Relationship Between Cell-Associated Matrix Metalloproteinase 9 and Psoriatic Keratinocyte Growth

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Primary cultures of psoriatic keratinocytes proliferated at a higher rate and produced lower amounts of matrix metalloproteinase 9 than normal keratinocytes cultured under similar conditions. Supplementation of psoriatic keratinocyte cell culture medium with batimastat or the use of a matrix metalloproteinase 9 blocking antibody further stimulated psoriatic keratinocyte growth. An increase in intracellular ceramide level enhanced matrix metalloproteinase 9 production and inhibited cell proliferation in parallel. Whether cells were treated with sphingomyelinase or not, however, conditioned media from psoriatic keratinocytes contained higher levels of tissue inhibitor of metalloproteinase-1 compared with matrix metalloproteinase 9 and secreted only the proenzyme form. Pro-matrix metalloproteinase 9, as well as active matrix metalloproteinase 9, was identified in membrane preparations of psoriatic keratinocytes, and enzyme amounts were greatly elevated following sphingomyelinase action. As (i) tissue inhibitor of metalloproteinase-1 antibody nearly totally abrogated keratinocyte growth and (ii) complexes of tissue inhibitor of metalloproteinase-1 and matrix metalloproteinase 9 were recovered in membrane extracts of sphingomyelinase-treated psoriatic keratinocytes, we postulate that an increased level of cell-associated matrix metalloproteinase 9 might compete for tissue inhibitor of metalloproteinase-1 binding to its receptor. As a consequence, the increased levels of matrix metalloproteinase 9 will decrease keratinocyte growth. ***Key words: ceramide/keratinocyte/matrix metalloproteinase/psoriasis. J Invest Dermatol 115:213–218, 2000***

Matrix metalloproteinases (MMPs) belong to a family of zinc-dependent neutral proteinases capable of degrading extracellular matrix components (Nagase and Woessner, 1999). Collectively, MMPs have been shown to participate in the migration of keratinocytes (Madlener *et al*, 1998). Interstitial collagenase plays a pivotal function for keratinocyte migration over type I collagen during wound healing (Petersen *et al*, 1989). A recent investigation revealed that gelatinase A was localized in laminin-5-rich structures at the advanced edge of mucosal keratinocytes (Mäkelä *et al*, 1999). The important role of gelatinase B (MMP-9) in the invasive function of keratinocytes was also delineated and growth-factor-mediated migration of keratinocytes was found to coincide with overproduction of this endopeptidase (McCawley *et al*, 1998).

MMP activity is controlled by specific tissue inhibitors, i.e., TIMPs (Gomez *et al*, 1997). TIMPs are multifunctional proteins. Besides interacting with the active site of MMPs, TIMP-1 and TIMP-2 can bind to the hemopexin-like domain of pro-MMP-9 and pro-MMP-2, respectively. We initially showed that TIMP-1 could bind to human skin keratinocytes in a receptor-mediated pathway, leading to the stimulation of cell proliferation, both in primary cultures, on a plastic support, and in skin equivalent (Bertaux *et al*, 1991). TIMP-1, as well as TIMP-2, were further found to exhibit growth-promoting activity for a wide range of cells (Hayakawa *et al*, 1992), and TIMP-1 was considered to be an autocrine growth factor in scleroderma fibroblasts (Kikushi *et al*, 1997).

We recently reported that the ceramide-mediated inhibition of human keratinocyte growth coincided with an overproduction of MMP-9 (Buisson-Legendre *et al*, 1999). In this investigation, we demonstrates the importance of MMP-9 in the control of psoriatic keratinocyte proliferation. The data support the contention that cell-associated MMP-9 might compete for TIMP-1 binding to its receptor leading to the inhibition of psoriatic keratinocyte growth.

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Abbreviations: MMP, matrix metalloproteinase; TIMP, tissue inhibitor of metalloproteinase; Smase, sphingomyelinase.

MATERIALS AND METHODS

Materials Neutral sphingomyelinase (Smase, from *Staphylococcus aureus*), N-acetyl-D-sphingosine (C2 ceramide), N-hexanoyl-D-sphingosine (C6 ceramide), and gelatin (from porcine skin) were obtained from Sigma (St. Quentin Fallavier, France). Recombinant MMP-9 (r-MMP-9) and the fluorogenic substrate (7-methoxycoumarin-4 yl) acetyl-L-Pro-Leu-Gly-Leu [N-3-(2,4 dinitrophenyl)-L-2,3-diaminopropionyl]-Ala-Arg-NH_2 (Mca-Pro-Leu-Gly-Leu Dpa-Ala-Arg-NH_2) were from Calbiochem (France Biochem, Meudon, France). Batimastat, generously provided by British Biotechnology (Oxford, U.K.), was dissolved in ethanol. The final concentration of ethanol in the culture medium never exceeded 0.01%. Enzyme-linked immunosorbent assay (ELISA) kits for quantitative determination of MMP-9 and TIMP-1 were purchased from Calbiochem-Oncogene Research Products (France Biochem). The MMP-9 Pro-Blot Western Blotting Kit, which recognizes only the latent form of enzyme under reducing conditions, and the MMP-9 monoclonal blocking antibody (ref: IM09L) came from Calbiochem-Oncogene Research Products.

Characteristics of psoriatic patients and controls Patients (age range 30–78 y) had a history of stable psoriasis vulgaris of at least 6 mo duration

involving 20%–40% of total skin surface area. No evidence of active bacterial or viral superinfections was noticeable and only patients who had not been under treatment with methotrexate, cyclosporine, or phototherapy for at least 3 mo or with local steroids for at least a week were taken into consideration. A punch biopsy specimen (3–6 mm) was taken from a typical skin lesion for each patient. For one patient, a biopsy was also taken from nonlesional skin. All patients and controls gave their consent and the study was approved by the local ethical committee.

Cell cultures Human keratinocytes were isolated from skin biopsies of healthy individuals (n = 3; age range 30–47 y) and psoriatic patients (n = 5) and cultured in keratinocyte serum-free medium containing 0.09 mM Ca^{2+} and supplemented with 20 ng per ml bovine pituitary extract and 0.2 ng per ml recombinant epidermal growth factor (Gibco-BRL, Life Technologies SARL, Cergy Pontoise, France), according to the manufacturer's instructions. The concentration of keratinocytes was determined using a Mallasez cell and primary cells were seeded into T-75 flasks at a cell density of approximately 2×10^6 cells per flask in 10 ml keratinocyte serum-free medium. On reaching 75% confluency, keratinocytes were trypsinized and plated into 96 well plates at an initial seeding density of $(1–2) \times 10^4$ cells per well and grown in keratinocyte serum-free medium. On day 1 of culture, the keratinocyte serum-free medium was supplemented with Smase (100 mU per ml) or cell permeable C2 or C6 ceramides (1–5 μM). Batimastat (100 nM), MMP-9 antibody (100 ng per ml), and anti-TIMP-1 antibody (1–5 μg per ml) were also added to the keratinocyte culture medium after 1 d of culture.

Viability and cell proliferation Keratinocyte serum-free medium was supplemented with either Smase (100 mU per ml), C2 (1–5 μM), or C6 (1–5 μM) ceramide. Keratinocyte viability, following ceramide or Smase treatment, was assessed by the trypan blue exclusion technique. After incubation for 24–96 h, the cells were washed twice with phosphate-buffered saline (PBS) and cell proliferation was determined by the crystal violet assay as described by Kueng *et al* (1989).

Variation of intracellular ceramide level Following Smase treatment, ceramide concentration was determined by the diacylglycerol kinase assay (Buisson-Legendre *et al*, 1999).

Preparation of serum-free conditioned media and plasma membrane extracts At different times of keratinocyte growth (from 1 to 5 d), conditioned media were harvested and centrifuged at 500*g* for 10 min at 20°C to remove cell debris. Cell layers were washed three times with cold 50 mM Tris/HCl, 150 mM NaCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$ (TBS), pH 7.6, scraped with 2×1 ml cold PBS, and centrifuged for 5 min at 1000*g*. After discarding the supernatant, the pellet was extracted with TBS containing 1.5% Triton X-114 for 15 min at 4°C. Cell extracts were centrifuged at 10,000*g* to remove insoluble particles and the supernatants were phase partitioned into detergent and aqueous phases following incubation at 37°C for 5 min. Phases were separated by centrifugation at 5000*g* for 1 min. To concentrate gelatinases, either TBS-diluted detergent phase or aqueous phase was incubated with gelatin-Sepharose beads (Sigma). Enzymes were eluted from beads with 2×20 μl of electrophoresis sample buffer.

MMP and TIMP determination

Gelatin zymography and reverse zymography Sodium dodecyl sulfate (SDS) substrate polyacrylamide gel electrophoresis (PAGE) was performed as previously described (Brassart *et al*, 1998). Gels for zymography comprised 0.1% (wt/vol) gelatin and 10% (wt/vol) polyacrylamide, and for reverse zymography 0.1% (wt/vol) gelatin, 15% (wt/vol) polyacrylamide, and 20 ng per ml purified activated MMP-2 (Calbiochem, France Biochem). Zymography revealed proteolytic activity, which appeared as clear zones, demonstrating lysis of the gelatin in the gel, against the blue background of stained gelatin. Reverse zymography revealed inhibitory activity that appeared as blue zones, demonstrating inhibition of lysis of the gelatin in the gel, against a clear background.

Fluorometric activity assay for MMPs The enzymatic activity of MMPs in keratinocyte-cell-conditioned medium was assayed using the fluorescence quenching substrate Mca-Pro-Leu-Gly-Leu Dpa-Ala-Arg-NH_2, dissolved in dimethylsulfoxide (DMSO). Briefly, 100 μl of conditioned medium was mixed with 400 μl of 50 mM HEPES, 150 mM NaCl, 5 mM $CaCl_2$, pH 7.5, and DMSO (1% vol/vol), containing 2 μM substrate. Each assay was carried out at 37°C for 24 h, in the dark. After incubation, substrate hydrolysis was measured using a Perkin Elmer LS 50B spectrofluorimeter (Lyon, France), with excitation and emission wavelengths set at 325 nm and 387 nm, respectively.

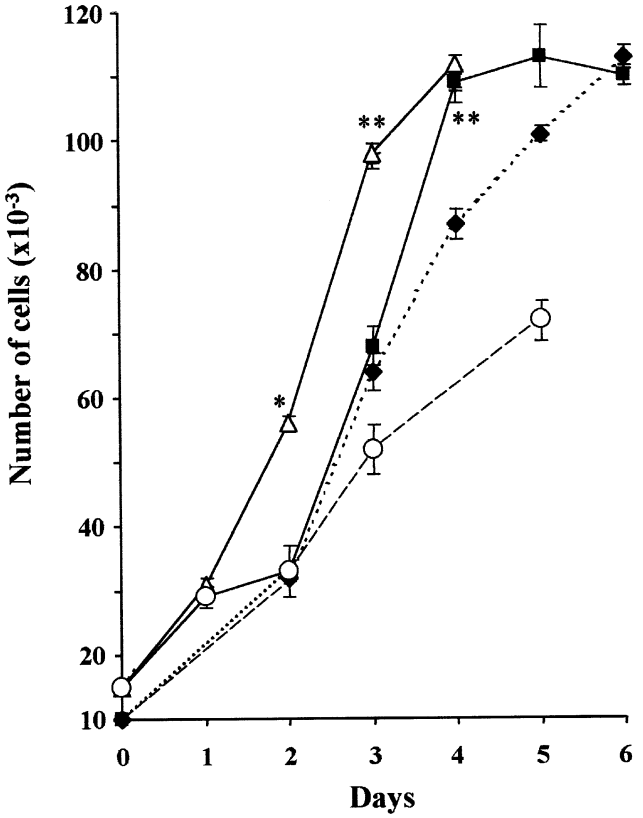

Figure 1. Growth rate of first passage keratinocytes from two psoriatic patients and two healthy individuals. ■, Psoriatic patient, aged 38 y; △, psoriatic patient, aged 35 y; ◆, healthy individual, aged 30 y; ○, healthy individual, aged 47 y. Cell counts represent the mean ± SEM of three triplicate samples. *Significantly different from both control values at $p < 0.01$; **$p < 0.001$.

Western blotting Membrane extracts were transferred from SDS gels to nitrocellulose membrane (Bio-Rad, Ivry/Seine, France). Under reducing conditions, pro-MMP-9, but not active MMP-9, was stained by the human MMP-9 Pro-Blot Western Blotting Kit with a monoclonal antibody directed against enzyme pro-domain according to the manufacturer's instructions (Calbiochem-Oncogene Research Products). Both forms (pro-MMP-9 and MMP-9) were detected by this antibody when membrane extracts were separated by SDS-PAGE under nonreducing conditions.

Quantification of MMP-9 and TIMP-1 by ELISA The manufacturer's instructions for assay protocols and data processing were followed. Results were expressed as picograms of enzyme or inhibitor per cell.

Statistical analysis of data Experiments were reproduced at least three times using three control and four different psoriatic keratinocyte cell strains. Statistical differences were analyzed using the Student-Fisher *t* test.

RESULTS

Growth rate and MMP-9 production of human keratinocytes: comparison between control and psoriatic cells Human keratinocytes from healthy individuals or psoriatic patients, at first passage, were seeded at $(1–2) \times 10^4$ cell density and allowed to proliferate in keratinocyte serum-free medium for 1–6 d. Psoriatic cells were found to grow at a faster rate than their normal counterparts, for all pairs of keratinocyte isolates analyzed, but differences became significant only at a later stage of culture (**Fig 1**).

As we recently reported that MMP-9 production was related to keratinocyte growth (Buisson-Legendre *et al*, 1999), we determined the levels of secreted MMP-9 as a function of cell growth for both control and psoriatic keratinocytes. Gelatin zymography analysis indicated that psoriatic keratinocytes secreted lower amounts of MMP-9 than controls (**Fig 2*A***). Production of MMP-9 protein was quantified by ELISA (**Fig 2*B***). On average,

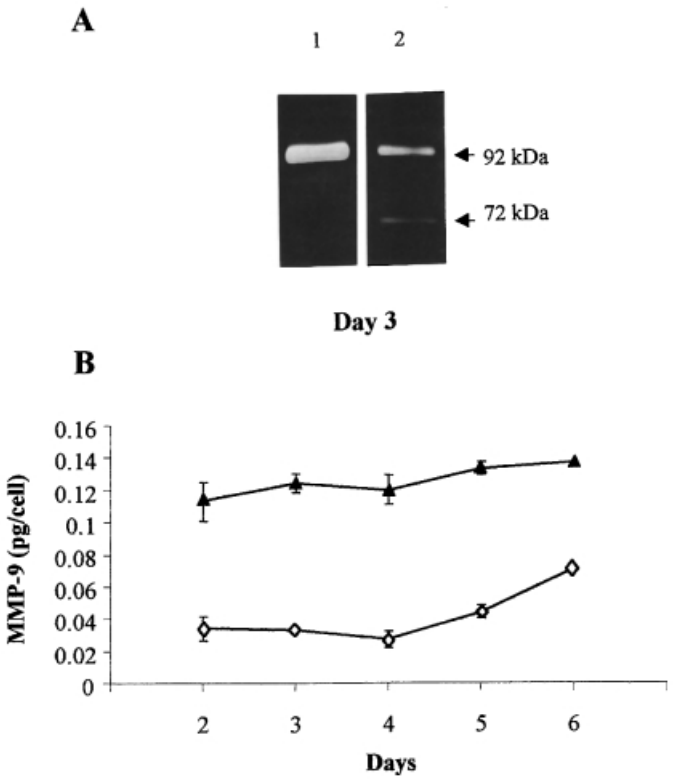

Figure 2. MMP-9 production by keratinocytes as a function of cell growth. (*A*) Gelatin zymography: *lane 1*, conditioned medium from control keratinocytes (age 30 y), 3 d of culture; *lane 2*, conditioned medium from psoriatic keratinocytes (age 38 y), 3 d of culture. (*B*) ELISA: ▲, conditioned medium from control keratinocytes (age 30 y); ◇, conditioned medium from psoriatic keratinocytes (age 38 y). Data are means of three separate determinations. Bars represent SEM values. Similar differences between control and psoriatic keratinocytes have been obtained with two other pairs.

control keratinocytes from three different strains secreted 0.18 ± 0.036 and 0.15 ± 0.03 pg MMP-9 per cell at day 3 and day 4 of culture, respectively. Psoriatic keratinocytes secreted 50%–20% of control MMP-9 levels (**Fig 2*B***).

Modulating MMP-9 activity and/or production influenced the growth of psoriatic keratinocytes To more directly substantiate the inverse relationship between the extent of growth rate and MMP-9 levels, psoriatic keratinocytes were first cultured in the presence of batimastat (100 nM), a peptide hydroxamate MMP inhibitor that binds to the active site of metallo-enzyme (Botos *et al*, 1996), or MMP-9 blocking antibody. Both compounds were found to significantly increase cell proliferation (**Fig 3**).

Alternatively, keratinocyte serum-free medium was supplemented with truncated ceramides or Smase to trigger the intracellular ceramide pathway (Hannun, 1996; Testi, 1996). When psoriatic keratinocytes were treated with C2 ceramide (1 μM) or C6 ceramide (1–5 μM), a significant 10%–20% decrease in keratinocyte growth was observed (**Fig 4**). At these concentrations, truncated ceramides exerted no influence on the proliferation of keratinocytes from healthy individuals (Buisson-Legendre *et al*, 1999). As in control keratinocytes, Smase treatment led to a 3-fold increase in intracellular ceramide concentration and induced a significant inhibitory effect on psoriatic keratinocyte proliferation (**Fig 4**). Inhibition of cell growth, by triggering the ceramide pathway, was associated with a marked increase in MMP-9 production (**Fig 5*A*,** *inset*). Depending on the psoriatic patient, a 2–7-fold enhancement in MMP-9 secretion was observed (**Fig 5*A***).

MMP-9 activity is controlled by both TIMP-1 and TIMP-2 (Birkedal-Hansen *et al*, 1993). Following treatment of keratinocytes

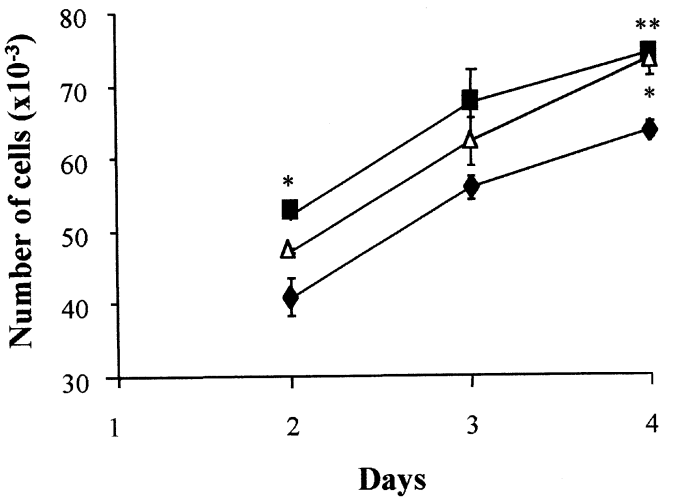

Figure 3. Influence of batimastat and MMP-9 blocking antibody on the proliferation of psoriatic keratinocytes (78-y-old). Keratinocytes were seeded at 1×10^4 cell density and effectors were added at day 1 of culture: ◆, cell growth in the absence of added effector; △, cell growth in the presence of MMP-9 blocking antibody (100 ng per ml); ■, cell growth in the presence of batimastat (100 nM). Data are means of three separate determinations; bars represent SEM values. *Significantly different from control at $p < 0.01$; **$p < 0.001$. Batimastat and MMP-9 blocking antibody also induced significant differences in cell growth using two other psoriatic keratinocyte strains.

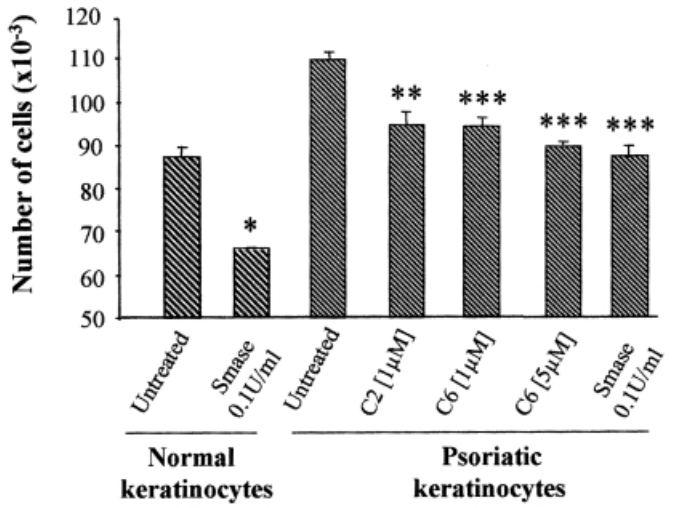

Figure 4. Effect of truncated ceramides (C2 or C6 ceramides) and bacterial Smase on normal and psoriatic keratinocyte growth. Cells were seeded at 1×10^4 density and treated on day 2 of culture with Smase or ceramide. On day 4 of culture, after 48 h of treatment with ceramide or Smase, cell number was determined by the crystal violet assay. Significantly different from control (absence of treatment) at *$p < 0.02$; **$p < 0.01$; ***$p < 0.001$.

by Smase, variations in the production of inhibitors were first assessed by gelatin reverse zymography (**Fig 5*B***, *inset*). Increasing ceramide levels of psoriatic keratinocytes led to an enhanced production of TIMP-1. TIMP-2 levels, which in many instances appeared regulated in a coordinated manner with MMP-2 (Brassart *et al*, 1998), were only moderately affected by such a treatment. TIMP-1 protein production was not greatly modified with cell growth. Smase treatment induced a 1.2- to 1.8-fold increase in TIMP-1 secretion, depending on cell strains analyzed (**Fig 5*B***). Thus, the ceramide-induced MMP-9 production was far more

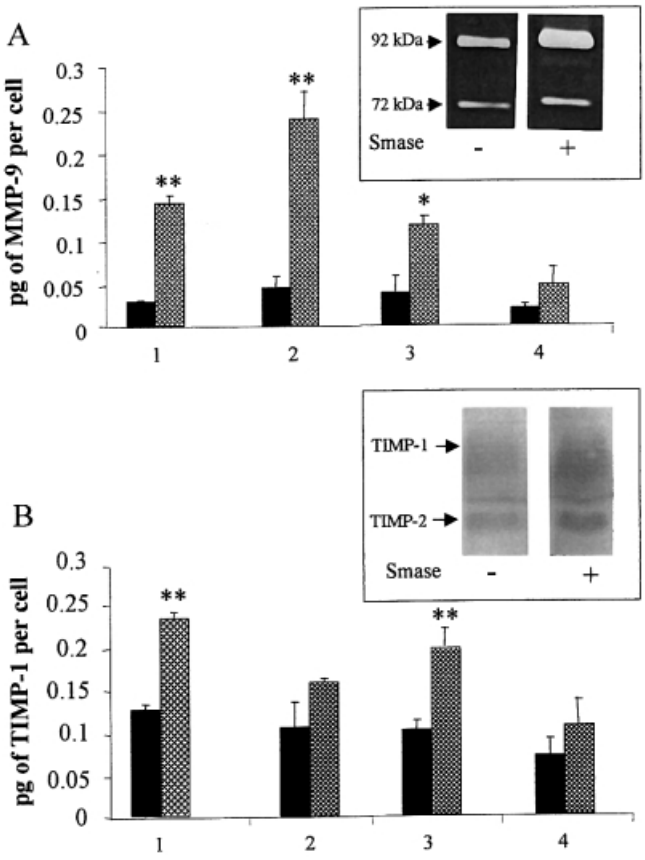

Figure 5. Increased production of MMP-9 and TIMP-1 following treatment of psoriatic keratinocytes with bacterial Smase. (*A*) MMP-9; (*B*) TIMP-1. Cells seeded at 1×10^4 density were treated on day 2 of culture with Smase (100 mU per ml). MMP-9 and TIMP-1 secreted levels were determined by ELISA following 48 h of culture and expressed as pg per cell: 1, psoriatic patient (38-y-old), lesional skin; 2, psoriatic patient (78-y-old), lesional skin; 3, psoriatic patient (35-y-old), lesional skin; 4, psoriatic patient (35-y-old), nonlesional skin. (*A*) MMP-9: control; in presence of Smase; *inset*, gelatin zymography (from patient 1). (*B*) TIMP-1: ■, control; ▨, in presence of Smase; *inset*, gelatin reverse zymography (from patient 1). Data are means of three separate determinations. Significantly different from control at $^*p < 0.01$; at $^{**}p < 0.001$.

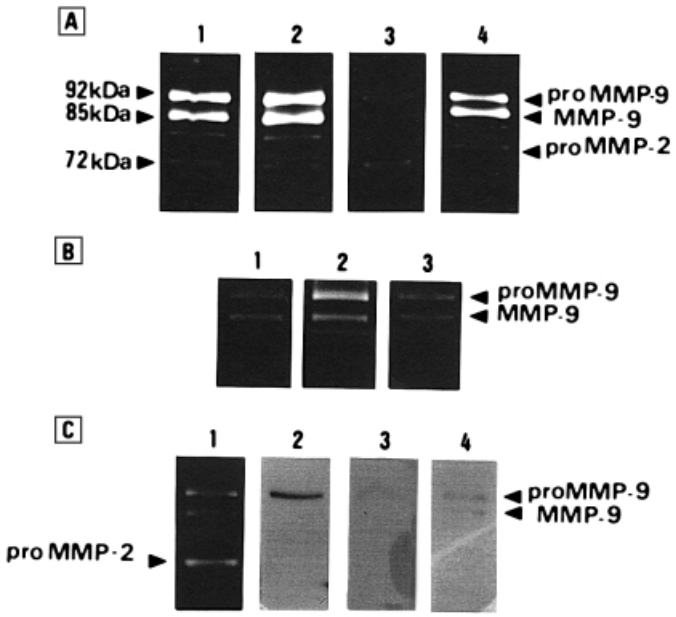

Figure 6. Presence and regulation of MMP-9 in cell membrane extracts of normal and psoriatic keratinocytes. (*A*) Normal (*lanes 1* and *2*) or psoriatic (*lanes 3* and *4*) keratinocytes were either treated with bacterial Smase (100 mU per ml) (*lanes 2* and *4*) or left untreated (*lanes 1* and *3*). (*B*) Control keratinocytes (*lane 1*) were treated with 100 mU per ml of Smase alone (*lane 2*) or in combination with 5 μM of batimastat (*lane 3*). After 24 h of treatment, the aqueous phase of the membrane extract of each set of keratinocytes was analyzed by gelatin zymography. (*C*) The electrophoretic position of pro-MMP-9 was identified by Western blotting of 10 ng purified pro-MMP-9 (*lane 2*). The aqueous phase of membrane extracts from psoriatic keratinocytes was also subjected to gelatin zymography (*lane 1*) or Western blotting after electrophoretic separation under reducing (*lane 3*) or nonreducing (*lane 4*) conditions.

important compared with TIMP-1. On a molar basis, however, irrespective of cell treatment or keratinocyte strains, inhibitor amounts exceeded enzyme levels. When conditioned media were analyzed for MMP activity using a specific fluorescent substrate, no activity could be demonstrated even following APMA activation (not shown).

Treatment of psoriatic keratinocytes with Smase increases cell-associated MMP-9 The absence of any detectable MMP-9 activity was contrary to the effect of batimastat and MMP-9 blocking antibody on cell growth. It is now generally assumed that MMP activation is a focal event, mostly occurring at the pericellular environment (Basbaum and Werb, 1996). We isolated keratinocyte plasma membranes and analyzed the preparations for MMP-9. Low levels of M_r 92,000, M_r 85,000 and M_r 72,000 species could be identified in the aqueous phase of membrane extracts from psoriatic keratinocytes. Membrane extracts from control cells contained higher amounts of these lysis bands. When cells were treated with Smase, increased amounts M_r 92,000 and M_r 85,000 but not M_r 72,000 lysis bands were observed (**Fig 6*A***). All forms were inhibited by ethylenediamine tetraacetic acid as well as by batimastat, and the M_r 72,000 band was identified as pro-MMP-2 by immunoblotting (not shown). Therefore, Smase treatment of keratinocytes, which significantly inhibited cell growth, led to increases in cell-associated pro- and active forms of MMP-9; such an effect was more significant for psoriatic cells, and importantly, the level of cell-associated MMP-9 could be substantially reduced following treatment of keratinocytes with batimastat (**Fig 6*B***). Only the M_r 92,000 molecular species was recognized by an antibody directed against the pro-domain of MMP-9, when analyzed under reducing conditions. Under nonreducing conditions, however, pro-MMP-9 and MMP-9 were detected by this antibody by Western blotting (**Fig 6*C***).

Binding of TIMP-1 to cell-associated MMP-9 can influence keratinocyte growth TIMP-1 had been previously found to exhibit growth-promoting activity for keratinocytes (Bertaux *et al*, 1991). As such a property was distinct from its inhibitory capacity (Hayakawa *et al*, 1994), we hypothesized that TIMP-1 binding to MMP-9 at the cell plasma membrane could hamper the binding of the growth factor to its receptor. We first confirmed the importance of TIMP-1 in keratinocyte growth. When keratino cytes from healthy individuals or psoratic patients were cultured in the presence of TIMP-1 antibody, cell proliferation was almost totally abolished (**Fig 7**). To determine whether TIMP-1 could be associated with MMP-9 at the cell plasma membrane, extracts from Smase-treated keratinocytes were incubated with gelatin beads. Bound and unbound fractions were analyzed by reverse zymography (**Fig 8**). TIMP-1 was identified in both fractions, indicating that a substantial part of the inhibitor was cell-associated with MMP-9 (**Fig 8**, *lane b*).

DISCUSSION

The prominent role of MMP-9 in the migratory or invasive potential of human epidermal cells has been well documented (Madlener *et al*, 1998; Mäkelä *et al*, 1998, 1999; Legrand *et al*, 1999). In normal human epidermal keratinocyte cultures, the

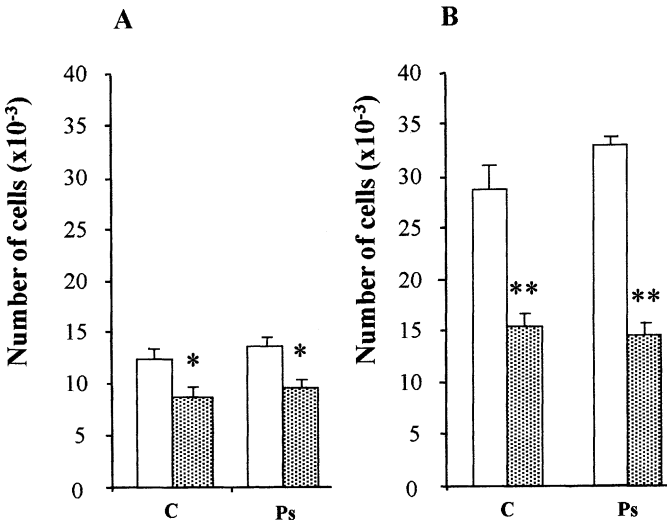

Figure 7. Influence of TIMP-1 on keratinocyte growth. Psoriatic (Ps) as well as control (C) keratinocytes were seeded at a density of 10^4 cells per dish. On day 2 of culture, they were treated with 5 μg per ml anti-TIMP-1 antibody or left untreated. Cell proliferation was analyzed at 48 h (*A*) or 72 h (*B*). Significantly different from control at *$p < 0.01$; **$p < 0.001$.

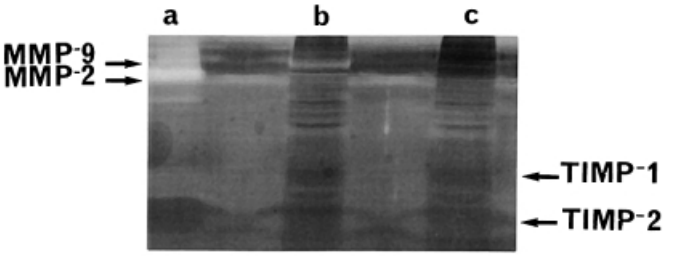

Figure 8. Presence of TIMP-1 in cell membrane extracts of psoriatic keratinocytes. The aqueous phase of membrane extracts from 1×10^5 psoriatic keratinocytes treated with 100 mU per ml of Smase for 48 h was incubated with gelatin beads. Both bound and unbound fractions were analyzed by gelatin reverse zymography. *Lane a*, MMP-2-TIMP-2 complex; *lane b*, bound fraction (note the presence of both MMP-9 and TIMP-1 in the gel); *lane c*, unbound fraction.

MMP-9-enhanced expression and cell migratory response brought about by epidermal growth factor and hepatocyte growth factor were found to be coordinated (McCawley *et al*, 1998). We recently reported that MMP-9 could also be involved in the ceramide-mediated inhibition of human keratinocyte growth (Buisson-Legendre *et al*, 1999). Increased MMP-9 production coincided with a decreased rate of cell proliferation. We therefore assumed that a hyperproliferative state of keratinocytes could be associated with decreased MMP-9 expression. Psoriatic keratinocytes, *in situ*, are characterized by a hyperproliferation state. Conflicting *in vitro* proliferation data have been reported in the literature (Liu and Parsons, 1983; Detmar *et al*, 1990). This can be accounted for by unstandardized experimental conditions and the transient character of the *in vitro* hyperproliferative behavior of psoriatic keratinocytes (Detmar *et al*, 1990). Under our experimental conditions, psoriatic keratinocytes in primary cell culture proliferate at a higher rate than normal cells. Any differences were far less important than those observed *in situ*, however. The increased growth rate of psoriatic keratinocytes was associated with a decreased level of MMP-9 production, at both the enzyme and protein levels. To further delineate the role of MMP-9 in the growth of psoriatic cells, we modulated its activity or its production. Supplementing the cell culture medium with batimastat or more specifically with an MMP-9 blocking antibody led to a further increase in keratinocyte proliferation. Alternatively, we increased intracellular levels of ceramide either with truncated ceramides (C2 or C6 ceramide) or with Smase, which induced sphingomyelin breakdown and triggered the ceramide pathway. These agents had been previously found to inhibit cell growth (Testi, 1996) and to increase MMP-9 expression (Buisson-Legendre *et al*, 1999) in normal keratinocytes in culture. A dual effect of ceramide was also seen in psoriatic keratinocytes. Such an effect is consistent with the beneficial influence in psoriasis of a topical treatment with calcipotriol, a noncalcemic vitamin D_3 analog that induces sphingomyelin breakdown in keratinocytes (Geilen *et al*, 1996). MMP-9 activity is controlled by both TIMP-1 and TIMP-2. Also, TIMP-1 could interact with the hemopexin-like domain of pro-MMP-9 via its carboxyl terminal domain, further hindering zymogen activation (O'Connell *et al*, 1994). In many instances, MMP-9 overexpression is accompanied by a simultaneous overproduction of TIMP-1 (Gomez *et al*, 1997). Ceramide was also found to stimulate TIMP-1 production from keratinocytes, and on a molar basis, TIMP-1 levels, irrespective of the conditions, i.e., the presence or absence of Smase, exceeded MMP-9 levels. In all cases, MMP-9 was secreted as a proenzyme and no MMP activity could be detected in concentrated conditioned media.

It is now considered that pro-MMP activation occurs mainly at the pericellular environment (Strongin *et al*, 1995; Mäkelä *et al*, 1998). The presence of MMP-9 at the membrane of a variety of cells, including nonmalignant and malignant breast epithelial cells, has been reported (Toth *et al*, 1999). In transformed cells, a membrane-anchored cysteine-rich protein with Kazal motifs was found to modulate both MMP-9 secretion and activity (Takahashi *et al*, 1998). Here, we showed that human keratinocytes in culture exhibited cell-associated MMP-9, present as proenzyme and active forms, with approximate M_rs of 92,000 and 85,000, respectively. These MMP-9 forms have also been identified in the plasma membrane of human MCF 10 A breast epithelial cells but found to correspond to different glycosylated forms of the proenzyme (Toth *et al*, 1997). In membrane extracts of keratinocytes, however, antibodies raised against the N-terminal pro-domain of MMP-9 only recognized the 92,000 M_r form. Thus, in these cells, the association of pro-MMP-9 to membranes correlated with enzyme activation but the mechanism leading to the formation of active cell-associated MMP-9 is at present unknown. Triggering the ceramide pathway with Smase greatly enhanced the levels of cell-associated MMP-9. We initially proposed that active MMP-9 could degrade a cell surface component involved in keratinocyte growth. Treatment of cells with a large excess of exogenous MMP-9, however, had no significant effect on keratinocyte proliferation (not shown). Alternatively, the presence of MMP-9 at the cell surface might somehow interfere with the binding of TIMP-1 to its cognate receptor, resulting in inhibition of keratinocyte growth.

Here we confirmed the importance of TIMP-1 in the growth of both normal and psoriatic keratinocytes. Almost complete suppression of cell growth was observed in the presence of 5 μg per ml anti-TIMP-1 antibody. We previously showed that 10^6 normal keratinocytes could be saturated with 14 ng of r-TIMP-1 at 22°C (Bertaux *et al*, 1991). Under our present experimental conditions, the amount of free TIMP-1 available for cell binding ranged from 34 to 140 ng per 10^6 keratinocytes, indicating that, in the presence or absence of Smase, TIMP-1 could saturate its receptor sites. Increasing the levels of cell-associated MMP-9 and TIMP-1, however, coincided with an inhibition of psoriatic keratinocyte growth following Smase treatment. This suggested that the amount of free TIMP-1 available for receptor binding could largely be decreased under such conditions. We could not directly affirm this, however, due to lack of information on the characteristics of the TIMP-1 receptor and the fate of TIMP-1-receptor complexes (Ritter *et al*, 1999). Overall, our data suggest that increased MMP-9 activity at the pericellular environment of psoriatic keratinocytes could exhibit a dual function. It could favor cell migration by degrading pericellular matrix macromolecules

(McCawley *et al*, 1998) or inhibit keratinocyte growth by competing with the binding of TIMP-1 to its receptor.

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