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Transforming growth factor-β1 modulates matrix metalloproteinase-9 production through the Ras/MAPK signaling pathway in transformed keratinocytes

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Abstract

Mouse transformed keratinocytes cultured in the presence of transforming growth factor-β1 (TGF-β1) acquire a set of morphological and functional properties giving rise to a more motile phenotype that expresses mesenchymal markers. In this work, we present evidence showing that TGF-β1 stimulates cellular production of MMP-9 (Gelatinase B), a metalloproteinase that plays an important role in tumoral invasion. Our results demonstrate that TGF-β1stimulates MMP-9 production and MMP-9 promoter activity in a process that depends of the activation of the Ras–ERK1,2 MAP kinase pathway. The latter was demonstrated by cellular transfection of TGF-β1-sensitive cells with a RasN17 mutant gene, using PD 098059, a MEK 1,2 inhibitor, and treating cells with anti-sense oligodeoxinucleotides. The enhanced MMP-9 production proved to be an important factor in the acquisition of migratory and invasive properties as shown by the use of a specific inhibitor of MMP-9 (GM6001) that inhibits the TGF-β1-stimulated invasive and migratory properties of these transformed keratinocytes.

Keywords: TGF-β1; MMP-9; Keratinocytes; Ras–ERK; Migration

Degradation of the basal membrane and the collagenous extracellular matrix (ECM) is a critical step in tumor invasion [1]. Matrix metalloproteinases (MMPs), a family of zinc-dependent neutral endopeptidases known for their ability to cleave one or several ECM constituents as well as non-matrix proteins, play an important role in this process [2]. In particular, the 92 kDa enzyme (MMP-9) is expressed in a variety of cancers and has been suggested to be directly involved in tumor dissemination [3].

The expression of MMP-9 is modulated by cytokines and growth factors which induce cellular responses activating intracellular signaling cascades including mitogen-activated protein kinases (MAPKs) or JAK–STAT pathways [4]. Many cellular mechanisms that play a role in the regulation of MMP-9 activity have been proposed. In human glioma cells, it has been demonstrated that actin polymerization transduces the signals that modulate the expression of MMP-9 [5]. During macrophage differentiation it has been demonstrated that fibronectin-mediated cell adhesion is required for MMP-9 induction [6]. Some authors have also demonstrated that rat mucosal keratinocytes respond by increasing the expression of MMP-9 to the stimulus of a diversity of growth factors including IL-1α and β, EGF and TGF-α [7]. The induction of MMP-9 by growth factors seems to operate mainly through transcriptional stimuli that target the MMP-9 gene promoter [8]. The induction of MMP-9 by *ras* oncogen has been proposed in a human ovarian cancer cell line using an antisense approach [9]. Also, cells transfected with the activated form of *H-ras* stimulate over 10-fold the activity of MMP-9 promoter operating a MEK-1-independent signaling pathway [10].

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Previous work of our laboratory has demonstrated that in mouse transformed keratinocytes (PDV cell line), TGF-β1 induces an epithelial–mesenchymal transdifferentiation associated with the loss of the differentiated phenotype of the tumors and the acquisition of metastatic abilities [11,12]. Culturing this cell line in the presence of TGF-β1, we demonstrated that the factor enhanced cell invasiveness and the production of urokinase-like plasminogen activator (u-PA) [13] in a phenomenon that required the activation of the Ras–ERK 1,2 pathway [14].

TGF-β1 exerts a dual action in carcinogenesis, acting as a suppressor of tumor formation in early stages of carcinogenesis and stimulating invasiveness and metastasis of carcinoma cells later on [15]. It has been demonstrated that TGF-β1 signals through transmembrane serine/threonine kinase receptors that activate mainly either the Smad or the Ras–MAPK pathway [16,17]. At present, the signaling pathway that mediates the TGF-β1-dependent stimulus of MMP-9 production in transformed keratinocytes is not well established. In human prostate cancer cell lines, it has been shown that TGF-β1 induces MMP-9 proenzyme, but this induction does not result from a direct effect on gene transcription but, instead, by increasing MMP-9 mRNA stability [18].

In this work we investigate the role of the TGF-β1-stimulated signaling pathway on MMP-9 regulation and the importance of TGF-β1-enhanced activity on keratinocyte migration. Our results demonstrate that TGF-β1-dependent MMP-9 production is regulated through the Ras–ERK MAPK pathway and also provide evidence that MMP-9 plays an important role in factor-dependent cell migration and invasion.

Materials and methods

Cell cultures and treatment conditions. Cell lines used in this study (MCA3D, PDV, and CarC) have been described elsewhere [11]. Cells were cultured in Ham's F-12 medium supplemented with aminoacids and vitamins (Gibco, Paisley, Scotland), 10% fetal bovine serum (FBS), and 80 μg/ml Gentamycin) and maintained on plastic flasks at 37 °C in a 5% CO_2 humidified atmosphere.

For TGF-β1 treatment, human recombinant TGF-β1 (Calbiochem–Novabiochem, La Jolla, CA) was used at a final concentration of 10 ng/ml for the times indicated. MAPK inhibitors PD98059, SB203580 (Calbiochem, La Jolla, CA), and Curcumin (Sigma, St. Louis, MO) were added to cells 30 min before the TGF-β1 treatment. GM6001 (llomastat) was obtained from Chemicom, Temecula, CA.

Stable transfection procedure. MCA3D cells were transfected with 1 μg of either the pUSEamp (+) plasmid containing an active form of Ha-Ras (Q61L) or the empty vector (Upstate Biotechnology, New York). PDV cells were transfected with the pMEXneo plasmid containing a dominant negative Ha-Ras gene (Ras N17) [14], or empty vector, using Lipofectamine-Plus (Gibco, Rockville, MD) following manufacturer's protocol. Stable cell clones were selected by adding 400 μg/ml G418 in culture medium. After two weeks of selection, G418-resistant clones were isolated by cloning rings.

Western blot and activation of ERK1,2. Semi confluent cultures were serum starved by 4 h before TGF-β1 stimulus. Thereafter, cells were lysed with 300 μl of lysis buffer (100 mM phosphate buffer at pH 7.2, 1% NP-40, 150 mM NaCl, 5 mM EDTA, 10 mM β-glicerophosphate, 1 mM sodium orthovanadate, 5 mM NaF, 10 μg/ml leupeptin, 1 μg/ml pepstatin, and 1 mM PMSF). After boiling, lysates were separated on a 10% SDS–PAGE gel [19]. Afterwards, gels were blotted onto PVDF membrane (BioRad, Hercules, CA) and ERK1,2 activation was assayed as previously described [14] using an antibody that recognized the phosphorylated form of ERK,2 (Santa Cruz Biothec, Santa Cruz, CA). Duplicated membranes were probed with antibody to both the phosphorylated and the unphosphorylated forms of ERK1,2 to verify equal loading.

Oligodeoxinucleotide treatment. Semi confluent cultures of PDV cells were pre-treated for 48 h with 5 μM of either ERK antisense (5′-GCC GCC GCC GCC GCC AT-3′) or sense (5′-ATG GCG GCG GCG GCG GC-3′) phosphorotioate-modified oligodeoxinucleotides synthesized and purified by Isogen Bioscience (Maarssen, The Netherlands) as previously described [14]. Afterwards, cells were changed into serum-free medium, exposed to TGF-β1 (10 ng/ml) for 24 h. Thereafter, conditioned medium was harvested for measurements of MMP-9 activity.

Zimogram for gelatinase. MMP activity was assayed in serum-free media conditioned by 24 h in the presence or absence of TGF-β1 and evaluated by SDS–PAGE zymograms containing 1 mg of gelatin per milliliter of gel as previously reported [14].

MMP-9 promoter activity. Cells (3×10^4) were co-transfected with 500 ng of MMP-9 promoter-luciferase plasmid and 200 ng of RSV-Bgal as an internal control, using Lipofectamine Plus (Gibco, Rockville, MD). After transfection, cells were cultured 24 h with complete medium before TGF-β1 stimulation (10 ng/ml), and reporter activities were analyzed after another 24 h. Firefly luciferase activities were standardized for β-galactosidase activity.

Migration and invasion assays. The capacity of the cells to invade was evaluated using a Matrigel-coated Transwell system and cellular migration was assayed by an in vitro wound assay, as previously described [14].

Results

Basal levels of MMP9 production and TGF-β1-mediated stimulus of MMP-9 secretion and promoter activity in skin carcinoma cell lines

In this study, we used three mouse epidermal cell lines, MCA3D, PDV, and CarC, that express an increasingly invasive phenotype that is directly associated with their basal MMP-9 production in culture. Fig. 1A shows that only the spindle CarC cell line (the most invasive) produces both the 72 kDa MMP-2 and the 105 kDa mouse MMP-9. The ability of these non-stimulated epidermal cells to produce MMP-9 was also evaluated by measuring the basal MMP-9 promoter activity. To do this, the three cell lines were transiently transfected with a vector containing the luciferase gene under the control of the MMP-9 promoter. As shown in Fig. 1B, the basal promoter activity mirrored the expression of malignancy and MMP-9 production, i.e. only CarC cells show a significant promoter activity. A 24 h TGF-β1 treatment produces no change in MCA3D cells, a significant enhancement of MMP-9 activity in PDV cells and no further increase in MMP-9 activity—

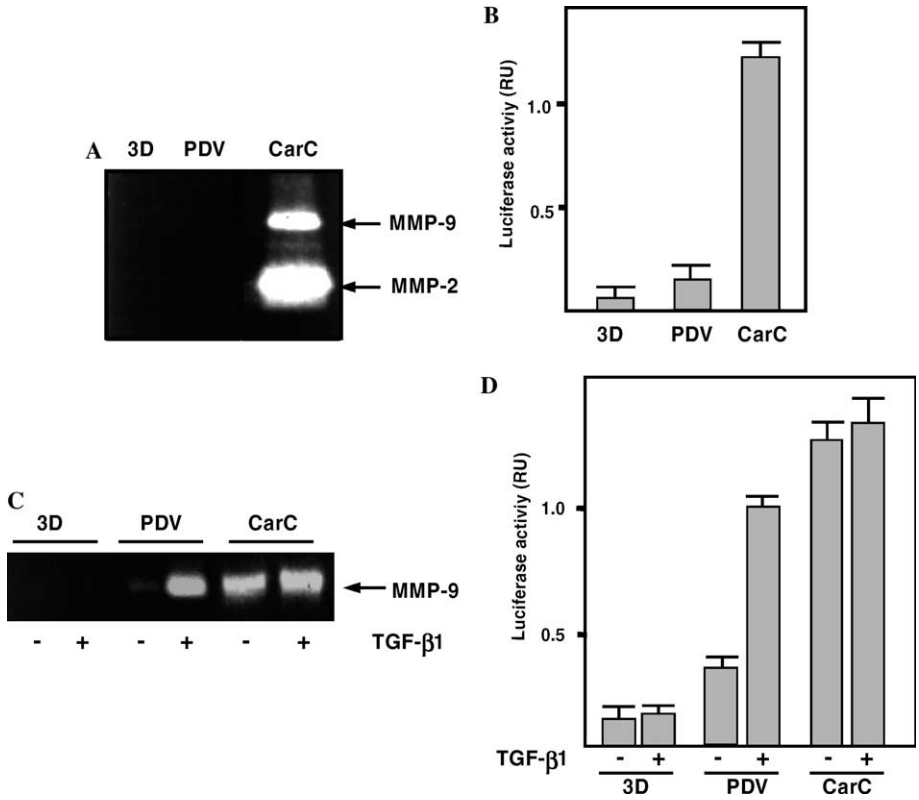

Fig. 1. Basal levels of MMP9 production and TGF-β1-mediated stimulus of MMP-9 secretion and promoter activity in skin carcinoma cell lines. (A) MMP-9 activity measured by zymography in conditioned media by non-tumorigenic (MCA3D), transformed keratinocytes (PDV), and highly invasive (CarC) cells. (B) Basal MMP-9 promoter activity in the same cells assayed in (A), measured as luciferase activity **C** MMP-9 activity in MCA3D and PDV cells stimulated by 10 ng/ml TGF-β1 for 24 h. (D) TGF-β1-stimulated MMP-9 promoter activity in MCA3D, PDV, and CarC cells measured as luciferase activity.

already high—in CarC cells (Fig. 1C). We also investigated if MMP-9 promoter activity was stimulated by TGF-β1 treatment. As Fig. 1D shows, only PDV cells respond to the growth factor by activating the MMP-9 promoter. TGF-β1 treatment of spindle shape CarC cells does not produce significant effects in their capacity to produce MMP-9 nor in the MMP-9 promoter response.

Effects of a dominant negative Ras-N17 mutant gene in TGF-β1-mediated stimulation of MMP-9 secretion and promoter activity

To investigate the role of Ras activation in the TGF-β1-induced MMP-9 production, a vector containing a dominant-negative RasN17 mutant gene was transfected into the TGF-β1-responsive PDV cells. As Fig. 2A shows, TGF-β1 treatment of parental PDV cells or of cells transfected with empty vector (EV) induces MMP-9 production. This stimulation of enzyme production was almost completely blocked in RasN17 transfected cells (referred to as RN2 and RN7 clones). Measurement of the MMP-9 promoter activity in RasN17 transfected cells shows that EV and RasN17 clones display similar levels of MMP-9 promoter activity in the absence of TGF-β1 treatment. However, while the addition of factor increased the MMP-9 promoter activity in EV cells 3-fold, no effect was observed in RasN17 transfected cells (Fig. 2B). Downstream effects of RasN17 transfection were evaluated measuring the activation of ERK1,2 in transfected clones. Fig. 2C shows that parental and EV PDV cells show a strong response to a 15-min TGF-β1 treatment of ERK1,2 activation while RN2 and 7 cells does not respond to the stimulus.

To further assess the role of Ras on MMP-9 production we transfected the non-tumorigenic MCA3D cell line, that, unlike PDV cells, express the normal version of Ras [20], with an oncogenic form of Ras (Q61L). Fig. 2D shows that two positive clones (R7 and R10) acquire the capacity to produce MMP-9 that is absent in parental MCA3D cells. This result was further sustained by measurement of MMP-9 promoter activity; R7 and R10 cells express five and four times, respectively, more luciferase activity than parental MCA3D cells (Fig. 2E).

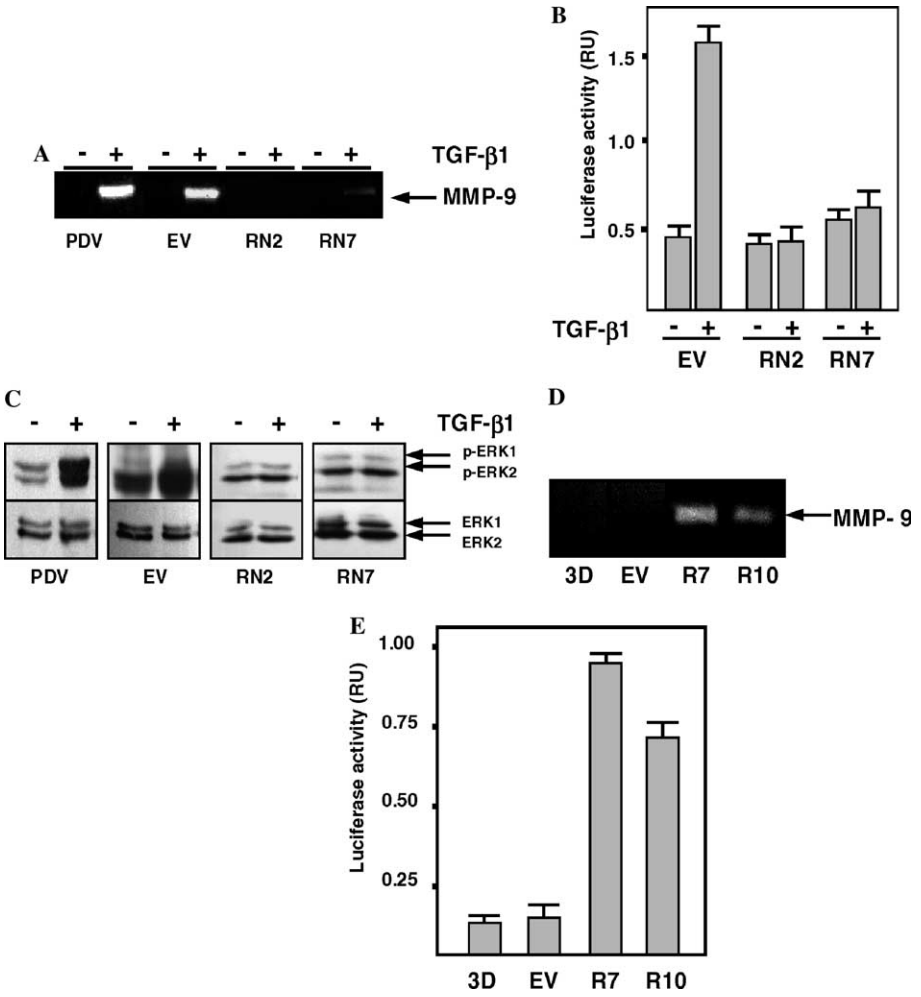

Fig. 2. Effects of a dominant negative Ras-N17 mutant gene in TGF-β1-mediated stimulation of MMP-9 secretion and promoter activity. (A) Zymographic analysis of secreted MMP-9 activity of parental PDV cells (PDV), cells transfected with the empty vector (EV), or transfected with Ras-N17 dominant negative mutant gene (RN2 and RN7). (B) MMP-9 promoter activity assayed in the same cells as in (A). (C) Western blot of ERK activation in cells after a 15-min treatment with 10 ng/ml TGF-β1. (D) Zymographic analysis of MMP-9 activity of non-tumorigenic parental MCA3D, cells transfected with empty vector (EV), and cells transfected with an active form of Q61L-Ras (R7 and R10). (E) MMP-9 promoter activity of non-tumorigenic parental MCA3D, cells transfected with empty vector (EV) and cells transfected with an active form of Q61L-Ras (R7 and R10).

ERK1,2 MAP kinase signaling pathway is involved in TGF-β1-mediated modulation of MMP-9

Previous results of our group and the results presented in Fig. 2C strongly suggest that transformed PDV keratinocytes respond to TGF-β1 by activating the ERK1,2 MAP kinase pathway [14,21]. To assess the involvement of ERK1,2 MAP kinase signaling pathway in the TGF-β1-mediated activation of MMP-9 production, we tested PD98059, an inhibitor of MEK1,2 activity, SB203580, a p-38 inhibitor and curcumin, a Jun kinase inhibitor, on the enzyme production. As Fig. 3A shows, only incubation of PDV cells with 25 μM PD98059 blocked the TGF-β1-mediated MMP-9 production. Addition of SB203580 or curcumin does not affect the TGF-β1-mediated MMP-9 stimulation. The inhibitory effect of PD98059 on MMP-9 production is reflected by the inhibition of ERK1,2 activation (Fig. 3B).

To further assess the direct involvement of the ERK1,2 MAPK in TGF-β1-mediated MMP-9 stimulation, we pretreated PDV cells with antisense oligodeoxynucleotides (AS) directed against ERK1,2 isoforms. As we have previously demonstrated, this procedure induces about 90% depletion of ERK1,2 proteins at the cellular level [14]. As Fig. 3C shows, 5 μM AS oligodeoxinucleotide strongly blocked the TGF-β1-mediated MMP-9 production while sense (S) oligo showed no difference with respect to parental cells. The participation of the ERK1,2 pathway in MMP-9 production was also assayed in the strongly invasive CarC cells that do not respond to TGF-β1 by increasing MMP-9 production,

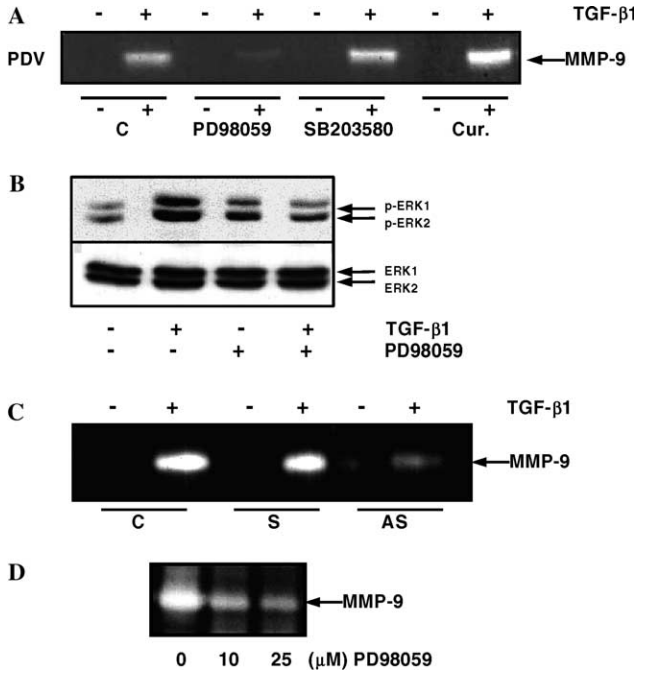

Fig. 3. Involvement of MAP kinase signal pathway in TGF-β1-mediated modulation of MMP-9. (A) Zymographic analysis TGF-β1-stimulated MMP-9 activity in PDV cells treated with MEK 1,2 inhibitor (PD 098059), p38 inhibitor (SB 203580), and JNK inhibitor curcumin (cur). (B) Western blot of ERK activation in cells after a 15-min treatment with TGF-β1 in the presence or absence of PD 098059. (C) Zymographic analysis of TGF-β1-stimulated MMP-9 activity in parental PDV cells (C) and cells pre-treated with sense (S) or anti-sense (AS) oligodeoxinucleotides for ERK1,2. (D) Zymographic analysis of MMP-9 activity of CarC cells in the presence of 10 and 25 μM PD 098059.

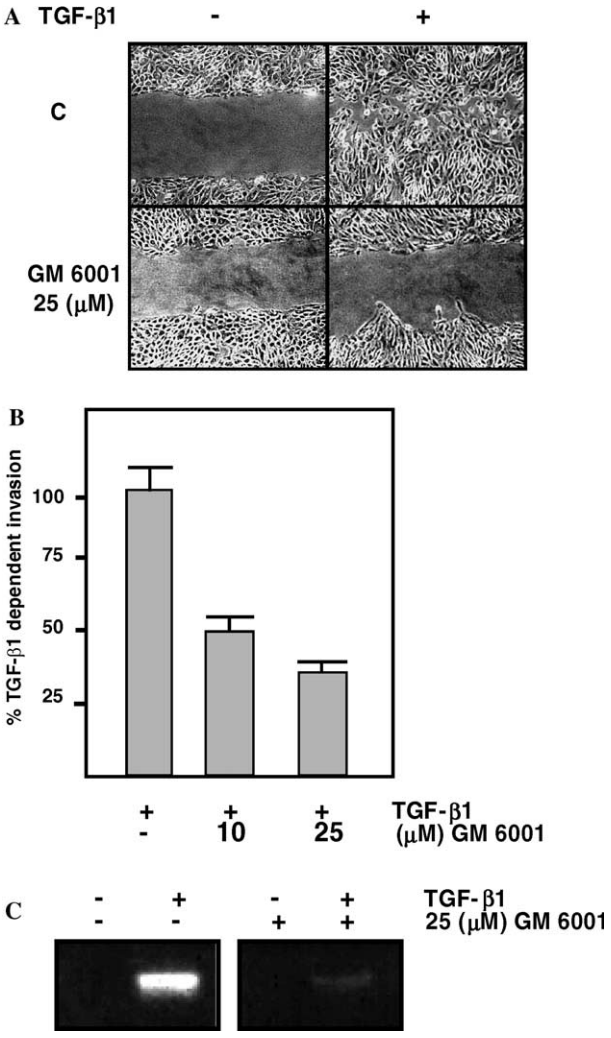

Fig. 4. TGF-β1-mediated enhancement of motility and invasiveness of PDV cells are blocked by a MMP-9 inhibitor. (A) TGF-β1-stimulated wound migration assay in the presence or absence of 25 μM MMP-9 inhibitor GM6001. (B) Inhibition of TGF-β1-stimulated Matrigel invasion assay by 10 and 25 μM GM6001. (C) In vitro inhibition of TGF-β1-stimulated MMP-9 activity by 25 μM GM6001.

and, as shown in Fig. 1A, secrete a high amount of enzyme activity in the basal state. Fig. 4D shows that a 10 and 25 μM PD98059 treatment inhibits in a dose-dependant manner the constitutive production of MMP-9.

The TGF-β1-mediated enhancement of motility and invasiveness in PDV cells is blocked by an MMP-9 inhibitor

To evaluate the role of MMP-9 in the invasive and migratory abilities of PDV cells, we exposed subject cells to a co-treatment with TGF-β1 and GM6001, a potent inhibitor of MMPs, [22]. PDV cells were stimulated to migrate in a wound healing assay and to invade through a Matrigel layer [14]. As Fig. 4A shows, TGF-β1 treatment induces control PDV cells to quickly close the wound area by 36 h of addition of the growth factor. However, treatment with 25 μM GM6001 (Ilomastat) strongly inhibited the migration of PDV cells stimulated by TGF-β1. Next, we studied whether GM6001 inhibited the invasive response of PDV cells to TGF-β1. As shown in Fig. 4B, GM6001 at concentrations of 10 and 25 μM reduced by about 48% and 67%, respectively, the TGF-β1-stimulated invasive capacity of PDV as measured by the Matrigel-coated Transwell assay. As Fig. 4C shows, TGF-β1-induced MMP-9 activity in PDV cells was almost completely abolished by 25 μM GM6001.

Discussion

The expression of MMP-9 has been implicated in cell motility and invasion and represents one of the major features of the expression of the invasive phenotype [3]. It has been proposed that this process is regulated in a paracrine manner by growth factors and cytokines secreted by tumor infiltrating inflammatory cells as well as tumor stromal cells [4]. Soluble factors such as epider-

mal growth factor (EGF) and scatter factor/hepatocyte growth factor (SF/HGF), both ligands of tyrosine kinase receptors, have been implicated in MMP-9 expression stimuli and in the expression of migratory properties [23]. These factors signal mainly through the MAPK pathway and it has been demonstrated that a sustained ERK activation is required for growth factor-dependent migration and MMP-9 induction [24].

In this report we present evidence that strongly suggest that TGF-β1 stimulates the production of MMP-9 in transformed PDV keratinocytes, thus activating the Ras–MAPK pathway. These results are in agreement with previous data from our laboratory showing that TGF-β1 was able to stimulate u-PA production and cellular invasiveness in transformed keratinocytes by activating the same route of signaling [13,14]. Also, further results of our group show that in PDV cells, TGF-β1 stimulates phosphorylation and nuclear translocation of ERK1,2 as a consequence of a rapid activation of H-Ras [21]. The direct TGF-β1-mediated activation of Ras–MAPK pathway (independent of other factors) has been also broadly demonstrated and reviewed by Hartsough and Mulder [17].

The present results show that in mouse keratinocytes, the expression of MMP-9 is directly associated with cellular malignancy, being undetectable in non-tumorigenic and transformed cells (MCA3D and PDV cells) and strongly expressed in highly anaplastic cells (CarC). These differences are associated with marked differences in promoter activity among the cell lines (Figs. 1A and B). A 24-h treatment with TGF-β1 stimulates MMP-9 production and promoter activity in transformed keratinocytes (PDV) while non-tumorigenic MCD 3D cells do not respond to the growth factor stimulus. Spindle-shaped invasive CarC cells already showing high levels of MMP-9 promoter activity under basal conditions, show no further increase in response to TGF-β1.

The growth factor stimuli on MMP-9 production and promoter activity were completely blocked in PDV cells transfected with a dominant-negative RasN17 mutant gene. These clones were also unable to activate ERK1,2 after the TGF-β1 stimulus (Figs. 2A–C). We assessed the relevance of *ras* expression in MMP-9 production by transfecting non-tumorigenic keratinocytes (MCA3D cells) with an oncogenic form of Ras (Q61L), which is presumed to maintain the molecule in a permanently active form. In this case, positive clones (R7 and R10) express MMP-9 and activate the enzyme promoter even in the absence of any added factor (Figs. 2D and E). Similar results have been reported in Ras-transfected NIH3T3 cells that express MMP-9 [25] and in OVCAR cells transfected with an activated version of Ha-*ras*. [10].

The requirement of activation of the MAPK pathway in the TGF-β1-mediated MMP-9 production was supported by experiments using MAPKs specific inhibitors and antisense oligonucleotides directed against ERK1,2. Both experimental approaches clearly indicate that the MAPK/ERK1,2 pathway represents an important route of signaling that allows transformed keratinocytes to produce MMP-9 under TGF-β1 stimulus. The regulatory mechanisms responsible for the expression of MMPs are still poorly understood. Some authors have proposed that protein kinases belonging to the MAPK family are involved in the regulation of MMP-9 production through the activation of ERKs, JNKs, and p38 pathway [26–28] Our data using specific inhibitors, demonstrate that JNK and p38 pathways are not involved in MMP-9 production in mouse keratinocytes (Fig. 3A). Other workers have shown that cells that express a constitutive version of MEK1 acquire a metastatic potential and a modest enhancement of MMP-9 expression [29]. Interestingly, the MMP-9 production in the highly invasive CarC cell line, that express a mutant version of *H-ras* and the loss of the normal allele [30,31], was also inhibited with the MEK inhibitor PD 098059 in a dose-dependent manner. These results demonstrate that MAPK/ERK1,2 pathway is also involved in the basal production of MMP-9 by these cells.

The contribution of MMP-9 to the acquisition of metastatic potential has been largely demonstrated [4]. Our results show that the TGF-β1-dependent stimulation of the invasive capacity of PDV cells can be reverted by GM6001 (Ilomastat), a specific inhibitor of MMP activity (Fig. 4). Also, the MMP inhibitor was able to significantly block the acquired motile properties of transformed keratinocytes (Fig. 4A) suggesting that the gelatinase activity is an important factor in these two phenomena.

Taken together, our results are in line with two main propositions: (i) MMP-9 expression is a significant feature in the TGF-β1-dependent epithelial–mesenchymal transdifferentiation in transformed keratinocytes that allow cells to migrate and invade, and (ii) the effect of TGF-β1 in this process depends of the functionality of the Ras–ERK1,2 MAPK pathway. These results also suggest that the selective inhibition of either Ras–ERK1,2 or MMP-9 activity may serve as a specific target to suppress TGF-β1-induced tumoral progression.

Acknowledgments

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