The Activity of Collagenase-1 Is Required for Keratinocyte Migration on a Type I Collagen Matrix

Brian K. Pilcher,* Jo Ann Dumin,* Barry D. Sudbeck,* Stephen M. Krane,§ Howard G. Welgus,* and William C. Parks*‡

*Department of Medicine (Dermatology), ‡Department of Cell Biology and Physiology, Washington University School of Medicine, St. Louis, Missouri 63110; and §Department of Medicine, Harvard Medical School and Massachusetts General Hospital, Boston, Massachusetts 02114

Abstract. We have shown in a variety of human wounds that collagenase-1 (MMP-1), a matrix metalloproteinase that cleaves fibrillar type I collagen, is invariably expressed by basal keratinocytes migrating across the dermal matrix. Furthermore, we have demonstrated that MMP-1 expression is induced in primary keratinocytes by contact with native type I collagen and not by basement membrane proteins or by other components of the dermal or provisional (wound) matrix. Based on these observations, we hypothesized that the catalytic activity of MMP-1 is necessary for keratinocyte migration on type I collagen. To test this idea, we assessed keratinocyte motility on type I collagen using colony dispersion and colloidal gold migration assays. In both assays, primary human keratinocytes migrated efficiently on collagen. The specificity of MMP-1 in promoting cell movement was demonstrated in four distinct experiments. One, keratinocyte migration was completely blocked by peptide hydroxymates, which are potent inhibitors of the catalytic activity of MMPs. Two, HaCaTs, a line of human keratinocytes that do not express MMP-1 in response to collagen, did not migrate on a type I collagen matrix but moved efficiently on denatured type I collagen (gelatin). EGF, which induces MMP-I production by HaCaT cells, resulted in the ability of these cells to migrate across a type I collagen matrix. Three, keratinocytes did not migrate on mutant type I collagen lacking the collagenase cleavage site, even though this substrate induced MMP-1 expression. Four, cell migration on collagen was completely blocked by recombinant tissue inhibitor of metalloproteinase-1 (TIMP-1) and by affinity-purified anti–MMP-1 antiserum. In addition, the collagen-mediated induction of collagenase-1 and migration of primary keratinocytes on collagen was blocked by antibodies against the α2 integrin subunit but not by antibodies against the α1 or α3 subunits. We propose that interaction of the α2β1 integrin with dermal collagen mediates induction of collagenase-1 in keratinocytes at the onset of healing and that the activity of collagenase-1 is needed to initiate cell movement. Furthermore, we propose that cleavage of dermal collagen provides keratinocytes with a mechanism to maintain their directionality during reepithelialization.

N ORMAL cutaneous wound healing, as well as healing in essentially all tissues, involves an orderly progression of events to reestablish the integrity of the injured tissue. The initial injury starts a programmed series of independent yet separate responses that includes reepithelialization and epithelial proliferation, inflammation, angiogenesis, fibroplasia, matrix accumulation, and eventually resolution. During each stage in this process, proteinases are needed to remove or remodel extracellular matrix components in both the epithelial and interstitial compartments, thereby accommodating cell migration and tissue repair (Mignatti et al., 1996).

Although extracellular matrix proteins can be degraded by various proteinases, fibrillar type I collagen, the most abundant protein in the dermis, is resistant to degradation by most enzymes. Collagen degradation is initiated by the catalytic activity of collagenases, a subgroup of the matrix metalloproteinase (MMP)[1] gene family, with the unique ability to cleave fibrillar collagen types I, II, and III at a specific locus in their triple-helical domain. At physiological temperature, cleaved collagen molecules denature into gelatin and become susceptible to further digestion by other proteinases. Of the three known human metallocollagenases, collagenase-1 (MMP-1) seems to be the enzyme

1. *Abbreviations used in this paper*: HU, hydroxyurea; MMP, metalloproteinase; SCID, severe combined immunodeficiency; TIMP-1, tissue inhibitor of metalloproteinases-1.

that is principally responsible for collagen turnover in most tissues. In a variety of normal and disease-associated tissue remodeling events, collagenase-1 may be expressed by epithelial cells, fibroblasts, endothelial cells, chondrocytes, and macrophages (Saarialho-Kere et al., 1992, 1993*a*; Stricklin et al., 1993; Wolfe et al., 1993; Fisher et al., 1994; Galis et al., 1994). Collagenase-2 (MMP-8) is found only in neutrophils and chondrocytes (Hasty et al., 1990; Chubinskaya et al., 1996), and collagenase-3 (MMP-13), originally cloned from a breast carcinoma line (Freije et al., 1994), is also expressed in articular cartilage (Mitchell et al., 1996; Reboul et al., 1996) and developing bone (Gack et al., 1995).

In a thorough examination of normally healing wounds and a variety of chronic ulcers, we found that collagenase-1 is invariably expressed by basal keratinocytes at the leading edge of repairing tissue and that expression of this MMP in the epidermis diminishes rapidly away from the wound margin (Saarialho-Kere et al., 1992, 1993*a*). In a time-course study of reepithelialization in human skin, Inoue et al. (1995) reported that collagenase-1 expression is detectable as early as 4 h after wounding, when migration is first apparent, and shuts off upon reformation of a contiguous epidermis. In chronic ulcers, collagenase-1 is expressed at higher levels and over a greater length of epidermis compared to that seen in acute wounds (Saarialho-Kere et al., 1993*a*), suggesting that overexpression of this MMP may contribute to the inability of certain lesions to heal. Importantly, this enzyme is always expressed by migrating keratinocytes that have moved off of an intact basement membrane and are in contact with dermal and provisional matrices (Saarialho-Kere et al., 1993*a*). Conversely, wound epidermal cells on an intact basement membrane, such as seen in certain forms of blisters (Saarialho-Kere et al., 1995), and on nonwounded epidermis do not express this MMP.

Our in vivo findings suggest that altered cell–matrix interactions influence collagenase-1 expression during reepithelialization. Indeed, we have shown that native type I collagen selectively supports primary human keratinocyte attachment and induces collagenase-1, whereas denatured collagen (gelatin), purified or combined basement membrane proteins, or other dermal-wound bed matrix components do not (Saarialho-Kere et al., 1993*a*; Sudbeck et al., 1994). Furthermore, we showed that contact with native type I collagen induces collagenase-1 transcription and is blocked by tyrosine kinase antagonists (Sudbeck et al., 1994). Thus, contact with dermal type I collagen is an important determinant in regulating the response of basal keratinocytes to injury. Indeed, in this report, we show that blocking antibodies to the $\alpha 2$ subunit of the $\alpha 2 \beta 1$ integrin inhibits collagen-mediated induction of collagenase-1 in keratinocytes.

The invariable expression of collagenase-1 in all forms of wounds and the confinement of its expression to periods of active reepithelialization suggests that this enzyme plays a critical role in keratinocyte migration during wound repair. Beyond directly remodeling structural proteins, MMPs are thought to break down extracellular matrix barriers that impede cell migration. Clearly, this is a reasonable role for these proteinases in facilitating cell movement through a three-dimensional matrix, as is seen during blastocyst invasion (Librach et al., 1991), angiogenesis

(Fisher et al., 1994), and extravasation and infiltration of inflammatory cells (Shipley et al., 1996). During normal reepithelialization, however, keratinocytes migrate along a path of least resistance, dissecting underneath the scab while remaining superficial to the underlying dermis and wound bed (Stenn and Malhotra, 1992). Thus, epidermal repair involves cell migration in a two-dimensional plane rather than through a three-dimensional matrix-rich environment. As reported here, our findings indicate that keratinocytes use collagenase-1 to cleave collagen to gelatin, thereby providing a substrate that is more conducive to migration. Without collagenolytic activity, these cells do not migrate on a collagen-containing matrix. We propose that the ability of keratinocytes to bind tightly to collagen and subsequently cleave the fibrils provides a mechanism that maintains the directionality of reepithelialization.

Materials and Methods

Cell Culture

Primary keratinocytes were isolated from full thickness skin obtained from reductive mammoplasty or lateral abdominoplasty and grown in DME (1.8 mM Ca^{2+}) as described (Pentland and Needleman, 1986; Sudbeck et al., 1994). Cells were plated on dishes or slides precoated with 1.0 mg/ml solution bovine type I monomeric collagen (Vitrogen; Collagen Corp., Palo Alto, CA) or with 1.0 μg/ml gelatin made by heating type I collagen at 80°C for 10 min. The HaCaT human keratinocyte cell line (Boukamp et al., 1988) was provided by Dr. Norbert Fusenig (German Cancer Research Center, Heidelberg, Germany) and was grown in DME containing 10% heat-inactivated FCS, 1% Hepes, and antibiotics. Collagenase-1 accumulation in the medium was quantified by competitive ELISA and normalized to total cellular protein (Sudbeck et al., 1994). RNA isolation and detection of collagenase-1 mRNA by reverse transcriptase–PCR were done as described in detail (Sudbeck et al., 1997). Total protein synthesis was determined in cultured keratinocytes as described (Sudbeck et al., 1994).

In Situ Hybridization

Collagenase-1 mRNA was detected in formalin-fixed tissue samples and cultured cells by hybridization with ^{35}S-labeled antisense RNA as described (Prosser et al., 1989; Saarialho-Kere et al., 1993*b*). For in situ hybridization of acute wounds, we obtained sections of intact and 2-d wounded neonatal human foreskin grafted onto severe combined immunodeficiency (SCID) mice from Drs. Horace DeLister and Steven Albelda (University of Pennsylvania, Philadelphia, PA). The creation of these grafted mice and the incisional wounding and biopsy procedures have been described in detail (Juhasz et al., 1993). Keratinocytes were grown on collagen-coated Lab-Tek chamber slides (Nunc, Naperville, IL) and were hybridized with 2.5×10^4 cpm/μl of ^{35}S-labeled antisense or sense RNA overnight at 57°C. After hybridization, slides were washed under stringent conditions, including RNase A treatment, and were processed for autoradiography.

Colloidal Gold Migration Assay

Keratinocytes were plated on chamber slides precoated with a mixture of 100 μg/ml type I collagen or gelatin or no matrix and colloidal gold particles in serum-containing DME with or without 30 ng/ml EGF (R&D Systems Inc., Minneapolis, MN). Colloidal gold–coated chamber slides were prepared as described (Albrecht-Buehler, 1977) with modifications for keratinocytes and the inclusion of matrix proteins (Woodley et al., 1988; Kim et al., 1994). Primary or HaCaT keratinocytes (about 330 cells in 0.3 ml) were added to each chamber, and 20 min later, nonadherent cells were removed and the medium was replaced. 20 h after plating, cultures were fixed in 1× Histochoice tissue fixative (Amresco, Solon, OH) for 1 min, washed in PBS, and dehydrated through graded ethanols. Paths of cell migration (phagokinetic tracks) were identified by areas devoid of gold particles. A migration index was determined using image analysis software by

measuring the area of phagokinetic tracks associated with cells in randomly chosen fields under dark-field illumination at 100×. All cells in a field were counted, and 20 cells were counted for each chamber. For each experiment, all conditions were done in duplicate, and all experiments were repeated at least four times with keratinocytes from different donors. The migration index of keratinocytes on colloidal gold with no matrix was essentially zero and was subtracted from experimental points.

Colony Dispersion Migration Assay

Keratinocyte migration was also assessed in a colony dispersion assay by plating 10^4 cells suspended in 150 μl DME within a siliconized cloning cylinder (6-mm internal diameter; Bellco Glass, Inc., Vineland, NJ) onto collagen- or gelatin-coated dishes. After a 24-h incubation period to allow the cells to attach and become confluent, the cylinder was removed, and cell colonies were allowed to migrate for 0, 24, 48, 96, or 120 h at 37°C in a 5% CO_2 humidified incubator. Keratinocytes were fixed and stained with 1.5% Coomassie blue, and the area of the colony was determined by digitized scanning analysis. Migration is expressed as the increase in colony area relative to 0-h controls.

Various reagents were added to promote or inhibit keratinocyte migration. In studies with HaCaT keratinocytes, collagenase-1 expression was induced with 30 ng/ml EGF (R&D Systems, Inc.). HaCaT proliferation in response to EGF was inhibited by treating cells with 100 mM hydroxyurea (HU; Sigma Chemical Co., St. Louis, MO) 24 h before addition of EGF. Peptide hydroxymate compounds SC44463 and SC44201 were used at 25 μM and were provided by Monsanto-Searle, Inc. (St. Louis, MO). Collagenase-1 antibody was affinity-purified and characterized as described (Saarialho-Kere et al., 1993a). This polyclonal antibody recognizes both the zymogen and activated forms of collagenase-1, free or bound to TIMP-1, and fully inhibits the catalytic activity of collagenase-1. In an in vitro degradation assay, a 1:20 dilution of collagenase antibody completely inhibited the cleavage of type I collagen monomers by 5 μg/ml of purified human collagenase-1 (data not shown). Ammonium sulfate–precipitated nonimmune IgG (1:10 dilution) was used as a control in the antibody-blocking experiments. Recombinant human TIMP-1 (50 μg/ml), which has full MMP-inhibitory activity, was provided as a gift from Dr. David Carmichael (Synergen Corporation, Boulder, CO). Wild-type and collagenase-1–resistant mouse type I collagens were used at 100 μg/ml. Generation of the mice and characterization of the mutant collagen were reported earlier (Wu et al., 1990; Liu et al., 1995). Monoclonal blocking antibodies to integrin subunits were purchased from Chemicon International Inc. (Temecula, CA) and were mAB1973 (anti-α1), mAB1950 (anti-α2), and mAB1952 (anti-α3). Integrin antibodies were added to a final concentration of 10 μg/ml. To prevent blocking of cell adhesion, keratinocytes were plated 2 h before addition of integrin-blocking antibodies.

Thiopeptolide Assay

Inhibition of MMP degradation of the thiopeptolide substrate Ac-Pro-Leu-Gly-thioester-Leu-Leu-Gly-Oet (BACHEM Bioscience, King of Prussia, PA) (Shipley et al., 1996) was used to determine the activity of SC44463 in tissue culture. We obtained recombinant 92-kD gelatinase catalytic domain, which is spontaneously active, from Dr. J. Michael Shipley (Washington University, St. Louis, MO) and used this protein as a source of MMP activity to generate an initial cleavage rate. The inhibitory activity of fresh 25 μM SC44463 in DME was compared to that of SC44463 in 48- or 72-h keratinocyte-conditioned medium.

Results

Collagenase-1 Is Expressed by Migrating Keratinocytes In Vivo and In Vitro

The epidermis of neonatal human skin grafted onto SCID mice heals by 4 d after wounding, and reepithelialization is associated with the expression of identical integrins and basement membrane proteins produced in actual human wounds (Juhasz et al., 1993). As demonstrated by in situ hybridization, collagenase-1 was expressed by human basal keratinocytes only at the leading edge of reepithelialization (Fig. 1, B and C), identical to the location of en-

zyme expression seen in a variety of human wounds and ulcers (Saarialho-Kere et al., 1992, 1993a, 1995). Collagenase-1 mRNA was not expressed in intact skin (Fig. 1 A). The precise, spatially confined expression of collagenase-1 by migrating keratinocytes suggests that altered cell–matrix interactions modulate enzyme expression, and indeed, collagenase-1-expressing keratinocytes were seen in intimate contact with dermal fibrillar collagen (Fig. 1 C).

When grown in DME medium, which contains 1.8 mM $CaCl_2$, cultured keratinocytes form subpopulations of migrating, proliferating, and differentiating cells (Pentland and Needleman, 1986). Differentiating keratinocytes are seen as blurred foci when viewed from above and are surrounded by an apron of hyperproliferative cells (Fig. 2 A). Bordering the hyperproliferative cells, and often detached from them, are migrating keratinocytes. Reflecting the phenotype of basal cells involved in reepithelialization in vivo, collagenase-1 mRNA was expressed only in keratinocytes migrating from the colonies of proliferating and differentiating cells (Fig. 2 A). No signal was seen in cultures hybridized with a ^{35}S-labeled sense RNA probe (data not shown). With time, proliferating cells and their migrating daughter cells cover, or "heal," the tissue culture surface, and collagenase-1 expression ceases (data not shown).

Collagenase-1–expressing Keratinocytes Migrate on Type I Collagen

To assure that collagenase-1–positive keratinocytes were migrating, we plated cells on chamber slides coated with a colloidal gold–type I collagen mixture and fixed the cultures 20 h later. Cells were treated with 30 ng/ml EGF to augment collagenase-1 production over the relatively short experimental period. Phagokinetic tracks created were identified as areas lacking gold particles and were often seen associated with single cells migrating along the collagen-coated surface (Fig. 2 B). Migration tracks were also associated with single cells and doublets proximal to keratinocyte foci and were apparently moving away from them (Fig. 2 C, arrows). In addition, gold particles were removed at the borders of cell foci (Fig. 2 C), suggesting zones of collagenolysis. The dark clumps in keratinocytes seen under phase (Fig. 2 C′) are phagocytized gold particles. Internalized gold particles were not present in keratinocytes within organized foci (Fig. 2 C′, large arrows), where collagenase-1 is not expressed (Fig. 2 A). Thus, the gold–collagen substrate was removed only in areas that corresponded to those where high levels of collagenase-1 expression were seen.

To verify that gold particle clearance was dependent on metalloproteinase activity, we treated keratinocytes with a peptide hydroxymate inhibitor. This compound is a substrate-based inhibitor containing a hydroxamic acid moiety that chelates the active site zinc cation and renders MMPs catalytically inactive. SC44463 has a K_i of about 1 nM for pure collagenase-1 in solution (Moore and Spilburg, 1986). Treatment with SC44463 completely blocked the formation of phagokinetic tracks or zones of lysis (Fig. 2, D and D′). These observations demonstrate that collagenase-1–expressing keratinocytes are migrating and that this process is inhibited by compounds that block the catalytic activity of this MMP.
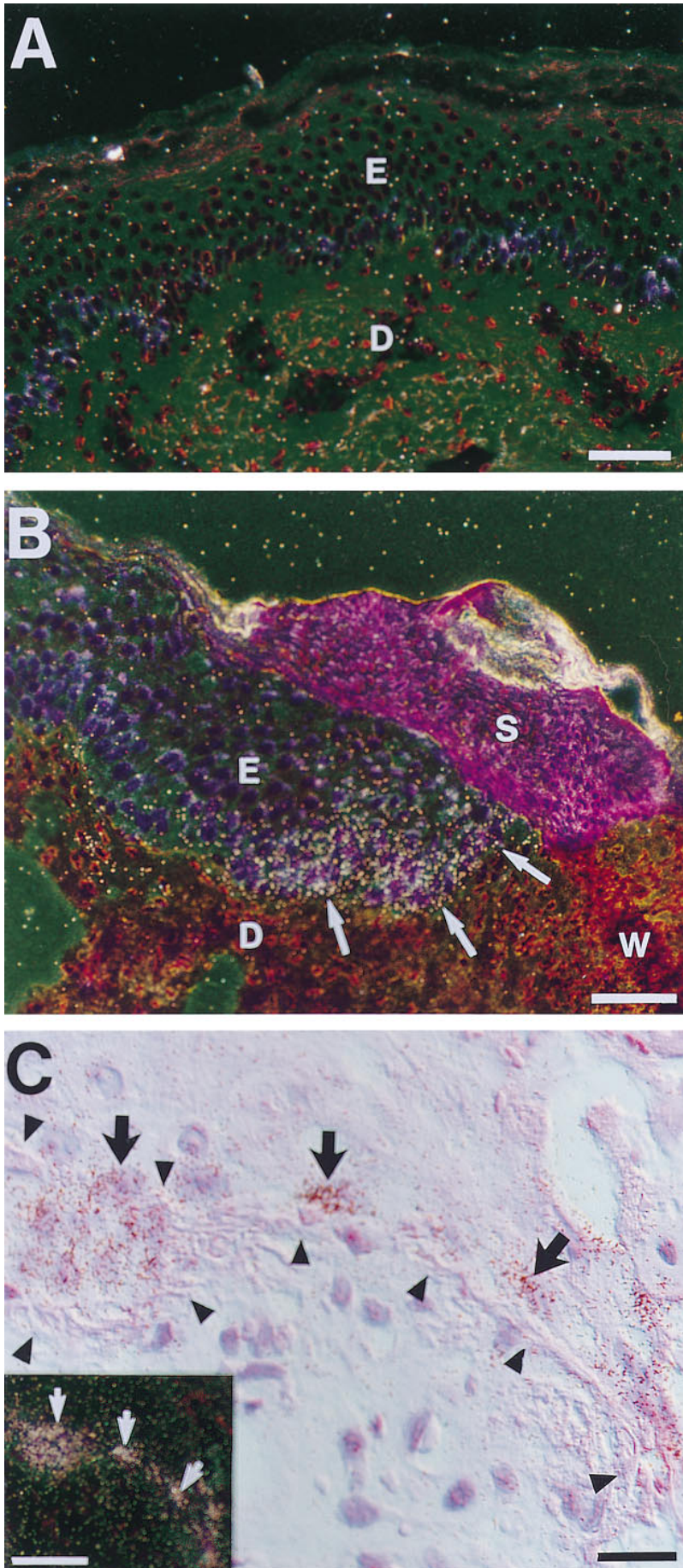
Figure 1. Collagenase-1 mRNA is expressed by migrating keratinocytes in contact with dermal collagen. (*A*) A section of uninjured neonatal foreskin grafted onto a SCID mouse was hybridized with a ^{35}S-labeled antisense RNA probe specific for collagenase-1 mRNA. No detectable signal for collagenase-1 mRNA was seen in the epidermis (*E*) or the dermis (*D*) of unwounded skin. Mouse skin and the graft junctions are not seen in this field. Under dark-field illumination, pigmented cells are iridescent and appear purplish. (*B*) In a section of a biopsy taken 2 d after wounding grafted human neonatal skin, autoradiographic signal for collagenase-1 mRNA was seen in basal keratinocytes at the leading edge of reepithelialization (*arrows*). Signal was confined to keratinocytes migrating into the wound area (*W*), under the scab (*S*), and over the dermal wound bed (*D*). No signal was detected in intact epidermis (*E*) or in any cell in the dermis. (*C*) In a section of pyogenic granuloma, which displays many features of an acute wound (Saarialho-Kere et al., 1992), collagenase-1 mRNA was seen in basal keratinocytes (*arrows*) along the dermal–epidermal junction (*arrowheads*) at the epidermal front. Collagenase-1–positive basal keratinocytes at the migrating front are in direct contact with fibrillar collagen (*arrowheads*), which appear as thick iridescent fibers under Nomarski optics. (*Inset*) Under dark-field illumination, autoradiographic signal for collagenase-1 mRNA is seen in basal keratinocytes demarcated by arrows in the larger photomicrograph. Autoradiographic exposure was 21 d for all sections. Bars: (*A* and *B*) 100 μm; (*C*) 25 μm; (*Inset*) 50 μm.
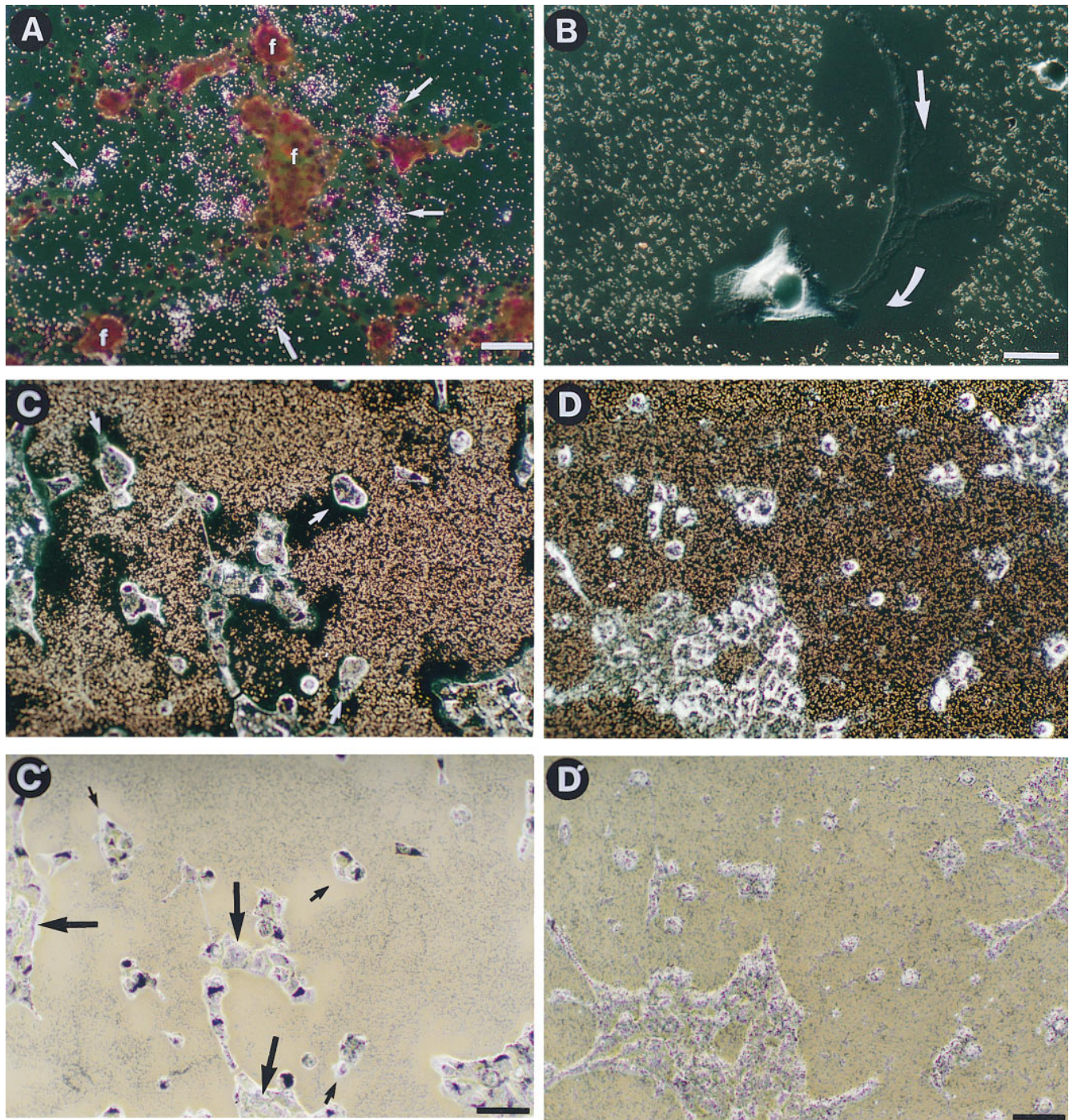
Figure 2. Collagenase-1 mRNA is expressed by migrating keratinocytes in culture. (*A*) Keratinocytes were plated on dishes precoated with native type I collagen and 24 h later were processed for in situ hybridization using a ^{35}S-labeled collagenase-1–specific antisense RNA probe. Signal for collagenase-1 mRNA was seen only in migrating keratinocytes (*arrows*), whereas no autoradiographic signal was detected in hyperproliferative or differentiating keratinocytes, which appear as blurred foci (*f*) when viewed from above. Autoradiographic exposure was 14 d. (*B–D*) Primary human keratinocytes were plated on culture slides coated with a mixture of type I collagen and colloidal gold particles and were fixed 20 h later. With Nomarski optics (*B*) or under dark-field illumination (*C*), tracks of keratinocyte migration were seen as areas devoid of gold particles (*arrows*). (*B*) Often, single cells were seen migrating along large bundles of collagen fibers. (*C*) Gold was also removed along the border of keratinocyte foci and in association with cells (*arrows*) that had apparently migrated from the keratinocyte islands. Under phase (*C′*), internalized gold particles were seen as dense accumulations in keratinocytes. No internalized gold was evident in cells within foci (*large arrows*). (*D* and *D′*) No migration tracks or areas of lysis around cell foci were seen in cultures treated with SC44463. Bars: (*A*) 50 μm; (*B*) 12 μm; (*C–D′*) 25 μm.
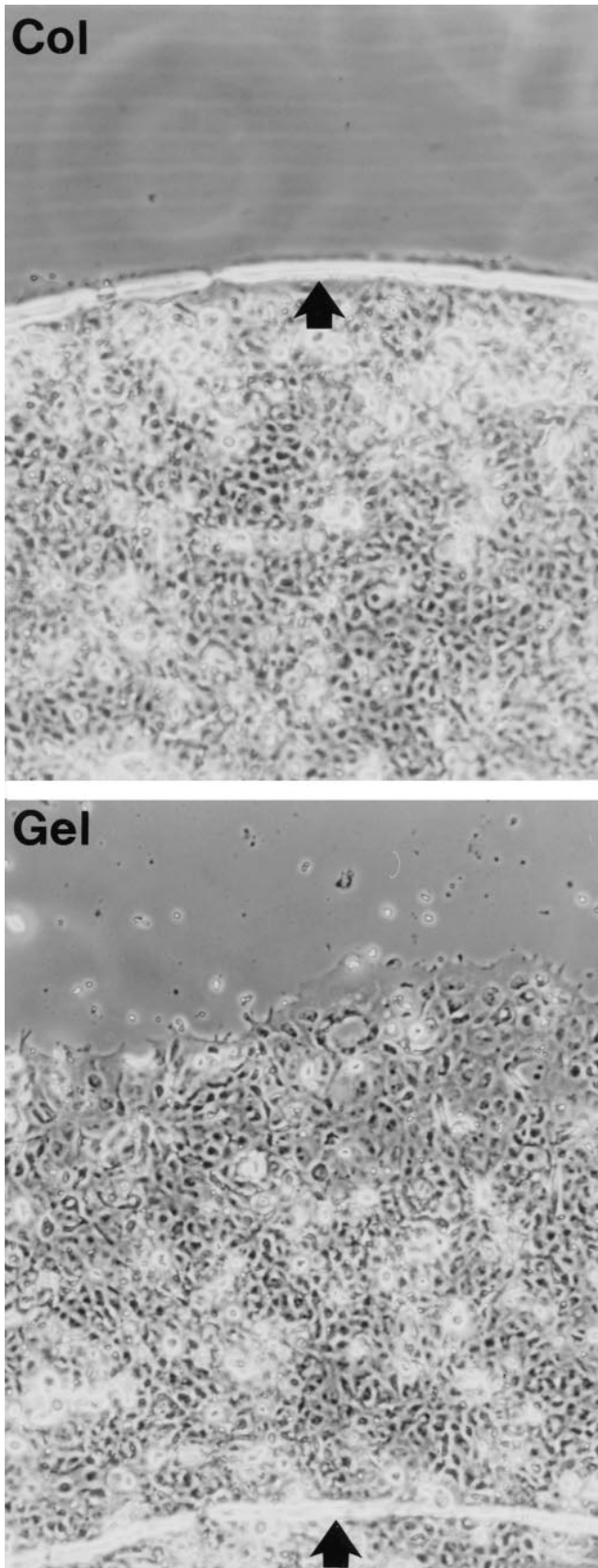
Collagenase-1–deficient HaCaT Keratinocytes Migrate on Gelatin but Not on Native Type I Collagen

To begin to assess if keratinocyte migration specifically requires the activity of collagenase-1, we assessed the ability of HaCaT cells, which do not express collagenase-1 in response to native type I collagen (see Fig. 4 *A*), to migrate on native or denatured collagen. HaCaT cells were plated within cloning cylinders on dishes coated with type I collagen or gelatin (heat-denatured type I collagen). The cylinders were removed 24 h later, and the cells were allowed to migrate for 48 h. HaCaT cells cultured on native type I collagen did not migrate from the colony border, whereas cells grown on gelatin-coated dishes migrated efficiently from the cell colony (Fig. 3). MMP inhibitors did not affect HaCaT migration on gelatin (data not shown). Because the primary cleavage products of collagenase-1 denature at 37°C but are not further degraded, we hypothesized that collagenase-1–mediated cleavage of collagen followed by denaturation to gelatin is required for effective migration.

Induction of Collagenase-1 Expression by HaCaT Keratinocytes Induces Migration on Type I Collagen

High levels of collagenase-1 production were induced in HaCaT keratinocytes after exposure to 30 ng/ml EGF (Fig. 4 *A*). As we saw in the primary keratinocyte cultures (Fig. 2 *A*), HaCaT cells formed tightly packed colonies, and collagenase-1 mRNA was expressed only by cells at the periphery of the foci (Fig. 4 *B*). In contrast, HaCaT cells within the cell colony had no detectable collagenase-1 mRNA. Although EGF potently induced collagenase-1 expression, enzyme production was confined only to migrating cells, or at least to cells with the potential to migrate.

Supporting the idea that collagenase-1 is required for keratinocyte motility on dermal matrix, EGF-treated cells migrated progressively on a type I collagen–coated surface over a 4-d period (Fig. 4 *D*). At 96 h, EGF-stimulated cells covered an area four times greater than that of untreated HaCaT cells, which did not produce collagenase-1 (Fig. 4 *A*) and which had not progressed far from the border of the cloning cylinder (Fig. 4, *C* and *D*). The small increase in colony area evident at 72 and 96 h in control HaCaT cells was likely due to cell proliferation.

HaCaT Migration on Type I Collagen Requires MMP Activity

Induction of HaCaT cell migration on type I collagen by EGF suggests that the activity of collagenase-1 facilitates cell movement on this matrix. Indeed, treatment of HaCaT cells with SC44463 inhibited EGF-mediated cell migration ∼50% in the colony dispersion assay (Fig. 4 *E*, −*HU*). The incomplete inhibition of cell migration in SC44463-treated cultures was likely due to EGF-stimulated cell proliferation, thereby increasing colony size independent of cell

Figure 3. Collagenase-1–negative HaCaT keratinocytes migrate on gelatin but not on native type I collagen. HaCaT cells were grown within cloning cylinders on dishes precoated with type I collagen (*Col*) or gelatin (*Gel*). The cylinders were removed 24 h later, and cells were allowed to migrate for 48 h. The arrows designate the border of the culture when the cloning cylinder was removed. The micrographs shown are representative of three experiments.
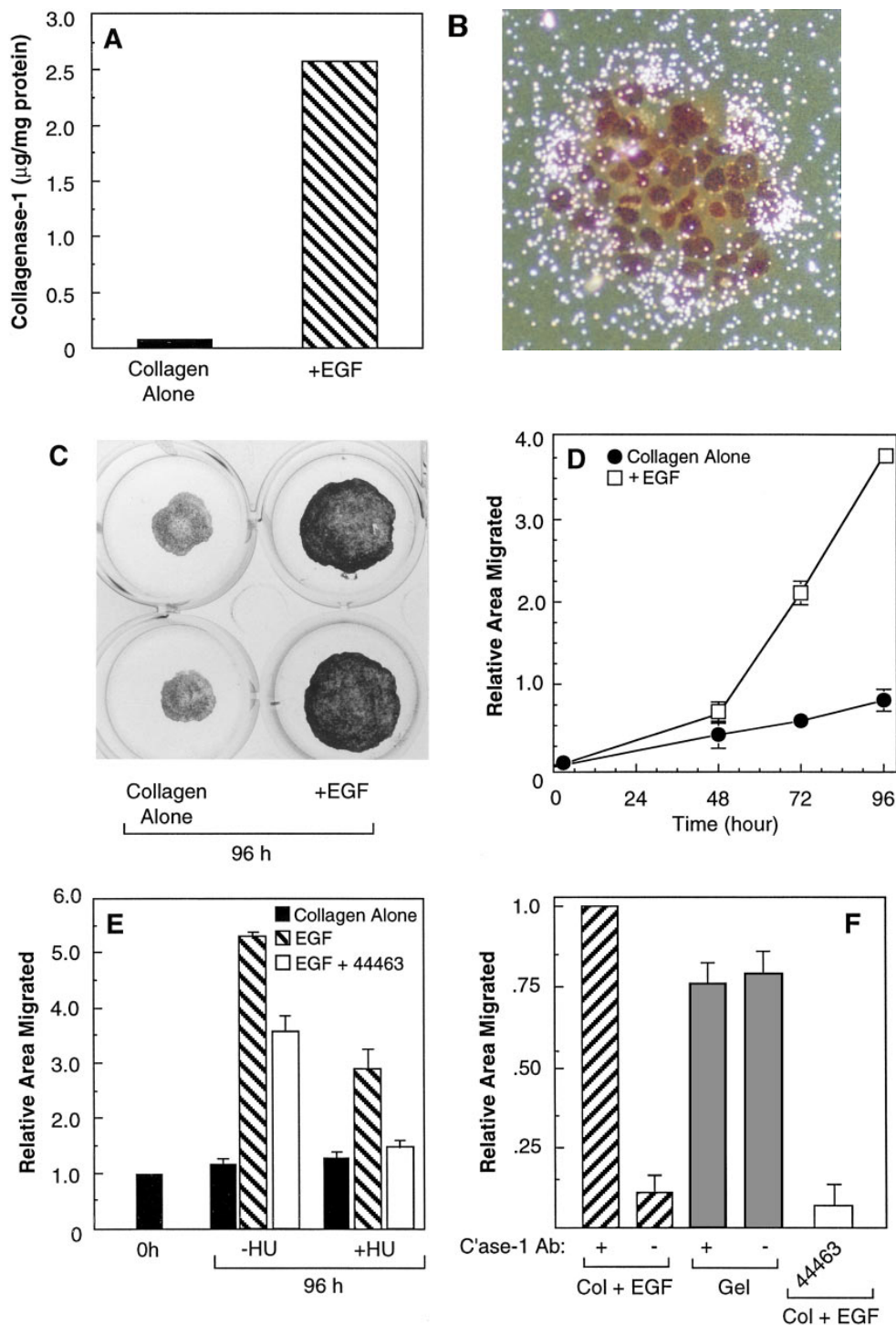
Figure 4. HaCaT migration on native type I collagen is MMP dependent. (*A*) HaCaT keratinocytes were grown on type I collagen–coated dishes and treated with or without 30 ng/ml EGF. Collagenase-1 accumulation in the medium was assessed 48 h later by ELISA and normalized to total cellular protein. (*B*) HaCaT cells were plated on collagen-coated slides, stimulated with EGF, and 24 h later were processed for in situ hybridization with a collagenase-1 ^{35}S-labeled antisense RNA probe. Only HaCaT cells at the periphery of cell clusters expressed collagenase-1 mRNA. Autoradiographic exposure was 14 d. (*C–E*) HaCaT cells were plated within cloning cylinders on collagen-coated dishes. After 24 h, the cylinders were removed, and the cells were allowed to migrate on collagen alone or in the presence of EGF for 48, 72, or 96 h. Cells were stained, and the area migrated was quantified by scanning densitometry. (*E*) During the initial 24-h culture period, some HaCaTs were treated with 100 mM HU to inhibit EGF-mediated proliferation. The cylinders were removed, and the cells were given fresh medium with or without 30 ng/ml EGF or EGF plus 25 μM peptide hydroxymate inhibitor SC44463. After 96 h, cultures were washed and stained, and migration was quantified by image analysis. Migration data for HaCaT cells pretreated with (*+HU*) or without (*−HU*) HU are shown. The data in *D* and *E* are the means ± SD or triplicate wells and are expressed in arbitrary units relative to 0-h controls. (*F*) HaCaT keratinocytes were plated on culture slides coated with a mixture of colloidal gold particles and type I collagen (*Col*) or gelatin (*Gel*). Cells on collagen-coated chambers were treated with 30 ng/ml EGF. To inhibit collagenase-1 activity, cells were treated with (+) or without (−) collagenase-1 affinity-purified antibody or 25 μM SC44463 and were fixed 20 h later. Keratinocyte migration was quantified as described under Materials and Methods, and the data shown are the means ± SEM of duplicate samples from four experiments.

movement per se. To neutralize the proliferative effects of EGF, cell colonies were pretreated for 24 h with HU, an effective inhibitor of DNA synthesis and cell division. The antiproliferative effect of HU pretreatment was dose dependent and persisted up to 96 h as determined by [^{3}H]thy-midine incorporation (data not shown). Under these conditions, EGF-stimulated cells migrated on collagen, but they did not cover an area as large as EGF-treated HaCaT cells not preexposed to HU (Fig. 4 *E*, *+HU*). Addition of SC44463 completely blocked EGF-mediated cell migration

on type I collagen (Fig. 4 *E*, *+HU*). These results demonstrate that EGF-mediated HaCaT migration on type I collagen is an MMP-dependent process.

Because the colony dispersion assay requires treatment with EGF for 96 h to see appreciable migration, we cannot conclude that cell movement is due specifically and solely to collagenase-1 activity. To address this concern, we used the colloidal gold assay to assess keratinocyte migration over a relatively shorter period of time (20 h). Duplicating the findings of the colony dispersion assay, EGF-treated HaCaTs migrated efficiently as single cells on a colloidal gold–collagen substratum, and this activity was blocked by SC44463 (Fig. 4 *F*). Migration was also completely inhibited by treatment with affinity-purified anti–collagenase-1 antibody, which blocks the catalytic activity of this MMP. Untreated HaCaT keratinocytes migrated efficiently on gelatin, and cell movement on this substrate was not affected by the blocking antibody (Fig. 4 *F*). These findings indicate that EGF-mediated migration of HaCaT keratinocytes on native collagen requires the activity of collagenase-1.

Migration of Primary Human Keratinocytes

In contrast to HaCaT cells, collagenase-1 is induced in primary human keratinocytes grown on native type I collagen (Sudbeck et al., 1994). 120 h after plating, primary keratinocytes cultured on collagen alone migrated well beyond the original cell colony border (Fig. 5), and addition of the MMP inhibitor SC44463 completely blocked this response (Fig. 5). The ability of SC44463 to inhibit cell migration was dose dependent over a range of 0.1 to 25 μM, demonstrating an ~50% reduction in cell movement with 1.0 μM in both EGF-stimulated HaCaT keratinocyte and primary human keratinocyte cultures (data not shown). Compound

SC44201, a stereoisomer of 44463 differing only in the plane of a single hydrogen atom, is a weak inhibitor of MMP activity, with a relative K_i for all MMPs over 1,000-fold greater than that of SC44463 (Moore and Spilburg, 1986). Consistent with the idea that keratinocyte migration on type I collagen is MMP dependent, SC44201 did not affect cell movement (Fig. 5). As determined by a [^{3}H]leucine pulse and trichloroacetic acid precipitation, the hydroxymate compounds at concentrations up to 25 μM did not affect the synthesis of total secreted protein (Table I). Furthermore, as determined by an in vitro MMP activity assay, the inhibitory activity of SC44463 was not diminished after 72 h in culture (Table II).

Primary Keratinocytes Do Not Migrate on Collagenase-resistant Type I Collagen

Inhibition of keratinocyte migration on type I collagen by the peptide hydroxymate compound demonstrated that MMP activity was required for cell motility. This compound, like other hydroxymates, inhibits a broad spectrum of metalloproteinases (Moore and Spilburg, 1986). Therefore, based on these data alone, we cannot conclude that collagenase-1 is the specific MMP required for migration on collagen. Because primary keratinocytes have the potential to express other MMPs, namely 92-kD gelatinase (Sudbeck et al., 1997), stromelysin-1, and stromelysin-2 (Windsor et al., 1993; Saarialho-Kere et al., 1994), we used more specific reagents to determine which enzyme is required for migration. Northern hybridization demonstrated that human keratinocytes express only collagenase-1 and do not produce collagenase-2 or -3 (data not shown).

To determine if the proteolytic activity of collagenase-1 is required for cell motility, we cultured primary human keratinocytes on a collagenase-resistant mutant type I collagen. Human collagenase-1 cleaves fibrillar type I collagen at Gly$_{775}$-Ile$_{776}$ in the $\alpha 1$(I) chains and at Gly$_{775}$-Leu$_{776}$ in the $\alpha 2$(I) chain, and these sites are conserved among mammalian type I collagens. This cleavage renders the molecule thermally unstable and susceptible to further degradation by other proteinases. The mutant collagen used in this study contains a double substitution of Pro for Glu$_{774}$ and Ala$_{777}$ and Met for Ile$_{776}$ in the region of the collagenase cleavage site of the $\alpha 1$(I) chain, rendering the molecule resistant to proteolysis by collagenase-1 (Wu et al., 1990; Liu et al., 1995). Collagenase-1 expression was induced in primary keratinocytes grown on wild-type native
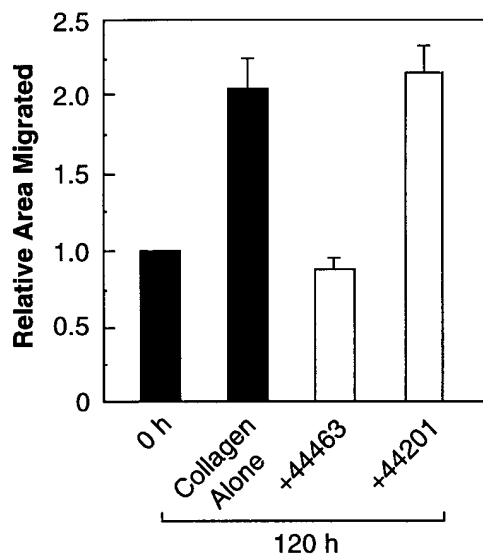

Figure 5. Keratinocyte migration on native type I collagen is MMP dependent. Primary human keratinocyte migration was assessed by the colony dispersion assay. Cells were cultured on collagen with or without peptide hydroxymate compounds SC44463 or SC44201. The data shown are the means ± SD of triplicate wells and are expressed in arbitrary units relative to 0-h controls.

Table I. Protein Synthesis

Treatment	Protein synthesis
	cpm/10^6 cells
Collagen alone	2836 ± 551
+ SC44463	2783 ± 816
+ SC44201	3627 ± 353

Keratinocytes were plated on type I collagen and treated with 25 μM of the indicated peptide hydroxymate compound for 24 h. Cultures were then incubated for 1 h in leucine-free medium to deplete intracellular pools and then pulsed for 24 h in leucine-free medium containing 1 μCi/ml L-[^{3}H]leucine in the presence or absence of hydroxymate compounds. Radioactivity incorporated into acid precipitable material in the conditioned medium was determined 24 h later. The data presented are the means ± standard deviations of triplicate determinations from three separate plates per treatment.

Treatment	Percent inhibition of 92-kD gelatinase catalytic domain (CD) activity
92-kD CD Alone	0
92-kD CD + 0 h CM	99.73
92-kD CD + 48 h CM	99.59
92-kD CD + 72 h CM	99.41

Keratinocytes were plated on type I collagen in medium containing 25 μM SC44463 for 0, 48, or 72 h. The inhibitory activity of SC44463 in conditioned medium (*CM*) was compared to that of fresh (*0 h*) SC44463 by assessing the ability of 92-kD gelatinase catalytic domain to hydrolyze a thiopeptolide substrate.

type I collagen, and cells migrated efficiently across this matrix (Fig. 6, *A* and *B*). In contrast, keratinocytes cultured on collagenase-resistant mutant collagen did not migrate, although enzyme expression was induced (Fig. 6, *A* and *B*). As an internal control, inhibition of all MMP activity with SC44463 completely inhibited the ability of these cells to migrate on native type I collagen (Fig. 6 *A*). Keratinocytes plated on mutant type I collagen adhered equally to those on wild-type collagen and expressed collagenase-1, indicating that the cell-recognition site is distinct from the enzyme cleavage site.

Migration of Primary Keratinocytes on Type I Collagen Is Blocked by Collagenase Antibodies

In both the colony dispersion and colloidal gold assays, anti–collagenase-1 antibodies inhibited keratinocyte migration in a dose-dependent fashion, whereas cell motility was not affected by nonimmune IgG (Fig. 7, *A* and *B*). The concentrations of collagenase-1 antibody that inhibited cell migration are the same as those that blocked activity of pure enzyme in solution (data not shown). In addition, human recombinant TIMP-1, a naturally occurring inhibitor of collagenase-1 and other MMPs, inhibited cell migration (Fig. 7 *A*).

In the colloidal gold assay, most keratinocytes showed evidence of migration on collagen (Fig. 7 *C*). Collagenase-1 antibody blocked keratinocyte migration on native type I collagen proportionally to the concentration of antibody added (Fig. 7 *B*). Similar to the inhibition seen with SC44463 (Fig. 7 *B*), essentially no migration was seen in cultures treated with the highest concentration of antibody (Fig. 7, *B* and *D*). In contrast, collagenase-1 antibody did not affect keratinocyte migration on gelatin (Fig. 7, *B* and *E*). Keratinocyte migration on gelatin was slightly reduced compared to that on collagen, likely because the cells did not adhere as efficiently to the heat-denatured substrate. These findings demonstrate that the proteolytic activity of collagenase-1, and not that of other MMPs, is required for keratinocyte migration on native type I collagen.

Integrin-mediated Induction of Collagenase-1

To assess which integrin–matrix interactions transduce the collagen-mediated induction of collagenase-1, we treated primary keratinocytes plated on native collagen with α1-, α2-, or α3-blocking antibodies. Each of these subunits is expressed by basal keratinocytes in intact and wounded skin and complex with the β1 subunit to form potential collagen-binding receptors (Larjava et al., 1993; Lange et al., 1994). Collagen-mediated induction of collagenase-1 mRNA was potently inhibited by treatment with the α2 antibody but not by antibodies against the α1 or α3 subunits (Fig. 8). Blocking collagenase-1 expression with the α2β1 antibody inhibited keratinocyte migration on collagen by ~75% relative to untreated controls, whereas cell movement was unaffected by the α1 or α3 antibodies (Fig. 8). To prevent potential disruption of cell attachment to collagen, we added the integrin-blocking antibodies 2 h after keratinocytes were plated, and migration and collagenase-1 mRNA levels were assessed 20 h later. Although collagenase-1 mRNA levels were completely repressed by 20 h, some migration was evident, which may be because of
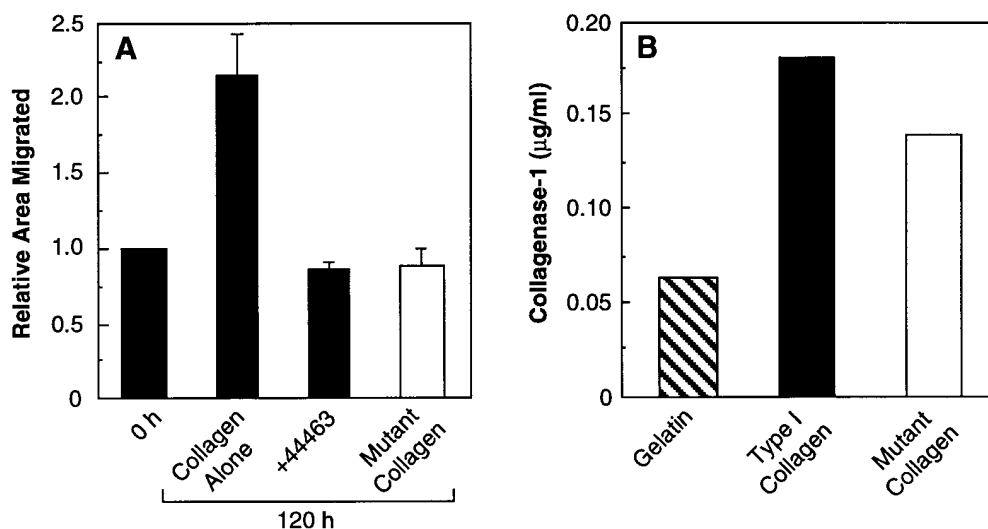

Figure 6. Keratinocytes do not migrate on a collagenase-resistant type I collagen. (*A*) Primary human keratinocyte migration on native type I collagen or on collagenase-resistant type I collagen was assessed by the colony dispersion assay. After 24 h, the cylinders were removed, and the cells were allowed to migrate for 120 h. Some cultures on native type I collagen were exposed to 25 μM SC44463 during the 120-h migration period. The data are the means ± SD of triplicate wells, and migration is expressed in arbitrary units relative to 0-h controls. (*B*) Keratinocytes were grown to confluence on gelatin, type I collagen, or mutant type I collagen. Collagenase-1 accumulation in conditioned medium at 72 h was determined by ELISA and normalized to total protein. Gelatin was included as a substrate that does not stimulate collagenase-1 expression in keratinocytes.
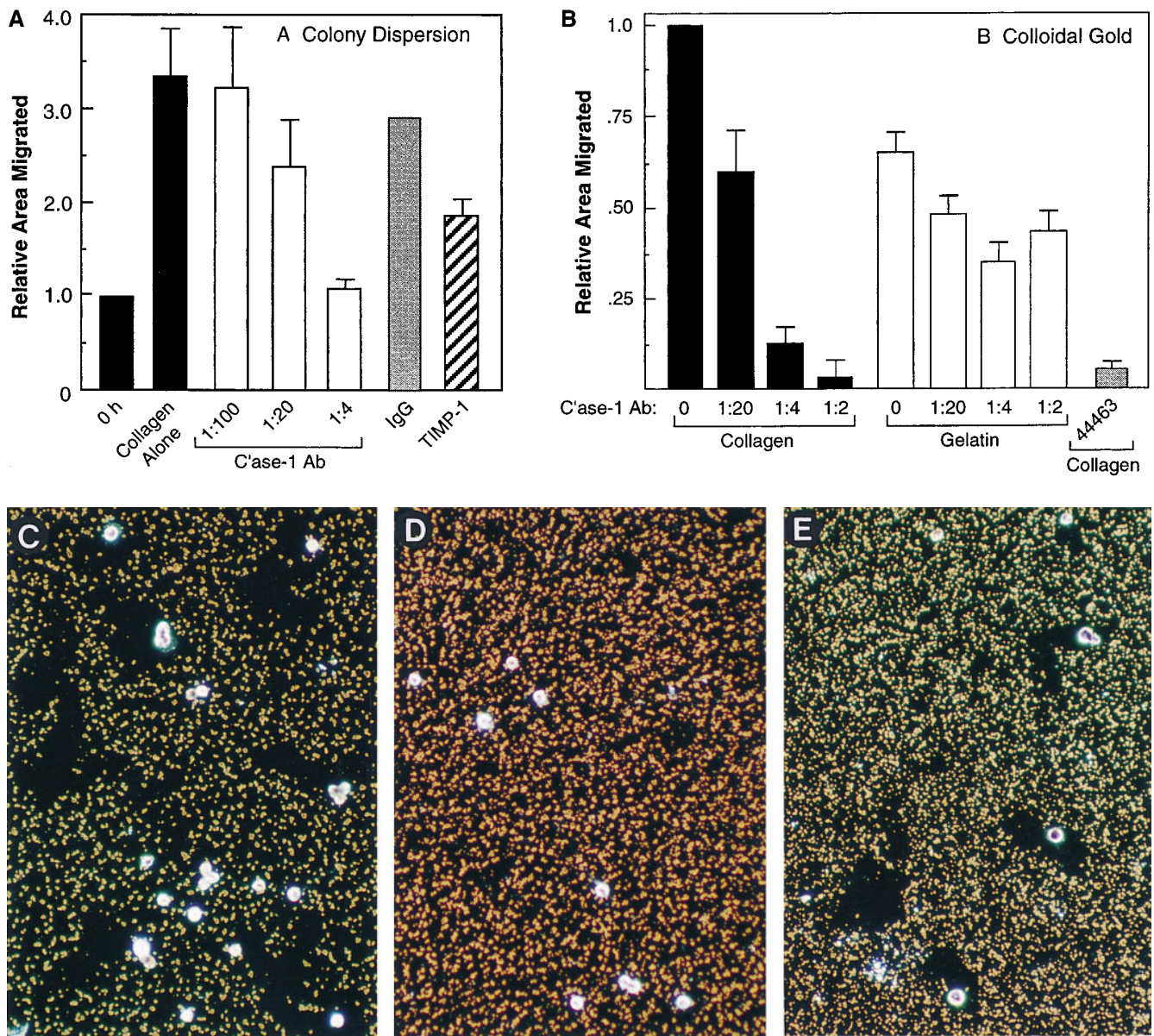
Figure 7. Keratinocyte migration on type I collagen is collagenase-1-dependent. (*A*) Colony dispersion. Primary human keratinocytes were grown for 24 h within cloning cylinders on type I collagen. The cloning cylinders were removed, and cells were allowed to migrate for 120 h on collagen in the presence of affinity-purified collagenase-1 antibody (*C'ase-1 Ab*) at the indicated dilutions or nonimmune IgG or 50 µg/ml recombinant human TIMP-1. The data presented are means ± SD of values from three separate wells per treatment group, and migration is expressed in arbitrary units relative to 0-h controls. (*B*) Colloidal gold. Primary human keratinocytes were plated on culture slides coated with colloidal gold particles and type I collagen or gelatin and the indicated titers of affinity-purified collagenase-1 antibody or 25 µM SC44463 and were fixed 20 h later. Keratinocyte migration was quantified as described under Materials and Methods, and the data shown are the means ± SEM of duplicate samples from four experiments. The lower panels are dark-field illuminated micrographs of keratinocytes on collagen alone (*C*), on collagen plus collagenase-1 antibody at 1:2 dilution (*D*), or on gelatin plus collagenase-1 antibody at 1:2 dilution (*E*).

some collagenase-1 produced during the 2-h attachment period.

Discussion

In this report, we demonstrate that the production and activity of collagenase-1 is required for keratinocyte migration on native type I collagen. In response to cutaneous injury, basal keratinocytes at the edge of the wound dis-

lodge, dissolve, or loosen their cell–cell and cell–matrix contacts, move from the basement membrane, and migrate in an organized front over the viable dermis and wound bed. Initiation of keratinocyte migration is one of the earliest responses of the epidermis to wounding and precedes by hours stimulation of cell proliferation (Garlick and Taichman, 1994). As we have shown in several different types of human wounds (Saarialho-Kere et al., 1992, 1993*a*, 1995), collagenase-1 is invariably expressed by
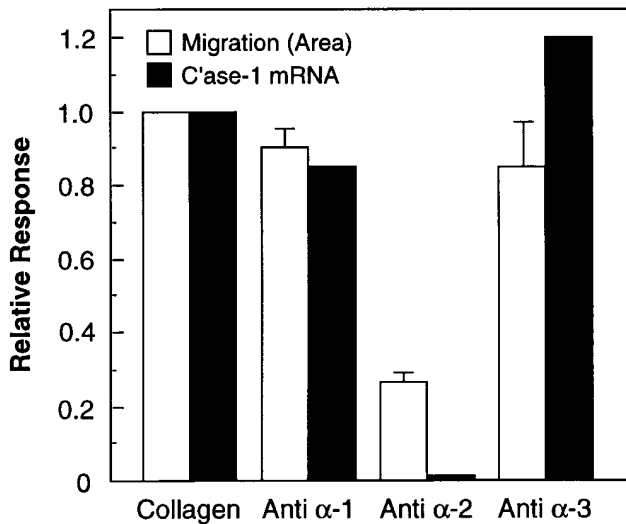
Figure 8. Collagen-mediated induction of collagenase-1 is α2 integrin dependent. Primary human keratinocytes were plated on collagen-coated slides with or without colloidal gold particles, and 2 h later, blocking antibodies (10 µg/ml) against the indicated integrin subunits were added. 20 h after plating, migration was assessed on gold-coated slides, and cells on slides without gold were harvested for RNA isolation. Collagenase-1 mRNA was assessed by reverse transcriptase–PCR, and densitometry was used to quantify relative signal strength. For both assays, migration area and collagenase-1 mRNA levels were normalized to the levels detected in cells on collagen alone.

keratinocytes that have moved off of the basement membrane and onto the underlying matrix, and studies by Inoue et al. (1995) demonstrated that collagenase-1 is induced as soon as 4 h after wounding. The confinement of collagenase-1 expression to a spatially defined subpopulation of keratinocytes suggests that altered cell–matrix contacts mediate induction of this MMP. Indeed, as we demonstrate here, contact with dermal type I collagen induces collagenase-1 expression only by migrating keratinocytes (Fig. 2 *A*). Other components of the dermal and provisional matrices, such as type III collagen, fibronectin, and fibrin, do not induce or affect collagenase-1 production or are not even recognized by keratinocytes (Sudbeck, B.D., B.K. Pilcher, H.G. Welgus, and W.C. Parks, unpublished observations). Consistent with the idea that migration from basement membrane onto a type I collagen–containing matrix is required for collagenase-1 expression, this MMP is not induced in keratinocytes grown on basement membrane proteins (Saarialho-Kere et al., 1993*a*; Sudbeck et al., 1994). Thus, altered cell–matrix interactions, and in particular, contact with dermal type I collagen, may initiate the keratinocyte's response to injury, which is characterized by the expression of collagenase-1 at the migrating front.

We propose that collagenase-1 acting on its principal substrate in the dermis, type I collagen, provides migrating keratinocytes with a mechanism to maintain their course and directionality in the wound environment during reepithelialization. In intact skin, basal keratinocytes constitutively express the type I collagen–binding integrin α2β1 along their basolateral surfaces (Hertle et al., 1991; Sym-

ington et al., 1993). In wounded epidermis, migrating keratinocytes continue to express their collagen-binding receptors (Cavani et al., 1993; Hertle et al., 1992; Juhasz et al., 1993), but α2β1 becomes redistributed and concentrated at the frontobasal end of the cells (Guo et al., 1991). This redistribution places α2β1 where it would likely come into intimate contact with dermal type I collagen. Although basal keratinocytes also express α1β1 and α3β1, keratinocytes preferentially use α2β1 to bind to type I collagen (Lange et al., 1994). Indeed, we demonstrate that blocking the ability of this integrin to bind type I collagen inhibits collagenase-1 expression and, consequently, keratinocyte migration, whereas blocking ligand interaction to α1β1 or α3β1 did not affect enzyme production or cell movement (Fig. 8). In contrast, Zhang and Kramer (1996) reported that blocking antibodies to α3β1 inhibited keratinocyte migration on collagen. However, in their assays, inhibition of cell movement was seen at a concentration of collagen at which we have demonstrated to be a weak stimulator of collagenase-1 expression (Saarialho-Kere et al., 1993*a*). At higher concentrations of collagen, cell migration was barely inhibited (Zhang and Kramer, 1996). Still, their data suggest that α3β1 plays a role in keratinocyte migration, but our findings indicate that this integrin does not influence collagenase-1 expression or collagenase-1–dependent motility.

α2β1 binds native collagen with high affinity (Staatz et al., 1989), and thus, clustering of this integrin at the forward edge of keratinocytes may actually tether the cells to the matrix, rendering them unable to migrate. Therefore, the proteolytic activity of collagenase-1 may aid in dissociating keratinocytes from these high-affinity attachments to a collagen matrix. As stated, collagenase-1 does not degrade fibrillar type I collagen but rather makes a single, site-specific cleavage through the triple helix about 3/4 the length from the NH_2 terminus. The resultant fragments, called TC^A and TC^B fragments, are thermally unstable at body temperature and may spontaneously denature into gelatin. Besides being highly susceptible to complete degradation by different proteinases, gelatin binds α2β1 with a much lower affinity than does native collagen (Staatz et al., 1989). Thus, we speculate that by cleaving type I collagen, which then denatures into gelatin, collagenase-1 effectively mediates the loosening of the tight contacts keratinocytes may establish with the dermal matrix. This function is distinct from the often suggested idea that migrating cells use metalloproteinases to remove matrix barriers that may physically impede movement.

Although keratinocytes may actually migrate on other molecules found in the provisional wound matrix, such as fibronectin and vitronectin, the proteolytic activity of collagenase-1 is still needed to initiate and maintain this process. Basal HaCaT cells did not express collagenase-1 (Fig. 4 *A*) and did not migrate on a collagen matrix, even though serum adhesive proteins were present (Figs. 3 and 4). These cells did, however, migrate easily on a gelatin matrix (Fig. 3), and stimulation of collagenase-1 expression by exogenous EGF induced migration of HaCaT cells on type I collagen (Fig. 4). Furthermore, we were able to block migration of primary human keratinocytes by any method that perturbed the ability of collagenase-1 to cleave collagen, be it addition of hydroxymate inhibitors,

collagenase-1–blocking antibodies, or TIMP-1 or plating cells on collagenase-resistant collagen (Figs. 5, 6 and 7). In addition, blocking the induction of collagenase-1 expression in primary keratinocytes by treatment with α2 antibody inhibited keratinocyte migration. We do not interpret these data to indicate that α2β1 binding to collagen is directly required for keratinocyte migration, but rather that this cell–matrix interaction mediates induction of collagenase-1, which, in turn, is essential for cell movement.

As stated, we propose that collagenase-1 facilitates keratinocyte migration by affecting the conformation of type I collagen and, consequently, the avidity with which cells interact with it. One may argue that this is an inherently inefficient mechanism. If activated keratinocytes migrate over the viable dermis interacting with provisional matrix proteins, then why do they need to cleave type I collagen? Why would they adhere to the dermis with high affinity if their objective is to close the wound as quickly as possible? The answer, we believe, is that the process of interacting with and then cleaving type I collagen provides keratinocytes with a mechanism to determine and maintain their directionality during reepithelialization.

An important observation relevant to our directionality hypothesis is that collagenase-1 production is induced in keratinocytes by native type I collagen but not by denatured forms of the molecule (see Fig. 6 B and Sudbeck et al., 1994). Thus, collagenase-1 acting on collagen creates a mediator that does not support or maintain its own production. The conversion of collagen to gelatin would replace the inductive stimulus with a neutral substrate (gelatin), and in stationary cells, collagenase-1 expression would decline. Indeed, collagenase-1 expression is rapidly turned off at the completion of reepithelialization (Inoue et al., 1995). Although cell–cell contacts may be involved in this process, the initial expression of collagenase-1 may mediate cleavage of the collagen substrate, thereby neutralizing the inductive effect of the underlying matrix. If keratinocytes continue to interact with type I collagen, presumably by migrating, then they would continue to express collagenase-1.

During wound healing in vivo, collagenase-1 cleavage of collagen would leave a trail of denatured collagen (gelatin) that would not attract keratinocyte attachment. Using high-affinity interactions with native type I collagen as a "molecular compass," keratinocytes could then bind to components of the provisional matrix to support motility. Because gelatin does not induce or maintain collagenase-1 expression, keratinocytes would not have the proteinase needed to invade into the dermis if they would begin to stray. The activity of collagenase-1 would allow keratinocytes to break away from collagen while it continually extends and interacts with new native collagen molecules present in the superficial plane of the viable dermis. In a stratified epithelium, cell migration is thought to proceed in a leap-frog fashion, whereby the cell at the front extends along and attaches to the matrix and is replaced by cells coming from behind and above (see Stenn and Malhotra, 1992). Thus, these interrelated collagenolytic and migratory processes may occur within a limited microenvironment.

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