Stromelysin-2 is Upregulated During Normal Wound Repair and is Induced by Cytokines

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Stromelysin-2 is a matrix metalloproteinase that degrades *in vitro* several protein components relevant to wound repair such as collagens III and IV, gelatin, nidogen, laminin-1, proteoglycans, and elastin. Furthermore, it can activate other matrix metalloproteinases, such as collagenase-1 (matrix metalloproteinase-1) and collagenase-2 (matrix metalloproteinase-8), as well as 92 kDa gelatinase. The aim of this study was to determine in a large variety of wounds (normally healing dermal and mucosal wounds, suction blisters, *ex vivo* cultures, diabetic, decubitus, rheumatic, and venous ulcers) and keratinocyte cultures, which factors contribute to stromelysin-2 expression and how it is induced in relation to other matrix metalloproteinases. Our results show that stromelysin-2 mRNA and protein are upregulated later (at 3 d) than matrix metalloproteinase-1 in normally healing wounds and *ex vivo* explants, in which stromelysin-2 is invariably expressed by keratinocytes migrating over dermal matrix. The number of keratinocytes expressing stromelysin-2 was greatest in chronic inflamed diabetic and venous ulcers compared with rheumatoid and decubitus ulcers, six of which had no signal. In keratinocyte cultures, tumor necrosis factor-α, epidermal growth factor, and transforming growth factor-β1 induced stromelysin-2 expression as measured by quantitative reverse transcriptase–polymerase chain reaction, whereas different matrices did not upregulate the mRNA. Immunostaining demonstrated stromal transforming growth factor-β1 in contact with the stromelysin-2-positive keratinocytes. Our results suggest that stromelysin-2 expression is important for the normal repair process and is upregulated by cytokines rather than cell–matrix interactions. Stromelysin-2 is most likely to participate in the remodeling of the newly formed basement membrane, and is not overexpressed in retarded wound healing.

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Cutaneous wound healing is a complex process involving clot formation, inflammation, re-epithelialization, angiogenesis, fibroplasia, wound contraction, and connective tissue remodeling (Martin, 1997). Proteolytic degradation of the extracellular matrix and basement membranes (BM) is implicated in many of these processes and it is well established that spatially and temporally controlled expression of several distinct matrix metalloproteinases (MMP) is associated with wound repair (Martin, 1997; Parks *et al*, 1998). Stromelysin-2 (MMP-10) belongs to the MMP subgroup of stromelysins that *in vitro* has a very broad substrate specificity, being able to degrade several protein components relevant to wound repair, such as collagens III and IV, gelatin, nidogen, laminin, proteoglycans, and elastin (Chandler *et al*, 1997; Nagase, 1998). Furthermore, it can activate collagenase-1 (MMP-1), neutrophil collagenase (MMP-8, collagenase-2), and 92 kDa gelatinase (MMP-9) (Nagase, 1998; Nakamura *et al*, 1998).

Keratinocyte migration across the dermal matrix is dependent on MMP-1 (Pilcher *et al*, 1997), which is the best characterized MMP in the context of wound repair. We have previously shown that MMP-10 mRNA is expressed by basal keratinocytes at the migrating front of epidermis, the same population of cells that also produces MMP-1 mRNA (Saarialho-Kere *et al*, 1994; Vaalamo *et al*, 1996). When MMP-1 is invariably expressed at the wound edge keratinocytes in normally healing and chronic wounds (Saarialho-Kere *et al*, 1993), however, MMP-10 was only detected in 50–70% of the chronic venous and decubitus ulcers examined (Saarialho-Kere *et al*, 1994; Vaalamo *et al*, 1996). Furthermore, we were able to find MMP-10 mRNA in a small number of basal keratinocytes in some normally healing wounds (Vaalamo *et al*, 1996). The aim of this study was to determine in a greater variety of wound tissue which factors contribute to MMP-10 expression in healing wounds and whether MMP-10 is essential or detrimental to normal wound repair, as excess proteolysis has been implicated as one of the causes of chronic ulcerations (Grinnell *et al*, 1992; Wysocki *et al*, 1993; Bullen *et al*, 1995).

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Abbreviations: BM, basement membrane; MMP, matrix metalloproteinase; MMP-10, stromelysin-2; MMP-1, collagenase-1; MMP-8, neutrophil collagenase or collagenase-2.

Table I. MMP-10 mRNA-positive cells in normally healing and different chronic wound types as assessed by *in situ* hybridization

Diagnosis	Total no. of samples	No. of positive cells			
		0	1–19	20–39	40+
Acute wounds					
1–2 d	3	3	–	–	–
3–4 d	8	1	4	3	–
5–6 d	4	1[a]	–	1	2
7–9 d	4	2[a]	–	2	–
Chronic wounds					
Venous ulcer	11	3	3	4	1
Diabetic ulcer	6	–	1	1	4
Rheumatoid ulcer	4	2	–	2	–
Decubitus ulcer	6	2	2	1	1
Well granulating	6	1	2	3	–

[a]Were already re-epithelialized.

MATERIALS AND METHODS

Tissues Samples of 27 chronic ulcers of various etiologies were collected from patients at the Department of Plastic Surgery, Helsinki University Central Hospital. The samples included venous, decubitus, diabetic, and rheumatoid ulcers (**Table I**). Six biopsies from clinically well granulating ulcers that required skin grafting because of their large size, were also examined (**Table I**). Biopsies of normally healing donor areas of anterior thigh were obtained 1–9 d after wounding from 19 patients of the Department of Dermatology, University of Helsinki, undergoing a pinch grafting procedure (Vaalamo *et al*, 1996). These specimens were used as samples of normally healing wounds (**Table I**). Suction blisters were induced on the abdominal skin of four healthy young volunteers, using a Dermovac device as described previously (Kiistala, 1968). Biopsies were performed 2, 4, and 9 d after induction of the blister. All tissue samples were formalin fixed and paraffin embedded, and contained both epithelium and ulcer bed.

Full thickness gingival wounds were generated as described (Ravanti *et al*, 1999). The wound samples were obtained by punch biopsy after 3, 7, 14, and 28 d, embedded in Tissue-Tek, frozen in liquid nitrogen, and stored at –70°C. Frozen sections (6 μm) were prepared, fixed in acetone, and used for immunohistochemical staining. Informed consent was obtained from each subject for all procedures.

Tissue cultures Biopsies (5 mm) of normal skin from different parts of the body from patients of the Department of Dermatology (age range 23–81 y) were placed in 35 mm culture dishes and cultured in Dulbecco's modified Eagle's medium (Gibco BRL, Life Technologies, Paisley, Scotland) supplemented with 5% fetal bovine serum. At indicated times (24, 48, or 72 h) the tissue samples were fixed in 10% formalin and processed for paraffin embedding.

RNA probes The production and specificity of the anti-sense human MMP-10 and MMP-1 cRNA probes have been described (Saarialho-Kere *et al*, 1992, 1994). As a control for nonspecific hybridization, sections were hybridized with ^{35}S-labeled sense RNA from a bovine tropoelastin cDNA. The validity of this probe as a negative control has been confirmed by northern blot analysis (Prosser *et al*, 1989) and by *in situ* hybridization (Saarialho-Kere *et al*, 1992).

***In situ* hybridization** *In situ* hybridization was performed on 4 μm sections as described in detail (Prosser *et al*, 1989). All the samples were treated with proteinase K and were washed in 0.1 mol per liter triethanolamine buffer containing 0.25% acetic anhydride. The sections were covered with 25–70 μl of hybridization buffer containing 2.5–4×10^4 cpm per μl of ^{35}S-labeled anti-sense or sense RNA probe, and incubated at 50°C to 55°C for 18 h in a humidified chamber. After hybridization, the slides were washed under stringent conditions, including treatment with RNase A to remove unhybridized probe. Following 15–45 d of autoradiography, the photographic emulsion was developed, and the slides were stained with hematoxylin and eosin. Cutaneous wounds previously positive for MMP-10 and MMP-1 were used as positive controls and each sample was hybridized in at least two experiments. The number of MMP-10-positive cells was counted under dark-field illumination by two independent investigators and rechecked under the bright-field view of the microscope.

Antibodies Immunohistochemical analysis using polyclonal sheep anti-human MMP-10 antibodies (Hembry *et al*, 1995) was performed to localize MMP-10 protein on wound sections. A monoclonal Mib-1 antibody (0505, Immunotech, Marseilles, France), which reacts with the Ki-67 nuclear antigen, was used to differentiate the proliferating cells from the quiescent epithelial cells (Cattoretti *et al*, 1992). A monoclonal antibody to type IV collagen (M785, Dako, Glostrup, Denmark) was used to stain the epithelial BM. Fibronectin was stained by using a monoclonal antibody (MS-426-P1, NeoMarkers, Union City, CA). MMP-9 and 72 kDa gelatinase (MMP-2) proteins were detected by using monoclonal antibodies (GE213, Diabor, Oulu, Finland; IM33L, Calbiochem, Cambridge, MA). Laminin-5 producing cells were identified with polyclonal rabbit antibodies against the γ-2 chain of laminin-5 (Pyke *et al*, 1994; Airola *et al*, 1997). Transforming growth factor-β1 (TGF-β1) was immunostained using a monoclonal antibody (MAB1032, Chemicon). E-cadherin was localized with a monoclonal anti-E-cadherin antibody (HECD-1, Zymed, South San Francisco, CA). Tissue macrophages were identified using a monoclonal antibody (KP-1, M814, Dako, Carpinteria, CA) to a specific macrophage marker, CD68 (Pulford *et al*, 1989).

Immunohistochemistry Immunostaining of the sections serial to those used for *in situ* hybridization and frozen sections of gingival wounds was performed by the avidin–biotin–peroxidase complex technique (Vectastain ABC Kit; Vector Laboratories, Burlingame, CA). Diaminobenzidine or aminoethylcarbazole (MMP-10, Ki-67) were used as chromogenic substrates and Harris hematoxylin as counterstain, as described in detail (Saarialho-Kere *et al*, 1993). If needed, the sections were pretreated with 10 mg per ml trypsin (MMP-10, CD68, type IV collagen, fibronectin, MMP-9, MMP-2, and laminin-5) or by antigen retrieval (Ki-67 and E-cadherin) as described (von Boguslawski, 1994). Type IV collagen antibody was diluted 1:75, E-cadherin, and MMP-2 1:200, CD68 1:400, and Ki-67, fibronectin, MMP-9, laminin-5, and TGF-β1 1:500, and MMP-10 1:2000–1:3000. Controls were performed with mouse preimmune ascites fluid or with rabbit and goat preimmune serum.

Cell cultures Human keratinocytes were isolated from normal adult skin obtained from reductive mammoplasties and laparotomies. The subcutaneous fat and the deep dermis were removed, and the remaining tissue was incubated overnight at 0.25% trypsin in solution A (Gibco BRL, Life Technologies). Following the incubation, the keratinocytes were scraped off from the epidermis with a scalpel and suspended in keratinocyte growth medium (KGM, Gibco BRL, Life Technologies), supplemented with 5 ng per ml epidermal growth factor (EGF) and 50 μg per ml bovine pituitary extract (supplied by the vendor), and containing 2% decalcified FBS (Biological Industries, Kibbutz Bet Haemek, Israel). The keratinocytes were maintained in KGM with supplements, and passages 3–6 were used in the experiments.

To study the regulation of MMP-10 expression, keratinocytes were seeded in 24-well plates and allowed to attach for 24 h. Then the cells were repeatedly washed with PBS and treated with tumor necrosis factor-α (TNF-α, 10 ng per ml, Sigma, St Louis, MO), interleukin-1β (10 ng per ml; Roche Molecular Biochemicals, Mannheim, Germany), TGF-β1 (1 ng per ml, Roche Molecular Biochemicals), EGF (10 ng per ml, Sigma), keratinocyte growth factor (KGF, 10 ng per ml, Sigma), hepatocyte growth factor (HGF, 10 ng per ml, Sigma), or platelet-derived growth factor (PDGF, 20 ng per ml, Sigma). Keratinocytes were also plated on to 35 mm dishes precoated with type I collagen, fibronectin, type IV collagen, and laminin-1 (all from Becton Dickinson, Bedford, MA), and 24-well plates precoated with Matrigel (Becton Dickinson) and gelatin (0.2 mg per cm^2, Sigma). All experiments were performed both in KGM (Ca^{2+} 0.09 mM) and in Dulbecco's modified Eagle's medium (Ca^{2+} 1.8 mM), without supplements or FBS. After 24 h total RNA was extracted from the cells.

PCR primers and probes PCR primers (Sigma Genosys, Cambridge, UK) and a probe (PE Biosystems, Warrington, UK) for MMP-10 were designed using the computer program Primer Express (PE Biosystems). The fluorogenic probe contained a reporter dye (FAM) covalently attached at the 5′ end and a quencher dye (TAMRA) covalently attached at the 3′ end. The fluorogenic probes were labeled and high-pressure liquid chromatography purified by PE Biosystems. Pre-developed TaqMan assay reagents for endogenous control human GAPDH labeled with the VIC reporter dye (PE Biosystems) were used for the amplification of the control gene.

Quantitative reverse transcriptase–PCR Total cellular RNA was extracted from keratinocytes by using RNeasy miniprep-kit (Qiagen, Chatsworth, CA) following the manufacturer's instructions. Reverse transcription of RNA to cDNA was performed with TaqMan Reverse Transcription Reagents (PE Biosystems), following the manufacturer's instructions. The reaction volume was 20 μl, and random hexamers were used as primers. The quantitative PCR assays were performed with the ABI PRISM 7700 Sequence Detector System (PE Biosystems), which monitors the quantitative PCR in real-time as described (Heid *et al*, 1996). The quantitative PCR amplifications were performed in a total volume of 50 μl, containing 5 μl cDNA sample, 25 μl TaqMan Universal PCR Master Mix (PE Biosystems), and 2.5 μl human GAPDH endogenous control reagents or 100 nM of each primer and 100 nM of fluorogenic probe. MicroAmp Optical 96-Well Reaction Plates and Optical Caps (PE Biosystems) were used. The reaction conditions were as follows: initially, 2 min at 50°C and 10 min at 94°C, followed by a total of 40 cycles of 15 s at 94°C and 1 min at 60°C.

RESULTS

MMP-10 mRNA is expressed in the migrating epithelial tip starting at day 3 in normally healing wounds A total of 19 samples of normally healing full-thickness wounds were examined for the expression of MMP-10 mRNA. MMP-10 mRNA was expressed at the leading edge of migrating epithelium beginning the third postoperative day (**Figs 1*a*** and **2*a***). The expression continued until the re-epithelialization was complete at 7–9 d, after which it was gradually turned off (data not shown). There were six to 45 MMP-10-positive cells depending on the day of biopsy (**Table I**); this number was not essentially different from the number of positive cells in chronic wounds (**Table I**). Normal epidermis further away from the wound remained constantly negative. No MMP-10 mRNA was detected in the wound stroma (**Figs 1*a*** and **2*a***). A subset of samples positive for MMP-10 mRNA were stained immunohistochemically for MMP-10. The protein colocalized with the mRNA in the migrating epidermal tip (**Fig 1,** inset b′). As described previously (Saarialho-Kere *et al*, 1993; Vaalamo *et al*, 1996), MMP-1 mRNA expression was detected in the migrating epithelium in all the samples (n = 11) investigated. Expression could be detected on the first postoperative day (**Fig 1*d***), thus preceding the expression for MMP-10 (**Fig 1*c***), and continuing until the re-epithelialization was complete. MMP-1 mRNA was expressed by both the basal and the suprabasal keratinocytes, and the positive cells reached generally more distal from the wound edge than those positive for MMP-10. Also, stromal fibroblast-like or macrophage-like cells expressed MMP-1 in all the samples, confirming previous results (Saarialho-Kere *et al*, 1993; Vaalamo *et al*, 1996).

Migrating keratinocytes also express MMP-10 in mucosal wounds Very much like in dermal wounds, MMP-10 was expressed by keratinocytes in 3 d gingival wounds (**Fig 1*e***). MMP-10 was not any more detected intracellularly in basal keratinocytes of 7 d wounds but in a band-like pattern at the BM zone (**Fig 1*f***). When the re-epithelialization was complete (2 and 4 wk wounds), the expression of MMP-10 was shut down (**Fig 1*g***).

MMP-10 is also expressed in suction blisters Experimental suction blisters provide a model of skin injury, in which the lamina densa remains intact (Stenn and Malhotra, 1992). We examined nine samples of suction blisters taken 2, 4, or 9 d after induction of the blister from four different patients. Most of the samples were negative for MMP-10 mRNA; only one of four 4 d samples showed signal for MMP-10 in keratinocytes (**Fig 1*h–j***). Three of three 2 d suction blisters had MMP-1-positive basal keratinocytes (data not shown), which is in agreement with previously published findings (Saarialho-Kere *et al*, 1995) that MMP-1 expression is associated with keratinocyte migration even in the presence of a partially disrupted BM.

MMP-10 expression is induced later than that of MMP-1 in *ex vivo* cultures Twenty skin biopsies were cultured *ex vivo* in order to determine the temporal pattern of MMP-10 expression after injury. The samples cultured for 24 h did not have any signal for MMP-10 (**Fig 3*a***), although they had migrating cells based on laminin-5 staining (**Fig 3*b***). MMP-10 could be detected in few migrating keratinocytes in the samples harvested at 48 (**Fig 3*c***) and 72 h (**Fig 3*f***). Interestingly, some of the MMP-10 mRNA-positive cells also expressed MMP-9 (**Fig 3*d***, ***h***). Epithelial and stromal MMP-1 expression was detected in all the samples (24, 48, and 72 h) (**Fig 3*e***).

MMP-10 is expressed in chronic wounds at the ulcer edge Chronic wounds of different etiologies were investigated for the presence of MMP-10 mRNA (**Table I**). It was detected in the majority of the samples (25 of 33; 75%) in keratinocytes at the wound edge (**Fig 4*a***, ***c***, ***e***, ***f***). Most of the MMP-10 expressing cells were negative for Ki-67 (marker for proliferating keratinocytes) (**Fig 4*a***, ***b***). In addition, absence of type IV collagen staining under the MMP-10-positive keratinocytes (data not shown), and abnormal E-cadherin staining in the same area confirmed the migrating phenotype of MMP-10-positive keratinocytes (**Fig 4*d***). MMP-1 mRNA expression was found in the keratinocytes at the wound edge in all the samples investigated (n = 14), and was generally more intense and widespread than the expression for MMP-10 mRNA (**Fig 4*e***, ***f***). Abundant stromal expression for MMP-1 mRNA was detected in all samples (**Fig 4*g***), whereas no signal for MMP-10 was found there (**Fig 4*e***, ***f***). Histologically, the wounds expressing MMP-10 mRNA were deeper, and more inflammatory than the ones negative for MMP-10 mRNA (either decubitus or rheumatoid ulcers). The greatest number of MMP-10-positive keratinocytes was detected in diabetic ulcers (**Table I**; **Fig 4*c***, ***g***).

MMP-10-positive cells express laminin-5 To characterize the phenotype of MMP-10-positive cells, laminin-5 production was assessed immunohistochemically in sections serial to those used for MMP-10 *in situ* hybridization. In acute wounds and cultured skin biopsies, laminin-5 was produced by migrating keratinocytes at the wound edge (**Fig 2*b***), and the protein expression was detected as early as 24 h after injury. The cells positive for MMP-10 mRNA all showed intracellular staining for laminin-5 (**Fig 2*a–d***); however, compared with MMP-10, the laminin-5 producing cells extended further back from the wound edge (**Fig 2*b***, ***d***).

Almost all the chronic wound samples stained for laminin-5 (11 of 13) had positive basal keratinocytes bordering the ulcer (**Fig 2*d***). Five of the chronic samples did not express MMP-10 mRNA by the epidermal tip. One of these five samples had a unique staining pattern for laminin-5 by showing abundant stromal staining, and two samples did not have epithelial staining at all, indicating that the bordering keratinocytes were nonmigratory.

As MMP-2 has been shown to degrade laminin-5 (Giannelli *et al*, 1997), 10 samples were also stained for MMP-2 protein. It was detected in stromal fibroblasts in all samples, but generally staining was not detected under the MMP-10- and laminin-5-positive cells (**Fig 5*a***, ***b***).

A subset of chronic wound sections (n = 5) was stained immunohistochemically for fibronectin, which is an *in vitro* substrate of MMP-10 (Chandler *et al*, 1997; Murphy and Knäuper, 1997). Fibronectin was detected in all the layers of the wound bed, and the staining was the most intense in the upper wound bed in the vicinity of the MMP-10 epidermal edge (data not shown).

As our cell culture studies showed that TGF-β1 may be an inducer of MMP-10 expression, we stained for TGF-β1 protein in chronic wounds. It was detected in all samples (n = 8) in fibroblast-like cells in the stroma and as extracellular staining of the wound area as well. The stromal staining was often very intense close to the MMP-10-positive epidermal tip (**Fig 5*c***, ***d***).

Neutrophils are detected near MMP-10-positive keratinocytes in chronic wounds As MMP can be induced by cell–cell interactions (Lacraz *et al*, 1994), we histologically investigated the matrix surrounding MMP-10-positive keratinocytes. As assessed by morphologic criteria and

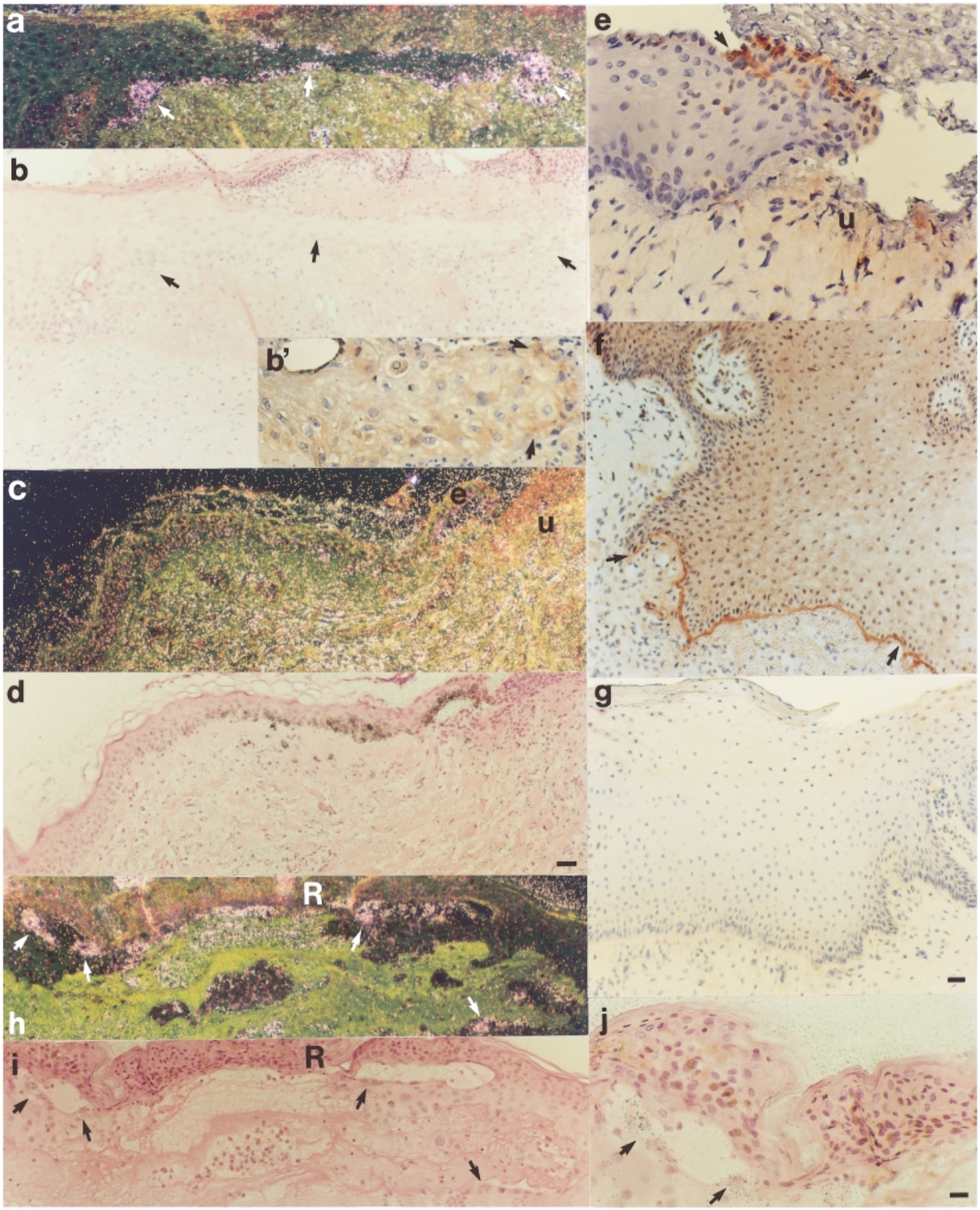

Figure 1. MMP-10 mRNA and protein are expressed by migrating keratinocytes of cutaneous and mucosal wounds beginning at 3 d after wounding. (*a*) Expression of MMP-10 mRNA in a 3 d normally healing wound. (*b*) Corresponding bright-field image. *Arrows* depict corresponding areas. (*Inset b′*) Immunostaining for MMP-10 in a 3 d wound (corresponds **Fig 4*a***) using polyclonal sheep antibodies. *Arrows* depict immunopositive cells. (*c*) *In situ* hybridization dark-field for MMP-10 mRNA in a 1 d acute wound. (*d*) *In situ* hybridization bright-field for MMP-1 mRNA in a serial section. Immunostainings for MMP-10 in gingival wound samples obtained at 3 d (*e*), 7 d (*f*), and 2 wk (*g*). *Arrows* depict positive immunostaining. (*h*) *In situ* hybridization dark-field image of MMP-10 in a 4 d suction blister. (*i*) Corresponding bright-field image. R = blister roof. *Arrows* depict corresponding spots. (*j*) Higher magnification of the area depicted at the left side of part (*h*). Epidermis is marked with the letter e and the ulcer bed with the letter u. Counterstaining with hematoxylin and eosin (*a–c*, *d*, *h–j*) or with hematoxylin (inset *b′*, *e–g*). *Scale bars*: (*a–d*, *f–i*) 50 μm; (*inset b′*, *e*, *j*) 25 μm.

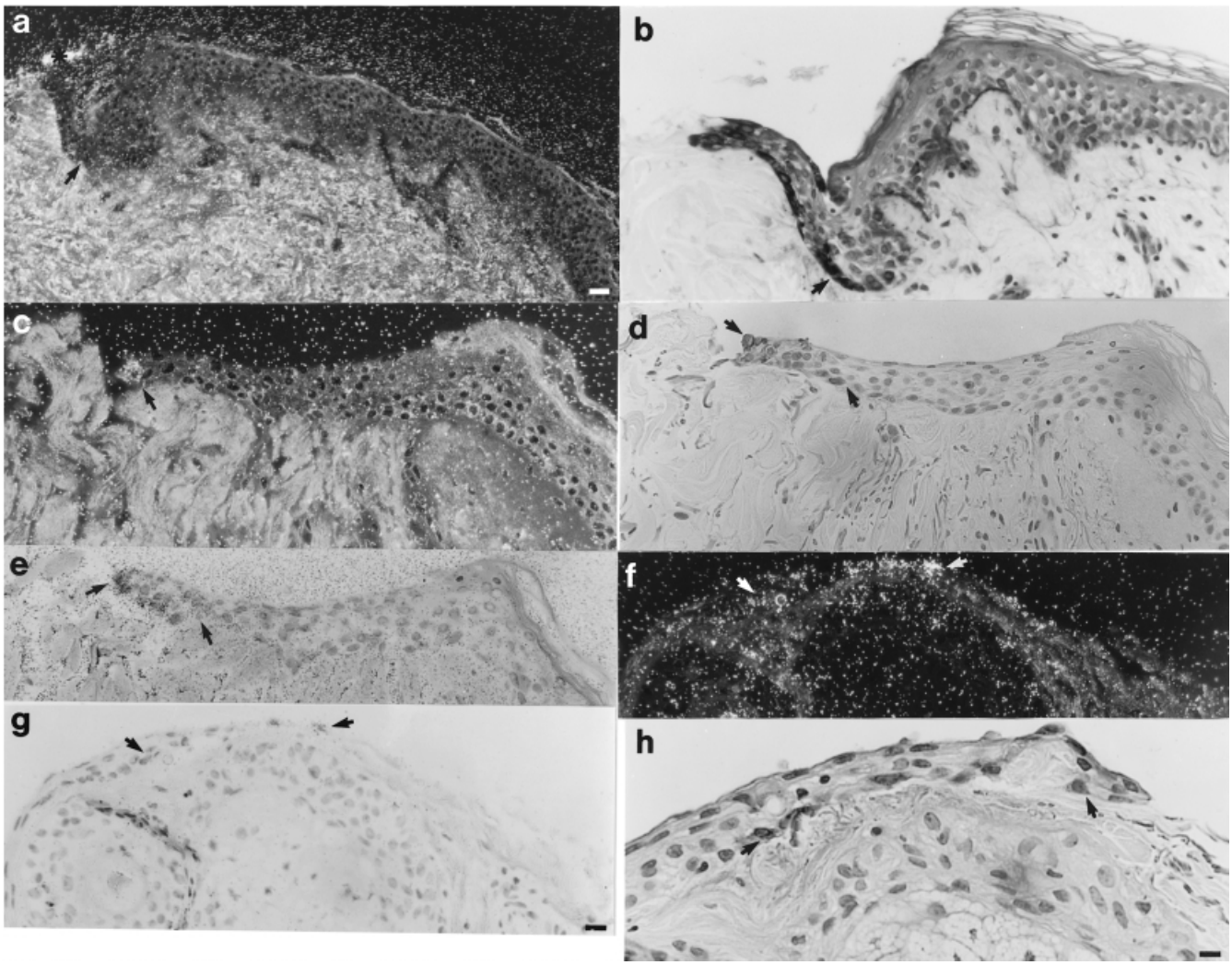

Figure 2. MMP-10 and MMP-9 expression is induced after that of MMP-1 in *ex vivo* skin explants. (*a*) A skin biopsy cultured for 24 h and hybridized for MMP-10 mRNA. The *star* marks a tissue edge artifact. (*b*) Immunostaining for laminin-5 in the same sample. *Arrows* mark corresponding spots. (*c*) A skin biopsy cultured for 48 h hybridized with a MMP-10 cRNA probe. (*d*) Immunostaining for MMP-9 in the same sample. (*e*) A serial bright-field section hybridized with an MMP-1 cRNA probe. *Arrows* depict the location of signal. (*f*) A 72 h cultured biopsy hybridized for MMP-10 mRNA. (*g*) Corresponding bright-field image. (*h*) Immunostaining for MMP-9 in the same specimen. *Arrows* depict positive cells. Counterstaining with hematoxylin and eosin (*a*, *c*, *e–g*) or with hematoxylin (*b*, *d*, *h*). *Scale bars*: (*a*) 50 μm; (*b–g*) 25 μm; (*h*) 13 μm.

immunostaining for MMP-9, a great number of polymorphonuclear leukocytes were often found either within or in close proximity to the MMP-10 mRNA expressing epithelium in the chronic wounds having the most MMP-10-positive cells (**Fig 3*h***). Staining for CD68 (n = 17) demonstrated that the chronic wounds have numerous macrophages within their stroma, and no particular spatial relation exists between the MMP-10-positive keratinocytes and macrophages. The presence of lymphocytes was assessed histologically (nuclear morphology and size) and they did not associate with MMP-10-positive areas.

MMP-10 is upregulated in keratinocytes by TGF-β1, EGF, and TNF-α, but not by matrix In order to characterize agents that may contribute to the induction of MMP-10, normal human keratinocytes were cultured in the presence of various cytokines and growth factors. Total RNA from these cells was analyzed with quantitative reverse transcriptase–PCR. Low basal level expression of MMP-10 mRNA was detected by control keratinocytes cultured on plastic without treatments (**Fig 6**) in accordance with previous data by Windsor *et al* (1993). In keratinocytes cultured in KGM, TGF-β1, EGF, and TNF-α upregulated MMP-10 mRNA expression, whereas interleukin-1β, KGF, HGF, and PDGF did not have any effect (**Fig 6**). In keratinocytes cultured in Dulbecco's modified Eagle's medium, interleukin-1β also induced MMP-10 expression (data not shown). Keratinocytes cultured in Dulbecco's modified Eagle's medium expressed MMP-10 at higher levels than those cultured in KGM, but generally the expression patterns were similar in both media. To assess whether components of the provisional matrix or basement membranes induce MMP-10 expression, keratinocytes were plated on different matrices (type I collagen, fibronectin, type IV collagen, laminin, Matrigel, and gelatin); however, compared with plastic, none of these matrices significantly upregulated the expression of MMP-10 mRNA as assessed by quantitative reverse transcriptase–PCR (data not shown).

DISCUSSION

During the course of re-epithelialization, keratinocytes at the wound edge migrate across the wound bed and cover it. Initiation of keratinocyte migration is one of the earliest responses of the epidermis to wounding and precedes cell proliferation by hours (Sarret *et al*, 1992). As shown in this study, in normally healing cutaneous wounds, MMP-10 is invariably expressed by keratinocytes migrating over the dermal matrix; however, MMP-10 is turned on later than MMP-1, which is already detectable 6–12 h after wounding (Inoue *et al*, 1995). MMP-10 may facilitate migration by degrading noncollagenous matrix or by remodeling

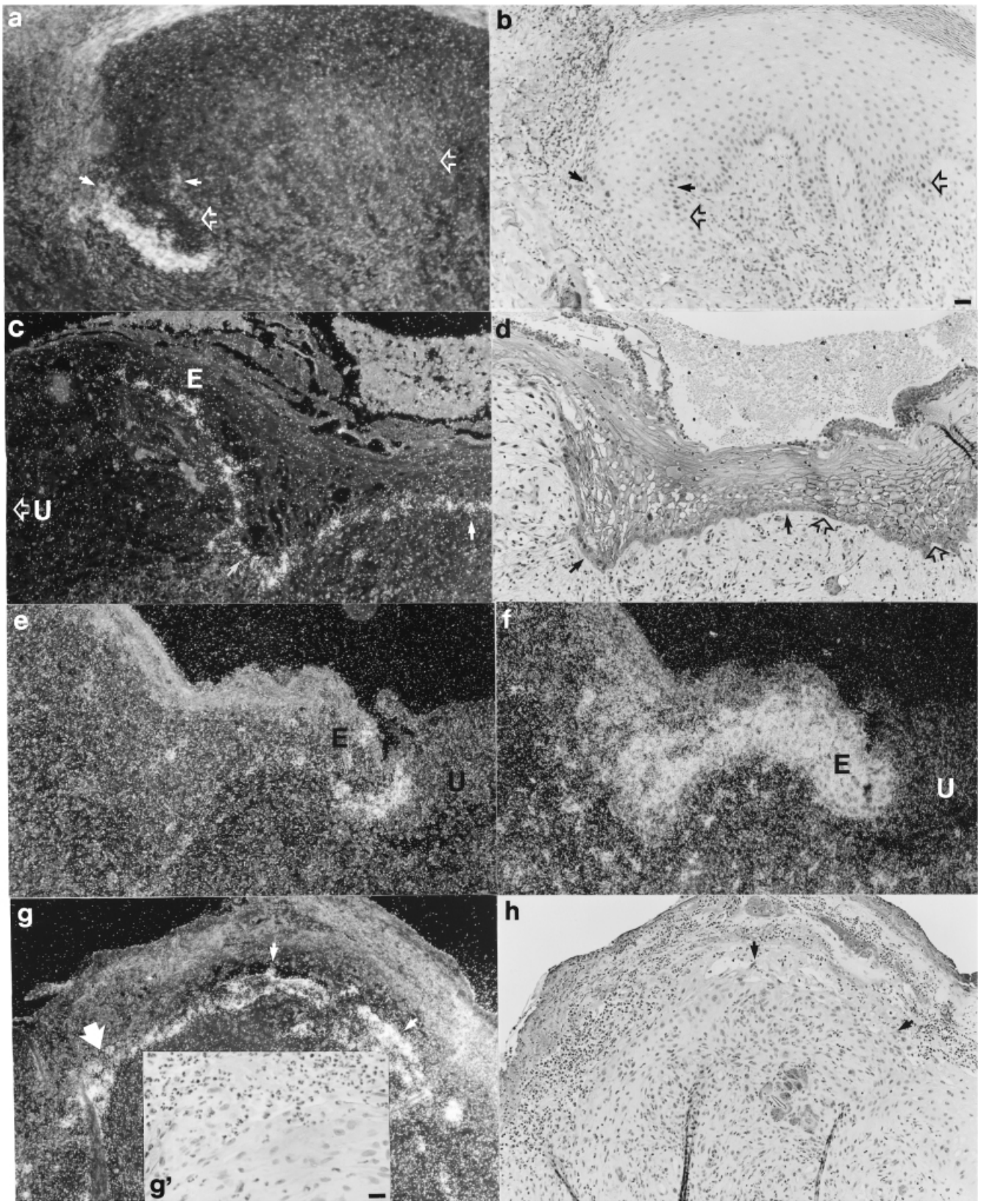

Figure 3. Cells expressing MMP-10 in chronic wounds have a migrating phenotype. (*a*) *In situ* hybridization dark-field for MMP-10 mRNA in a 1 y old venous ulcer. (*b*) Immunostaining for Ki-67 in a serial section. *Arrows* border the area of MMP-10-positive keratinocytes, *open arrows* the area of most Ki-67-positive cells. (*c*) *In situ* hybridization dark-field for a 3 mo old diabetic ulcer. (*d*) Immunostaining for E-cadherin in the same sample. *Arrows* depict corresponding spots and *open arrows* the area of normal E-cadherin staining. (*e*) *In situ* hybridization for MMP-10 in a 7 y old decubitus ulcer. (*f*) Serial section hybridized for MMP-1 mRNA. (*g*) A 9 mo old diabetic ulcer with signal for MMP-10 mRNA. (*h*) An adjacent section immunostained with monoclonal antibody for MMP-9. *Arrows* depict corresponding spots, *large arrow* the area shown in high magnification from (*h*) (*inset g′*). Epidermis is marked with the letter E and the ulcer bed with the letter U. Counterstaining was performed with hematoxylin and eosin (*a*, *c*, *e*, *f*, *g*) and with hematoxylin (*b*, *d*, *g′*, *h*). *Scale bars*: (*a–h*) 50 μm; (*inset g′*) 25 μm.

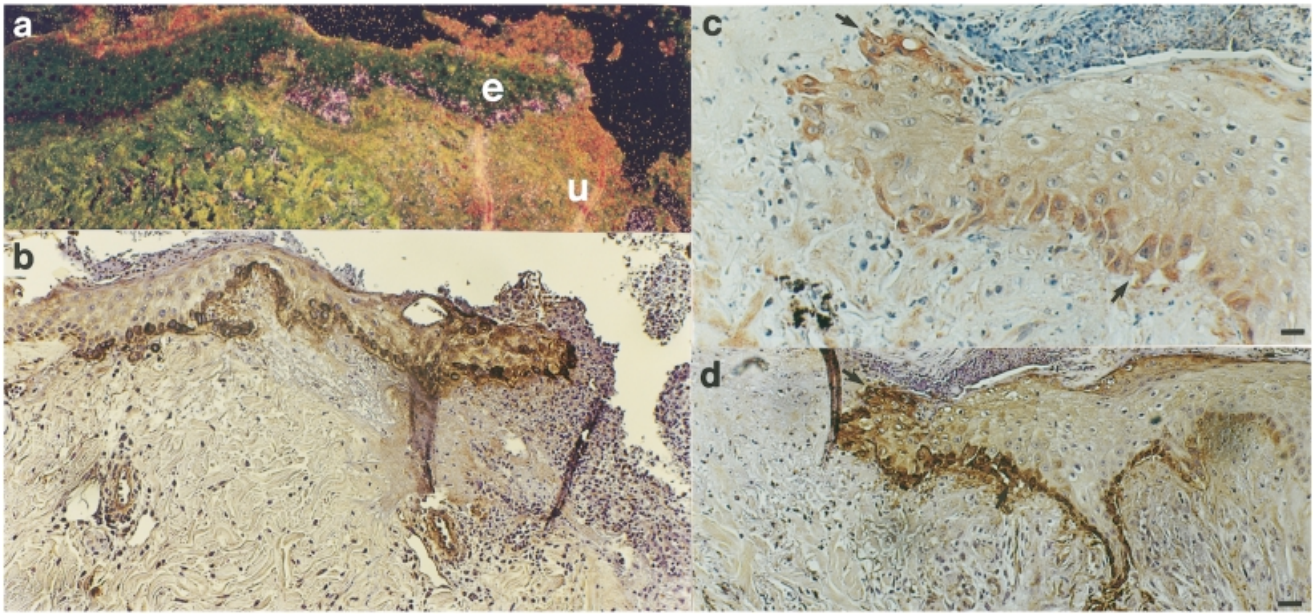

Figure 4. Laminin-5 partly colocalizes with MMP-10 in acute and chronic wounds. (*a*) Expression of MMP-10 in a 3 d normally healing wound. (*b*) Immunostaining for laminin-5 in the same specimen. (*c*) Immunostaining for MMP-10 in a 6 mo old chronic venous ulcer. (*d*) Staining for laminin-5. *Arrows* depict corresponding spots. Counterstaining was performed by hematoxylin and eosin (*a*) and with hematoxylin (*b–d*). *Scale bars*: (*a*, *b*, *d*) 50 μm; (*c*) 25 μm.

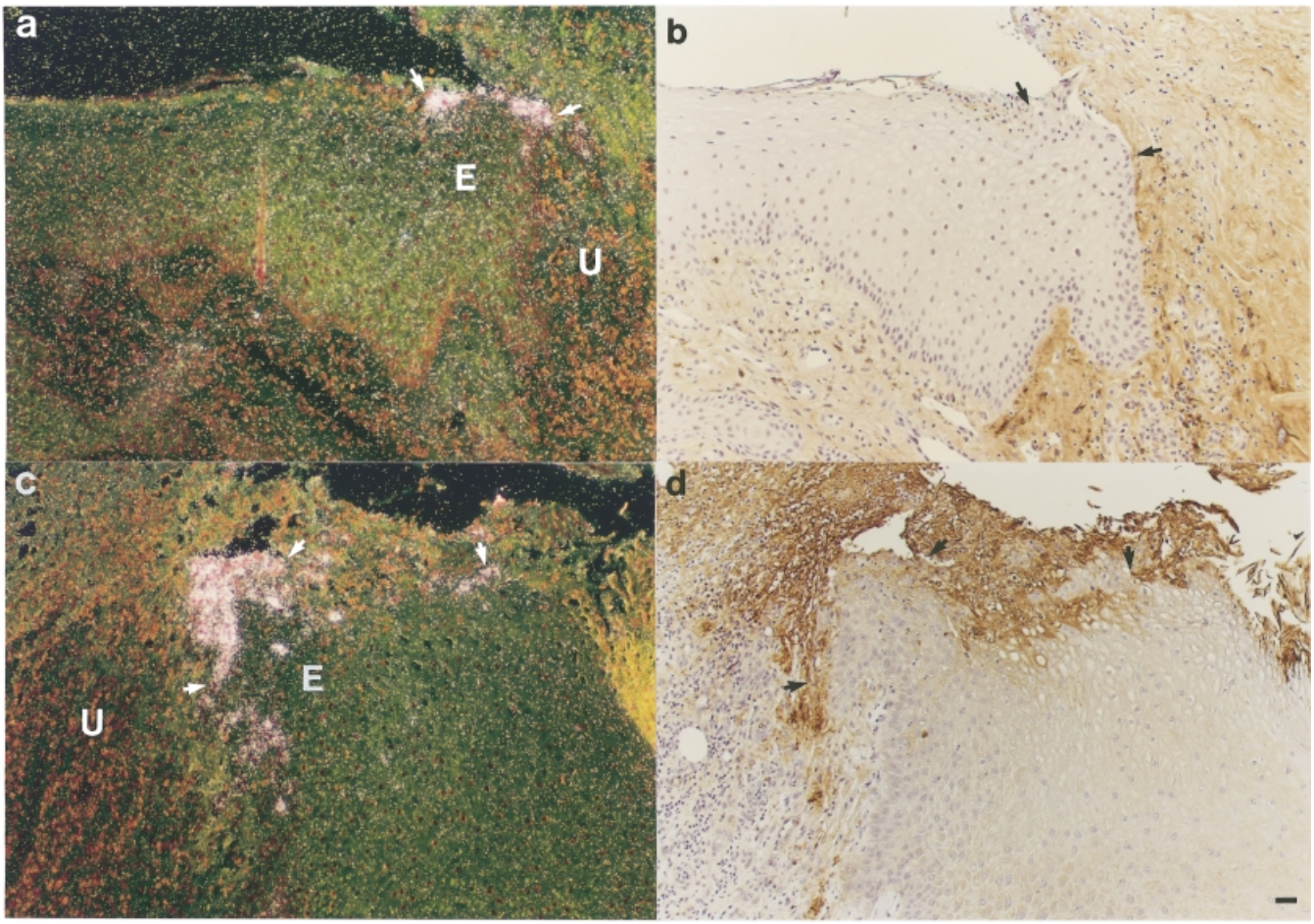

Figure 5. TGF-β, but not MMP-2, is detected in contact with MMP-10-positive keratinocytes. (*a*) *In situ* hybridization for MMP-10 in a 6 mo old decubitus ulcer. (*b*) Immunostaining for MMP-2 in the same sample. (*c*) *In situ* hybridization for MMP-10 in a 4 mo old diabetic ulcer. (*d*) Immunostaining for TGF-beta. *Arrows* depict MMP-10-positive areas. Epidermis is marked with the letter E and the ulcer bed with the letter U. Counterstaining was performed with hematoxylin and eosin (*a*, *c*) and with hematoxylin (*b*, *d*). *Scale bar*: 50 μm.

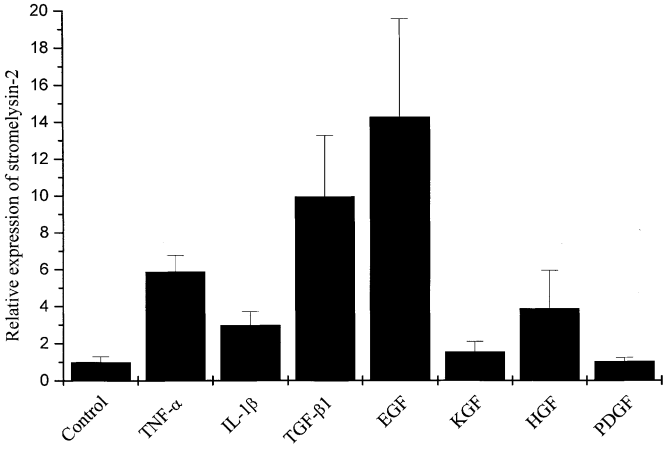

Figure 6. MMP-10 mRNA expression is induced in keratinocytes by TGF-β1, EGF, and TNF-α. Human keratinocytes were cultured in KGM and stimulated with TNF-α (10 ng per ml), interleukin-1β (10 ng per ml), TGF-β1 (1 ng per ml), EGF (10 ng per ml), KGF (10 ng per ml), HGF (10 ng per ml), and PDGF (20 ng per ml) for 24 h in triplicate wells. Cells without treatments were used as a control. Total cellular RNA was extracted and MMP-10 mRNA levels were analyzed by quantitative reverse transcriptase–PCR. Results are shown relative to mRNA levels from control keratinocytes. The values given are mean ± SD.

damaged BM (**Fig 1*f***). Like MMP-1 (Inoue *et al*, 1995), MMP-10 expression is rapidly turned off at the completion of re-epithelialization (**Fig 1*e–g***).

This is one of the first studies showing MMP-10 protein in migrating keratinocytes of wounds by immunohistochemistry that suggests MMP-10 mRNA and protein colocalize. Based on previous data, MMP-10-positive cells in normally healing wounds colocalize with urokinase plasminogen activator (Vaalamo *et al*, 1996) and as plasmin is known to activate MMP-10 *in vitro* (Nagase, 1998), it may also have this function in our wound setting.

Compared with dermal wound healing, gingival wounds heal more rapidly and with minimal scarring (Yang *et al*, 1996). As shown here for MMP-10 and elsewhere for MMP-1, –2, and –9 (Garlick *et al*, 1996; see Mäkelä *et al*, 1999), the temporal and spatial regulation of these MMP during re-epithelialization seems to be very similar in dermis and gingiva. In fact, at least collagenase-3 (MMP-13) is differentially regulated in dermal and gingival fibroblasts (Ravanti *et al*, 1999) and it may be the stromal healing component that leads to the clinical differences of dermal and oral wound healing.

The epithelial cells producing MMP-10 mRNA in this study also synthesized laminin-5. Interestingly, also intestinal enterocytes that produce MMP-10 in inflammatory bowel disease produce laminin-5 (Vaalamo *et al*, 1998). It is possible that during *in vivo* re-epithelialization, proteolysis of laminin-5 by abundant MMP reveals previously inaccessible domains of laminin-5 and switches it from being a potent inhibitor of migration to a migratory substratum (Giannelli *et al*, 1997; O'Toole *et al*, 1997). MMP-2 and membrane type 1 (MT1)-MMP are the only MMP with laminin-5 as a documented *in vitro* substrate (Giannelli *et al*, 1997; Koshikawa *et al*, 2000). As MMP-2 was detected immunohistochemically only occasionally under laminin-5- and MMP-10-positive keratinocytes and as MT1-MMP has not been detected in migrating keratinocytes during wound repair (Okada *et al*, 1997), we cannot exclude the possibility that laminin-5 is degraded by MMP-10. The novel results of our study also show that acute and chronic wound edges do not generally differ in their laminin-5 expression.

During wound healing, the remaining BM is degraded and replaced by a newly formed BM, and a temporary matrix is formed rich in, e.g., fibronectin, tenascin, and vitronectin (Juhasz *et al*, 1993; Larjava *et al*, 1993). Based on its substrate specificity MMP-10 may participate in the degradation of these matrix components. As fibronectin is so widely distributed, and MMP-10 is expressed only in the epidermal tip, it is most probable that there are also other enzymes responsible for fibronectin degradation. Furthermore, previous studies provide evidence that, in particular, neutrophil elastase is responsible for excessive degradation of fibronectin in the chronic wound environment (Rao *et al*, 1995; Grinnell and Zhu, 1996). The time of MMP-10 induction during normal wound repair (3 d), however, coincides with the upregulation of tenascin binding αvβ6 integrin (Haapasalmi *et al*, 1996), and we cannot exclude tenascin being a substrate for MMP-10.

MMP-10 activates MMP-1, MMP-8, and MMP-9 *in vitro* (Nakamura *et al*, 1998). Activation of MMP-1 is probably not its function in wound healing, as based on the present results, in normally healing wounds and *ex vivo* cultures, MMP-1 is turned on earlier during migration than MMP-10 (**Figs 1*c***, ***d*** and **2*c***, ***e***); however, the MMP-10-positive epithelial cells also seem to make MMP-9, which could thus be fully activated.

Our results on suction blisters suggest that total disruption of BM, leading to keratinocyte contact to dermal matrix, is not necessary for MMP-10 upregulation. The laminin-5 positivity of MMP-10 producing cells in suction blisters imply that MMP-10 is associated with the migration of keratinocytes and does not require total disruption of the BM to be upregulated. Interestingly, the only suction blister with MMP-10-positive cells was the one with an intact roof, suggesting that the cytokines trapped in the blister fluid might have enhanced MMP-10 production.

In this study, 75% of the chronic ulcers were positive for MMP-10. In particular, the rheumatoid and decubitus ulcers expressed only small amounts of MMP-10 mRNA, whereas the number of positive cells was higher in diabetic ulcers (**Table I**; **Fig 4*c***, ***e***). Interestingly, also clinically well granulating ulcers expressed MMP-10 (**Table I**), confirming our results on normally healing pinch graft wounds, which suggests that MMP-10 expression is needed for wound healing to succeed. When assessed histologically, the amount of inflammation correlated with the upregulation of MMP-10, suggesting that growth factors and cytokines released by inflammatory cells are more likely inducers of MMP-10 than cell–matrix interactions. Various MMP are also known to be induced by cell–cell interactions (Lacraz *et al*, 1994). As in skin cancers (Kerkelä *et al* unpublished), chronic wounds with most MMP-10-positive keratinocytes had abundant neutrophils in the vicinity of positive areas.

Our *in vitro* studies on cultured keratinocytes using quantitative reverse transcriptase–PCR demonstrated that MMP-10 can be upregulated by TNF-α and EGF, in agreement with the findings of Windsor *et al* (1993). In addition, we also found induction of MMP-10 by interleukin-1β and particularly by TGF-β1; however, cytokines relevant in wound repair, such as KGF, HGF, and PDGF, had no effect. In agreement with our findings, Madlener *et al* (1996) found TGF-β1 to induce MMP-10, whereas their finding of KGF induction differs from ours. This may be due to the fact that those experiments were done in transformed HaCaT cells and it is well established that there are differences in their production of MMP compared with normal keratinocytes (Johansson *et al*, 1997; Sudbeck *et al*, 1997). Based on our immunostaining results on TGF-β1 in chronic ulcers, it may well be the cytokine behind MMP-10 induction. TGF-β1 stimulates wound re-epithelialization via induction of keratinocyte migration (Zambruno *et al*, 1995) and MMP-10 functions in that event. Despite our *in vitro* results, however, KGF is another good candidate for the upregulation of MMP-10, because it stimulates keratinocyte migration and its expression has previously been localized to the fibroblasts at the wound edge (Werner *et al*, 1992). The fact that, although MMP-10 is expressed by keratinocytes grown on type I collagen (Saarialho-Kere *et al*, 1994), fibronectin, type IV collagen, laminin, Matrigel, and gelatin, no upregulation was obtained on different matrices in

this study, further substantiates the role of soluble factors in the upregulation of keratinocyte MMP-10 during wound healing.

During impaired murine wound healing, MMP-10 expression was increased compared with untreated control mice, suggesting that high levels of MMP-10 retard wound healing (Madlener *et al*, 1996). Based on our findings in MMP-10 negative decubitus and rheumatoid ulcers that still do not heal at all, we would only conclude that a temporally and spatially correct regulation of MMP-10 expression is important for the normal repair process. Also in murine excisional skin wounds, MMP-10 is expressed in the advancing epithelial tip, but unlike in skin, its temporal pattern is the same as that of MMP-1 (Madlener *et al*, 1996).

In summary, we conclude that MMP-10 mRNA and protein production are induced later than MMP-1 in normal cutaneous and mucosal wound healing in laminin-5-positive migrating keratinocytes. MMP-10 is not upregulated by keratinocyte contact with the dermal matrix but by the inflammation and action of cytokines. It also most likely participates in the remodeling of the newly formed BM.

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