In response to cutaneous injury, expression of collagenase-1 is induced in keratinocytes via α2β1 contact with native type I collagen, and enzyme activity is essential for cell migration over this substratum. However, the cellular mechanism(s) mediating integrin signaling remain poorly understood. We demonstrate here that treatment of keratinocytes cultured on type I collagen with epidermal growth factor receptor (EGFR) blocking antibodies or a specific receptor antagonist inhibited cell migration across type I collagen and the matrix-directed stimulation of collagenase-1 production. Additionally, stimulation of collagenase-1 expression by hepatocyte growth factor, transforming growth factor-β1, and interferon-γ was blocked by EGFR inhibitors, suggesting a required EGFR autocrine signaling step for enzyme expression. Collagenase-1 mRNA was not detectable in keratinocytes isolated immediately from normal skin, but increased progressively following 2 h of contact with collagen. In contrast, EGFR mRNA was expressed at high steady-state levels in keratinocytes isolated immediately from intact skin but was absent following 2 h cell contact with collagen, suggesting down-regulation following receptor activation. Indeed, tyrosine phosphorylation of the EGFR was evident as early as 10 min following cell contact with collagen. Treatment of keratinocytes cultured on collagen with EGFR antagonist or heparin-binding (HB)-EGF neutralizing antibodies dramatically inhibited the sustained expression (6–24 h) of collagenase-1 mRNA, whereas initial induction by collagen alone (2 h) was unaffected. Finally, expression of collagenase-1 in *ex vivo* wounded skin and re-epithelialization of partial thickness corneal burn wounds was blocked following treatment with EGFR inhibitors. These results demonstrate that keratinocyte contact with type I collagen is sufficient to induce collagenase-1 expression, whereas sustained enzyme production requires autocrine EGFR activation by HB-EGF as an obligatory intermediate step, thereby maintaining collagenase-1-dependent migration during the re-epithelialization of epidermal wounds.

Efficient repair of a cutaneous wound requires a programmed series of spatially and temporally regulated events. Among these, effective proteolytic degradation of extracellular matrix (ECM) macromolecules is thought to be necessary to remodel the damaged tissue, promote neovascularization, and facilitate efficient migration of cells during re-epithelialization (1). Matrix metalloproteinases (MMPs) constitute a family of zinc-dependent enzymes with the collective capacity to degrade virtually all ECM components (2). Although most MMPs can degrade many ECM proteins with overlapping substrate specificities, degradation of fibrillar type I collagen is initiated only by the catalytic activity of collagenases, a subgroup of the MMP gene family.

Previous studies from our group and others have shown that, in both normally healing wounds and chronic ulcers, basal keratinocytes at the leading edge of re-epithelialization invariably express collagenase-1 (3–6). Collagenase-1 expression is rapidly induced in wound-edge keratinocytes after injury, persists during the healing phase, and ceases following complete re-epithelialization (7, 8). We demonstrated that induction of collagenase-1 by basal keratinocytes is mediated via α2β1 interaction with native type I collagen (9, 10), requires tyrosine kinase activity (11), and that the activity of this MMP is essential for cell migration over this matrix protein (10). Although much is known about the role of cell:matrix interactions regulating collagenase-1 expression by keratinocytes, the signaling mechanism(s) following collagen binding and integrin receptor occupancy remain poorly understood.

The epidermal growth factor receptor (EGFR; c-erbB1/HER1) is a transmembrane cell-surface tyrosine kinase that, upon ligand binding, phosphorylates downstream effector molecules, leading to changes in cell function (12). EGFR-null mice demonstrate involvement of the receptor in a broad range of developmental processes, and these mice have pronounced defects in epithelial cell proliferation and differentiation (13–15). Activation of the EGFR by members of the EGF family of growth factors (i.e. EGF, TGF-α, HB-EGF, and amphiregulin) is associated with multiple keratinocyte functions during wound repair, including cell proliferation, migration, and stimulation of α2β1 integrin expression (16, 17). Keratinocyte migration is essential for effective re-epithelialization, and expression of the EGFR and its ligands is up-regulated following...
injury in vivo (17, 18). Indeed, exogenously administered EGFR ligands (EGF, TGF-α) stimulate keratocyte motility in vitro and re-epithelialization in vivo (19–23), and wound healing–specific keratins (K6 and K16) contain upstream regulatory sequences responsive to EGFR activation (24). Furthermore, EGFR overexpression in cultured keratocytes enhances ligand-mediated motility (25).

Autocrine signaling mechanisms often regulate cell function and behavior, and, in keratocytes, autocrine activation of the EGFR can influence epithelial homeostasis and cutaneous repair. Indeed, recent evidence from Stoll et al. (51) demonstrates that heparin-binding ligands, namely HB-EGF and amphiregulin, mediate autocrine activation of the EGFR in a skin organ culture model, suggesting that these ligands play an important role in the amplification and transmission of the wound healing signal. Because collagenase-1 production is tyrosine kinase–dependent (11) and because keratocytes express both the EGFR and its various ligands during wound repair, we reasoned that an autocrine loop mechanism may regulate keratocyte collagenase-1 expression following α.β1 integrin–mediated collagen binding. Here, we report that the initial induction of collagenase-1 by keratocytes following contact with type I collagen requires only integrin receptor activation. However, autocrine activation of the EGFR by HB-EGF is required for the sustained expression of collagenase-1 by keratocytes in vitro and during the full re-epithelialization process in vivo.

**EXPERIMENTAL PROCEDURES**

**Materials**

Recombinant human EGF, HB-EGF, amphiregulin, and polyclonal neutralizing antiserum to HB-EGF were obtained from R & D Systems, Minneapolis, MN. Recombinant human TGF-α was purchased from Collaborative Biomedical Products, Becton Dickinson, Inc., Bedford, MA. Human EGFR antagonist PD153035 was obtained as a generous gift from Dr. Robert Panek (Parke Davis, Inc., Ann Arbor, MI). Another specific EGFR tyrosine kinase inhibitor, tyrphostin 1478, was purchased from Calbiochem, San Diego, CA. Recombinant IL-1α receptor antagonist (IL-1Ra) was a generous gift from Dr. David Carmichael (Synergen, Inc.). This compound is a soluble IL-1α receptor that competitively binds, blocking ligand binding and receptor activation (26). Polyclonal neutralizing antiserum to TGF-α (clone 189-2130.1) (27), a monoclonal EGFR blocking antibody (clone 528) (28), and polyclonal EGFR Ab-4 (used for EGFR immunoprecipitation) (28) were purchased from Oncogene Research Products, Cambridge, MA. Anti-phosphotyrosine monoclonal antibody (PY-20) and anti-mouse IgG-horseradish peroxidase conjugate were purchased from Transduction Laboratories, Lexington, KY. Bovine type I collagen (Vitrogen-100) was obtained from Celltrix Laboratories, Palo Alto, CA.

**Isolation and Culture of Human Keratocytes**

Human keratocytes were harvested from healthy adult skin from reduction mammoplasties or abdominoplasties as described (11, 29). Briefly, the subcutaneous fat and deep dermis were removed, and the remaining tissue was incubated in 0.25% trypsin in PBS. After 16 h, the epidermis was separated from the dermis with forceps, and the keratocytes were scraped into DMEM. The keratinocyte suspension was added to fresh DMEM supplemented with 5% fetal calf serum and 0.1% penicillin/streptomycin. Under these culture conditions, keratocytes proliferate, migrate, differentiate, and cornify similarly to cells in vivo (29). A specified amount of keratocyte suspension was then plated onto tissue culture dishes coated with 1 mg/ml type I collagen, which is necessary for induction of collagenase-1 and keratinocyte adhesion (5, 9, 11).

**In Situ Hybridization**

Collagenase-1 mRNA was detected in formalin-fixed tissue samples by hybridization with 35S-labeled antisense RNA as described (30, 31). Punch biopsies (2 mm) of human skin were obtained and grown as explant cultures in serum-containing DMEM for 4 days. Following treatment, the tissue was fixed in neutral buffered formalin for 24 h followed by washing in PBS and dehydration in graded ethanol. Sections of tissue were hybridized with 2.5 × 10⁸ cpm/ml 35S-labeled antisense or sense RNA overnight at 57 °C. After hybridization the slides were washed under stringent conditions, including RNase A treatment, and were processed for autoradiography. After development of the photographic emulsion, slides were stained with hematoxylin-eosin.

The specificity of the antisense RNA probe for collagenase-1 and the complete lack of reactivity by the sense probe have been demonstrated in previous studies (3, 7).

**Migration Assays**

Primary human keratocytes were plated on chamber slides precoated with a mixture of 100 µg/ml type I collagen and colloidal gold particles in serum-containing DMEM. Keratocytes (~300 cells) were added to each chamber, and 20 min later, nonadherent cells were removed and the medium was replaced. Twenty hours after plating, cultures were fixed in 1× Histochoice tissue fixative (Amresco, Solon, OH), washed in PBS, and dehydrated through graded methanol and ethanol and embedded in paraffin. Sections were cut (5 µm), mounted on glass slides, and stained with methylene blue, and the area of the colony was determined by digitized image analysis software. Migration is expressed as the increase in colony area relative to 0-h controls.

**Colloidal Gold—**Primary human keratocytes were plated on chamber slides precoated with a mixture of 100 µg/ml type I collagen and colloidal gold particles in serum-containing DMEM. Keratocytes (~300 cells) were added to each chamber, and 20 min later, nonadherent cells were removed and the medium was replaced. Twenty hours after plating, cultures were fixed in 1× Histochoice tissue fixative (Amresco, Solon, OH), washed in PBS, and dehydrated through graded methanol and ethanol and embedded in paraffin. Sections were cut (5 µm), mounted on glass slides, and stained with methylene blue, and the area of the colony was determined by digitized image analysis software. Migration is expressed as the increase in colony area relative to 0-h controls.

**Enzyme-linked Immunosorbent Assay (ELISA)**

The amount of collagenase-1 accumulated in keratocyte conditioned medium was measured by indirect competitive ELISA (32). This ELISA is completely specific for collagenase-1, has nanogram sensitivity, and detects active and zymogen enzyme forms, as well as collagenase-1 bound to TIMP or bound to substrate. Results were obtained from triplicate determinations and were normalized to total cell protein as quantified by the BCA protein assay (Pierce) using bovine serum albumin as a standard.

**Metabolic Labeling**

Post-confluent keratocytes plated on type I collagen were cultured for 24 h in the presence of serum-containing DMEM control or experimental solutions. The culture wells were then washed and replaced with methionine-free DMEM containing 1% dialyzed fetal calf serum (to remove free amino acids), 1 mM sodium pyruvate, 2 mM L-glutamine, 0.1 mM each of non-essential amino acids, 50 µCi/ml [35S]methionine (ICN Radiochemicals, Irvine, CA), and the identical concentrations of experimental reagents. Conditioned medium was collected 24 h later and analyzed by immunoprecipitation.

**Immunoprecipitation and Western Immunoblotting**

A specific polyclonal antiserum (33) was used to immunoprecipitate collagenase-1 from keratocyte conditioned medium as described (34). To immunoprecipitate the EGFR, cell layers were washed with PBS and treated for 10 min at room temperature with cell lysis buffer (1.5% Triton X-100, 50 mM Tris, pH 7.5, 150 mM NaCl, 1 mM CaCl₂, 1 mM MnCl₂, 1 mM MgCl₂, 2 mM phenylmethylsulfonyl fluoride, 0.02 mg/ml aprotinin, and 0.01 mg/ml leupeptin). All samples were precleared with protein G-Sepharose (Zymed Laboratories Inc., San Francisco, CA), and supernatants were incubated with collagenase-1 or EGFR polyclonal (35) antibodies for 1 h at 37 °C then overnight at 4 °C. Immune complexes were precipitated with protein A-Sepharose and washed extensively. For visualization of collagenase-1, radiolabeled protein was resolved by polyacrylamide gel electrophoresis and visualized by fluorography as described previously (36). Immunoprecipitated EGFR was resolved by polyacrylamide gel electrophoresis and electrophoretically transferred to Immobilon-P polyvinylidene difluoride membrane (Millipore Corp., Bedford, MA) using a semidy blanking apparatus (Bio-Rad). Tyrosine phosphorylation of the EGFR was then visualized by incubating the membrane with anti-PY-20 (primary) and anti-mouse IgG-horseradish peroxidase (secondary) antibodies followed by detection using the ECL system (Amersham Corp., Arlington Heights, IL).
according to the manufacturer's instructions. Total incorporated radioactivity (new protein synthesis) was determined from keratinocyte conditioned medium by trichloroacetic acid precipitation as described previously (37).

RNA Analysis

Collagenase-1, EGFR, EGF, TGF-α, amphiregulin, and GAPDH mRNAs were detected by modification of previously described reverse transcription-polymerase chain reaction (RT-PCR) assays (9, 38, 39). HB-EGF mRNA was amplified using primers designed with GeneWorks software (Oxford Molecular Group Inc., Campbell, CA). The 5′-sense primer was complementary to bases 791–810 in exon 4, and the 3′-antisense primer was complementary to bases 1017–1036 in exon 6 of human HB-EGF (40). These primers amplify a fragment across three exons; thus, the 246-base pair cDNA produced from HB-EGF mRNA would be easily distinguished from contaminating DNA or preprocessed mRNA. In addition, all resultant cDNAs from each of the primer pairs contain restriction sites specific to the mRNA amplified and each product was subject to digestion analysis to verify specificity. The primers used to amplify each mRNA, annealing temperatures, and resulting product sizes are listed in Table I.

Total RNA was isolated from cultured keratinocytes by phenol-chloroform extraction (41) and treated with RQ1 RNase-free DNase (Promega, Madison, WI) to remove any contaminating DNA as described (42). DNase-treated RNA was reverse transcribed with random hexamers using kit reagents and under the manufacturer's recommended conditions (GeneAmp RNA PCR kit, Perkin Elmer Cetus, Norwalk, CT). Signal strength for each RT-PCR cDNA product increased exponentially between 1 and 25 ng of total RNA at 25 cycles. To amplify each mRNA, we have used 10 ng of total RNA and 25 cycles. PCR products were separated through a 2% agarose gel and visualized by ethidium bromide staining. Specificity was determined by overnight transfer to Hybond N+ membrane (Amersham Corp.) followed by Southern hybridization with a radiolabeled product-specific oligonucleotide probe (EGF family members and EGFR) or radiolabeled cDNA probes (collagenase-1 and GAPDH). The probes used to detect EGF family members and EGFR transcripts following RT-PCR were developed to recognize only specific sequences that were amplified in the PCR reaction. For each oligonucleotide, a BLAST search was performed, and no sequence similarity to other known cDNAs was found. In addition, a parallel reaction was run without reverse transcriptase to exclude contamination from contaminating DNA or preprocessed mRNA. Under the conditions used, we routinely achieve 85% transcription efficiency of keratinocytes (9, 11, 37).

TABLE I

| Transcript | Forward primer | Reverse primer | Annealing temperature | Product length |
|------------|----------------|----------------|-----------------------|---------------|
| C'ase-1    | 5′-GGGCTGTCAGGGAGCAGATTCTGTC-3′ | 5′-GGGCTGTCAGGGAGCAGATTCTGTC-3′ | 60°C | 400 base pairs |
| EGF        | 5′-AGGGCTGTCAGGGAGCAGATTCTGTC-3′ | 5′-AGGGCTGTCAGGGAGCAGATTCTGTC-3′ | 45°C | 253 base pairs |
| TGF-α      | 5′-ATGGTCCCTCCGCTTGGAGACA-3′ | 5′-CTCGGACCTGCCAGCTTGGAGACA-3′ | 58°C | 182 base pairs |
| AR         | 5′-GCTGTCAGGGAGCAGATTCTGTC-3′ | 5′-GCTGTCAGGGAGCAGATTCTGTC-3′ | 50°C | 268 base pairs |
| HB-EGF     | 5′-ATGGTCCCTCCGCTTGGAGACA-3′ | 5′-CTCGGACCTGCCAGCTTGGAGACA-3′ | 55°C | 246 base pairs |
| EGFR       | 5′-GAGCCGCTTGGAGCAGATTCTGTC-3′ | 5′-GAGCCGCTTGGAGCAGATTCTGTC-3′ | 45°C | 221 base pairs |
| GAPDH      | 5′-GCTGTCAGGGAGCAGATTCTGTC-3′ | 5′-GCTGTCAGGGAGCAGATTCTGTC-3′ | 55°C | 610 base pairs |

In Vivo Studies

Partial thickness thermal burns were created on the dorsal skin of pigs as described (43). Briefly, two domestic male pigs, each weighing 35 pounds, were anesthetized with ketamine and xylazine, and anesthesia was maintained with halothane inhalation. The dorsal skin was chemically depilated, and six partial thickness burns measuring 3 × 3 cm were created for each pig by contact with 714 g heated previously in a water bath maintained at 70 °C. The six burns were arranged in two rows of three burns on each side of the spine. Blister roofs were removed and two burns were treated topically with 3 ml of Silvadene® cream (Marion Laboratories, Kansas City, MO) containing 20 μg/ml tyrophostin 1478 (Calbiochem) (44). Two burns were treated with Silvadene® cream alone, and two burns were left untreated. Each wound was individually covered with a 6 × 6-cm3 adhesive occlusive dressing (Ster-Drape™2, 3M, St. Paul, MN). Dressings were removed daily, the burns were retreated, and fresh dressings were applied. Five days after injury, the pigs were sacrificed and a full-thickness biopsy was taken diagonally across each burn, fixed in 10% buffered formalin, and embedded in paraffin. Sections were stained with hematoxylin and eosin. The extent of epithelial healing for each burn was calculated by measuring the distance between intact epithelial edges divided by the total width of the wound and was expressed as percentage of re-epithelialization. Epithelial healing for each of the three treatment groups was averaged and compared for statistical significance by analysis of variance and Tukey's HSD post-test.

RESULTS

Blockade of EGFR Signaling Inhibits Migration on Type I Collagen—We used specific inhibitors of EGFR occupancy and tyrosine kinase activity to determine if signaling through this receptor was related to collagenase-1 induction and keratinocyte migration across type I collagen. Migration was assessed using colloidal gold and colony-dispersion motility assays as described (10). In the colloidal gold assay, keratinocytes were plated on chamber slides coated with a colloidal gold type I collagen mixture, and migration was quantified 20 h later. In the colony dispersion assay, cells were cultured within cloning cylinders for 24 h. After cell attachment to the matrix, the cloning cylinders were removed and migration was assessed at 96 h. As we have shown (10), keratinocytes migrated efficiently over type I collagen in both migration assays (Fig. 1, A and D).

As we reported (10), treatment of keratinocytes with an affinity-purified collagenase-1 antibody (1:4 antisera dilution), which blocks enzymatic activity, markedly inhibited cell migration across type I collagen, reconfirming that collagenase-1 activity is required for motility over this matrix (Fig. 1D).

Similar to collagenase-1 antisera, the addition of PD153035 (500 nM), a highly specific EGFR tyrosine kinase antagonist (45), inhibited keratinocyte migration by about 80% (Fig. 1, A versus B and D). EGFR phosphorylation and EGFR-dependent cellular functions in vitro are inhibited by this compound at concentrations of 40–300 nM, whereas other tyrosine kinase inhibitors are unaffected below 10 μM (45). In addition, anti-EGFR mAb 528 (1.0 μg/ml), which blocks ligand binding and subsequent receptor activation, was equally effective at inhibiting keratinocyte motility on collagen (Fig. 1, C and D). Addition of an IL-1 α receptor antagonist (IL-1 RA) (500 ng/ml) did not affect keratinocyte migration (Fig. 1D). Results for all conditions...
paralleled one another in each migration assay (Fig. 1D). Thus, inhibition of EGFR function blocks keratinocyte motility across a type I collagen matrix.

**Blockade of EGFR Function Inhibits Keratinocyte Collagenase-1 Production**—Because keratinocyte migration across type I collagen is dependent on both collagenase-1 activity and EGFR function, we determined if EGFR signaling was required for collagen-mediated collagenase-1 production. Human keratinocytes were plated on culture slides coated with colloidal gold and type I collagen (1.0 mg/ml) in the presence of vehicle control (collagen alone), affinity-purified collagenase-1 antiserum (1:4 dilution) or PD153035 (500 nM), EGFR blocking Ab 528 (1.0 μg/ml), or IL-1 RA (500 ng/ml) and were fixed 20 h later. Keratinocyte migration was quantified as described under “Experimental Procedures,” and the data are shown as means ± S.D. of triplicate samples from three experiments. For colony dispersion, primary human keratinocytes were cultured for 24 h within cloning cylinders on type I collagen (1.0 mg/ml). After 24 h, the cloning cylinders were removed and the cells were allowed to migrate for an additional 96 h in the presence of vehicle control (collagen alone), collagenase-1 antiserum (1:4 dilution), PD153035 (500 nM), EGFR blocking Ab 528 (1.0 μg/ml), or IL-1 RA (500 ng/ml). The data presented are means ± S.D. of values from three separate wells per treatment group, and migration is expressed as arbitrary units relative to 0-h controls.

**Fig. 1.** Blockade of EGFR signaling inhibits keratinocyte migration on type I collagen. A–C, primary human keratinocytes were plated on culture slides coated with a mixture of type I collagen (1.0 mg/ml) and colloidal gold particles, treated with vehicle control (collagen alone) (A), PD153035 (500 nM) (B), or EGFR blocking antibody 528 (1.0 μg/ml) (C), and fixed after 20 h. D, colloidal gold. Primary human keratinocytes were plated on culture slides coated with colloidal gold and type I collagen (1.0 mg/ml) in the presence of vehicle control (collagen alone), affinity-purified collagenase-1 antiserum (1:4 dilution), PD153035 (500 nM), EGFR blocking Ab 528 (1.0 μg/ml), or IL-1 RA (500 ng/ml) and were fixed 20 h later. Keratinocyte migration was quantified as described under "Experimental Procedures," and the data are shown as means ± S.D. of triplicate samples from three experiments. For colony dispersion, primary human keratinocytes were cultured for 24 h within cloning cylinders on type I collagen (1.0 mg/ml). After 24 h, the cloning cylinders were removed and the cells were allowed to migrate for an additional 96 h in the presence of vehicle control (collagen alone), collagenase-1 antiserum (1:4 dilution), PD153035 (500 nM), EGFR blocking Ab 528 (1.0 μg/ml), or IL-1 RA (500 ng/ml). The data presented are means ± S.D. of values from three separate wells per treatment group, and migration is expressed as arbitrary units relative to 0-h controls.

Keratinocyte Collagenase-1 Expression Requires EGFR Activation

We also found that other known stimulators of collagenase-1 expression by keratinocytes required intermediate signaling through the EGFR. Collagen-mediated induction of collagenase-1 expression was augmented by transforming growth factor-β1 (TGF-β1), hepatocyte growth factor/scatter factor, phorbol ester, and interferon-γ (IFN-γ). However, in the presence of PD153035 (Fig. 2, B and C), all such soluble factor-stimulated enzyme production was inhibited. In contrast, IL-1 RA had no effect on collagenase-1 stimulation by each of these factors (data not shown). Therefore, agents that stimulate collagenase-1 production by keratinocytes, whether matrix or soluble, appear to require an obligatory intermediate EGFR signaling step.

**EGFR Blockade Inhibits Collagen-induced Collagenase-1 mRNA Levels and Promoter Activity in Keratinocytes**—To gain an understanding of the molecular level at which EGFR blockade inhibits collagen-directed collagenase-1 production, total RNA was harvested from keratinocytes grown on collagen alone, or on collagen and treated with PD153035 or EGFR mAb.
Keratinocyte Collagenase-1 Expression Requires EGFR Activation

**Fig. 2. Blockade of EGFR signaling inhibits matrix- and soluble factor-induced collagenase-1 production by keratinocytes.**

Primary human keratinocytes were cultured on type I collagen (collagen alone) or heat-denatured collagen (gelatin) until confluent. A, cells on collagen were treated with PD153035 (100 or 500 nM), EGFR blocking antibody 528 (0.1 or 1.0 μg/ml), or IL-1 RA (250 or 500 ng/ml). B and C, keratinocytes on collagen were treated with TGF-β (25 ng/ml), hepatocyte growth factor (25 ng/ml), phorbol ester (20 ng/ml), or IFN-γ (1000 units/ml) in the presence or absence of PD153035 (500 nM). Collagenase-1 protein in the conditioned medium after 72 h was quantified by ELISA, and values were normalized to total protein content. Data shown are the means ± S.D. of triplicate observations from the same cell preparation and are representative of up to four separate experiments.

528 for 24 h. To detect collagenase-1 mRNA present in each sample, we used a semiquantitative RT-PCR assay (9). Only collagenase-1-specific products are detected by Southern hybridization or by ethidium bromide staining. Collagenase-1 mRNA was readily observed in keratinocytes cultured on collagen alone (Fig. 3A). In contrast, and paralleling the protein data, treatment of keratinocytes with PD153035 (500 nM) or EGFR mAb 528 (1.0 μg/ml) inhibited collagenase-1 mRNA expression by 96% and 92%, respectively, indicating pretranslational regulation.

To determine if collagenase-1 transcription was affected following inhibition of EGFR signaling, keratinocytes on collagen were transfected with a CAT expression construct containing 2.2 kilobase pairs of the human collagenase-1 promoter. As shown in previous studies, changes in the activity of this promoter construct parallel changes in the activity of the endogenous collagenase-1 gene (48), and the activity of the promoter construct is about 5-fold greater in keratinocytes plated on native collagen than in cells on heat-denatured collagen (gelatin) (9). The normalized level of CAT activity detected in keratinocytes plated on collagen was reduced in cells treated with PD153035 or EGFR Ab 528 by 75% and 87.5%, respectively (Fig. 3B), indicating that inhibition of EGFR function blocks collagenase-1 production by a transcriptional mechanism.

**Keratinocyte Contact with Type I Collagen Induces EGFR Phosphorylation**—Because keratinocyte contact with collagen is the primary and requisite inductive event for collagenase-1 expression (9, 10) and because EGFR block inhibits enzyme production, we reasoned that EGFR activation should follow initial matrix binding. To assess this temporal relationship, we analyzed changes in keratinocyte steady-state mRNA levels of collagenase-1, EGF family members, and the EGFR at different time points after plating on collagen (0–48 h) (Fig. 4A). As we reported previously (9), no collagenase-1 mRNA was detected in keratinocytes prior to matrix contact (Fig. 4A, Case-1, 0-h). Low levels of collagenase-1 mRNA were observed as early as 2 h after contact with collagen, and expression increased markedly and progressively over the next 8 h. Collagenase-1 mRNA dropped to lower levels between 24 and 48 h after plating, coincident with the keratinocytes reaching confluence. Expression of the mRNAs for EGF, TGF-α, and amphiregulin paralleled that of matrix-stimulated collagenase-1 expression (Fig. 4A).

In contrast, mRNAs for HB-EGF and EGFR were constitutively expressed at high levels in keratinocytes prior to collagen binding (Fig. 4A). EGFR mRNA became undetectable following 2 h of contact with collagen (Fig. 4A, EGFR), but then increased progressively from 4 to 24 h, diminishing slightly at 48 h. HB-EGF mRNA was expressed at near-constant levels throughout the entire time course, except for a single drop at 4 h.

We next determined whether collagen binding induced EGFR activation. Primary keratinocytes were plated on collagen, and total cell lysates were harvested over the next 120 min (Fig. 4B). Phosphorylated species were immunoprecipitated with an anti-phosphotyrosine monoclonal antibody, and phosphorylated EGFR was detected by Western immunoblotting.
EGFR was not phosphorylated in freshly isolated keratinocytes from normal skin (Fig. 4B, 0 min). In contrast, marked phosphorylation of the EGFR was observed as early as 10 min following contact with collagen and persisted up to 120 min (Fig. 4B). Therefore, keratinocyte contact with native type I collagen rapidly induces phosphorylation of the EGFR. Furthermore, despite EGFR mRNA down-regulation at 2 h following matrix contact (Fig. 4A), the receptor remains present and functional on the cell surface as evidenced by continued phosphorylation (Fig. 4B).

EGFR Activity Mediates Sustained, but Not Early Collagen-directed Keratinocyte Collagenase-1 Expression—In the experiments shown in Figs. 1–3, inhibitors of EGFR function blocked collagen-mediated collagenase-1 expression as measured by enzyme protein and mRNA levels at 24 h. We next performed a time course of collagenase-1 expression in the presence of EGFR inhibitors to determine if the initial induction of collagenase-1 by collagen was mediated by EGFR signaling. Freshly isolated keratinocytes from normal skin were incubated with vehicle control or PD153035 (500 nM) for 2 h prior to plating on collagen. After plating, total RNA was isolated over a time course (0–24 h), and collagenase-1 mRNA was assessed by RT-PCR. Surprisingly, the initial matrix-induced expression of collagenase-1 at 4 h was unaffected by inhibition of EGFR signaling (Fig. 5a, A and B, +PD153035). In contrast, collagenase-1 mRNA was slightly diminished at 6 h and almost completely absent at 24 h in cells treated with PD153035 when compared with vehicle controls (Fig. 5, A and B). To ensure that PD153035 inhibited EGFR activation, keratinocytes were plated on collagen and treated with vehicle, 30 ng/ml EGF, or EGF + PD153035 (500 nM). Stimulation of collagenase-1 expression by EGF was inhibited by PD153035 (data not shown). Therefore, the sustained expression of collagenase-1 mRNA requires EGFR signaling, whereas matrix contact alone is sufficient to induce enzyme expression.

Heparin-binding Epidermal Growth Factor Is the Ligand That Mediates EGFR-dependent Sustained Collagenase-1 Expression—We then determined if collagenase-1 expression was mediated via EGFR signaling. Keratinocytes were isolated from normal human skin as described under “Experimental Procedures.” Results presented in A and B are representative of data obtained from at least three individual skin donors.

**Fig. 4.** Collagenase-1, EGF, TGF-α, amphiregulin, HB-EGF, EGFR expression, and EGFR activation in keratinocytes cultured on type I collagen. Normal human skin was processed for keratinocyte isolation as described under “Experimental Procedures.” As the trypsin-dispersed cell suspension was prepared, some cells were collected for RNA isolation (0 h). The remaining keratinocytes were plated on collagen-coated dishes. A, total RNA was isolated over a time course (0–48 h) and the mRNAs for collagenase-1, EGF, TGF-α, amphiregulin, HB-EGF, EGFR, and GAPDH were amplified by RT-PCR and analyzed by Southern hybridization with product-specific cDNA (‘case-1 and GAPDH) or oligonucleotide probes (all others). Duplicate samples were processed without reverse transcriptase (−RT) to assure RNA purity. B, total proteins were isolated from keratinocytes grown on collagen (1 mg/ml) at the indicated time points and processed for immunoprecipitation of tyrosine-phosphorylated species followed by Western immunodetection of the EGFR as described under “Experimental Procedures.” Results presented in A and B are representative of data obtained from at least three individual skin donors.

**Fig. 5.** Sustained keratinocyte collagenase-1 expression is mediated via EGFR signaling. Keratinocytes were isolated from normal human skin as described under “Experimental Procedures” and pretreated for 2 h with vehicle control or PD153035 (500 nM). After pretreatment some keratinocytes were collected for RNA isolation (0 h). The remaining keratinocytes were plated on collagen-coated dishes in the continued presence of vehicle control or PD153035 and total RNA was isolated over a time course (0–24 h). A, collagenase-1 mRNA was amplified by RT-PCR analysis of DNase-treated RNA and visualized by Southern hybridization with a radiolabeled cDNA probe. Duplicate samples were processed without reverse transcriptase (−RT) to assure RNA purity. B, collagenase-1 mRNA hybridization bands were quantified by scanning digitized densitometry and values were plotted over the time course.
Keratinocyte Collagenase-1 Expression Requires EGFR Activation

expression—Members of the EGF family, namely EGF and TGF-α, up-regulate matrix metalloproteinase expression in several cell types, including keratinocytes (49, 50). Furthermore, collagen-directed collagenase-1 expression in keratinocytes is enhanced by treatment with EGF (9, 10). Recent evidence has shown that autocrine production of HB-EGF is likely responsible for potentiation and transmission of the healing response during epidermal wound repair (51), yet effects of this cytokine on collagenase-1 expression have not been studied. Because HB-EGF was the only EGFR ligand constitutively expressed in keratinocytes from intact skin (Fig. 4A), we hypothesized that sustained collagen-mediated production of keratinocyte collagenase-1 is regulated via a HB-EGF/EGFR autocrine loop mechanism. We treated primary keratinocytes with several EGF family members to determine their effect on collagen-directed collagenase-1 expression (Fig. 6A). Collagenase-1 production, as assessed by immunoprecipitation of metabolically labeled proteins, was induced in keratinocytes cultured on collagen alone, and this expression was slightly enhanced in cells treated with EGF (Fig. 6A). Collagenase-1 production was substantially increased in keratinocytes treated with HB-EGF or TGF-α, whereas cells treated with amphiregulin showed no stimulation above that induced by collagen. Thus, of the EGF family members tested, HB-EGF and TGF-α were the most potent modulators of matrix-mediated collagenase-1 production by keratinocytes, making them likely candidates for autocrine activation of the EGFR following contact with collagen.

To address this issue, keratinocytes were isolated from normal human skin and pre-incubated for 2 h with vehicle control or neutralizing antibodies to HB-EGF or TGF-α prior to plating on collagen. After plating, total RNA was harvested over the next 0–24 h, and collagenase-1 mRNA was assessed by RT-PCR. The expression of collagenase-1 by keratinocytes was inhibited by treatment with the HB-EGF neutralizing antibody at both time points tested (Fig. 6B, 8 and 24 h). In contrast, collagenase-1 expression at 24 h was unaltered by TGF-α neutralizing antiserum (Fig. 6C). Therefore, the sustained production of keratinocyte collagenase-1 in the presence of collagen requires an intermediate HB-EGF/EGFR autocrine signaling step.

Blockade of EGFR Signaling Inhibits ex Vivo Epidermal Collagenase-1 Expression and Prevents Re-epithelialization of Porcine Burn Wounds—We treated ex vivo wounded human skin explants with inhibitors of EGFR tyrosine kinase activity to determine the requirement, at the tissue level, of EGFR activation for keratinocyte collagenase-1 expression. Punch biopsies of normal human skin were cultured for 4 days with vehicle control or PD153035 (500 nM). As demonstrated by in situ hybridization of the control specimens, collagenase-1 mRNA was expressed by migrating keratinocytes at the leading edge of re-epithelialization (Fig. 7, A and A’). Collagenase-1 was also expressed by some dermal fibroblasts. In marked contrast to control injured skin, no expression of collagenase-1 mRNA was evident in wound-edge keratinocytes in specimens treated with PD153035, whereas fibroblast production was unaffected (Fig. 7, B and B’).

Because EGFR signaling is required for sustained keratinocyte collagenase-1 expression both in vitro and ex vivo, and because the activity of this enzyme is essential for cell migration across type I collagen (10), we next determined if EGFR inhibition affected re-epithelialization of in vivo wounds. Partial thickness burn wounds were created in pigs and were treated with Silvadene® cream containing tyrphostin 1478 (20 μg/ml), a highly potent and specific EGFR tyrosine kinase inhibitor (52), vehicle control (Silvadene® cream), or occlusive dressing only. All wounds were covered with occlusive dressing. In tyrphostin 1478-treated wounds, epithelial healing was significantly impaired when compared with burns treated with vehicle control or covered with occlusive dressing only (Fig. 8, A–D). Specifically, wounds treated with tyrphostin 1478 had re-epithelialized only 22 ± 16% (mean ± standard error) of the surface of the burn, whereas wounds treated with vehicle control had closed an average of 90 ± 5% of the burn area (p = 0.008). Burns covered with occlusive dressing only had re-epithelialized an average of 82% ± 12% of the wound surface (p = 0.016 versus 1478-treated wounds). There was no significant difference between epithelial healing of burns treated with vehicle control or covered with occlusive dressing (p = 0.88).

To verify that tyrphostin 1478 blocked collagenase-1 expression, human keratinocytes plated on type I collagen were treated with PD153035 (100–500 nM) or tyrphostin 1478 (0.1–
1.0 μM) for 72 h and collagenase-1 secreted into conditioned medium was quantified by ELISA (Fig. 8E). Both PD153035 and tyrphostin 1478 blocked collagen-induced collagenase-1 expression in a dose-dependent manner (Fig. 8E). Taken together, these findings support the hypothesis that inhibition of re-epithelialization in porcine burn wounds was due, at least in part, to blocking collagenase-1 production.

**DISCUSSION**

Activation of the EGFR in basal keratinocytes following injury provides several critical signals required for proper healing of the tissue defect. For example, the wound-associated keratins 6 and 16 are markedly up-regulated (53), and their promoters contain regulatory elements that are responsive to EGFR activation (24). Continued signaling through the EGFR promotes Bcl-X-L-mediated prevention of keratinocyte apoptosis, thereby potentiating re-epithelialization by maintaining survival of the migrating cells (54). We show here that the sustained expression of collagenase-1 in two systems that mimic epidermal healing (keratinocyte migration on type I collagen and ex vivo skin explants) requires an EGFR autocrine loop mechanism. In previous studies, we showed that collagenase-1 expression is induced at the onset of re-epithelialization as keratinocytes migrate onto dermis, and that collagenase-1 activity is required for cell migration over a type I collagen substratum (10, 55). Thus, EGFR activation is central to yet another important phase of the wound repair process.

Our findings that both matrix- and soluble factor-induced collagenase-1 expression in keratinocytes require autocrine signaling through the EGFR (Fig. 2) draw interesting parallels to reports in human fibroblasts showing that all up-regulators of collagenase-1 production operate via an IL-1α/IL-1α receptor autocrine loop. Fini and colleagues (46, 47) have demonstrated that only IL-1α directly stimulates collagenase-1 gene transcription in fibroblasts. All other inducers, ranging from cytokines such as TNF-α to phorbol esters to agents causing cytoskeletal rearrangement, must first stimulate IL-1α gene transcription and subsequent release of IL-1α protein. This cytokine then binds to the cell-surface IL-1α receptor, resulting in triggering of collagenase-1 expression. Consequently, the addition of an IL-1α receptor antagonist to fibroblast cultures blocks collagenase-1 gene expression by any stimulating agent. Our findings suggest an analogous role for HB-EGF/EGFR in keratinocytes and offer the possibility that autocrine signaling pathways may represent a general biologic mechanism for inducing the expression of collagenase-1, and perhaps even other MMPs, in different cell types.

Our results implicate HB-EGF as the key ligand that mediates autocrine signaling through the EGFR, resulting in sustained keratinocyte production of collagenase-1. Evidence demonstrating multiple effects of HB-EGF on keratinocytes has accumulated through studies of its role in epidermal wound repair. This newer member of the EGF family is synthesized by multiple cell types, including vascular endothelium, smooth muscle, and keratinocytes (56). Exogenously added HB-EGF,
TGF-α, or EGF promotes autoinduction of HB-EGF mRNA, suggesting that this protein is an autocrine growth factor for these cells (56). The requirement for activation of the EGFR to sustain matrix-induced collagenase-1 production is an example of wound keratinocyte phenotype modulation by HB-EGF. Although HB-EGF mRNA is expressed in freshly isolated keratinocytes from normal skin, the membrane-bound form of this protein may not be processed and released in a soluble form until later in the wound healing response. In fact, other groups have shown that soluble HB-EGF does not appear in conditioned medium from excisional wounds or human skin explants until 24 h after injury (51, 57). These findings correlate with our observations that complete inhibition of collagenase-1 expression by blocking EGFR or HB-EGF activity does not occur until 24 h following keratinocyte contact with collagen.

Our findings indicate that matrix-induced keratinocyte collagenase-1 expression involves two distinct pathways, an initial response not requiring EGFR activation and a sustained response obligatory to EGFR activation. Both of these responses are integrin-mediated, but early collagenase-1 expression is EGFR-independent. Our data demonstrate that αβ3 integrin activation alone is sufficient to induce the early (0–8 h) expression of collagenase-1 in keratinocytes following contact with type I collagen. However, the sustained production (>8 h) of this MMP requires signaling through the EGFR in addition to αβ3 binding (Figs. 5 and 6B). Indeed, blocking αβ3 activity blocks all matrix-induced collagenase-1 production (10), whereas only the sustained expression is inhibited when EGFR activity is blocked (this report) (Fig. 5). Stimulation by both integrin adhesion and cell binding of a soluble factor are required for a variety of cellular responses during tissue morphogenesis. Unique to our system, however, is that both integrin and EGFR signaling are required only after prolonged exposure to type I collagen (the inducing stimulus).

Similar to our findings, EGFR autophosphorylation is induced by contact of glomerular epithelial cells with type I collagen (58). Our data demonstrate that EGFR (and HB-EGF) mRNA is expressed in keratinocytes freshly isolated from intact skin and that the receptor is rapidly autophosphorylated following contact with type I collagen. These findings represent further examples of how keratinocytes in unwounded skin are "primed" to respond to injury. Indeed, keratinocytes in intact skin express endogenous αβ3 integrin (59). Interestingly, although the EGFR is autophosphorylated rapidly following keratinocyte binding to collagen, collagenase-1 expression does not require EGFR signaling until later in the healing response. We speculate, however, that other early functions of the wound keratinocyte, such as non-enzymatic migration-related events or cell proliferation may require early EGFR signaling, although this remains to be determined.

In addition to our in vitro data, we also show a role for EGFR signaling in the re-epithelialization of porcine burn wounds in vivo. Blocking receptor activity with a specific inhibitor of the EGFR tyrosine kinase (tyrphostin 1478) inhibited keratinocyte collagenase-1 production and markedly delayed re-epithelialization when compared with normal controls. Similarly, treat-
ment of ex vivo human skin punch biopsies with PD153035 inhibited collagenase-1 production in keratinocytes at the wound edge. Taken together, these data suggest that blocking EGFR activity inhibits keratinocyte migration across the dermal matrix and that this is due, at least in part, to inhibition of collagenase-1 expression. Although blocking EGFR signaling may inhibit other biological events necessary for the complete wound repair (e.g. cell proliferation), we suggest that EGFR-dependent collagenase-1 expression is critical for sustained keratinocyte migration and normal re-epithelialization.

Our findings in this report add substantially to understanding the mechanisms that regulate collagenase-1 expression during wound repair. Previously, we found that collagenase-1 gene transcription is induced following injury when keratinocytes move off their underlying basement membrane and contact type I collagen of the dermal matrix (3, 7, 5). We identified the cell-surface recognition integrin as α6β1 and showed that collagenase-1 activity was requisite for keratinocyte migration over a type I collagen substrate (10). Our present data indicate that intact skin is primed and ready to respond to injury with high endogenous levels of keratinocyte EGFR and HB-EGF mRNA. Upon keratinocyte α6β1 binding to type I collagen, HB-EGF phosphorylation occurs within minutes. Collagenase-1 mRNA levels are induced within 2 h, however, by a mechanism that is EGFR-independent. Nevertheless, as collagenase-1 expression continues, the sustained high levels of enzyme production from ≥8 h following contact with type I collagen are dependent on an EGFR/HB-EGF autocrine signaling loop. Since re-epithelialization of even minor wounds takes days, this sustained collagenase-1 expression is likely essential for most keratinocyte migration and for complete re-epithelialization. Important unanswered questions raised by our findings include: 1) what signaling pathway does α6β1 use to transmit the rapid initial, EGFR-independent induction of collagenase-1?; and 2) upon completion of re-epithelialization, does the cessation of collagenase-1 production involve the dismantling of the EGFR/HB-EGF autocrine loop? Studies to address these important questions are currently in progress.

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