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Modulation of autocrine TNF-α-stimulated matrix metalloproteinase 9 (MMP-9) expression by mitogen-activated protein kinases in THP-1 monocytic cells*

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*Dedicated to Professor Hans Fritz on the occasion of his 70th birthday

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Abstract

Matrix metalloproteinase 9 (MMP-9) is implicated in various physiological processes by its ability to degrade the extracellular matrix (ECM) and process multiple regulatory proteins. Normally, MMP-9 expression is tightly controlled in cells. Sustained or enhanced MMP-9 secretion, however, has been demonstrated to contribute to the pathophysiology of numerous diseases, including arthritis and tumor progression, rendering this enzyme a major target for clinical interventions. Here we show that constitutive MMP-9 secretion was abrogated in THP-1 monocytic leukemia cells by addition of neutralizing antibodies against tumor necrosis factor α (TNF-α) or TNF receptor type 1 (TNF-R1), as well as by inhibition of TNF-α converting enzyme (TACE). This indicates that MMP-9 production in these cells is maintained by autocrine stimulation, with TNF-α acting via TNF-R1. To investigate the intracellular signaling routes involved in MMP-9 gene transcription, cells were treated with different inhibitors of major mitogen-activated protein kinase (MAPK) pathways. Interruption of the extracellular signal-regulated kinase pathway 1/2 (ERK1/2) using PD98059 significantly downregulated constitutive MMP-9 release. In contrast, blockage of p38 kinase activity by addition of SB203580 or SB202190, as well as inhibition of c-Jun N-terminal kinase (JNK) using L-JNK-I1, clearly augmented MMP-9 expression and secretion by an upregulation of ERK1/2 phosphorylation. Moreover, exogenously added TNF-α augmented MMP-9 synthesis and secretion in THP-1 cells via enhancement of ERK1/2 activity. Taken together, our results indicate that ERK1/2 activity plays a pivotal role in TNF-α-induced MMP-9 production and demonstrate its negative modulation by p38 and JNK activity. These findings suggest ERK1/2 rather than p38 and JNK as a reasonable target to specifically block MMP-9 expression using MAPK inhibitors in therapeutic applications.

Keywords: cross-talk; ERK; gelatinase B; JNK; leukemia; p38.

Introduction

The matrix metalloproteinases (MMPs) constitute a family of 24 zinc-dependent proteinases that play fundamental roles in the turnover and remodeling of tissues by degrading proteins of the extracellular matrix (ECM) (Sternlicht and Werb, 2001). MMPs have also been implicated in a variety of pathological conditions, including arthritis (Vincenti and Brinckerhoff, 2002), multiple sclerosis (Chandler et al., 1997), and tumor progression (Egeblad and Werb, 2002).

The MMPs are divided into several subgroups according to their structure and substrate specificity. The two so-called gelatinases, MMP-9 and MMP-2, are key enzymes in the degradation of basement membranes (Stetler-Stevenson, 1990). Recent work has elucidated a much more complex role of these enzymes provided by the ability of MMP-9 to cleave the intercellular adhesion molecule-1 (ICAM-1), to process cytokines, such as transforming growth factor-β (TGFβ), interleukin 8 (IL-8), and IL-1β, and to enhance the bioavailability of vascular endothelial growth factor (VEGF) (Schonbeck et al., 1998; Bergers et al., 2000; Van den Steen et al., 2000; Yu and Stamenkovic, 2000; Fiore et al., 2002). Because of these versatile functions, MMP-9 is thought to play important regulatory roles during the immune response (Opdenakker et al., 2001), in angiogenesis (Hamano et al., 2003), and in malignant diseases (McCawley and Matrisian, 2001).

After secretion from cells, the proteolytic activity of MMP-9 is mainly regulated by zymogen activation and interaction with the tissue inhibitor of metalloproteinase 1 (TIMP-1) (Ramos-DeSimone et al., 1999). MMP-9 gene expression is controlled by a promoter containing AP-1, PEA3 and NF-κB binding sites that tightly regulate MMP-9 transcription in response to distinct extracellular stimuli such as TNF-α and IL-1β (He, 1996; Ries and Petrides, 1995; Westermarck and Kahari, 1999). TNF-α is one of the strongest physiological inducers of MMP-9 expression. The biological and cellular effects of TNF-α are mediated through two cell surface receptors, TNF receptor type 1 (TNF-R1) and type 2 (TNF-R2). TNF-R1 is stimulated by soluble TNF-α, while TNF-R2 is activated by the membrane-bound precursor preTNF-α (Hehlgans and Mannel, 2002). Shedding of preTNF-α from the cell surface is accomplished by the membrane-associated TNF-α converting enzyme (TACE) (Black et al., 1997).

Intracellular transmission of TNF-α signaling has been reported to occur via three major members of the mito-

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gen-activated protein kinases (MAPKs) family: ERK1/2 (extracellular signal-regulated kinase), JNK (c-Jun N-terminal kinase), and p38 (Lewis et al., 1998; Kyriakis and Avruch, 2001). ERK1/2 is phosphorylated by sequential activation of the Ras-Raf-MEK1/2 cascade upon stimulation of the cells with mitogen-like epidermal growth factor (EGF), whereas the pathways of JNK and p38 appear to be primarily induced by inflammatory cytokines (Baud and Karin, 2001). Phosphorylation of the MAPKs results in their translocation to the nucleus, where they modulate gene expression by phosphorylating targets such as downstream kinases and transcription factors (Pearson et al., 2001).

Recent data indicate that inflammatory cells that infiltrate neoplastic tissues favor tumor growth by secretion of TNF-α and MMP-9 (Coussens et al., 2000; Coussens and Werb, 2002). Moreover, breast tumor cells release TNF-α, which stimulates MMP-9 production in stromal fibroblasts (Stuelten et al., 2005). Therefore, because of their multiple regulatory roles, MAPKs have been proposed as valuable targets for anti-cancer and anti-inflammatory therapies (Hilger et al., 2002; Waetzig et al., 2002; Sebolt-Leopold and Herrera, 2004). However, little is known about specific MAPK signal transduction pathways responsible for MMP-9 expression.

We used THP-1 monocytic cells that constitutively synthesize and secrete MMP-9 to study the implication of three major MAPK pathways in this process. Our results indicate complex cross-talk between ERK1/2 on one hand, and p38 and JNK on the other, regulating MMP-9 expression in opposite manners. For therapeutic applications, these data suggest the choice of MAPK inhibitors should be carefully considered to achieve MMP-9 downregulation but to avoid induction of MMP-9 biosynthesis.

Results

Constitutive MMP-9 expression in THP-1 cells is maintained by autocrine stimulation with TNF-α

THP-1 monocytic leukemia cells were used to assess the role of potential TNF-α-induced signaling in the constitutive expression of MMP-9. The cells were incubated under serum-free conditions for 24 h with or without different concentrations of an antibody that is capable of neutralizing the biological activity of TNF-α. Analysis of MMP-9 mRNA expression by qRT-PCR showed that addition of anti-TNF-α antibodies significantly decreased MMP-9 transcription in a dose-dependent manner (Figure 1A). Analysis of the conditioned medium by gelatin zymography revealed that basal secretion of the latent form of MMP-9 was blocked with increasing concentrations of anti-TNF-α antibody (Figure 1B). These findings on MMP-9 protein correlate with the mRNA expression data, indicating that endogenous TNF-α is responsible for continuous MMP-9 expression and secretion in THP-1 cells. In contrast to MMP-9, constitutive MMP-2 release was not affected under these conditions (Figure 1B).

To determine which TNF-α receptor is involved in the autocrine regulation of MMP-9 production, THP-1 cells were treated with neutralizing monoclonal antibodies against TNF-R1 or TNF-R2. Quantitative analysis of MMP-9 mRNA revealed that blockage of TNF-R1 downregulated MMP-9 gene transcription much better than blockage of TNF-R2 (Figure 1C). Consistent with these data, zymographic analysis of conditioned media showed that treatment of the cells with antibodies against TNF-R1 abrogated MMP-9 secretion in a dose-dependent manner, whereas antibodies against TNF-R2 had only a marginal inhibitory effect on MMP-9 release (Figure 1D, upper panel). Appropriate isotype control antibodies showed no influence on MMP-9 secretion (Figure 1D, lower panel).

To investigate whether TACE, which is responsible for the release of TNF-α from the plasma membrane, is involved in basal MMP-9 secretion, THP-1 cells were treated with Ro32-7315, a highly specific inhibitor of TACE activity (Beck et al., 2002). Zymographic analysis of secreted enzymes showed that increasing concentrations of Ro32-7315 led to a decrease in MMP-9 release from the cells (Figure 1E). Ro32-7315 had no cytotoxic effect at concentrations up to 10 μg/ml as determined by trypan blue staining of the cells (data not shown).

Autocrine TNF-α-mediated MMP-9 expression is maintained via ERK1/2 activity

To elucidate the signaling cascades involved in basal MMP-9 production, we first examined the role of the ERK1/2 pathway. The cell-permeable inhibitor PD98059 selectively blocks the activity of MEK1/2, which is the upstream activator of ERK1/2. Incubation of THP-1 cells with this inhibitor induced a dose-dependent downregulation of continuous MMP-9 mRNA expression and enzyme secretion, as determined by real-time PCR and zymography, respectively (Figure 2A,B). Maximum inhibition of MMP-9 mRNA expression by 85% was observed at a concentration of 10 μM PD98059 and was not further enhanced at a higher inhibitor concentration. MMP-2 secretion was not affected under these conditions. These data suggest that ERK1/2 activity essentially contributes to basal production and release of MMP-9 in THP-1 cells.

Inhibitors of p38 upregulate constitutive MMP-9 expression and secretion

To study potential implication of the p38 MAPK pathway in constitutive MMP-9 expression, cells were incubated with SB202190 or SB203580, representing specific inhibitors of p38. Both compounds consistently led to an augmentation of MMP-9 mRNA expression and protein secretion, as determined by qRT-PCR and zymography, respectively (Figure 3A–D). The stimulatory effect on MMP-9 production was approximately five- to six-fold at 25 μM SB202190 (Figure 3A,B) and three-fold at 25 μM SB203580 (Figure 3C,D). MMP-2 secretion was not affected by either of the inhibitors (Figure 3B,D). Application of an inactive p38 MAPK inhibitor analogue (SB202474) up to a concentration of 25 μM had no influence on MMP-9 mRNA expression or protein secretion (data not shown). These findings suggest that p38 activ-

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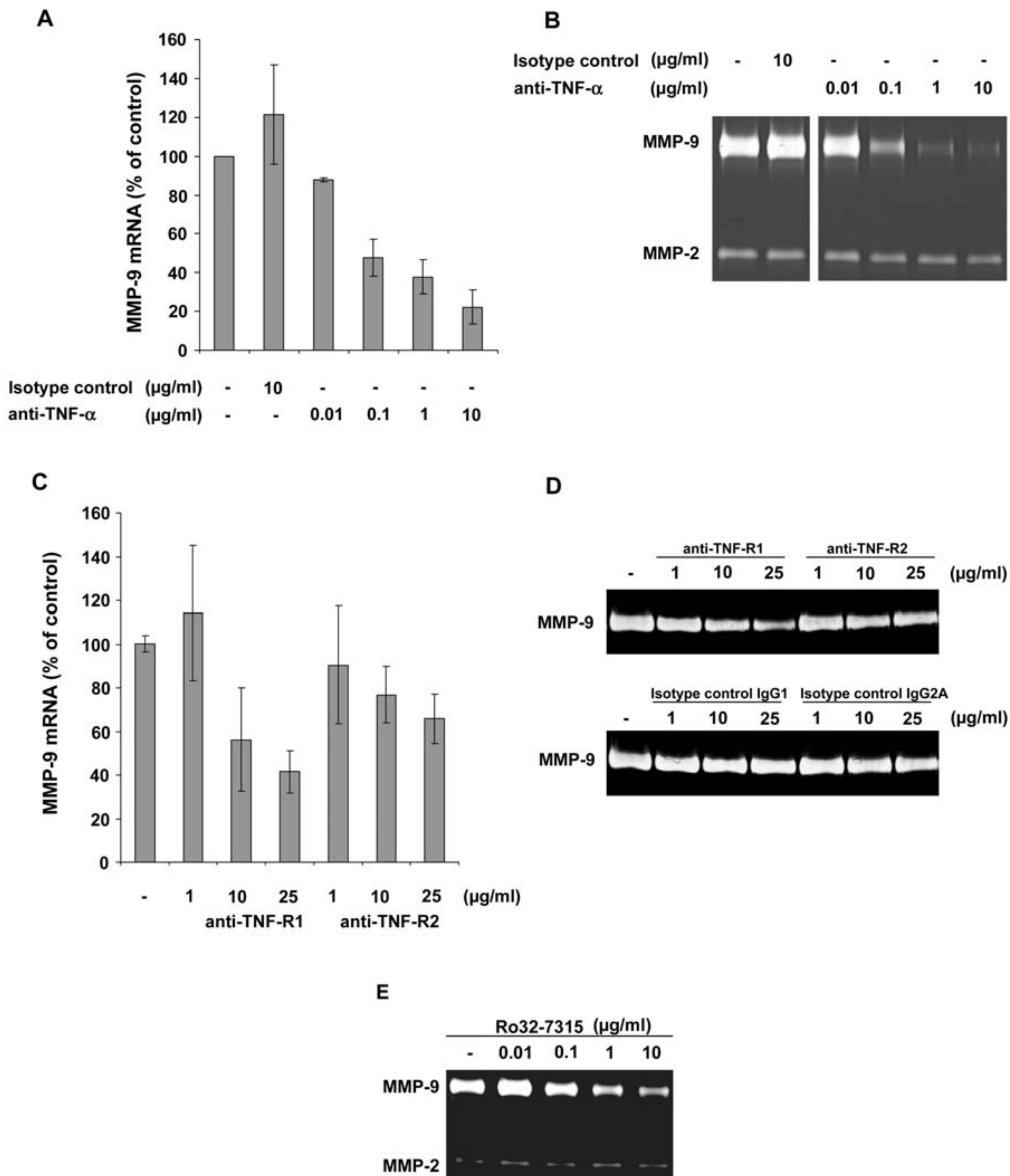

Figure 1 Neutralizing antibodies against TNF-α and TNF-R1 downregulate MMP-9 synthesis and secretion.
THP-1 cells were incubated for 24 h under serum-free conditions in the presence and absence of different concentrations of antibodies that (A,B) neutralize soluble TNF-α or (C,D) block TNF-R1 and TNF-R2. (A,C) MMP-9 mRNA was quantified by qRT-PCR and (B,D) culture supernatants were examined for MMP-9 secretion by zymography. Results of mRNA quantification (A,C) are expressed as a percentage of the unstimulated control normalized to cyclophilin B and represent one single experiment representative of three determinations, each performed in triplicate. (E) Cells were incubated with different concentrations of the TACE inhibitor Ro 32-7315 under serum-free conditions. Conditioned media were collected after 24 h and analyzed by zymography.

ity has a negative regulatory effect on the constitutive MMP-9 production in THP-1 cells.

Inhibition of p38 upregulates MMP-9 expression by increasing the phosphorylation of ERK1/2

Our results suggest a differential role of ERK1/2 and p38 MAPK activity in the regulation of MMP-9 expression. To examine the effect of simultaneous inhibition of both kinases, we added the MEK1/2 inhibitor PD98059 and the p38 inhibitor SB202190 at optimal effective concentrations of 25 μM to the cells. Zymographic analysis of conditioned media revealed that the combined application of these inhibitors resulted in abrogation of MMP-9 secretion similar to the effect of ERK1/2 inhibition alone (Figure 4A). These results suggest that ERK1/2 activity is required for the stimulatory effect on MMP-9 expression achieved by inhibition of p38 activity.

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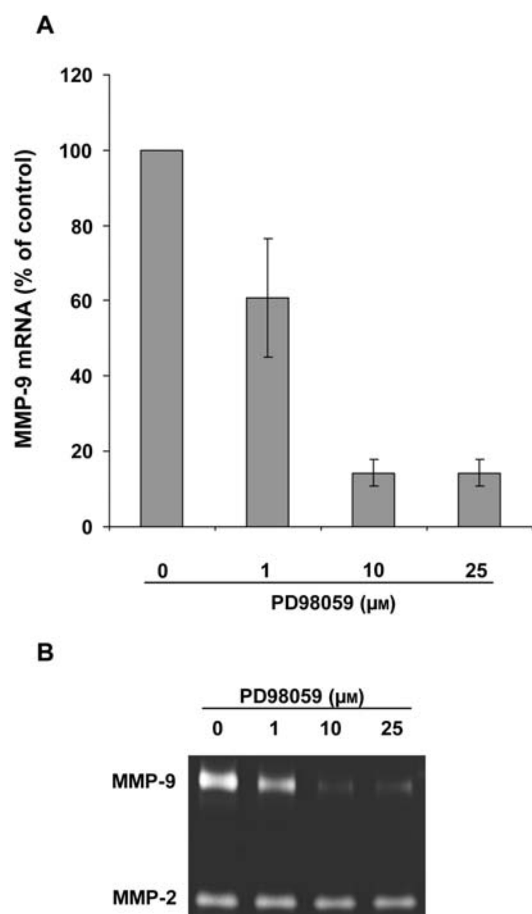

Figure 2 Inhibition of MEK1/2 blocks basal MMP-9 expression and secretion.
THP-1 cells were incubated with different concentrations of PD98059 for 24 h. (A) MMP-9 mRNA expression was analyzed by qRT-PCR and the results are expressed as a percentage of the unstimulated control normalized to cyclophilin B. Data represent one of three independent experiments, each performed in triplicate. (B) Cell culture supernatants were analyzed by zymography.

To verify our hypothesis of potential interactions between the two pathways, we examined the level of ERK1/2 phosphorylation after incubating the cells with the p38 inhibitor SB202190. Western blotting analysis of cell lysates showed a significant increase in the phosphorylation of ERK1/2 within a 15–60-min period after treatment with the p38 inhibitor (Figure 4B, upper panel). To confirm that equal amounts of protein were loaded in each lane, we applied antibodies that detected total ERK1/2, both the phosphorylated and the non-phosphorylated protein (Figure 4B, lower panel). These data indicate that stimulation of MMP-9 production induced by inhibition of p38 is mediated via activation of ERK1/2.

Inhibition of the JNK pathway upregulates constitutive MMP-9 expression alone and in combination with inhibition of the p38 pathway

To investigate a potential role of the JNK pathway in autocrine TNF-α-stimulated MMP-9 expression, THP-1 cells were incubated with the water-soluble cell-permeable inhibitor peptide (L)-JNK-I1 that specifically blocks JNK activity. As a control, cells were also incubated with a non-inhibitory peptide analog. Inhibition of JNK activity led to dose-dependent stimulation of MMP-9 mRNA expression and enzyme secretion. MMP-9 mRNA expression was enhanced by approximately three-fold at an inhibitor concentration of 10 μM and ~4.5-fold at an inhibitor concentration of 25 μM (Figure 5A). Consistent with the mRNA expression, MMP-9 release was augmented to similar extents in the presence of 10 and 25 μM (L)-JNK-I1, as determined by zymography (Figure 5B). The control peptide exhibited no effect on MMP-9 mRNA or protein at a maximum concentration of 25 μM (Figure 5A,B). To assess if JNK inhibition did influence ERK1/2 activity, cells were treated with L-JNK-I1 and examined for ERK1/2 phosphorylation. Analysis of cell extracts by Western blotting revealed that blockage of JNK enhanced phosphorylation of ERK1/2 (Figure 5C).

To clarify whether p38 and JNK cooperate in their stimulating influence on MMP-9 production, THP-1 cells were treated with the respective inhibitors SB202190 and L-JNK-I1 alone and in combination at a concentration of 10 μM. Analysis of MMP-9 mRNA expression and enzyme release showed that combined application of inhibitors for p38 and JNK enhanced MMP-9 biosynthesis and secretion in an additive manner without affecting MMP-2 release (Figure 6A,B).

Exogenous addition of TNF-α stimulates MMP-9 expression by activation of the ERK1/2 signal transduction pathway

To examine the implication of MAPK pathways in soluble TNF-α-initiated signaling and induction of MMP-9 expression, THP-1 cells were preincubated with or without the MEK1/2 inhibitor PD98059 and subsequently stimulated by exogenous addition of 50 ng/ml TNF-α. Western blotting analysis of cell extracts showed that in the absence of MEK1/2 inhibitor, TNF-α leads to enhanced phosphorylation of ERK1/2 and p38 as early as 5 min after treatment, which persisted for at least 60 min (Figure 7A, upper panel). Preincubation with PD98059 prevented TNF-α-stimulated activation of ERK1/2, but had no effect on p38 phosphorylation (Figure 7A, upper panel). To control application of equal amounts of protein in the samples, total ERK1/2 was also measured (Figure 7A, lower panel). Analysis of MMP-9 synthesis demonstrated that incubation of THP-1 cells with TNF-α for 24 h significantly stimulated MMP-9 mRNA expression and protein secretion, which was highly significantly blocked upon preincubation of the cells with PD98059 (Figure 7B,C). Taken together, our data demonstrate the importance of the ERK1/2 signaling pathway for enhanced MMP-9 expression upon exogenous stimulation of THP-1 cells with TNF-α.

Discussion

Based on their central roles in regulating growth and survival of cells, the growth factor-mediated MAPK signaling pathways have become interesting targets for the development of clinical therapies. For the treatment of cancer, compounds that block MMP gene transcription by inhi-

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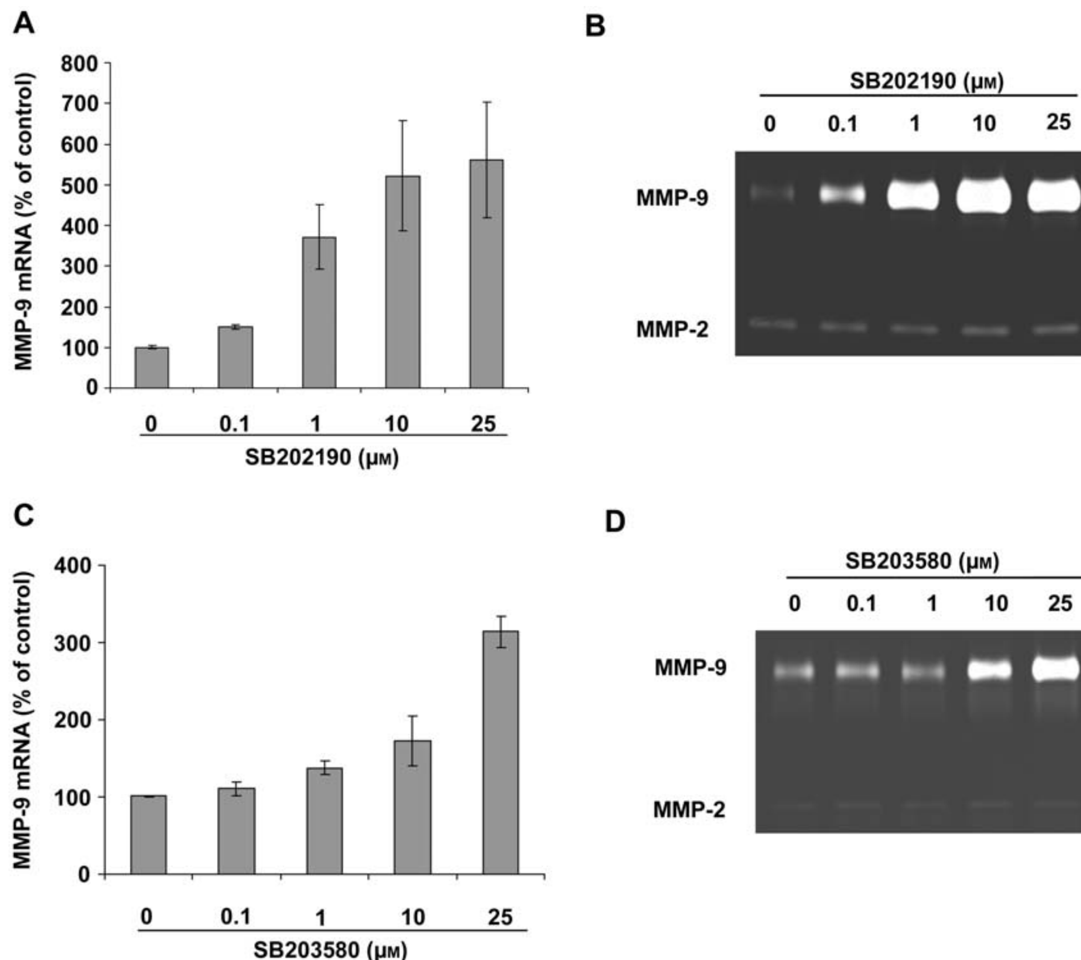

Figure 3 Inhibitors of p38 enhance MMP-9 production.
THP-1 cells were treated with different concentrations of the p38 inhibitors (A,B) SB202190 or (C,D) SB203580. (A,C) MMP-9 mRNA was quantified by qRT-PCR. The data represent one of three independent experiments, each performed in triplicate. Results are shown as a percentage of the unstimulated control and normalized to cyclophilin B. (B,D) Secretion of gelatinases into the cell culture supernatants was monitored by zymography.

bition of MAPKs have been proposed as potential antitumor agents (Overall and Lopez-Otin, 2002) and several MAPK inhibitors have already entered clinical cancer trials (Sebolt-Leopold and Herrera, 2004). Since MMP-9 is known as a key player in the pathophysiology of tumor progression (Coussens et al., 2000), the purpose of this study was to analyze the role of major MAPKs on the cellular expression and secretion of this enzyme. We have shown in monocytic leukemia cells that inhibition of the ERK1/2 pathway blocks autocrine and paracrine TNF-α-induced MMP-9 production, whereas blockage of p38 and JNK lead to an augmentation of MMP-9 synthesis and secretion. Our data support the utility of inhibitors against the ERK1/2 pathway to target MMP-9 for therapeutic interventions and suggest that blocking of other MAPK pathways should be critically examined to avoid potential opposite effects.

It has been shown that inflammatory mechanisms such as macrophage infiltration and TNF-α release are correlated with MMP-9 expression, angiogenesis, and tumor growth (Coussens et al., 2000; Coussens and Werb, 2002). Moreover, the primary source of MMP-9 in many malignant tumors are inflammatory or stromal cells, including monocytes/macrophages, neutrophils, and endothelial cells (Egeblad and Werb, 2002). It can be hypothesized that targeting MMP-9 production by inhibition of specific MAPK pathways could represent a possible mechanism to suppress tumor growth and angiogenesis. In our study, we used THP-1 monocytic cells, characterized by constitutive release of MMP-9, as a model system to examine the role of MAPK in MMP-9 gene expression. We found that continuous secretion of MMP-9 in these cells was mediated by autocrine TNF-α stimulation via TNF-R1, consistent with previous results obtained in HL-60 and NB4 leukemic cells (Ries et al., 1994; Ismair et al., 1998). The importance of released TNF-α in this context was also shown by others using the unspecific metalloproteinase inhibitor Marimastat (Robinson et al., 2002). Applying the specific TACE inhibitor Ro32-7315 (Beck et al., 2002), we demonstrated that TACE activity is essential for autocrine TNF-α-stimulated MMP-9 expression. Slight impairment of MMP-9 gene expression was also observed upon blocking of TNF-R2, which may be caused by 'ligand passing' from TNF-R2 to TNF-R1.

Various cytokines and growth factors are known to activate the ERK1/2 pathway, which controls multiple cellular processes and is frequently found to be active in

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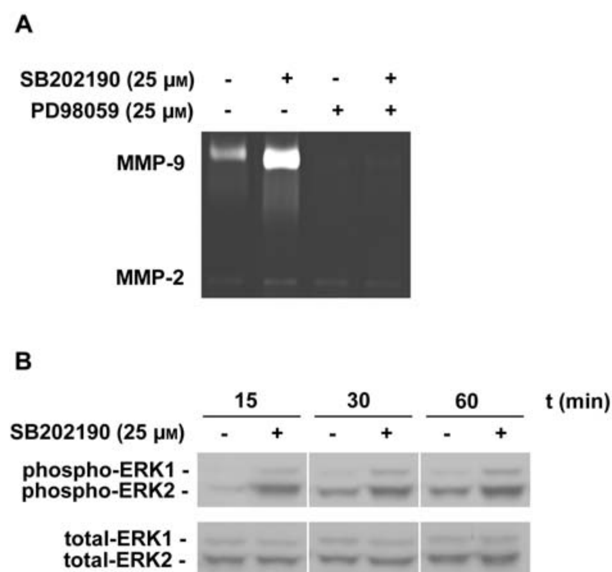

Figure 4 The p38 blockage-stimulated MMP-9 expression depends on phosphorylation of ERK1/2.
(A) THP-1 cells were incubated with or without 25 μM of the MEK1/2 inhibitor PD98059 and the p38 inhibitor SB202190, alone or in combination. Conditioned media were collected and gelatinolytic activity was analyzed by zymography. (B) Phospho-ERK1/2 and total ERK1/2 were examined by Western blotting of cell extracts after treatment of the cells with 25 μM SB202190 for different time intervals.

tumor cells (Giehl, 2005; Rennefahrt et al., 2005). As we have demonstrated here, inhibition of the ERK1/2 pathway in THP-1 leukemic cells caused a marked decrease in continuous MMP-9 production, which was maintained by endogenous TNF-α. Likewise, we investigated HL-60 and NB4 leukemic cells, which are also known to secrete MMP-9, by autocrine stimulation with TNF-α (Ries et al., 1994; Ismair et al., 1998). In agreement with our findings in THP-1 cells, blockage of MEK1/2 activity also down-regulated constitutive MMP-9 production in both HL-60 and NB4 cells (data not shown in the results section to avoid redundancy). Furthermore, we demonstrated here that stimulation of THP-1 cells with exogenous TNF-α significantly upregulated MMP-9 synthesis and release by enhanced activity of ERK1/2. Taken together, these findings indicate that the ERK1/2 pathway plays a central role in TNF-α-induced MMP-9 expression under both autocrine and paracrine stimulatory conditions.

Whereas knowledge about the contribution of the ERK1/2 pathway in TNF-α-initiated gene expression is limited, abundant information is available on the involvement of NF-κB in this process (MacEwan, 2002; Sanceau et al., 2002). Recently it was reported that ERK1/2 mediates TNF-α-stimulated MMP-9 expression via NF-κB activity in human smooth muscle cells (Moon et al., 2004). Our own unpublished results in THP-1 cells, however, gave no clear evidence for the implication of NF-κB in autocrine TNF-α-induced MMP-9 expression, although the exact role of NF-κB remains to be resolved in detail. So far, our findings clearly demonstrate that TNF-α-reg-

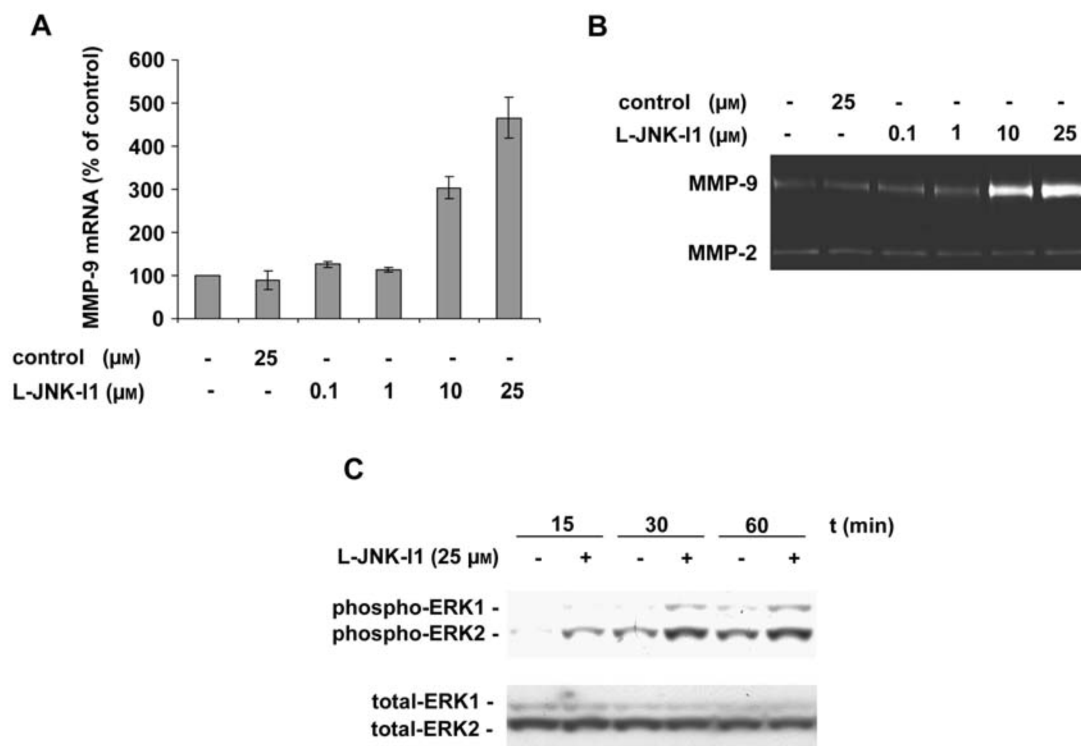

Figure 5 Inhibition of JNK augments MMP-9 production and phosphorylation of ERK1/2.
THP-1 cells were incubated for 24 h with or without different concentrations of the JNK inhibitor L-JNK-I1 or 25 μM of a control peptide. (A) MMP-9 mRNA was monitored by qRT-PCR. The data were normalized to cyclophilin B and are expressed as a percentage relative to the unstimulated control. Results reflect one of three independent experiments performed in triplicate. (B) Cell culture supernatants were analyzed by zymography. (C) Cells were treated with 25 μM L-JNK-I1 or the control peptide and lysed after different time intervals. Phosphorylation and total amount of ERK1/2 were analyzed by Western blotting.

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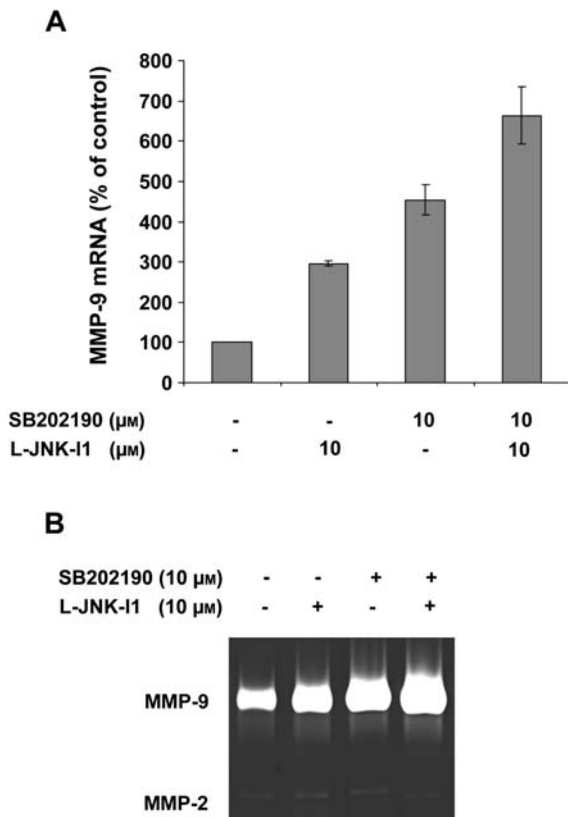

Figure 6 Combined blockage of p38 and JNK upregulates MMP-9 production in an additive manner.
THP-1 cells were incubated for 24 h in the absence or presence of the p38 kinase inhibitor SB202190 and the JNK inhibitor L-JNK-I1 alone or in combination. (A) MMP-9 mRNA was quantified by qRT-PCR. The results are expressed as a percentage of the unstimulated control and normalized to cyclophilin B. Data represent one of three independent experiments, each performed in triplicate. (B) Cell culture supernatants were analyzed by zymography.

ulated MMP-9 expression in THP-1 cells is essentially mediated via ERK1/2 signaling, which is in agreement with data obtained in endothelial cells (Genersch et al., 2000), keratinocytes (Holvoet et al., 2003), rat astrocytes (Arai et al., 2003), and vascular smooth muscle cells (Moon et al., 2004).

Besides ERK1/2, the JNK and p38 MAPK pathways are typically activated by TNF-α, thus contributing to various inflammatory pathophysiologies (Baud and Karin, 2001). Recent data have also suggested a negative regulatory effect of p38 on the ERK1/2 pathway in THP-1 cells cultivated in serum-containing medium (Numazawa et al., 2003). When we blocked p38 activity in THP-1 cells under serum-free conditions, we also observed enhanced ERK1/2 phosphorylation, which resulted in significant upregulation of MMP-9 gene expression and protein secretion. These findings indicate that p38 acts as a negative regulator of MMP-9 expression by influencing ERK1/2 activity in a reciprocal manner, whereas constitutive MMP-2 expression and release was not influenced at all. As shown in isolated primary monocytes, lipopolysaccharide (LPS)-stimulated MMP-9 expression was repressed by inhibition of the ERK1/2 pathway, while blocking of p38 resulted in enhanced ERK1/2 phosphorylation and MMP-9 expression (Lai et al., 2003). This is in agreement with our findings in THP-1 leukemic monocytic cells, demonstrating the pivotal role of ERK1/2 in MMP-9 expression and its regulation via p38 MAPK cross-talk. Moreover, in accordance with our findings on MMP-9, previous work has also demonstrated a negative effect of p38 activity via ERK1/2 on MMP-1 and MMP-3 expression in different cell systems (New et al., 2001; Endo et al., 2003). Thus, taken together, this cross-talk seems to represent a common phenomenon in the regulation of gene expression of certain but not all MMPs. Finally, we were able to demonstrate that JNK activity,

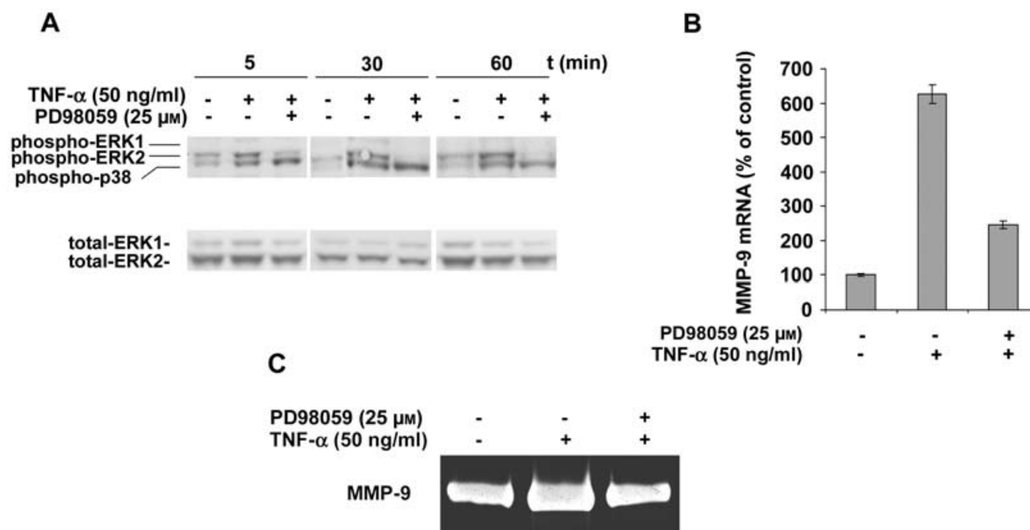

Figure 7 Exogenously added TNF-α enhances MMP-9 expression via activation of ERK1/2.
THP-1 cells were preincubated for 5 min with or without 25 μM PD98059 and then stimulated by addition of 50 ng/ml TNF-α. (A) After different time intervals the cells were lysed and phosphorylation of MAPK was analyzed by Western blotting using antibodies against ERK1/2 in combination with antibodies against p38. (B) mRNA was isolated after 24-h incubation and MMP-9 expression was quantified by RT-PCR. The results are expressed as a percentage of the unstimulated control and normalized to cyclophilin B. Data represent one of three independent experiments, each performed in triplicate. (C) MMP-9 secretion was examined by zymographic analysis of THP-1-conditioned media after a 24-h incubation period.

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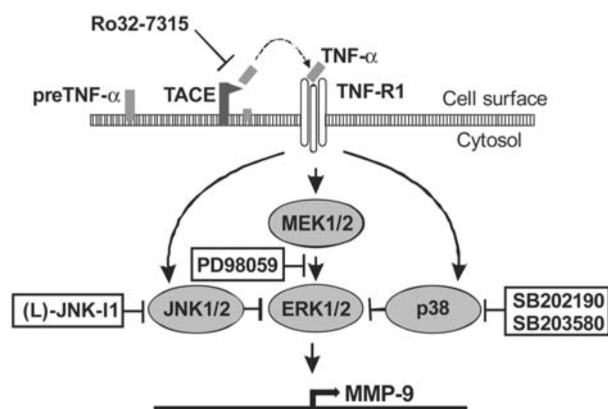

Figure 8 Schematic depiction showing the possible interaction of signal transduction pathways involved in the regulation of autocrine TNF-α-induced MMP-9 synthesis.
Endogenous TNF-α is shed from the cell surface by TACE activity, which can be prevented by addition of a specific TACE-inhibitor (Ro32-7315). Soluble TNF-α binds to TNF-R1, thereby inducing MMP-9 mRNA transcription and protein secretion. Inhibition of MEK1/2 by PD98059 interrupts intracellular signaling via the ERK1/2 pathway and blocks MMP-9 mRNA expression. The kinases p38 and JNK in their activated forms act as negative regulators of MMP-9 transcription by down-modulating ERK1/2 activity. Blockage of either p38 or JNK by specific inhibitors leads to an upregulation of MMP-9 synthesis. Thus, complex cross-talk mechanisms between these MAPK pathways may allow control and fine tuning of TNF-α-stimulated MMP-9 biosynthesis.

similar to p38, negatively controls ERK1/2 phosphorylation and thereby regulates MMP-9 biosynthesis. Interestingly, very recent data also indicated a negative cross-talk by demonstrating that activity of the JNK pathway suppressed ERK1/2 phosphorylation (Rangaswami et al., 2005).

As summarized in the proposed cross-talk model shown in Figure 8, our data clearly demonstrate that inhibition of JNK or p38 upregulates MMP-9 production by stimulating ERK1/2 in THP-1 cells. This indicates that ERK1/2 represents a key regulator for MMP-9 expression in these cells that can be modulated via p38 and JNK, allowing precise regulation of the synthesis and secretion of this enzyme in response to extracellular stimuli.

Although MMPs, particularly MMP-9, have been recognized as a promising target in the treatment of malignant diseases, clinical studies with compounds blocking MMP activity produced disappointing results and novel strategies emerged to inhibit the expression of distinct MMPs (Coussens et al., 2002). Therefore, identification of intracellular signaling cascades responsible for the biosynthesis of individual MMPs represents a major challenge in the development of kinase inhibitors intended for clinical application. According to our data, inhibitors of the ERK1/2 signal transduction pathway may be useful for blocking MMP-9 expression in malignant diseases, whereas inhibitors of p38 or JNK pathway might evoke unwanted effects by inducing MMP-9 upregulation. The latter aspect is of major importance, since compounds that block p38 or JNK have already entered clinical trials for treatment of malignant tumors or inflammatory diseases (Kumar et al., 2003; Bogoyevitch et al., 2004).

Materials and methods

Antibodies and reagents

Monoclonal neutralizing antibodies against TNF-α, TNF-R1, TNF-R2, and control antibodies IgG_1 and IgG_{2A} were purchased from R&D Systems (Wiesbaden-Nordenstadt, Germany). The following antibodies were used for Western blotting: anti-ERK1/2, anti-phospho-ERK1/2, anti-phospho-p38 and anti-mouse IgG-HRP (all purchased from Cell Signaling Technology Inc., Beverly, MA, USA). Soluble TNF-α was obtained from PAN systems (Nürnberg, Germany). Inhibitors used were as follows: SB203580, SB202190, SB202474, PD98059, cell-permeable JNK-inhibitor (L-form), negative control for cell-permeable JNK-inhibitor (L-form), (all from Calbiochem, Bad Soden, Germany). The TACE inhibitor Ro32-7315 was kindly provided by Dr. H.W. Krell (Roche Diagnostics, Penzberg, Germany). Stock solutions of SB203580, SB202190, SB20474 and PD98059 were prepared in dimethylsulfoxide (DMSO). All other compounds were dissolved in culture medium.

Cell culture and treatment of cells

THP-1 monocytic cells originally derived from a patient with acute monocytic leukemia were obtained from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). The cells were cultured in RPMI-1640 supplemented with 10% fetal calf serum (FCS) (PAA Laboratories, Pasching, Austria), 100 U/ml penicillin-streptomycin 100 μg/ml, and 2 mM of glutamine (Biochrom AG, Berlin, Germany) at 37°C under a humidified atmosphere of 5% CO_2. For all experiments, the cells were centrifuged, washed three times with PBS and resuspended in serum-free RPMI-1640 supplemented with 1% Nutridoma SP (Roche, Mannheim, Germany). Thereafter, the cells were counted using a Neubauer chamber and adjusted to a concentration of 1×10^6 /ml. Cells were left untreated or treated with concentrations of antibodies, MAPK inhibitors or the respective control molecules, as indicated in the corresponding figures, followed by incubation for 24 h.

Preparation of cell extracts and Western blotting

Cells were lysed in lithium dodecyl sulfate containing buffer (NuPAGE™ LDS sample buffer 4×) (Invitrogen GmbH, Karlsruhe, Germany) and then sonicated on ice for 15 s using a B-12 Branson sonifier (Danbury, CT, USA) fitted with a microtip at an intensity of 7 on the output control. The cell lysates were heated for 5 min at 99°C and subjected to SDS-PAGE on a precast 4–12% Bis-Tris polyacrylamide gel (Invitrogen). See-Blue™ prestained standard and Magic Mark™ (Invitrogen) were used for assessment of Western blotting transfer efficiency and for estimation of molecular masses, respectively. Proteins were blotted onto PVDF membranes (Millipore, Schwalbach, Germany) for 60 min at 250 mA and a maximum of 25 V. After transfer, the membranes were blocked in TBS blocking buffer (137 mM NaCl, 20 mM Tris, pH 7.6) containing 0.1% Tween-20 and 5% non-fat dry milk powder (Roth, Karlsruhe, Germany) for 1 h before incubation with primary antibodies at a concentration of 0.5 μg/ml for 24 h.

The membranes were then washed in TBS containing 0.1% Tween-20 (TBS-Tween buffer) and incubated with horseradish peroxidase-conjugated secondary antibodies at a dilution of 1:1000 in TBS-Tween buffer for 15 min. Detection of bound antibodies was accomplished using substrate enhanced chemoluminescence reagent (ECL system, Amersham Bioscience, Little Chalfont, UK). For sequential detection of different antigens, antibodies were removed from the blot by incubating the membrane in stripping buffer (62.5 mM Tris-HCl, 2% SDS, and

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100 mM 2-mercaptoethanol, pH 6.7) for 45 min at 50°C, with subsequent washing in TBS-Tween buffer.

Gelatin zymography

Cell culture supernatants were analyzed for the presence of gelatinolytic enzymes by zymographic analysis using precast 10% polyacrylamide minigels containing 0.1% gelatin as substrate (Invitrogen). The samples were mixed 1:1 with a non-reducing buffer containing 126 mM Tris-HCl, 20% glycerol, 4% SDS and 0.005% bromophenol blue (Invitrogen). As a marker for electrophoretic mobility of gelatinases in zymograms, we used conditioned medium from PMA-treated HT1080 fibrosarcoma cells (Moll et al., 1990) containing the latent form of MMP-9 (ca. 92 kDa), the latent form of MMP-2 (ca. 72 kDa) and the activated form of MMP-2 (ca. 66 kDa).

After electrophoresis, gels were washed twice for 15 min in 2.7% Triton X-100 on a rotary shaker to remove SDS and to allow proteins to renature. The gels were then incubated in a buffer containing 50 mM Tris, pH 7.5, 200 mM NaCl, 5 mM $CaCl_2$ and 0.2% Brij35 (Invitrogen) for 18 h at 37°C. The zymograms were stained for 90 min with 0.02% Coomassie Blue R-350 in a 30% methanol/10% acetic acid solution using PhastGel-Blue-R tablets (Amersham Biosciences, Freiburg, Germany). Zymograms were scanned using a Umax ImageScanner driven by MagicScan software (Amersham Biosciences).

RNA isolation, reverse transcription polymerase chain reaction (RT-PCR) and real-time quantitative RT-PCR (qRT-PCR)

Total RNA from differently treated or untreated cells was extracted using the RNeasy mini kit (Qiagen, Hilden, Germany). Samples of 1 μg of purified RNA were reverse transcribed using the First Strand cDNA Synthesis Kit for RT-PCR (AMV) (Roche) according to the manufacturer's instructions. Reverse transcription was performed for 60 min at 42°C, followed by incubation at 99°C for 5 min to avoid interference with subsequent reactions. For all experiments we used Lightcycler™-FastStart DNA Master SYBR Green I (Roche). Standards and primers were all purchased from Search LC (Heidelberg, Germany). The qRT-PCR was performed on a LightCycler system (Roche). PCR conditions were 95°C for 10 min, followed by 35 cycles consisting of 95°C for 10 s, 58°C for 10 s (secondary target temperature), 68°C for 10 s (target temperature) and 72°C for 16 s. RNA levels of target genes were normalized by the level of the housekeeping gene cyclophilin B, used as an endogenous control to correct the efficiency of the RT-PCR. Results are expressed as a percentage of the untreated control.

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