Inhibition of the p38 Mitogen-activated Protein Kinase by SB 203580 Blocks PMA-induced Mr 92,000 Type IV Collagenase Secretion and in Vitro Invasion

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Abstract

Although the p38 mitogen-activated protein kinase (MAPK) has been implicated in signal transduction events, its role in regulating the Mr 92,000 type IV collagenase matrix metalloprotease-9 (MMP-9) and in vitro invasiveness in cancer has not yet been determined. We made the surprising observation that, in a human squamous cell carcinoma cell line (UM-SCC-1), phorbol ester-enhanced MMP-9 secretion and in vitro invasiveness were associated with a strong activation of the p38 MAPK and its downstream target, MAPK-activated protein kinase-2. To determine the role of p38 activation in these events, we investigated the effect of SB 203580, a novel specific p38 inhibitor, on protease expression and in vitro invasion of these cells. We found that inhibition of p38 by SB 203580 resulted in the almost complete reduction of phorbol myristate acetate-induced MMP-9 secretion but not of urokinase-type plasminogen activator secretion. In contrast, the activation of a transiently transfected wild-type MMP-9 promoter by MEKK-1, a specific c-Jun NH2-terminal kinase activator, was only marginally inhibited by the compound, arguing for the specificity of SB 203580. Moreover, phorbol myristate acetate-enhanced in vitro invasion was completely blocked by SB 203580, whereas p38 inhibition had little effect on growth. These findings suggest that activation of p38 may contribute to a more invasive phenotype in vitro, possibly via the expression of MMP-9, and that targeting of p38 using SB 203580 may provide a novel means of controlling invasion of cancers in which this MAPK is activated.

Introduction

Secretion of the Mr 92,000 type IV collagenase MMP-9 by some cancers, such as squamous cell carcinomas of the head and neck, is an important determinant of their invasive phenotype. The enzyme degrades the basement membrane, a structure that is largely composed of type IV collagen and separates the epithelial and stromal compartments (1). At present, the regulatory mechanisms responsible for the expression of this matrix metalloprotease are poorly understood. We previously demonstrated that two protein serine-threonine kinases, ERK and JNK, are involved in the regulation of MMP-9 (2, 3). These protein kinases belong to the MAPK family, which is composed of the ERKs, JNKs, and the p38 MAPK. However, the function of the third subfamily of MAPKs, the p38 MAPK, in regulating this protease and in vitro invasiveness has not yet been determined.

The p38 MAPK is a protein activated by a wide spectrum of stimuli, such as cellular stress, lipopolysaccharides (4), and cytokines such as TNF-α (5). We found that, in the squamous cell carcinoma cell line UM-SCC-1, the tumor promoter PMA, regarded in the past as a specific ERK activator (6, 7), activates all three MAPK subfamilies, in particular p38, and this activation was associated with the induction of MMP-9 secretion and a more invasive phenotype in vitro. Because p38 can activate c-Fos via phosphorylation of the ternary complex factor Sap-la (8), which may induce expression of AP-1-regulated proteins, such as MMP-9 (3), we hypothesized that the activation of p38 may contribute to the induction of MMP-9 secretion and the more invasive phenotype in vitro observed after exposure to PMA. To investigate this hypothesis, we used a highly specific p38 inhibitor SB 203580, a pyridinyl imidazol belonging to the CSAID family, which prevents activation of the kinase by binding into its ATP acceptor pocket (9). Our results indicate that the activation of p38 is involved in PMA-induced secretion of the protease MMP-9 and enhanced in vitro invasion.

Materials and Methods

Tissue Culture and Materials. UM-SCC-1 cells (obtained from Dr. Thomas Carey, University of Michigan, Ann Arbor, MI) were maintained in McCoy's 5A culture medium supplemented with 10% FBS. For the collection of conditioned medium for zymography, 80% confluent UM-SCC-1 cells were incubated in serum-free medium (McCoy's 5A medium supplemented with 5 μg/ml insulin, 10 ng/ml epidermal growth factor, and 4 μg/ml transferrin) for 48 h with and without PMA (Sigma Chemical Co., St. Louis, MO) and various amounts of SB 203580 (kindly provided by Dr. Peter R. Young, Smith Kline Beecham Pharmaceuticals, King of Prussia, PA) or carrier (DMSO), added at the same time. The culture medium was collected and proliferation determined after cells were incubated in 0.2 mg/ml MTT vital stain and aliquots of DMSO-dissolved formazan crystals were read by spectrophotometry at 570 nm. Growth curves were constructed as described previously (2), using various amounts of SB 203580 and PMA, added at the same time, after allowing 12 h for cell attachment. TNF-α was purchased from Life Technologies, Inc. (Gaithersburg, MD). PD 098059 was a generous gift from Dr. Alan Saltiel (Parke Davis, Ann Arbor, MI). The abbreviations used are: MMP-9, matrix metalloprotease-9; ERK, extracellular signal-regulated kinase; JNK, c-Jun NH2-terminal kinase; MAPK, mitogen-activated protein kinase; TNF-α, tumor necrosis factor α; PMA, phorbol myristate acetate; CSAID, cytokine-suppressive anti-inflammatory drug; FBS, fetal bovine serum; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; u-PA, urokinase-type plasminogen activator; MAPKAPK-2, MAP-activated protein kinase-2; GST, glutathione S-transferase; CAT, chloramphenicol acetyltransferase.

Received 1/6/98; accepted 1/26/98.

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1This work was supported by NIH Grants ROI DE10845, ROI CA58311, and P50 DE11906 (to D. B.) and Deutsche Forschungsgemeinschaft Fellowship Si 634/1-1 (to C. S.).

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3The abbreviations used are: MMP-9, matrix metalloprotease-9; ERK, extracellular signal-regulated kinase; JNK, c-Jun NH2-terminal kinase; MAPK, mitogen-activated protein kinase; TNF-α, tumor necrosis factor α; PMA, phorbol myristate acetate; CSAID, cytokine-suppressive anti-inflammatory drug; FBS, fetal bovine serum; MTI, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; u-PA, urokinase-type plasminogen activator; MAPKAPK-2, MAP-activated protein kinase-2; GST, glutathione S-transferase; CAT, chloramphenicol acetyltransferase.

Zymography. Zymography was performed exactly as described previously (2, 3), using SDS-PAGE containing 0.1% (w/v) gelatin to assay for MMP-9 or casein and 10 μg/ml plasminogen to assay for u-PA. MMP- and plasminogen-dependent proteolytes were detected as white zones in a dark field.

In-Gel Kinase Assays for p38 and ERK Activity and Western Blotting. Kinase assays were performed as described previously (2). Briefly, cells were extracted with buffer A (1% Nonidet P-40 (octylphenoxypolyethoxy ethanol), 25 mM Tris-HCl (pH 7.4), 25 mM NaCl, 1 mM sodium vanadate, 10 mM NaF, 10 mM sodium pyrophosphate, 10 mM o-phenylenediamine, 0.5 mM EGTA, and 1 mM phenylmethylsulfonyl fluoride). Extracted protein was either incubated with 2 μg of the anti-ERK antibody (no. 93, Santa Cruz Biotechnology, Santa Cruz, CA) or anti-p38 antibody (no. 535, Santa Cruz Biotechnology) and protein A-agarose beads (2 μg) for immunoprecipitation or resolved by SDS-PAGE.
and transferred to a nitrocellulose filter for Western analysis (10 μg). For kinase assay, the beads were washed with buffer A and resuspended in 2× sample buffer, and the immune complexes were electrophoresed in a polyacrylamide gel containing myelin basic protein. The gel was treated sequentially with buffers containing 20% 2-propanol, 5 mM 2-mercaptoethanol, 6 mM guanidine-HCl, and 0.04% Tween 40-5 mM 2-mercaptoethanol. The gel was then incubated at 25°C for 1 h with 10 μM ATP and 25 μCi of [γ-32P]ATP in a buffer containing 2 mM DTT, 0.1 mM EGTA, and 5 mM MgCl2, washed in a solution containing 5% trichloroacetic acid and 1% sodium pyrophosphate, dried, and autoradiographed. For Western analysis, the membrane was blocked with 3% BSA before incubation with primary antibody and horseradish peroxidase-conjugated antirabbit IgG; bands were visualized using an enhanced chemiluminescence detection system (Amersham, Arlington Heights, IL).

JNK and MAPKAPK-2 Activity Assay. JNK and MAPKAPK-2 activities were determined as described elsewhere (3, 10). Cell extracts were prepared in a buffer containing 1% Triton X-100, 25 mM HEPES (pH 7.6), 300 mM NaCl, 1.5 mM MgCl2, 0.2 mM EGTA, 0.5 mM DTT, 0.5% sodium deoxycholate, 0.1% SDS, 20 mM β-glycerophosphate, 20 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, and 1 mM sodium orthovanadate (for JNK) or in a buffer containing 20 mM Tris-HCl (pH 7.4), 1% Triton X-100, 10% glycerol, 150 mM NaCl, 2 mM EDTA, 25 mM β-glycerophosphate, 20 mM NaF, 1 mM sodium orthovanadate, 2 mM pyrophosphate, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml leupeptin (for MAPKAPK-2). Experiments were then incubated at 4°C for 2 h with 1 μg of an anti-JNK antibody (no. 474, Santa Cruz Biotechnology) or 2.5 μg of an anti-MAPKAPK-2 antibody (no. 06-534, Upstate Biotechnology). Lake Placid, NY) and protein A- and protein G-agarose beads (for JNK and MAPKAPK-2, respectively). The beads were rinsed several times and then incubated in 40 μl of kinase buffer [for JNK, 12.5 mM 3-(N-morpholino)propanesulfonic acid (pH 7.5), 7.5 mM MgCl2, 12.5 mM β-glycerophosphate, 0.5 mM EGTA, 0.5 mM NaF, and 0.5 mM sodium orthovanadate; for MAPKAPK-2, 25 mM HEPES (pH 7.4), 25 mM MgCl2, 25 mM β-glycerophosphate, 2 mM DTT, and 0.1 mM sodium orthovanadate] with 1 and 2 μCi of [γ-32P]ATP (for JNK and MAPKAPK-2, respectively), 2 μg of GST-c-Jun protein (Santa Cruz Biotechnology) and 2.5 μg of heat shock protein 27 (Stress Gene Biotechnologies, Victoria, BC, Canada; for JNK and MAPKAPK-2, respectively), and 20 μM cold ATP at 30°C. The reaction was terminated 20 min later by adding 2× reducing sample buffer and heating to 100°C for 5 min. The beads were removed by centrifugation. The supernatant (80 μl) was electrohoresed in a 12% polyacrylamide gel. The gel was dried and autoradiographed for 16 h.

In Vitro Invasion Assays. Invasion assays were performed as described previously (2) using Matrigel-coated filters (Becton Dickinson, Bedford, MA). The amount of invasion was determined on the basis of the MTT activity on the lower side of the filter as a percentage of the total activity in the chamber.

Results

PMA Activates not only ERK-1 but also JNK-1/2 and p38 in UM-SCC-1 Cells. Exposure of UM-SCC-1 cells to PMA for 20 min resulted in the activation of all three MAPK subfamilies, ERK-1, JNK-1/2, and p38 (Fig. 1). After immunoprecipitation of endogenous p38, ERK 1/2, and JNK-1/2 from UM-SCC-1 cells after treatment with PMA or carrier (DMSO) alone, followed either by in-gel kinase assay (p38 and ERK) or by immunocomplex reaction using a GST-c-Jun fusion protein as a substrate (JNK), densitometric analysis revealed about a 7-fold induction of p38 activity (Fig. 1A), compared with a 3-fold JNK-1/2 activation (Fig. 1C) and a 4-fold activation for ERK (Fig. 1D). Because PMA caused a rapid (20-min) increase in p38 activity, it is unlikely that the increase in activity of this MAPK was secondary to ERK activation. To exclude the possibility that the strong activation of p38 and ERK-1 was due to alterations in the relative amounts of p38 and ERK-1 protein in the samples, we performed Western analysis for p38 and ERK-1 on the same samples that had been used for the in-gel kinase assay. We found that the dose-dependent increase in kinase activity of p38 and ERK-1 was not a consequence of a change in protein levels (Fig. 1, B and E). PMA Activates MAPKAPK-2 through p38. To confirm that p38 is activated by PMA, we performed two additional experiments. First, we immunoprecipitated MAPKAPK-2, a kinase that lies immediately downstream of p38 (13), and performed immunocomplex reactions after incubation of UM-SCC-1 cells with various concentrations of PMA for 20 min using heat shock protein 27 as a substrate. TNF-α, a known activator of MAPKAPK-2 (14), was used as a positive control. We found that a 100 nM PMA treatment resulted in a 4-fold induction of MAPKAPK-2 activity (Fig. 2A), whereas a quantitatively...
Fig. 2. PMA activates MAPKAPK-2 through p38. A. UM-SCC-1 cells were incubated with various amounts of PMA, SB 203580, or PD 098059 but the same concentration of carrier (0.2% DMSO) for 20 min, and protein extracts were prepared. Extracts (equal protein) were incubated with or without (1× activation, no antibody Ab) anti-MAPKAPK-2 antibody, and immunoprecipitates were subjected to kinase reaction with heat shock protein 27 (HSP 27). B. UM-SCC-1 cells were incubated with various amounts of TNF-α. PD 098059 or SB 203580 was added where indicated, and the concentration of carrier (DMSO) was kept constant (0.2%). Protein extracts were incubated with or without (1× activation) anti-MAPKAPK-2 antibody, and immunoprecipitates were subjected to kinase reaction with heat shock protein 27 (HSP 27). The experiment was conducted on two separate occasions.

Similar induction was observed with 100 ng/ml TNF-α (Fig. 2B). Second, the specific p38 inhibitor (SB 203580) was used (15). The activation of MAPKAPK-2 was entirely countered using 10 μM of the inhibitor (Fig. 2A). Similarly, the stimulation of MAPKAPK-2 by a known p38 activator (TNF-α) was also blocked by SB 203580 (Fig. 2B). In contrast, PMA-induced activation of MAPKAPK-2 was only marginally inhibited by the addition of 10 μM PD 098059, which prevents MAPK kinase 1 activation (Fig. 2). Together, these findings suggest that PMA, indeed, activates p38.

Increase of MMP-9 Activity by PMA Is p38 Dependent. Recently, it was found that p38 activates c-Fos transcription by activating ternary complex factor members, such as SAP-1a (8). Because a number of protease promoters, including the MMP-9 promoter, contain AP-1-binding sites, which were shown to be important for its transcriptional regulation (3), we hypothesized that p38 activation caused by PMA may be involved in PMA-induced MMP-9 expression. We, therefore, incubated UM-SCC-1 cells with 10 nM PMA for 48 h, with and without the specific p38 inhibitor SB 203580 (15), in serum-free medium. PMA treatment resulted in about a 16-fold induction of a gelatinolytic activity in the harvested condition medium, which comigrated with a Mr 92,000 molecular weight marker, consistent with secreted MMP-9.
INHIBITION OF INVASION AND MMP-9 SECRETION BY SB 203580

Fig. 4. PMA enhances in vitro invasion of UM-SCC-1 cells through a p38-dependent mechanism. A, UM-SCC-1 cells were plated out on filters precoated with Matrigel in serum-free medium and incubated with 10 nM PMA and various amounts of SB 203580 for 72 h. The concentration of the carrier (DMSO) was maintained at 0.2%. B, UM-SCC-1 cells were grown in 10% FBS for 5 days with various amounts of PMA and SB 203580. Number of viable cells was determined with MTT vital stain and consecutive reading of DMSO-dissolved formazan crystals at 570 nm on the indicated days. Data points, typical of duplicate experiments.

The gelatinase activity was substantially reduced upon addition of 10 μM SB 203580 (Fig. 3A), suggesting that the activation of p38 is required for enhanced MMP-9 secretion.

To determine whether the p38 inhibitor specifically inhibits MMP-9 expression, we zymographically assayed the same conditioned medium for u-PA activity and found that the PMA caused a 2-fold induction of caseinolytic activity, which comigrated with a Mr 55,000 molecular weight marker, consistent with active u-PA. This was only 5% inhibited by 10 μM SB 203580 (Fig. 3B). These data suggested that the effect of SB 203580 was specific for MMP-9 secretion.

Recently, it has been shown that SB 203580 at high concentrations (10 μM) may inhibit the activity of two JNK-2 isoforms (16). We have shown that constitutive MMP-9 expression can be down-regulated upon introduction of a dominant-negative JNK-1 mutant in UM-SCC-1 cells, suggesting that JNK-1 is required for constitutive activity of the MMP-9 promoter (3). To demonstrate that the CSAID does not inhibit JNK-mediated induction of MMP-9 expression, we transiently transfected a constitutively activated MEKK-1, a specific upstream activator of JNK-1 and JNK-2 but not of p38 (17), into UM-SCC-1 cells along with a CAT reporter construct driven by the wild-type MMP-9 promoter. Upon introduction of MEKK-1 into UM-SCC-1 cells, the wild-type MMP-9 promoter was activated 3-fold. Addition of 10 μM SB 203580 decreased promoter activation by about 15% (Fig. 3C). These data suggest that the inhibition of MMP-9 gelatinolytic activity by the CSAID is unlikely to reflect a block in JNK-mediated induction of MMP-9 expression.

PMA Enhances in Vitro Invasion of UM-SCC-1 Cells through a p38-dependent Mechanism. MMP-9 contributes to the in vitro invasiveness of UM-SCC-1 cells (1). Because our data so far indicated that this protease is regulated in part via a p38-dependent mechanism, we investigated whether interfering with p38 activation by PMA countered enhanced in vitro invasiveness. We incubated UM-SCC-1 cells with 10 nM PMA, with and without SB 203580, and assayed for in vitro invasion using extracellular matrix-coated filters. Upon incubation with PMA, in vitro invasiveness of UM-SCC-1 cells increased from 7 to 16% invading cells. Addition of 1 μM SB 203580 resulted in a 25% reduction of invasiveness, whereas the 5 μM concentration eliminated the PMA-dependent stimulation (Fig. 4A). Because in vitro invasiveness has been shown to depend on the number of cells attached to the Matrigel (18), which serves as the invasion barrier, we generated growth curves to exclude the possibility that the observed inhibition of PMA-induced in vitro invasiveness was due to a growth effect. As shown in Fig. 4B, neither PMA at a 10 nM concentration nor concentrations of the CSAID as high as 10 μM caused any significant growth effect over a period of 5 days, indicating that the inhibition of invasion caused by SB 203580 is not due to inhibition of growth.

Discussion

To our knowledge, this is the first study to demonstrate a role for the p38 subfamily of MAPKs in the regulation of MMP-9 expression and in vitro invasion. In a previous study, we demonstrated that constitutive MMP-9 promoter activity requires both an active ERK and an active JNK (3). Considering that p38 activation is also required for PMA-induced MMP-9 induction, it is now clear that all three MAPK cascades are involved in regulating MMP-9.

The fact that inhibition of p38 suffices to almost completely counter MMP-9 activation by PMA suggests that p38 activation by PMA is a critical requirement for the elevation in MMP-9 activity induced by the phorbol ester. However, it is unlikely that p38 activation by PMA alone is sufficient for the elevation because the expression of a constitutively active MKK-6 was unable to reproduce the effect of
PMA on MMP-9 levels (data not shown). Thus, it is probable that all three MAPK subfamilies act in concert to regulate MMP-9 production. We can only speculate that the requirement for all three MAPK subsets may allow the cell to fine-tune its MMP-9 production in response to a diverse set of environmental cues.

Although it has previously been demonstrated that PMA induces secretion of MMP-9 and also in vitro invasiveness (1), these effects were considered to be due to activation of ERK because PMA has, in the past, been regarded as a specific ERK activator (6). However, our findings that not only ERK-1 but also JNK-1/2 and p38 were activated by PMA, together with the observation that SB 203580 countered the ability of the phorbol ester to stimulate MMP-9 production, strongly suggest the involvement of p38. Thus, the idea that PMA is a specific ERK activator may have to be reevaluated.

Interestingly, an important role of p38 is the promotion of growth inhibition and apoptosis in cancer cell lines (19–21). Thus, for a role of this MAPK in the regulation of MMP-9 production by phorbol ester was surprising because, in most, if not all, cases, increased MMP-9 production is often brought about by mitogens such as epidermal growth factor or transforming growth factor &alpha; (22), fibroblast growth factor (23), and keratinocyte growth factor (24). Thus, p38 activation may have multiple functions in cancer cells, depending on the cell type or the availability of downstream substrates. This dual (and opposing) effect of MAPK is not unprecedented. Indeed, a similar phenomenon has been described for the ERKs, in which MAPK can induce either cell growth or growth inhibition in smooth muscle cells (25).

Our findings may also have bearing on a previous study by this laboratory that showed that the regulation of MMP-9 expression by Ras was mediated through a MAPK kinase 1-insensitive pathway (26). It is interesting to speculate that this induction by the GTP-binding protein made use of a p38-dependent pathway.

In this study, we showed the reductive effect of the pyridyl imidazol derivative SB 203580 on MMP-9 production and in vitro invasion of a head and neck cancer cell line. Studies are underway to determine the in vivo anti-invasive effect of this compound on head and neck squamous cell cancers, in which p38 is constitutively activated.

Acknowledgments

We are grateful to Dr. P. Young (Smith Kline Beecham Pharmaceuticals, King of Prussia, PA) for SB 203580, to Dr. A. Saltiel (Parke Davis, Ann Arbor, MI) for the generous gift of PD 098059, to Dr. M. Seiki (Cancer Research Institute, Kanazawa University, Ishikawa, Japan) for the 670-bp MMP-9 promoter CAT construct, to Dr. J. Han (Scripps Research Institute, La Jolla, CA) for the constitutively activated MKK-6 construct, to Dr. D. Templeton (Case Western Reserve University, Cleveland, OH) for the constitutively activated MEKK-1 construct, and to Drs. R. Bauer and A. Nemechek (The University of Texas M. D. Anderson Cancer Center, Houston, TX) for their valuable input on this manuscript.

References

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