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The additive effect of TGF-ß with TNF-α on type VII collagen gene expression to activate the expression of an extracellular matrix-related gene

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Abstract

Type VII collagen is the predominant, if not the exclusive, component of the anchoring fibrils, attachment structures stabilizing the association of the cutaneous basement membrane to the underlying dermis. In the skin, type VII collagen is synthesized by both dermal fibroblasts and epidermal keratinocytes. Alterations in the type VII collagen protein structure or lack of its expression due to mutations in the corresponding gene COL7A1 are the hallmark of dystrophic epidermolysis bullosa, a mechano-bullous skin disease characterized by extreme fragility of the skin and leading to development of sub-lamina densa blisters. In this study, we have examined whether the additive effect of TGF-ß with TNF-α on type VII collagen gene expression is exerted at the transcriptional level by activation of the corresponding a promoter. Specifically, we demonstrate that the TNF-α effect is mediated by NF-kB1/RelA (p50/p65) and RelA/RelA (p65/p65) NF-kB complexes binding the TNF-α response element (TaRE) located in the region [7252/7230], with RelA acting as the transcriptional activator. We provide definitive evidence for the role of both TGF-ß and TNF-α response elements as enhancer sequences, functioning in the context of a heterologous promoter in an additive manner in response to TGF-ß and TNF-α. This study provides the functional interaction between the two immediate-early transcription factors, SMAD and NF-kB, to activate the expression of an extracellular matrix-related gene, COL7A1.

Key words: Type VII collagen; TGF-Beta; SMAD; NF-kB; TNF-α

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Introduction

Type VII collagen is the predominant, if not the exclusive, component of the anchoring fibrils, attachment structures stabilizing the association of the cutaneous basement membrane to the underlying dermis. In the skin, type VII collagen is synthesized by both dermal fibroblasts and epidermal keratinocytes. Alterations in the type VII collagen protein structure or lack of its expression due to mutations in the corresponding gene COL7A1 are the hallmark of dystrophic epidermolysis bullosa, a mechano-bullous skin disease characterized by extreme fragility of the

skin and leading to development of sub-lamina densa blisters [1]. The TGF-β super-family of growth factors includes the various forms of TGF-β, bone morphogenic proteins (BMP), nodals, activins, the anti-Mullerian hormone, and many other structurally related factors found in vertebrates, insects, and nematodes [2]. There are three mammalian isoforms of TGF-β (TGF-β1–3), structurally nearly identical, with a knot motif composed of six cysteine residues joined together by three intrachain disulfide bonds that stabilize β-sheet bands. One free cysteine forms an interchain disulfide bond with an identical monomeric chain to generate the mature TGF-β dimer.

TGF-β are secreted as latent precursor molecules (LTGF-β) requiring activation into a mature form for receptor binding and subsequent activation of signal transduction pathways. The LTGF-β molecules consist of 390–414 amino acids. They contain an amino-terminal hydrophobic signal peptide region, the latency-associated peptide (LAP) region, of 249 residues, and the C-terminal, potentially bioactive region that contains 112 amino acids per monomer. LTGF-β is usually secreted as a large latent complex covalently bound via the LAP region to LTGF-β-binding protein [3] or as a small latent complex without LTBP. The LAP confers latency to the complex, whereas LTBP serves to bind TGF-β to the ECM and to enable its proteolytic activation [4].

Activation of TGF-β is a complex process involving conformational changes of LTGF-β, induced by either cleavage of the LAP by various proteases such as plasmin, thrombin, plasma transglutaminase, or endoglycosylases, or by physical interactions of the LAP with other proteins, such as thrombospondin-1, leading to the release of bioactive, mature, TGF-β [5].

Furthermore; TNF-α is a potent pro-inflammatory cytokine exerting pleiotropic effects on various cell types and plays a critical role in the pathogenesis of chronic inflammatory diseases, such as RA [6]. Accumulating evidence suggests that not only soluble TNF-α, but also its precursor form, transmembrane TNF-α, is involved in the inflammatory response. Transmembrane TNF-α exerts its biological function in a cell-to-cell contact fashion, which is distinct from the feature of soluble TNF-α, which acts at sites remote from the TNF-α-producing cells [7]. In transgenic mice, transmembrane TNF-α was shown to be sufficient to induce arthritis with synovial hyperplasia and inflammation [8].

Transmembrane TNF-α acts as a ligand by binding to TNF-α receptors as well as functioning as a receptor that transmits outside-to-inside (reverse) signals back into the transmembrane TNF-α-bearing cells (TNF-α-producing cells) [9]. It is therefore considered that transmembrane TNF-α plays a critical role in local inflammation [10]. Anti-TNF agents have been successfully introduced for the treatment of chronic inflammatory diseases. However, clinical features against granulomatous inflammation are not similar among these agents. For example, all the anti-TNF agents are effective against RA, but not all of them against Crohn's disease [11]. The binding and neutralizing activities against soluble TNF-α are the critical and common mechanisms of

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action of these anti-TNF-agents. On the other hand, recent studies have shown that these agents have differential effects against transmembrane TNF-α and TNF-α-producing cells [12]. In the light of a growing body of evidence for the involvement of transmembrane TNF-α in inflammation, such as granulomatous inflammation, it would be important to summarize the biology of transmembrane TNF-α in health and disease as well as its interaction with anti-TNF agents. We would like to review the following issues: (i) biological function of transmembrane TNF-α as a ligand, (ii) biological function of transmembrane TNF-α as a receptor and (iii) different effects of anti-TNF agents on transmembrane TNF-α-bearing cells (TNF-α-producing cells) that would help to understand the different clinical effects of the anti-TNF agents.

Materials and methods

Cell cultures

Human dermal fibroblast cultures, established by explanting tissue specimens obtained from neonatal foreskins, were utilized in passages 3 ± 6. Cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 mM glutamine, and antibiotics (100 units/ml penicillin, 50 mg/ ml streptomycin-G and 0.25 mg/ml FungizoneTM). Human recombinant TGF-b2 was a kind gift from Dr David Olsen, Celtrix Co., (Palo Alto, CA, USA). Human recombinant TNF-a was purchased from Boehringer Mannheim, (Indianapolis, IN, USA).

Plasmid constructs

Various COL7A1 promoter 5' deletion/CAT constructs were cloned into promoter less pBS0CAT vector [13]. Cloning of wild-type and mutant COL7A1 SMAD binding sequence (SBS) and NF-kB fragments into pBLCAT5 was performed according to standard protocols [14]. The NF-kB1 and RelA expression vectors were obtained through the AIDS Research and Reference Reagent Program, NIAID, NIH: pRSV-NF-kB1 (p50) and pRSV-RelA (p65) from Dr Gary Nabel and Dr Neil Perkins [15].

Transient cell transfections and CAT assays

Transient cell transfections of human dermal fibroblasts were performed with calcium phosphate/DNA co-precipitation procedure [16]. Following appropriate incubation periods, the cells were rinsed once with phosphate burred saline, harvested by scraping and lysed in 200 ml of Reporter Lysis Buner (Promega, Madison, WI, USA). The b-galactosidase activities were measured according to a standard protocol [17]. Unless stated otherwise, aliquots corresponding to identical b-galactosidase activity were used for each CAT assay with [14C] chloramphenicol as substrate [18], using thin layer chromatography. Following autoradiography, the plates were cut and counted by liquid scintillation to quantify the acetylated [14C] chloramphenicol.

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Electrophoresis mobility shift assays

Promoter fragments spanning distinct regions of the COL7A1 promoter were generated by PCR amplification using 7722COL7A1/CAT as template, or by using annealed synthetic oligonucleotides, radiolabeled, and purified by electro-elution after electrophoresis in a 2% agarose gel, as described previously [19]. Nuclear extracts were isolated using a small-scale preparation [20], aliquoted in small fractions to avoid repetitive freeze-thawing, and stored at 7808C until use. The protein concentration in the extracts was determined using a commercial assay kit (Bio-Rad). For super shift experiments, the following antibodies were used: rabbit polyclonal antibodies against NF-kB1 and RelA [11], NF-kB2 (p52) (Lanoix et al., 1994), Rel (p75) [6], RelB (p68) (Nancy Rice, unpublished; raised against a synthetic 17-amino acid peptide covering the C-terminus of human RelB: REAAFGGGLLPGPEAT), a pan-NF-AT antibody, raised against an internal peptide of human NF-AT common to all members of the NF-AT family [21]. Nuclear extracts were incubated overnight with 1 ml of either antisera prior to the binding reaction.

Results

TNF-a and TGF-b exert an additive effect on the COL7A1 promoter To determine whether the additive effect of TGF-b and TNF-a on COL7A1 gene expression, as determined at both the mRNA and protein levels [22], was exerted, at least in part, at the transcriptional level through activation of the corresponding promoter, transient cell transfection experiments were performed in human dermal fibroblast cultures. These studies utilized a COL7A1 promoter/CAT reporter gene construct containing 722 bp of 5' end regulatory sequences of the human COL7A1 gene [11]. As shown in Figure 1, TGF-b and TNF-a both enhanced COL7A1 promoter activity (6.8- and 3.6-fold, respectively). An additive effect was observed (13.3-fold) when the two cytokines were added simultaneously to the fibroblast culture medium. These data establish that both cytokines exert their enhancing activity on COL7A1 gene expression, at least in part, at the transcriptional level.

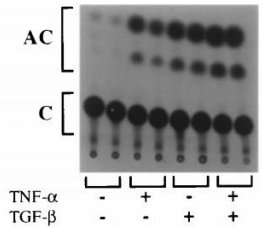

Figure 1

TNF-a and TGF-b exert an additive effect on the COL7A1 promoter. Confluent fibroblast cultures were transfected with the 7722COL7A1/CAT construct of the human COL7A1 promoter by the calcium phosphate/DNA co-precipitation procedure, as described in Materials and methods. After glycerol shock, the cultures were incubated in fresh medium containing 1% fetal calf serum. TNF-a and/or TGF-b (10 ng/ml) were added to the medium 4 h later. After 40 h of incubation, cell extracts were assayed for CAT activity with [14C] chloramphenicol as a substrate, using identical amounts of protein. Quantitation of CAT activity is expressed as the mean+SD. of independent experiments.

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Delineation of the TNF-a responsive region within the COL7A1 promoter We have recently demonstrated that the TGF-b responsive sequences reside between nucleotides 7496 and 7444, relative to the transcription start site of the COL7A1 gene [22]. To characterize the TNF-a responsive region of the COL7A1 promoter, fibroblast cultures were transfected with a series of 5' end deletion COL7A1 promoter/CAT constructs [23]. Cells were subsequently treated with TNF-a for 40 h, at which point CAT activity was determined. As shown in Figure 2, a stimulatory effect of TNF-a, varying from 2.5 ± 4.7-fold, was observed with constructs containing at least 7396 bp of COL7A1 5' regulatory sequences. TNF-a responsiveness was totally abolished by further 5' deletion to position 7230 of the promoters, indicating that the TNF-a responsive sequences are located between nucleotides 7396 and 7230.

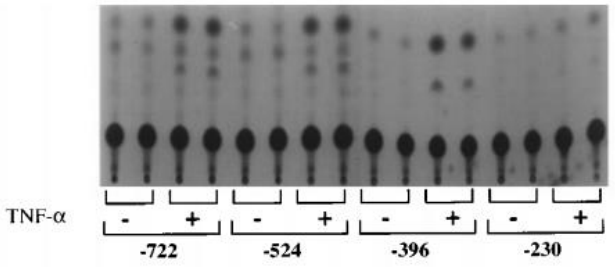

Figure 2.
Delineation of the TNF-a responsive region within the COL7A1 promoter. Fibroblast cultures were transfected with various 5' deletion/CAT constructs of the human COL7A1 promoter by the calcium phosphate/DNA co-precipitation procedure, as described in Materials and methods. After glycerol shock, the cultures were incubated in fresh medium containing 1% fetal calf serum, without (7) or with (+) TNF-a (10 ng/ml) added to the medium 4 h later. After 40 h of incubation, cell extracts were assayed for CAT activity with [14C] chloramphenicol as a substrate, using identical amounts of protein. A representative autoradiogram of the CAT assay, depicting the separation of acetylated (AC) and unacetylated (C) forms of [14C] chloramphenicol by a thin-layer chromatography is shown, together with the quantitation of CAT activity, expressed as the mean+SD. of seven separate experiments utilizing overlapping sets of 5' deletion constructs.

Point mutations within the NF-kB binding site of the COL7A1 promoter abolish the TNF-a responsiveness

To determine whether the NF-kB site was functional in the context of the COL7A1 promoter. For this purpose, novel deletion constructs were generated, with a 5' end at position 7252, keeping the NF-kB intact or mutating it (construct 7252 m) to render it incapable of binding NF-kB proteins (EMSAs not shown). Results of the transient cell transfections, shown in Figure 6, indicate that removal of the region between nucleotides 7396 and 7252 does not alter TNF-a responsiveness, the latter being lost after further deletion to 7230. These findings restrict the TNF-a responsive region to the fragment 7252/7230, a result which is in total agreement with the EMSA data presented above. In addition, it should be noted that functional mutations of the 7237/7227 NF-kB site (GGGAATTCCC to AAGAATTCAT; construct 7252 mCOL7A1/CAT) totally abolished the

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responsiveness of the CAT construct to TNF-a (Figure 3), attesting to its role as an element essential for TNF-a response of the COL7A1 promoter.

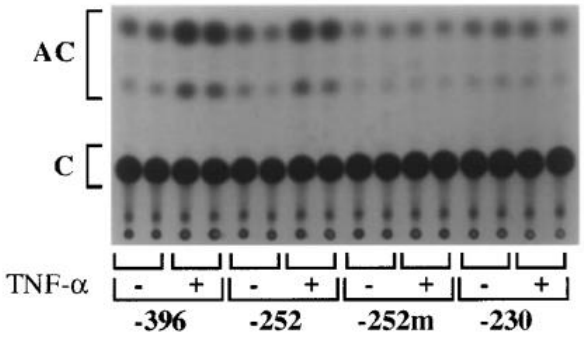

Figure 3.
Point mutations within the 7237/7227 NF-kB binding site of the COL7A1 promoter abolishes TNF-a responsiveness. Fibroblast cultures were transfected with the 7396, 7252, 7252 m, and 7230COL7A1/CAT constructs, as described in Materials and methods. The 7252 mCOL7A1/CAT constructs contain two double mutations within the 7237/7227 NF-kB binding site, which abolish NF-kB1/RelA binding (not shown). After glycerol shock, the cultures were incubated in fresh medium containing 1% fetal calf serum. TNF-a (10 ng/ml) was added to the medium 4 h later. After 40 h of incubation, cell extracts were assayed for CAT activity with [14C] chloramphenicol as a substrate, using identical amounts of protein. Quantitation of CAT activity is expressed as the mean+SD. of three independent experiments.

Combined tandem repeats of both TGF-b and TNF-a response elements confer cytokine additivity to a heterologous promoter in transient cell transfection experiments First, we aimed to determine the functionality of both the NF-kB element and the previously identified 7496/ 7444 SMAD binding sequence (SBS) corresponding to the TGF-b response element [23] outside the context of the COL7A1 promoter. For this purpose, a quadruple tandem repeat of a 22 bp oligonucleotide containing the COL7A1 NF-kB site identified above and a double tandem repeat of the SBS were cloned upstream of the thymidine kinase promoter, in the context of the plasmid pBLCAT5, either as wild-type, unaltered sequences, or as nonfunctional mutants. As shown in Figure 8a, four copies of the COL7A1 NF-kB site ((NF-kB)4-TK/CAT) conferred TNF-a but not TGF-b responsiveness to the otherwise unresponsive pBLCAT5 plasmid (not shown). This activation by TNF-a could be abolished by functional inactivation of the NF-kB site (not shown). (SBS)2-TK/CAT, on the other hand, was only inducible by TGF-b (Figure 8b). Next, both elements were cloned in combination as tandem repeats upstream of the TK promoter into pBLCAT5, either as wild-type or mutant sequences, to create the constructs (NF-kB)4-(SBS)2-TK/CAT and (NF-kBmut)4-(SBSmut)2-TK/CAT. As shown in Figure 8c, (NF-kB)4-(SBS)2-TK/CAT, not only responded to either one of the cytokines alone, but TGF-b and TNF-a exerted their additive effects, as observed in the context of the COL7A1 promoter. The effect of either cytokine, alone or in combination, was abolished when a functional mutation was introduced into its corresponding response element repeats (NF-kBmut)4- (SBSmut)2-TK/CAT, (Figure 8d).

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Together, these data attest for the fact that TGF-b and TNF-a exert their effect on (NF-kB)4-(SBS)2-TK/CAT through the SBS and NF-kB repeats, respectively.

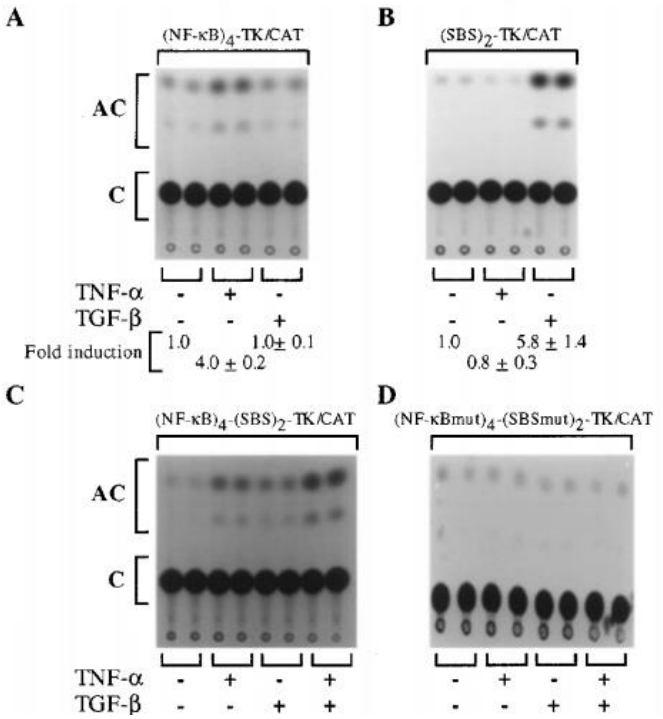

Figure 4.
Combined tandem repeats of both TGF-b and TNF-a response elements confer cytokine additivity to a heterologous promoter in transient cell transfection experiments. Fibroblast cultures were transfected with either (NF-kB)4-TK/ CAT, (SBS)2-TK/CAT, (NF-kB)4/(SBS)2-TK/CAT, or (NFkBmut)4/(SBSmut)2-TK/CAT constructs (a±d, respectively). After glycerol shock, the cultures were incubated in fresh medium containing 1% fetal calf serum for 4 h, prior to TNF-a and/or TGF-b addition (10 ng/ml). Cell extracts were assayed for CAT activity with [14C] chloramphenicol as a substrate, using identical amounts of protein. Note that the relative activities of the different constructs should not be compared, as each one of them was assayed with different protein amounts in order to optimally assess their respective growth factor responsiveness

Discussion

Type VII collagen assembly into anchoring provides the structural integrity to the stable attachment of the cutaneous basement membrane to the underlying dermis. Pathological conditions affecting type VII collagen deposition, such as structural mutations in the COL7A1 gene [24], are known as dystrophic forms of epidermolysis bullosa (DEB). Other studies have also suggested that decent expression of type VII collagen (ECM) remodeling, TGF-b promotes ECM accumulation, by both reducing the expression of metalloproteinases and by increasing the synthesis of ECM components [25]. In contrast, inflammatory cytokines such as IL-1 and TNF-a induce metalloproteinases gene expression and inhibit ECM synthesis [26]. We have recently demonstrated that TGF-b and TNFa exert their antagonistic activities on COL1A2 gene promoter through adjacent cis-elements [27]. Antagonism between TGF-b and TNF-a has been described on NF1/CTF-1- mediated transcription [28]. Specially, in the latter case, a single

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element, CTF-1, is the molecular target for mutually antagonistic regulation by TGF-b and TNF-a. The stimulatory effect of TNF-a, and its additive effect with TGF-b, on COL7A1 gene transcription is remarkable, as it contrasts its known inhibitory activity on the expression of collagen genes, such as COL1A1, COL1A2 or COL3A1 [29-32]. Contrary to these collagens whose excessive deposition leads to pathologic conditions such as hypertrophic scars, keloids, or fibrosis in general, disease states associated with type VII collagen result from decreased or absent expression of the COL7A1 gene. It may therefore be speculated that an adaptive phenomenon has taken place, allowing activation of COL7A1 gene expression in response to a variety of stimuli, including those by pro-inflammatory cytokines.

Conclusion

There have been major advances in our understanding of the TGF-β-induced intracellular signaling since the identification of SMAD proteins as direct links between the cell surface and the nucleus. The recent development of several SMAD pathway specific knockout mice and transgenic animals has confirmed the pivotal nature of the SMAD pathway in fibrogenesis and tumorigenesis. Still, several difficulties remain - 15 - before the TGF-β/SMAD pathway can be efficiently targeted in situations such as tissue fibrosis or impaired wound healing. In particular, the precise spatio-temporal role of each TGF-β/SMAD pathway component during the development of excessive ECM deposition leading to tissue fibrosis remains to be ascertained

Competing interests

The authors declare that they have no competing interests.

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