Synergistic upregulation of metalloproteinase-9 by growth factors and inflammatory cytokines: an absolute requirement for transcription factor NF-κB

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Abstract Matrix metalloproteinase (MMPs) enzymes are implicated in matrix remodelling during proliferative inflammatory processes including wound healing. We report here synergistic upregulation of MMP-9 protein and mRNA by platelet-derived growth factor (PDGF) or basic fibroblast growth factor (bFGF) in combination with interleukin-1α (IL-1α) or tumour necrosis factor-α (TNF-α) in primary rabbit and human dermal fibroblasts. The synergistic interaction between growth factors and cytokines implies that basement membrane remodelling is maximal physiologically when both are present together. The signalling pathways mediating this synergistic regulation are not understood, although analysis of the MMP-9 promoter has identified an essential proximal AP-1 element and an upstream nuclear factor kappa-B (NF-κB) site. Using electromobility shift assays, binding to the AP-1 site was only slightly increased by growth factors and cytokines. NF-κB binding was rapidly induced by IL-1α or TNF-α but was neither induced nor potentiated by bFGF or PDGF. Neither AP-1 nor NF-κB was therefore sufficient on its own for synergistic regulation. Using a recently developed adenovirus that overexpresses the inhibitory subunit, IκBα, we demonstrated an absolute requirement for NF-κB in upregulation of MMP-9. Activation of NF-κB binding by inflammatory cytokines was therefore necessary but not sufficient for synergistic upregulation of MMP-9.

Key words: Metalloproteinase; Nuclear factor kappa-B; Extracellular matrix; Fibroblast

1. Introduction

Basement membrane (BM) components perform both structural and regulatory functions in the proliferation and migration of connective tissue cells. The matrix degrading metalloproteinases (MMPs) are a family of structurally related enzymes that are capable of promoting physiological turnover of all extracellular matrix components, for example during wound healing. Deregulated degradation of BM has also been implicated in several pathologies including glomerular nephritis, atherosclerosis and tumour cell invasion [1]. Of the MMPs, the gelatinase subgroup, comprised of gelatinase-A (MMP-2) and gelatinase-B (MMP-9), efficiently degrade BM components laminin and type-IV collagen. MMP-2 is constitutively expressed by many cell types and thought to be regulated at the level of pro-enzyme activation [2–4]. MMP-9 is transcriptionally regulated by inflammatory cytokines (tumour necrosis factor-α (TNF-α), interleukin-1α (IL-1α)) [1,3,5–7]. Few studies have examined the interaction of connective tissue mitogens (e.g. platelet-derived growth factor (PDGF), basic fibroblast growth factor (bFGF) and EGF) and inflammatory cytokines, despite the fact that both are present together in healing wounds. MMP-9 expression in rabbit aortic smooth muscle cells is unaffected by PDGF on its own, submaximally increased by IL-1α alone, but synergistically upregulated by combinations of PDGF and IL-1α [3]. We sought here to determine whether this synergy applied to other combinations of growth factors and cytokines, and to dermal fibroblasts. We also investigated the molecular mechanisms underlying this synergistic regulation.

Several studies have highlighted the absolute requirement for a conserved proximal AP-1 binding site in the induction of MMP-9 [7–10]. A distal stimulatory region encompassing an NF-κB element and an additional AP-1 binding site [7,9] has also been identified although it is uncertain whether this is also essential or merely cooperates with the proximal AP-1 site. We used electromobility shift assays and adenoviral mediated overexpression of IκBα, the endogenous inhibitor of NF-κB, to determine the importance of NF-κB in upregulation of MMP-9.

2. Materials and methods

2.1. Materials

Phorbol 12-myristate 13-acetate (PMA) was obtained from Sigma, Poole, Dorset, UK. Human recombinant platelet-derived growth factor BB (PDGF BB) and basic fibroblast growth factor (bFGF) were purchased from Boehringer Mannheim, Lewes, East Sussex, UK. Human recombinant interleukin-1α (IL-1α) was a generous gift from Roche, Welwyn Garden City, Herts, UK. Tumour-necrosis factor-α (TNF-α) was purchased from R&D Systems, Abingdon, UK. Recombinant adenovirus capable of overexpressing porcine IκBα was a kind gift from Dr Rainer De Martin (Department of Vascular Biology and Thrombosis Research, University of Vienna, Austria). Recombinant adenovirus capable of overexpressing the bacterial *LacZ* gene [11] and a control virus with a silent expression cassette were kind gifts form Dr G. Wilkinson (Dept. Cardiology and Medicine, University of Wales College of Medicine, Heath Park, Cardiff, UK). Antibody to NF-κB/p65 subunit was obtained from Santa Cruz Biotechnology, Delaware Avenue, CA, USA.

2.2. Methods

2.2.1. Cell culture. Primary cultures of human foreskin fibroblasts and rabbit dermal fibroblasts were each prepared by modifications of the explant technique, as previously described in detail [12]. Cells

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Abbreviations: bFGF, basic fibroblast growth factor; BM, basement membrane; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IL-1α, interleukin-1α; MMP, metalloproteinase; PMA, phorbol myristate acetate; PDGF, platelet-derived growth factor; RAd, recombinant adenovirus; TNF-α, tumour necrosis factor-α

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between passage one to three were plated at a density of 7×10^4 cells/well into 12-well culture plates for zymography and Western blotting or 8×10^5 cells/75 cm^2 flask for RNA studies. For all experiments, subconfluent cells were rendered quiescent by incubation in serum-free DMEM supplemented with 0.25% (v/v) lactalbumin hydrolysate (Gibco-BRL, Paisley, UK) for 3 days. Cultures were then exposed to fresh serum-free medium containing the appropriate concentration of the agent under investigation for varying time intervals.

2.2.2. Zymography. Zymography was used for semi-quantitative analysis of gelatinase levels secreted into the culture medium as previously described [3,12]. Human fibroblasts conditioned media were concentrated 30-fold by ultrafiltration (Centricon 30, Amicon, Stonehouse, Gloucestershire, UK) prior to analysis. Gelatinolytic bands were size calibrated with a high molecular weight standard mixture of proteins (Sigma). Briefly, zymograms were loaded so as to yield bands of lysis that were in the linear range of measurement. Band intensities were quantified using a Bio-Rad GS 690 Image Analysis software system (Bio-Rad Laboratories, Hemel Hempstead, Hertfordshire, UK) and related to that of a standard mixture of MMP-2 and -9 run on each zymogram.

2.2.3. Northern analysis. The generation of the MMP-9 and GAPDH riboprobes has been described previously [3]. Total cellular RNA was prepared from 1×10^6 rabbit skin fibroblast cultures using Qiagen RNeasy total RNA extraction columns (Hybaid, Teddington, Middlesex, UK) according to the manufacturer's instructions. 10 μg of RNA was fractionated on 1% agarose/2.2 M formaldehyde gels and transferred onto Hybond-N membranes (Amersham International) by capillary blotting overnight using 20× SSC (3 M sodium chloride, 300 mM sodium citrate). RNA integrity and equality of loading was assessed by staining with 0.03% methylene blue in 0.3 M sodium acetate, pH 5.2.

Specifically hybridised DIG-labelled probe was detected using the DIG-detection system (Boehringer Mannheim). Bands were quantified using a Bio-Rad GS 690 Image Analysis software system.

2.2.4. Nuclear extracts and electromobility shift assay. Nuclear proteins were extracted from approximately 1×10^6 rabbit dermal fibroblast nuclei as previously described [13]. Binding reactions (20 μl) containing 2 μg poly(dI-dC), 10 mM HEPES, pH 7.9, 50 mM NaCl, 0.5 DDT, 2.5 mM EDTA, 7 mM $MgCl_2$, 4% glycerol, and 4–6 μg nuclear extract were incubated on ice for 30 min with 20 000 cpm of ^{32}P-labelled oligonucleotide corresponding to the rabbit MMP-9 NF-κB element (5′-..CCC CGG TGG AAT TCC CCA AAT CCT..-3′) or the proximal AP-1 element (5′-..CCG GCC CTG AGT CAG CAC TTG CCT G..-3′). Complexes were separated on 6% non-denaturing polyacrylamide gels.

3. Results

3.1. Combinations of cytokines and growth factors synergistically stimulate MMP-9 expression

As shown by zymography in Fig. 1, IL-1α but not TNF-α alone gave a barely detectable stimulation of MMP-9 secretion. PDGF or bFGF alone did not increase MMP-9 secretion. Combinations of IL-1α (Fig. 1B) or TNF-α (Fig. 1A) with PDGF acted synergistically and even greater effects were seen with combinations of bFGF with either IL-1α or TNF-α. TNF-α was consistently less effective than IL-1α. Combinations of PDGF and bFGF were ineffective in stimulating MMP-9 secretion, while combinations of IL-1α and TNF-α were at best additive (results not shown). MMP-9 secretion from human foreskin fibroblasts was also synergistically upregulated by PDGF or bFGF plus IL-1α (data not shown). In human cells, the effect of bFGF plus IL-1α was similar to that of PDGF plus IL-1α. No changes in MMP-2 levels were seen in either cell type under any of the conditions tested (Fig. 1A,B, and not shown).

Northern blotting (Fig. 2) showed that MMP-9 mRNA levels were also upregulated by combinations of PDGF or bFGF plus IL-1α, as early as 4 h after stimulation. bFGF was more effective than PDGF, consistent with the protein secretion data.

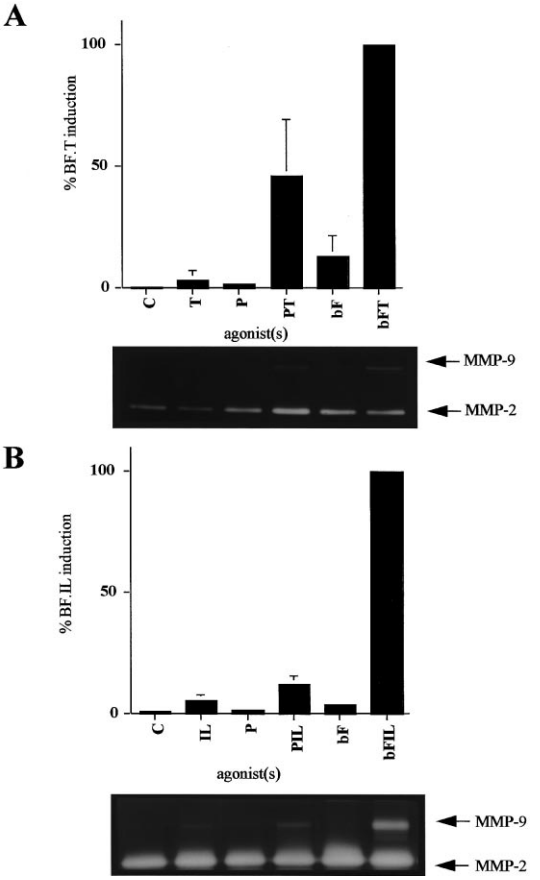

Fig. 1. Effect of IL-1α, TNF-α, bFGF and PDGF-BB on MMP-9 secretion. Conditioned media from quiescent rabbit dermal fibroblasts incubated for 16 h without serum. A: With combinations of TNF-α with PDGF or bFGF. Lane 1: control; lane 2: 10 ng/ml TNF-α; lane 3: 20 ng/ml PDGF-BB; lane 4: TNF-α+PDGF; lane 5: 20 ng/ml bFGF; lane 6: bFGF+TNF-α. B: With combinations of IL-1α with PDGF or bFGF. Lane 1: control; lane 2: 10 ng/ml IL-1α; lane 3: 20 ng/ml PDGF-BB; lane 4: IL-1α+PDGF; lane 5: 20 ng/ml bFGF; lane 6: bFGF+IL-1α was subjected to gelatin zymography and densitometric analysis. Values are means ± S.E.M. of 3 separate experiments.

3.2. Role of AP-1 and NF-κB transcription factors in MMP-9 upregulation

Electromobility shift assays (EMSA) demonstrated high basal levels of AP-1 binding that were only slightly increased by IL-1α, bFGF or their combination with no evidence of synergy (Fig. 3B). EMSA demonstrated strong and rapid NF-κB activation in response to IL-1α but not bFGF stimulation (Fig. 3A). bFGF did not enhance the binding in response to IL-1α. These results were confirmed by immunofluorescent staining using a p65 specific antibody (results not shown). Strong NF-κB nuclear translocation occurred in response to a 45-min stimulation with IL-1α or TNF-α; PDGF and bFGF neither stimulated nor enhanced NF-κB nuclear translocation. These results established that neither AP-1 nor NF-κB binding alone was sufficient to explain MMP-9 regulation.

To establish whether NF-κB activity was necessary or not for synergistic regulation of MMP-9, we employed a recombi-

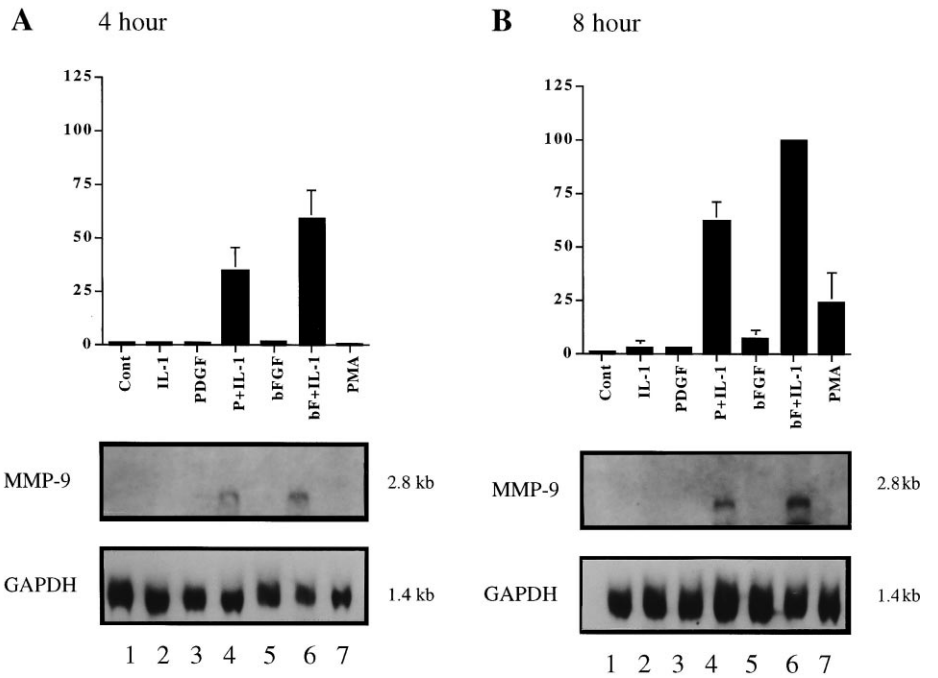

Fig. 2. Synergistic regulation of MMP-9 steady state mRNA expression. Total RNA from quiescent rabbit dermal fibroblasts incubated for 4 or 8 h without serum with: lane 1: unstimulated control; lane 2: 10 ng/ml IL-1α; lane 3: 20 ng/ml PDGF-BB; lane 4: IL-1α+PDGF-BB; lane 5: 20 ng/ml bFGF; lane 6: IL-1α+bFGF; lane 7: 10^{-7} M PMA was analysed for steady state MMP-9 (panel A) and GAPDH (panel B). Blots were analysed by densitometry and normalised as a percentage of the response to IL-1α plus bFGF after 8 h. Values are mean ± S.E.M. for 3 separate experiments.

nant adenovirus (RAd:IκBα) [14] to overexpress IκBα, the NF-κB inhibitory subunit. Using Rad35:βgal [11], a recombinant adenovirus that expressed the bacterial *LacZ* gene, demonstrated that a multiplicity of infection (MOI) of 250 pfu/ml was required to achieve a 100% infection efficiency (data not shown). Similar infection with RAd:IκBα but not RAd66: null virus increased expression of IκBα measured by Western blotting (not shown). RAd:IκBα but not RAd66:null completely inhibited IL-1α induced NF-κB DNA binding activity (Fig. 4A), which was further identified by antibody supershift (Fig. 4B). As a proof of specificity, RAd:IκBα was shown not to affect AP-1 DNA binding (Fig. 4C), cell viability or serum induced proliferation (data not shown). Infection with RAd: IκBα but not RAd66:null completely inhibited MMP-9 secretion (Fig. 5A) and steady state mRNA expression (Fig. 5B) in response to all agonists tested, alone or in combination. The

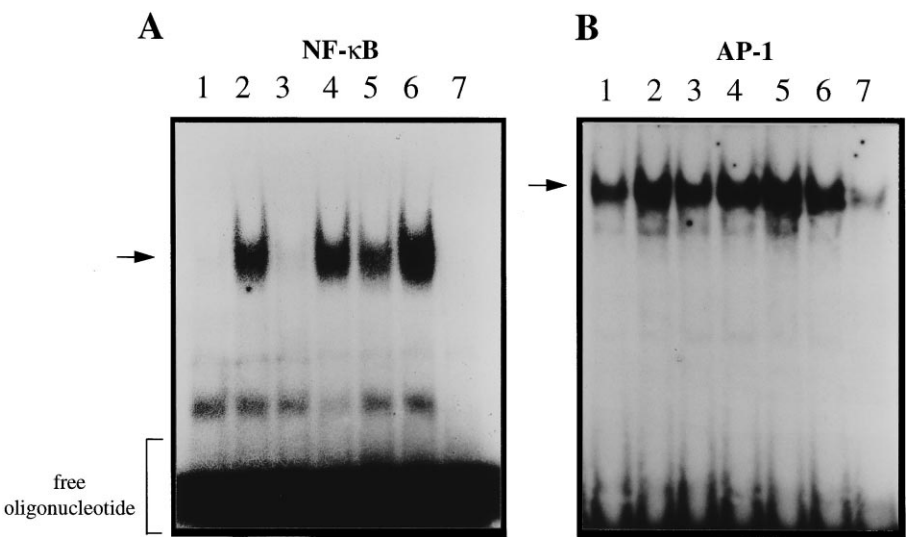

Fig. 3. Activation of NF-κB and AP-1 DNA binding by cytokines and growth factors. Nuclear extracts were prepared from quiescent rabbit dermal fibroblasts after 3-h stimulation with 10 ng/ml IL-1α, 20 ng/ml bFGF or 10^{-7} M PMA. Lane 1: control; lane 2: IL-1α; lane 3: bFGF; lane 4: IL-1α+bFGF; lane 5: PMA. Nuclear extracts were analysed for (A) NF-κB DNA binding activity and (B) AP-1 binding activity by EMSA (protein/DNA complexes indicated by arrows). Binding specificity was demonstrated with addition of 200-fold excess of unlabelled specific or irrelevant oligonucleotide to binding reactions containing IL-1α induced nuclear extracts. A: Lane 6: excess AP-1 oligonucleotide; lane 7: excess NF-κB oligonucleotide; B: lane 6: excess NF-κB oligonucleotide; lane 7: excess AP-1 oligonucleotide.

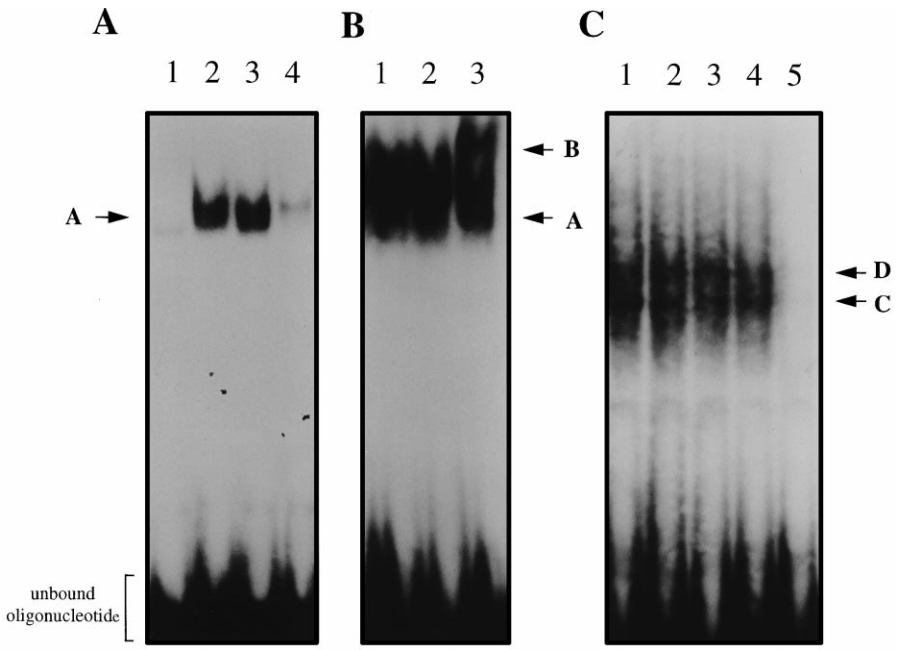

Fig. 4. Effect of IκBα overexpression on NF-κB and AP-1 DNA binding. Quiescent rabbit dermal fibroblasts were infected with 250 MOI of RAd66 control virus or RAd:IκBα virus and stimulated with 10 ng/ml IL-1α or 10^{-7} M PMA as indicated for 3 h, 3 days post infection. A: Nuclear extracts (approx. 5 μg) were analysed for NF-κB activity. Lane 1: uninfected, unstimulated control, IL-1α stimulated uninfected cells; lane 3: IL-1α stimulated RAd66:null control virus infected cells; lane 4: IL-1α stimulated RAd:IκBα infected cells. B: Binding activity was identified as NF-κB in IL-1α induced extracts by antibody supershift. Lane 1: control; lane 2: non-immune goat IgG; lane 3: anti-NF-κB/p65. C: Effect of viral infection on PMA induced AP-1 DNA binding. Lane 1: uninfected control; lane 2: RAd66 control virus infected; lane 3: RAd:IκBα virus infected. Binding specificity was confirmed with addition of 200-fold excess cold oligonucleotide. Lane 4: 200-fold excess NF-κB oligonucleotide; lane 5: 200-fold excess AP-1 oligonucleotide. The results are representative of 3 separate experiments.

constitutive secretion of MMP-2 and the steady state mRNA expression of the GAPDH, genes that lack NF-κB regulatory elements, were unaffected.

4. Discussion

We have demonstrated that the synergistic upregulation of MMP-9 secretion is not restricted to PDGF and IL-1α in smooth muscle cells [3] but is an example of a more general regulatory mechanism of potential physiological importance. IL-1α or TNF-α interacted synergistically with either PDGF or bFGF. Synergistic regulation occurs also at the mRNA level at early time points. This regulatory mechanism is common to both rabbit and human fibroblasts. Activation of AP-1 and NF-κB transcription factors is insufficient but absolutely required for upregulation of MMP-9.

Increased expression of gelatinase-B occurs in response to IL-1α and TNF-α in a number of cell types [1,5,15]. IL-1α and TNF-α are secreted by activated macrophages, leukocytes and connective tissue cells [16–18], with increased levels of these factors associated with several degradative inflammatory diseases and healing wounds [19,20]. The data presented here illustrate an additional requirement for growth factor stimulation to achieve maximal MMP-9 secretion. Several studies have reported increased levels of connective tissue mitogens such as PDGF and bFGF at sites of tissue damage and inflammation [17,21]. Activation of macrophages and cell injury can both result in release of bFGF and PDGF [17,22–24]. The synergistic pattern of MMP-9 upregulation reported here is therefore likely to be of physiological importance.

Synergistic interactions between growth factors and cytokines mediating various biological responses have been reported previously. Bonin et al. [25] reported that IL-1 enhances the growth promoting activity of PDGF and macrophage derived growth factor (MDGF). However, IL-1 induced proliferation of fibroblasts and smooth muscle cells has been reported to be due to induction and release of PDGF-AA, suggesting that this synergy may be due to initiation of autocrine or paracrine stimulation [26]. Chandrasekhar et al. [27,28] reported cooperative effects of IL-1 and bFGF on metalloproteinase gene expression in rabbit articular chondrocytes which may be due to increased numbers of IL-1 receptors. However, stimulation with 100 ng/ml bFGF (5-fold the concentration used here) only resulted in a doubling of IL-1 receptor numbers, while greatly enhancing MMP secretion, which implies the involvement of additional mechanisms such as those defined here [27]. Increase in IL-1 receptors and ensuing signal transduction cannot account for the increase in MMP-9 expression seen in this study. Firstly, the effect on MMP-9 mRNA expression occurred within 4 h, insufficient time for increased receptor expression. Secondly, we demonstrated that NF-κB activation, an early event downstream of cytokine receptor occupation, was not potentiated by addition of growth factor (Fig. 3). Finally, we have found that induction of another cytokine induced gene, VCAM-1, is not at all potentiated by PDGF or bFGF stimulation (M. Bond and A.C. Newby, unpublished observations). Taken together, these data imply that the synergistic interactions between cytokines and growth factors reported most probably results from activation of multiple interacting signal transduction pathways.

PDGF and bFGF typically activate the ERK-1/ERK2 MAP kinase pathway, leading ultimately to activation of AP-1 transcription factors. TNF-α and IL-1α activate recep-

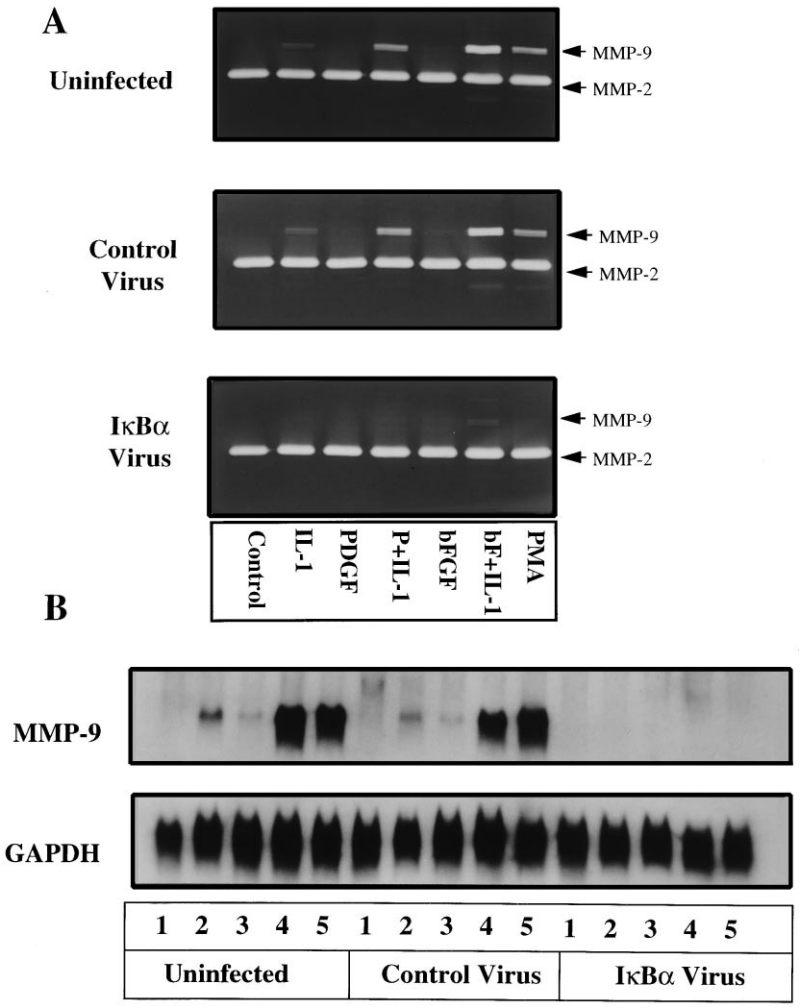

Fig. 5. Effect of IκBα overexpression on MMP-9 expression. Zymography was conducted on conditioned media from uninfected quiescent rabbit dermal fibroblasts (panel A, upper) or cells infected with 250 MOI of RAd66:null control virus (panel A, middle) or RAd:IκBα virus (panel A, lower) and stimulated with 10 ng/ml IL-1α, 20 ng/ml PDGFBB, 20 ng/ml bFGF or 10^{-7} M PMA as indicated for 16 h, 3 days post infection. Total RNA was extracted and analysed for MMP-9 (panel B, upper) and GAPDH (panel B, lower) mRNA expression by Northern blotting. Lane 1: unstimulated; lane 2: IL-1α; lane 3: bFGF; lane 4: bFGF+IL-1α; lane 5: PMA. The results are representative of 3 separate experiments.

tor tyrosine kinase independent pathways, leading to the rapid activation of NF-κB. From previous studies, the AP-1 transcription factor is known to be essential for MMP-9 expression in fibroblasts [7,9]. However, we showed here that in rabbit dermal fibroblasts AP-1 DNA binding is poorly regulated by cytokine and growth factor combinations implying that activation of AP-1 binding alone is insufficient for synergistic upregulation of gelatinase-B. This suggests the interaction with additional factors, of which NF-κB is an attractive possibility because a functional NF-κB binding element exists in the gelatinase-B promoter in a region that cooperates with the proximal AP-1 element [7,9]. Moreover, stable expression of a dominant negative NF-κB/p65 mutant partially inhibited IL-1β induced MMP-9 expression in mesangial cells [10]. In this study, cytokine stimulation of rabbit dermal fibroblasts resulted in a rapid and potent activation of NF-κB but little or no induction of MMP-9 expression. This implied either that NF-κB activity was unnecessary or insufficient on its own for full upregulation of the gene. To investigate whether NF-κB activation was essential for MMP-9 expression, we used a recombinant adenovirus that overexpresses IκBα, the endogenous inhibitor of NF-κB. Overexpression of IκBα completely inhibited NF-κB binding and both MMP-9 protein and mRNA expression, demonstrating an absolute requirement for NF-κB activity in upregulating MMP-9.

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