Interstitial Collagenase Is Expressed by Keratinocytes That Are Actively Involved in Reepithelialization in Blistering Skin Diseases

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Migrating keratinocytes actively involved in reepithelialization in dermal wounds acquire a collagenolytic phenotype upon contact with the dermal matrix. To determine whether this phenotype is associated with repair in other forms of wounds, we assessed collagenase expression in 50 specimens representing a variety of blistering skin diseases, including subtypes of epidermolysis bullosa, porphyria cutanea tarda, bullous pemphigoid, pemphigus, transient acantholytic dermatosis, and suction blisters. Distinct from that seen in chronic ulcers or in normal healing by second intention, reepithelialization in these blistering conditions was not necessarily associated with a complete loss of basement membrane, as determined by immunostaining for type IV collagen. Collagenase mRNA was detected in the basal keratinocytes of several specimens of epidermolysis bullosa simplex (six of 10) and of pemphigus (three of seven), as well as in one quarter of transient acantholytic dermatosis samples in the presence of an intact basement membrane. In contrast, three of nine porphyria cutanea tarda, one third of epidermolysis bullosa acquisita, and one of 10 bullous pemphigoid samples had collagenase-positive basal keratinocytes with the basement membrane disrupted. The collagenase-positive lesions generally represented older blisters with evidence of epithelial regeneration. Collagenase was also expressed in suction blisters at 2 and 5 d after induction of the blister, but was shut off when the epidermis had healed. Other metalloproteinases were expressed occasionally, if at all. Our results suggest that keratinocyte migration is associated with collagenase expression and that contact of keratinocytes with the dermal matrix is not necessarily needed for collagenase induction. ***Key words: metalloproteinase/bullous disease/in situ hybridization. J Invest Dermatol 104: 982–988, 1995***

The matrix metalloproteinase family consists of 11 structurally related, zinc-dependent enzymes that together can degrade essentially all extracellular matrix components. Besides participating in developmental remodeling, tissue involution, and wound healing [1], excess production of these proteinases contributes significantly to the tissue damage that occurs in tumor invasion [2] and in chronic inflammatory diseases [3]. Among the metalloenzymes, interstitial collagenase is the principal enzyme that can cleave types I, II, III, and X collagens [4,5]. Stromelysin-1 has a much broader substrate specificity, being able to degrade proteoglycans, types IV and IX collagens, laminin, fibronectin, and the globular domains of procollagens I and III [6], and it can also activate procollagenase [7]. These enzymes are synthesized by various cell types such as keratinocytes [8], fibroblasts [9], macrophages [10], and endothelial cells [11].

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Abbreviation: TAD, transient acantholytic dermatosis.

We have shown that basal keratinocytes at the migrating front of reepithelialization invariantly express collagenase and are the predominant source of collagenase during active healing in both normal wounds and chronic ulcers [1]. In the epidermis, collagenase may be used to promote keratinocyte migration rather than participating in the remodeling of dermal connective tissue. Stromelysin-1 is also abundantly expressed in wounds, typically in chronic ulcers and poorly healing wounds, but in the proliferating population of basal keratinocytes just behind the collagenase-producing cells [12].

In our earlier work, we showed that collagenase is expressed only by basal keratinocytes, which are not in contact with an intact basement membrane (BM), and that collagenase is induced in primary human keratinocytes grown on type I collagen [1,13]. To assess our hypothesis that contact with matrix is a critical determinant of collagenase expression in keratinocytes, we examined specimens of various blistering skin diseases in which, in contrast to dermal wounds, reepithelialization is not associated with a complete loss of BM. We detected collagenase mRNA in keratinocytes in blisters of various etiology, but generally expression was confined to older lesions in which partial reepithelialization was already present. As in dermal wounds, the signal for collagenase mRNA was restricted to basal keratinocytes, suggesting that only these

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cells, and not the more differentiated keratinocytes, are competent to respond to stimulators of collagenase production.

MATERIALS AND METHODS

Tissues Formalin-fixed, paraffin-embedded specimens were obtained from the Department of Dermatology, University of Helsinki, Finland. The specimens of blistering diseases studied included epidermolysis bullosa simplex (EBS; n = 10), EB dystrophica (n = 2), EB junctionalis (n = 2), EB acquisita (EBA; n = 3), porphyria cutanea tarda (PCT; n = 9), bullous pemphigoid (BP; n = 10), pemphigus (n = 7), and transient acantholytic dermatosis (TAD; n = 4). The diagnoses of the various EB specimens had been made on the basis of clinical, histopathologic, and electron microscopic findings. The diagnoses of BP and pemphigus were based on the well-known clinical, histologic, and immunofluorescent properties [14]. The diagnosis of PCT was confirmed clinically and by abnormal uroporphyrin levels. Suction blisters were induced on the abdominal skin of a healthy volunteer using a Dermovac device as described previously [15]. Biopsies were done 2, 5, and 9 d after induction of the blister.

***In Situ* Hybridization** *In situ* hybridization was performed on 5-μm sections as described in detail [16]. All samples were treated with proteinase K and were washed in 0.1 M triethanolamine buffer containing 0.25% acetic acid. Sections were covered with 25 μl of hybridization buffer containing 2.5×10^4 cpm/μl of ^{35}S-labeled antisense or sense RNA probe. Sections were incubated at 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, and were processed for autoradiography as described [16]. After 10 to 16 d of autoradiographic exposure, the photographic emulsion was developed, and slides were stained with hematoxylin and eosin. The sections chosen for presentation in this report were exposed for 13 d. Samples known to be positive for collagenase or stromelysin-1 mRNA were used as positive controls in each experiment.

RNA Probes The production and specificity of the antisense human interstitial collagenase, stromelysin-1, and of tissue inhibitor of metalloproteinases-1 RNA probes have been described [12,17]. As a control for nonspecific hybridization, tissue sections in each experiment were hybridized with ^{35}S-labeled sense RNA transcribed from a bovine tropoelastin cDNA. The validity of this probe as a negative control has been confirmed by Northern [16] and by *in situ* hybridization assays [1,17,18].

Immunohistochemistry On sections serial to those used for *in situ* hybridization, immunostaining for type IV collagen and laminin was done by the peroxidase-antiperoxidase technique using diaminobenzidine as chromogenic substrate and Harris hematoxylin as counterstain, as described in detail [1]. Controls were performed with rabbit preimmune serum or preimmune mouse ascites fluid.

RESULTS

Tissues To assess whether collagenase is expressed in various bullous diseases, we studied 47 samples using *in situ* hybridization **(Table I)**. The samples represented diseases in which cleavage occurs above (pemphigus, EBS, TAD), within (BP, EB junctionalis), or below the BM (EB dystrophica). In mild cases of PCT, the blisters arise within the junctional zone, whereas in severe cases they form beneath the BM [19]. The approximate ages of the blisters were assessed by light microscopy, and blisters with overt reepithelialization at any level were considered old. In intraepidermal blisters, the beginning of reepithelialization was detected as an alignment of flattened keratinizing cells at the bottom of the blisters **(Fig 1*B*)**. In subepidermal blisters, the beginning of reepithelialization was seen as small clusters of keratinocytes in follicular openings at the bottom or at the edges of the blister **(Fig 2*B*)**. For all samples, sections were stained for type IV collagen as a marker of BM integrity. In a subset of sections, the integrity of the epidermal BM was further confirmed by immunostaining for laminin, which coincided with the presence of type IV collagen in all samples examined (except in suction blisters).

Collagenase Is Expressed by Basal Keratinocytes in Blisters Showing Epidermal Regeneration Prominent signal for collagenase mRNA was detected in the basal keratinocytes in 60% of the EBS samples and in one third of the samples of EBA **(Table I)**. In all EB samples, regardless of the subtype, epithelial regeneration was detected whenever collagenase-positive keratinocytes were found **(Fig 1)**. In four specimens of EBS, migrating keratinocytes expressed collagenase mRNA **(Fig 1*A*)**, whereas in two other samples, signal was detected in basal keratinocytes covered with several layers of epithelium **(Fig 1*D*)**. However, in relatively recent EB lesions with no evidence of reepithelialization, no signal for collagenase mRNA was seen (data not shown). In addition, collagenase mRNA was detected in macrophage-like cells in the dermis of two EBA samples and one dystrophic specimen (data not shown). In contrast to our consistent findings in chronic ulcers and acute wounds [1], in which only basal keratinocytes that were not in contact with an intact BM expressed collagenase mRNA, staining for type IV collagen revealed that the BM was intact under collagenase-positive keratinocytes in all EBS samples **(Fig 1*E*)**. No signal was seen on sections hybridized with sense RNA **(Fig 1*C*)**.

Table I. Pathologic Features and Distribution of Interstitial Collagenase mRNA of the 47 Samples of Blistering Skin Diseases Used in These Studies

Number of Samples	Diagnosis[a]	Reepithelialization[b]	Signal for Collagenase mRNA in Basal Keratinocytes
6	EBS	+	+
4	EBS	−	−
2	EBD	−	−
2	EBJ	−	−
1	EBA	+	+
2	EBA	−	−
3	PCT	+	+
1	PCT	+[c]	−
5	PCT	−	−
1	PG	+	+
9	PG	−	−
3	PV	+	+
1	PV	+[c]	−
3	PV	−	−
1	TAD	+	+
3	TAD	−	−
1	SB (2 d)	+	+
1	SB (5 d)	+	+
1	SB (9 d)	+[c]	−

[a] EBD, EB dystrophica; EBJ, EB junctionalis; PG, pemphigoid; PV, pemphigus vulgaris; SB, suction blister.
[b] For definition, see *Results*.
[c] Blister bottom completely reepithelialized.

In three of nine samples of PCT, collagenase mRNA was detected in basal keratinocytes **(Fig 2*A*)**. Again, partial reepithelialization was detected in these samples. Samples that were devoid of signal for collagenase mRNA were typically fresh lesions with no evidence of keratinocyte migration **(Table I)**. In five PCT specimens, stromal signal for collagenase was also seen in occasional macrophage-like cells **(Fig 2*C*)**.

In three of seven samples of pemphigus, collagenase mRNA was detected in basal keratinocytes. This was found in both pemphigus vulgaris **(Fig 2*D*)** and erythematodes, and in all samples examined, the BM was intact as assessed by staining for type IV collagen **(Fig 2*E*)**. As before, no collagenase mRNA was detected in new lesions with no evidence of epidermal regeneration or in old lesions with complete reepithelialization.

Four samples of TAD were probed for collagenase mRNA to exclude acanthosis as a factor contributing to collagenase induction. No collagenase mRNA was found in the acantholytic epidermal areas of these samples, but in one specimen, reepithelializing basal keratinocytes at the bottom of a blister were positive **(Fig 2*F,G*)**.

Collagenase mRNA was detected in basal keratinocytes in only one of 10 BP samples (data not shown). This agrees with recent data demonstrating that 92-kDa gelatinase, produced by eosinophils, is the principal metalloenzyme in these lesions [20]. Collagenase mRNA was never detected in granulocytes, eosinophils, or lymphocytes. As reported previously, collagenase and

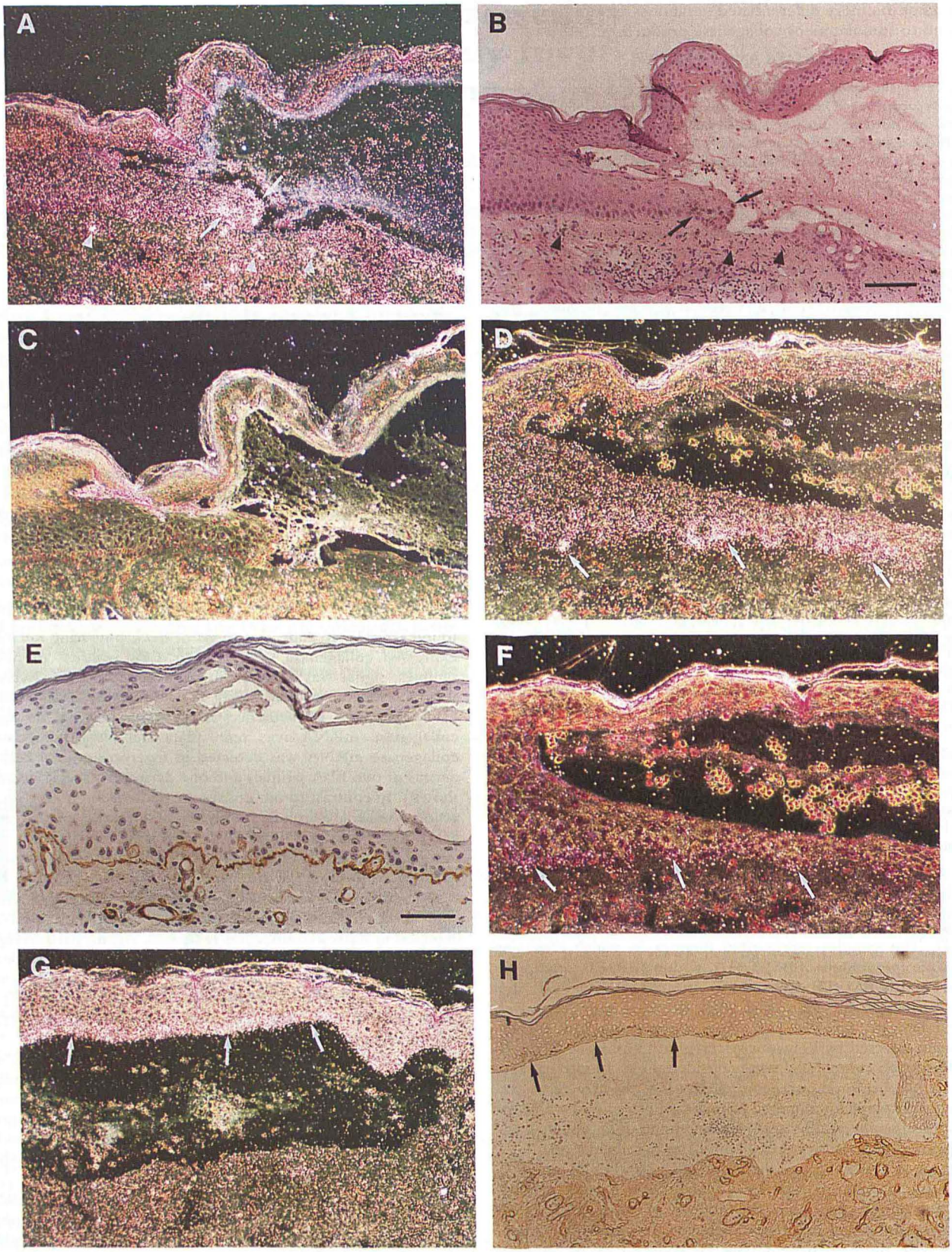

Figure 1. Expression of collagenase and stromelysin-1 by basal keratinocytes in EBS and EB dystrophica. Specimens of EBS (*A–F*) and EB dystrophica (*G,H*) were processed for *in situ* hybridization with RNA probes for collagenase and stromelysin-1 mRNAs, as described in *Materials and Methods*. *A, in situ* hybridization for collagenase mRNA shows prominent signal in migrating keratinocytes (*arrows*) and weaker signal in occasional stromal cells (*arrowheads*). *B*, bright-field illumination of *A* for histologic comparison. *C*, parallel section of that shown in *A* and *B* hybridized with a sense RNA probe. *D*, collagenase mRNA (*arrows*) was seen in basal keratinocytes of an old EBS blister. *E*, immunostaining with a monoclonal antibody to type IV collagen in a parallel section shows an intact BM. *F*, stromelysin-1 mRNA (*arrows*) was seen in basal keratinocytes in a section parallel to that shown in *D*. *G*, in EB dystrophica, signal for stromelysin-1 mRNA was seen in basal keratinocytes (*arrows*). *H*, in a section parallel to that in *G*, immunostaining for type IV collagen is abnormal (*arrows*). *Bars: A–C,G,H*, 38 μm; *D–F*, 19 μm.

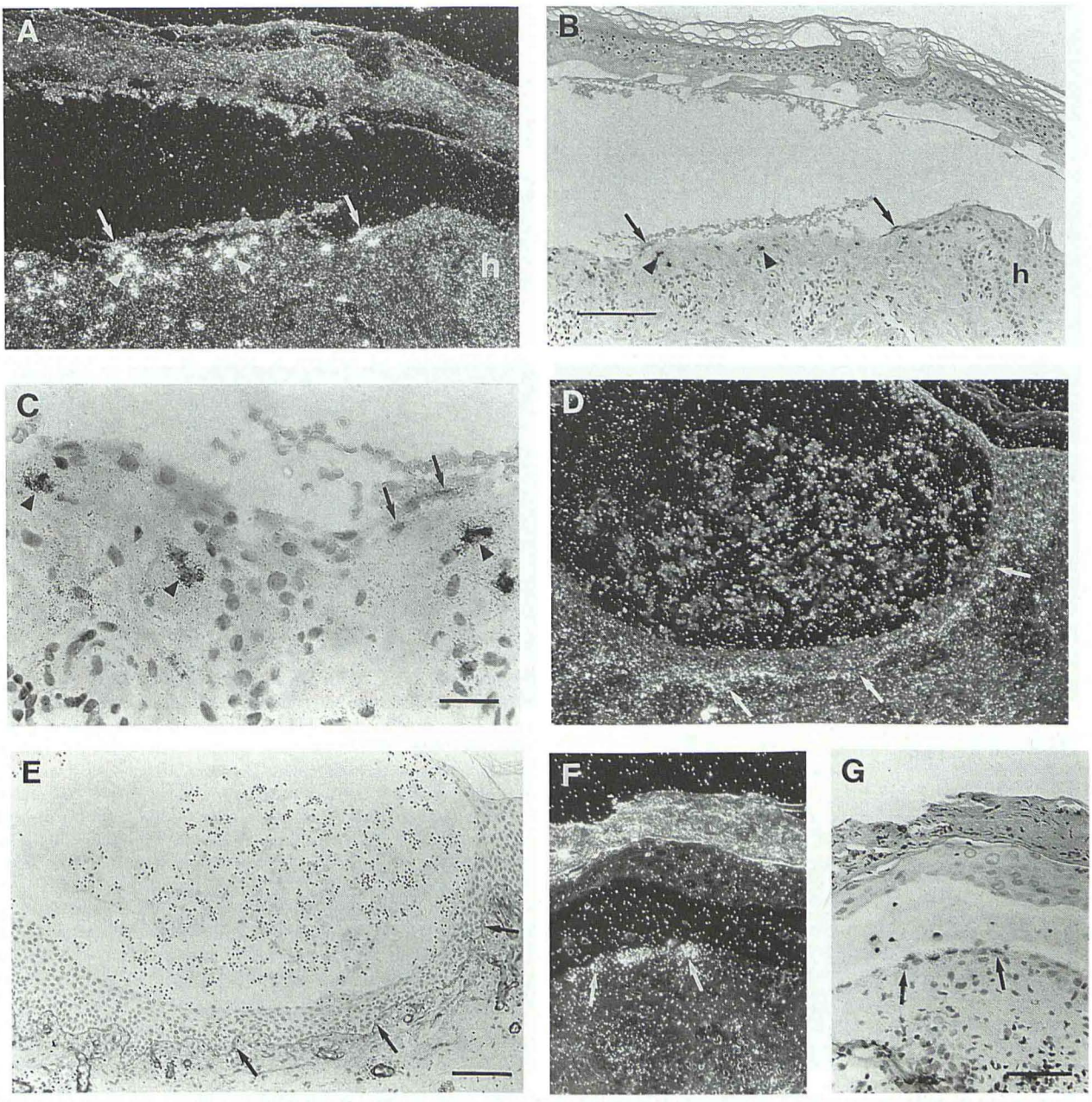

Figure 2. Collagenase mRNA is expressed in basal keratinocytes in PCT, pemphigus, and TAD. Sections of PCT (*A–C*), pemphigus vulgaris (*D,E*), and TAD (*F,G*) were hybridized for collagenase mRNA. *A,B*, paired dark-field and bright-field illuminated photomicrographs of sections of PCT. Signal for collagenase mRNA was seen in reepithelializing keratinocytes (*arrows*) and in stromal cells (*arrowheads*). *C*, high-power image from the underlined area in *B* shows collagenase mRNA in migrating keratinocytes (*arrows*) and in some stromal cells (*arrowheads*). *D*, in pemphigus, collagenase mRNA was seen in basal keratinocytes (*arrows*). *E*, immunostaining for type IV collagen in a parallel section showed an intact BM (*arrows*). *F, in situ* hybridization reveals collagenase mRNA in basal keratinocytes in a regenerating blister caused by TAD (*arrows*). *G*, bright-field illumination of *F* for histologic comparison. h, hair follicle. *Bars: A,B,D,E*, 38 μm; *F,G*, 19 μm; *C*, 9 μm.

stromelysin-1 are not expressed in the epidermis of healthy human skin [12,17].

Our results suggest that contact of keratinocytes with the dermal matrix is not necessarily needed for induction of collagenase production. To assess this further, we examined experimental suction blisters in which the lamina densa remains intact [21]. Biopsy specimens were taken 2, 5, and 9 d after induction of the blister, and type IV collagen immunostaining showed that the BM remained intact at all times **(Fig 3*B*)**. On day 5, many collagenase-mRNA–positive keratinocytes were detected at the edges and at the bottom of the bulla in migrating keratinocytes **(Fig 3*A*)**. However, on day 9, when epidermal continuity was re-established, keratinocytes ceased to express collagenase **(Fig 3*C*)**. This finding agrees with our previous observations in acute incisional wounds [1].

Stromelysin-1 Is Only Sporadically Expressed by Keratinocytes in Various Blisters Stromelysin mRNA was detected in basal keratinocytes in only half of the samples of dystrophic EB **(Fig 1*G*)** and in three of 10 specimens of EBS **(Fig 1*F*)**. It was also detected in one of nine samples of PCT in basal keratinocytes of the regenerating blister floor (data not shown). Five BP and three pemphigus samples were negative for stromelysin-1 and tissue inhibitor of metalloproteinases-1 mRNAs. No signal for stromely-

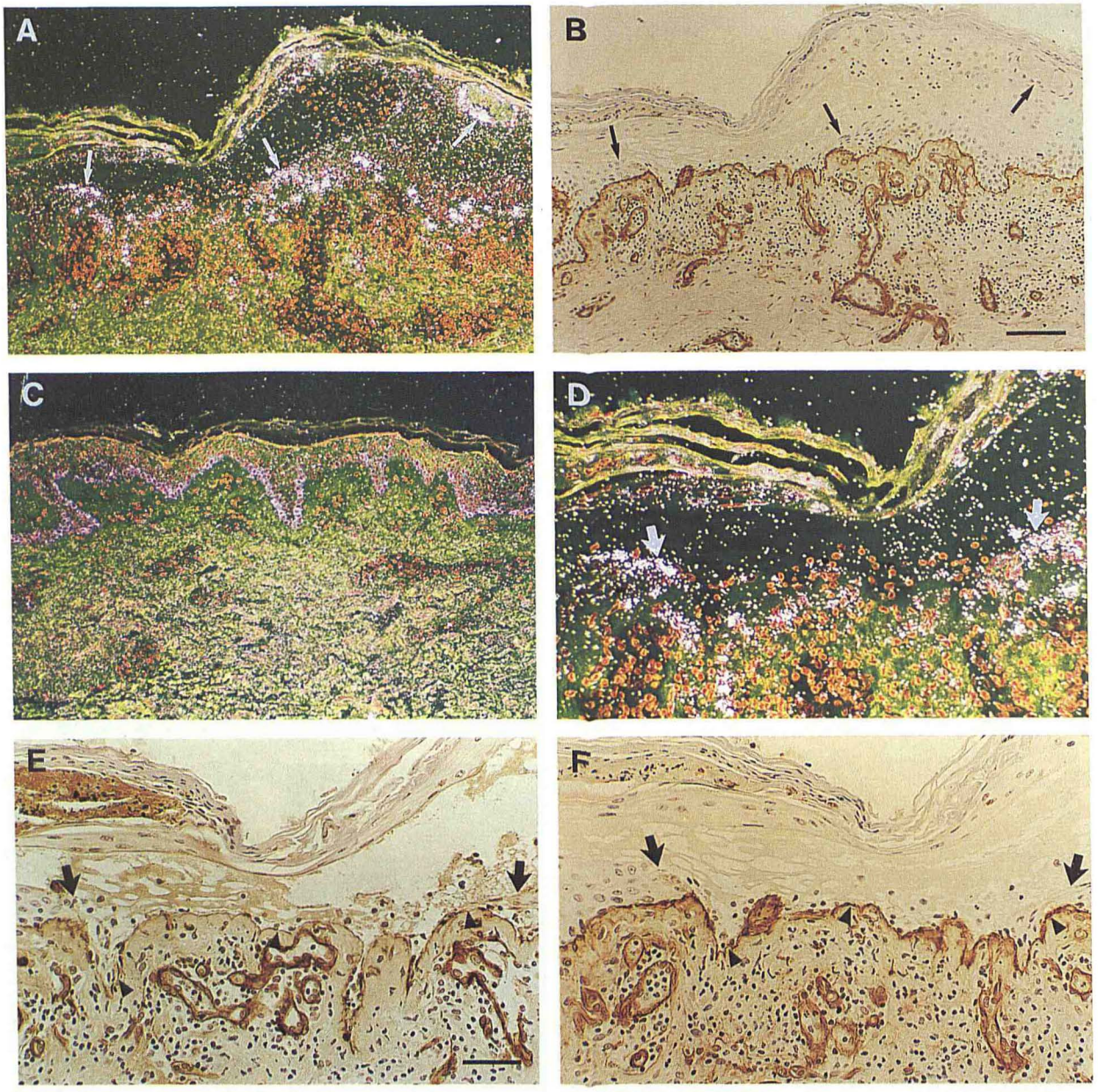

Figure 3. Expression of collagenase mRNA by migrating keratinocytes in an experimental suction blister. Biopsy specimens of suction blisters were hybridized with collagenase riboprobe and immunostained with type IV collagen and laminin antibodies, as described in *Materials and Methods*. *A, in situ* hybridization reveals collagenase mRNA in a 5-d–old blister in migrating keratinocytes and in keratinocytes around an intraepidermal blister (*arrows*), as well as a few positive cells in the dermis. *B*, immunostaining for type IV collagen in a corresponding section shows an intact BM (*arrows*). *C, in situ* hybridization for collagenase mRNA in a reepithelialized 9-d–old blister shows no signal in keratinocytes. *D*, higher magnification of *A* showing collagenase mRNA in migrating keratinocytes (*large arrows*). *E*, immunostaining for laminin reveals disruptions (*arrowheads*), whereas type IV collagen immunostaining in a parallel section (*F*) is intact (*arrowheads*). Strong immunostaining is evident in the BM of multiple blood vessels (*E,F*). *Thick arrows* indicate corresponding structures (*D,E,F*). *Bars: A–C*, 19 μm; *D–F*, 38 μm.

sin-1 or tissue inhibitor of metalloproteinases-1 mRNA was detected in any of the suction blisters biopsied.

DISCUSSION

In this study, collagenase production was detected in older blisters with clear evidence of reepithelialization regardless of the level of cleavage. This agrees with previous *in vitro* studies showing increased expression of keratinocyte-derived collagenase when the cells are stimulated to migrate [22]. Indeed, expression of metalloenzymes may be important for epithelial migration [23]. Collagenolytic activity has been detected in BP blister fluids and in lesions of dystrophic EB [24,25], but not in pemphigus [24,26]. These findings are not incompatible with our results because we assessed active expression, not the presence of protein. Because collagenase and stromelysin mRNAs were not expressed in all samples examined, it is unlikely that they contribute to the etiology of these bullous lesions.

In contrast to our findings in dermal wounds [1] or in wounds due to mechanical injury of vascular smooth-muscle cells [27], cell-matrix interactions may play a lesser role in regulating collagenase induction in blisters. Perhaps a particular pattern of cytokines or other soluble factors released from damaged epidermal cells or from migrating inflammatory cells modulates collagenase expression. Indeed, keratinocytes express several matrix metalloproteinases in response to growth factors and cytokines [8]. For example, transforming growth factor-alpha induces collagenase production in human epidermal raft cultures [28]. On the other hand, transforming growth factor-beta may increase expression of integrins,

thereby facilitating keratinocyte migration and collagenase production [29].

Although stromelysin-1 is a potent activator of procollagenase *in vitro* [30] and even though collagenase and stromelysin are often expressed coordinately [27], our results in blistering diseases, similar to those in chronic wounds [12], show that collagenase and stromelysin are not necessarily expressed by the same cells. Thus, these two metalloproteinases probably do not act in concert *in vivo*. Stromelysin-1 was expressed in keratinocytes in only four EB samples and in stromal cells in only one PCT specimen. However, collagenase and stromelysin-1 expression did spatially coincide in two samples of EBS and in one of PCT. In these specimens, these matrix metalloproteinases were detected in basal keratinocytes under the proliferating epithelium without clear evidence of ongoing migration **(Fig 1*D*)**. Stromelysin-1 may not be involved in reepithelialization *per se* but rather is needed to restructure the newly formed BM or to remodel the dermal stroma [12]. Alternatively, its expression may be aberrant and actually contribute to impaired healing.

Although the precise nature of the proteinase responsible for acantholysis in pemphigus is still unclear, metalloproteinases do not seem to play a role in the etiology of this disease. Indeed, collagenase mRNA was not found in fresh pemphigus lesions displaying only basal acantholysis of suprabasal keratinocytes with classic tombstone formation. Furthermore, no signal was detected in the acantholytic areas of TAD lesions. This suggests that collagenase is produced only in the later stages of these diseases in response to secondary changes, such as epidermal regeneration and inflammation.

In the repair of a suction blister, with the split at the lamina lucida, short tongues of epithelial cells grow out within 12–24 h from the residual epithelial structures [21]. Data on suction blisters suggest that keratinocytes migrate over an intact BM [31], but exactly what changes are associated with this migratory phenotype are not known. We showed collagenase induction, at least on day 5, and these findings agree with those of Welgus *et al* [24], who measured collagenase protein levels from suction-blister fluids. We found that as in dermal wounds, collagenase expression ceased when reepithelialization was complete. Although type IV collagen staining was intact in all the suction blisters examined, we cannot exclude microdisruptions in the BM, because staining for type IV collagen was performed with an antibody directed to the N-terminal domain only [1]. In fact, as our staining results show **(Fig 3*E*)**, the retention of laminin in suction blisters is variable [31,32]. Furthermore, inflammatory cells accumulating in the blister over time might release proteases that cause microdisruptions of type IV collagen. Thus, the migrating keratinocytes expressing collagenase in the blisters that we examined may not be in contact with a structurally complete BM.

Type IV collagen matrix induces keratinocytes to migrate [33] and to synthesize increased amounts of collagenase [34]. Thus, disrupted laminin membrane might lead to contact with type IV collagen deeper in the BM, leading to migration and collagenase production in different types of blisters. Alternatively, it is possible that contact with some other BM protein, such as entactin, proteoglycans, or laminin variants, may influence collagenase expression. However, findings by Petersen *et al* [35] have indicated that laminin is probably not the modulating factor, because it inhibits migration [36] and does not stimulate collagenase expression above the levels seen in cultured cells.

Keratinocyte migration and integrin expression are regulated differently depending on whether BM is present or absent [29]. Compared with normal skin, few changes in integrins are observed in suction blisters [32], other than reorganization of some receptors, whereas in dermal wounds, $\alpha 5$ is expressed on collagenase-positive, migrating keratinocytes [1]. Thus, in dermal wounds, collagenase production might be affected by cell-matrix interactions, whereas in epidermal wound repair, soluble factors may have a more prominent role.

In conclusion, our *in situ* hybridization studies show that basal keratinocytes are able to express interstitial collagenase even when they are not in contact with the dermal matrix and that their migration is generally associated with collagenase expression. Induction of collagenase mRNA in keratinocytes may be modulated by a suitable combination of both cytokines and extracellular matrix. Stromelysin does not seem to have a consistent role in the epidermal regeneration associated with healing of blisters.

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