Epilysin (MMP-28) Expression is Associated with Cell Proliferation During Epithelial Repair

Ulpu Saarialho-Kere, Erja Kerkelä, Tiina Jahkola,* Sari Suomela, Jorma Keski-Oja,† and Jouko Lohi†

Departments of Dermatology, *Plastic Surgery, and †Pathology and Virology, Biomedicum Helsinki, University of Helsinki, and Helsinki University Central Hospital, Finland

Epilysin (MMP-28) is the newest member of the matrix metalloproteinase enzyme family. Several members of this enzyme family have been associated with various aspects of wound repair and cancer invasion. The aim of this study was to characterize in different types of wounds, skin cancers, and keratinocyte cultures factors that contribute to epilysin expression *in vivo*, as well as how and where it is induced in relation to other matrix metalloproteinases. Our results indicate that epilysin is produced by the mitotic Ki-67-positive keratinocytes distal from the wound edge in both acute and chronic wounds and that it does not generally colocalize with collagenase-1, stromelysin-2, or 92 kDa gelatinase in migrating keratinocytes. An injury of epidermis was needed for epilysin induction as it was upregulated in ulcerated pyogenic granulomas and in suction blisters but was not detected in intact acanthotic or normal skin. Unlike many other matrix metalloproteinases, epilysin was not detected in the invading cancer cell nests of sclerosing basal or squamous cell cancers of various grades. When primary keratinocytes were stimulated with tumor necrosis factor α, upregulation of epilysin mRNA was evident within 24–48 h as measured by quantitative reverse transcription polymerase chain reaction. In primary keratinocyte, HaCaT, and A431 carcinoma cell cultures none of the 10 other growth factors or extracellular matrices studied were able to upregulate epilysin expression. Our results suggest that epilysin expression is tightly spatially and temporally regulated during wound repair. Although the *in vivo* substrates of epilysin are not known at present, its expression pattern suggests that it may be needed to restructure the basement membrane or to degrade adhesive proteins between keratinocytes to supply new cells for the migrating front. ***Key words: cancer/laminin-5/wound healing. J Invest Dermatol 119:14–21, 2002***

The matrix metalloproteinase (MMP) enzyme family nowadays comprises more than 20 members capable of degrading most extracellular matrix proteins (Nagase and Woessner, 1999; Uria and Lopez-Otin, 2000). Epilysin (MMP-28), the newest member of this family, was first cloned from human keratinocyte and testis cDNA libraries (Lohi *et al*, 2001) and also from a lung cDNA library (Marchenko and Strongin, 2001). It is a 59 kDa protein and three different transcripts (2.6, 2.0, and 1.2 kb) can be detected in various tissues due to alternative splicing by northern analysis (Lohi *et al*, 2001). Although hybridization of multiple tissue northern blots indicated that MMP-28 mRNA is expressed at high levels in testis and at lower levels in normal human lung, heart, colon, intestine, and brain, RNAse protection assays with various cell lines indicated that epilysin was selectively expressed by keratinocytes in culture. By reverse transcription polymerase chain reaction (RT-PCR) amplification it has been detected in several fetal tissues (lung, brain, skeletal muscle, and kidney) and in adult kidney and pancreas (Marchenko and Strongin, 2001). Comparison of the amino acid sequence of MMP-28 with the other MMPs indicated that it is most closely related to certain other recently cloned members of the family, such as MMP-19 (Lohi *et al*, 2001; Marchenko and Strongin, 2001). No physiologic substrates or activators of MMP-28 have been identified yet, however, although it has been shown to cleave casein *in vitro* (Lohi *et al*, 2001).

Several MMPs have been implicated in cutaneous wound healing during which they are strictly spatially and temporally regulated. Collagenase-1 (MMP-1), stromelysin-2 (MMP-10), and 92 kDa gelatinase (MMP-9) are expressed by migrating keratinocytes, whereas stromelysin-1 (MMP-3) is produced in keratinocytes just behind the migrating cells (see Parks, 1999). The same MMPs also participate in cancer cell migration occurring in basal and squamous cell cancers (see Kähäri and Saarialho-Kere, 1999; Kerkelä *et al*, 2001). Epilysin has been detected by PCR amplification in colon, pancreatic, and ovarian adenocarcinomas, and in prostate and lung cancer cell lines (Marchenko and Strongin, 2001). Furthermore, immunostaining of *ex vivo* wounds in cell culture has revealed that epilysin is induced during epithelial repair (Lohi *et al*, 2001). The aim of this study was to better define the role and cellular origin of epilysin in epithelial repair and skin cancer. We have analyzed epithelial tissues and specific cell types that express MMP-28 *in vivo*, which is an important step in elucidating the physiologic functions of this proteinase. We observe that during wound repair epilysin expression is tightly regulated. It is produced by the proliferating keratinocytes behind the migrating

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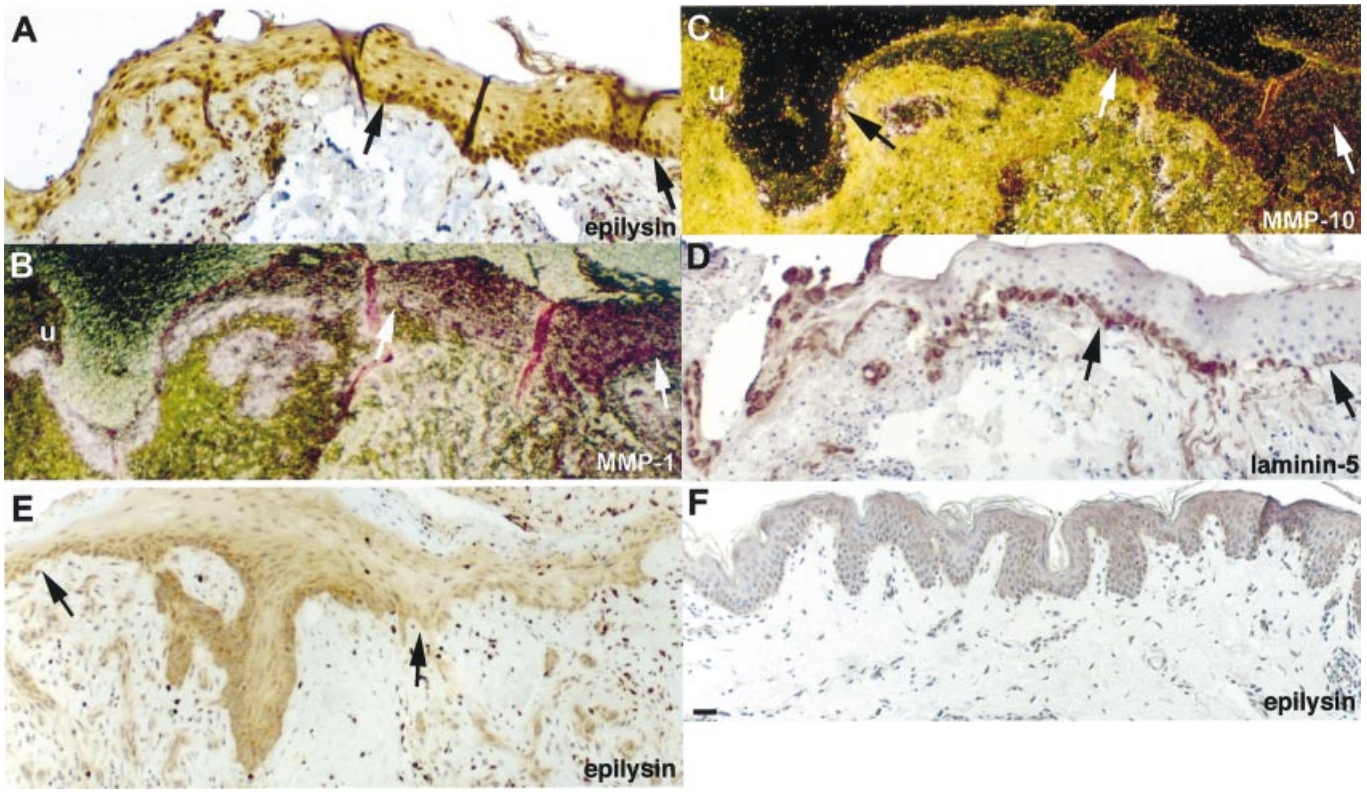
Reprint requests to: Dr. Ulpu Saarialho-Kere, Department of Dermatology, Helsinki University Central Hospital, Meilahdentie 2, 00250 Helsinki, Finland; Email: ulpu.saarialho-kere@helsinki.fi

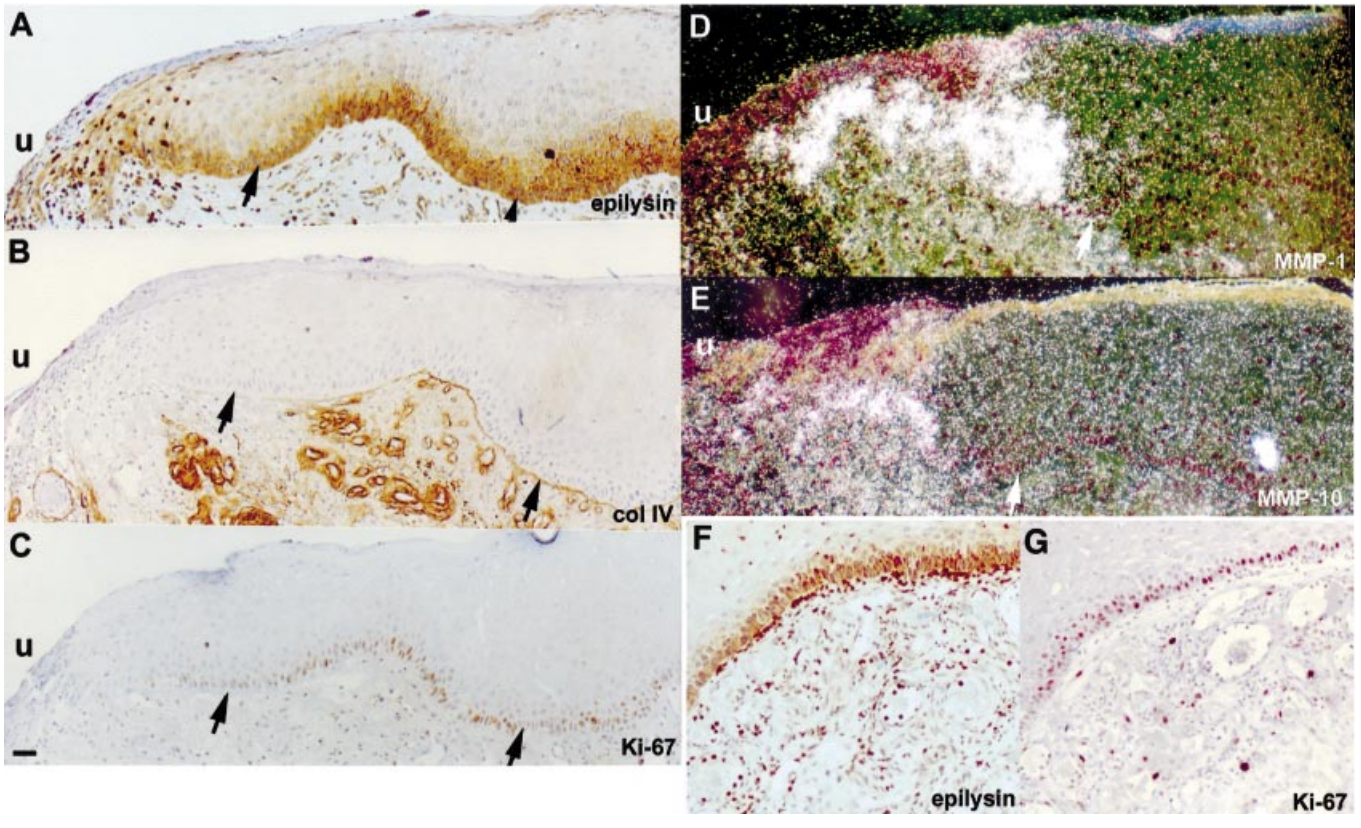
Abbreviation: LN-5, laminin-5.

cells in an area where intracytoplasmic laminin-5 (LN-5), a marker for migrating keratinocytes (Larjava *et al*, 1993), becomes confined to the basement membrane (BM) zone. Furthermore, unlike most other MMPs, epilysin does not seem to be induced in aggressive subtypes of basal or squamous cell carcinoma of the skin.

MATERIALS AND METHODS

Tissues Informed consent was obtained from individual subjects for all procedures. The study was approved by the Ethics Committees of the Departments of Dermatology and Plastic Surgery, Helsinki University





Central Hospital. Formalin-fixed, paraffin-embedded archival specimens from adult patients were obtained from the Department of Dermatopathology, Skin and Allergy Hospital, University of Helsinki. The following specimens were examined: normal skin ($n = 4$), ulcerated and nonulcerated pyogenic granulomas ($n = 5$), basal cell cancers (sclerosing subtype; $n = 7$), squamous cell carcinomas (grades II and III; $n = 9$), lichenoid eczema ($n = 4$), chronic wounds ($n = 15$), normally healing timed wounds ($n = 9$), and suction blisters ($n = 12$). Suction blisters were induced on healthy abdominal skin and harvested at 2, 4, and 9 d as previously described (Vaalamo *et al*, 1999). Samples of 15 chronic ulcers of various etiologies were collected from patients at the Department of Plastic Surgery undergoing excision and grafting for nonhealing ulcers. Five biopsies from clinically well granulating ulcers, that required skin grafting because of their large size, were also examined. Biopsies of normally healing donor areas of anterior thigh were obtained 1–9 d after wounding from nine patients of the Department of Dermatology undergoing pinch grafting, and they served as samples of normally healing wounds (Vaalamo *et al*, 1996).

Immunohistochemistry Immunostaining was performed by the avidin-biotin-peroxidase technique (Vectastain ABC Kit, Vector Laboratories, Burlingame, CA) as described previously (Saarialho-Kere *et al*, 1993; Rechardt *et al*, 2000) using pretested dilutions of affinity-purified epilysin rabbit antibodies (1:700; Lohi *et al*, 2001). Diaminobenzidine or aminoethylcarbazole were used as chromogenic substrates and Harris hematoxylin as counterstain. The sections were pretreated by antigen retrieval as described previously (Von Boguslawski, 1994) and endogenous peroxidase was inactivated by 0.3% H_20_2 for 30 min. A monoclonal Mib-1 antibody (1:500; 0505, Immunotech, Marseilles, France), which reacts with the Ki-67 nuclear antigen, was used to differentiate proliferating cells from quiescent epithelial cells. A monoclonal antibody to type IV collagen (1:75; M785, Dako, Glostrup, Denmark) was used to stain the BM. MMP-9 was detected using a monoclonal antibody (1:500; GE213, Diabor, Oulu, Finland). LN-5-producing cells were identified with polyclonal rabbit antibodies against the gamma2 chain of LN-5 (1:600; a gift from Professor Karl Tryggvason, Karolinska Institutet). For negative controls, we processed sections with preimmune serum, or we coincubated affinity-purified antibodies with excess peptide antigen to inhibit specific interactions with epilysin in the tissue.

***In situ* hybridization** *In situ* hybridization using ^{35}S-labeled probes for MMP-1, MMP-3, and MMP-10 was performed as previously described (Prosser *et al*, 1989; Rechardt *et al*, 2000).

Cell cultures Human keratinocytes were obtained from seven patients undergoing reductive mammoplasties as described previously (Rechardt *et al*, 2000) and cultured in keratinocyte growth medium (KGM, Gibco BRL, Life Technologies, Paisley, U.K.), supplemented with 5 ng per ml epidermal growth factor (EGF) and 50 μg per ml bovine pituitary extract. A431 cells (epidermoid carcinoma cell line, ATCC) and HaCaT cells (Boukamp *et al*, 1988), transformed human epidermal keratinocytes, were cultivated in Dulbecco's modified Eagle's medium (DMEM, Gibco BRL) containing 10% fetal bovine serum, 1% penicillin–streptomycin, and 1% sodium pyruvate.

Cytokines, growth factors, and matrices To study the regulation of epilysin expression, equal amounts of cells were plated on 24-well tissue culture plates. On the next day, the cells were repeatedly washed with phosphate-buffered saline and incubated in serum-free medium without supplements for 12–24 h. Analogously, all treatments were carried out without serum. The cells were subsequently treated with basic fibroblast growth factor (bFGF) (10 ng per ml, Oncogene, Calbiochem, Boston, MA), EGF (10 ng per ml, Sigma, St. Louis, MO), granulocyte–macrophage colony stimulating factor (GM-CSF) (10 ng per ml, R&D Systems, Minneapolis, MN), hepatocyte growth factor (HGF) (20 ng per ml, Sigma), interferon-γ (IFN-γ) (20 ng per ml or 100 U per ml, Sigma), interleukin-1β (IL-1β) (10 ng per ml, Roche Molecular Biochemicals, Mannheim, Germany), keratinocyte growth factor (KGF) (10 ng per ml, Sigma), platelet-derived growth factor (PDGF) (20 ng per ml, Sigma), transforming growth factor β1 (TGF-β1) (5 ng per ml, Sigma), tumor necrosis factor α (TNF-α) (20 ng per ml, Sigma), vascular endothelial growth factor (VEGF) (10 ng per ml; 293-VE, R&D Systems), and insulin-like growth factor 1 (IGF-1) (100 ng per ml; 291-G1, R&D Systems) for 24 or 48 h. Untreated cells were used as a control.

To characterize the role of the growth substratum, cells were cultivated on tissue culture dishes coated with different matrices, including collagen I, collagen IV, laminin, fibronectin, and Matrigel (Becton-Dickinson, Bedford, MA). Matrigel was diluted 1:10 using serum-free medium according to the manufacturer's instructions. After 24 or 48 h total RNA was extracted from the cells. All treatments were carried out in triplicate, and the results were confirmed in at least two independent experiments. For immunostaining, HaCaT cells and primary keratinocytes (in both KGM and DMEM) were also cultured on Laboratory-Tek chamber slides (plastic or type I collagen coated) and treated with TNF-α and KGF where indicated.

Quantitative RT-PCR PCR primers (Sigma Genosys, Cambridge, U.K.) and probes (PE Biosystems, Warrington, U.K.) for epilysin were designed using the computer program Primer Express (PE Biosystems). Primers used for amplification were 5′-GGCGTAAGAAACGCTT-TGCA-3′ (forward, nucleotides 765–784) and 5′-CAGTTCACCAG-GCGGTAGGA-3′ (reverse, nucleotides 834–816). Fluorogenic probes contained a reporter dye (FAM) covalently attached at the 5′ end and a quencher dye (TAMRA) covalently attached at the 3′ end. Human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) labeled with VIC reporter dye (Pre-developed TaqMan Control Reagents, PE Biosystems) was used as an endogenous control in PCR. Stromelysin-2, which can be induced by TGF-β1 in cultured keratinocytes, was used as a positive control as described previously (Rechardt *et al*, 2000).

Total cellular RNA from cell cultures was extracted by using RNeasy miniprep-kit (Qiagen, Chatsworth, CA) according to the manufacturer's instructions. RNA was then reverse transcribed to cDNA with TaqMan Multiscribe enzyme and random hexamers and used as a template in PCR. Real-time quantitative PCR were performed with the ABI PRISM 7700 Sequence Detector System (PE Biosystems) as previously described (Rechardt *et al*, 2000). PCR amplifications were performed in a total volume of 20 μl, containing 5 μl cDNA sample, 1 μl human GAPDH endogenous control reagents, 100 nm of each primer, and 100 nm of fluorogenic probe. The PCR was started with 2 min at 50°C and an initial 10 min denaturing temperature of 94°C, followed by a total of 40 cycles of 15 s of denaturing and 1 min of annealing and elongation at 60°C.

RESULTS

Expression of epilysin during normal wound healing In normally healing pinch graft wounds epilysin was detected in basal and suprabasal keratinocytes distal from the migrating edge (**Fig 1*A***). *In situ* hybridization of serial sections indicated that the epilysin-positive epidermis overlapped with the distal tip of MMP-1-positive cells to some extent (**Fig 1*B***); however, epilysin-positive cells reached much further away from the wound edge. MMP-10 mRNA (**Fig 1*C***) or MMP-9 protein (data not shown) were expressed by the migratory tip and did not colocalize with epilysin. LN-5, which is an adhesive ligand in epithelial BM and produced by migrating keratinocytes (Larjava *et al*, 1993), was used as a

Figure 1. Epilysin is differentially expressed from other MMPs in normally healing wounds. (*A*) Immunostaining for epilysin in a 4-d-old wound. (*B*) *In situ* hybridization for MMP-1. (*C*) *In situ* hybridization for MMP-10. *Black arrow* marks the distal tip of MMP-10 signal. (*D*) Immunostaining for LN-5. *Arrows* mark corresponding spots (*A–D*). U indicates the ulcer area. (*E*) Re-epithelialization occurring from a hair follicle with positive staining for epilysin (*arrows*). (*F*) Normal skin. Counterstaining with hematoxylin (*A*, *D–F*) and hematoxylin–eosin (*B*, *C*). *Scale bar*: 40 μm.

Figure 2. Epilysin protein is detectable in proliferating keratinocytes residing also on an intact BM. (*A*) Immunostaining for epilysin in a 1-mo-old rheumatoid ulcer. Immunostainings for type IV collagen (*B*) and Ki-67 proliferation marker (*C*) were carried out on nearby sections. *In situ* hybridizations for MMP-1 (*D*) and MMP-10 (*E*). *Arrows* mark corresponding spots. U marks the ulcer area. (*F*) Immunostaining for epilysin in an ulcerated pyogenic granuloma. (*G*) Immunostaining for Ki-67 in the same sample. Counterstaining with hematoxylin (*A–C*, *F*, *G*) and hematoxylin–eosin (*D*, *E*). *Scale bar*: 40 μm.

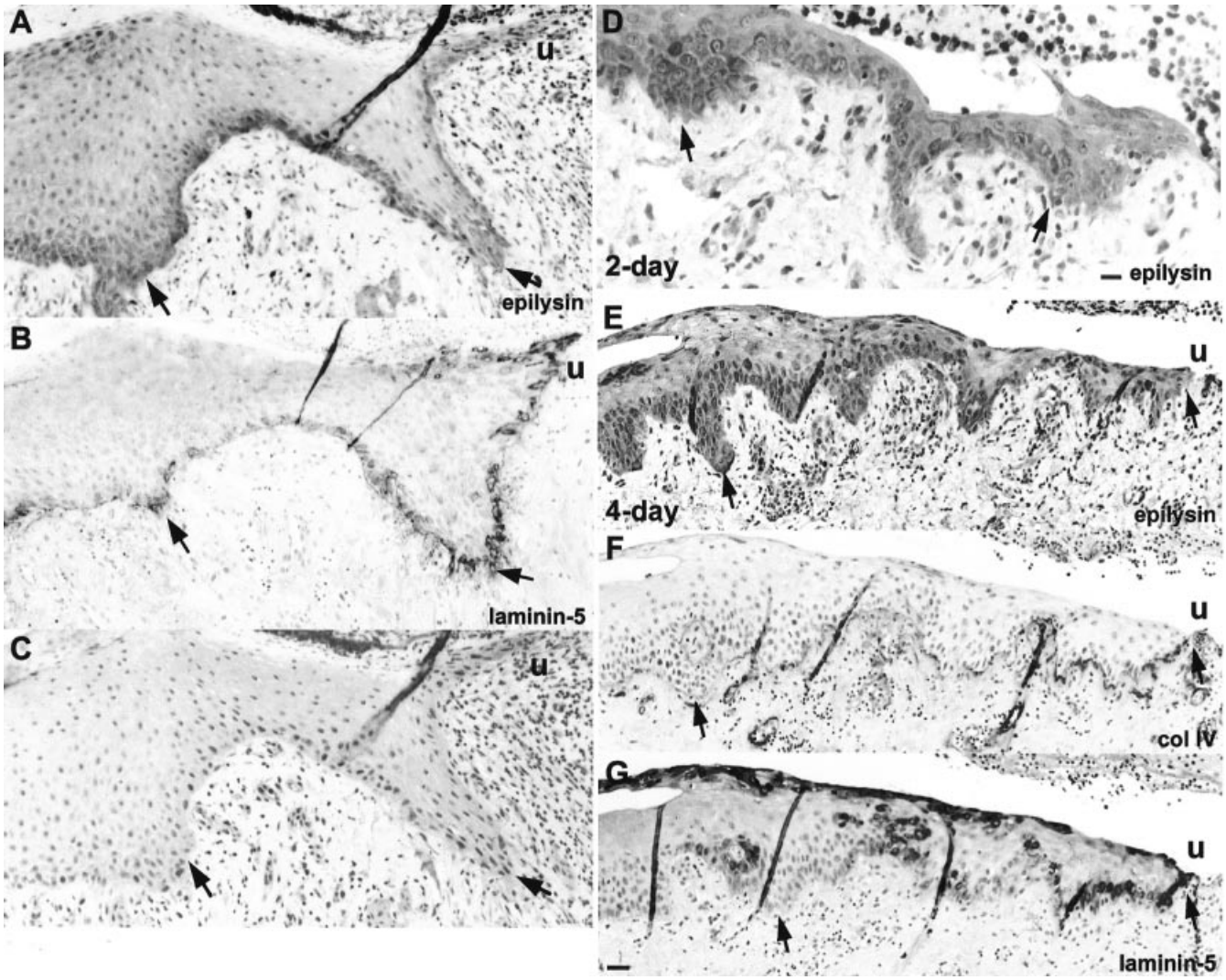


Figure 3. Epilysin-expressing cells are not migrating as assessed by LN-5 immunostaining and the protein is induced in suction blisters. (*A*) Immunostaining for epilysin in a well-granulating 2-wk-old post-traumatic wound. (*B*) Staining for LN-5 in the same sample. Note the change of LN-5 immunostaining from intracellular to BM-like in the epilysin-positive area. (*C*) Negative control for epilysin immunostaining performed by coincubating affinity-purified antibodies with excess peptide antigen. *Arrows* depict corresponding spots. U marks the ulcer area. A 2 d suction blister immunostained for epilysin (*D*). *Arrows* depict area of positive cells. A 4 d suction blister immunostained for epilysin (*E*), type IV collagen (*F*), and LN-5 (*G*). Counterstaining with hematoxylin (*A–G*). *Scale bars*: (*A*)–(*C*), (*E*)–(*G*) 40 μm; (*D*) 20 μm.

marker. Intracellular staining for LN-5 partly overlapped with the area of epilysin-positive keratinocytes (**Fig 1*A*, *D***), suggesting that the keratinocyte phenotype changed from migratory to stationary in this region. Also, when re-epithelialization had started from hair follicles, the pattern of epilysin protein expression was confined distal to the migrating tips (**Fig 1*E***). No epilysin was immunodetected in normal skin (**Fig 1*F***). The epithelial structures of the samples were always negative in controls performed with either preimmune serum or incubation with neutralizing excess of peptide antigen (**Fig 3*C***).

In larger well-granulating wounds several weeks old, epilysin was detected in basal and partly suprabasal keratinocytes away from the migrating edge (**Fig 2*A***) over a greater length of epidermis compared to that seen in acute wounds. Epilysin expression coincided with the area where BM type IV collagen started to show an intact pattern by immunostaining (**Fig 2*B***). Epilysin did not colocalize with MMP-10 mRNA or MMP-9 protein in these wounds (**Fig 2*E*** *and* data not shown), but it overlapped with the distal front of MMP-1-positive cells to some extent (**Fig 2*D***). In both wounds and pyogenic granulomas, the epilysin-positive area was clearly hyperproliferative as assessed by Ki-67 staining (**Fig 2*A*, *C*, *F*, *G***). Strong intracellular LN-5 staining, characteristic for migrating epithelial cells of the wound margin, was usually absent from epilysin-positive areas in both chronic cutaneous wounds (diabetic, decubitus, rheumatoid) (**Fig 3*A*, *B***) and ulcerated pyogenic granulomas (data not shown).

Epilysin in suction blisters To understand whether epilysin can be induced in keratinocytes without a direct contact with underlying extracellular matrix, suction blisters harvested at 2, 4, and 9 d were studied. If the blister roof was left intact, epilysin was detected at some basal and suprabasal keratinocytes distal from the migrating tip at day 2 (**Fig 3*D***). Epilysin was abundantly expressed by proliferating basal and suprabasal keratinocytes in both types of 4 d blisters, however. Epilysin-positive keratinocytes were generally not positive for LN-5 intracellularly (**Fig 3*E*, *G***), and they resided on an intact BM as assessed by type IV collagen staining (**Fig 3*E*, *F***). As in acute wounds, MMP-9 and MMP-10 mRNAs did not colocalize with epilysin (data not shown). Epilysin was not detected any more in the basal keratinocytes of re-epithelialized 9 d blisters (data not shown).

Epilysin in skin cancers No immunosignal for epilysin could be detected in basal keratinocytes of benign hyperproliferative skin disorders such as lichenoid eczema characterized by acanthotic

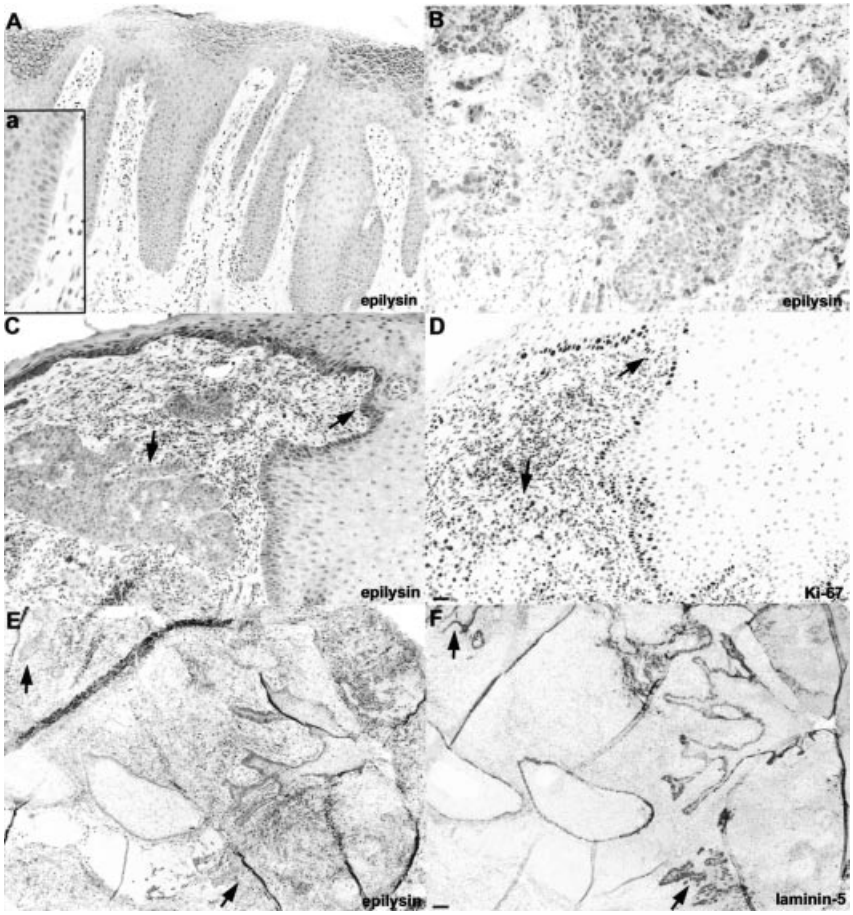


Figure 4. Epilysin is not expressed in invasive cancer cells. (*A*) Negative basal cell immunostaining for epilysin in a sample of lichenoid eczema with prominent acanthosis. *Inset a*: Higher magnification of epilysin-negative basal cell area. (*B*) Negative immunostaining for epilysin in a basal cell cancer. (*C*) Positive basal cell staining for epilysin at the surface epithelium of a well-differentiated squamous cell carcinoma developed from a chronic wound. (*D*) Immunostaining for Ki-67 in the same sample. *Arrows* depict corresponding spots. (*E*) Immunostaining for epilysin in an ulcerated area of another squamous cell carcinoma developed from a chronic wound. (*F*) Immunostaining for LN-5. *Arrows* depict epilysin-negative, but LN-5-positive, cancer islands. Counterstaining with hematoxylin (*A–F*). *Scale bars*: (*A*)–(*D*) 20 μm; (*E*), (*F*) 40 μm.

hyperproliferative epidermis (**Fig 4*A***, *inset a*), suggesting that tissue injury is needed for epilysin induction. Epilysin was not detected in aggressive cutaneous sclerosing basal (**Fig 4*B***) or squamous cell carcinomas (grades II and III) either. In four grade I squamous cell carcinomas that had arisen in chronic ulcers, however, epilysin could be detected in proliferating basal and suprabasal keratinocytes in the ulcerated area (**Fig 4*C***, ***D***). We also immunostained these samples for MMP-9 and found that it was present not in the epilysin-positive epithelium but deeper in epithelial cancer islands (data not shown). In contrast to results with various other cancer-associated MMPs including MMP-1, MMP-2, MMP-11, and MMP-13 (Airola *et al*, 1997; Johansson *et al*, 1999), epilysin was not detected in small invading cancer cell nests (**Fig 4*C–F***).

Epilysin was detected in the cytoplasm of HaCaT cells cultured on Laboratory-Tek chamber slides (**Fig 5*A***). When keratinocytes cultured on collagen I in DMEM were wounded, no staining was detected in migrating cells, supporting our *in vivo* observation that epilysin expression is associated with proliferating but not with migrating keratinocytes (**Fig 5*C***, ***D***).

Regulation of epilysin mRNA in epidermal cells *in vitro* To study the regulation of epilysin expression, we cultured primary keratinocytes isolated from seven different patients, transformed HaCaT cells, and A431 cells in the presence of various growth factors and cytokines and used quantitative real-time RT-PCR for analysis. Basal levels for epilysin mRNA in A431 cells were quite low, as it was detected only at cycle 31, whereas in HaCaT cells and human epidermal keratinocytes it was found already at cycles 26–27. None of the cytokines or growth factors studied (bFGF, EGF, GM-CSF, HGF, IFN-γ, IL-1β, KGF, PDGF, TGF-β1, VEGF, IGF-1) significantly upregulated the production of epilysin mRNA. TGF-β showed a slight inductive tendency, but the induction did not exceed 2.0-fold (**Fig 6**), which is considered the smallest significant value in the Taqman analysis. In contrast, significant upregulation of epilysin mRNA was seen when primary keratinocytes were stimulated with TNF-α (**Fig 6**). After this finding, we also studied the effect of this cytokine in subconfluent keratinocyte cultures and saw that the TNF-α upregulation was even higher than in confluent cells. Furthermore, stronger immunostaining of TNF-α treated cells cultured on Laboratory-Tek chamber slides compared with unstimulated control cells was observed (**Fig 5*E–G***). When cultured cells were stimulated by KGF, cell proliferation was evident but no changes in immunostaining for epilysin were observed compared to unstimulated cells (data not shown). Direct contacts between the cells and components of the extracellular matrix did not induce the production of epilysin, as analyzed by cells cultured on collagens I or IV, fibronectin, laminin-1, or the BM matrix Matrigel. Our results were confirmed in several experiments, and growth factor responsiveness was observed in every experiment. A clear induction of MMP-10 was observed in each Taqman run in keratinocytes treated with TGF-β1 (Rechardt *et al*, 2000).

DISCUSSION

In this work we find that epilysin is produced *in vivo* by nonmigrating, proliferating keratinocytes in response to skin injury. It is generally not expressed by the same keratinocyte population that synthesizes MMP-1, MMP-9, and MMP-10, thus emphasizing the strict spatial and temporal regulation of different MMPs during epithelialization (**Fig 7**). Much like epilysin, MMP-3 is expressed just behind the migrating front by keratinocytes still in contact with the underlying BM (Saarialho-Kere *et al*, 1994). MMP-3 is known to activate several other MMPs, and this cannot be excluded in the case of epilysin either.

Epilysin is a secretory MMP. Its *in vivo* substrates or activators are not known at present. Unlike in this study, Lohi *et al* (2001) found epilysin also in migrating keratinocytes. This may be due to the use

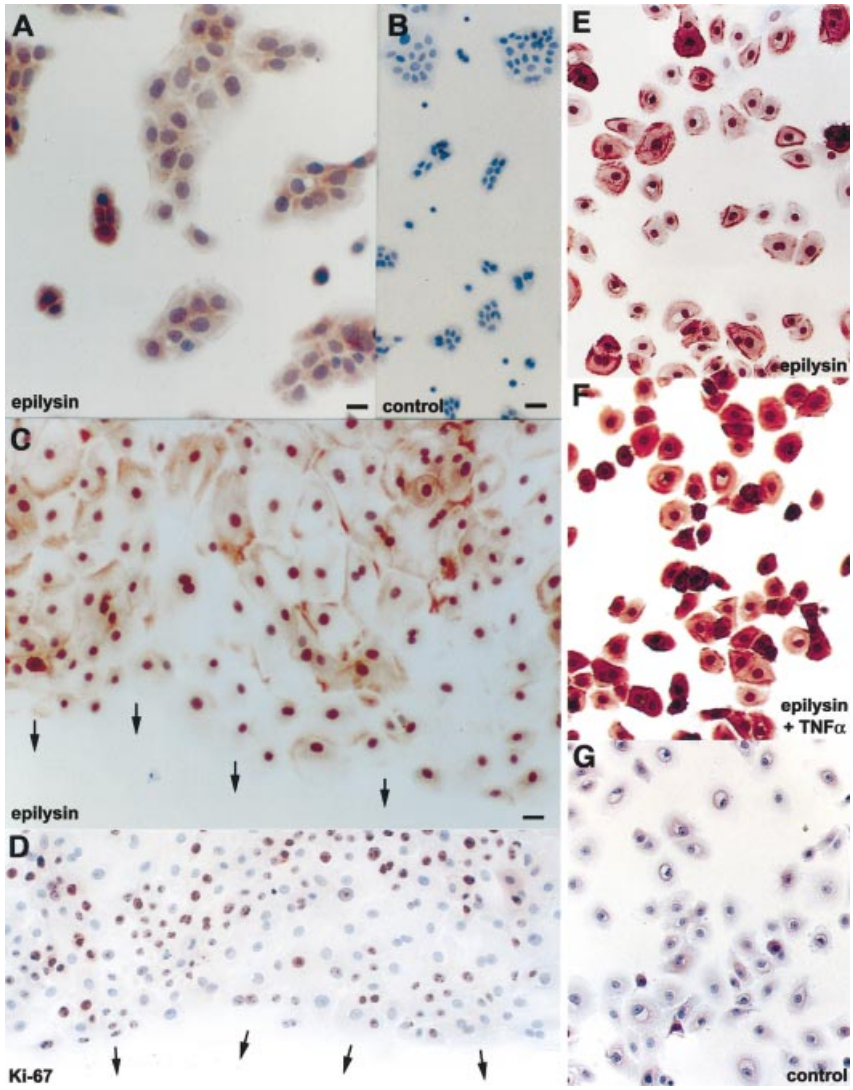


Figure 5. Migrating cells in culture are devoid of epilysin. (*A*) Intracytoplasmic staining for epilysin in cultured HaCaT cells. (*B*) Negative control (preimmune serum). (*C*) Keratinocytes (in DMEM) cultured on type I collagen at the migrating front do not express epilysin. (*D*) Immunostaining for Ki-67 in an adjacent chamber. *Arrows* depict the wounded area and the migration direction of the cells. (*E*) Epilysin staining of unstimulated primary keratinocytes cultured on plastic. (*F*) TNF-α-treated keratinocytes with enhanced staining. (*G*) Negative immunocontrol (preimmune serum). Aminoethylcarbazol was used as chromogenic substrate and counterstaining was performed with hematoxylin (*A*–*G*). *Scale bars*: (*A*) 10 μm; (*C*), (*E*)–(*G*) 20 μm; (*B*), (*D*) 40 μm.

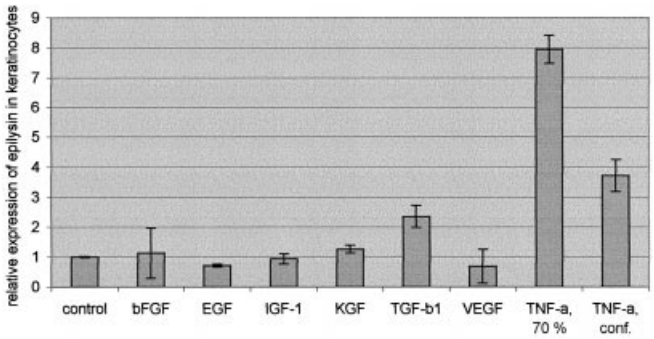


Figure 6. Epilysin mRNA expression is induced by TNF-α in primary keratinocytes. Cells were cultured in KGM and stimulated with the cytokines and growth factors indicated for 24 h. Total RNA was extracted, reverse transcribed, and analyzed by real-time quantitative RT-PCR (Taqman, PE Biosystems) using GAPDH as an endogenous control. Results are illustrated relative to the mRNA levels of control cells. Epilysin induction by TNF-α was much more prominent in subconfluent (70%) than confluent cells.

of *ex vivo* explants in culture and not wound biopsies. Although these *ex vivo* wounds mimic the MMP expression of normal wound repair in many respects (Pilcher *et al*, 1997), differing culture conditions and the lack of necrosis and thrombosis may modify MMP expression.

In addition to cell–matrix and cell–cell interactions (Saarialho-Kere *et al*, 1993), soluble factors present within the extracellular matrix may also play an important role in regulating the expression of MMPs by keratinocytes. Our results indicate that the expression of epilysin in cultured keratinocytes cannot be induced by keratinocyte mitogens such as EGF, bFGF, KGF, or IGF-1, or by HGF, IFN-γ, IL-1β, VEGF, PDGF, or TGF-β1 *in vitro*. The results were similar in both normal and immortalized (HaCaT) as well as transformed keratinocytes (epidermoid carcinoma A431). All the tested cytokines are instrumental in wound repair and variably upregulate MMP-1, MMP-3, MMP-9, and MMP-10 in keratinocytes during epithelialization (Salo *et al*, 1994; Dunsmore *et al*, 1996; Pilcher *et al*, 1997; Rechardt *et al*, 2000). In contrast, TNF-α constantly upregulated epilysin in primary keratinocytes obtained from several different patients.

TNF-α is a multifunctional pro-inflammatory cytokine that is mainly produced by macrophages. It is an angiogenic factor and capable of, for example, inhibiting tumor growth (Feiken *et al*, 1995). Expressed by neutrophils and macrophages subadjacent to the wound clot (Feiken *et al*, 1995), it is an important mediator during the inflammatory phase of wound healing and stimulates the secretion of several MMPs during skin repair (Han *et al*, 2001). TNF-α is also known to enhance invasive migration and mitogenesis of fibroblasts, and it in fact upregulates KGF gene expression in these cells, which may ultimately signal keratinocytes to proliferate (Tang and Gilchrest, 1996). In keratinocyte cultures TNF-α is not a mitogen but inhibits cell proliferation when

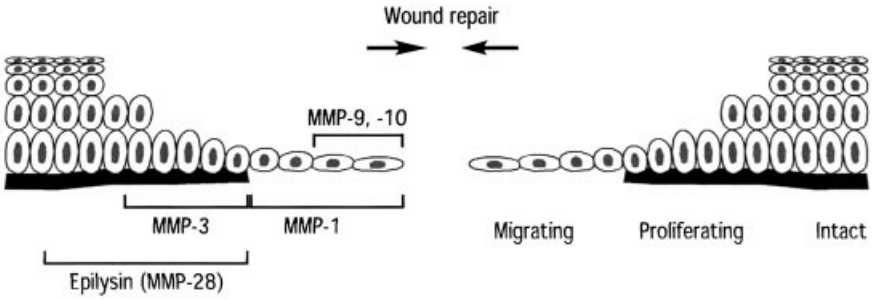


Figure 7. Spatial patterns of MMP expression in the epidermis during wound healing.

administered for several days (Pillai *et al*, 1989). This contrasts with our findings *in vivo* showing the expression of MMP-28 in Ki-67-positive cells. One of the explanations for this discrepancy could be that there exist many examples where *in vivo* epidermal keratinocytes respond to cytokine expression in a fashion quite distinct from that observed *in vitro* (Turksen *et al*, 1992).

According to our immunostaining results, epilysin is not associated with the migration of keratinocytes in skin cancers. Epithelial cells in both squamous cell carcinomas and adenocarcinomas of the lung produce epilysin, however (Saarialho-Kere *et al*, unpublished). This may be due to a specific cell type and/or tissue type governing its expression, which is a typical feature for many MMPs such as matrilysin (Dunsmore *et al*, 1998). Interestingly, the closely related metalloenzyme MMP-19 is not associated with invasive phenotype and neoplastic dedifferentiation (Djonov *et al*, 2001), and thus seems to possess the same kind of biologic pattern during transformation of mammary epithelium as epilysin has in skin cancer progression.

The promoter of epilysin contains a functional GT-box/Sp1 site that is necessary for basal promoter activity (Illman *et al*, 2001). Among other MMPs, also MMP-2, MMP-9, MT1-MMP, and MMP-19 promoters have similar elements (Mohan *et al*, 1998; Lohi *et al*, 2000; Muller *et al*, 2000; Price *et al*, 2001). In fact, Sp1 transcription factor is selectively induced in the corneal epithelium migrating to heal a wound (Mohan *et al*, 1998), and during cutaneous wound repair epilysin may also be regulated through this transcriptional pathway. TNF-α upregulates 92 kDa gelatinase in human skin through the NF-κB pathway (Han *et al*, 2001) and this cascade may mediate the induction of epilysin even though its promoter has not been reported to contain a consensus NF-κB binding site.

In agreement with the behavior of MMP-3, MMP-9, and MMP-10 in keratinocytes (Sudbeck *et al*, 1997), epilysin expression was not modulated by matrices in cell culture. Analogously, epilysin was upregulated *in vivo* in suction blisters despite the fact that in them keratinocytes are not in direct contact with dermal matrices (Kainulainen *et al*, 1998). Therefore, contact with a specific extracellular matrix component is not a prerequisite for epilysin induction, which is turned off soon after completion of re-epithelialization.

LN-5 immunohistochemistry was performed to investigate the migratory phenotype of epilysin-positive epithelial cells (Larjava *et al*, 1993). Intracellular staining for LN-5 reflects active synthesis of this protein needed for the deposition of normal BM and is usually encountered in migrating cells of suction blisters and acute wound edges (Kainulainen *et al*, 1998; Rechardt *et al*, 2000). Based on our serial specimens, keratinocytes expressing epilysin do not seem to be migratory. Instead, LN-5 staining becomes confined to the BM (**Fig 4*B***) in the epilysin-positive areas of the epidermis. Cleavage of the precursor molecule to mature LN-5, needed for hemidesmosome assembly, may signal the epithelial cells to become quiescent and form integrated tissue (Häkkinen *et al*, 2000). Unfortunately, our antibody does not distinguish between mature and precursor LN-5. To date, MMP-2 and MMP-14 are the only metalloenzymes known to cleave LN-5 *in vitro* (Giannelli *et al*, 1997; Koshikawa *et al*, 2000). Whether epilysin also belongs to that group awaits further studies.

Although the physiologic substrates of epilysin have not been identified yet, its expression pattern suggests that it may be needed to restructure the newly formed BM. Keratinocytes at the wound edge of tissue damage augment their basal proliferating phenotype, supplying new cells for the migrating front. Thus it may be that none of the BM components is the substrate of epilysin but that it is needed to degrade adhesive proteins like cadherins or type XIII collagen to release new cells for the migrating front.

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