Collagenase Expression Is Rapidly Induced in Wound-Edge Keratinocytes After Acute Injury in Human Skin, Persists During Healing, and Stops at Re-Epithelialization

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Collagenolytic activity has been reported previously in association with wounds. We used *in situ* hybridization and immunohistochemistry to localize cellular sites of interstitial collagenase production in acute wounds in human skin at days 1, 2, 4, 6, 9, and 14 after wounding. *In vivo*, collagenase expression peaked in migrating basal keratinocytes at the wound edge at day 1, then gradually decreased and was undetectable at day 9 when healing was complete. To minimize the effects of crust formation and inflammation, we examined the healing of wounds made with a 3-mm punch in organ-cultured skin. In these *in vitro* wounds, re-epithelialization occurred by 5–7 d in 10% serum, although remodeling of the connective tissue was minimal. Collagenase expression showed a similar pattern as in the *in vivo* wounds; it was detected in migrating keratinocytes already 4–6 h after wounding, peaked at 12–24 h, gradually decreased during the next few days, and subsided upon re-epithelialization. In dermal fibroblasts, on the other hand, expression of collagenase started considerably later, after 5–7 d in culture, and persisted after complete re-epithelialization, indicating that collagenase is differentially regulated in different cell types. Our findings also show that collagenase induction in keratinocytes does not require inflammation and occurs as a rapid response to wounding, suggesting that interstitial collagenase is not only necessary for remodeling of the extracellular matrix, but may also have a role in initiating migration of keratinocytes in wound healing. *Key words: wound healing/metalloproteinase/time course/skin wound. J Invest Dermatol 104:479–483, 1995*

Wound healing *in vivo* is a dynamic, complex, and highly regulated process. In response to injury, tissue starts exudation, re-epithelialization, and remodeling of the extracellular matrix, which requires the precise coordination of matrix degradation and synthesis. Then, unlike in tumors, these phenomena subside upon healing and become a stationary condition after scar formation.

Matrix metalloproteinases are a family of related proteolytic enzymes that are likely to play an important role in degrading and remodeling matrix proteins during wound healing [1]. Activities of these proteinases in physiologic conditions are strictly regulated by multi-step mechanisms on the levels of expression, activation, and binding to inhibitors. Although matrix metalloproteinases are structurally related, they are differentially regulated. Among them, interstitial collagenase has the unique ability to cleave the triple-helical structure of collagen bundles and is therefore crucial for initiating collagen degradation. Elevation of collagenolytic activity has been reported in association with wound healing [2–5]. Many cell types have the capacity to produce interstitial collagenase in culture and may be the source of collagenolytic activity in wound healing, including fibroblasts, macrophages, endothelial cells, and neutrophils [6–11]. Keratinocytes have collagenolytic activity when cultured on certain matrices, and early wound-healing studies using organ-culture methods suggested that collagenolytic activity was found in the epidermal wound edge [2,5]. However, cellular source and time course of production during the healing process were still vague. Recently, collagenase mRNA was detected at the epidermal edge of ulcerative pyogenic granuloma and various other chronic wounds, whereas no expression was found in the epidermis of healed acute wounds [12,13]. Taken together, these findings indicate a role for keratinocytes in collagenolysis in association with wounding.

Although *in situ* hybridization does not show protein or enzyme activity, it is a suitable method to investigate alterations in expression at the cellular level. To determine the localization and time course of collagenase expression, we carried out *in situ* hybridization for collagenase mRNA at various times during healing of acute wounds in human skin. To assess possible effects of crust formation and inflammation, we studied both inflammatory wounds *in vivo* and noninflammatory wounds cultured *in vitro*. In both wound models, we saw a similar pattern of collagenase expression. In migrating keratinocytes at the wound edge, the onset of expression was rapid, persisted during healing, but was undetectable when re-epithelialization was complete. Our findings suggest that collagenase is expressed as an early response to injury and is primarily a feature of the migratory keratinocyte phenotype.

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MATERIALS AND METHODS

Tissue Samples

In Vivo Wounds: Wounds were made with a 6-mm punch in the buttocks of three healthy volunteers after informed consent. The skin samples were used as "time point 0." Subsequently, 4-mm biopsy specimens of the wound area including adjacent skin were taken 1, 2, 3, 4, 6, and 14 d after wounding. The skin samples were fixed in formalin, embedded in paraffin, and sectioned at 5 μm on Superfrost Plus slides (Fisher Scientific).

In Vitro Wounds: Sterile human skin was obtained from routine breast reduction operations. Six separate wounds were analyzed for each culture condition. Under sterile conditions, 6-mm punch biopsy specimens were made, and in the center of each piece, a 3-mm partial-thickness wound was punched. The pieces were then transferred to 24-well plates (Costar). Each wound was covered with 1 ml of Dulbecco's modified Eagle's medium containing antibiotics (penicillin 50 U/ml and streptomycin 50 μg/ml), with the addition of either 2% or 10% fetal bovine serum (FBS). Media were changed every second day, and the wounds were harvested at different times (4–6, 12, 24, and 48 h; 3, 5, and 7 d), fixed in 4% formalin, embedded in paraffin, and sectioned at 5 μm on Superfrost Plus slides.

Probes A 550-base pair cDNA fragment from the 5′ end of mRNA of human skin fibroblast collagenase [7], subcloned in Bluescript KS transcription vector (Stratagene, La Jolla, CA), was kindly provided by Dr W.C. Parks (Department of Dermatology, Washington University, St. Louis, MO). After linearization, sense and antisense RNA probes were transcribed and labeled, as described previously [14,15]. To achieve higher specific activity, cold UTP was substituted for a-thioUTP (Amersham, UK), and the incubation time was prolonged to 4 h. The specificity of the collagenase probe has been shown [16].

***In Situ* Hybridization** *In situ* hybridization was carried out as described previously [14,15]. Briefly, deparaffinized and rehydrated sections were pretreated with 1 μg/ml proteinase K (Sigma, St. Louis, MO), and then with 0.1 M triethanolamine buffer (pH 8.0) containing 0.25% acetic anhydride. The sections were hybridized overnight with 2.0×10^6 cpm of ^{35}S-labeled RNA probes at 55°C. After hybridization, the slides were washed under stringent conditions including incubation with 50 μg/ml RNase-A (Sigma) for 30 min at 37°C. Washed slides were dipped in Kodak NTB-2 emulsion (Eastman Kodak, Rochester, NY), prediluted 1:1 with distilled water, and processed for autoradiography as described [14]. After development of the photographic emulsion, slides were stained with hematoxylin and eosin.

Immunostaining Affinity-purified rabbit anti-collagenase antibody was generously supplied by Dr. H.G. Welgus (Department of Dermatology, Washington University) [17]. Deparaffinized, rehydrated sections were digested with 0.1% trypsin (Sigma) at 37°C for 30 min, rinsed in phosphate-buffered saline, and treated with 0.75% H_2O_2 in methanol for 30 min at room temperature. Rinsed sections were stained with the avidin-biotin-peroxidase complex system (Vector Laboratories, Burlingame, CA), as described [18]. Rabbit anti-collagenase was diluted (1:250) in 1% FBS and incubated with the sections for 2 h at room temperature. As chromogenic substrate, we used 0.2 mg/ml 3-amino-9-ethylcarbazole in 0.1 M acetate buffer (pH 5.2) with 1 μl/ml of H_2O_2. For type IV collagen, deparaffinized sections were digested by 0.5% pronase in 0.5 M Tris-HCl buffer (pH 8.0) for 20 min and stained with 1:50 dilution of mouse monoclonal anti-human collagen IV (Dako, Glostrup, Denmark) using the peroxidase-antiperoxidase method and 3-amino-9-ethylcarbazole. Control slides were incubated with appropriate preimmune serum diluted to the same protein concentration as the primary antibody. After development, the slides were rinsed with water, counterstained with hematoxylin, and mounted with glycerol-gelatin.

RESULTS

Collagenase Expression in *In Vivo* Wounds In skin biopsy specimens at time point 0, there was no specific signal for collagenase mRNA **(Fig 1*a*)**. However, at day 1 after wounding, there was prominent signal for collagenase in basal keratinocytes at the edge of the wound **(Fig 1*c,d*)**, and in the wound there was an accumulation of mononuclear, macrophage-like cells positive for collagenase mRNA **(Fig 1*c*)**. After 2 d, expression of collagenase declined except in the leading edge of the keratinocytes. The signal in the wound edge remained during the course of healing **(Fig 1*e*)** but became undetectable at day 9–14 when re-epithelialization was complete **(Fig 1*f*)**. Sections hybridized with sense RNA probe had only background autoradiographic signal (not shown).

Immunohistochemically, the presence of collagenase protein paralleled the *in situ* hybridization signal, i.e., the staining was most pronounced in migrating keratinocytes in the epidermal outgrowth (data not shown). After re-epithelialization, we could not detect collagenase protein by immunostaining.

***In Vitro* Wounds** Re-epithelialization was complete within 5–7 d in wounds cultured in Dulbecco's modified Eagle's medium containing 10% FBS. After 4–6 h in culture, keratinocytes at the wound edge had already started migrating. On average, five to seven cells had migrated over the wound, forming a triangle-shaped edge with prominent autoradiographic signal for collagenase mRNA **(Fig 2*a,b*)**. Collagenase expression in migrating keratinocytes peaked at 12–24 h **(Fig 2*c*)** and remained high until day 2, when expression started to decrease. Specific signal for collagenase mRNA was still obvious in wound-edge keratinocytes at day 4 after wounding **(Fig 2*e*)**. However, at day 7, when the whole wound was covered by a sheet of flat epidermal cells, collagenase expression was not detected **(Fig 2*f*)**. Wounds cultured in only 2% FBS did not re-epithelialize, but in early wounds at 6–12 h, a few migrating keratinocytes showed specific signal for collagenase mRNA at the wound edge (data not shown). After 12 h, there was no evidence of further migration or of collagenase expression in these cells.

After 5 d in culture, most fibroblast-like spindle-shaped cells scattered throughout the dermis started to show strong positive signal for collagenase mRNA. The signal was especially prominent in cells surrounding blood vessels and appendages (data not shown).

Immunostaining confirmed that collagenase protein colocalized with the signal for collagenase mRNA **(Fig 2*d*)**. Already at the earliest time investigated, 4–6 h, there was faint positive staining for collagenase enzyme in the migrating keratinocytes at the wound edge (data not shown).

DISCUSSION

The present study clearly demonstrates the course of collagenase expression during normal wound healing after acute injury in human skin. Wounding rapidly induced expression of collagenase in keratinocytes at the wound edge, with evident hybridization signal 4–6 h after wounding, the earliest time investigated. These findings confirm data regarding the ulcer edge of pyogenic granulomas and various other chronic wounds [12,13]. In both *in vivo* and *in vitro* wounds, collagenase expression was intense in the early phase, declined during healing except for the keratinocytes in the leading edge, then completely subsided upon re-epithelialization. These findings are compatible with reported collagenolytic activity in healing pig wounds [4] and suggest that mechanisms controlling collagenase during re-epithelialization are highly regulated. The mononuclear cells expressing collagenase accumulating in the *in vivo* wound at day 1 had a macrophage-like appearance and were most likely infiltrating cells because of their location in the outer zone of the wound and because they could not be observed in the *in vitro* wounds. However, further characterization of these cells was not pursued.

Wounds cultured in both 10% FBS and 2% FBS consistently showed strong signal for collagenase mRNA in dermal fibroblast-like cells after 5–7 d in culture. Unlike in keratinocytes, collagenase expression in dermal cells persisted after completed re-epithelialization, suggesting that different mechanisms control collagenase expression in different cell types. However, in this system, collagenase expression in fibroblasts may be an effect of the culture condition because it was not observed in the *in vivo* wounds.

In both *in vivo* and *in vitro* wounds, injury and disruption of the basement membrane seemed to be a primary stimulus to induce collagenase expression in keratinocytes, which is in agreement with observations by Saarialho-Kere *et al* [13]. Previous *in vitro* studies have shown that cultured human keratinocytes migrating on col-

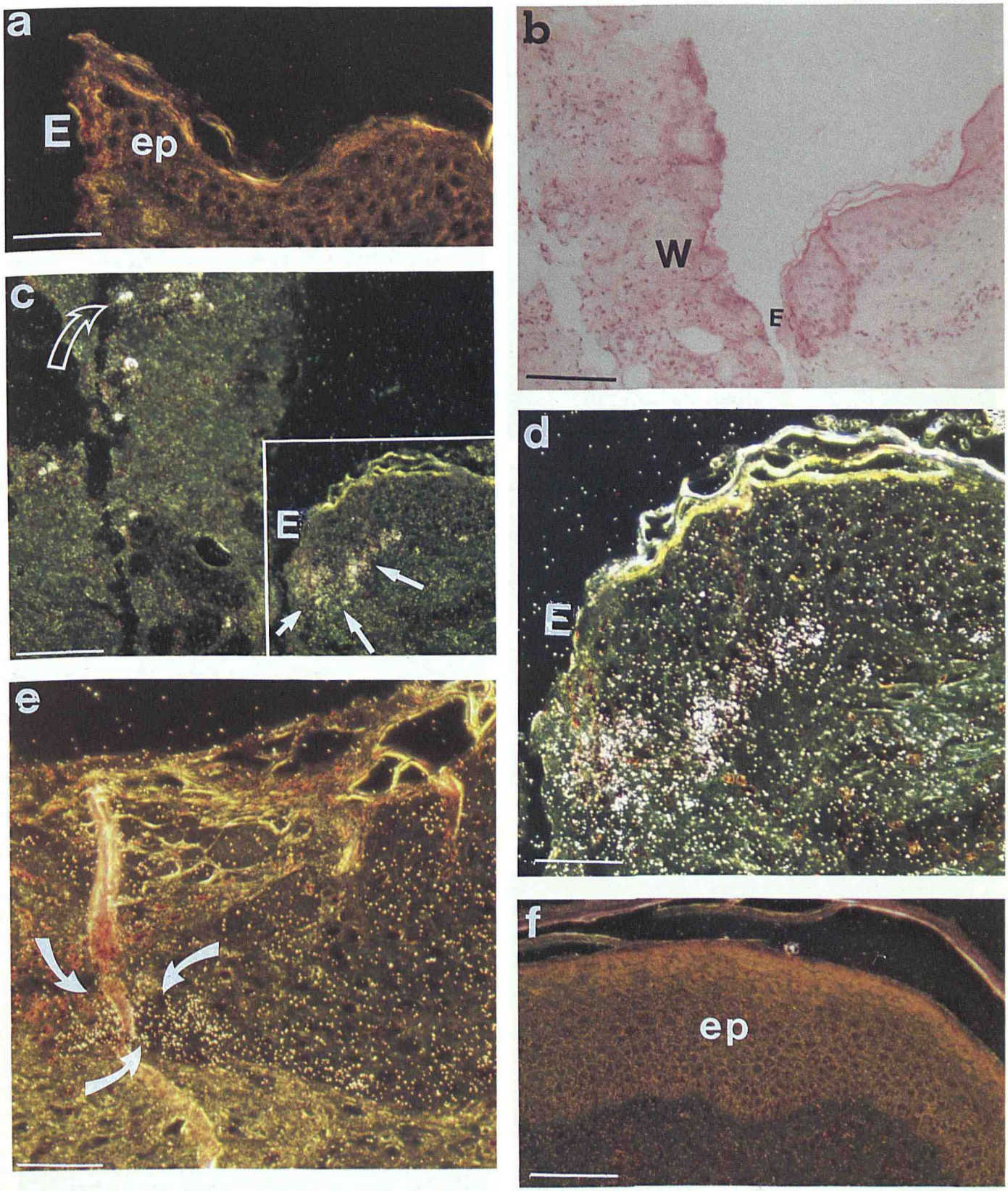

Figure 1. Time course of collagenase expression in human *in vivo* skin wounds. *a*) Dark-field photomicrograph showing wound at time 0 immediately after wounding. There is no signal for collagenase mRNA. E, wound edge; ep, epidermis. *Bar*, 60 μm. *b*) Wound at day 1 counterstained with hematoxylin and eosin to visualize tissue morphology. E, wound edge; W, wound bed. *Bar*, 125 μm. *c*) Same field of view as in *b*. Dark-field photomicrograph showing strong autoradiographic signal for collagenase mRNA at day 1 in keratinocytes at the wound edge (E; *small arrows*) and in mononuclear cells (*open arrow*) in the bed of the wound. *Bar*, 125 μm. *d*) High magnification of wound-edge area (E) within the box in *c* showing strong signal for collagenase mRNA in keratinocytes. *Bar*, 60 μm. *e*) Dark-field photomicrograph showing wound edge at day 4. There is obvious signal for collagenase mRNA in migrating keratinocytes in the leading front (*arrows*). *Bar*, 60 μm. *f*) Healed wound at day 14 after wounding. There is no specific signal for collagenase mRNA. ep, epidermis. *Bar*, 125 μm. Autoradiographic exposure was for 2 weeks and sections were stained with hematoxylin and eosin.

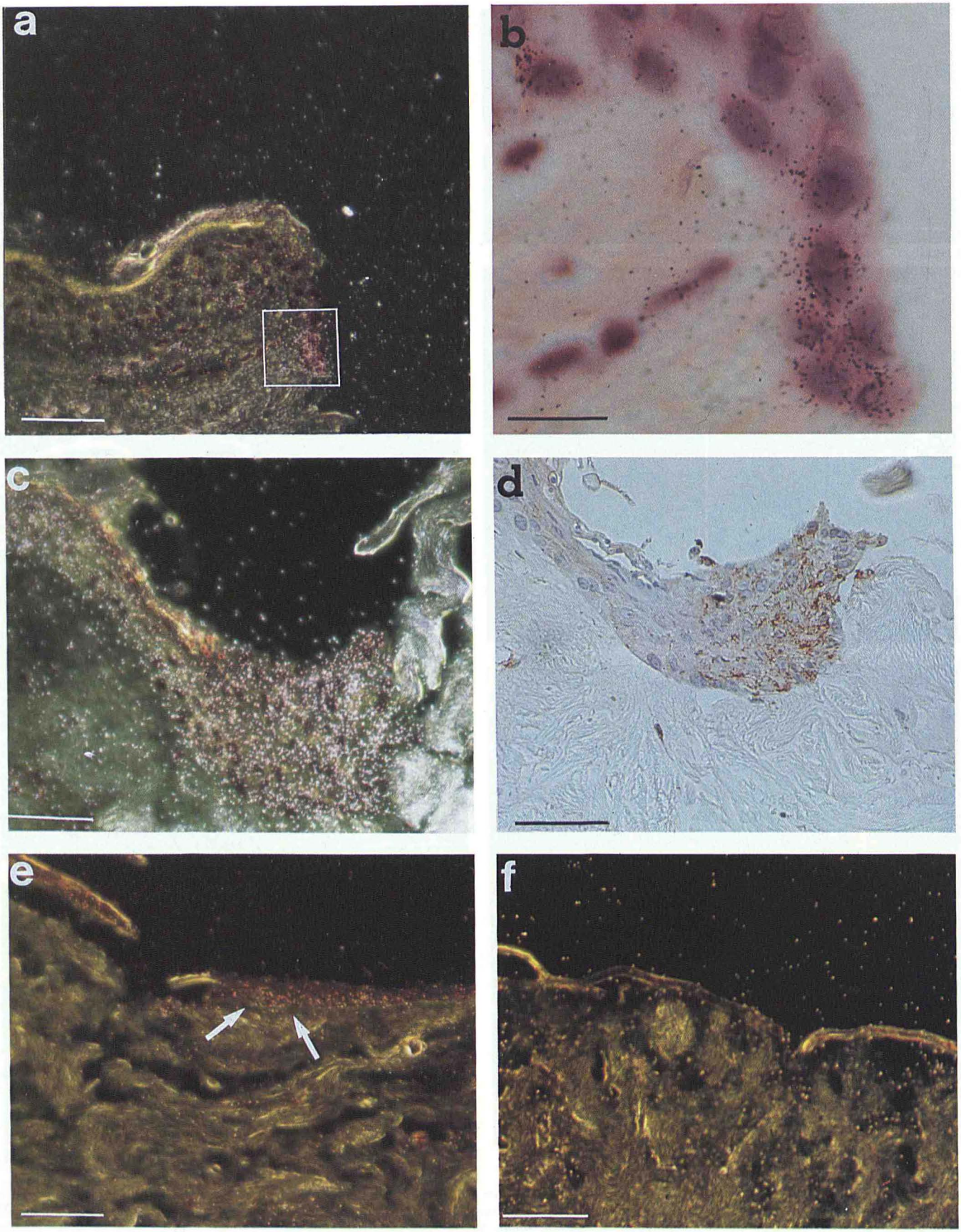
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Figure 2. Time course of collagenase expression in human skin wounds cultured in 10% FBS. *a*) Wound harvested 4 h after wounding. Dark-field photomicrograph showing evident autoradiographic signal for collagenase mRNA in keratinocytes at the wound edge (*box*). *Bar*, 125 μm. *b*) Bright-field photomicrograph showing area within the box in *a* at high magnification. Autoradiographic signal appears as black grains within keratinocytes at the wound edge. *Bar*, 15 μm. *c,d*) Serial sections of skin wound 24 h after wounding. *c*) Dark-field photomicrograph revealing intense signal for collagenase mRNA in wound-edge keratinocytes. *Bar*, 60 μm. *d*) Immunostaining shows the presence of collagenase protein in wound-edge keratinocytes. *e*) Dark-field view of wound at day 4 showing expression of collagenase mRNA in migrating keratinocytes (*arrows*). *Bar*, 60 μm. *f*) Re-epithelialized wound at day 7 without specific signal for collagenase mRNA in the epidermis. *Bar*, 60 μm. Autoradiographic exposure was for 2 weeks and sections were stained with hematoxylin and eosin.

lagen exhibit enhanced collagenolytic activity [19] or collagenase levels [13,20], whereas contact with laminin does not stimulate collagenolysis [20]. In intact skin, basal keratinocytes are normally in contact with laminin and not exposed to collagen. Thus, it was suggested that loss of contact with intact basement membrane and exposure of the cells to interstitial collagen stimulate collagenase production. This is compatible with the intense collagenase expression that we observed in wound-edge keratinocytes both *in vivo* and *in vitro*. However, our data show that collagenase expression stops when the two epithelial sheets meet, which is before reconstruction of the basement membrane is completed [21]. Thus, even though cell-matrix interaction may be critical for inducing collagenase expression, cell-cell contact is likely to influence expression, possibly through modulation of cell adhesion systems and/or secretion of signaling factors.

To investigate the complex process of wound healing, it is necessary to manipulate the microenvironment of cells. For this purpose, the *in vitro* wound-healing model provides a useful system. Under culture conditions that allowed the keratinocytes to complete re-epithelialization, i.e., 10% FBS, the expression pattern of collagenase mimicked that observed in *in vivo* wounds. When the concentration of FBS was lowered to 2%, only eight to ten keratinocytes migrated over the wound. In such suppressed wounds, collagenase expression was weak at 6 h and barely detectable 12 h after wounding (data not shown). Thus, collagenase expression may be closely related to the re-epithelialization process.

The known substrate specificity for interstitial collagenase, including fibrillar collagen types I, II, III, and X, makes it critical for matrix remodeling. However, the consistent finding of collagenase expression in wound-edge epithelium and its close association with keratinocyte migration and re-epithelialization strongly suggest a precise spatial and temporal role for collagenase in this process. Prevailing data offer no evident candidate function for collagenase in this context, but our understanding of the dermoepidermal zone is still incomplete and allows speculation. Interestingly, the recently cloned hemidesmosome protein 180-kD bullous pemphigoid antigen (also called BPAG2, HD4, and type XVII collagen) possesses a large collagenous extracellular domain extending into the basement membrane [22]. Even though interstitial collagenase does not cleave recombinant 180-kD bullous pemphigoid antigen *in vitro* [23], the complete structure of the native protein and its *in vivo* degradation profile are not known. The dermo-epidermal junction zone is complex, and future research may reveal novel proteins possibly serving as substrates for interstitial collagenase. Clearly, further investigations are needed to elucidate precise functions of this enzyme.

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