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Langerhans' cells produce type IV collagenase (MMP-9) following epicutaneous stimulation with haptens

Y. KOBAYASHI *R & D Headquarters, Sunstar Inc., Takatsuki, Osaka, Japan*

SUMMARY

For initiation of the contact hypersensitivity response, epidermal Langerhans' cells (LC) migrate from the epidermis to draining nodes via afferent lymphatics by passing through the basement membrane. In this study, we examined production of matrix metalloproteinases (MMPs) in LC-enriched epidermal cells to clarify the type of enzymes involved in LC transmigration through the basement membrane. Using gelatine enzymography and immunoblotting analysis, 95 000 MW type IV collagenase (MMP-9) was found to be produced by LC-enriched epidermal cells. Analysis of the kinetics of MMP-9 expression showed that its production was induced within 6 hr after application of 2,4,6-trinitrochlorobenzene (TNCB), substantially increased between 12 hr and 24 hr, and then decreased to the normal level by 7 to 10 days. Other haptens, such as 2,4-dinitrochlorobenzene and 2,4-dinitrofluorobenzene, also induced MMP-9 expression. Fluoroescence-activated cell sorter analysis revealed that LC were one of the major cell types to express MMP-9 in response to TNCB. In addition, highly enriched LC from sensitized skin were shown to express strong gelatinolytic activity. These results indicate that LC by themselves, as well as other epidermal cells, are capable of producing MMP-9, and suggest that MMP-9 may contribute to proteolysis associated with transmigration of LC in the induction phase of contact dermatitis.

INTRODUCTION

Epidermal Langerhans' cells (LC) reside in the supra-basal portion of the epidermis. They capture and process foreign antigens, move to the T-cell area in the draining lymphoid organs via afferent lymphatics, and present antigenic peptide in association with major histocompatibility complex (MHC) products to specific T cells.[1,2] It has been shown that hapten painting induces a decrease in LC number in the epidermis and an increase in number of hapten-loaded dendritic cells (DC) in the draining nodes.[3–6] In order to reach the draining nodes, LC first must cross the basement membrane of the dermo-epidermal junction, which consists of a variety of extracellular matrices, such as laminin, type IV collagen and heparan sulphate proteoglycans.[7] To do this, LC must attach to and degrade basement membrane proteins. Regarding cell adhesion, LC have been demonstrated to express adhesion molecules, including β_1 integrins, to matrix components.[8] However, little is known about the transmigration mechanism of LC.

Tumour cells, one of the most well-known cell types, are capable of migrating through the basement membrane. It has been shown that the invasive and metastatic capacities of several types of tumour cell are correlated with their ability to secrete matrix metalloproteinases (MMPs).[9–14] Invasive tumour cells produce large amounts of 72 000 MW type IV collagenase (MMP-2) and/or 92 000 MW type IV collagenase (MMP-9). MMP-2 degrades gelatine and type IV, V, VII and X collagens; likewise, MMP-9 degrades gelatine and type IV and V collagens.[15] Therefore, these two MMPs are regarded as key enzymes involved in invasion.

In this study, we examined whether gelatinases are produced by LC after the application of hapten, and we found that the epicutaneous application of hapten stimulated MMP-9 secretion from LC-enriched epidermal cells.

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Abbreviations: DC, dendritic cells; DNCB, 2,4-dinitrochlorobenzene; DNFB, 2,4-dinitrofluorobenzene; FITC, fluorescein isothiocyanate; LC, epidermal Langerhans cells; MMP, matrix metalloproteinase; PE, phycoerythrin; MFI, mean fluorescence intensity; TNCB, 2,4,6-trinitrochlorobenzene; TNF-α, tumour necrosis factor-α.

Correspondence: Y. Kobayashi, Skin Care Division, R&D Headquarters, Sunstar Inc., 3-1, Asahi-machi, Takatsuki, Osaka, 569–11 Japan.

MATERIALS AND METHODS

Animals

Female C3H/He mice (8–10-weeks old) were obtained from SLC Japan (Hamamatsu, Japan).

Reagents

Trinitrochlorobenzene (TNCB) was obtained from Kanto Chemical (Tokyo, Japan). Dinitrochlorobenzene (DNCB) and

dinitrofluorobenzene (DNFB) were purchased from Katayama Chemical (Osaka, Japan). A specific monoclonal antibody (mAb) against murine MMP-9[16] was a generous gift from Dr S. Saga (Aichi Human Service Centre, Japan). R-Phycoerythrin (PE)- and fluorescein isothiocyanate (FITC)-conjugated rat mAb against I-$A^{k,s}$ (clone OX6) were purchased from Life Technologies (Gaithersburg, MD).

Chemical treatment

The ears of mice were painted on the ventral side with 50 μl of TNCB (1% and 3%), DNCB (1% and 3%), or DNFB (1·0%) dissolved in acetone/olive oil (1:4) or with vehicle alone. These concentrations were chosen according to the results of previous studies.[17,18]

Cell preparation

Epidermal cells from the ventral side of mouse ears were obtained by trypsinization (0·25%) for 90 min at 37°. LC were partially enriched by density gradient centrifugation using Lymphoprep™ (Nycomed Pharma AS, Oslo, Norway). The purity of LC was determined by counting the percentage of I-A-positive cells under fluorescence microscopy (Olympus, Tokyo, Japan). In some experiments, LC were highly purified by magnetic cell sorter (MACS; Miltenyi Biotec GmbH, Gladbach, Germany) using a PE- or FITC-conjugated mAb against I-$A^{k,s}$, followed by MACS microbead-conjugated anti-rat IgG. Trapped cells (I-A-positive cells) were used as highly purified LC, and non-trapped cells (I-A-negative cells) were used as LC-depleted cells. The purity of LC was assessed by FACSCalibur™ (Becton Dickinson Immunocytometry Systems, San José, CA). Thioglycollate-elicited macrophages were prepared according to the method of Werb & Gordon.[19]

Zymograms

To determine the gelatine-degrading activities of the cells, gelatine-enzymography was performed.[20] Isolated, partially enriched LC were cultured in RPMI-1640 medium supplemented with 10% fetal calf serum (FCS) and 1 μM monensin that was used to augment accumulation of intracellular gelatinases.[21,22] After 4 hr incubation at 37°, recovered cells were sonicated in 50 mM Tris buffer containing 2% sodium dodecylsulphate (SDS) for 15 seconds. Aliquots of lysate prepared from the cells containing 5×10^3 I-A-positive cells were used to determine the gelatinase activity. In some experiments, conditioned medium was obtained from the culture of cells containing 1×10^4 I-A-positive cells in serum-free RPMI-1640 for 24 hr at 37°. Macrophage-conditioned medium was prepared by culturing 1×10^4 cells in serum-free RPMI-1640 for 24 hr. Both the cell lysates and the conditioned media were electrophoresed (without boiling and without reduction) in 1 mm of 10% SDS-polyacrylamide gels containing gelatine (0·5 mg/ml). After electrophoresis, SDS was removed from the gels by incubation in 2·5% Triton-X-100 for 60 min, and thereafter the gels were incubated for 24 hr at 37° in 50 mM Tris–HCl (pH 7·5) containing 10 mM $CaCl_2$. The gels were then stained with Coomassie brilliant blue and destained. Gelatine degradation was detected by the appearance of non-staining bands.

Immunoblotting

Conditioned medium was prepared from $2{\cdot}0 \times 10^5$ partially enriched LC obtained from 3% TNCB-treated or untreated mice, and macrophages as described above. These media were concentrated using Microcon-10 (Grace Japan, Tokyo, Japan), and electrophoresed in a 10% SDS–polyacrylamide gel. After electrophoresis, proteins were transferred onto a nylon membrane (MagnaGraph; Micron Separtious, Westboro, MA) which was then treated with anti-MMP-9 mAb (25 μg/ml) followed by incubation with biotinylated rabbit polyclonal antibody against rat IgG (Zymed, San Francisco, CA). MMP-9 was then visualized by staining with streptavidin-conjugated alkaline phosphatase (Amersham, Bucks., UK) plus 5-bromo-4-chloro-3-indolylphosphate p-Toluidine salt/nitroblue tetrazolium dye (BCIT/NBT) substrate (Amersham, Bucks., UK).

Immunocytological analysis

Two-colour labelling was used to detect the intracellular expression of MMP-9 by I-A-positive and negative cells. Partially enriched and monensin-treated LC suspensions were prepared from TNCB-treated or untreated mice as described above. One hundred thousand cells were fixed in 500 μl of 4% paraformaldehyde (10 min, 4°), and permeabilized in phosphate-buffered saline (PBS) containing 1·0% saponin (30 min, 4°). These cells were then sequentially incubated in the presence of 0·1% saponin with: (1) anti-MMP-9 mAb or isotype-matched control (rat IgG2b: Zymed); (2) biotinylated rabbit polyclonal antibody against rat IgG; and PE-conjugated streptavidin (Dako, Denmark). Cells were then labelled with FITC-conjugated anti-I-$A^{k,s}$ mAb. At least 10 000 cells were examined using FACSCalibur™.

RESULTS

Time-dependent changes in gelatinase secretion after sensitization

Epidermal cells were obtained at various times after painting the skin with 3% TNCB, and partially enriched LC suspensions (content of 12·3–26·1%) were prepared. To visualize the gelatinase activity, gelatine-substrate enzymography was performed. A representative result from seven different experiments is shown in Fig. 1. The kinetics of gelatinase production were examined in the cell lysates (Fig. 1a) and 24 hr-conditioned media of the cells (Fig. 1b). The epidermal cells were also prepared from unsensitized normal mice as a negative control, and thioglycollate-elicited macrophages served as a positive control.

Cell lysates obtained from untreated mice (0 hr) displayed little gelatinolysis. At 6 hr after TNCB painting, however, significant gelatinolytic activity was detected. This gelatinase expression increased substantially until 24 hr, but decreased gradually thereafter until day 4. No detectable activity remained at days 7 and 10. All gelatinolytic bands migrated to approximately the same position as a macrophage-derived gelatinase known as 95 000 MW type IV collagenase (murine MMP-9). Similar kinetics of gelatinase production were observed in the 24 hr-conditioned medium from LC-enriched cells of TNCB-treated mice, although clear gelatinolytic bands were detected even at day 10 (Fig. 1b). One possible reason for the detectable gelatinolytic activity in the supernatant at days 7 and 10 may be that LC-enriched cells were stimulated during the 24 hr-culture to prepare the conditioned medium.

In the conditioned medium, marked production of two gelatinases of 250 000–270 000 MW and 150 000 MW was

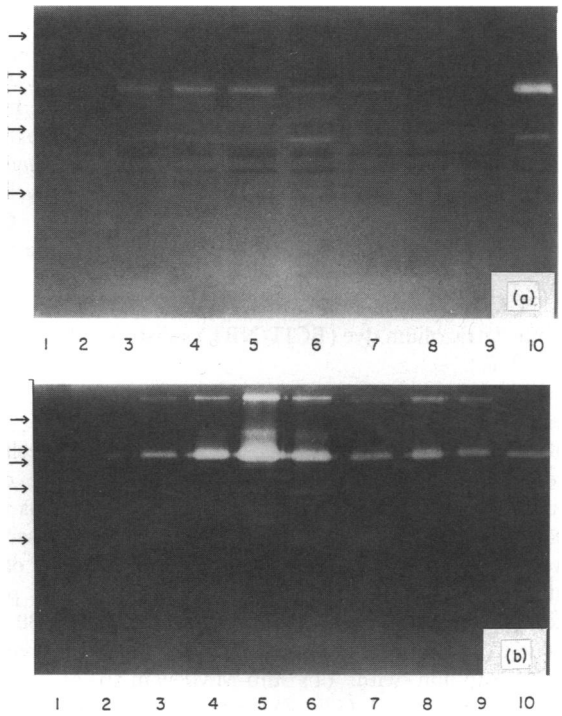

Figure 1. Kinetics of gelatinase activity from LC-enriched epidermal cells after application of 3% TNCB. LC-enriched epidermal cells were obtained at various times after TNCB painting. To examine gelatinase activity, aliquots of the cell lysates corresponding to 5×10^3 I-A-positive LC cultured for 4 hr in the presence of 1 μM monensin (a), and the 24 hr-culture supernatants of cells that contained 1×10^4 I-A-positive LC (b) were subjected to electrophoresis in a gelatine-copolymerized polyacrylamide gel. After electrophoresis, the gel was processed to visualize gelatinase activities, as described in the Materials and Methods. Lane 1, molecular weight standards are indicated by arrows: myosin (200 000 MW), β-galactosidase (116 250 MW), phosphorylase B (97 400 MW), bovine serum albumin (66 200 MW), and ovalbumin (45 000 MW); lane 2, 0 hr; lane 3, 6 hr; lane 4, 12 hr; lane 5, 24 hr; lane 6, 48 hr; lane 7, 4 days; lane 8, 7 days; lane 9, 10 days; lane 10, 24 hr-conditioned medium prepared from 1×10^4 macrophages.

observed in addition to the 95 000 MW band. As the kinetics of their gelatinase activities were mostly the same as that of the main gelatinolytic band, these two gelatinases appeared to be the dimerized products of the 95 000 MW gelatinase, as human polymorphonuclear leucocytes secrete several dimers of 92 000 MW gelatinase.[23] A 60 000 MW band showing faint gelatinolysis was also observed, especially at 48 hr, in both the cell lysates and the conditioned medium. This appeared to be another gelatinase, MMP-2. In this study, however, we did not analyse this band further because of its level of expression.

Effects of other haptens on the production of gelatinase

To investigate the effects of other haptens on gelatinase production, gelatinolytic activities in the cell lysates were determined at 12 hr after application of various agents. Representative results from five experiments are shown in Fig. 2. The acetone/olive oil vehicle had no effect. Not only 3% TNCB, but also 1% TNCB, 1% and 3% of DNCB, and 1% DNFB treatments markedly induced gelatinase production,

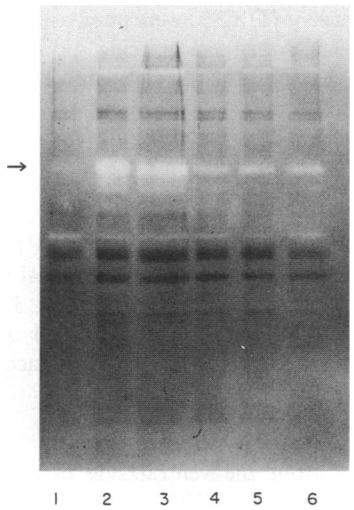

Figure 2. Effects of other sensitizers on the induction of gelatinase production of LC-enriched epidermal cells. Cell lysates were prepared from partially enriched LC of mice 12 hr after treatment with various haptens, as described in Fig. 1, and their gelatine-degrading activities were assessed. The arrow indicates the position of the gelatinolytic band. Lane 1, vehicle (acetone/olive oil); lane 2, 3% TNCB; lane 3, 1% TNCB; lane 4, 3% DNCB; lane 5, 1% DNCB; lane 6, 1% DNFB.

although DNCB and DNFB were less potent than TNCB. Similar results were obtained with the supernatant of LC-enriched cells (data not shown).

Identification of hapten-induced gelatinase

Since the gelatinase produced by LC-enriched epidermal cells migrated to approximately the same position as that from macrophages, it is possible that the hapten-induced gelatinase activity relies on MMP-9. To confirm this possibility, Western blotting analysis was performed using a specific mAb against murine MMP-9.

Figure 3 shows the results from four different experiments. A clear band at 95 000 MW was detected in the culture supernatant of LC-enriched cells from TNCB-treated mice, as in that of macrophages, while no significant bands were seen in the LC-enriched cells from untreated mice.

Determination of gelatinase-producing cells

To determine which cells in the epidermal cells produce MMP-9, fluorescence-activated cell sorter (FACS) analysis was carried out to examine the intracellular expression of MMP-9 by LC-enriched epidermal cells prepared 12 hr after treatment of mice with 3% TNCB. Identically prepared cell populations from normal mice were used as controls. As shown in Fig. 4, marked expression of MMP-9 by I-A-positive cells prepared from TNCB-treated mice was observed. Both I-A-positive and -negative populations were gated out, and fluorescence levels of MMP-9 were calculated separately for each population. Relative fluorescence index (=[mean fluorescence intensity of MMP-9 − mean fluorescence intensity of Control]/mean fluorescence intensity of Control) of I-A-positive cells from TNCB-treated mice was $3{\cdot}26 \pm 1{\cdot}91$ (mean $\pm$ SD, $n = 5$), and was significantly higher than that of the I-A-

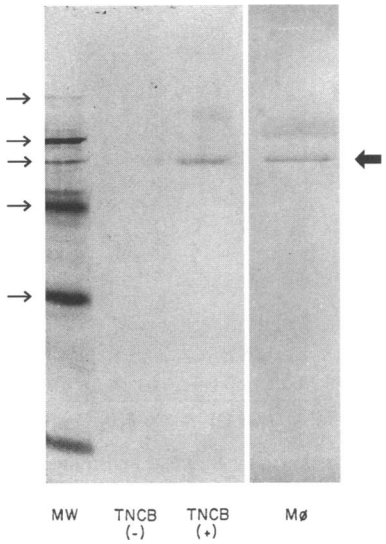

Figure 3. Immunoblot analysis of gelatinases in LC-enriched epidermal cells. Twenty-four-hour culture supernatants were prepared from the LC-enriched epidermal cells of normal mice (lane 2) and of mice 12 hr after treatment with 3% TNCB (lane 3). Macrophage culture medium is shown as a control (lane 4). Arrows indicate the molecular weight standards, as in Fig. 1. The thick arrow indicates the position of 95 000 MW type IV collagenase (MMP-9).

positive population from untreated mice ($1{\cdot}01 \pm 0{\cdot}77$, $n=5$, $P<0{\cdot}05$). Slight augmentation of gelatinase expression was observed in the I-A-negative population prepared from TNCB-treated mice compared to those from untreated controls. Relative fluorescence indexes were $0{\cdot}66 \pm 0{\cdot}64$ ($n=5$), and $0{\cdot}32 \pm 0{\cdot}18$ ($n=5$), respectively.

To confirm further whether LC express MMP-9, highly enriched LC and LC-depleted epidermal cells were prepared from mice 12 hr after treatment with 3% TNCB. Representative results from four experiments are shown in Fig. 5. Identically prepared cell populations from normal mice were used as controls. Cytofluorometric analysis indicated that the purities of I-A-positive cells were 95·4% and 87·3% in LC-enriched fractions and 0·4% and 3·6% in LC-depleted fractions from untreated and TNCB-treated mice, respectively (Fig. 5a). Gelatinase activities of each fraction are shown in Fig. 5b. Purified LC and LC-depleted fractions prepared from TNCB-treated mice showed markedly stronger gelatinolysis than those from untreated mice, demonstrating the increased production of gelatinase by hapten application. In addition, the LC-enriched fraction from TNCB-treated mice showed stronger gelatinolytic activity than the LC-depleted fraction from the same animals.

DISCUSSION

LC migration from the epidermis to the regional draining nodes is a crucial step in the induction of allergic contact dermatitis. The study presented here was undertaken to examine MMP production, particularly gelatinase production, in LC-enriched epidermal cells to clarify the types of enzymes involved in LC transmigration through the basement membrane in contact sensitization. The results of enzymography, immunoblotting and cytofluorography experiments demonstrated that treatment with haptens, such as TNCB, DNCB and DNFB, induced the production and secretion of MMP-9 by LC-enriched epidermal cells. In contrast, little expression of another gelatinase, MMP-2, was observed in hapten-treated and untreated cells.

Regarding the kinetics of change in the number of LC in the epidermis, Van Wilsem *et al.* reported that LC number in the epidermis declines sharply during the first 16 hr after rhodamine-B painting and that it takes over 2 weeks to return to normal levels.[24] In the meantime, Macatonia *et al.* showed that the maximum increase in DC number in the regional nodes occurred 2 days after FITC painting.[4] These results seem compatible with those shown in Fig. 1, indicating that gelatinase activity could be detected in cell lysates and culture supernatants of LC-enriched epidermal cells at 6 hr, followed by a substantial increase until 24 hr, and then a gradual decrease to the normal level by 7–10 days after TNCB application. These results indicate that stimulation with hapten induces epidermal cells to synthesize and secrete gelatinase prior to migration.

The gelatinase of LC-enriched epidermal cells was identified as MMP-9. FACS analysis revealed that MMP-9 expression of LC (I-A-positive population) prepared from sensitized mice was stronger than that of the I-A negative population from

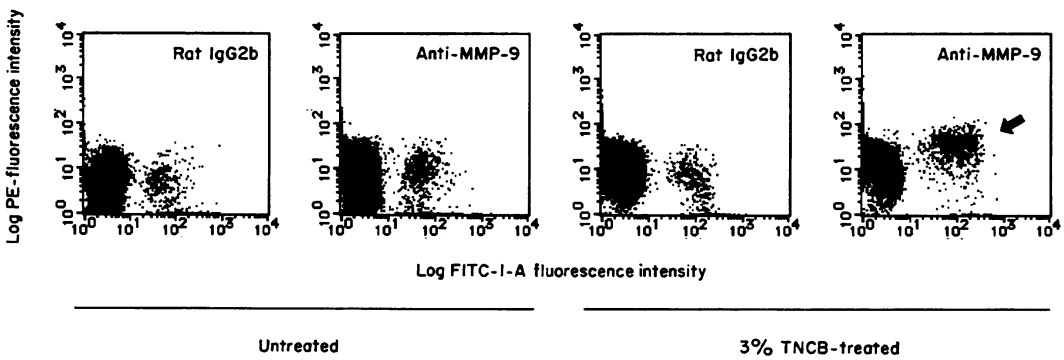

Figure 4. Intracellular expression of MMP-9 by LC-enriched cells prepared from TNCB-treated and untreated mice. Partially enriched and monensin-treated LC epidermal cells were obtained from TNCB-treated or untreated mice. They were labelled with anti-MMP-9 mAb or isotype-matched control IgG plus biotinylated rabbit polyclonal antibody against rat IgG and R-PE-conjugated streptavidin (y axes), and FITC-conjugated anti-I-$A^{k,s}$ mAb (x axes) as described in the Materials and Methods. The arrow indicates the position of I-A-positive LC.

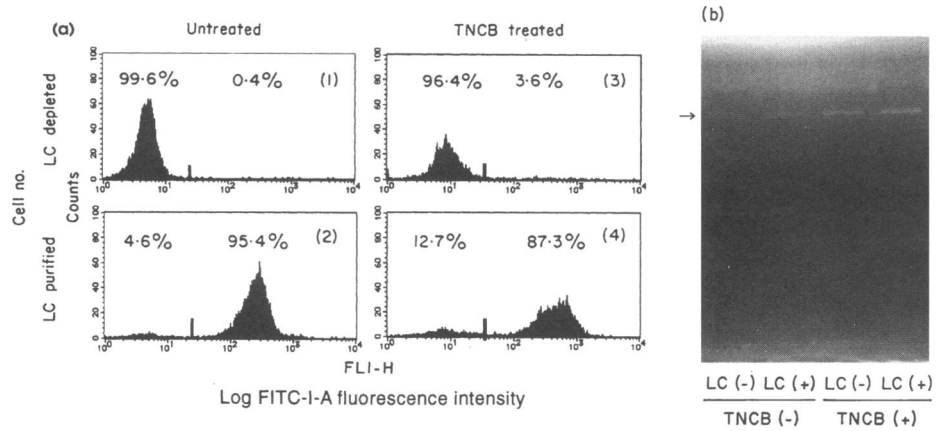

Figure 5. Gelatinase production in highly purified LC and LC-depleted epidermal cells. Partially enriched and monensin-treated LC epidermal cells were obtained from TNCB-treated or untreated mice, and stained with PE-conjugated rat anti-I-$A^{k,s}$ plus magnetic microbeads-conjugated goat anti-rat IgG (H+L), and then positively and negatively separated with MACS to obtain highly enriched LC and LC-depleted epidermal cells, respectively. (a) The purity of these populations was determined by FACS. (b) The cell lysates were prepared from the remaining cells, and then each aliquot corresponding to 5×10^3 cells was analysed for gelatinase activity. LC-depleted cells in lanes 1 and 3, and LC-enriched cells in lanes 2 and 4 were from normal and TNCB-treated mice, respectively.

the same animals and those of both populations from untreated mice. In addition, the gelatinolytic activity of the highly purified LC (content of 87%) from sensitized skin appeared more abundant than that of the LC-depleted epidermal cells and that of highly purified LC (content of 95·4%) from untreated mice. The highly enriched population of I-A-positive cells (content of 97%) which migrated from skin explants expressed the same gelatinase (data not shown). Therefore, it is conceivable that LC by themselves, as well as other epidermal cells, are capable of producing MMP-9 in response to hapten application. We thus speculate that this MMP-9 expression may in turn contribute to proteolysis associated with LC motility, such as degradation of basement membrane components and/or detachment of LC from the keratinocyte network in the epidermis.

Other cell types in the epidermis may also produce MMP-9. Thy-1^+ dendritic cells, in particular, have been demonstrated to migrate out of the epidermis when given such stimuli as ultraviolet B or PUVA irradiation or tumour-promoting agents.[25,26] This cell type was eventually present in the cell population migrating from skin explants (data not shown), although in this study we did not attempt to purify the cells to examine their gelatinase-producing capacities. Keratinocytes, fibroblasts, granulocytes and macrophages may also secrete gelatinase. With the exception of fibroblasts, these cell types have been shown to produce MMP-9, whereas fibroblasts are primarily responsible for producing MMP-2.[27] In a recent study, Varani *et al.* reported that MMP-9 was produced by the epithelial component in human neonatal foreskin organ culture.[28] Thus, MMP-9 secreted from many cell types may aid LC migration by loosening the interstitial extracellular matrix and the intercellular attachment between LC and keratinocytes.

There is accumulating evidence suggesting that epidermal cytokines may play an important role in mediating immune responses in the skin. Among these cytokines, tumour necrosis factor-α (TNF-α) may act as a stimulus for MMP-9 production from LC. Previous studies have shown that MMP-9 secretion is induced or stimulated by TNF-α in several cell types.[29–31] In the mean time, it has been demonstrated that intradermal injection of TNF-α induces the reduction of LC numbers in the epidermis and the accumulation of DC in lymph nodes.[32,33] The augmentation of TNF-α by cutaneous sensitization might induce and stimulate MMP-9 production from LC and surrounding cells, and this in turn could provide a signal for LC migration from the epidermis to the dermis.

Cell migration through the basement membrane has been postulated to proceed in three steps: first, cell attachment via specific receptors to the matrix of the basement membrane; second, the local degradation of the matrix by proteinases, such as MMPs; and third, the enhanced motility required for penetration.[34,35] This model is based on the results of analysis of the invasive and metastatic capacities of tumour cells. Regarding LC migration, Le Varlet *et al.* showed that LC express several types of β_1 integrins,[8] of which $\alpha5\beta_1$ and $\alpha6\beta_1$ integrins are known to bind fibronectin and laminin, respectively.[36] We have also demonstrated that the chemo-invasive activity and the random-migration capability of LC are stimulated by hapten application.[37,38] Along with the results presented here, the three-step theory of cell invasion appears to be compatible with LC emigration from the epidermis to the afferent lymphatic system following contact sensitization. The role of MMP-9 in LC motility should be clarified in future studies, but its expression in LC and other epidermal cells suggests that it may play an important role in LC emigration in the induction phase of contact sensitization.

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