p38 mitogen-activated kinase is a bidirectional regulator of human fibroblast collagenase-1 induction by three-dimensional collagen lattices

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When fibroblasts are cultured in contracting collagen matrices, matrix metalloproteinase-1 (MMP-1, collagenase-1) is induced. In the present study we demonstrate that p38 α mitogen-activated protein kinase (p38 α MAPK) plays a bi-directional role in the MMP-1 response to contracting floating collagen lattices (fl-coll). fl-coll, but not attached collagen lattices (att-coll), coordinately increased expression of MMP-1 and activities of p38 α and MKK3/6 (MAPK kinase 3/6). However, treatment of primary fibroblasts cultured in fl-coll with increasing doses of SB203580, an inhibitor of p38 α and p38 β , caused a bipolar pattern of MMP-1 expression. Partial inhibition of p38 MAPK activity resulted in the lowest level of MMP-1 expression, whereas total inhibition of p38 activity led to MMP-1 levels as high as in the absence of inhibitor. The activation/inhibition of p38 α was apparently responsible for the observed phenomena, as supported by three lines of evidence. (1) p38 α was the predominant isoform sensitive to SB203580 in primary fibroblasts. (2) Fibroblasts transfected with increasing dose of a dominant negative p38 α

(p38DN) similarly demonstrated the bipolar pattern of MMP-1 expression induced by fl-coll. (3) The bipolar MMP-1 expression occurred during the gradual, linear inhibition of p38 α kinase activity by both inhibitors, SB203580 and p38DN. Nuclear factor- κ B (NF- κ B), a previously identified positive regulator of MMP-1 expression induced by fl-coll [Xu, Zutter, Santoro and Clark (1998) *J. Cell Biol.* **140**, 709–719] was mediated by fl-coll-activated p38 α . However, the fl-coll-induced expression of MMP-1 facilitated by p38 α suppression was maintained independent of NF- κ B activity, suggesting the existence of a p38 α -dependent antagonistic pathway. We conclude that fl-coll-induced MMP-1 expression is the net outcome of opposing effects mediated by p38 α . Therefore, the level of p38 α kinase activity may provide a fine-tuned control of MMP-1 gene expression in response to biomechanical signals.

Key words: MMP-1, contraction, p38 MAPK, nuclear factor κ B.

INTRODUCTION

Type I collagen is the most abundant extracellular matrix (ECM) protein in the human body. Floating and attached three-dimensional collagen lattices (fl-coll and att-coll) are considered *in vitro* models for fibroblast–collagen interactions at different stages of wound repair [1]. Fibroblasts cultured in fl-coll and att-coll demonstrate distinct cell morphologies, cytoskeletal organization and cellular functions, such as gene expression and growth-factor responses. Matrix metalloproteinase-1 (collagenase-1; MMP-1), a secreted proteinase that cleaves native fibrillar collagens of types I, II, III and V, is thought to play a critical role in the remodelling of extracellular matrix. Indeed, MMP-1 is expressed by activated fibroblasts in the granulation tissue of normal and chronic wounds [2]. fl-coll [3], as well as other signals that induce changes in cell morphology and actin reorganization, stimulate MMP-1 expression. The other signals include mechanical strain [4] and direct mechanical wounding [5] in human vascular smooth-muscle cells, exposure to cytochalasin D in human and rabbit synovial fibroblasts [6,7] and specific cell–matrix interactions [8].

One of the events leading to MMP-1 gene expression mediated by ECM signals, such as fl-coll [9] and matrix contacts [8], is the activation of the transcription factor nuclear factor κ B (NF- κ B),

a homo- or hetero-dimer of members of the Rel family of proteins which consist of p50/p105, p52/p100, p65/RelA, c-Rel, and RelB [10]. Several studies have reported that p38 mitogen-activated protein kinase (p38 MAPK) regulates NF- κ B activation [11–13]. The p38 group of MAPKs is activated through phosphorylation of specific tyrosine and threonine residues in response to physical and chemical stress signals, such as hyperosmolarity and H₂O₂ [14,15]. Pyridinylimidazole compounds, such as SB203580, were found to act as specific inhibitors of p38 α and p38 β [16], two members of the p38 MAPK family, and have been widely used to investigate the physiological functions of p38 α and p38 β . The p38 MAPK has been implicated in the signalling of type I collagen. For example, p38 MAPK activity is required for collagen-induced platelet aggregation [17] and for collagen-lattice-induced MMP-13 expression in fibroblasts [18]. Integrin $\alpha_2\beta_1$, a collagen-binding integrin that regulates the contraction of collagen lattices, mediates p38-dependent collagen gene transcription [19]. Several studies have also reported that the p38 MAPK is required for the induction of MMP-1 gene expression by ceramide and tumour-derived extracellular matrix metalloproteinase inducer (EMMPRIN) [20,21]. Interestingly, although contracting collagen lattices induce both activation of p38 MAPK [18] and expression of MMP-1 [3,22] in human fibroblasts, the complete inhibition of p38 MAPK by SB203580

Abbreviations used: MAPK, mitogen-activated protein kinase; MMP-1, matrix metalloproteinase-1; EMMPRIN, extracellular matrix metalloproteinase inducer; CAT, chloramphenicol acetyltransferase; β gal, β -galactosidase; IL, interleukin; ECM, extracellular matrix; ATF2, activating transcription factor-2; fl-coll, floating collagen lattices; att-coll, attached collagen lattices; NF- κ B, nuclear factor κ B; mAb, monoclonal antibody; GST, glutathione S-transferase; MAPKAP-K2, MAPK-activated protein kinase-2; MKK, MAPK kinase (also called MEK); RSV, rous sarcoma virus; I κ B α , NF- κ B inhibitor α ; FCS, fetal-calf serum; DMEM, Dulbecco's modified Eagle's medium; HA, haemagglutinin; CTF, CCAAT-box-binding transcription factor; NF-1, nuclear factor 1; Oct-1, octamer factor-1; AS- α_2 , antisense integrin α_2 ; SS- α_2 , sense integrin α_2 ; C/EBP, CAAT-enhancer binding protein.

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at 10 μ M does not result in appreciable suppression of MMP-1 expression [18].

Here we report that p38 MAPK has a bidirectional role in human fibroblast MMP-1 expression induced by the mechanical properties of contracting collagen lattices. We found that mechanical, instead of three-dimensional structural, signals of fl-coll co-ordinately regulate the expression of MMP-1 and the activation of p38 MAPK in adult human dermal fibroblasts. However, inhibition of p38 MAPK by SB203580 resulted in a bipolar pattern of MMP-1 expression induced by fl-coll. The p38 α activity was apparently responsible for this non-linear pattern. The p38 α activity was also required for the activity of NF- κ B, a transcription factor necessary for the fl-coll-induced expression of MMP-1 [1], implicating p38 α in NF- κ B-dependent MMP-1 expression. However, NF- κ B was not required for the MMP-1 expression facilitated by p38 α suppression in the fl-coll environment, suggesting the existence of an antagonist pathway, comprising factors other than NF- κ B, yet still mediated by p38 α . Our data suggest that fl-coll-induced MMP-1 expression is the net outcome of opposing effects mediated by p38 α . Therefore the antipodal p38-dependent effects on MMP-1 gene expression in response to the mechanical signals of a contracting collagen gel may provide an elaborate mechanism to control MMP-1 expression during the latter phase of wound healing.

EXPERIMENTAL

Cell culture and reagents

Human fibroblast cultures were maintained as described in [23]. Polyclonal antibodies against phospho-activating transcription factor-2 (phospho-ATF2), total ATF2, phospho-MAPK kinase 3/6 (phospho-MKK3/6) and phospho-MKK4 were purchased from New England BioLab (Beverly, MA, U.S.A.). Monoclonal antibody (mAb) against haemagglutinin (HA) tag (a peptide sequence derived from human-influenza-virus haemagglutinin protein) was purchased from Boehringer-Mannheim (Indianapolis, IN, U.S.A.). Polyclonal antibodies against phospho-p38, total p38, p38 α (SC-535), p38 β (SC-6176), p50 and p65/RelA were from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). The mAb against p38 and MAPK-activated protein kinase-2 (MAPKAP-K2) and the purified proteins glutathione S-transferase (GST)-MAPKAP-K2, GST-ATF2 and GST-p38 β were purchased from Upstate Biotechnology (Lake Placid, NY, U.S.A.). mAbs against integrins α_2 and β_1 were from Chemicon (La Jolla, CA, U.S.A.). SN50 and SM, the control peptide of SN50, have been described previously [9]. [14 C]Chloramphenicol, [γ - 32 P]ATP and [α - 32 P]dCTP were obtained from DuPont-NEN (Boston, MA, U.S.A.). [γ - 32 P]Orthophosphate was purchased from Amersham Pharmacia Biotech, Piscataway, NJ, U.S.A.

Plasmids

Plasmids rous sarcoma virus (RSV)- β -galactosidase, human α_2 cDNA probe, full-length human α_2 cDNA, antisense construct of human α_2 cDNA, wild-type NF- κ B inhibitor α (I κ B α), mutant I κ B α ^{S32A,S36A} and GST-p38 (p38 α) were kindly provided by Dr Lorne Taichman (State University of New York at Stony Brook, Stony Brook, NY, U.S.A.), Dr Yoshikazu Takada (The Scripps Research Institute, La Jolla, CA, U.S.A.), Dr Martin Hemler (Dana-Farber Cancer Institute, Boston, MA, U.S.A.), Dr Samuel Santoro (Washington University, St. Louis, MO, U.S.A.), Dr Warner Greene (The Gladstone Institute of Virology and Immunology, San Francisco, CA, U.S.A.) and Dr Michael Karin (University of California at San Diego, San Diego, CA, U.S.A.) respectively. Human MMP-1 cDNA was purchased from the

A. T. C. C. (formerly at Rockville, MD, U.S.A., but now at Manassas, VA, U.S.A.). The dominant negative p38 α , p38^{T180A/Y182F}, was generated from the wild-type by site-directed mutagenesis using the QuickChange kit from Stratagene (La Jolla, CA, U.S.A.). The sense α_2 cDNA was generated by insertion into the same vector as the antisense construct. The 2.2 kb fragment of MMP-1 promoter-chloramphenicol acetyltransferase (CAT) construct (pCL2278CAT) [24] and NF- κ B reporter plasmid [9] have been described previously.

Preparation of collagen lattices

Collagen gels were prepared according to a procedure previously described [23]. For experiments measuring kinase activities, cells were starved in 1% fetal-calf serum (FCS)/Dulbecco's modified Eagle's medium (DMEM) for 24 h prior to subculture in collagen lattices. For fl-coll and att-coll, the collagen-cell suspension was immediately placed on to 100-mm-diameter Petri dishes coated with 2% BSA (ICN, Costa Mesa, CA, U.S.A.) and tissue-culture plates respectively, and incubated at 37 °C for 1 h before addition of FCS/DMEM at the indicated FCS concentration. After incubation at 37 °C in air/CO₂ (19:1) and 100% humidity for the indicated time, cultures were carefully washed twice in DMEM and processed for various analyses.

Cell incubation with antibodies

Cells were preincubated with mAbs at 5 μ g/ml for 20 min. For experiments in collagen lattices, the final antibody concentration in the lattices as well as in the medium was 5 μ g/ml. For ligation experiments, cells were cultured with antibodies in solution or on immobilized antibodies. In some experiments, inhibitors were present as indicated. Incubation was continued for 18–24 h. All experiments were conducted using serum-free DMEM.

Northern analysis of total cellular RNA

Total RNA was isolated as previously described [23]. All results shown are representative of at least two independent experiments.

Preparation of cell lysates, subcellular fractions and Western blotting

Fibroblasts grown in collagen gels were released by digestion of gels with collagenase D (Boehringer-Mannheim, Indianapolis, IN, U.S.A.). Total cell lysates and nuclear extracts were prepared as described previously [9]. Samples of protein (2–7 μ g each) were subjected to Western analysis and detected with enhanced chemiluminescence (ECL[®]; Amersham Pharmacia Biotech).

ELISA assay of MMP-1

Serum-free medium collected after culture of fibroblasts in collagen gels as described above was analysed using a human MMP-1 ELISA system (Calbiochem, San Diego, CA, U.S.A.) according to manufacturer's protocol. The final values were the averages of triplicate determinations.

In vitro and in vivo kinase assays

For measurement of p38 MAPK activity *in vitro*, p38 α or p38 β was immunoprecipitated by incubation of isoform-specific polyclonal antibodies with cell lysates at 4 °C overnight with continuous rotation. Then 20 μ l of IgG-conjugated agarose beads (Sigma, St. Louis, MO, U.S.A.) was added and the incubation was extended for an additional 2 h. The p38 immunoprecipitates were washed and assayed for the associated kinase activity using either purified GST-MAPKAP-K2 or GST-ATF2. An identical

Figure 1 Inhibition of p38 by SB203580 both represses and enhances MMP-1 expression by fibroblasts cultured in fl-coll

(A, B and C) Normal human dermal fibroblasts were pretreated with SB203580 at indicated concentration for 20 min at 37 °C before and during culture into collagen lattices in 1% FCS/DMEM for 24 h. Cells were treated in serum-free medium for 24 h for the detection of MMP-1 secretion by Western analysis and ELISA (A and B) or in 1% FCS/DMEM for 24 h for Northern analysis (C). The inhibitors were continuously present in both culture conditions. Results represent four (A and B) and two independent experiments (C) respectively. (D) Fibroblasts were co-transfected with pCL2278CAT and a plasmid containing RSV- β gal before culture in fl-coll containing SB203580 at the indicated concentrations. Cells were harvested 2 days after subculture, and cell extracts were assayed for CAT activity after normalization with β -galactosidase activity. The results are representative of those obtained in four independent experiments. Abbreviations: f, fl-coll; a, att-coll.

sample of immunoprecipitates was used for Western blotting detected with mAb against p38. For the *in vivo* kinase assay, fibroblasts transfected with expression construct HA-ATF2 were labelled with 1 mCi/ml of [32 P]orthophosphate in phosphate-free medium supplemented with 1% dialysed FBS and 15 mM Hepes, pH 7.2, overnight. Cells were lysed as described previously [9]. After immunoprecipitation of total cell extracts with anti-HA antibody, beads were washed four times and subjected to SDS/PAGE. A fraction of the sample was assayed by Western blotting and detected with anti-ATF2 antibody.

Gel-mobility-shift assay

The assay of the DNA-binding proteins in nuclear extracts by gel-mobility-shift assay was described previously [25]. The consensus sequences of NF- κ B, nuclear factor 1(NF-1)/CCAAT-box-binding transcription factor (CTF) and octamer factor 1 (Oct-1) were purchased from Promega (Madison, WI, U.S.A.).

DNA transfection and reporter assay

DNA transfection was performed as previously described [9]. Cells were plated at 5×10^5 /100-mm-diameter plate. Transfection

was performed 22–24 h after passage. After 24 h, transfected cells were subcultured into collagen lattices. For promoter activity measurements, the CAT reporter plasmids, pCL2278CAT, containing the 2.2 kb promoter region of MMP-1 gene, or 4 $\times$ (four tandem repeats) NF- κ B, were used. CAT activity was analysed using an assay kit from Promega. The butyrylated chloramphenicol products were counted for radioactivity in a liquid-scintillation counter. For the *in vivo* ATF2 phosphorylation assay, fibroblasts were co-transfected with HA-ATF2 expression plasmid and p38 $\alpha^{T180A/Y182F}$ or vector control at indicated amount. Cell extracts containing an equal amount of radioactivity were used for immunoprecipitation.

RESULTS

Human dermal fibroblasts treated with SB203580 results in a bipolar pattern of MMP-1 expression induced by fl-coll

Recently, three-dimensional collagen lattices were demonstrated to stimulate MMP-13 and collagen gene expression via activation of p38 MAPK [18,19]. Thus we used SB203580, a specific inhibitor of p38 (p38 α) and p38 β [26], to study the role of p38 MAPK in the expression of MMP-1, another injury-related gene

Figure 2 Endogenous expression of p38 α and p38 β MAPKs in normal human dermal fibroblasts under different culture conditions

Normal human dermal fibroblasts were starved for 24 h in 1% FCS/DMEM before subculture on plastic tissue-culture plates or in collagen lattices. The incubation in 1% FCS/DMEM continued for 18–24 h. (A) Total cellular lysates were analysed for p38 α and p38 β with specific antibodies. (B) p38 α was detected in cytosolic and nuclear fractions. Abbreviations: f, fl-coll; a, att-coll; tcp, plastic tissue-culture plates; control, positive control for p38 α (Jurkat-cell lysates) and p38 β (purified GST-tagged p38 β); c-p38 α , cytosolic p38 α ; n-p38 α , nuclear p38 α . The results are representative of those obtained in three independent experiments.

regulated by three-dimensional collagen [27]. Four concentrations of SB203580 (0.01, 0.1, 1.0 and 10 μ M) that span its IC₅₀ (0.5 μ M) were chosen [28]. Surprisingly, SB203580 mediated an antipodal effect on MMP-1 expression induced by fl-coll (Figure 1A). MMP-1 protein secretion was inhibited by SB203580 at 0.01 and 0.1 μ M, but increased gradually at 1 and 10 μ M. An inactive derivative of SB203580, SB202474, had no effect at these concentrations (results not shown). As a control for fl-coll, MMP-1 expression by fibroblasts in att-coll was slightly inhibited by SB203580 at 0.01 μ M and remained unchanged at other concentrations (Figure 1A). To define better the SB203580 concentration that effected the fl-coll-induced MMP-1 expression, we assessed MMP-1 protein secretion in the presence of SB203580 at 0.005, 0.01, 0.05, 0.1, 1 and 10 μ M by ELISA (Figure 1B). After a slight decrease of MMP-1 secretion at 0.005 μ M SB203580, a steep decline was observed at 0.01 μ M. The MMP-1 expression remained suppressed and was gradually restored at concentrations higher than 0.1 μ M. Aliquots from one set of samples were also analysed by Western blotting to monitor the appearance of MMP-1.

Because SB203580 at 0.1, 1, and 10 μ M was used to inhibit p38 MAPK in a dose-dependent manner, as determined by *in vitro* kinase assay [28,29], we decided to focus on these three concentrations for further study. Figures 1(C) and 1(D) demonstrated that both MMP-1 mRNA level (Figure 1C) and promoter activity (Figure 1D) induced by fl-coll were similarly regulated in a bipolar pattern by SB203580 over the 0.1–10 μ M range. The consistency between MMP-1 promoter activity and its mRNA steady-state level suggest that transcriptional control is responsible for this effect. Collectively these data suggest that SB203580 has both negative and positive function in MMP-1 expression by fibroblasts cultured in fl-coll.

p38 α MAPK is differentially activated in human dermal fibroblasts by fl-coll and att-coll

Ectopic expression of p38 α and p38 β cDNA in HeLa cells has opposite effects on cell apoptosis [30]. Therefore we wondered whether the antipodal response to SB203580 was affected by opposing effects of the two p38 isoforms. Primary fibroblasts expressed abundant p38 α , but minimal p38 β (Figure 2A). The total protein levels of each p38 isoforms were similar in cells cultured on tissue-culture plastic, in fl-coll or in att-coll (Figure

Figure 3 Differential activation of p38 MAPK in human dermal fibroblasts by fl-coll and att-coll

Normal human dermal fibroblasts were starved for 24 h in 1% FCS/DMEM before subculture in collagen lattices. (A) After 0.5, 1, 3, 24, or 48 h, cell extracts were prepared and assayed for the phosphorylation of p38, MKK3/6 and MKK4. The antibodies against phospho-p38 and total p38 are specific for p38 α and p38 β . The results are representative of at least four individual experiments. (B) The p38 α MAPK activity was measured with an *in vitro* immune complex kinase assay. The p38 α immunoprecipitates from fibroblast extracts were prepared after incubation with collagen matrices for 24 h and were assayed for the phosphorylation of purified MAPKAP-K2, a substrate. The results are representative of at least two individual experiments. Abbreviations: f, fl-coll; a, att-coll; tcp, plastic tissue-culture plates; pep, immunoprecipitation in the presence of a blocking peptide to p38 α -specific antibody; pi-, phospho-. (C) Normal human dermal fibroblasts were pretreated with SB203580 at the indicated concentrations for 20 min at 37 °C before culture into collagen lattices in 1% FCS/DMEM for 18 h. The p38 α kinase activity was determined in an *in vitro* immune complex kinase assay using ATF2 as substrate.

2A). p38 α was distributed in both cytosolic (c-p38 α) and nuclear (n-p38 α) compartments (Figure 2B). The three-dimensional collagen environment slightly altered the cellular distribution, with a modest increase in the nuclear fraction and a small decrease in the cytosolic fraction after 24 h of incubation in fl-coll (Figure 2B). p38 β in subcellular fractions was nearly undetectable (results not shown).

Figure 4 Effects of dominant negative p38 α on MMP-1 expression in fibroblasts cultured in fl-coll and att-coll

(A) Normal human dermal fibroblasts were co-transfected with HA-ATF2 and kinase-negative p38 α ^{T180A/Y182F} (p38DN) or 200 ng of vector (0) before subculture into collagen lattices for 24 h in the presence of [³²P]orthophosphate with 1% dialysed FBS as described in the Experimental section. Cell extracts containing equal level of radioactivity were used for immunoprecipitation. ATF2 was precipitated with anti-HA antibody and subjected to SDS/PAGE and Western-blot analysis using an ATF2 antibody. The experiment represents two individual experiments. (B) Normal human dermal fibroblasts were co-transfected with p38DN, pRSV- β gal and pCL2278CAT before subculture into collagen lattices for 24 h. CAT activity was analysed, and the butyrylated chloramphenicol products were counted in a liquid-scintillation counter. Results, which are normalized to β gal activity, are representative of four individual experiments. Abbreviations: a, att-coll; f, fl-coll; tcp, plastic tissue-culture plates; pi-, phospho-.

We then examined the activity of both p38 isoforms. Because MMP-1 expression was differentially regulated by fl-coll and att-coll (lanes 1 and 6, Figure 1A), the modulation of fl-coll-induced MMP-1 expression by SB203580 suggests that p38 MAPK activity might also respond to mechanical signals from the collagen lattices. Within 48 h after fibroblasts were seeded into collagen lattices, p38 MAPK was differentially phosphorylated by fl-coll and att-coll, as detected by an antibody raised against phosphorylated p38 α and p38 β . While the level of p38 phosphorylation in cells grown on conventional plastic tissue-culture plates for 24 h did not change (lanes 1 and 2, Figure 3A), p38 activation was slightly lower in cells in fl-coll than those in att-coll at 0.5 h and 1 h (lanes 3–6), but increased after 3 h and was maximal at 24 h (lanes 7–12, Figure 3A). This differential activation persisted for 48 h. The total protein level of p38 remained similar between fl-coll and att-coll (Figure 3A), indicating that p38 phosphorylation, but not protein synthesis, was regulated by mechanical properties of collagen lattices. An unidentified band with a molecular mass at 55 kDa co-detected

Figure 5 Differential activation of NF- κ B by fl-coll and att-coll

(A) Normal human dermal fibroblasts starved in 1% FCS/DMEM were cultured into collagen lattices in 1% FCS/DMEM for 24 h. A gel-mobility-shift assay was performed to detect the DNA binding activities of NF- κ B, NF-1 and Oct-1. Results are representative of three individual experiments. Arrows indicate the specific DNA binding complexes. Abbreviation: cold oligomer, unlabelled oligonucleotides in 10-fold excess. (B) Fibroblasts were co-transfected with a CAT reporter plasmid that contains 4 $\times$ κ B consensus sequence and a plasmid containing RSV- β gal before culture in collagen lattices for 48 h. Cell extracts were assayed for CAT activity and the levels were normalized to β gal activity. The results shown are representative of four independent experiments. Abbreviations: f, fl-coll; a, att-coll; tcp, plastic tissue-culture plates; NF- κ B and mNF- κ B, CAT reporter plasmid construct containing four tandem repeats of wild-type or mutated NF- κ B consensus sequence.

with phosphorylated p38 remained unchanged, serving as an internal control for the level of p38 phosphorylation.

The differential activation of p38 α and p38 β by fl-coll and att-coll was further assessed by an *in vitro* kinase assay using MAPKAP-K2 as a substrate. MAPKAP-K2 was effectively phosphorylated by anti-p38 α immunoprecipitates from cells grown in fl-coll. In contrast, lower levels of phosphorylation

Figure 6 Effects of p38 inhibition on NF- κ B activity induced by fl-coll

(A) Normal human dermal fibroblasts were starved for 24 h before culture in collagen lattices for an additional 24 h in the presence of SB203580 at indicated concentrations. Nuclear extracts were subjected to the gel-mobility-shift assay. The DNA binding activity to NF- κ B was determined. (B) Western blotting was performed to examine NF- κ B subunits p50 and p65 in the nuclear extracts. The results in (A) and (B) are representative of three individual experiments. Two unspecific bands were marked by stars. (C) Fibroblasts were co-transfected with a CAT reporter plasmid that contains 4 $\times$ κ B consensus sequence and a plasmid containing RSV- β gal before culture in collagen lattices for 48 h in the presence of SB203580 at indicated concentrations. Cell extracts were assayed for CAT activity after normalization with β -gal activity. The results are representative of four independent experiments.

were mediated by immunoprecipitates from cells grown on conventional plastic tissue-culture plates or in att-coll (Figure 3B). Furthermore, MAPKAP-K2 was only minimally phosphorylated by p38 β immunoprecipitates (results not shown), supporting the results from Western analysis (Figure 2A). A blocking peptide to the p38 α antibody completely abrogated the kinase activity (Figure 3B), confirming the specificity of the kinase reaction. Taken together, these results suggest that fl-coll, but not att-coll, induced sustained p38 α activity in human primary fibroblasts.

We also examined the collagen-mediated activity of MKK3 and MKK6, the dual-specificity kinase activators of p38 α [26,31]. An antibody raised against phosphorylated MKK3/6 detected higher levels of phosphorylated MKK3/6 in cells grown in fl-coll than cells in att-coll for 24 h and 48 h, similar to that of p38 α (Figure 3A). In contrast, phosphorylation of MKK4, a weak kinase activator of p38 α [26], was not affected in response to fl-coll and att-coll (lanes 7–12, Figure 3A). Taken together, these data show that the mechanical properties of three-dimensional collagen lattices signal the sustained activation of both MKK3/6 and p38 α .

To determine whether SB203580 treatment simultaneously caused the non-linear bipolar pattern of fl-coll-induced MMP-1 expression and the linear dose-dependent inhibition of p38 α kinase activity, we analysed the kinase activity associated with

p38 α in the presence of SB203580. Phosphorylation of ATF2, a transcription-factor substrate, by p38 α immunoprecipitates was inhibited by SB203580 in a one-way concentration-dependent fashion (Figure 3C), indicating that the linear reduction of the p38 α activity by the increasing SB203580 concentration gradient coincided with the bipolar expression of MMP-1 in a fl-coll environment.

Expression of dominant negative p38 α in human dermal fibroblasts also produced a bipolar pattern of MMP-1 expression induced by fl-coll

The abundance of p38 α , as compared with p38 β , in these cells suggests that the antipodal effects of SB203580, an inhibitor for both isoforms, on fl-coll-induced MMP-1 expression was largely due to the inhibition of p38 α . To assess further the specificity of SB203580 effects on MMP-1 expression, we used a dominant negative approach. A kinase-inactive mutant of p38 (p38 α), p38^{T180A/Y182F}, has been reported to have a dominant negative effect on p38 (p38 α) activity [32]. Thus we used this mutant (designated p38DN) to assess the role of p38 α in MMP-1 expression. After extensive titration, we found that p38DN at different DNA concentrations, while inhibiting the phosphorylation of ATF2 (Figure 4A), similarly caused a bipolar pattern of MMP-1 promoter activity in fibroblasts cultured in fl-coll (Figure

4B). These data further support the results obtained with SB203580 and indicate that the inhibition of p38 α caused an antipodal effect on MMP-1 gene expression. Additionally they suggest that p38 α mediates both positive and negative downstream events that modulate MMP-1 gene expression signalled by fl-coll.

NF- κ B is a downstream target of p38 α in fibroblasts surrounded by fl-coll

Inhibition of p38 has been reported to have both positive and negative impact on tumour-necrosis-factor- α regulation of NF- κ B activation [13]. Since NF- κ B is a positive regulator of fl-coll-induced MMP-1 expression [1], we speculated that an increasing SB203580 concentration gradient might result in the inhibition and the subsequent activation of NF- κ B, leading to the bipolar pattern of MMP-1 expression. To assess this possibility, we first examined whether NF- κ B, like MMP-1 and p38 α , responded to the mechanical signals, but not the three-dimensional organization, of collagen lattices. The DNA binding activity of NF- κ B was significantly induced by fl-coll, but not att-coll, as compared with tissue-culture plates (Figure 5A), indicating the contracting collagen lattice is a stimulatory signal for NF- κ B activation. In contrast, binding to NF-1/CTF and Oct-1 DNA elements did not differ appreciably between fl-coll and att-coll (Figure 5A), thus confirming the specificity for the differential DNA binding of NF- κ B between fl-coll and att-coll. Consistent with the DNA binding activity, transactivation activity of NF- κ B was also differentially regulated by fl-coll and att-coll (Figure 5B), suggesting that mechanical properties of collagen lattices coordinately regulate the activities of MKK3/6, p38 α , NF- κ B and the expression of MMP-1.

Next we examined the effects of p38 α inhibition on NF- κ B activity. The inhibition of p38 α by SB203580 at concentrations from 0.1 μ M to 10 μ M blocked transactivation activity of NF- κ B in a one-way concentration-dependent manner (Figure 6C), suggesting that NF- κ B activation by fl-coll requires p38 α activity. Depending on the extracellular signals, p38-mediated NF- κ B activation is manifested by nuclear translocation [12], phosphorylation of p65 itself [13] or association of NF- κ B with basal transcription factors such as TFIIB and TATA-binding protein [11]. Because we have shown that fl-coll induces NF- κ B nuclear translocation [1], the effects of p38 α on DNA binding of NF- κ B and the nuclear presence of p50 and p65, two subunits previously identified present in the NF- κ B DNA-binding complex in fl-coll-induced fibroblasts [1], were examined. SB203580 inhibited DNA binding activity of NF- κ B (Figure 6A) and blocked nuclear translocation of p50 and p65 (Figure 6B) in fibroblasts induced by fl-coll. Two unspecified bands that accompanied the p65, as shown in Figure 6(B), were also detected by the antigen-neutralized antibody against p65 (results not shown), thereby serving as internal controls. It is apparent that p38 α induced by fl-coll is an upstream activator of NF- κ B, implicating its role in NF- κ B-dependent MMP-1 expression. It is also clear that NF- κ B activity was suppressed by SB203580 at concentrations found to facilitate MMP-1 expression.

MMP-1 expression facilitated by p38 α suppression in fl-coll environment does not require NF- κ B activity

The above finding suggests that the MMP-1 expression facilitated by fully inhibited p38 α employs a NF- κ B-independent regulatory mechanism. Two inhibitors of NF- κ B activation, SN50, a peptide inhibitor of NF- κ B nuclear translocation, and I κ B α ^{S32A,S36A}, a

Figure 7 p38-suppression-facilitated MMP-1 expression in fl-coll is independent of NF- κ B activity

(A) Normal human dermal fibroblasts starved for 24 h in 1% FCS/DMEM were pretreated with SB203580 at 10 μ M and SN50 at 50 μ g/ml, separately or together, for 30 min at 37 $^{\circ}$ C before culture into fl-coll in 1% FCS/DMEM for 24 h. Cells were further cultured in serum-free medium for 24 h. The inhibitors were continuously present in both culture conditions. MMP-1 secretion was analysed by Western blotting. Results are representative of two individual experiments. Abbreviation: SM, the control peptide of SN50. (B) Fibroblasts were co-transfected with a dominant negative I κ B α ^{S32A,S36A}, pCI2278CAT and pRSV- β gal before culture in fl-coll containing SB203580 for 48 h. Cell extracts were assayed for CAT activity after normalization with β -gal activity. The results are representative five independent experiments. Abbreviations: I κ Bwt, wild-type I κ B α ; I κ BDN, I κ B α ^{S32A,S36A}.

mutant that has greater stability than wild-type I κ B α , were previously shown to inhibit NF- κ B-mediated MMP-1 expression induced by fl-coll [1]. We tested whether these two inhibitors might affect the MMP-1 expression at high SB203580 concentration. As seen in Figure 7(A), SN50 alone inhibited MMP-1 secretion by fibroblasts in fl-coll (compare lanes 1 and 3, Figure 7A), whereas the control peptide had no inhibitory effect (compare lanes 1 and 2, Figure 7A). In contrast, SN50 did not inhibit MMP-1 secretion in the presence of SB203580 (lanes 4–6, Figure 7A). These observations were further evaluated by co-transfection of I κ B α ^{S32A,S36A} (I κ Bmut) and pCL2278CAT into fibroblasts. Although I κ B α ^{S32A,S36A} inhibited MMP-1 promoter activity induced by collagen lattices, it did not affect the MMP-1 promoter activity restored by SB203580 at 10 μ M (Figure 7B). Therefore, a NF- κ B-independent pathway apparently mediated MMP-1 expression when p38 α was suppressed by SB203580.

Integrin $\alpha_2\beta_1$ is required for fl-coll-induced MMP-1 expression in the absence or presence of SB203580

Integrin $\alpha_2\beta_1$ has a pivotal role in the regulation of MMP-1 expression induced by contracted three-dimensional collagen

Figure 8 Integrin $\alpha_2\beta_1$ mediates MMP-1 expression and NF- κ B activation by fl-coll

(A) MMP-1 was detected in the serum-free medium after fibroblasts were grown in fl-coll in the presence of a control (Con) mouse IgG, mAb to integrin α_2 (α_2) or combination of mAb to integrins α_2 and β_1 ($\alpha_2+\beta_1$). SM and SN50 were added as described in Figure 7. (B) Fibroblasts were co-transfected with pRSV- β gal, pCL2278CAT and one of the plasmids (vector, AS- α_2 or SS- α_2) before subculture into collagen lattices for 48 h. Analysis of CAT activity was performed as described in Figure 1(D). Results are representative of five individual experiments. (C) Fibroblasts were co-transfected with a CAT reporter plasmid that contains $4 \times \kappa$ B consensus sequence, pRSV- β gal, and one of the plasmids (vector, AS- α_2 , or SS- α_2) before culture in collagen lattices for 48 h. Analysis of CAT activity was performed as described in Figure 1(D). Results shown are representative of three independent experiments. NF- κ B and mNF- κ B are as described in Figure 5.

lattices [33]. The role of integrin $\alpha_2\beta_1$ in the antipodal effects of SB203580 on fl-coll-induced MMP-1 expression was thus assessed. In the absence of SB203580, the fl-coll-induced MMP-1 expression, a NF- κ B-dependent process, was greatly enhanced by the combination of mAb against α_2 and β_1 integrin (Figure 8A). In contrast, individual mAb against α_2 integrin (Figure 8A) and β_1 integrin (results not shown) did not have obvious effects. Therefore our result confirmed the findings previously made by Langholz et. al. [33]. The inhibition of NF- κ B by SN50 prevented MMP-1 expression induced by either fl-coll alone or together with mAb against α_2 and β_1 (Figure 8A), suggesting that integrin $\alpha_2\beta_1$ mediates MMP-1 expression through NF- κ B. To further

Figure 9 Integrin $\alpha_2\beta_1$ mediates the p38-suppression-facilitated MMP-1 expression

(A) Integrin α_2 was detected in cell extracts prepared from fibroblasts after culture in fl-coll with or without SB203580 at $10 \mu\text{M}$. The photograph of the fl-coll was taken before cell extracts were prepared. (B) Fibroblasts were co-transfected with pRSV- β gal, pCL2278CAT and one of the plasmids (vector, AS- α_2 or SS- α_2) before subculture into collagen lattices for 48 h in the absence or presence of SB203580 at $10 \mu\text{M}$. Analysis of CAT activity was performed as described in Figure 1(D). Results are representative of three individual experiments. (C) Fibroblasts were cultured with mAb to integrin α_2 or integrin β_1 in solution (Sol) or on immobilized mAbs (Sub) in serum-free medium for 24 h. MMP-1 was detected by Western analysis of the medium.

determine the role of $\alpha_2\beta_1$ in regulating NF- κ B activation by fl-coll, we examined MMP-1 promoter activity (Figure 8B) and NF- κ B transactivation activity (Figure 8C) in fibroblasts transfected with antisense integrin α_2 plasmid (AS- α_2). The AS- α_2 similarly decreased MMP-1 promoter and NF- κ B transactivation activity induced by fl-coll, whereas the control, vector or sense integrin α_2 plasmid (SS- α_2), did not. Therefore integrin α_2 apparently mediates NF- κ B-dependent MMP-1 expression by fl-coll in the absence of SB203580.

The role of integrin α_2 in fl-coll-induced MMP-1 expression in the presence of SB203580 at $10 \mu\text{M}$ was examined next. The protein level of integrin α_2 and the contraction of fl-coll were

slightly reduced by SB203580 (Figure 9A). However, the co-transfection of cells with AS- α_2 plasmid and pCL2278 resulted in a significant decrease in MMP-1 promoter activity (Figure 9B), indicating that integrin α_2 is also necessary for fl-coll-induced MMP-1 expression in the presence of SB203580. To substantiate this finding, we tested various integrin- α_2 -activating conditions using mAbs that could demonstrate the direct role of integrin α_2 in SB203580-relieved MMP-1 expression. By culturing fibroblasts with mAb to integrin α_2 in solution or on immobilized anti-(integrin α_2), we found that SB203580 treatment increased MMP-1 expression when integrin α_2 was ligated with its mAb in solution (lanes 1 and 2, Figure 9C). In contrast, SB203580 did not have a similar effect when cells were plated on the immobilized mAb as a substrate (lanes 3 and 4, Figure 9C). Control mAb to integrin β_1 did not have any effects on MMP-1 expression either in solution or as a substrate (lanes 5–8, Figure 9C). These data suggest that SB203580 treatment of cells is not sufficient to induce MMP-1 expression and that ligation of integrin α_2 to its ligand in fl-coll (Figures 1A and 9B) or to mAb in solution (Figure 9C) is necessary for the positive effect of SB203580 on MMP-1 expression.

DISCUSSION

We present results on the function of p38 α MAPK in MMP-1 gene expression by human fibroblasts embedded in three-dimensional collagen lattices. Three-dimensional structure of collagen lattices has been reported to activate p38 MAPK [18]. Our finding that fl-coll, but not att-coll, co-ordinately activated p38 α and its upstream kinase activator, MKK3/6, indicates that the mechanical properties, instead of the three-dimensional organization of collagen lattices, trigger p38 α activation. Because cell shrinkage caused by hypertonicity is a regulatory signal for the p38 MAPK activation [14,34], the cell size difference between fibroblasts cultured in fl-coll and att-coll might be one of the signals that activate the p38 MAPK. In fact, a number of physiological or disease processes that involve morphological alterations, such as ischaemic and hypertensive myocardial disease, also require p38 MAPK activation [12,35]. The specific activation of fibroblast p38 α by fl-coll is consistent with observations that p38 MAPK mediates mitotic arrest [36] and apoptosis [37], two cellular functions associated with fibroblasts cultured in contracting collagen matrices, but not att-coll [2,38].

On the basis of studies showing that MMP-1 expression similarly depends on the mechanical signals by three-dimensional collagen lattices [22] and that integrin $\alpha_2\beta_1$ mediates both p38 α activation [19] and MMP-1 expression induced by three-dimensional collagen lattices [33], we hypothesized that p38 α activation and MMP-1 expression might be related. To our surprise, the inhibition of p38 α by SB203580 resulted in the bipolar pattern of MMP-1 expression induced by fl-coll (Figure 1). The SB203580 dose titration demonstrated a 'valley-shaped' inhibitory pattern of MMP-1 expression. The MMP-1 level reached the lowest point at SB203580 concentrations ranging between 0.01 and 0.1 μ M, and recovered at higher concentration. Because the only known target of SB203580 inhibition is p38 MAPK, this finding suggests that p38 MAPK has both positive and negative function on MMP-1 expression. This interpretation was further supported by the evidence that transfection with dominant negative p38 α also caused bipolar regulation of MMP-1 expression (Figure 4). We postulate that the valley-shaped pattern of MMP-1 expression is attributed to the opposing action among p38 α -dependent positive and negative regulatory signals for MMP-1 expression.

The involvement of p38 α in the positive regulation of MMP-1 expression probably requires NF- κ B, a known mediator of MMP-1 expression induced by a variety of signals, including three-dimensional collagen [8,9,39]. On the basis of the two regulatory events, p38 α $\rightarrow$ NF- κ B (Figure 6) and NF- κ B $\rightarrow$ MMP-1 (Figure 7) [8,9,39], a positive regulatory pathway, fl-coll $\rightarrow$ p38 α $\rightarrow$ NF- κ B $\rightarrow$ MMP-1, may be constructed in the absence of SB203580. MMP-1 expression at a high concentration of SB203580 that clearly inhibited p38 α and NF- κ B uncovered a novel mechanism, suggesting a negative function of p38 α in counteracting an NF- κ B-independent MMP-1 expression induced by fl-coll. The complete inhibition of p38 α , while impairing its positive functions, including NF- κ B-dependent MMP-1 expression, apparently relieved the suppression of an independent pathway with an unknown identity. This observation was substantiated using an I κ B α mutant that serves as a dominant negative inhibitor of NF- κ B (Figure 7). We did not detect an obvious effect of wild-type I κ B α on the NF- κ B-dependent MMP-1 expression, in contrast with the findings of Yokoo et al. [41] and Wrighton et al. [40]. Presumably the dominant negative inhibition of NF- κ B in our system may require a stable I κ B α .

It should be pointed out that SB203580 at low dose appeared more effective in inhibiting MMP-1 than p38 α and NF- κ B, as demonstrated by the far lower concentration of SB203580 required to inhibit the fl-coll-induced MMP-1 expression by 50% in the descending phase (5–10 nM) than that found to inhibit p38 α and NF- κ B (100–500 nM). While our data did not rule out that an unknown kinase sensitive to SB203580 might be responsible in the descending phase, a most likely explanation is that MMP-1 expression is under a non-linear control by the p38 α /NF- κ B pathway during that phase. Negative effects from the partial inhibition of p38 α can be amplified by the subsequent suppression of an array of p38 α -inducible transcription factors in addition to NF- κ B, such as ATF2, cyclic AMP-responsive-element binding protein ('CREB') and CAAT-enhancer binding protein (C/EBP) homologous protein ('CHOP') [42–44]. These factors are leucine zipper proteins that can form dimers to influence transcription activity by NF- κ B, AP-1 or C/EBPs, factors that mediate MMP-1 gene expression. The signal amplification can also occur downstream of NF- κ B. For example, NF- κ B can both directly [45] and indirectly regulate MMP-1 gene expression through intermediate regulators such as interleukin (IL)-1 [9]. IL-1 can enhance NF- κ B activity through an autocrine feedback regulation [46]. As a result, the disturbance in p38 α and NF- κ B activity is likely to produce a far more potent effect in MMP-1 gene expression than one might predict.

Integrin $\alpha_2\beta_1$ is known to mediate three-dimensional collagen-stimulated MMP-1 expression [33]. Because fibroblast integrin α_2 gene expression induced by fl-coll is mediated by NF- κ B activity [1], we tested whether NF- κ B may induce MMP-1 expression by increasing integrin α_2 (Figure 8). By using mAbs to integrin α_2 and β_1 and AS- α_2 plasmid, we showed the requirement of integrin $\alpha_2\beta_1$ for MMP-1 expression in the absence of SB203580, confirming the findings of Langholz et al. [33]. Furthermore, we also showed that NF- κ B activation depended on the presence of integrin α_2 , indicating the presence of a positive feedback mechanism, $\alpha_2 \leftrightarrow$ NF- κ B, in this system. Indeed, untransfected fibroblasts had a substantial basal level of integrin $\alpha_2\beta_1$ protein before subculture into fl-coll (results not shown). Incidentally, a similar autocrine feedback mode, IL-1 $\leftrightarrow$ NF- κ B, also exists in primary fibroblasts stimulated by signals including cytochalasin B and regulates MMP-1 expression [46]. Therefore, an integrin $\alpha_2\beta_1$ -initiated signalling pathway apparently activated NF- κ B, leading to the increased expression of both α_2 and MMP-1.

We also examined the role of integrin α_2 in MMP-1 expression

at high SB203580 concentration. Integrin α_2 protein level was only slightly reduced by SB203580, despite the inhibition of NF- κ B activity. It is thus likely that a NF- κ B-independent regulatory mechanism may co-ordinately mediate the expression of integrin α_2 and MMP-1 at high SB203580 concentration. Interestingly, integrin α_2 was also required by fl-coll-induced MMP-1 expression at high SB203580 concentration (Figure 9B). By examining MMP-1 expression after the ligation of integrin α_2 to its mAb in solution or on immobilized surface as a substrate, we found that the mAb to integrin α_2 in solution, but not as a substrate, potentiated the MMP-1 expression in the presence of SB203580 (Figure 9C). NF- κ B activation was not observed under this condition (results not shown), consistent with the NF- κ B-independent nature of MMP-1 expression relieved by p38 α inhibition. We postulate that the integrin α_2 ligation to its mAb in solution may cause two antagonistic events – NF- κ B-independent MMP-1 expression and p38 α -dependent MMP-1 suppression – resulting in an undetectable net MMP-1 expression. As a result, the MMP-1 expression became evident when p38 α is inhibited by SB203580 (Figure 9C). While fl-coll is a far more complex system than the mAb to integrin α_2 in solution, cells cultured in these two systems share certain similar characteristics, such as shape and actin cytoskeletal organization, as opposed to cells either spread on mAb to integrin α_2 as a substrate or embedded in att-coll. It is likely that at least three integrin- α_2 -dependent antagonistic pathways may exist in cells surrounded by fl-coll: p38 α /NF- κ B/MMP-1 expression, NF- κ B-independent MMP-1 expression and p38 α -dependent MMP-1 suppression. The balance among these pathways caused by p38 α inhibition could conceivably result in the non-linear pattern of MMP-1 expression.

In summary, we describe a novel bidirectional role of p38 α in fl-coll-induced MMP-1 gene expression. The findings are in line with the knowledge about three-dimensional collagen signalling and the complex control of MMP-1 expression by positive and negative signals. Cells cultured in three-dimensional collagen receive integrated signals, such as integrin–ECM interaction, spatial arrangement and/or cell shape change. As a result, fl-coll, the single signal as we perceive, may mediate a combination of multiple signals, which could include both stimulatory and suppressive regulators for MMP-1 expression. Further identification of these pathways is currently under way.

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