levels have not been investigated.

IL-4 Regulates Tenascin Expression at the Transcriptional Level To establish whether the increase in tenascin mRNA levels after IL-4 treatment involves transcriptional activation, we tested the magnitude of IL-4 induction in the presence of actinomycin D. Newborn foreskin fibroblasts were used for these experiments. They were shown to produce similar amounts of tenascin as compared with human adult dermal fibroblasts (Rettig *et al*, 1994), and we have established that they respond to IL-4 with kinetics similar to those of adult dermal fibroblasts (data not shown). Foreskin fibroblasts were treated with IL-4 for 6 h in the presence or absence of a transcriptional inhibitor, actinomycin D **(Fig 5)**. Actinomycin D completely blocked the IL-4–mediated increase in tenascin mRNA, suggesting that IL-4 stimulation of tenascin mRNA is not mediated via increased message stability, but rather involves direct activation of transcription of the tenascin gene and/or other genes involved in tenascin gene regulation. In addition, we asked whether this stimulation requires the synthesis of new proteins. Cycloheximide, an inhibitor of protein synthesis, was added to foreskin fibroblasts together with IL-4 for 6 h **(Fig 5)**. Cycloheximide increased basal tenascin mRNA expression and superinduced tenascin mRNA in IL-4–treated fibroblasts, suggesting that repressor protein(s) regulate transcription of the tenascin gene in human fibroblasts.

DISCUSSION

Previous studies have identified several cytokines as inducers of tenascin expression in various tissue culture systems. For example, TGF-β increased tenascin synthesis in chick embryo fibroblasts (Pearson *et al*, 1988; Chiquet-Ehrismann *et al*, 1989); PDGF BB and angiotensin II induced tenascin mRNA and protein in rat smooth muscle cells (Sharifi *et al*, 1992; La Fleur *et al*, 1994); bFGF induced tenascin synthesis in several cell types, including Swiss 3T3 cells (Tucker *et al*, 1993); and IL-1 increased tenascin production in human synovial fibroblasts (McCachren and Lightner, 1992). The combination of IL-1, IL-4, and TNF-α induced high levels of tenascin synthesis in human fetal conjunctival fibroblasts (Rettig *et al*, 1994). In this study, we demonstrate that only some of these cytokines are capable of stimulating tenascin expression in human dermal fibroblasts. Among the cytokines tested, IL-4 was the most potent inducer of tenascin synthesis in human fibroblasts. IL-4 increased tenascin mRNA levels more than 3-fold and tenascin protein levels more than 10-fold. Tenascin protein was also moderately induced by PDGF and bFGF. Moreover, we have shown recently that all three PDGF isoforms are equally potent in stimulating tenascin synthesis in newborn foreskin fibroblasts.[1] In contrast to IL-4, however, PDGF did not affect tenascin mRNA levels. This observation suggests that different regulatory mechanisms are used by these two cytokines, which may be relevant to the observed differences in their potencies.

Surprisingly, we found no significant effects of TGF-β on tenascin expression in human fibroblasts. A recent study by Rettig *et al* (1994) also demonstrated that TGF-β has various effects on tenascin expression in fibroblasts and that TGF-β reduced tenascin secretion in newborn fibroblasts. In the same study (Rettig *et al*, 1994), tenascin secretion was analyzed in fetal conjunctival fibroblasts after stimulation with IL-1, IL-4, and TNF-α. In agreement with the data presented herein, IL-4 induced tenascin expression; however, those investigators found that IL-1 and TNF-α also induced tenascin synthesis. Moreover, the combination of IL-1, IL-4, and TNF-α was shown to cause a synergistic increase in tenascin expression in conjunctival fibroblasts, whereas in our system, this combination increased tenascin levels only slightly in comparison with IL-4 alone (data not shown). The observed differences are not due to variations in cytokine concentrations,

[1] Stepniakowska J, LeRoy EC, Trojanowska M: Platelet-derived growth factor -AA, -AB, and -BB isoforms stimulate tenascin production equivalently in human fibroblasts. *Mol Biol Cell* 6:125, 1995 (abstr.).

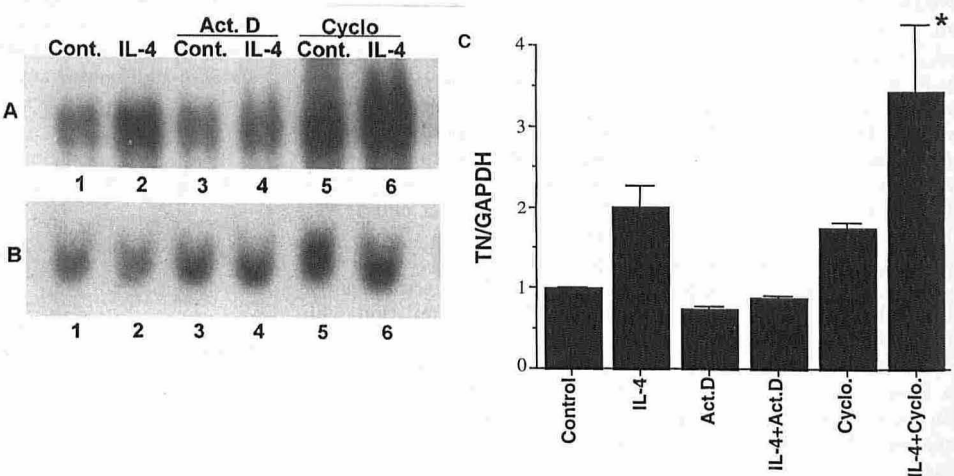

Figure 5. Actinomycin D blocks the transcription of tenascin (TN) gene in foreskin fibroblasts stimulated with IL-4. Foreskin fibroblasts were stimulated for 6 h with IL-4 *(lane 2)*, actinomycin D (Act. D; *lane 3*), IL-4 and actinomycin D *(lane 4)*, cycloheximide (Cyclo; *lane 5*), or IL-4 and cycloheximide *(lane 6)*. Control cells received serum-free medium (Cont., *lane 1*). Membranes were hybridized with tenascin cDNA probe *(A)* and rehybridized with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe *(B)*. *C*, TN/GAPDH ratio in fibroblasts (n = 3). *Statistically significant difference from control, $p < 0.05$. *Error bars*, mean ± SEM.

because in the study by Rettig *et al* (1994), a dose-response curve for tenascin induction with TNF-α and IL-1 reached a plateau with 10 ng per ml and 1 ng per ml, respectively; similar cytokine concentrations were used in our study. Our results also differ from those of a study of human synovial fibroblasts in which IL-1 induced tenascin protein and mRNA (McCachren and Lightner, 1992). Together, these observations suggest that tenascin expression by fibroblasts may be regulated in a tissue-specific manner.

In conclusion, this study demonstrates that human dermal fibroblasts display a novel pattern of responses to cytokines with regard to tenascin synthesis. Most significantly, two of the cytokines, IL-4 and PDGF, which stimulate the synthesis of tenascin *in vitro*, have been found to be elevated in the skin of patients with SSc (Yamakage *et al*, 1992)[2] and therefore may be directly responsible for the tenascin production in SSc lesions. In conjunction with our observation of similar levels of tenascin expression by passaged control and SSc dermal fibroblasts in the absence or presence of IL-4, these findings suggest that control and SSc fibroblasts may not be intrinsically different in their responses to IL-4. Rather, the high level of IL-4 in SSc, secreted by mast cells, which are present in high numbers and are activated in SSc lesions (Claman, 1989; Gruber, 1995), may lead to a high level of tenascin expression. In addition, Il-4 is present at elevated levels in SSc serum (Needleman *et al*, 1992). Further studies are needed to determine the effects of tenascin on the cells present in fibrotic lesions and to understand the roles of IL-4 and tenascin in the pathogenesis of SSc.

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