Suppression of matrix metalloproteinase-9 transcription by transforming growth factor-β is mediated by a nuclear factor-κB site

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TGF-β (transforming growth factor-β) plays a critical role in modulating the inflammatory response and other biological processes through its regulation of the production of MMPs (matrix metalloproteinases). In both Mono-Mac-6 and RAW264.7 monocyte/macrophage cells, TGF-β abrogated lipopolysaccharide-induced increases in the enzymic activity and mRNA level of MMP-9. A fragment of the human MMP-9 promoter was used to characterize its regulation by TGF-β signalling. In RAW264.7 cells, TGF-β or its downstream signalling protein, Smad3 (Sma- and Mad-related protein 3), inhibited lipopolysaccharide-stimulated promoter activity. The suppressive activity of TGF-β on the MMP-9 promoter was abrogated by an inhibitory Smad, Smad7. The MMP-9 promoter contains a putative TIE (TGF-β inhibitory element). However, neither mutation nor deletion of the TIE had any effect on the inhibitory activity of TGF-β on MMP-9 transcription, indicating that the consensus TIE is not required for this effect of TGF-β. Analysis using a series of deletion mutants of the MMP-9 promoter revealed that a region containing a consensus NF-κB (nuclear factor-κB) site is required for the basal activity and TGF-β-mediated suppression of the promoter. Mutation of the putative NF-κB site not only markedly reduced the basal transcriptional activity of the promoter, but also abrogated the responsiveness of the promoter to TGF-β. In addition, a minimal promoter containing one copy of the NF-κB sequence was responsive to TGF-β treatment. Furthermore, an electrophoretic mobility shift assay was performed with the nuclear extracts from RAW264.7 cells, and it was found that TGF-β treatment did not disrupt the binding of NF-κB p50 and p65 proteins to the NF-κB sequence. Taken together, these studies indicate that the NF-κB site is indispensable for the suppressive activity of TGF-β in the regulation of MMP-9 transcription.

Key words: immunity, inflammation, matrix metalloproteinase-9, Smad, transcription.

INTRODUCTION

Monocytes and macrophages play a central role in the inflammatory response and in innate immunity through their production of MMPs (matrix metalloproteinases), which represent a family of major matrix-degrading enzymes [1,2]. MMPs share certain biochemical properties, yet each has a distinct substrate specificity. The expression of MMPs is regulated by a variety of factors, including cytokines, growth factors, chemical agents, physical stress, cell transformation, etc. [3]. Treatment of human macrophages with LPS (lipopolysaccharide), a potent inflammatory stimulus, induces a variety of MMPs, including intestinal collagenase (MMP-1), which degrades native collagen types I and III [4], stromelysin (MMP-3), which degrades proteoglycans, laminin and fibronectin [5], and type IV collagenases (MMP-2 and MMP-9), which cleave basement membrane collagen types IV and V [5,6]. In addition, pro-inflammatory cytokines, such as TNF-α (tumour necrosis factor-α) and IL-1β (interleukin-1β), selectively up-regulate the macrophage expression of MMP-9 [7]. The combination of TNF-α or IL-1β with granulocyte/macrophage colony-stimulating factor was found to induce MMP-1 in monocytes [7]. On the other hand, TGF-β (transforming growth factor-β) has an inhibitory effect on the expression of several MMPs, including MMP-1, MMP-3 and MMP-9 in fibroblasts [8]. In monocytes or macrophages, TGF-β has been reported to exert a suppressive effect on LPS- or TNF-α-induced MMP-9 production [9,10].

TGF-β is a multifunctional immune modulator that plays an essential role in maintaining the homoeostasis of the immune system [11]. TGF-β co-ordinates events critical to the progression and resolution of inflammatory responses. This complex action involves recruitment of inflammatory cells, activation of lymphocytes, modulation of macrophage function, and expression of adhesion molecules by endothelial cells, monocytes and lymphocytes. The immune-suppressive function of TGF-β is best illustrated by *in vivo* studies in which TGF-β1 or Smad3 (Sma- and Mad-related protein3), one of the critical signalling proteins downstream of TGF-β receptors, is genetically disrupted in mouse. Targeted disruption of TGF-β1 or Smad3 in mice results in a severe and multifocal inflammatory response in all pups that are born alive [12–15]. TGF-β exerts its biological effects by interacting with two transmembrane receptors, type I and type II, that have serine/threonine kinase domains in the intracellular region [16]. After ligand binding, the activated type I receptor

Abbreviations used: ALK, activin receptor-like kinase; AP-1, activator protein-1; BMP, bone morphogenetic protein; CA-ALK, constitutively active ALK; DMEM, Dulbecco's modified Eagle's medium; EGF, epidermal growth factor; EMSA, electrophoretic mobility shift assay; FBS, fetal bovine serum; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; IL-1β, interleukin-1β; LPS, lipopolysaccharide; MM6 cells, Mono-Mac-6 cells; MMP, matrix metalloproteinase; NF-κB, nuclear factor-κB; RT-PCR, reverse transcription–PCR; SBE, Smad-binding element; Smad, Sma- and Mad-related protein; TGF-β, transforming growth factor-β; TIE, TGF-β inhibitory element; TNF-α, tumour necrosis factor-α.

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relays the signal to Smad2 and Smad3 (pathway-specific Smads), which are activated by the kinase activity of the TGF-β type I receptor through phosphorylation at the C-terminal end [17,18]. Upon phosphorylation, the pathway-specific Smads form hetero-oligomeric complexes with Smad4, the common mediator Smad [19,20]. These complexes then migrate to the nucleus and activate gene transcription either through direct DNA binding by the Smad proteins or by their association with other sequence-specific transcription factors [21]. The DNA-binding ability of Smad proteins is achieved mainly by their MH1 (Mad homologous domain 1) domains, as indicated by the crystal structure of the Smad MH1 domain [22]. For example, Smad3 and Smad4 have been shown to associate with the palindromic sequence GTCTAGAC [23], as well as with GTCT or AGAC motifs in many promoters [24–30]. The transactivating activity of Smad proteins is achieved by their MH2 domains, which interact with two closely related transcriptional co-activators, CBP [CREB (cAMP response element binding protein) binding protein] and p300, that link specific transcription factors with the basal transcriptional machinery [31–33].

The molecular mechanism underlying the inhibition of MMP expression by TGF-β has been a focus of studies for over a decade. Kerr et al. [34] reported that a *cis*-acting element is involved in the repression of the rat MMP-3 gene by TGF-β. This element, named TIE (TGF-β inhibitory element), contains the core sequence GNNTTGGtGa (N denotes any nucleotide, and lower-case letters denote preferred nucleotides), which is required for the inhibitory effects of TGF-β on MMP-3 gene expression induced by EGF (epidermal growth factor). TIE binds the c-*fos* proto-oncogene product specifically to mediate the inhibitory effect of TGF-β in fibroblasts. It was proposed that a consensus TIE is responsible for the repressive function of TGF-β on the transcription of many other genes, including those encoding transin, urokinase, elastase, proliferin and c-*myc* [34]. In addition, TIE has been found in the human and rabbit MMP-1 promoters, and it functions both as a constitutive repressor of MMP-1 gene expression and as an antagonist of transcriptional induction by phorbol esters [35]. In addition, an AP-1 (activator protein-1) site was found to be crucial for the repressive activity of TGF-β in the MMP-1 promoter [36], and the signalling protein of TGF-β receptors, Smad3, was shown to be involved in this regulation [37]. A similar AP-1-mediated suppression of gene expression was also observed for MMP-12, whereby Smad3 is involved in inhibiting the cytokine-induced transcription of the MMP-12 gene [38].

MMP-9 (gelatinase B) is an important member of the MMP family, and is involved in the cleavage of denatured collagens of all types and of native basement membrane components [1,2]. Studies have found that MMP-9 is also able to cleave fibrin [39], serpin α_1-proteinase inhibitor [40], IL-1β [41,42] and TGF-β [43], indicating that MMP-9 is involved in various biological processes, including tissue remodelling, the inflammatory response and oncogenesis. The gene structure and promoter of MMP-9 have been characterized [44,45], although how TGF-β represses the transcription of MMP-9 is still unclear. To elucidate the molecular mechanisms by which TGF-β inhibits MMP-9 gene expression, we have analysed the human MMP-9 promoter in macrophages and monocytes, and addressed how Smad proteins and other factors are involved in this regulation.

MATERIALS AND METHODS

Reagents

RPMI 1640 medium, DMEM (Dulbecco's modified Eagle's medium), FBS (fetal bovine serum), and penicillin/streptomycin were purchased from Bio Whittaker (Walkersville, MD, U.S.A.). Macrophage-SFM medium was purchased from Life Technologies (Rockville, MD, U.S.A.). Recombinant human TGF-β1 was obtained from R&D Systems (Minneapolis, MN, U.S.A.). Recombinant human activin A was provided by the National Hormone and Pituitary Program (NHPP; Rockville, MD, U.S.A.). LPS was purchased from Sigma (St. Louis, MO, U.S.A.). Antibodies against NF-κB (nuclear factor-κB) p50 and p65 were from Upstate USA, Inc. (Charlottesville, VA, U.S.A.) and the anti-Smad2/3 antibody was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, U.S.A.).

Cell culture

MM6 (Mono-Mac-6) cells, a human monocytic leukaemia cell line (DSMZ no. ACC-124; German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany), was cultured in RPMI 1640 medium supplemented with 2 mM glutamine, 100 units/ml penicillin, 100 mg/ml streptomycin and 10 % (v/v) heat-inactivated (56 °C, 30 min) FBS. RAW264.7 cells, a mouse macrophage cell line (A.T.C.C. no. TIB-71; A.T.C.C., Manassas, VA, U.S.A.) was cultured in DMEM containing 10 % FBS supplemented with penicillin and streptomycin. For gelatin zymography, MM6 or RAW264.7 cells were cultured at 2×10^5 cells/ml in serum-free Macrophage-SFM medium. Cells were seeded in 24-well plates (Greiner, Lake Mary, FL, U.S.A.) at 1 ml/well and cultured for 48 h with LPS, TGF-β and/or activin. For RT-PCR (reverse transcription–PCR), MM6 cells were seeded at 5×10^5 cells/ml in 24-well plates and treated with LPS and/or TGF-β for various incubation periods. For transient transfection, RAW264.7 cells were cultured at 3×10^5 cells/ml in 24-well plates. Cells were cultured at 0.5 ml/well overnight, and then transfection was performed using SuperFect reagent (Qiagen, Valencia, CA, U.S.A.).

Gelatin zymography

Cell-conditioned medium was subjected to substrate gel electrophoresis [46] with some modifications. The samples were applied without reduction to a 10 % (w/v) polyacrylamide gel impregnated with 1 mg/ml gelatin (Sigma). After electrophoresis, the gel was washed in washing buffer (50 mM Tris/HCl, pH 7.5, 5 mM $CaCl_2$, 1 μM $ZnCl_2$, 2.5 % Triton X-100) for 30 min at room temperature, and then incubated overnight at 37 °C with shaking in the same buffer, except that Triton X-100 was present at 1 %. The gel was stained with a solution of 0.1 % Coomassie Brilliant Blue R-250. In this assay, clear zones against the blue background indicate the presence of gelatinolytic activity. The gelatinolytic activity was quantified using densitometric analysis (NIH Image program). For inhibition studies, gel slices were incubated overnight at 37 °C in the presence of 20 mM EDTA. The appearance of gelatinolytic bands was completely inhibited by 20 mM EDTA.

RNA isolation and cDNA synthesis

RNA from MM6 cells was isolated using RNeasy (Qiagen). A sample of 1 μg of the recovered RNA was treated with RNase-free DNase I (Invitrogen, Rockville, MD, U.S.A.) to remove the residual DNA, and reverse-transcribed in a 25 μl volume reaction with oligo(dT) primer using the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen) to generate first-strand cDNA. Products were diluted to a final volume of 1 ml. Diluted cDNA was stored at − 80 °C until used for PCR.

Table 1 Oligonucleotide primers used in RT-PCR

Transcript	Primer	Location	Sequence (5′ to 3′)	Product (bp)
MMP-2	5′	1276–1295	ACCTACACCAAGAACTTCCG	654
	3′	1910–1929	TTGGTTCTCCAGCTTCAGGT	
MMP-9	5′	380–399	ACTACTCTGAAGACTTGCCG	707
	3′	1067–1086	GGTACAGGAAGAGTACTGCT	
G3PDH	5′	301–322	GTCTTCACCACCATGGAGAAGG	683
	3′	962–983	ATGAGGTCCACCACCCTGTTGC	

PCR

The oligonucleotides used for PCR reactions to detect the expression of human MMP-2, MMP-9 and G3PDH (glyceraldehyde-3-phosphate dehydrogenase) are shown in Table 1. PCR reactions were performed in a total volume of 10 μl containing 10 mM Tris/HCl, 50 mM KCl, 2.5 mM $MgCl_2$, 0.2 μM each dNTP, 1 μM each primer, 1.0 unit of *Taq* DNA polymerase (Promega, Madison, WI, U.S.A.) and 2 μl of previously diluted reverse transcription reaction. The thermal cycling parameters consisted of denaturation at 94 °C for 30 s, annealing at 56 °C for 30 s and extension at 72 °C for 45 s, for 30 cycles. The PCR products were separated on 2 % (w/v) agarose gels in 0.5 × TAE (40 mM Tris/acetic acid and 1 mM EDTA) buffer and visualized with ethidium bromide.

Plasmid construction

A DNA fragment corresponding to 689 bp of the 5′-flanking region of the human MMP-9 gene (−670 to +19) was isolated from a CAT (chloramphenicol acetyltransferase)–reporter construct of the MMP-9 promoter [45] and cloned into the pGL3-basic luciferase vector (Promega). Mutant constructs of TIE (GGTTTGGGGA being replaced by GGTTcGaGGA), SBE (Smad-binding element; AGACAG being replaced by AtcgAG) and the NF-κB site (GGAATTCCCC being replaced by GtcAacagCC) were generated by a PCR-based method. A series of deletion constructs was generated by overlap-extension PCR followed by subcloning into pGL3-basic. Expression plasmids for Smad3, Smad4, Smad5, Smad7, CA-ALK3 (constitutively active activin receptor-like kinase 3) and CA-ALK5 expression have been described previously [29].

Promoter assay

RAW264.7 cells were transfected with different combinations of plasmid DNA. The *Renilla* luciferase vector phRL-SV40 (Promega) was co-transfected to serve as an internal control for transfection efficiency. The cells were harvested 30–32 h after transfection by lysis with 120 μl of lysis buffer. For the LPS- and/or TGF-β-treated groups, cells were treated with LPS (100 ng/ml) and/or TGF-β1 (1.25 ng/ml) for 6 or 8 h before harvest. Aliquots of 10 μl of the lysate were used for the dual-luciferase assay. The samples were counted for 10 s in an FB12 luminometer (Zylux), and the data were represented as relative light units/s.

EMSA (electrophoretic mobility shift assay)

Double-stranded oligonucleotides were labelled with [γ-^{32}P]dATP by T4 polynucleotide kinase. For analysis of NF-κB binding, the sequence CCAGTGGAATTCCCCAGCCT, which contains the consensus NF-κB site of the human MMP-9 promoter, was used. For Smad binding, the sequence CAGGGTGTC-TAGACGGCCACG, based on the mouse Smad7 promoter, was used [29]. The nuclear extracts were prepared as described [47]. The labelled probes (approx. 5×10^4 c.p.m.) were incubated with 2 μg of nuclear extract in a buffer containing final concentrations of 4 % (v/v) glycerol, 10 mM Tris (pH 7.5), 1 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM dithiothreitol, 50 mM NaCl and 0.1 μg/μl poly(dI-dC). For supershift EMSA, antibodies against Smad2/3, NF-κB p50 and NF-κB p65 were incubated individually with nuclear extracts at room temperature for 10 min before addition of the probe. The binding reaction was incubated on ice for 60 min, separated on a non-denaturing 6 % (w/v) polyacrylamide gel in 0.5 × TBE buffer (1 × TBE = 45 mM Tris/borate/1 mM EDTA) and detected by autoradiography.

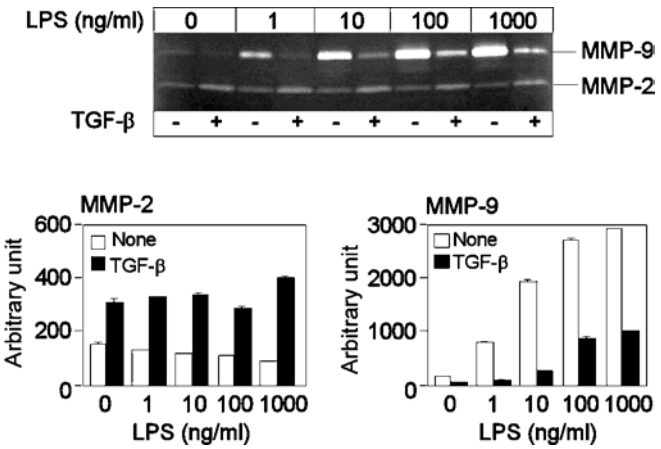

Figure 1 Effects of TGF-β on MMP-2 and MMP-9 production in MM6 cells

MM6 cells were incubated with various concentrations of LPS (0–1000 ng/ml) in the presence or absence of TGF-β1 (1.25 ng/ml). After treatment for 48 h, the cell supernatant was subjected to gelatin zymography (upper panel). The intensity of the gelatinolytic bands for MMP-2 and MMP-9 was measured using NIH Image. The relative intensity of the bands is shown (lower panels). Three independent experiments were performed with similar results.

RESULTS

TGF-β decreases MMP-9 production in monocyte/macrophage cell lines

To evaluate the role of TGF-β in regulating MMP-9 production in monocytes/macrophages, we analysed gelatinase activity in the culture medium of human monocytic leukaemia MM6 cells. MM6 cells were treated or not with LPS and/or TGF-β and the enzymic activity of MMPs was measured by gelatin zymography. As shown in Figure 1, LPS treatment significantly increased MMP-9 activity in the culture medium of MM6 cells in a dose-dependent manner, whereas it did not affect MMP-2 activity. TGF-β treatment decreased both basal and LPS-induced MMP-9 activity in the culture supernatant of MM6 cells. In contrast, the gelatinase activity of MMP-2 was increased in MM6 cells by exogenous TGF-β. On the other hand, activin A, a closely related member of the TGF-β family, did not have any effect on either MMP-2 or MMP-9 in the presence or the absence of LPS stimulation (results not shown).

We performed a similar experiment using the mouse macrophage cell line RAW264.7. LPS treatment significantly increased MMP-9 activity in the culture medium of RAW264.7 cells in a

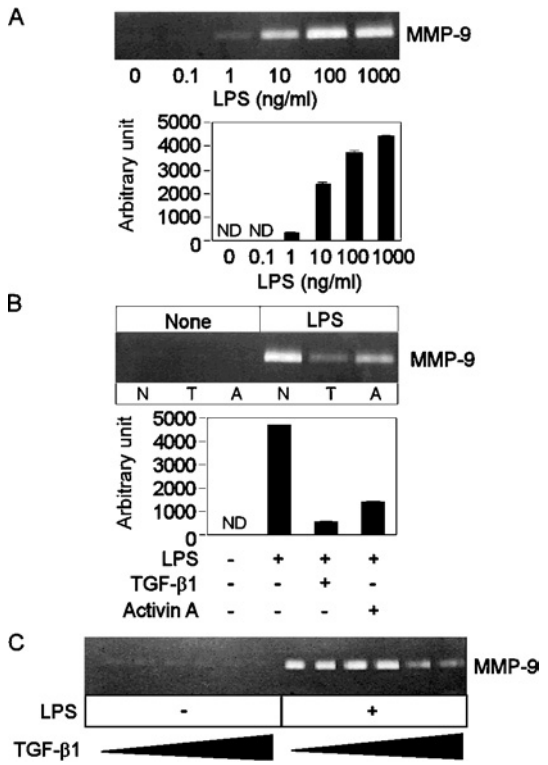

Figure 2 Effects of TGF-β and activin on MMP-9 production in RAW264.7 cells

(**A**) Dose-dependent stimulation of MMP-9 activity in RAW264.7 cells by LPS. RAW264.7 cells were incubated with various concentrations of LPS. After 48 h of incubation, the gelatinolytic activity of the culture medium was assayed by gelatin zymography (upper panel). The intensity of the band for MMP-9 was measured by NIH Image (lower panel). (**B**) Effects of TGF-β and activin on MMP-9 production in RAW264.7 cells. RAW264.7 cells were incubated with TGF-β (1.25 ng/ml) or activin A (20 ng/ml) in the presence or absence of LPS (100 ng/ml). The culture medium was collected 48 h later and used in gelatin zymography (upper panel), and the intensity of each band was determined (lower panel). Three independent experiments were performed with similar results. ND, not detectable; N, no treatment; T, TGF-β; A, activin A. (**C**) Effects of various concentrations of TGF-β1 on LPS-induced MMP-9 production. RAW264.7 cells were incubated with TGF-β1 (0, 0.005, 0.0125, 0.025, 0.05 or 0.125 ng/ml) in the presence or absence of LPS (100 ng/ml). Note the dose-dependent inhibition of MMP-9 by TGF-β1.

dose-dependent manner (Figure 2A). MMP-2 activity was not detectable in the culture medium of RAW264.7 cells even after LPS stimulation (results not shown). Similar to the findings in MM6 cells, LPS-induced MMP-9 production in RAW264.7 cells was suppressed by TGF-β (Figure 2B). In addition, activin A inhibited MMP-9 activity. Furthermore, TGF-β inhibited LPS-induced MMP-9 production in a dose-dependent manner; maximal inhibition occurred at 0.125 ng/ml TGF-β (Figure 2C). Taken together, these experiments confirm that TGF-β suppresses LPS-induced MMP-9 production in both of these monocyte/macrophage cell lines.

TGF-β decreases the level of MMP-9 mRNA in MM6 cells

We next used RT-PCR to determine if the TGF-β-mediated regulation of MMP-9 production is due to an effect on gene expression. MM6 cells were treated with different combinations of LPS and TGF-β1 and the total RNA was isolated at various times after treatment. The RNA samples were subjected to RT-PCR with specific primers for MMP-2, MMP-9 and G3PDH (used as a control to monitor the relative RNA level used in the experiment) (Table 1). LPS treatment increased the MMP-9 mRNA level in a dose-dependent manner, and this increase was lowered by TGF-β1 (Figure 3). Thus TGF-β suppresses MMP-9 production at the mRNA level. LPS did not change the amount of MMP-2 mRNA in MM6 cells. However, TGF-β1 treatment elevated MMP-2 mRNA levels, consistent with the increase in enzymic activity indicated by gelatin zymography analysis (Figure 1).

Regulation of the MMP-9 promoter by TGF-β signalling

Our experiments indicated that TGF-β affects the expression of MMP-9 in monocyte/macrophage cell lines at the mRNA level. Activation of TGF-β receptors by ligand binding elicits downstream signalling events via the phosphorylation of Smad proteins. In particular, Smad2 and Smad3 have been postulated to be the cognate Smad proteins activated by TGF-β receptors; they form a complex with Smad4, followed by nuclear translocation and regulation of target genes [21]. To determine if Smad proteins specific for the TGF-β signalling pathway are involved in the suppression of MMP-9 transcription, we analysed regulation of the human MMP-9 promoter by TGF-β treatment or Smad proteins downstream of the TGF-β receptor. The human MMP-9 promoter has been characterized previously [45]. Based on this information, we used a 689 bp human MMP-9 promoter region that spans positions -670 to $+19$ relative to the transcription initiation site. This fragment was linked to a firefly luciferase reporter and used in transient transfection experiments. RAW264.7 cells were transfected with this reporter, followed by treatment with TGF-β1. LPS increased promoter activity, and the LPS-induced stimulation was suppressed significantly by TGF-β treatment (Figure 4A). We also tested the activity of a constitutively active TGF-β type I receptor (CA-ALK5), which was made by replacement of threonine-204 with aspartic acid, yielding a type I receptor that has elevated kinase activity independent of ligand stimulation [48]. CA-ALK5 with Smad4 and Smad3, the cognate Smad proteins downstream of the TGF-β receptor, strongly suppressed both basal and LPS-induced promoter activity (Figure 4B). These data indicate that the human MMP-9 promoter is under negative regulation by TGF-β receptor activation in RAW264.7 cells.

We next examined whether the MMP-9 promoter is specifically responsive to TGF-β signalling. We analysed promoter activity in RAW264.7 cells after transfection with TGF-β- or BMP (bone morphogenetic protein)-specific Smads combined with constitutively active type I receptors for either TGF-β or BMP (Figure 4C). The combination of the TGF-β type I receptor (CA-ALK5) with TGF-β-specific Smad3 suppressed LPS-stimulated MMP-9 promoter activity, whereas the constitutively active BMP type I receptor (CA-ALK3) did not affect promoter activity in the presence of Smad5, a BMP-specific Smad. To further confirm that the Smad proteins downstream of TGF-β receptors are involved in the suppression of MMP-9 promoter activity, we analysed the effect of Smad7, an inhibitory Smad that suppresses TGF-β/activin signalling by associating with the type I receptor and preventing association and activation of pathway-specific Smads by the receptor [49–51]. As shown in Figure 4(D), expression of Smad7 diminished the suppressive effect of CA-ALK5 on LPS-induced MMP-9 promoter activity in a dose-dependent manner. However, Smad7 expression alone had only a minimal effect on basal MMP-9 promoter activity. In control experiments, we analysed the activities of CA-ALK5 and CA-ALK3 plus respective Smad proteins in RAW264.7 cells. In one experiment, we examined whether CA-ALK5 could stimulate TGF-β signalling in the presence of Smad3 and Smad4. CA-ALK5

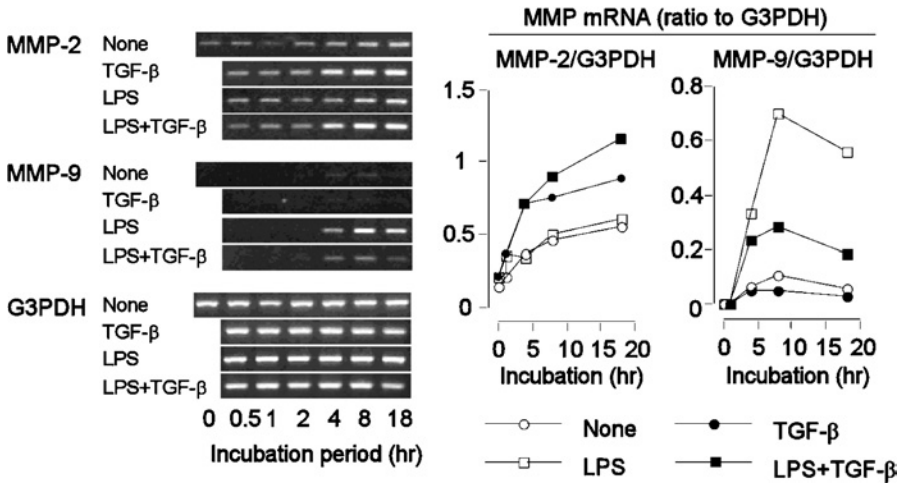

Figure 3 Effects of TGF-β on levels of MMP-2 and MMP-9 mRNAs

Total RNAs from MM6 cells treated with LPS (100 ng/ml) and/or TGF-β (1.25 ng/ml) were isolated at various time points and used for RT-PCR with specific primers for MMP-2, MMP-9 and G3PDH. The PCR products were run in a 2 % (w/v) agarose gel and stained with ethidium bromide (left panel). The intensities of the PCR products of MMP-2 and MMP-9 were normalized to G3PDH levels (right panels).

strongly stimulated a TGF-β-responsive reporter that contained tandem repeats of SBE [29] (Figure 4E). In addition, we used a BMP-responsive reporter to examine the activity of CA-ALK3. As shown in Figure 4(F), CA-ALK3 together with Smad5 and Smad4 stimulated the activity of the Id1 promoter, which has been reported to be responsive to BMP signalling [52,53]. Therefore these control experiments confirmed that the constitutively active receptors and the Smads used in transfections are functioning in the RAW264.7 cells.

TIE is not required for the suppression of MMP-9 transcription by TGF-β

To address the molecular mechanism underlying the suppressive effect of TGF-β on MMP-9 expression, we first analysed the possible involvement of a consensus element, TIE, found in the human MMP-9 promoter. Analysis of the human MMP-9 promoter sequence revealed the presence of a putative TIE at positions -474 to -465 of the promoter (Figure 5A). This sequence, GGTTTGGGA, matches the proposed consensus TIE sequence very well. We mutated the putative TIE in the MMP-9 promoter, with the wild-type sequence GGTTTGGGGA site being replaced by GGTCGGAGA; a similar mutation abrogated TIE activity in the MMP-3 promoter [34]. In addition, a promoter construct lacking the region -505 to -401, covering the TIE, was also generated. These mutant MMP-9 promoters were transfected into RAW264.7 cells, which were either treated with TGF-β1 or co-transfected with CA-ALK5 and Smad3/4 to activate TGF-β signalling. As shown in Figure 5(B), activation of TGF-β signalling suppressed LPS-stimulated transcriptional activity with the wild-type MMP-9 promoter. However, neither mutation nor deletion of the TIE had any effect on the suppressive activity of TGF-β signalling on the MMP-9 promoter. These experiments indicate that the consensus TIE found in the human MMP-9 promoter is not required for the inhibitory activity of TGF-β on MMP-9 gene transcription.

Characterization of TGF-β responsive region in the MMP-9 promoter

In order to localize the region of MMP-9 promoter that is required for the suppressive activity of TGF-β, we generated a series of deletion mutants of the MMP-9 promoter (Figure 6A). The putative transcription factor binding sites in the promoter region were detected using Transcription Element Search Software (TESS; http://www.cbil.upenn.edu/tess). These deletion mutants were linked to a luciferase reporter and used for transient transfection in RAW264.7 cells. Deletion of the regions $-670/-591$, $-554/-505$ and $-150/+19$ greatly reduced the basal transcriptional activity of the MMP-9 promoter (Figure 6B). In contrast, deletion of the region $-505/-401$, which contains the putative TIE, increased basal promoter activity. When the fold change in luciferase activity was analysed, the region $-670/-591$ was implicated in the repressive activity of TGF-β, as TGF-β treatment could no longer inhibit LPS-stimulated promoter activity.

A NF-κB site is required for the suppression of MMP-9 transcription by TGF-β

Sequence analysis revealed that the TGF-β-responsive region identified here contains a consensus NF-κB site (GGAATTCCCC) at positions -600 to -591. In addition, a putative SBE (with the sequence AGACAG, at positions -620 to -615) is located immediately upstream of the NF-κB site in the MMP-9 promoter (Figure 7A). To characterize further the possible contributions of the putative SBE and NF-κB sites to the TGF-β-mediated transcriptional suppression of the MMP-9 promoter, we generated two mutation constructs, with one site being disrupted in each. The putative SBE was replaced with ATCGAG and the putative NF-κB site was changed to GTCAACAGCC (Figure 7A). The full-length MMP-9 promoter ($-670/+19$) containing these mutations was linked to a luciferase reporter and transfected into RAW264.7 cells for transcriptional studies. As shown in Figure 7(B), mutation of the putative SBE had no significant effect on the repressive activity of TGF-β. However, mutation of the putative NF-κB site not only decreased the basal transcriptional activity of the promoter, but also abrogated the responsiveness of the promoter to TGF-β. These data strongly suggest that the NF-κB site is implicated in the repressive activity of TGF-β on the MMP-9 promoter.

To analyse further the contribution of the putative NF-κB site to the repressive activity of TGF-β, we generated a construct that contained one copy of the NF-κB sequence (GGAATTCCCC)

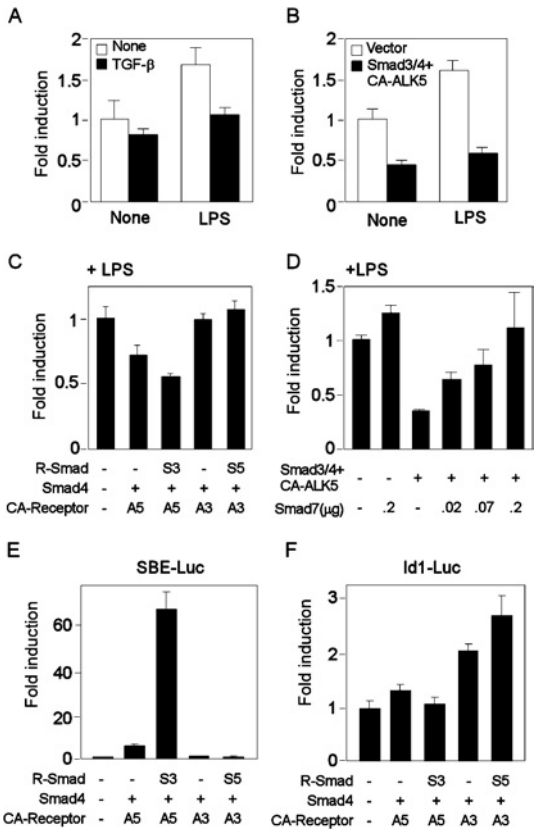

Figure 4 Regulation of the MMP-9 promoter by TGF-β signalling in RAW264.7 cells

(**A**) Repression of the MMP-9 promoter by TGF-β. RAW264.7 cells were transiently transfected with a MMP-9 promoter construct ($-670/+19$) and a *Renilla* luciferase vector. At 24 h after transfection, cells were treated with LPS (100 ng/ml) and/or TGF-β (1.25 ng/ml) for an additional 8 h. The whole-cell lysate was used in a dual-luciferase assay. Fold changes in luciferase activity normalized to *Renilla* luciferase activity are shown as means $\pm$ S.D. (**B**) Repression of MMP-9 transcription by Smad3. RAW264.7 cells were transfected with MMP-9 promoter construct plus Smad3, Smad4 and a constitutively active TGF-β type I receptor (CA-ALK5) as indicated. At 24 h after transfection, cells were treated with LPS for 8 h and used in luciferase assays. The fold induction of transcriptional activity was calculated as the ratio of values obtained following transfection with Smad3/Smad4/CA-ALK5 expression plasmids and with empty vectors (means $\pm$ S.D.). (**C**) Activation of the TGF-β receptor, but not the BMP receptor, affects MMP-9 transcription. Cells were transfected with the MMP-9 promoter construct and various combinations of expression plasmids to activate TGF-β signalling [Smad3 (S3), Smad4 and CA-ALK5 (A5)] or BMP signalling [Smad5 (S5), Smad4 and CA-ALK3 (A3)]. The transfected cells were treated with LPS for 8 h and the cell lysate was used to measure luciferase activity. (**D**) Smad7 abrogates regulation of the MMP-9 promoter by TGF-β signalling. RAW264.7 cells were transfected with the MMP-9 promoter and Smad3, Smad4 and CA-ALK5 to activate TGF-β signalling. Different amounts of Smad7 (0.02, 0.07 or 0.2 μg/well) were co-transfected as indicated. The cells were treated with LPS at 24 h after transfection. After 8 h of additional incubation, luciferase activity was measured. (**E**) Control experiment showing that CA-ALK5 stimulates TGF-β signalling. RAW264.7 cells were transfected with a SBE-containing luciferase reporter, which is responsive to TGF-β activation. This promoter is profoundly stimulated by CA-ALK5 in the presence of Smad3 and Smad4. (**F**) Control experiment showing that CA-ALK3 stimulates BMP signalling. RAW264.7 cells were transfected with a luciferase reporter driven by an Id1 promoter that is responsive to BMP activation. Note that this promoter is activated by CA-ALK3 in the presence of Smad4 and Smad5.

directly upstream of a TATA box. This construct was linked to a luciferase reporter and transfected into RAW264.7 cells. LPS treatment activated this minimal promoter, and TGF-β treatment greatly suppressed the LPS-mediated stimulation of transcription (Figure 8). Taken together, these data provided further evidence that the repressive activity of TGF-β on the MMP-9 promoter is mediated by the NF-κB site.

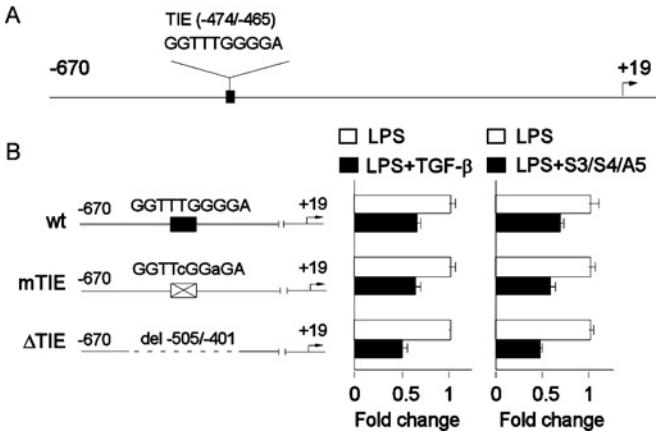

Figure 5 A putative TIE in the MMP-9 promoter is not necessary for TGF-β-mediated repression

(**A**) Graphic illustration to show the location and sequence of the putative TIE within the MMP-9 promoter. (**B**) Effect of mutation (m) or deletion (Δ) of TIE on MMP-9 transcription. Mutation of the putative TIE in the MMP-9 promoter was performed using a PCR-based method; the wild-type (wt) GGTTTGGGGA sequence was mutated to GGTCGGAGA (mutated nucleotides are underlined). In addition, deletion of the region $-505/-401$, containing the putative TIE, was carried out. The wild-type and mutant promoters were transfected into RAW264.7 cells. TGF-β signalling was activated by either treatment of the transfected cells with TGF-β1 (1.25 ng/ml) or co-expression of Smad3, Smad4 and CA-ALK5 (S3/S4/A5). All cells were treated with LPS for 8 h before luciferase assay. The fold change in luciferase activity normalized to *Renilla* luciferase activity is shown (means $\pm$ S.D.).

TGF-β does not interfere with binding of the NF-κB p50 and p65 proteins to the NF-κB site in the MMP-9 promoter

Five NF-κB/rel family members, i.e. p50, p52, p65, relB and c-rel, are involved in the binding and activation of NF-κB elements in mammalian cells [54]. One of the potential mechanisms whereby TGF-β might suppress MMP-9 transcription is to disrupt the binding of NF-κB proteins to their cognate sites. To address this possibility, we characterized the protein complex that binds the consensus NF-κB site in the MMP-9 promoter. Nuclear extracts were isolated from RAW264.7 cells by various treatments and used in an EMSA. LPS treatment for 0.5 h induced complex formation (Figure 9, lanes 3 and 9 compared with lanes 1 and 7 respectively). Inclusion of an anti-p50 antibody lowered the intensity of the complex and induce a supershifted band (lanes 4 and 6), indicating that the p50 subunit is involved in binding to the NF-κB sequence in these cells. In addition, an anti-p65 antibody disrupted the shifted band (lanes 10 and 12), suggesting the existence of p65 in the NF-κB binding complex. However, TGF-β1 treatment appeared not to significantly alter binding of the p50 and p65 subunits to the NF-κB sequence (lanes 5, 6, 11 and 12). These data suggest that the suppression of NF-κB transactivation by TGF-β in the MMP-9 promoter is not likely to be caused by disruption of the binding of the NF-κB subunits to the consensus sequence.

To analyse whether or not Smad proteins downstream of TGF-β receptors are implicated in the complex that binds the NF-κB sequence, we employed an anti-Smad2/3 antibody in the gel mobility shift assay. This antibody did not supershift or change the NF-κB binding complex in the nuclear extracts of RAW264.7 cells before or after TGF-β treatment (Figure 10, upper panel), indicating that Smad2 and Smad3 are not directly involved in the binding to the NF-κB site in the MMP-9 promoter. As a positive control, we used the same nuclear extracts in a gel mobility shift assay with a SBE from the Smad7 promoter [29] as a probe (Figure 10, lower panel). TGF-β1 treatment induced

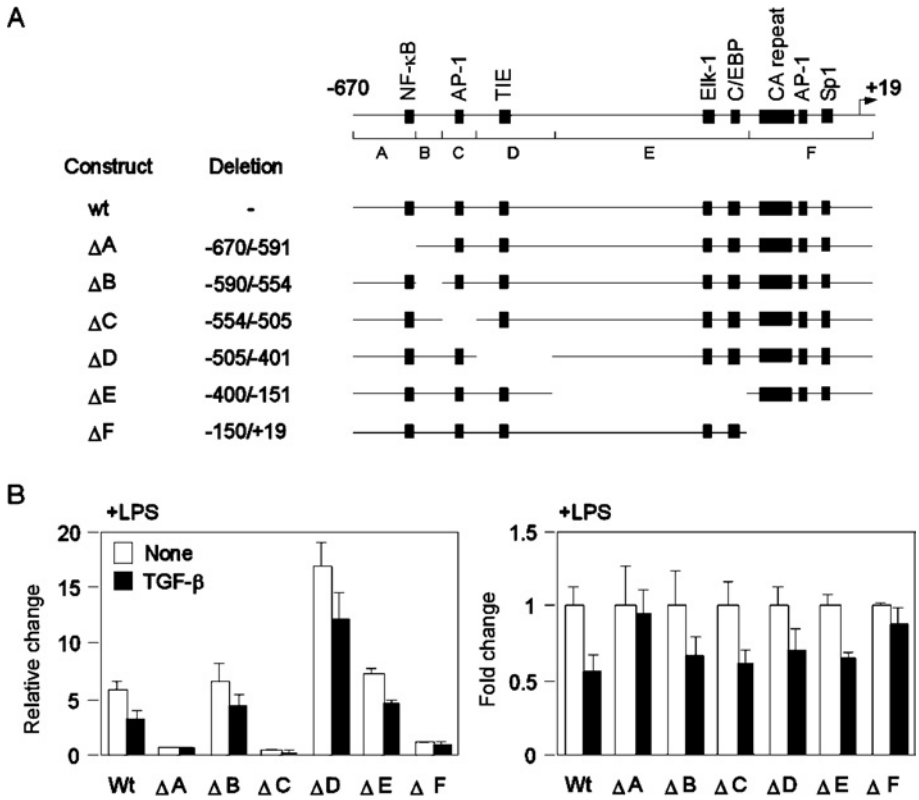

Figure 6 Characterization of the TGF-β-responsive region in the MMP-9 promoter

(**A**) Schematic representation of the mutant constructs of the human MMP-9 promoter. The promoter was divided into six subregions (A–F), based on physical distance. A series of deletion mutants of the human MMP-9 promoter was generated by PCR-based methods. The putative transcription factor binding sites conserved in both human and mouse genomic sequences are indicated. wt, wild type. (**B**) Transactivating activity of the mutant MMP-9 promoters. RAW264.7 cells were transfected with various deletion constructs of the MMP-9 promoter as indicated. The cells were treated with LPS (100 ng/ml) with or without TGF-β (1.25 ng/ml) for 8 h before cell harvest. The luciferase activities of the promoter constructs were measured after normalization to *Renilla* luciferase activity, and both relative change (left panel) and fold change (right panel) are shown as means $\pm$ S.D.

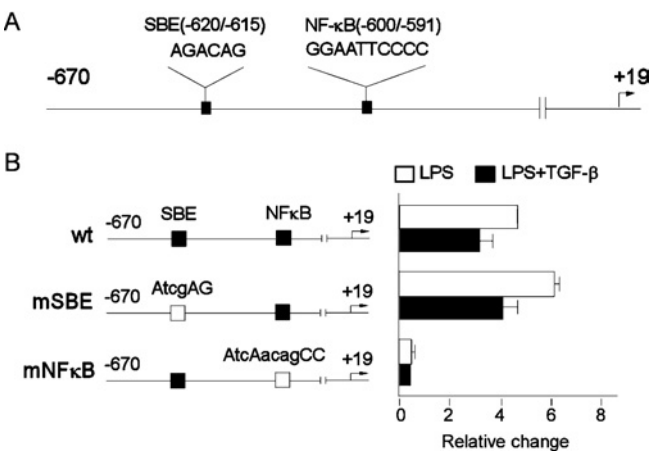

Figure 7 A NF-κB site in the MMP-9 promoter is required for the suppressive activity of TGF-β

(**A**) Schematic representation to illustrate the locations and sequences of the putative SBE and NF-κB sites in the human MMP-9 promoter. (**B**) Effects of mutations of the putative SBE and NF-κB sites on MMP-9 promoter activity. Mutation of the putative SBE and NF-κB consensus sites was carried out using a PCR-based method; the mutated nucleotides are shown in lower case. The mutant (m) and wild-type (wt) MMP-9 promoters were transfected into RAW264.7 cells. At 24 h after transfection, cells were treated with LPS (100 ng/ml), with or without TGF-β1 (1.25 ng/ml) for 8 h. The luciferase activity was measured and normalized to *Rellina* luciferase activity. The relative change in MMP-9 promoter activity is shown (means $\pm$ S.D.).

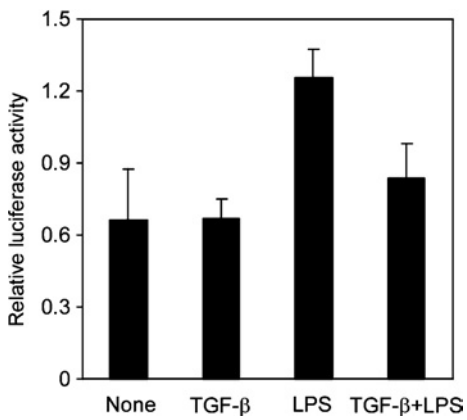

Figure 8 A minimal promoter containing the NF-κB site is sufficient to mediate repression by TGF-β

A minimal promoter construct was generated to contain one copy of the NF-κB site from the MMP-9 promoter upstream of a TATA box and luciferase reporter. This construct was transfected into RAW264.7 cells. At 24 h after transfection, cells were treated with LPS (100 ng/ml), with or without TGF-β1 (1.25 ng/ml) for 8 h. The luciferase activity of the promoter was normalized to *Rellina* luciferase activity. The relative change in luciferase activity is shown (means $\pm$ S.D.).

a SBE-binding complex in the absence (lane 6) or presence (lanes 7 and 9) of LPS. The anti-Smad2/3 antibody completely supershifted the SBE-binding complex (lanes 8 and 10). Taken together, these data suggest that the TGF-β-mediated inhibition

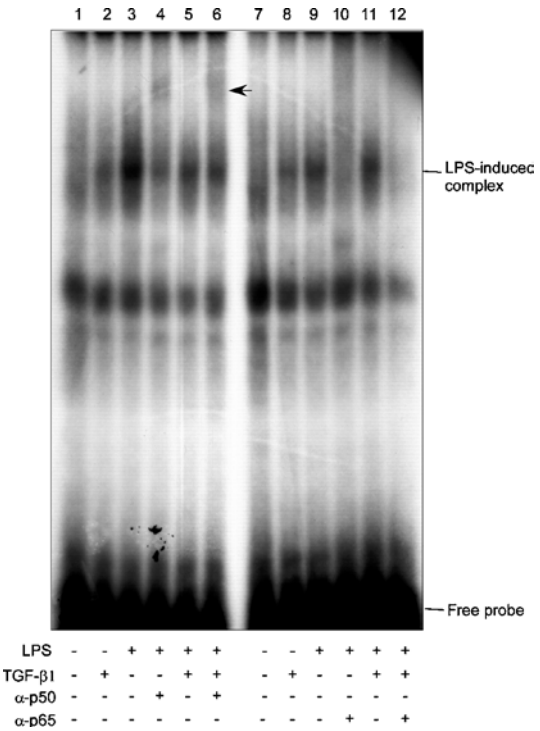

Figure 9 Participation of p50 and p65 in a LPS-induced complex that binds the NF-κB site of the MMP-9 promoter, and effect of TGF-β1 treatment

RAW264.7 cells were treated with or without LPS for 0.5 h, and TGF-β1 (1.25 ng/ml) was added 1 h before LPS treatment as indicated. Nuclear extracts were isolated from the cells and used in EMSAs with a ^{32}P-labelled NF-κB sequence found in the MMP-9 promoter. For antibody supershift assay, antibodies specific for p50 (α-p50) or p65 (α-p65) were included in the binding reaction as indicated. The arrow indicates the band supershifted by the anti-p50 antibody.

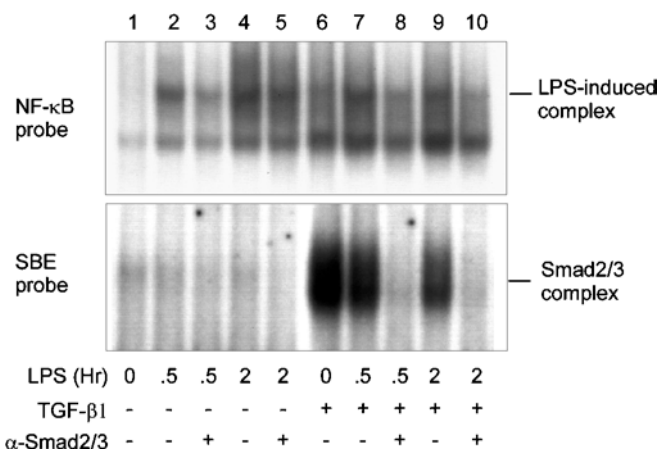

Figure 10 Smad2/3 are not involved in formation of the NF-κB-binding complex

RAW264.7 cells were treated with LPS and TGF-β1 as indicated, and the nuclear extracts were used in EMSAs with a NF-κB probe from the human MMP-9 promoter (upper panel) and a SBE probe from the mouse Smad7 promoter (lower panel). An anti-Smad2/3 antibody (α-Smad2/3) was included as indicated to analyse the presence of Smad proteins in the binding complex. The LPS-induced complex and Smad-binding complex are indicated.

of MMP-9 transcription through the NF-κB site is not due either to decreased binding of NF-κB subunits or to binding by activated Smad proteins.

DISCUSSION

The present study has characterized the negative regulation of the MMP-9 promoter by TGF-β in monocytes/macrophages. In both the human monocyte cell line MM6 and the mouse macrophage cell line RAW264.7, TGF-β suppressed LPS-stimulated MMP-9 production, manifested by changes in both enzymic activity and mRNA level. These findings are consistent with previous reports that MMP-9 activity induced by LPS was suppressed by TGF-β in mouse peritoneal macrophages [9]. Thus the negative regulation of LPS-stimulated MMP-9 expression by TGF-β is considered to be a physiological phenomenon observed not only in primary monocytes/macrophages, but also in cell lines. In the present study, we have shown that LPS stimulation of MMP-9 is negatively regulated by TGF-β at the transcriptional level. LPS increased MMP-9 promoter activity, but this was significantly suppressed by TGF-β treatment in RAW264.7 cells. Furthermore, expression of a constitutively active TGF-β type I receptor in the presence of Smad3 and Smad4 also strongly suppressed MMP-9 promoter activity. Meanwhile, an inhibitory Smad, Smad7, abrogated such repressive activity. All of these findings indicate that the human MMP-9 promoter is under the negative regulation of the TGF-β signalling pathway. In contrast, the gelatinase activity of MMP-2 was increased in MM6 cells by TGF-β. MMP-9 and MMP-2 share structural and catalytic similarities, but the mechanisms of regulation of their transcription are different, due to distinct structures in the regulatory elements of the promoters [9,45]. The transcriptional regulation of MMP-2 by TGF-β is currently under investigation in our laboratory.

The transactivation of target genes by TGF-β is mediated mostly by direct binding of Smad proteins to their binding element, SBE, in the promoter region [21]. However, the negative regulation of gene expression by TGF-β has not been characterized fully. Previous studies revealed that a *cis*-acting element, TIE, is involved in the repression of certain genes by TGF-β [34,35]. TIE contains the core sequence GAGTTGGTGA that is required for the inhibitory effects of TGF-β on MMP-3 transcription induced by EGF [34]. TIE specifically binds complexes of nuclear proteins from TGF-β-treated fibroblasts, and the complex contains the c-*fos* proto-oncogene product. A consensus TIE sequence (GNNTTGGtGa) was found in several genes whose expression was down-regulated by TGF-β, such as those encoding transin, urokinase, elastase, proliferin and c-Myc [34]. A TIE sequence was also found in the human and rabbit MMP-1 promoters [34,35]. Similar to the MMP-3 promoter, suppression of phorbol ester-induced MMP-1 expression by TGF-β is mediated through a TIE [35]. A putative TIE is also localized in the human MMP-9 promoter, suggesting that this element may be involved in the suppression of MMP-9 transcription by TGF-β. In the present study, we found that neither mutation nor deletion of the TIE had any effect on the inhibitory activity of TGF-β signalling on the MMP-9 promoter, indicating that the putative TIE in the MMP-9 promoter is not implicated in the action of TGF-β, unlike the findings with the MMP-1 and MMP-3 gene promoters.

Our further characterization of the MMP-9 promoter indicated that a NF-κB site is indispensable for TGF-β-mediated transcriptional regulation. To localize the region of the MMP-9 promoter that is required for the suppressive activity of TGF-β, we generated a series of deletion mutants of the promoter. Deletion of the regions containing the NF-κB site (−670/−591) and AP-1 sites (−554/−505 and −150/+19) greatly reduced the basal transcriptional activity of the MMP-9 promoter. This is consistent with previous study showing that NF-κB and AP-1 sites are important for both basal and PMA-induced transcription

of human MMP-9 [45]. Furthermore, deletion of the putative NF-κB site was able to abrogate the suppressive activity of TGF-β. This was explored further in transcriptional studies using a minimal promoter construct that contained one copy of the NF-κB site linked to a TATA box, which provided further evidence that the repressive activity of TGF-β on the MMP-9 promoter is mediated by the NF-κB site. Interestingly, we found that deletion of the putative TIE-containing region ($-505/-401$) increased the basal promoter activity. A previous study showed that the TIE in the rabbit MMP-1 promoter has dual functions as a repressor of basal transcription and as a mediator of the biological effects of TGF-β [35]. Thus the TIE in the human MMP-9 promoter may have a role in the repression of basal transcription, but not in TGF-β-induced repression.

Our studies indicated the importance of the NF-κB site in the negative regulation of MMP-9 transcription by TGF-β. We found that activation of the TGF-β pathway did not disrupt binding of the p50 and p65 subunits to the NF-κB sequence, and that Smad2/3 is not directly involved in the formation of the NF-κB binding complex. These data are consistent with previous studies on MMP-1 regulation [37]. The IL-1β-induced transactivation of MMP-1 was repressed by TGF-β via the Smad pathway in fibroblasts via a NF-κB site. It was proposed that such repression is caused by competition between the Smad and NF-κB pathways for a limiting pool of transcription co-activators, e.g. p300 [37]. Consistent with this theory, activation of the NF-κB pathway can reduce Smad-induced transactivation [55], and we found that overexpression of p300 was able to partly overcome TGF-β-mediated suppression of the MMP-9 promoter (results not shown). It is conceivable that the activated Smad proteins after TGF-β treatment compete with NF-κB for limiting amounts of transcriptional co-activators to prevent LPS-stimulated MMP-9 transactivation. However, we found only a modest recovery of the TGF-β-mediated repression of MMP-9 transcription on overexpression of p300. TGF-β is very potent in inhibiting the production of MMP-9 in MM6 and RAW264.7 cells (Figures 1 and 2). It therefore remains to be determined whether or not other mechanisms are implicated in the repressive activity of TGF-β, at the levels of translation, post-translation and/or secretion. Smad3 has been shown to interact with the NF-κB subunit p52, and this interaction is involved in the transcriptional regulation of the *junB* promoter by TGF-β via a NF-κB site [56]. However, the gel mobility shift assay in our present study did not reveal the presence of Smad2/3 in the protein complex that bound the NF-κB site of the MMP-9 promoter, indicating that a direct interaction between Smad and the NF-κB member(s) is not likely to be involved in the regulation of MMP-9 transcription. Nevertheless, our studies highlight the importance of cross-talk between the Smad and NF-κB pathways in the regulation of MMP-9 production, which plays an important role in inflammatory and other biological processes such as tissue remodelling and cancer progression.

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