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Sustained ERK phosphorylation is necessary but not sufficient for MMP-9 regulation in endothelial cells: involvement of Ras-dependent and -independent pathways

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SUMMARY

Endothelial expression of matrix metalloproteinase-9 (MMP-9), which degrades native type IV collagen, was implicated as a prerequisite for angiogenesis. Therefore, the aim of this study was to determine signaling requirements that regulate MMP-9 expression in endothelial cells. Both, primary and permanent human umbilical vein endothelial cells (HUVEC and ECV304, respectively) were stimulated with phorbol 12-myristate 13-acetate (PMA) and the cytokine tumor necrosis factor-α (TNFα) to induce MMP-9 expression. While both cell types responded to PMA at the protein, mRNA and promoter level by induction of MMP-9, TNFα caused this response only in ECV304. Inhibitors specific for mitogen-activated protein/ERK kinase 1/2 (MEK1/2), protein kinase C (PKC), and Ras and co-transfections of wild-type and mutant Raf were used to elucidate the signaling cascades involved. Thus, we could show that the Raf/MEK/ERK cascade is mainly responsible for MMP-9 induction in endothelial cells and that this cascade is regulated independently of PKC and Ras subsequent to TNFα stimulation and in a PKC-dependent manner as a result of PMA treatment. In addition, PMA triggers a Ras-dependent signal transduction pathway bypassing the phosphorylation of ERK. Finally, we provide evidence that sustained phosphorylation of ERK1/2 is necessary but not sufficient for expression of MMP-9.

Key words: ECV304, HUVEC, Regulation of MMP-9, Signal transduction

INTRODUCTION

Angiogenesis, the formation of new capillaries from preexisting blood vessels, occurs during various physiological as well as pathological processes such as development, wound healing, tumor growth, and artheriosclerosis. Angiogenesis involves complex signaling events that cause the endothelial cells to acquire a proliferative and migratory phenotype. As a first step in angiogenesis the sprouting endothelial cells have to locally degrade their basement membrane, invade the surrounding interstitial matrix, migrate along different extracellular matrix components, and finally form a new, patent capillary. Matrix metalloproteinases (MMPs) secreted by endothelial cells are hypothesized to play a key role in the processes of matrix remodeling and endothelial cell migration during angiogenesis (Fisher et al., 1994; Schnaper et al., 1993).

MMPs are a family of proteases with diverse substrate specificity, ranging from multiple extracellular matrix components as their primary targets to growth factors, cytokines and other proteinases. MMPs can be divided into four subgroups, the collagenases, the stromelysins, the gelatinases, and the membrane-type MMPs. The combined and strictly regulated action of various MMPs is essential for controlled matrix remodeling. Thus, studies of the mechanisms that regulate expression of MMPs are important for understanding the processes of tissue structure formation and matrix remodeling. Particularly the gelatinases, MMP-2 and MMP-9, the only MMPs capable of degrading native collagen type IV, the major constituent of basement membranes, are involved in vascular cell migration and invasion (Kräling et al., 1999; Puyraimond et al., 1999). Recently, the analysis of homozygous mice with a null mutation in the MMP-9 gene exhibited a defect in growth plate angiogenesis establishing a role for MMP-9 in controlling angiogenesis (Vu et al., 1998).

Tumor necrosis factor-α (TNFα) is a proinflammatory cytokine, which is sometimes also regarded as a growth factor, playing a major role not only in inflammation but also in embryogenesis and in a wide variety of tumors. It is known for its dual and often opposite effects (Ferrari, 1999). It potently inhibits accumulation of extracellular matrix by

stimulating degradation of connective tissue components. In fibroblasts, TNFα induces the expression of MMP-1, MMP-3 (Brenner et al., 1989; MacNaul et al., 1990), and MMP-9 (Singer et al., 1999). MMP-9 is also induced by TNFα in keratinocytes (Makela et al., 1998) and endothelial cells (Hanemaaijer et al., 1993; Mackay et al., 1992; Nelimarkka et al., 1998). The cellular effects of TNFα are mediated by two distinct cell surface receptors: TNF-RI and TNF-RII, both of which are expressed by endothelial cells (Introna and Mantovani, 1997).

A major mechanism through which signals from extracellular stimuli are transmitted to the nucleus involves activation of kinases related to the mitogen-activated protein kinase (MAPK) superfamily (for review see Robinson and Cobb, 1997). To date, the involvement of at least three subgroups of MAPK family members have been identified in a wide range of cellular responses to extracellular signals. The enzymes in the first subgroup, named extracellular signal-regulated kinases (ERKs), are activated through sequential phosphorylation of the upstream kinases Raf and MEK. Activation of Raf, in turn, is achieved through its interaction with membrane bound farnesylated Ras. The classical Raf/MEK/ERK mitogenic cascade is strongly activated upon stimulation of cells with growth factors, serum, and phorbol esters like phorbol 12-myristate 13-acetate (PMA). For the other two MAPK subgroups, c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK) and p38MAPK, homologous signal transduction pathways have been described. These latter two subgroups of the MAPK family are only weakly activated by mitogens, but are highly stimulated on exposure to inflammatory cytokines such as TNFα and IL-1 and a wide variety of environmental stress inducers.

Several reports have examined the signal transduction pathways responsible for MMP regulation in fibroblasts, keratinocytes, and tumor cells suggesting involvement of different signaling cascades depending on stimulus, cell type, and MMP isoform (Gum et al., 1997; McCawley et al., 1999; Reunanen et al., 1998). Far less is known about the signal transduction pathways and transcription factors that regulate endothelial MMP gene regulation. The postulated role of MMP-9 in angiogenesis therefore urged for a characterization of the signal transduction pathways involved in the induction of MMP-9 in endothelial cells.

Here we unravel for the first time the signal transduction pathways that activate MMP-9 expression in endothelial cells. We show that TNFα is able to induce MMP-9 expression in ECV304, a spontaneously immortalized HUVEC line (Takahashi et al., 1990), whereas primary HUVEC failed to respond to TNFα with synthesis of MMP-9. We demonstrate that Raf, MEK1/2, and ERK1/2 are involved in endothelial regulation of MMP-9 expression in response to TNFα as well as PMA, and that a sustained initial phosphorylation of ERK1/2 is necessary but not sufficient for MMP-9 induction. This is, therefore, the first report showing TNFα stimulation being transmitted through sustained activity of ERK, although cytokines are known to act mainly via JNK/SAPK or p38MAPK pathways. Furthermore, we provide evidence that the ERK phosphorylation after PMA but not after TNFα stimulation is PKC-dependent and that Ras signaling is involved in PMA but not in TNFα induction of MMP-9 expression.

MATERIALS AND METHODS

Reagents

Phorbol-12-myristate-13-acetate (PMA) and protein kinase inhibitors Ro31-8220, PD98059, and SB203580 were obtained from Calbiochem. Cerivastatin® was purchased from Bayer, geranylgeranylpyrophosphate and farnesylpyrophosphate from Sigma. Human tumor necrosis factor-α (TNFα) was obtained from Roche. The following human growth factors were from Biomol: acidic fibroblast growth factor (aFGF), basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF), epithelial growth factor (EGF), platelet-derived growth factor AA (PDGF AA), and platelet-derived growth factor BB (PDGF BB). Polyclonal antibodies specific for ERK1 and ERK2 and for the phosphorylated isoforms of ERK1 and ERK2 were obtained from New England Biolabs. Expression vectors for wild-type c-Raf-1, dominant negative mutant Raf-1, and constitutively active mutant Raf-1 were a generous gift from S. Ludwig (Ludwig et al., 1996).

Cell culture

The human endothelial cell line ECV304 was obtained from the European Collection of Cell Cultures (ECACC). Human umbilical vein endothelial cells (HUVEC) were isolated from umbilical cords by chymotrypsin treatment as previously described (Haller et al., 1994; Haller et al., 1996). Briefly, the cords were cleaned with isotonic NaCl at room temperature and incubated for 25 minutes at 37°C with 1% chymotrypsin in PBS. Endothelial cells were collected by centrifugation (400 ***g*** for 10 minutes), and the pellet was resuspended in EGM (Clonetics) with 2% fetal bovine serum (PAA Laboratories GmbH) and 0.4% bovine brain extract (Clonetics). Primary cells were grown for 3 to 4 days and then subcultured. Subcultures 3 to 4 were used for the experiments. ECV304 and HUVEC were routinely grown on gelatin-coated plastic in M199 medium (Biochrom KG) containing 5% fetal calf serum (PAA Laboratories GmbH) and in EGM containing 2% fetal bovine serum and 0.4% bovine brain extract, respectively. Prior to stimulation, ECV304 and HUVEC were starved for 16 hours in serum-free M199 containing 5% lactalbumin hydrolysate (Gibco Life Technologies), 10 μg/ml transferrin (Sigma) and in EGM without supplements, respectively. The following concentrations of PMA, TNFα, and growth factors were used for stimulation: PMA 50 nM, TNFα 25 ng/ml, aFGF 5 ng/ml, bFGF 5 ng/ml, VEGF 50 ng/ml, EGF 10 ng/ml, PDGF AA 10 ng/ml, and PDGF BB 10 ng/ml. For further analysis 24 hours following stimulation, conditioned media were collected, cellular protein extracts were prepared or total cellular RNA was extracted.

Zymography

The presence of MMP-2 and MMP-9 in endothelial cell conditioned medium was analyzed by zymography in 10% SDS-polyacrylamide gels containing 1 mg/ml gelatin (Sigma) according to the method of Hibbs (Hibbs et al., 1985). Cells were seeded to confluency (1×10^5 cells/cm^2), allowed to attach and spread for 6 hours in the presence of serum and then maintained in serum-free medium. After 16 hours of starvation cells were stimulated as indicated. The conditioned media were collected 24 hours after stimulation, clarified by centrifugation, normalized to cell number, mixed with non reducing Laemmli sample buffer, heated for 5 minutes at 96°C and subjected to SDS-PAGE. The gels were incubated for 90 minutes at 37°C in 2% Triton X-100 and subsequently for further 16 hours at 37°C in 50 mM Tris-HCl, pH 7.9, 5 mM $CaCl_2$. Finally gels were stained with Coomassie Brilliant Blue R250.

Real-time quantitative RT-PCR (TaqMan analysis)

For analyzing mRNA levels of MMP-9, we developed a real-time quantitative RT-PCR assay using TaqMan methodology (Perkin Elmer). In this system, a one-tube RT-PCR reaction is performed. In the first step, total RNA isolated using the RNeasy MiniKit

(QIAGEN) was transcribed into cDNA using oligo-dT18 primers and MultiScribe® Reverse Transcriptase according to the protocol of Perkin Elmer. The following PCR contained conventional primers (5′-GCTCACCTTCACTCGCGTG and 5′-CGCGACACCAAACTG-GATG, starting at positions 478 and 538, respectively, of the coding sequence of MMP-9, accession number J05070) and a probe (5′-FAMACAGCCGGGACGCAGACATCGTAMRA, starting at position 498 of the coding sequence of MMP-9) labeled with reporter and quencher molecules, FAM and TAMRA, respectively. RT-PCR was performed and samples were analyzed using an ABI Prism 7700 Sequence Detection System (Perkin Elmer). Data were analyzed and validated using the relative standard curve method and the delta delta Ct method as described in the PE Applied Biosystems User Bulletin #2 (1997). During specific amplification the probe is cleaved causing separation of reporter and quencher molecules and emission of fluorescence. Fluorescence intensity increases in direct proportion to the amount of specific amplified product and cycle-by-cycle changes are measured. Results are scored during the exponential phase of the reaction when the fluorescence emission is proportional to the input number of template copies. Data are initially expressed as Ct (threshold cycle) values; the Ct corresponds to the cycle number at which the amplification plot for a given sample crosses the threshold, which is normally set at the point where the fluorescence signal equals 10 times the standard deviation of background fluorescence. Ct values were recorded for serial dilutions of each RNA sample. For quantitative analysis only those experiments were chosen where the Ct values within the dilution series strictly corresponded to the dilution. As control for equal amount of starting material TaqMan analysis with GAPDH primers were run in parallel for each experiment. The relative amount of starting mRNA can be calculated using the fact that each decrease in Ct value by 1 equals a twofold increase in specifically amplified mRNA. Therefore for comparison of different samples run in the same experiment threshold values can be transformed into relative amount of mRNA by the formula 2^(ΔCt), with ΔCt=Ct(unstimulated control) – Ct(sample).

Cloning of the MMP-9 promoter and construction of promoter-reporter gene plasmids

By cloning of PCR products generated from genomic DNA of ECV304 various 5′-end deletions of the MMP-9 promoter were produced. Genomic DNA was purified from ECV304 using the QIAamp Blood Kit (QIAGEN) according to the manufacturer's protocol. Primers were designed according to the MMP-9 promoter sequence (accession number D10051). All PCR products were generated with the same lower primer (5′-TGGTGAGGGCAG-AGGTGTCT-3′) corresponding to positions +20 up to +1. Upper primers used for PCR were as follows: 5′-AAGGCTGTCAGGG-AGGGAAAAAGA-3′ beginning at position –523 in the 5′-flanking region of the MMP-9 gene; 5′-AGAGGAAGCTGAGTCAAAGA-AGGC-3′ beginning at position –540; 5′-CATTCCTTCCGCCC-CCAGAT-3′ beginning at position –571; 5′-TACTGTCCCCTT-TACTGCCCTGAA-3′ beginning at position –657; and 5′-GGGAGGGAGGCTTGGCATAA-3′ beginning at position –1285. PCR products (QIAGEN Taq PCR core kit) were purified by agarose gel electrophoresis and cloned into pGEM-TEasy (Promega) making use of the addition of an extra single deoxyadenosine to the 3′-ends of the amplified fragments. Afterwards MMP-9 promoter fragments were subcloned into pBLCAT5, a promoterless CAT reporter gene vector (Jonat et al., 1990). Promoter fragments were cut out of pGEM-TEasy with *Sph*I and *Sal*I and ligated to pBLCAT5 linearized with *Sph*I and *Sal*I. The correct orientation and sequence fidelity of all plasmids was determined by restriction enzyme digestion and electrophoresis, as well as by sequencing.

Transient transfection and CAT assay

Cells were seeded to confluency (1×10^5cells/cm^2) into 6-well plates and subjected to transient transfection at the next day. Each construct was transfected in triplicate. Plasmids were introduced into the cells using Superfect (QIAGEN) as transfection vehicle according to a modified manufacturer's protocol. Briefly, per each well 2 μg DNA and 6 μl Superfect were added to 60 μl M199 without serum and without antibiotics. The mixture was vortexed vigorously, incubated for 10 minutes at room temperature and then added to the cells in fresh, serum containing medium. The cells were incubated with the complexed DNA for 3 hours, allowed to recover for another 3 hours in serum containing medium and starved in serum-free medium 16 hours prior to stimulation. Transcriptional response as determined by expression of CAT enzyme in extracts of cells transfected with CAT reporter gene plasmids was measured 24 hours after stimulation using a colorimetric ELISA for the quantitative determination of CAT in transfected eukaryotic cells (Roche). The assay was performed essentially according to the manufacturer's protocol where CAT expression is normalized to the amount of extracted cellular protein determined by BCA protein measurement assay (Pierce). Every transfection experiment was performed at least three times. The promoterless control vector pBLCAT5 always gave negative CAT expression values and therefore was unsuitable for quantification and comparison of relative CAT expression in our experiments. Instead, the promoter construct p523GelBCAT, which showed nearly no promoter activity and acted like a minimal promoter in our system, served as reference to quantify our results and was arbitrary set to 1. Therefore in each experiment, the promoter construct p523GelBCAT was transfected to serve as the base-line indicator of CAT-expression, and the expression of each promoter construct is expressed relative to this minimal promoter construct.

Western blotting and immunodetection

For western blotting cells were seeded to confluency (1×10^5 cells/cm^2), stimulated as indicated and total cellular proteins were extracted at the time points given. Cells were washed with ice-cold PBS, scraped in PBS with complete protease inhibitor cocktail (Roche) and 2 mM sodium orthovanadate. After brief centrifugation cells were lysed in lysis buffer (20 mM Tris-HCl, pH 8.0, 138 mM NaCl, 10% glycerol, 2 mM EDTA, 1% Triton X-100, complete protease inhibitor cocktail (Roche), 2 mM sodium orthovanadate) for 30 minutes on ice. Cell debris was removed by centrifugation (20,000 ***g*** for 5 minutes) and protein concentration of the lysate was determined by BCA assay (Pierce). Equal amounts of cell lysates (10 μg protein per lane) were subjected to SDS-PAGE (10% polyacrylamide) under reducing conditions and electroblotted for 2.5 hours at 225 mA onto PVDF membrane (Roche) using 10 mM sodium borate as transfer buffer in a tank blot apparatus. Blocking of the membrane and probing with the indicated antibodies were performed according to the ECL western blotting protocol from Amersham. Reactions with antibodies were visualized using an enhanced chemiluminescence kit and Hyperfilm ECL (both Amersham).

RESULTS

Secretion of MMP-9 in endothelial cells is induced with TNFα and PMA

The proinflammatory cytokine TNFα, the tumor promoter PMA and several growth factors were reported to have differential impacts on the expression of gelatinases (MMP-2 or MMP-9) in several cells (Fabunmi et al., 1996; Kurogi et al., 1996; Lamoreaux et al., 1998; Makela et al., 1998; McCawley et al., 1998; Robbins et al., 1999; Singer et al., 1999). Therefore, we first determined by zymography which external stimuli could induce gelatinase synthesis in endothelial cells. ECV304, a spontaneously immortalized

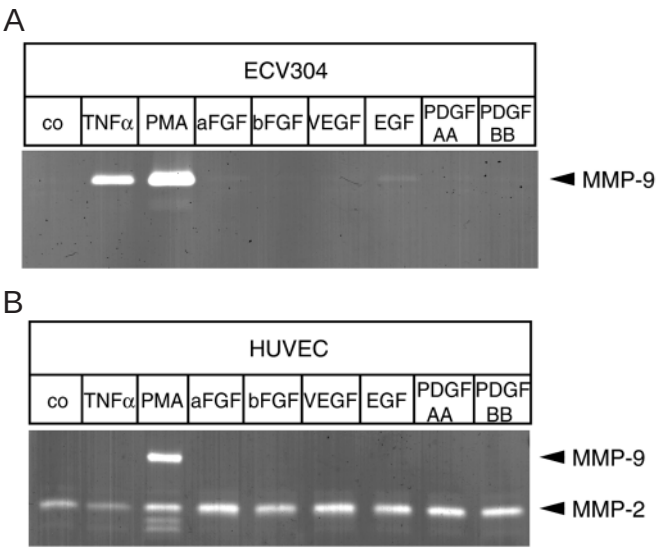

Fig. 1. Induction of MMP-9 secretion in ECV304 and HUVEC. Zymography of serum-free conditioned medium of ECV304 (A) and HUVEC (B). ECV304 and HUVEC were seeded to confluency (1×10^5 cells/cm^2). For stimulation, the cells were incubated with TNFα (25 ng/ml), PMA (50 nM), aFGF (5 ng/ml), bFGF (5 ng/ml), VEGF (50 ng/ml), EGF (10 ng/ml), PDGF AA (10 ng/ml), and PDGF BB (10 ng/ml), or left untreated as a control. Conditioned media were collected after 24 hours, normalized to cell number, and aliquots corresponding to equal amounts of cells were subjected to gelatin zymography. The positions of gelatinases MMP-2 and MMP-9 are indicated.

HUVEC line (Takahashi et al., 1990), and subcultured primary HUVEC were exposed to a panel of extracellular stimuli (TNFα, PMA, aFGF, bFGF, VEGF, EGF, PDGF-AA, PDGF-BB) under serum-free conditions (Fig. 1A,B). Although HUVEC constitutively expressed MMP-2 (72-kDa gelatinase A) no constitutive synthesis of MMP-9 (96-kDa gelatinase B) was detectable in untreated ECV304 or HUVEC. TNFα, described to induce MMP-9 expression in endothelial cells (Mackay et al., 1992; Nelimarkka et al., 1998), unexpectedly had no effect on MMP-9 synthesis in HUVEC. In contrast, marked MMP-9 activity was detectable in the conditioned medium of TNFα-stimulated ECV304. Upon stimulation with PMA, a well known angiogenic promoter (Montesano and Orci, 1985; Montesano and Orci, 1987), both ECV304 and HUVEC showed strong expression of MMP-9. In addition, two faster migrating bands, corresponding to the activated forms of MMP-2 and therefore indicative of activation of membrane-type MMPs (Murphy et al., 1999), were seen with HUVEC after PMA stimulation. Except for EGF, which sometimes gave rise to a very faint MMP-9 activity in ECV304, none of the other growth factors tested had an effect on MMP-9 expression in both cell types. These data demonstrate that synthesis of MMP-9 is inducible by TNFα only in ECV304, but in both cell types by PMA.

Transcription of MMP-9 in endothelial cells is induced with TNFα and PMA

We next analyzed the steady state levels of MMP-9 mRNA as a result of TNFα and PMA stimulation by real-time quantitative RT-PCR. In Fig. 2A amplification plots of MMP-9 mRNA are shown for control and PMA or TNFα stimulated endothelial cells. Fluorescence signals generated during each PCR cycle were plotted as relative fluorescence intensity against the number of cycles. The maximal relative fluorescence intensity is related to the amount of specific mRNA copies present in the starting material. Quantification and comparison of results (Fig. 2B) were performed as described in Materials and Methods.

In accordance with our data on protein level, no MMP-9 mRNA was detected in untreated HUVEC and ECV304. Specific induction of MMP-9 mRNA expression was achieved in both cell types by stimulating the cells with PMA, whereas TNFα strongly induced MMP-9 expression only in ECV304 (Fig. 2A). Identical curves generated with primers specific for GAPDH (Fig. 2A, insets) for all RNAs used proved that the amount of total RNA was equal in all reactions. Quantification of the data (Fig. 2B) revealed that PMA caused an increase in the abundance of MMP-9 mRNA in the range of 37,000- and 15,000-fold over control in ECV304 and HUVEC, respectively. TNFα resulted in a 1,700-fold increase of MMP-9 mRNA over controls in ECV304 and had almost no effect in HUVEC. In these cells MMP-9 mRNA was elevated only 3.5-fold substantiating the finding that TNFα is not able to induce MMP-9 expression in HUVEC. Hence, real-time quantitative RT-PCR analysis showed that markedly enhanced levels of MMP-9 mRNA correlate well with the demonstration of secreted protein. Since in control cells no MMP-9 mRNA was detectable, mRNA levels observed after stimulation cannot be explained solely by altered mRNA stability. Therefore, the differential stimulation of MMP-9 expression by TNFα and PMA must be due to a transcriptional upregulation of the MMP-9 gene.

Synergistic action of several transcription factors regulates expression of MMP-9

Transient transfection assays of various promoter deletion constructs were used to determine the cis-acting elements mediating TNFα and PMA induced up-regulation of MMP-9 gene expression in endothelial cells. Starting with the construct p1285GelBCAT, in which a 1285 bp human MMP-9 promoter segment was linked to the promoterless CAT reporter gene, we made progressive 5′-end deletion mutants of the MMP-9 promoter down to 523 bp (p523GelBCAT). MMP-9 promoter/CAT constructs were transiently transfected into ECV304 and HUVEC (Fig. 3). In unstimulated control cells, all transfected promoter constructs showed equally no or only weak activity. Treatment of cells transiently transfected with p1285GelBCAT with PMA potently (51-fold for ECV304, 45-fold for HUVEC) enhanced the activity of this promoter construct. In ECV304 as well as HUVEC a gradual decrease in PMA-stimulated promoter activity from p1285GelBCAT down to p540GelBCAT was detected. In general the same pattern of promoter activity was observed when the cells were stimulated with TNFα although overall promoter activity was lower than with PMA as were levels of mRNA and secreted protein. Therefore, regulation seem to involve synergistic action of trans-acting factors like NF-κB, Sp1, and PEA3/AP-1, binding at positions −600, −558, and −540/−533, respectively, together with other not yet characterized factors binding upstream of position −657. Our data, therefore,

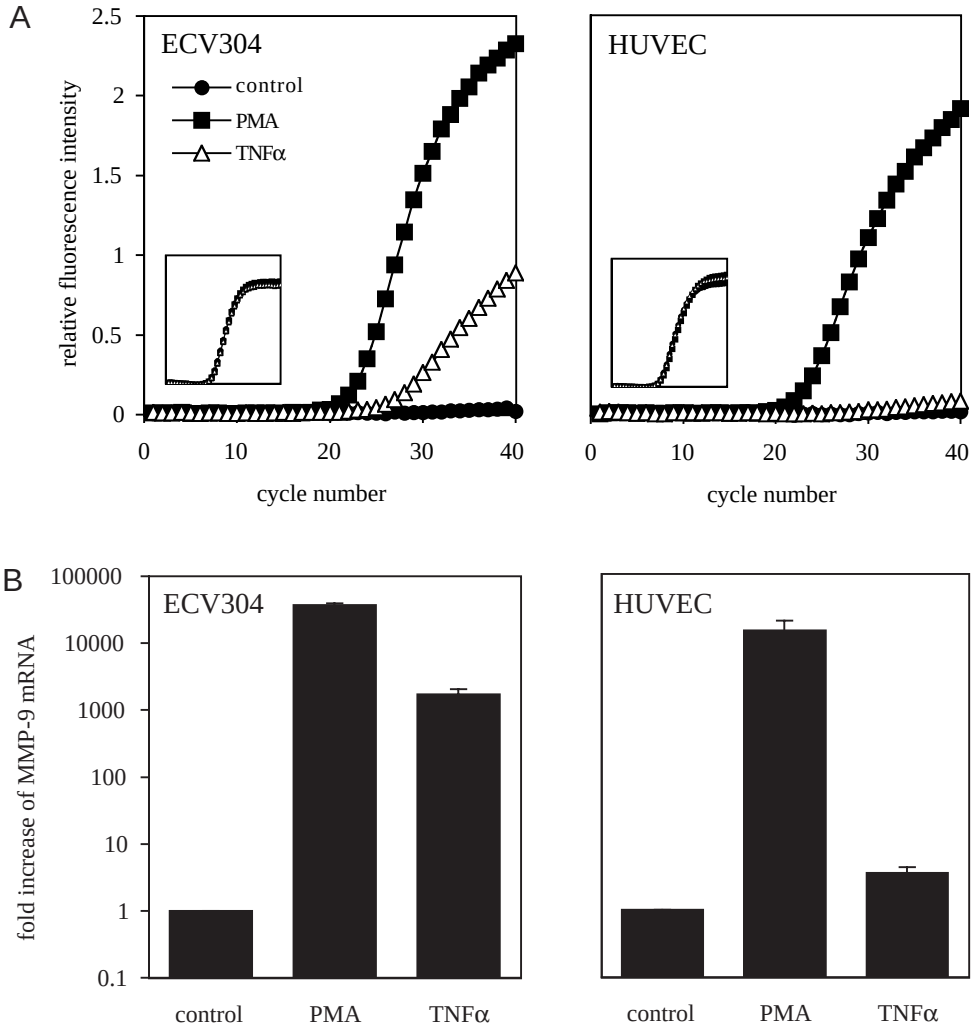

Fig. 2. Effect of PMA and TNFα on mRNA levels for MMP-9 in ECV304 and HUVEC. For real time-quantitative RT-PCR (TaqMan analysis), cells were treated essentially as for gelatin zymography and total RNA was extracted. To control for equal amounts of RNA TaqMan analysis using mouse GAPDH primers was performed in parallel (curves shown as inserts). (A) Exemplary TaqMan curves of one RNA dilution of PMA-, TNFα-treated, and untreated ECV304 and HUVEC are shown. Fluorescence intensity is given in relative units. (B) Quantification of the data underlying the curves shown in A as given in Materials and Methods. MMP-9 mRNA of unstimulated control cells (equals background level) was arbitrary set to 1 and PMA and TNFα induction of MMP-9 mRNA is expressed as fold increase (± s.d.) of MMP-9 mRNA over control.

demonstrate that MMP-9 promoter activity in endothelial cells is regulated in a similar manner as described for several other cell lines (Gum et al., 1996; Gum et al., 1997; Sato and Seiki, 1993). Surprisingly, at the level of promoter activity, TNFα was able to increase CAT expression driven by the MMP-9 promoter not only in ECV304 but also in HUVEC, where CAT expression was even higher than in ECV304. This result agrees with the notion that transfected promoter constructs do not always serve as appropriate 'stand-ins' for their endogenous counterpart (Smith and Hager, 1997; Zedlacher et al., 1999).

PKC and MEK are differentially involved in MMP-9 expression

The subsequent experiments were designed to elucidate the signaling cascades, which turn on the expression of the MMP-9 gene in endothelial cells, in response to stimulation by PMA and TNFα.

First, the effect of specific kinase inhibitors on the expression of MMP-9 was analyzed. Prior to stimulation with TNFα or PMA the cells were incubated with Ro31-8220, PD98059 or SB203580, specific inhibitors for PKC, ERK-activator MEK1/2 or p38MAPK, respectively. The expression level of MMP-9 was determined both at the protein (Fig. 4A) as well as mRNA (Fig. 4B) level. Inhibition of PKC by pre-incubation with Ro31-8220 did not affect TNFα induced MMP-9 protein and mRNA expression in ECV304 ruling out an involvement of PKC in this signaling. In contrast, PD98059 totally and SB203580 partially abolished MMP-9 expression. This indicated that ERK and p38MAPK signaling cascades should be involved in the TNFα mediated expression of MMP-9 in ECV304. Interestingly, inhibition of PMA stimulation in ECV304 gave a different picture. PKC inhibitor Ro31-8220 totally inhibited PMA-induced MMP-9 expression, whereas the MEK1/2 inhibitor PD98059 caused a marked decrease in MMP-9 protein and mRNA level and the inhibition of p38MAPK by SB203580 did not show any effect. We therefore concluded that PMA stimulates both PKC and ERK signaling in ECV304. PMA treated HUVEC failed to induce MMP-9 expression when pretreated with Ro31-8220 or PD98059, but rather increased the synthesis of MMP-9 under the influence of SB203580. This revealed a complex interplay of positive (PKC and ERK) and negative (p38MAPK) signaling cascades involved in the regulation of MMP-9 expression in this primary endothelial cells. Again, all our data correlated at the protein and mRNA level, demonstrating that induction as well as inhibition of induction is regulated at the level of transcription.

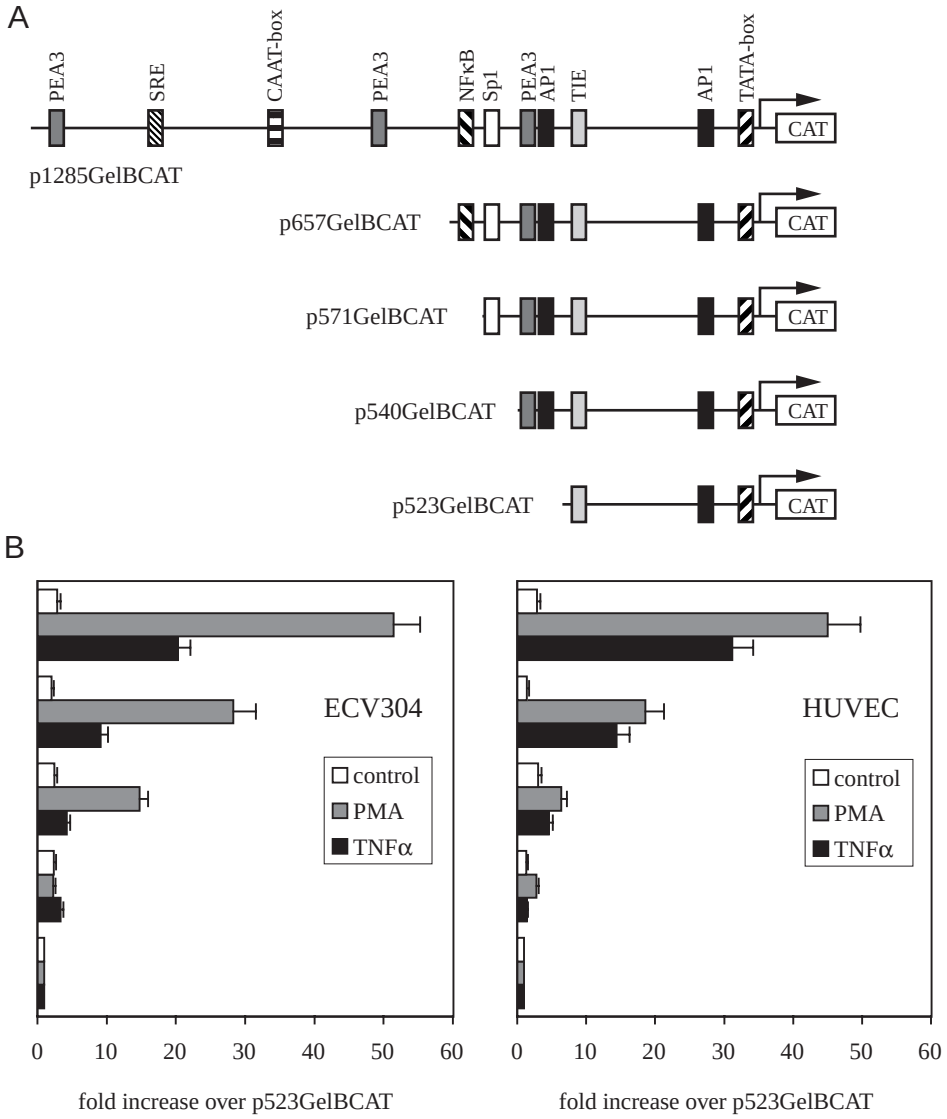

Fig. 3. Activity of different MMP-9 promoter constructs in ECV304 and HUVEC. A series of 5′-end deletion mutants of the MMP-9 promoter was fused to a CAT reporter gene and used for transient transfection assays. The organization of each construct used is depicted in the schematic. ECV304 and HUVEC were transfected with the indicated reporter gene constructs. Subsequent to serum starvation for 16 hours cells were stimulated with TNFα (25 ng/ml) and PMA (50 nM) or left untreated as a control. After another 24 hours, CAT expression of each construct was determined and is expressed relative to the base-line CAT expression of our minimal promoter-CAT construct p523GelBCAT arbitrary set to 1 (see Materials and Methods). Values are given as fold increase (± s.d.) over control construct p523GelBCAT.

Sustained initial phosphorylation of ERK 1/2 is necessary for MMP-9 expression

Considering that EGF- and HGF-induced MMP-9 expression in the tumor cell line SCC-12F was shown to depend on sustained ERK phosphorylation (McCawley et al., 1999), we investigated the role of ERK phosphorylation in endothelial MMP-9 expression in greater detail. First we confirmed by western blotting analysis that there is no difference in the MAPK expression pattern between ECV304 and HUVEC (data not shown). Subsequently, the phosphorylated isoforms of ERK1 and ERK2 and the kinetics of phosphorylation following stimulation with TNFα and PMA were analyzed in both cell types using antibodies specific for phosphorylated ERK1 and 2 (Fig. 5). In ECV304, ERK1 and ERK2 were constitutively phosphorylated in control cells, with ERK 2 always showing a higher abundance of the phosphorylated isoform than ERK1. In contrast, no ERK1- or ERK2-phosphorylation was detectable in HUVEC control cells. Stimulation with PMA resulted in a rapid (within 15 minutes) phosphorylation of ERK1 and ERK2 in both cell types, which sustained for several hours. Although HUVEC showed a small decline in phosphorylation of ERK1 and ERK2 after 6 hours, control level were not yet reached after 24 hours in both cell types. This very similar ERK1/2 phosphorylation pattern observed in ECV304 and HUVEC is in accordance with the fact that in both cell types PMA induced the expression of MMP-9, which could be inhibited by inhibiting ERK phosphorylation. In contrast, TNFα caused a sustained initial ERK-phosphorylation only in ECV304. Like with PMA, although less prominent, ERK phosphorylation was stimulated within 15 minutes and sustained for more than 2 hours but in contrast to PMA was back to basal level at time point 24 hours. In HUVEC, ERK phosphorylation was biphasic with a rapid phosphorylation peak after 15 minutes followed by a decline to a nearly basal level and a second peak around 6 hours which then persisted for at least 24 hours. These findings clearly demonstrate that the crucial point for induction of MMP-9 synthesis in endothelial cells is not whether or not there is still phosphorylated ERK1/2 at later times after stimulation. Rather, it is the sustained initial phosphorylation of ERK1/2, which is necessary for activating MMP-9 expression.

Raf, the upstream activator of MEK1/2, is involved in MMP-9 expression

The results obtained above prompted us to analyze whether or not Raf, the protein kinase classically activating MEK1/2, which then in turn activates ERK1/2, is involved in the TNFα or PMA induced MMP-9 expression. ECV304 and HUVEC were therefore transiently transfected with the promoter construct p1285GelBCAT, co-transfected with control vector or wild-type Raf and various Raf mutants and stimulated with TNFα and PMA or only PMA, respectively (Fig. 6). In ECV304 and in HUVEC, co-transfection of wild-type Raf or constitutively active Raf showed an increase in p1285GelBCAT activity already in unstimulated control cells

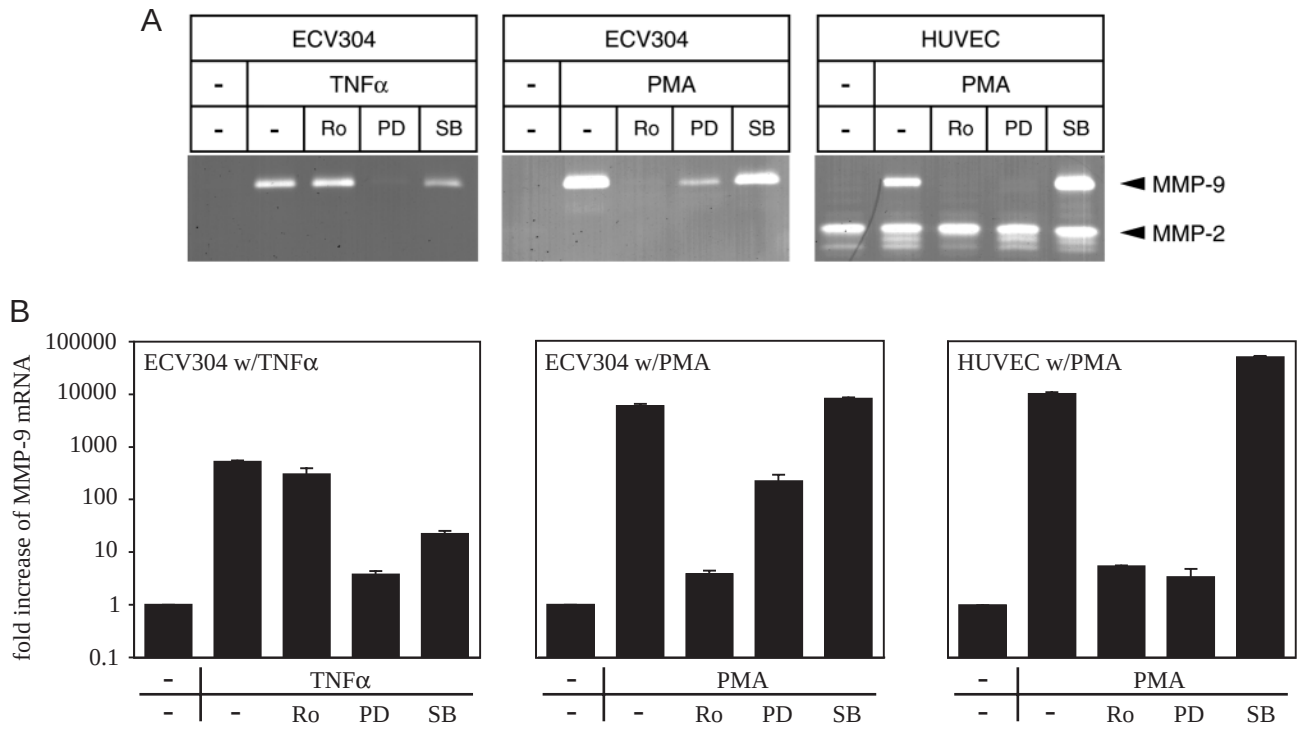

Fig. 4. Effect of protein kinase inhibition on PMA and TNFα stimulated MMP-9 expression in ECV304 and HUVEC. (A) Zymography of conditioned media of ECV304 stimulated with TNFα (25 ng/ml) and PMA (50 nM) or of HUVEC stimulated with PMA. 30 minutes prior to stimulation cells were incubated with PKC inhibitor Ro31-8220 (1 μM, 'Ro'), MEK 1/2 inhibitor PD98059 (10 μM, 'PD'), and p38MAPK inhibitor SB203580 (10 μM, 'SB'). Control cells were left unstimulated and uninhibited or stimulated but not inhibited. The positions of MMP-2 and MMP-9 are indicated. (B) Analysis of MMP-9 mRNA levels in stimulated ECV304 and HUVEC following protein kinase inhibition. Quantitative real time RT-PCR of total RNA from ECV304 and HUVEC treated essentially as for (A) was performed and results were quantified as given in Materials and Methods. MMP-9 mRNA levels are expressed as fold increase (± s.d.) of MMP-9 mRNA over untreated control cells arbitrary set to 1.

demonstrating that an enhanced level or activity of Raf alone is able to cause MMP-9 promoter up-regulation. In ECV304 overexpression of wild-type Raf strongly enhanced the stimulatory effect of TNFα and PMA on MMP-9 promoter activity. Expression of a dominant negative or constitutively active mutant of Raf decreased or increased induction of promoter activity, respectively. HUVEC strongly responded to co-expression of Raf mutants. Wt Raf as well as constitutively active Raf caused a marked increase in PMA stimulated MMP-9 promoter activity whereas expression of dominant negative Raf nearly abolished PMA stimulation. These results clearly showed that Raf must be involved in both TNFα and PMA induced MMP-9 expression in endothelial cells.

Ras, the upstream activator of Raf, is involved in PMA-induced MMP-9 expression

To test whether Ras or Rho signal transduction is involved in either PMA or TNFα activation of MMP-9 expression we used a HMG-CoA reductase inhibitor, Cerivastatin®, to block function of those small GTPases through inhibition of isoprenylation (Bassa et al., 1999; Negre-Aminou et al., 1997). In the presence of the inhibitor induction of MMP-9 could be almost or totally abolished in PMA stimulated ECV304 or HUVEC, respectively. Interestingly, Cerivastatin® had no effect on the TNFα induced synthesis of MMP-9 in ECV304 (Fig. 7A). These results indicate that only PMA-induced MMP-9 expression is positively regulated by isoprenoid synthesis. To identify the GTPase involved in PMA-induced MMP-9 expression ECV304 pretreated with Cerivastatin® were then stimulated with PMA in the presence of isoprenoid intermediates, geranylgeranylpyrophosphate (GGPP) and farnesylpyrophosphate (FPP), to suppress the effect of Cerivastatin® (Laufs and Liao, 1998; Takeuchi et al., 2000). Cotreatment with FPP concentration dependently reversed the suppressive effect of Cerivastatin® (Fig. 7B) indicating that PMA-induced MMP-9 expression is positively regulated by farnesyl synthesis pointing to Ras as the responsible GTPase. GGPP at a concentration of 10 μM also showed a slight reversion of the effect of Cerivastatin®. Therefore an additional involvement of Rho in PMA-induced MMP-9 expression cannot be ruled out. These experiments revealed that Ras is part of the PMA-stimulated signal transduction pathways in both ECV304 and HUVEC, whereas it is not involved in TNFα signaling in ECV304. These results were further supported by confocal microscopy on TNFα- and PMA-stimulated ECV304 where strong staining for Ras was detecable in PMA- but not in TNFα-stimulated cells. This staining was not visible in serumstarved control cells and when cells were treated with Cerivastatin® prior to PMA stimulation (data not shown).

Sustained initial phosphorylation of ERK 1/2 is necessary but not sufficient for MMP-9 expression

The experiments described above suggested that sustained

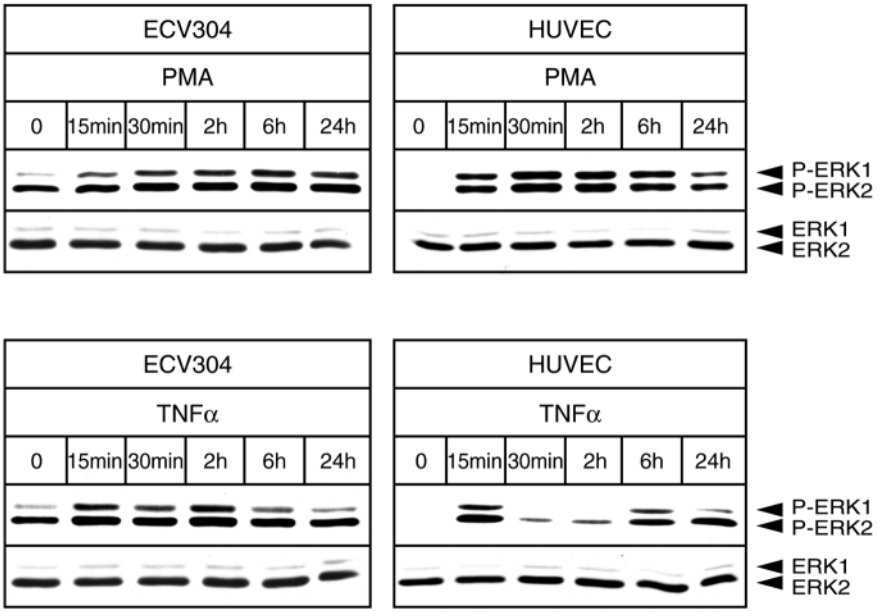

Fig. 5. Effect of PMA and TNFα stimulation on the phosphorylation of ERK1 and ERK2 in ECV304 and HUVEC. Cells were stimulated with TNFα (25 ng/ml) and PMA (50 nM) for the given period of time. 10 μg of total cellular protein extracted after stimulation at the time point indicated were subjected to western blot analysis and analyzed for ERK1 and ERK2 expression or phosphorylation using antibodies specific for ERK1/2 or the phosphorylated isoforms of these protein kinases. The positions of ERK1 and ERK2 or phosphoERK1 and phosphoERK2 are indicated.

initial ERK phosphorylation is the key to MMP-9 induction. We therefore analyzed the state of ERK phosphorylation under the influence of the different inhibitors at time points of sustained phosphorylation (Fig. 8). As expected, PD98059, the direct and specific inhibitor of the ERK activator MEK1/2 inhibited ERK phosphorylation after TNFα stimulation of ECV304 and decreased ERK phosphorylation after PMA stimulation of both cell types. This correlates with the inhibition of MMP-9 expression through PD98059. Inhibition of MMP-9 induction through the PKC inhibitor Ro31-8220 also correlated with inhibition of ERK phosphorylation through Ro31-8220. In TNFα stimulated ECV304, where Ro31-8220 does not affect MMP-9 synthesis, ERK phosphorylation is also not affected. In contrast, PMA induced stimulation of ERK phosphorylation is inhibited in ECV304 and drastically reduced in HUVEC by Ro31-8220 which correlates with the inhibitory effect of Ro31-8220 on PMA stimulated MMP-9 expression in both cells. These findings clearly indicate that PKC and MEK/ERK are members of the same PMA-initiated signaling cascade. Cerivastatin® as an inhibitor of Ras function had no effect on sustained ERK phosphorylation independent of cell type (ECV304, HUVEC) and stimulus (TNFα, PMA), although Cerivastatin® did inhibit PMA-induced MMP-9 expression in both ECV304 and HUVEC. These findings demonstrate firstly that in our system Ras does not feed into the Raf/MEK/ERK signaling cascade no matter whether initiated by TNFα or PMA. Secondly, although sustained initial phosphorylation of ERK1/2 is

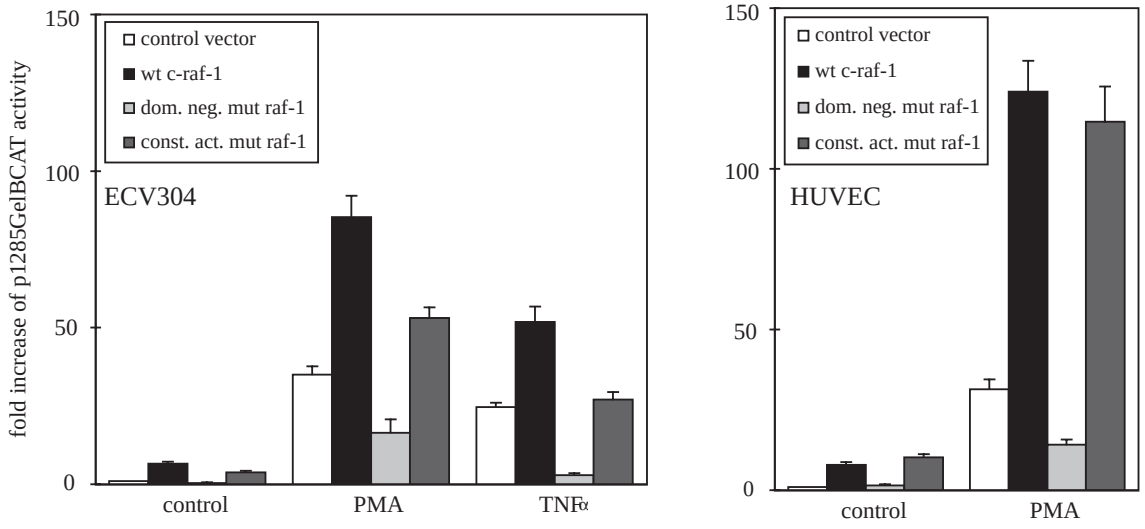

Fig. 6. Influence of Raf on the activity of the MMP-9 promoter construct p1285GelBCAT in stimulated ECV304 and HUVEC. Cells transiently transfected with the promoter construct p1285GelBCAT were cotransfected with an expression vector carrying wild-type c-Raf-1, dominant negative mutant Raf, and constitutively active mutant Raf, or with an empty expression vector as a control vector. ECV304 were stimulated with PMA (50 nM) and TNFα (25 ng/ml) and HUVEC were stimulated only with PMA (50 nM). As a control for stimulatory effects of PMA and TNFα cells were left untreated. After 24 hours, CAT expression was determined. Values are given as fold increase (± s.d.) of p1285GelBCAT acitvity in unstimulated control cells co-transfected with the empty control vector. This activity was arbitrary set to 1.

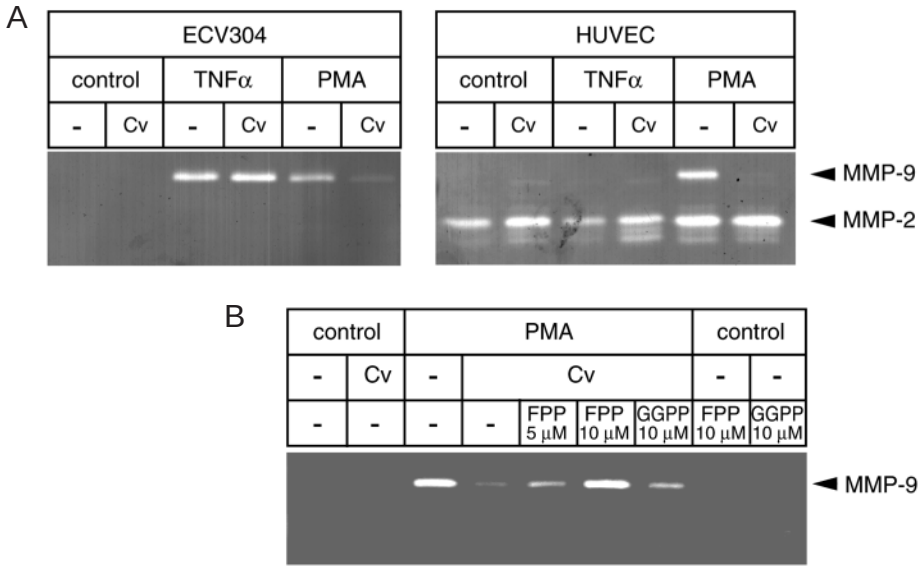

Fig. 7. Effect of inhibition of isoprenoid-dependent signaling on PMA and TNFα stimulated MMP-9 expression in ECV304 and HUVEC. (A) Zymography of conditioned media of ECV304 and HUVEC stimulated with TNFα or PMA. 30 minutes prior to stimulation cells were incubated with a HMG-CoA reductase inhibitor, Cerivastatin® (5 μM, 'Cv'). Control cells were left unstimulated and uninhibited or stimulated but not inhibited. The positions of MMP-2 and MMP-9 are indicated. (B) Zymography of conditioned media of ECV304 pretreated with Cerivastatin® ('Cv') and stimulated with PMA in the presence of farnesylpyrophosphate (5 μM and 10 μM, 'FPP') or geranylgeranylpyrophosphate (10 μM, 'GGPP'). Control cells were treated as given in the figure. The position of MMP-9 is indicated.

necessary for MMP-9 expression it is not sufficient, since Cerivastatin® blocked MMP-9 expression without affecting ERK phosphorylation.

DISCUSSION

It is firmly established that MMP expression by endothelial cells is a prerequisite for angiogenesis (Fisher et al., 1994; Kräling et al., 1999). In particular, MMP-9 expressed by endothelial cells seems to play a critical role in angiogenesis by virtue of its ability to hydrolyze native type IV collagen, a major constituent of basement membranes. As a result, the sprouting endothelial cells are capable of initiating the angiogenic process by escaping their basement membrane and invading the underlying interstitial matrix (Qian et al., 1997; Vu et al., 1998).

Several studies have identified signal transduction pathways involved in the regulation of MMP-1 expression in fibroblasts (Reunanen et al., 1998) or MMP-9 expression in keratinocytes (McCawley et al., 1999; Zeigler et al., 1999) and tumor cells (Gum et al., 1996; Gum et al., 1997; Simon et al., 1998). The signaling cascades that mediate the expression of MMP genes in endothelial cells have, however, remained elusive. We were interested in elucidating the individual steps that act in transforming events at the cell surface into responses that modulate transcription of MMP-9. Here we present data showing that MMP-9 expression is differentially induced in primary (HUVEC) versus permanent (ECV304) endothelial cells as determined at protein, mRNA, and promoter level. Thus, both cell types did not exhibit constitutive expression of MMP-9 under serum-free conditions but they started to express MMP-9 upon stimulation with PMA. In contrast, TNFα stimulation led to MMP-9 expression only in ECV304 cells but not in HUVEC.

Previous reports, which tried to analyze the signaling requirements for MMP-9 expression, did not yield a uniform picture. Instead, results varied depending both on the cell type and the nature of the stimulus. The Ras-dependent expression of MMP-9 in OVCAR-3 cells, as studied by the effect of transient expression of activated Ha-ras on MMP-9 expression, was reported to be mediated through a MEK1-independent signaling pathway (Gum et al., 1996). In contrast, disruption of ERK- and JNK-dependent signaling was shown to decrease endogenous constitutive MMP-9 expression in UM-SCC-1 cells (Gum et al., 1997). In keratinocytes, JNK and p38MAPK cascades were described not to be sufficient for growth factor induced MMP-9 expression. Instead, prolonged activation of ERK signaling was suggested to be necessary for induction of MMP-9 expression by growth factors (McCawley et al., 1999; Zeigler et al., 1999).

Our data presented here show that in endothelial cells ERK1/2 represent the convergence point for TNFα as well as

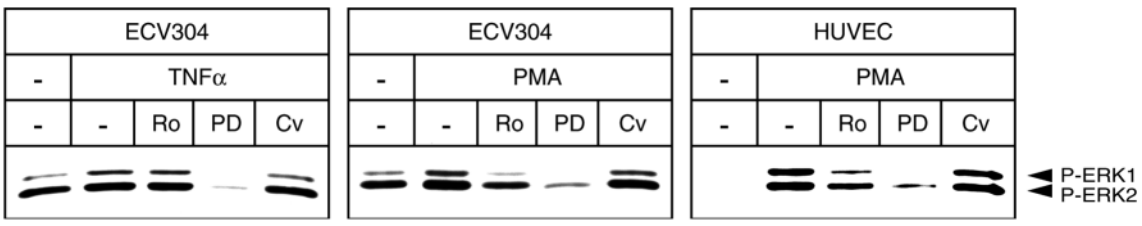

Fig. 8. Effect of Ro31-8220, PD98059, and Cerivastatin® on ERK phosphorylation in PMA and TNFα stimulated ECV304 and HUVEC. Prior to stimulation with PMA (50 nM) and TNFα (25 ng/ml) ECV304 and HUVEC were incubated for 30 minutes with Ro31-8220 (1 μM, 'Ro'), PD98059 (10 μM, 'PD'), and Cerivastatin® (5 μM, 'Cv'). 2 hours subsequent to stimulation total cellular protein was extracted. 10 μg of each protein sample were subjected to western blot analysis and analyzed for ERK1 and ERK2 phosphorylation using antibodies specific for the phosphorylated isoforms of these protein kinases. The positions of phosphoERK1 and phosphoERK2 are indicated.

PMA induced MMP-9 expression. Using PD98059, a selective MEK1 inhibitor, we were able to block induction of MMP-9 mRNA and protein expression subsequent to TNFα treatment in ECV304 and PMA stimulation in both cells. An analysis of the kinetics of ERK phosphorylation revealed that both, PMA and TNFα activate the classical mitogenic MEK/ERK cascade in ECV304 and HUVEC, since in both cell types stimulation with PMA and TNFα is followed by the rapid phosphorylation of ERK1/2.

However, whereas in both cells PMA stimulation results in a prolonged initial ERK phosphorylation, such a sustained activation of ERK1/2 is only evident in ECV304 subsequent to TNFα treatment. In HUVEC, where in contrast to ECV304 ERK is not activated constitutively, TNFα causes a bisphasic ERK phosphorylation where the initial phosphorylation is only transient. Thus, the induction of the endogenous MMP-9 gene strongly correlates with sustained initial ERK phosphorylation. Both can be inhibited by PD98059. Therefore, these results indicate that such a sustained ERK activation may indeed be necessary for induction of MMP-9 expression not only through growth factors (McCawley et al., 1999; Zeigler et al., 1999) but also as a result of induction by cytokines and phorbol esters.

However, the transcriptional requirements for this regulation have not been characterized yet. It was speculated that one of the potential cis-acting elements identified within the first 670 bp of the promoter region (McCawley et al., 1999; Sato et al., 1993; Sato and Seiki, 1993), for instance the closely spaced AP-1 and PEA3 elements (Gum et al., 1997), might provide a mechanism by which sustained MAPK activation could result in MMP-9 induction. Contradictory to the regulation of the endogenous gene, in HUVEC all promoter fragments analyzed could be activated by TNFα, although only transient ERK phosphorylation occurred. Therefore, we can rule out the 1285 bp 5′ upstream region of the MMP-9 gene to be involved in regulation by sustained ERK phosphorylation. The pattern of promoter activity was identical to that observed with sustained ERK activity after PMA stimulation or in ECV304 after TNFα treatment. Therefore, for activation of this promoter region, initial transient ERK phosphorylation is sufficient and sustained ERK activity is not necessary, causing the inconsistency between activity of the transiently transfected promoter constructs and expression of the endogenous MMP-9 gene in TNFα-stimulated HUVEC. This results agrees with a growing number of reports showing similar differences between the activity of transiently transfected promoters and their endogenous counterparts (Kitabayashi et al., 1992; Smith and Hager, 1997; Zedlacher et al., 1999).

With respect to the general transcriptional control of the MMP-9 gene, we could show that the already extensively studied cis-acting elements, the AP1-, PEA3-, SP1-, and NF-κB motifs located between bp –520 and –670 (Bond et al., 1998; Gum et al., 1996; Sato et al., 1993; Sato and Seiki, 1993), together with not yet characterized elements downstream of –670, are synergistically involved in endothelial MMP-9 regulation. The only difference in promoter activity observed with endothelial cells compared to what is reported so far with tumor cells was that all promoter fragments smaller than 523 bp had faint if any activity (data not shown). Measurable promoter activity stopped once the closely spaced AP1/PEA3 sites (Gum et al., 1996) were deleted. Our results suggest that the AP1 site located at –79, which was described to be important for v-src and phorbol ester specific induction of MMP-9 expression in tumor cells (Gum et al., 1996; Sato et al., 1993; Sato and Seiki, 1993), is unlikely to play a role in endothelial cells.

Initially, we were surprised by the requirement of ERK1/2 in the regulation of TNFα induced MMP-9 expression in ECV304, because cytokines are known to act predominantly through the other two MAPK subgroups, JNK/SAPK and p38MAPK. Although TNFα was found to be also an agonist of the p38MAPK cascade in ECV304, only the ERK pathway was identified to be of major relevance for TNFα-induced expression of the MMP-9 gene. By transient co-transfection of wild-type Raf and constitutively active mutant Raf, by selective inhibition of MEK1 and by analysis of the kinetics of ERK phosphorylation, we could clearly demonstrate that the classical mitogenic Raf/MEK/ERK cascade resulting in sustained ERK activity is responsible for TNFα mediated MMP-9 expression in ECV304.

Indeed, it is now becoming apparent that TNFα may be implicated in the activation of all three MAPK cascades. In endothelial cells, for instance, TNFα initiated inflammatory events were shown to be mediated through ceramide-independent activation of p38MAPK and JNK and through activation of Raf-1, which in turn could be ceramide dependent (Modur et al., 1996). Further, Goebeler and co-workers (Goebeler et al., 1999) recently reported that TNFα caused activation of p38MAPK, JNK, and ERK in HUVEC, with p38MAPK being mainly responsible for the up-regulation of monocyte-chemoattractant protein-1 (MCP-1). These studies, together with our findings, suggest that although TNFα triggers all three MAPK pathways, the diverse cellular responses can be addressed to certain pathways, like activation of MCP-1 or MMP-9 to activation of p38MAPK or ERK, respectively.

Ras is known to interact predominantly with Raf thus influencing the ERK kinase cascade. There are also reports showing that PKC activates Raf-1 kinase in a Ras-independent manner (Ueda et al., 1996; Zou et al., 1996). Therefore, we initially hypothesized that triggering the Raf/MEK/ERK cascade was accomplished via Ras or PKC. However, Cerivastatin, a member of the statin-family of HMG-CoA reductase inhibitors, known to impair Ras-signaling by interfering with its farnesylation (Bassa et al., 1999), failed to inhibit both MMP-9 expression and sustained ERK phosphorylation caused by TNFα, as did Ro98059, a selective PKC inhibitor. Thus, TNFα regulates the expression of MMP-9 in ECV304 cells via PKC and Ras independent Raf/MEK/ERK signaling.

So far, we can only speculate as to the identity of the upstream mediator of Raf in TNFα signaling. A likely candidate is certainly ceramide, since TNFα was shown to stimulate sphingomyelinase activity, thereby inducing hydrolysis of sphingomyelin to ceramide, which in turn activates MAPK pathways (Spiegel and Merrill, 1996). However, initial experiments involving C2-and C6-ceramide as well as sphingomyelinase were not convincing (data not shown), consistent with the notion, that the small amount of ceramide evoked by TNFα in endothelial cells is incapable of stimulating a functional response from these cells (Modur et al., 1996). We cannot, however, exclude the possibility that sphingosine 1-phosphate (SPP), a metabolite of ceramide generated via sphingosine kinase (SKase) activation, might be

mediating the TNFα response in ECV304. Although it was suggested that SPP acts as an intracellular second messenger in growth factor signaling pathways (Spiegel et al., 1998), other investigators demonstrated that in HUVEC the SKase pathway through generation of SPP is critically involved in mediating TNFα-induced NF-κB dependent adhesion molecule expression (Xia et al., 1998).

It is well established that phorbol esters mediate their effects through PKC and that downstream mediators of PKC may include all three MAPKs (Arnould et al., 1998; Schultz et al., 1997; Sozeri et al., 1992) as well as effectors other than MAPK (Osborn et al., 1999). Phorbol esters like PMA are known to stimulate MMP-9 promoter activity (Sato et al., 1993; Sato and Seiki, 1993) and PMA-induced expression of MMP-9 in tumor cells has been described to be mediated through p38MAPK activation (Simon et al., 1998).

Our results obtained with kinase inhibition experiments and co-transfections of wild-type Raf and mutants of Raf now provide evidence that in endothelial cells solely the classical mitogenic Raf/MEK/ERK cascade is mainly responsible for PMA induced MMP-9 expression and not p38MAPK. Further analysis of the upstream mediators of Raf in PMA signaling revealed that PKC but not Ras activates Raf, because inhibition of PKC but not inhibition of Ras prevented sustained ERK phosphorylation corresponding to prevention of MMP-9 expression. The hypothesis that in endothelial cells a PKC/Raf/MEK/ERK cascade is involved in PMA-induced MMP-9 expression is supported further by the studies of Ueda and co-workers (Ueda et al., 1996), which show that PKC indeed activates the MEK/ERK pathway in a Raf-dependent way but independent of Ras. Further, Zou and co-workers (Zou et al., 1996) demonstrated that in cardiac myocytes PKC but not Ras plays a critical role in angiotensin-II induced activation of Raf and ERK.

However, PMA induced MMP-9 expression in endothelial cells is Ras-dependent since interfering with Ras-signaling blocked MMP-9 expression although it had no effect on ERK phosphorylation. We therefore propose that PMA triggers two independent signaling pathways in endothelial cells which lead to MMP-9 expression, one being the PKC/Raf/MEK/ERK cascade, and the other pathway involving Ras but not ending in ERK activation. In tumor cells, MMP-9 expression was shown to be dependent on Ras rather than MEK1 (Gum et al., 1996). Indeed, it is now apparent that Ras can modulate gene expression through a number of independent pathways other than the best-studied Raf/MEK/ERK cascade (al-Alawi et al., 1995). Since interfering with either one pathway in PMA-stimulated endothelial cells blocks MMP-9 production none of the pathways alone is sufficient to mediate PMA-induced MMP-9 expression. More importantly, our results show that sustained ERK activation is necessary but not sufficient to induce MMP-9 expression since inhibition of Ras-signaling blocks MMP-9 expression despite sustained ERK phosphorylation.

In conclusion, we have shown that ERK phosphorylation is a convergence point for both TNFα- and PMA-induced MMP-9 expression in endothelial cells. TNFα activates the classical mitogenic Raf/MEK/ERK cascade in ECV304 in a Ras-independent manner resulting in MMP-9 expression. In contrast, PMA activates MMP-9 expression in endothelial cells via a Ras-dependent signaling cascade bypassing ERK-phosphorylation and via a Ras-independent PKC/Raf/MEK/ERK pathway. Both pathways are necessary but not sufficient for MMP-9 induction by PMA. Sustained phoshorylation of ERK is shown to be a general but not sufficient requirement for MMP-9 expression.

Since MMP-9 was implied in angiogenesis, these findings may be important for the development of anti-angiogenic agents for tumor treatment, which modulate MMP-9 expression by interfering with these endothelial MMP-9 regulating pathways.

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