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Induction by IL-1β of Tissue Inhibitor of Metalloproteinase-1 in Human Orbital Fibroblasts: Modulation of Gene Promoter Activity by IL-4 and IFN-γ[1]

Rui Han and Terry J. Smith[2]

Thyroid-associated ophthalmopathy (TAO), an autoimmune component of Graves' disease, is associated with profound connective tissue remodeling and fibrosis that appear to involve the selective activation of orbital fibroblasts. Accumulation of extracellular matrix molecules is a hallmark of this process. Here we report that orbital fibroblasts treated with IL-1β express high levels of tissue inhibitor of metalloproteinase-1 (TIMP-1), an important modulator of matrix metalloproteinase activity. These high levels are associated with increased TIMP-1 activity. The induction is mediated at the pretranslational level and involves activating the TIMP-1 gene promoter. IL-1β activates the ERK 1/2 pathway in these fibroblasts and interrupting this signaling either with PD98059, a chemical inhibitor of MEK, or by transfecting cells with a dominant negative ERK 1 plasmid results in the attenuation of TIMP-1 induction. Surprisingly, treatment with IL-4 or IFN-γ could also block the IL-1β induction by attenuating TIMP-1 gene promoter activity. These findings suggest that TIMP-1 expression in orbital fibroblasts following activation with IL-1β could represent an important therapeutic target for modifying the proteolytic environment. This might alter the natural course of tissue remodeling in TAO. ***The Journal of Immunology,*** **2005, 174: 3072–3079.**

Severe thyroid-associated ophthalmopathy (TAO),[3] an autoimmune component of Graves' disease, is characterized by dramatic tissue reactivity (1, 2). Inflammation dominates the early histopathology, followed by profound remodeling of orbital connective tissue and scar formation. Fibrosis can impair the motility of the extraocular muscles and accounts for much of the substantial morbidity associated with TAO (1). In some patients, fibrosis occurs rather early in the disease and few therapeutic options can be offered currently to alter the course of this process. Histological examination of affected tissues reveals an often impressive infiltration of several types of immunocompetent cells, including $CD4^+$ and $CD8^+$ T cells, B lymphocytes, and mast cells (2–5). It is currently believed that cytokines and other molecules generated by these cells drive connective tissue activation (6). Evidence has been introduced suggesting that both Th1 and Th2 cytokines can predominate in TAO, depending on the stage and duration of the orbital disease (7, 8). Metachromatically staining material accumulates in TAO, much of which is susceptible to digestion with hyaluronidase, indicating that the nonsulfated glycosaminoglycan, hyaluronan, is an abundant component (9). Other extracellular matrix (ECM) materials accumulating in TAO are poorly characterized (6). The dramatic nature of tissue remodeling found in TAO results in substantial morbidity, yet virtually nothing is currently known about the pericellular proteolytic environment existing in the human orbit or surrounding its derivative cells.

Orbital fibroblasts differ from most others in the human body in that they derive from neural crest and thus exhibit substantial plasticity (10, 11). They are believed to play an important role in the pathogenesis of TAO (6). Orbital fibroblasts exhibit unique phenotypic attributes, including exaggerated responses to proinflammatory cytokines. These peculiarities may underlie the susceptibility of the orbit to this disease (10, 12). They synthesize relatively high levels of hyaluronan following activation by cytokines such as leukoregulin (13), IL-1β (14), CD154 (15), or IgG from patients with Graves' disease (16). Unusually high levels of plasminogen activator inhibitor type 1 have been found in orbital fibroblast cultures treated with IFN-γ, TGF-β, and leukoregulin when compared with their extraorbital counterparts (17–19). This suggests that the pericellular proteolytic environment surrounding these cells might favor the accumulation of macromolecules comprising the ECM. Orbital fibroblasts represent a heterogeneous population of cells, subsets of which exhibit discreet phenotypes (20–23). They appear to actively participate in the tissue remodeling that occurs in several inflammatory and fibrotic disease processes found in the orbit, including TAO.

Degradation of ECM proteins, such as collagen, results from the actions of zinc-dependent enzymes known as matrix metalloproteinases (MMPs) (24). Although a wide array of proteinases contribute to the turnover of matrix proteins, MMPs appear to dominate that function. The activity of MMPs is regulated by an endogenous group of proteins, including α-macroglobulins and the tissue inhibitors of metalloproteinases (TIMPs) (25, 26). TIMPs comprise a family of four small (21–30 kDa) homologous proteins. They form complexes with the catalytic domains of MMPs and in so doing modulate the activities of those enzymes and the activation of pro-MMPs. Interactions between TIMPs and MMPs have been extensively characterized (27). TIMPs have two domains, an

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[3] Abbreviations used in this paper: TAO, thyroid-associated ophthalmopathy; ECM, extracellular matrix; MMP, matrix metalloproteinase; TIMP, tissue inhibitor of metalloproteinase; DRB, 5,6-dichlorobenzimidazole; DN, dominant negative.

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N terminus of ~125 amino acids and a C terminus of ~65 residues. The domains are stabilized by three disulfide bonds. TIMP-1 is the most thoroughly studied member of this group. It can inhibit the activities of all MMPs thus far identified. TIMP-1 also exhibits growth factor-like effects in several cell-types including keratinocytes (28, 29). These activities may be mediated through TIMP-1 binding to the cell surface and potentially translocating to the nucleus of target cells (30, 31). Cell apoptosis can also be modulated by TIMPs (32). The expression of TIMP-1 in orbital fibroblasts has not been explored previously, despite its potentially important role in modulating the turnover of ECM molecules in this anatomic region. We hypothesize that the important balance between MMPs and TIMPs in the human orbit might become disrupted in TAO and result in the fibrosis that occurs in that disease process.

In this study, we demonstrate that IL-1β can induce TIMP-1 expression in orbital fibroblasts and that this up-regulation involves elevated TIMP-1 gene promoter activity. The induction can be attenuated by glucocorticoids. Surprisingly, IL-4, and IFN-γ can also block the up-regulating action of IL-1β on TIMP-1 expression. These cytokines work by down-regulating IL-1β-dependent TIMP-1 gene promoter activity. Our findings suggest a potential mechanism through which multiple cytokines, from both Th1 and Th2 classes, might influence TIMP-1 expression and thereby alter macromolecular turnover in the orbit.

Materials and Methods

Materials

The cDNA encoding human TIMP-1 was purchased from American Type Culture Collection. Anti-human TIMP-1 Ab, cycloheximide, 5,6-dichlorobenzimidazole (DRB) and dexamethasone (1,4 pregnadiene-9-fluoro-16α-methyl-11β,17α,21 triol-3,20-dione) were purchased from Sigma-Aldrich. PD98059 was from Calbiochem. A dominant negative (DN) mutant expression vector for ERK 1 was a gift from Dr. M. Cobb (University of Texas Southwestern, Dallas, TX). Anti-ERK and anti-phospho-ERK Abs were purchased from Santa Cruz Biotechnology. All cytokines used in these studies were purchased from BioSource International. Neutralizing Abs against IL-4 and IFN-γ were supplied by R&D Systems.

Cell culture

Human orbital fibroblasts were cultivated from connective tissue obtained as surgical waste during decompression surgery for severe TAO. Control fibroblasts were obtained from individuals undergoing ophthalmic surgery for some other condition not involving the orbit. We have used seven different orbital fibroblast culture strains in studies concerning the induction of TIMP-1 by cytokines. The Institutional Review Board of Harbor-University of California Los Angeles Medical Center has approved these activities. Fibroblasts were maintained in a humidified 5% CO_2 incubator at 37°C covered with DMEM (Invitrogen Life Technologies) supplemented with 10% FBS, glutamine, and antibiotics. Medium was changed every 3–4 days. Cultures were screened for cellular contaminants and were devoid of cells expressing factor VIII, smooth-muscle specific actin, or cytokeratin (20). Monolayers were serially passaged by gentle trypsin/EDTA treatment and were used between the 2nd and 14th passage. We have determined that the cellular phenotype remains constant over this number of population doublings.

Western blot analysis

Relative levels of TIMP-1 protein released into the fibroblast culture medium were determined by Western blot analysis using mAbs generated against human TIMP-1. Equal volumes of conditioned culture medium were mixed with 2× sample buffer. Samples were denatured by heating to 95°C for 5 min and were then cooled on ice. They were subjected to separation on 10% SDS-PAGE. Separated proteins were transferred to Immobilon-P membrane (Millipore). Primary Ab (1:1000) was added and incubated with the membrane at 4°C overnight. Membranes were washed extensively and incubated with a second, peroxidase-labeled Ab (DakoCytomation) for 1 h. Following extensive washing, the ECL (Amersham Biosciences) detection system was used to generate specific signals. Resulting bands were quantified with a densitometer (Bio-Rad).

Northern blot analysis

Total cellular RNA was extracted using the method of Chomczynski and Sacchi (33) from near-confluent 100-mm diameter plastic plates of fibroblasts. Monolayers were covered with ULTRASPEC solution (Biotecx Laboratories) and harvested with a rubber policeman. Lysates were transferred into 1.5-ml autoclaved, RNase-free, centrifuge tubes. A total of 0.2 ml of chloroform was added to each tube which was then vortexed. Tubes were cooled on ice for 15 min and centrifuged at 4°C. RNA was precipitated from the aqueous phase by addition of isopropanol, washed with 75% ethanol, and solubilized in diethyl pyrocarbonate water. Equal amounts of RNA (10–20 μg) were subjected to electrophoresis in 1% agarose-formaldehyde gels and transferred to Zeta-Probe (Bio-Rad) membranes. The integrity of the electrophoresed RNA was verified by UV inspection following ethidium bromide staining. The [^{32}P]dCTP random-primed (Bio-Rad) TIMP-1 cDNA probe was allowed to hybridize to immobilized RNA in ExpressHyb hybridization solution (Clontech Laboratories) at 68°C for 1 h. Membranes were washed under high stringency conditions and exposed to X-Omat AR film (Eastman Kodak) at −70°C with intensifier screens. To normalize the amounts of RNA transferred, membranes were stripped according to the manufacturer's instructions and rehybridized with a radiolabeled human GAPDH probe. Radioactive DNA/RNA hybrids were quantified by subjecting autoradiographs to densitometric analysis.

Transient transfection of orbital fibroblasts with plasmids containing fragments of the TIMP-1 promoter and DN mutant ERK-1

A 981-bp fragment spanning −920 to +61 nt of the putative TIMP-1 gene promoter was cloned with the Human Genome Walker kit (Clontech Laboratories), according to the instructions from the supplier. Two primer sequences used for the PCR include 5′-TCCCAATGCTCATTCAT TCA-3′ and 5′-GGGCCCTGCTTACCTCTG-3′. The amplified fragment was sequenced and subcloned from the pCR2.1 TOPO vector (Invitrogen Life Technologies) into a promoter-less pGL-2 luciferase reporter vector (Promega). Serial deletions were generated by restriction enzyme digestions with *Kpn*I and *Sma*I, which yielded the fragments −733/+61 and −194/+61, respectively.

Promoter constructs and a DN mutant ERK-1 were transiently transfected into orbital fibroblasts using the LipofectAMINE PLUS system (Invitrogen Life Technologies). Both 1 μg of DNA and 0.1 mg of pRL-TK vector DNA (Promega), serving as a transfection efficiency control, were mixed with PLUS reagent for 15 min at room temperature before being combined with diluted LipofectAMINE for another 15 min. The DNA-lipid mixture was added to 80% confluent cultured cells in fresh serum-free DMEM for 3 h at 37°C. DMEM containing 10% FBS then replaced the transfection mixture. Transfected cells were serum-starved overnight and treated with or without IL-1β (10 ng/ml) for 1–4 h. Cellular material was harvested in lysis buffer (Promega) and stored at −80°C. Luciferase activity was assayed with the Dual-Luciferase Reporter Assay System (Promega) in an FB12 tube luminometer (Zylux). Values were normalized to internal controls, and each experiment was performed at least three times.

Detection of TIMP activity by reverse zymography

TIMP activity was measured by quantitative reverse zymography as described by Oliver et al. (34). A volume of 30 μl of 10× concentrated conditioned culture medium was mixed with 10 μl of 3× sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, and 0.00125% bromphenol blue). Samples were then subjected to electrophoresis through 15% gels (1.25 ml of 1.5M Tris-HCl, pH 8.8, 2.5 ml of protogel solution (National Diagnostic, Atlanta, GA), 125 μl of 100 mg/ml gelatin A (Sigma-Aldrich), 0.1 ml of 10% SDS, and 4 μl of active gelatinase A (0.1 μg/μl; Calbiochem). After electrophoresis, gels were washed for 30 min at room temperature in 2.5% Triton X-100 to remove SDS and then incubated in developing buffer (pH 7.6) containing 200 mM NaCl, 0.5 mM CaCl, and 0.02% Brij-35 for 17 h at 37°C. Gels were stained in 0.25% Coomassie blue (methanol/acetate acid/H_20, 40:10:50) for 30 min and destained.

Results

IL-1β increases TIMP-1 protein expression and steady-state mRNA levels in orbital fibroblasts

Orbital fibroblasts, cultured under standard growth conditions, express low basal levels of TIMP-1 protein, which is released from the cell layer (Fig. 1*A*). IL-1β (10 ng/ml) induces TIMP-1 production in these cells in a dose-dependent manner with a small increase at the lowest concentration tested (0.1 ng/ml) and a maximal

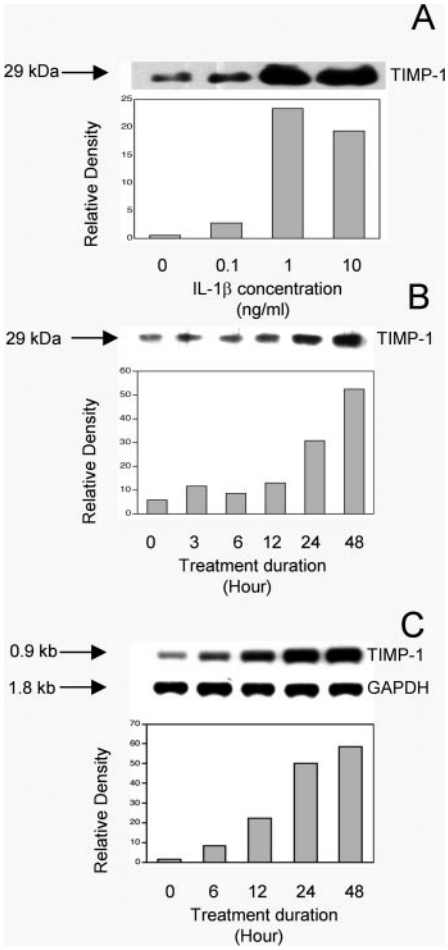

FIGURE 1. IL-1β induces TIMP-1 protein and mRNA in a concentration- and time-dependent manner in orbital fibroblasts. Cultures, in this case from patients with severe TAO, were allowed to proliferate to confluence as described in *Materials and Methods*. Graded concentrations of the cytokine were incubated in the culture medium for 48 h (*A*), or IL-1β (10 ng/ml) was incubated for the intervals indicated along the abscissas (*B* and *C*). TIMP-1 protein was quantified in the medium by Western blot analysis (*A* and *B*) or mRNA was extracted and subjected to Northern blot analysis (*C*) using the techniques described. Signals were quantified by subjecting exposed films to densitometry.

effect seen at 1 ng/ml. No further increase is found at the highest concentration used (10 ng/ml). The induction is also time-dependent; a small increase in TIMP-1 protein level is found at 3–12 h of cytokine treatment. It continues to rise so that by 48 h, TIMP-1 protein is 11-fold higher than in controls (Fig. 1*B*). The Northern blot shown in Fig. 1*C* demonstrates a similar pattern of TIMP-1 mRNA induction by IL-1β. It is near maximal at 24 h and is sustained for the duration of the study (48 h). Thus, IL-1β up-regulates TIMP-1 expression substantially in orbital fibroblasts at the pretranslational level.

Induction of TIMP-1 by IL-1β is particularly robust in cultures derived from patients with TAO

To assess whether the induction of TIMP-1 was peculiar to orbital fibroblasts from patients with TAO or could be detected in fibroblasts from normal orbital connective tissue as well, multiple strains from each source were subjected to Northern analysis. As the blot shown in Fig. 2 demonstrates, all four strains of TAO fibroblasts exhibited induction of TIMP-1 mRNA by IL-1β after 16 h of treatment. The magnitude of induction varied from strain

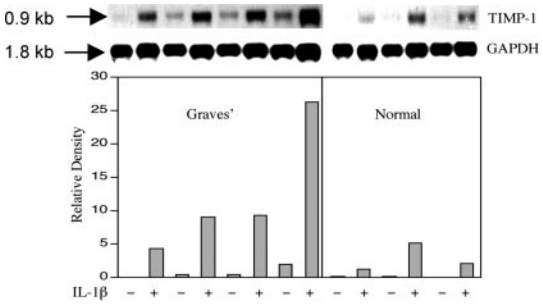

FIGURE 2. IL-1β can induce TIMP-1 mRNA in orbital fibroblasts from control tissues and those from patients with severe TAO. Four strains of fibroblasts, each from a different patient with TAO and three from donors without orbital disease were cultured to confluence and then the medium was supplemented with nothing or IL-1β (10 ng/ml) for 16 h. Cell layers were collected, and RNA was extracted and subjected to Northern blot analysis for TIMP-1. Columns represent the respective TIMP-1 mRNA signal strengths, each corrected for its respective GAPDH.

to strain (5- to 20-fold). As the *right panel* in Fig. 2 exhibits, the TIMP-1 transcript was also induced in orbital fibroblasts from normal tissue, albeit at a generally lower magnitude (1.8- to 4.5-fold).

Induction of TIMP-1 involves an up-regulation of its gene promoter

Transcriptional regulation of TIMP-1 by cytokines has been reported in other cell types (35–37). In our initial studies, the TIMP-1 promoter fragment spanning −920/+61 was cloned into the pGL-2 luciferase reporter vector and transfected into orbital fibroblasts. There is a small 2-fold increase in the level of basal activity found under untreated conditions, when compared with the empty-vector control (Fig. 3*A*). Some cultures were treated with IL-1β for the graded time intervals indicated. Promoter activity increased ~2-fold after 1 h and 12-fold after 2 h following addition of IL-1β (10 ng/ml) compared with the untreated control samples. The cytokine effect was transient and activity had fallen to 2-fold above control values at 4 h. We then transfected truncated fragments, resulting from 5′ serial deletions of the promoter sequence. These included fragments spanning −733/+61 nt and −194/+61 nt. As the data shown in Fig. 3*B* indicate, reporters fused to either of these truncated fragments were considerably less active than the longest (−920/+61) construct, both under basal conditions and following treatment with IL-1β. These data suggest that an important response element(s) for the up-regulation by 1L-1β of TIMP-1 promoter activity lies between −920 and −733 nt. Of particular note are the two PEA3 binding sites in this sequence that are lost in both shorter fragments (Fig. 3*C*). These elements, located from −802 to −796 nt and from −768 to −763 nt have been demonstrated previously to play an important role in the regulation of TIMP-1 gene expression (36).

To determine whether posttranscriptional regulation is also involved in the induction of TIMP-1 by IL-1β, we examined the stability of its mRNA. Confluent orbital fibroblast cultures were treated with IL-1β (10 ng/ml) for 3 h and were then shifted to medium containing DRB (20 μg/ml, an inhibitor of gene transcription) without or with IL-1β for the times indicated in Fig. 3*D*. Levels of TIMP-1 mRNA appear to drop gradually over the course of the study (8 h), regardless of whether IL-1β was present. Levels had dropped by ~50% at the 8 h time point from those at time "0" in both the treated and untreated cultures. It would appear that the up-regulation of TIMP-1 by IL-1β is largely mediated at the level of gene transcription. Moreover, the transcript exhibits appreciable

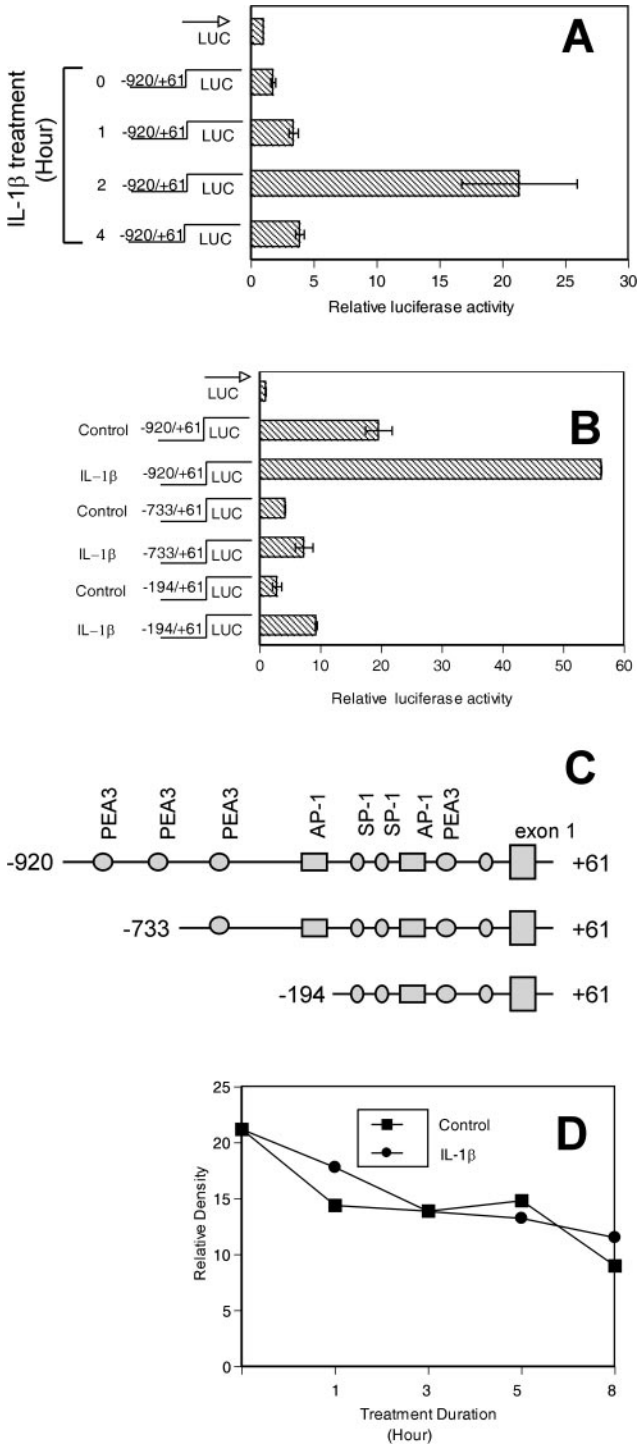

FIGURE 3. *A,* IL-1β treatment of orbital fibroblasts results in a time-dependent activation of TIMP-1 gene promoter activity. Subconfluent fibroblasts, in this case from a patient with severe TAO, were transiently transfected with a plasmid containing a luciferase reporter gene linked to nothing or a 981-bp fragment spanning −920/+61 nt of the TIMP-1 gene promoter, as described in *Materials and Methods*. Cultures were then treated without or with IL-1β (10 ng/ml) for the times indicated; then the monolayers were harvested and luciferase activity was determined. Data are presented as the mean ± SD of three replicates. *B,* Subconfluent orbital fibroblast cultures were transiently transfected with either empty vector or one of the three fragments from the putative TIMP-1 gene promoter, each fused to a luciferase reporter gene. Cultures were then treated with nothing or with IL-1β for 2 h, and the cell layers were harvested and analyzed. Data are expressed as the mean ± SD of three replicates. *C,* Schematic of the three TIMP-1 gene promoter fragments generated in these studies and demonstrating several recognizable response elements. *D,* IL-1β fails to alter the stability of TIMP-1 mRNA. Confluent cultures were treated first with IL-1β (10 ng/ml) for 3 h and then with DRB (20 μg/ml) without or with IL-1β (10 ng/ml) for the duration indicated along the abscissa. Cultures were harvested and subjected to Northern blot analysis by hybridizing immobilized RNA with a probe generated from TIMP-1 cDNA. Signals were normalized against those generated with a GAPDH probe.

stability under both untreated and IL-1β-treated conditions in orbital fibroblasts. Thus, even modest increases in the rate of TIMP-1 gene transcription can yield substantial elevations in the steady-state mRNA levels seen in Northern analysis.

We next examined whether the induction of TIMP-1 represents a primary gene induction by IL-1β or is dependent on the ongoing synthesis of an intermediate protein. Cultures were treated with the cytokine or cycloheximide (10 μg/ml, a concentration that blocks >95% of protein synthesis in these cells (38)) alone or in combination for 16 h. Addition of cycloheximide to the culture medium alone resulted in a 3-fold increase in TIMP-1 mRNA levels compared with controls (Fig. 4*A*). IL-1β treatment increased the transcript level by 5-fold. When cycloheximide and IL-1β were added together, the inhibitor attenuated the induction of the cytokine by 65%. These results suggest that TIMP-1 acts as an immediate early gene because disrupting protein synthesis enhances its transcript levels. Moreover, TIMP-1 mRNA induction by IL-1β is partially dependent on de novo protein synthesis.

Dexamethasone can partially block TIMP-1 induction by IL-1β

Glucocorticoids are widely used as a therapy for TAO. Moreover, they have been found by many investigators to attenuate the inflammatory component of this disease (1). Thus we tested the ability of dexamethasone, a potent synthetic steroid, to influence cytokine-dependent TIMP-1 expression. Addition of the steroid (100 nM) to cultures treated with IL-1β resulted in a 65% reduction of the TIMP-1 protein induction compared with those cultures receiving the cytokine alone (Fig. 4*B*). A similar magnitude of inhibition was found with regard to the impact of dexamethasone treatment on IL-1β-induced TIMP-1 mRNA levels (Fig. 4*A*). These results suggest a potential mechanism through which some of the beneficial effects of steroids are mediated in patients with TAO.

Induction of TIMP-1 involves activation of the ERK signaling pathway

MAP kinase pathways are key mediators of several actions of IL-1β in orbital fibroblasts, including the induction of PG endoperoxide H synthase-2 and microsomal PGE_2 synthase (39). Moreover, when cultures were treated with IL-1β, there was a transient activation of ERK 1/2, as the Western blot of phosphorylated ERK demonstrates in Fig. 5*A*. The response to IL-1β was rapid and phosphorylation of ERK was apparent within 5 min and appeared maximal by 15 min. Levels of activated phospho-ERK began to decay at 30 min, the duration of the study. In contrast, levels of ERK protein were abundant and remained unaffected by treatment with the cytokine throughout the study. We next examined whether the ERK pathway was involved in the induction of TIMP-1 by IL-1β. Cultures were treated with PD98059 (10 μM), a specific inhibitor of MEK (40) that therefore influences activity immediately upstream from ERK. The compound almost completely blocked the TIMP-1 induction by IL-1β, as is evident from the data shown in Fig. 5*B*. We then transiently transfected fibroblasts with an ERK1 DN mutant kinase and found that this approach also attenuated the induction of TIMP-1 (Fig. 5*C*). These findings in aggregate demonstrate the congruence of multiple approaches for

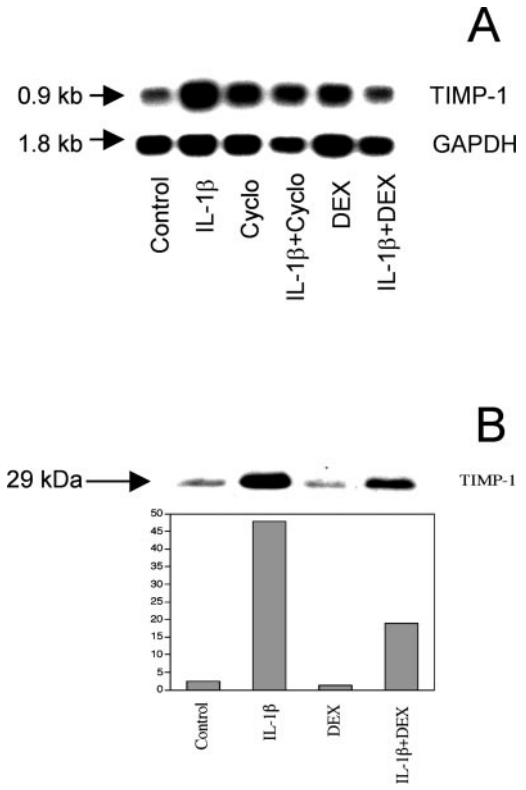

FIGURE 4. Dexamethasone and cycloheximide partially attenuate the induction of TIMP-1 in orbital fibroblasts by IL-1β. *A,* Confluent cultures were treated with IL-1β (10 ng/ml) without or with cycloheximide (10 μg/ml) or dexamethasone (100 nM) for 16 h and then the cell layers were subjected to RNA extraction and Northern blot analysis for TIMP-1 mRNA. *B,* Confluent orbital fibroblast cultures were treated with IL-1β or dexamethasone alone or in combination for 48 h. The media were harvested and subjected to Western blot analysis.

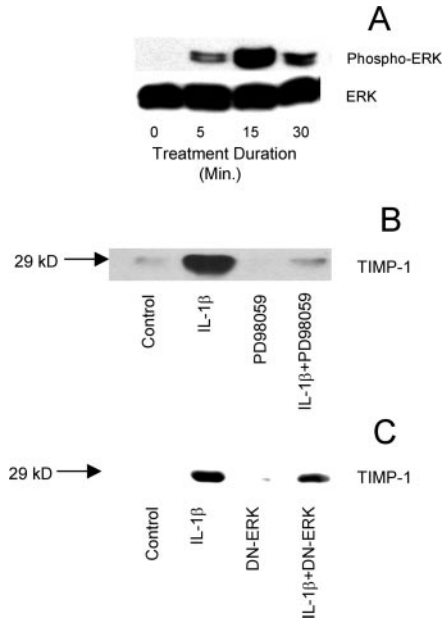

FIGURE 5. IL-1β activates the ERK pathway in orbital fibroblasts. Blocking this pathway results in an attenuation of the TIMP-1 induction by the cytokine. *A,* Confluent cultures were treated with IL-1β (10 ng/ml) for the duration indicated along the abscissa; then monolayers were processed and subjected to Western blot analysis for ERK and phospho-specific ERK. *B,* Cultures were treated with IL-1β without or with the MEK inhibitor PD98059 (10 μM) for 48 h. Aliquots of media were subjected to Western blot analysis with anti-TIMP-1 Abs. *C,* Cultures were transiently transfected with DN ERK1 and then some were treated with nothing or with IL-1β (10 ng/ml) for 48 h. Aliquots of media were Western blotted with anti-TIMP-1.

interrupting the ERK pathway. They strongly implicate signaling through that pathway in the mediation of the induction of TIMP-1 by IL-1β.

IFN-γ and IL-4 can attenuate the induction by IL-1β of TIMP-1

Several cytokines have been demonstrated in active TAO (1). Moreover, both Th1 and Th2 cytokines have been detected in affected orbital tissue from patients with this disease (7, 8). We thus investigated the impact of either Th1 cytokines, such as IFN-γ, or the Th2 cytokine, IL-4 on the induction of TIMP-1 by IL-1β. Confluent cultures were treated for 48 h with IL-1β (10 ng/ml) alone or in combination with IFN-γ (100 U/ml) or IL-4 (10 ng/ml). As the Western analysis in Fig. 6*A* demonstrates, either IFN-γ or IL-4 can markedly attenuate the TIMP-1 induction by IL-1β. This finding suggests that both Th1 and Th2 immune responses might modulate TIMP-1 expression in the orbit. We then determined whether these cytokines were acting at the pretranslational level. As the Northern blot shown in Fig. 6*B* indicates, both IFN-γ and IL-4 substantially attenuate the induction of TIMP-1 mRNA. The magnitude of the inhibition was ~60%. We next examined the potential for these same cytokines to influence TIMP-1 gene promoter activity. Both completely blocked the promoter activation by IL-1β (Fig. 6*C*). These findings are somewhat surprising, considering the often opposing actions of cytokines from Th1 and Th2 classes. We surveyed a wide array of other cytokines for their abilities to induce TIMP-1 expression. As the Western blot in Fig. 6*D* indicates, IL-1β stands out with regard to the magnitude of induction it elicits. IL-1α (10 ng/ml) could also induce TIMP-1 expression, as expected (data not shown). The magnitude was comparable to that following treatment with IL-1β. In contrast, TNF-α (10 ng/ml) and TGF-1β (10 ng/ml) had little or no effect on TIMP-1 expression in orbital fibroblasts. These cytokines have been shown to exert powerful up-regulatory effects in other cell types (41, 42). In addition, IL-5, IL-12, IL-13, IL-17, and IL-18 (all at a final concentration of 10 ng/ml) failed to substantially influence TIMP-1 protein expression. As Fig. 7, *A* and *B,* indicate, addition of IL-4 and IFN-γ neutralizing Abs can reverse the blockade imposed by these respective cytokines.

IL-1β induction of TIMP-1 expression results in inhibition of protease activity

The reverse zymogram pictured in Fig. 8 demonstrates the impact of IL-1β-treatment on the activity of TIMP released by orbital fibroblasts. Conditioned medium from control and IL-1β-treated orbital fibroblasts was electrophoresed through a gel containing recombinant MMP-9 and gelatin. As the analysis reveals, a discrete band of diminished proteolytic activity can be seen at 29 kDa. This indicates that the IL-1β-treated cultures are releasing an inhibitory protein that attenuates protolytic activity of the gelatinase. The M_r of this activity coincides with the electrophoretic mobility of TIMP-1 and suggests that the IL-1β dependent enzyme is active.

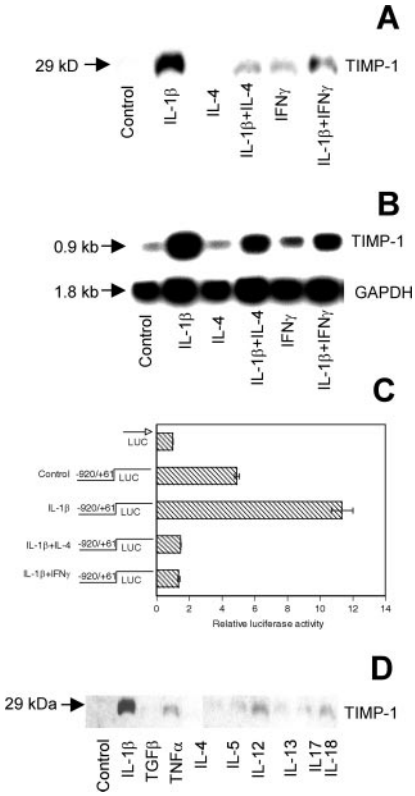

FIGURE 6. IL-4 and IFN-γ can attenuate the induction of TIMP-1 in orbital fibroblasts by IL-1β. This is mediated through the down-regulation of TIMP-1 gene promoter activity. Cultures were treated with nothing (control), IL-1β (10 ng/ml), IL-4 (10 ng/ml) or IFN-γ (100 U/ml) alone or in the combinations indicated for 48 h. Medium samples were subjected to Western blot analysis (*A*) or extracted RNA to Northern analysis (*B*) for TIMP-1 protein or mRNA, respectively. *C,* The 981-bp fragment spanning −920/+61 of the TIMP-1 gene promoter fused to a luciferase reporter gene was transiently transfected in orbital fibroblasts, and cultures were treated with nothing, IL-1β alone, or IL-1β in combination with either IL-4 or IFN-γ for 2 h. *D,* Confluent orbital fibroblast cultures were treated with nothing or the cytokine indicated (all cytokine concentrations were 10 ng/ml) for 48 h. Medium samples separated by electrophoresis were then subjected to Western blot analysis for TIMP-1 protein levels.

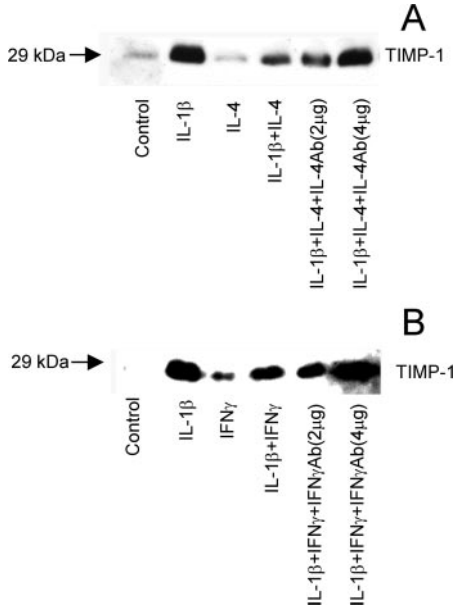

FIGURE 7. Anti-IL-4 and anti-IFN-γ Abs can reverse the blockade imposed by the respective cytokines on IL-1β induction of TIMP-1 in orbital fibroblasts. Confluent cultures were treated with nothing, IL-1β (10 ng/ml) alone, or IL-1β in combination with the compounds indicated for 48 h. The medium was then subjected to Western blot analysis for TIMP-1 content.

Discussion

TIMPs play important roles in maintaining the delicate balance between ECM production and disposal, in states of health. Derangements in their expression are associated with tissue dysfunction (26, 27). High levels of TIMP-1 expression, such as those found in IL-1β-activated orbital fibroblasts, are often associated with fibrosis. Our current findings suggest that TIMP-1 is induced to high levels of expression in IL-1β-activated orbital fibroblasts and that those strains from patients with severe TAO exhibit particularly robust induction. This is notable in light of the other exaggerated responses to IL-1 and other proinflammatory cytokines we and others have documented in these cells (12, 13, 43, 44). It would appear that orbital fibroblasts, by virtue of the diverse repertoire of regulatory molecules they express, are positioned to influence the nature and magnitude of tissue reactions occurring in the orbit. An earlier report indicated that IL-1α can up-regulate collagen degradation in rabbit corneal stromal fibroblasts (keratocytes) (45). This activity was attributable to increases in the expression of pro-MMP-1, -3, and -9. In preliminary studies, we detected MMP-2 in untreated orbital fibroblasts and its levels did not appear appreciably altered following treatment with IL-1β (data not shown). We have also found relatively high levels of TIMP-1 mRNA in several strains of untreated dermal strains, and their response to cytokines may not be as vigorous as that found in orbital fibroblasts. Further studies comparing orbital fibroblasts with those from other anatomic regions will be necessary before conclusions about the general applicability of our current findings can be made. The large capacity to produce and export TIMP-1 suggests that under the appropriate conditions, orbital fibroblasts might act as an important source of MMP inhibition. Thus the balance between ECM synthesis and degradation could be altered in states such as TAO, where high levels of IL-1 may provoke TIMP-1 expression in these fibroblasts. IL-1 has been detected in orbital tissue from patients with active TAO (46). Fibrosis is a firmly established consequence of chronic TAO. Thus our current findings suggest that TIMP-1 expression in orbital fibroblasts might be very high in the context of an inflammatory response and could contribute to a profibrotic microenvironment. The morbidity associated with fibrosis, occurring late in the course of TAO (1), makes our current findings potentially relevant to the clinical disease.

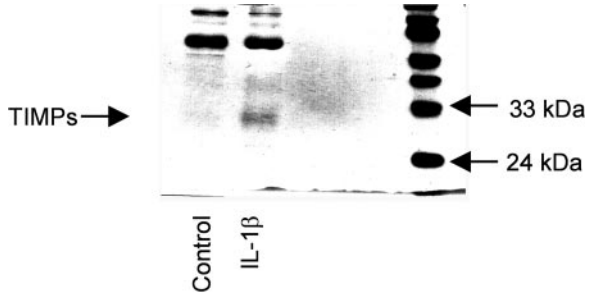

FIGURE 8. IL-1β induces the release of TIMP activity from orbital fibroblasts. Confluent orbital fibroblasts, in this case from a patient with severe TAO, were treated with nothing or IL-1β (10 ng/ml) for 48 h; then the medium was collected and subjected to reverse zymography as described in *Materials and Methods*.

The relationship between fibrosis and inhibition of ECM degradation has been studied extensively in chronic liver disease models where hepatic fibrosis develops (47, 48). Removal of TIMP-1 from HSC cell culture supernatants by chromatography resulted in a 20-fold increase in MMP activity. In human liver disease, significant increases of TIMP-1 have been observed in vivo and in vitro, unassociated with changes in MMP activity. Further studies have shown that regression of fibrosis is associated with significantly decreased TIMP-1 expression. It is tempting to speculate that the balance between TIMP-1 expression and the MMPs produced locally in the orbit may become altered in TAO and result in the tissue remodeling associated with the disease.

From the findings presented here, it would appear that IL-1β provokes TIMP-1 expression largely by enhancing transcriptional activity. In fact, those studies examining TIMP-1 mRNA stability suggest that the cytokine exerts no important influence on transcript turnover in orbital fibroblasts (Fig. 3*D*). Transcriptional regulation of the TIMP-1 gene has been studied previously and shown to involve multiple response elements (35–37, 49–51). Our findings suggest that substantial differences exist with regard to both cytokine- and cell type-specific transcriptional regulation. For instance, in HepG2, the induction of TIMP-1 by oncostatin M uses an AP1 site at −59/−53 (37) while in synovial fibroblasts, Egr-1 appears to participate in the activation of the TIMP-1 promoter (35). Of interest is the finding that this same transcriptional factor can activate collagen synthesis in fibroblasts (52). In human foreskin fibroblasts transfected with a series of both 3′ and 5′ deletions of a fragment spanning −738/+95, substantial basal promoter activity appears to be lost between the −102/+95 and −80/+95 fragments (36). From our studies reported here, it would appear that important promoter activity, inducible with IL-1β, is lost in 5′ deletions between −920/+61 and −733/+61. Notable among the potential sites lost are two PEA3 binding signatures (Fig. 3*C*; Ref. 36).

The pathogenesis of TAO is associated with the activities of both Th1 and Th2 cytokines (7, 8). Multiple cytokines have been detected in affected connective tissue and are produced in culture by clones of T lymphocytes isolated from these tissues (8). From several studies using a variety of techniques, it appears that in the early disease stages, Th1 cytokines predominate, while at later stages, Th2 cytokines are more abundant. Thus, cellular behavior in vivo may be directly influenced by multiple cytokines, either produced by infiltrating immune cells or by residential cells such as fibroblasts. The attenuation of IL-1β-induced TIMP-1 expression by both Th1 and Th2 cytokines in vitro may reflect the proximate regulation of TIMP-1 expression in situ, albeit at different stages of TAO. Although no precedent exists regarding Th1 cytokines influencing TIMP-1 synthesis, IL-4 has been shown previously in monocytes to exert a substantial attenuation of cytokine-dependent expression (42). That study failed to demonstrate the mechanism through which IL-4 might be exerting its down-regulatory effect. In view of the central role postulated for TIMP-1 over-expression in the pathogenesis of fibrosis, the findings we report here suggest that a wide spectrum of endogenous factors, including cytokines, may modulate the expression of this protein. It is the aggregate of several cytokine effects that likely drives the pattern of tissue remodeling. Based on our findings in vitro, it would appear that both Th1 and Th2 predominant immune responses might blunt protease activity and therefore limit the disposal of ECM in affected orbital connective tissue. Orbital fibroblasts express extremely high levels of certain proinflammatory cytokines such as IL-1α, IL-6, and IL-8 (43), and key prostanoid biosynthetic enzymes leading to PGE_2 production when activated (39, 44). Considering the substantial impact of that prostanoid on Th0 differentiation (53, 54), its exaggerated synthesis by these fibroblasts might serve to bias the inflammatory cell profile in TAO. Thus, it is possible that Th1 or Th2 cytokines can influence the proteolytic environment in orbital connective tissue. Therefore, these molecules and the complex cellular signaling pathways they activate may represent attractive therapeutic targets for modifying disease outcome.

Disclosures

The authors have no financial conflict of interest.

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