

## Collagen-stimulated Induction of Keratinocyte Collagenase Is Mediated via Tyrosine Kinase and Protein Kinase C Activities\*

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We have previously shown that during wound healing migrating keratinocytes, which are in contact with the dermal matrix, express interstitial collagenase, whereas basal epidermal cells, which reside on an intact basement membrane, do not. Duplicating this *in vivo* pattern, collagenase production was induced in primary human keratinocytes grown on native type I collagen, but only background levels of enzyme were detected in cells cultured on denatured type I collagen or on Matrigel<sup>™</sup>. Using genistein, herbimycin A, and sodium orthovanadate, we show that tyrosine kinase activity was required for collagen-mediated induction of keratinocyte collagenase. Similarly, collagenase steady-state mRNA levels and the activity of a transfected human collagenase-promoter CAT construct were inhibited by genistein and enhanced by orthovanadate. Staurosporine and H-7 also blocked collagenase production, indicating that protein kinase C activity was also required for collagen-mediated induction of keratinocyte collagenase. All inhibitory effects were dose-dependent, and no compound significantly affected total protein synthesis. Furthermore, both tyrosine kinase and protein kinase C inhibitors blocked phorbol ester-mediated induction of collagenase, but only protein kinase C antagonists abrogated phorbol ester-mediated induction of *c-fos* mRNA. These data suggest that similar signal transduction pathways are used by various agonists to mediate the stimulation of interstitial collagenase production.

Wound healing involves an orderly progression of events that lead to re-epithelialization of the epidermis and the formation and resolution of granulation and scar tissues in the underlying interstitium. During each stage of this process, matrix is removed or remodeled to facilitate tissue repair (1). The breakdown of pre-existing extracellular matrix components occurs by the activity of various matrix-degrading enzymes which are released by resident and inflammatory cells (1, 2). To determine the function of matrix remodeling during wound healing, we have studied the pattern of expression and the regulation of matrix metalloproteinases in a human wound repair model.

Metalloproteinases constitute a family of matrix-degrading enzymes with the combined capacity to degrade nearly all components of the extracellular matrix (3, 4). Members include interstitial and neutrophil collagenases, the recently described collagenase-3 (5), 72-kDa and 92-kDa gelatinases, stromelysins-1, -2, and -3, matrilysin, metalloelastase, and a putative

cell surface metalloproteinase that may be involved in proenzyme activation (6). Metalloenzymes are activated after secretion, and their enzymatic activity can be regulated by soluble inhibitors, such as the tissue inhibitors of metalloproteinases (TIMP-1, -2, and -3)<sup>1</sup> (3, 4, 7). Interstitial collagenase catalyzes the initial step in the degradation of fibrillar type I and III collagens (8), which together comprise the most abundant components of the dermal extracellular matrix. Because collagenases are the only known mammalian proteinases that can cleave fibrillar collagen in their triple helical domains, their activity is essential for effective interstitial matrix remodeling. Typical of most metalloproteinases, expression of interstitial collagenase is under strict regulatory control, and the enzyme is produced only by cells actively involved in remodeling during development, tissue involution, and disease (3, 4).

During epithelial repair, keratinocytes at the wound border detach from the underlying basement membrane and migrate over the dermal interstitium of the wound bed. During this period, migrating keratinocytes dissociate their cell:cell and cell:matrix contacts and begin producing matrix degrading enzymes, principally interstitial collagenase (9-12). We have shown by *in situ* hybridization that collagenase is prominently expressed by keratinocytes at the migrating epithelial front and that these cells are not in contact with an intact basement membrane (12, 13). This spatially precise pattern of expression suggests that cell:matrix interactions are an important determinant in regulating interstitial collagenase production. Indeed, we have shown that collagenase expression is induced in primary keratinocytes grown on native type I collagen but not in cells cultured on basement membrane proteins (12).

Our *in vivo* findings suggest that the invariant and prominent expression of collagenase in migrating basal keratinocytes is needed for efficient re-epithelialization and that type I collagen is the key inducer of keratinocyte collagenase synthesis. To understand the intermediate signaling steps that regulate collagenase production, we have begun to define the signal transduction pathways involved in collagen-mediated induction of keratinocyte collagenase. We report here that the collagen-mediated increase in collagenase protein and mRNA is dependent upon tyrosine kinase and protein kinase C activities. Because we also found that TPA-mediated induction of *c-fos* is dependent on protein kinase C activity but not on tyrosine kinase activity, the collagen-mediated signaling pathway appears distinct from that induced by exposure to TPA.

### EXPERIMENTAL PROCEDURES

**Materials**—Genistein, sodium orthovanadate, 8-bromo-cAMP, pertussis toxin, and 12-*O*-tetradecanoylphorbol-13-acetate (TPA) were

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<sup>1</sup> The abbreviations used are: TIMP, tissue inhibitor of metalloproteinases; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; PGE<sub>2</sub>, prostaglandin E<sub>2</sub>; EGF, epidermal growth factor; Me<sub>2</sub>SO, dimethyl sulfoxide; kb, kilobase(s); ELISA, enzyme-linked immunosorbent assay; CAT, chloramphenicol acetyltransferase.

from Sigma. Tyrphostin-1, tyrphostin-23, staurosporine, cardiotoxin, KT5823, and herbimycin A were from Calbiochem, and H-7 and H-89 were from Biomol (Plymouth Meeting, PA).

**Cell Culture**—Keratinocytes were isolated from healthy, full thickness skin obtained from reduction mammoplasty or abdominoplasty and were cultured in Dulbecco's modified Eagle's medium supplemented with 5% fetal calf serum, 1% HEPES, and penicillin/streptomycin (14). Under these conditions, keratinocytes differentiate and stratify similar to cells *in vivo*, but the cells are not amenable to passage. Thus, essentially each experiment was done with cells derived from a different individual. The primary cell suspension was plated on tissue culture dishes coated with either native rat tail type I collagen, prepared by differential salt precipitation (15), or bovine native type I collagen (Vitrogen; Collagen Corp., Palo Alto, CA), heat-denatured type I collagen (gelatin), or Matrigel™ (Becton Dickinson) as described (12). Gelatin was prepared by heating Vitrogen at 80 °C for 10 min. The cells were grown to visual confluence, which required about 3 to 5 days, and were then treated with various agents at different concentrations (see figure legends) for 48 h. The effector compounds were dissolved in dry Me<sub>2</sub>SO, and control cultures received an equal concentration of Me<sub>2</sub>SO (0.2%). The medium and cell layer were collected for measurement of collagenase secretion and steady-state mRNA levels. In some experiments, keratinocytes were treated with 30 ng/ml epidermal growth factor (EGF; Sigma) for 48 h or with  $5 \times 10^{-8}$  M TPA for 24 or 48 h. Adult human fibroblasts were isolated by explant culture of healthy skin (16).

**Quantitative Immunoassays**—The concentration of interstitial collagenase in keratinocyte-conditioned, serum-containing medium was determined using a competitive binding ELISA (17). This assay has nanogram sensitivity, is specific for human interstitial collagenase, and measures total enzyme present whether free or bound to inhibitor or substrate or whether in an inactive or active form. Other than dilution, samples were assayed without modification. Standard curves using purified human collagenase were included in each assay. Results were normalized to total cellular protein content determined by the bicinchoninic acid microassay using bovine serum albumin as a standard (Pierce). Prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) concentration in keratinocyte conditioned medium was determined by radioimmunoassay using [<sup>3</sup>H]PGE<sub>2</sub> as described (18).

**Total Protein Synthesis**—Confluent keratinocytes were treated with 40 μM genistein, 1.0 μM herbimycin A, 0.5 μM staurosporine, or vehicle for 24 h in standard culture medium. Cultures were then incubated for 1 h in leucine-free medium to deplete the intracellular pool of leucine and were then pulsed for 3 h in leucine-free medium containing 1 μCi/ml L-[<sup>3</sup>H]leucine (42 Ci/mmol). The medium and cell layer were collected, and macromolecules were precipitated with trichloroacetic acid and processed for scintillation spectrophotometry as described (19).

**mRNA Analysis**—Total RNA was isolated and Northern hybridization was performed as described (20). Probes were prepared by labeling a 2-kb human collagenase cDNA (21), a 313-base pair AccI-EcoRI fragment of the 5' end of human TIMP cDNA (22), or a 3.56-kb human c-fos cDNA (23) with α-[<sup>32</sup>P]dCTP by random priming.

**Transfection and Assessment of Promoter Activity**—An expression construct, pGLCAT, was obtained from Dr. Steven Frisch and contains the chloramphenicol acetyltransferase (CAT) gene downstream of a 2.2-kb fragment (−2280 to +34) of the human collagenase promoter. To correct for transfection efficiency, cells were co-transfected with a plasmid containing the luciferase gene driven by the thymidine kinase promoter (provided by Dr. Richard Pierce). Expression vectors were purified by repeated CsCl density centrifugation. Primary keratinocytes were plated on collagen-coated tissue culture dishes (28.3 cm<sup>2</sup>) and were transfected at 50–70% confluency using lipofectamine (Life Technologies, Inc.). Cells were washed with 2 ml of serum-free Dulbecco's modified Eagle's medium and were incubated in 1 ml of serum-free Dulbecco's modified Eagle's medium containing 2 μg of collagenase-promoter plasmid DNA, 0.1 μg of luciferase-expression plasmid, and 10 μl of lipofectamine. After 16 h, the medium was replaced with standard culture medium, and the cells were incubated for an additional 24 h and then harvested. CAT activity was assayed in lysed cells using [<sup>14</sup>C]chloramphenicol and acetyl-CoA, and radiolabeled products were detected by thin layer chromatography as described (24) and quantified using a BetaScope 603 (Betagen, Mountain View, CA). Luciferase activity was assayed in the same cell lysates as described (25).

## RESULTS

**Contact with Native Type I Collagen Induces Collagenase Production in Human Keratinocytes**—During wound healing, collagenase is expressed by basal keratinocytes in contact with

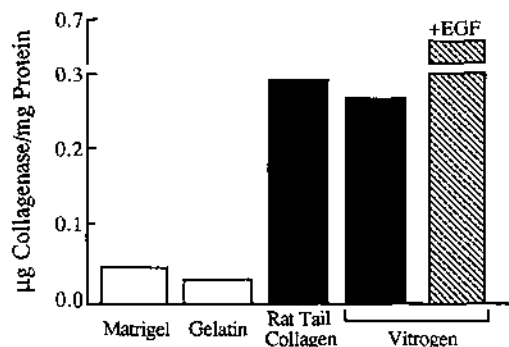


**FIG. 1. Native type I collagen induces collagenase production in keratinocytes.** Primary human keratinocytes were plated on dishes precoated with Matrigel™, bovine type I gelatin, or rat tail or bovine (Vitrogen) native type I collagen. When the cells reached visual confluency, the medium was changed, and some cells plated on Vitrogen were treated with 30 ng/ml EGF (+EGF). Conditioned medium was collected 48 h later, and the accumulation of collagenase in the medium was determined by ELISA and normalized to total protein.

the dermal matrix (12, 13). Reflecting this regulation by the extracellular matrix, collagenase production was stimulated about 6-fold in primary keratinocytes grown on native type I collagen, whereas only background levels of enzyme were released from cells cultured on basement membrane proteins (Matrigel™) or on heat-denatured type I collagen (gelatin) (Fig. 1). EGF, which potently stimulates basal collagenase expression in fibroblasts (26, 27), augmented the collagen-mediated stimulation of collagenase production in keratinocytes by about 2.5-fold above that in cells on collagen alone (Fig. 1). Although the magnitude of the matrix-induced collagenase response varied from about 3- to 10-fold among cells isolated from different individuals (cumulative data not shown), the inductive effect of native type I collagen and the lack of stimulation by Matrigel™ or gelatin were consistently seen in numerous experiments (>15).

**Collagen-mediated Induction of Keratinocyte Collagenase Is Dependent upon Tyrosine Kinase and Protein Kinase C Activities**—Cell-matrix receptors likely mediate the matrix-dependent regulation of keratinocyte collagenase expression. Because many matrix receptors, including integrins, either possess intrinsic tyrosine kinase activity or interact with non-receptor tyrosine kinases (28, 29), we assessed if inhibitors of tyrosine kinase activity influence the collagen-stimulated production of collagenase. Genistein, which inhibits tyrosine kinase activity by competing for ATP binding (30, 31), effectively blocked collagen-mediated induction of collagenase in a dose-dependent manner (Fig. 2). As expected, genistein also inhibited EGF-mediated induction of keratinocyte collagenase (data not shown). A second tyrosine kinase inhibitor, herbimycin A, which inactivates tyrosine kinases by reacting with free thiol groups (32), also blocked the collagen-mediated induction of collagenase in a dose-dependent manner (Fig. 2). A third tyrosine kinase inhibitor, tyrphostin-23 (25 to 100 μM), which inactivates the tyrosine kinase activity of a subset of receptors, including the EGF receptor (33), did not block the collagen-mediated induction of collagenase, even at doses as high as 100 μM (data not shown). Because each of the inhibitors tested blocks tyrosine kinase activity by a different mechanism, the inability of tyrphostin-23 to block the collagen response may be due to its narrow substrate specificity.

An involvement of tyrosine kinases during signal transduction can also be assessed by inhibiting tyrosine dephosphorylation, thereby allowing the accumulation of phosphorylated regulatory proteins. Consistent with the data using tyrosine kinase inhibitors, the phosphatase inhibitor sodium orthovan-

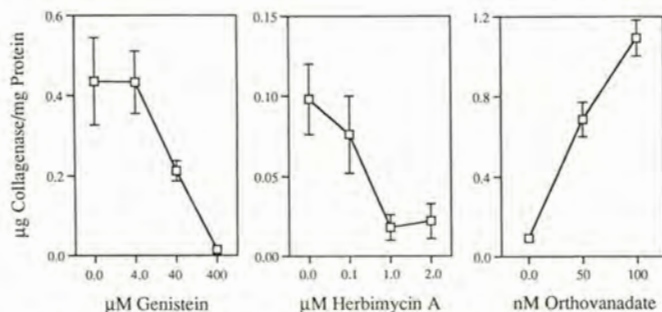


**FIG. 2. Tyrosine kinase activity is involved in the collagen-mediated induction of keratinocyte collagenase.** Primary keratinocytes were grown to confluence on collagen-coated dishes and were treated with the indicated concentrations of genistein, herbimycin A, or orthovanadate. Controls received 0.2% Me<sub>2</sub>SO. After 48 h, medium samples were collected, and collagenase accumulation was determined by ELISA and normalized to total protein. The data presented are the mean  $\pm$  S.D. of values from 3 separate plates per compound of cells from a single donor. The differences in scales for the y-axes in the genistein and herbimycin A panels indicates the difference in stimulation of collagenase production among primary keratinocytes derived from separate individuals.

date markedly augmented (>10-fold) the collagen-mediated increase in collagenase accumulation (Fig. 2). Together, these observations indicate that tyrosine kinase activity is involved in the regulation of collagen-initiated signal transduction.

To determine if tyrosine kinases affect collagenase gene transcription, primary keratinocytes were transiently transfected with a CAT expression construct containing 2.2 kb of the human collagenase promoter and were then exposed to genistein or orthovanadate. Numerous and diverse models have shown that collagenase expression is controlled at the level of transcription (34–38) and that this CAT construct accurately reflects endogenous transcriptional activity of the collagenase gene (38–43). In close agreement with the effects on collagenase protein production at similar doses (Fig. 2), incubation with 80 μM genistein caused about a 4.5-fold decrease in CAT activity, while exposure to 10 nM orthovanadate mediated about a 2.25-fold increase in CAT activity over control levels (Fig. 3).

We also assessed if other signaling pathways are involved in the collagen-mediated stimulation of keratinocyte collagenase. Similar to tyrosine kinase inhibition, the protein kinase C inhibitors staurosporine and H-7 blocked the collagen-mediated induction of keratinocyte collagenase (Fig. 4). The cause of the apparent stimulation of collagenase at low doses of protein kinase C antagonists is unclear, but similar effects on collagenase expression have been seen in other studies (44, 45).

Because tyrosine kinase and protein kinase C activities are necessary for numerous cellular events, we measured total protein synthesis to assess the overall metabolism of these cells. Genistein and herbimycin A, at concentrations which significantly inhibited collagenase production, did not markedly affect the total incorporation of [<sup>3</sup>H]leucine in trichloroacetic acid-precipitable material relative to that detected in keratinocytes plated on collagen (Table I).

**Collagen-mediated Induction of Keratinocyte Collagenase Is Not Dependent on Prostaglandins or cAMP-dependent Protein Kinase, cGMP-dependent Protein Kinase, or G-protein Activities**—In fibroblasts and alveolar macrophages, matrix-dependent stimulation of interstitial collagenase is dependent on increased PGE<sub>2</sub> synthesis (46–49). To determine if a similar requirement is needed for the collagen-mediated induction of keratinocyte collagenase, we exposed cells to indomethacin (3 ng/ml, replenished daily for 3 days), an inhibitor of prostaglandin synthesis. Although all three tyrosine kinase inhibitors blocked PGE<sub>2</sub> accumulation and the phosphatase inhibitor or-

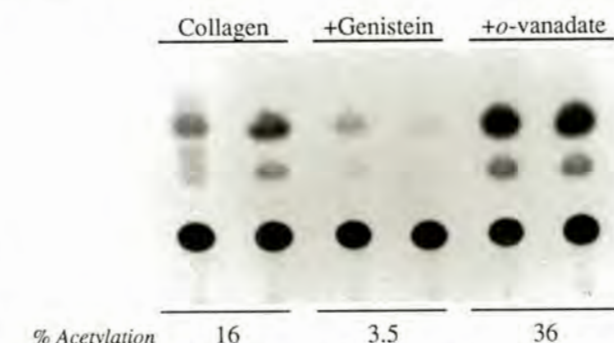


**FIG. 3. Genistein decreases and orthovanadate stimulates the activity of a collagenase-promoter expression construct.** Keratinocytes were grown to about 70% confluence and were co-transfected with a human collagenase promoter-CAT construct and a luciferase expression plasmid. Duplicate cultures were then treated with vehicle (0.2% Me<sub>2</sub>SO) (Collagen), 80 μM genistein, or 10 nM sodium orthovanadate (o-vanadate). Relatively low, yet effective doses of genistein and sodium orthovanadate were used to minimize any cytotoxic effects on the transfection assay. After 24 h, soluble proteins were isolated, and CAT activity was quantified as the percent of radioactivity in the acetylated bands normalized to luciferase activity.

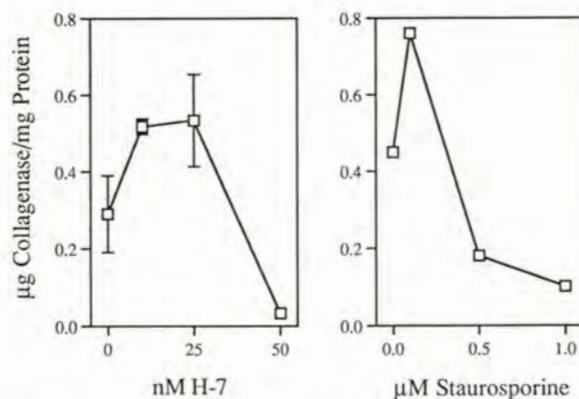


**FIG. 4. Protein kinase C agonists block the collagen-mediated induction of keratinocyte collagenase.** Keratinocytes were grown to confluence on collagen-coated dishes and were treated with the indicated concentrations of H-7 or staurosporine for 48 h. Collagenase accumulation in the medium was determined by ELISA and normalized to total protein. The data presented are the mean  $\pm$  S.D. of values from 3 separate plates.

TABLE I  
Total protein synthesis

Keratinocytes were plated on collagen and treated with genistein, herbimycin A, staurosporine, or vehicle for 24 h before addition of [<sup>3</sup>H]leucine as described under "Experimental Procedures." Radioactivity incorporated into trichloroacetic acid-precipitable material was determined in the culture medium and the isolated cells. The data presented are the means  $\pm$  S.D. of triplicate determinations from three separate plates per treatment.

| Treatment            | Protein synthesis           |                 |
|----------------------|-----------------------------|-----------------|
|                      | Cell layer                  | Medium          |
|                      | cpm / 10 <sup>6</sup> cells |                 |
| Collagen alone       | 22,466 $\pm$ 3,230          | 1,107 $\pm$ 312 |
| 40 μM genistein      | 27,052 $\pm$ 1,810          | 574 $\pm$ 51    |
| 1.0 μM herbimycin A  | 32,422 $\pm$ 2,572          | 1,314 $\pm$ 175 |
| 0.5 μM staurosporine | 19,388 $\pm$ 1,408          | 1,440 $\pm$ 134 |

thovanadate augmented PGE<sub>2</sub> accumulation (data not shown), collagenase production was not affected in cells treated with indomethacin (Table II). These findings indicate that increased collagenase expression by keratinocytes in response to contact with native type I collagen is not dependent on PGE<sub>2</sub> but rather is under cell-specific controls.

TABLE II

Involvement of different signal transducing pathways required for collagen-mediated induction of keratinocyte collagenase

Presented here is a summary of various experiments in which primary human keratinocytes were plated on native type collagen and exposed to different antagonists or agonists of various signaling pathways. Collagenase production was assessed by ELISA of conditioned medium. Compounds that mediated no effect (NE) had levels of collagenase protein that were at least 90% of those in the control (collagen-alone) cultures.

| Signaling pathway       | Antagonist                                                          | Effect <sup>a</sup> | Agonist                        | Effect <sup>a</sup> |
|-------------------------|---------------------------------------------------------------------|---------------------|--------------------------------|---------------------|
| Tyrosine kinase         | Genistein (5) <sup>b</sup><br>Herbimycin A (2)<br>Tyrphostin-23 (2) | ↓<br>↓<br>NE        | Orthovanadate <sup>c</sup> (2) | ↑                   |
| Protein kinase C        | Staurosporine (5)<br>H-7 (3)                                        | ↓<br>↓              | TPA (<15)                      | ↑                   |
| Protein kinase A        | H-89 (2)<br>Cardiotoxin (1)                                         | NE<br>NE            | 8-bromo-cAMP (2)               | NE                  |
| Protein kinase G        | KT5823 (2)                                                          | NE                  |                                |                     |
| G Protein               | Pertussis toxin (2)                                                 | NE                  |                                |                     |
| Prostaglandin synthesis | Indomethacin (2)                                                    | NE                  |                                |                     |

<sup>a</sup> The various compounds either inhibited (↓), stimulated (↑), or had no effect (NE) on collagen-mediated induction of collagenase by primary keratinocytes.

<sup>b</sup> The number in parentheses indicates the number of dose-response experiments done for each compound with a different strain of primary human keratinocytes.

<sup>c</sup> Orthovanadate is a tyrosine phosphatase inhibitor and not a tyrosine agonist *per se*.

Inhibitors and agonists of other intracellular signaling enzymes, namely cAMP-dependent protein kinase, cGMP-dependent protein kinase, and G-proteins, were also tested to determine if they participate in collagen-stimulated production of collagenase. The cAMP-dependent protein kinase inhibitors H-89 (50 to 200 nM) and cardiotoxin (0.5 to 50 µg/ml), the cAMP-dependent protein kinase agonist 8-bromo-cAMP (0.01 to 1 µg/ml), the cGMP-dependent protein kinase inhibitor KT5823 (0.125 to 0.5 mM), and the G-protein antagonist pertussis toxin (10 to 100 ng/ml) did not affect collagen-mediated stimulation of keratinocyte collagenase production (Table II). In summary, our results indicate that tyrosine kinases and protein kinase C are the primary signal transduction activities involved in collagen-mediated stimulation of keratinocyte collagenase.

**TPA-stimulated Production of Keratinocyte Collagenase Is Dependent on Tyrosine Kinase Activity**—To determine the order of tyrosine kinase and protein kinase C activation during collagen-stimulated synthesis of collagenase, we treated keratinocytes with TPA, which acts independently of cell surface receptors (50). TPA is a potent stimulator of collagenase, other metalloproteinases, and TIMP-1 in a variety of cells (39, 41, 43, 51–53), and, as expected, it markedly enhanced collagenase production in keratinocytes (Fig. 5). Since the TPA-mediated increase in enzyme synthesis was independent of the plating substratum (Fig. 5), the specificity of native type I collagen-mediated signal transduction must occur at a stage prior to protein kinase C activation. Treatment of cells with the inactive phorbol analog 4- $\alpha$ -phorbol had no effect on the collagen-stimulated induction of collagenase (data not shown), indicating that the TPA effect was protein kinase C-dependent. Similar to that seen in keratinocytes plated on collagen alone (Fig. 2), TPA-mediated induction of collagenase was markedly inhibited by genistein and herbimycin A but was increased about 2-fold by orthovanadate (Fig. 6). These findings indicate that tyrosine kinase activity is involved in the intracellular signaling at some stage following protein kinase C activation. However, these data do not discount the possibility that a distinct tyrosine kinase acts prior to protein kinase C activation. TPA-mediated stimulation of collagenase expression was also inhibited by the protein kinase C antagonists H-7 and staurosporine (Fig. 6).

**Genistein and Staurosporine Block the TPA-mediated Induction of Collagenase mRNA**—To assess the mechanism of inhi-

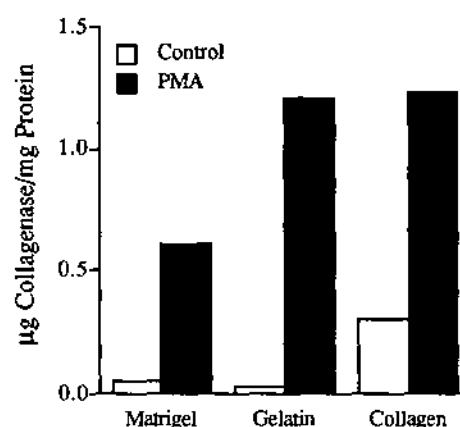


FIG. 5. **TPA induces collagenase production independently of the matrix substratum.** Primary keratinocytes were grown on dishes precoated with Matrigel<sup>TM</sup>, gelatin, or native type I collagen. At confluence, half of the dishes were treated with  $5 \times 10^{-6}$  M TPA (PMA on figure). After 48 h, collagenase accumulation in the medium was determined by ELISA and normalized to total protein.

bition, we determine if genistein and staurosporine reduced the steady-state levels of collagenase mRNA proportionately to the decreases detected in secreted protein. By 8 h, TPA had stimulated collagenase protein and mRNA levels above those detected in keratinocytes plated on type I collagen alone, and both parameters were similarly reduced by co-exposure to genistein (Fig. 7A). In contrast, genistein did not significantly affect the TPA-mediated induction of TIMP-1 mRNA in keratinocytes (Fig. 7A), thereby providing further evidence of the selective regulation of collagenase gene expression by tyrosine kinase activation in keratinocytes. Similarly, staurosporine significantly inhibited TPA-stimulated collagenase mRNA levels (Fig. 7B). These data demonstrate that staurosporine and genistein inhibit collagenase production at a point prior to transcription.

We also assessed if the TPA-mediated induction of collagenase was dependent on tyrosine kinase activity in other cell types. Adult human dermal fibroblasts grown on tissue culture plastic produced little collagenase, but, in agreement with findings by others (51, 54, 55), TPA markedly stimulated the expression of collagenase protein and mRNA and TIMP-1 mRNA in these cells (Fig. 8). As we detected in keratinocytes, genistein

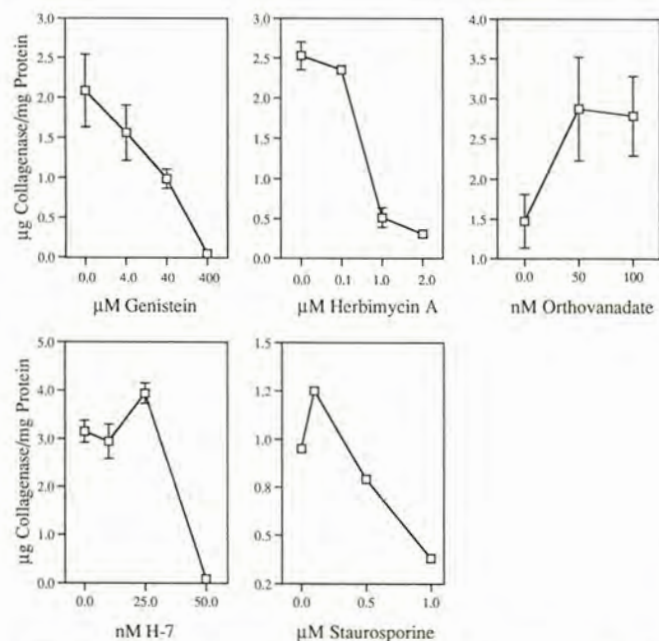


**FIG. 6. Tyrosine kinase and protein kinase C antagonists inhibit and orthovanadate increases TPA-mediated stimulation of keratinocyte collagenase.** Primary keratinocytes were grown to confluence on collagen-coated dishes and were treated with the indicated concentration of the various compounds for 48 h. Collagenase accumulation in the medium was determined by ELISA and normalized to total protein. The data presented are the mean  $\pm$  S.D. of values from 3 separate plates per compound of cells from a single donor. The differences in the level of collagenase among the vehicle-treated controls reflects the disparity in the magnitude of collagenase production among primary keratinocytes derived from separate individuals.

markedly inhibited the induction of collagenase expression in fibroblasts and did not significantly affect TIMP-1 mRNA levels (Fig. 8). These data suggest that collagenase induction is dependent on tyrosine kinase activity in various cell types.

**Inhibition of TPA-mediated Collagenase Transcriptional Activation by Genistein Is Not Due to Decreased *c-fos* Induction**—The collagenase promoter contains a single AP-1 site which is required but not sufficient for TPA induction of collagenase (41). The AP-1 transcription complex is a *c-Fos/c-Jun* heterodimer, although homodimers and other combinations of related components have been described (56, 57). As shown in other cell types, collagenase induction is *c-fos*-dependent (57, 58). To assess the effects of genistein and staurosporine on the expression of this early response gene, *c-fos* mRNA levels in keratinocytes were determined after TPA treatment with or without genistein or staurosporine. Steady-state levels of *c-fos* mRNA peaked between 1 and 2 h post-TPA and decreased rapidly thereafter (Fig. 9). Although staurosporine completely blocked the TPA-mediated induction of *c-fos* mRNA, genistein had little to no effect on the expression of this *trans*-acting factor (Fig. 9).

#### DISCUSSION

Our previous results showed that interstitial collagenase is invariably expressed by migrating basal keratinocytes during cutaneous wound repair (12, 13). Using immunohistochemical and *in situ* hybridization assays, we found that these collagenase-producing cells are not in contact with an intact basement membrane but rather are migrating over the dermal and/or provisional wound matrix. As proposed by Woodley *et al.* (2) and by us (12), this alteration in cell:matrix interactions suggests that some component of the interstitial matrix provides a determinant for the induction of interstitial collagenase. Indeed,

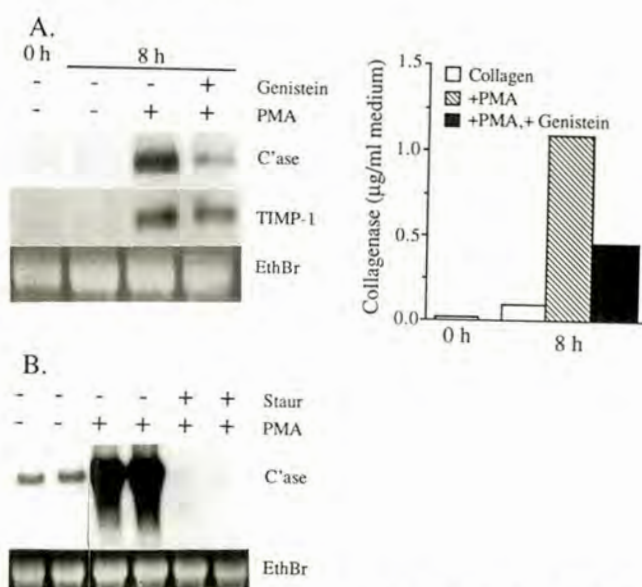


**FIG. 7. Genistein and staurosporine block TPA-mediated stimulation of keratinocyte collagenase protein and mRNA.** Keratinocytes were grown on collagen-coated dishes. At confluence, the medium was replaced, and the cells were pretreated for 30 min with 0.2% Me<sub>2</sub>SO (Collagen), 40  $\mu$ M genistein, or 0.5  $\mu$ M staurosporine. Half of the cultures were then stimulated with  $5 \times 10^{-9}$  M TPA (PMA on figure). A, conditioned medium and total RNA from cells treated with genistein were harvested at 0 and 8 h. Collagenase protein levels were determined by ELISA (right panel), and collagenase (*C'ase*) and TIMP-1 mRNA levels were assessed by Northern blot analysis (10  $\mu$ g/lane) with <sup>32</sup>P-labeled human cDNAs (left panel). Equivalent loading among lanes is demonstrated by ethidium bromide staining (*EthBr*) of the 28 S rRNA subunit. Autoradiographic exposure was 48 h for collagenase mRNA and 24 h for TIMP-1 mRNA. B, total RNA was isolated from primary keratinocytes after an 8-h exposure to vehicle, TPA, or TPA plus 0.5  $\mu$ M staurosporine (*Staur*). Collagenase (*C'ase*) mRNA levels were assessed by Northern hybridization (10  $\mu$ g/lane). Equivalent loading among lanes is demonstrated by ethidium bromide staining (*EthBr*) of the 28 S rRNA subunit. Autoradiographic exposure was 48 h.

we found that contact with native type I collagen, the most abundant component of dermal matrix, induces keratinocyte collagenase expression in primary cell cultures, whereas components of the basement membrane (4) and other proteins of the interstitial matrix do not.<sup>2</sup> Our work extends the observations of Petersen *et al.* (59) who demonstrated that keratinocytes recognize and migrate on type I collagen substratum, and that this interaction results in enhanced collagenase production. Collectively, these studies demonstrate a key role for type I collagen in initiating keratinocyte collagenase synthesis in the epithelial response to wounding. We report here that tyrosine kinase and protein kinase C activities are required for collagen-mediated induction of interstitial collagenase by keratinocytes and that the distal portion of the signaling pathway is common with the TPA-mediated response (Fig. 10).

During wound repair, basal keratinocytes move from the basement membrane onto dermal and provisional matrix proteins, of which type I collagen is an abundant component. Interestingly, basal keratinocytes constitutively express collagen binding integrins, such as  $\alpha 1\beta 1$  and  $\alpha 2\beta 1$  (60–62). The interaction of keratinocytes with the dermal matrix, and in particular type I collagen, may provide an early and critical signal to initiate the epithelial response to wounding. Binding of integrins with their matrix ligands activates intrinsic or non-receptor tyrosine kinases (29, 63, 64), and a similar mechanism may occur in keratinocytes in contact with type I collagen (Fig.

<sup>2</sup> B. D. Sudbeck, A. P. Pentland, H. G. Welgus, and W. C. Parks, unpublished observations.

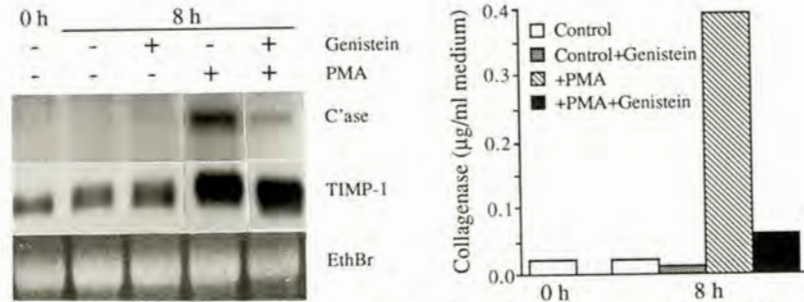


FIG. 8. **Genistein blocks TPA-induced fibroblast collagenase protein and mRNA accumulation.** Adult human skin fibroblasts were grown to confluence on tissue culture plastic and were then pretreated for 30 min with 0.2% Me<sub>2</sub>SO or 40 µM genistein. Half of the cultures were stimulated with  $5 \times 10^{-9}$  M TPA (PMA on figure). Conditioned medium and total RNA were harvested at 0 and 8 h, collagenase protein levels were determined by ELISA (right panel), and collagenase (C'ase) and TIMP-1 mRNA levels were assessed by Northern blot analysis (10 µg/lane) (left panel). Equivalent loading among lanes is demonstrated by ethidium bromide staining (EthBr) of the 28 S rRNA subunit. Autoradiographic exposure was 48 h for collagenase mRNA and 24 h for TIMP-1 mRNA.

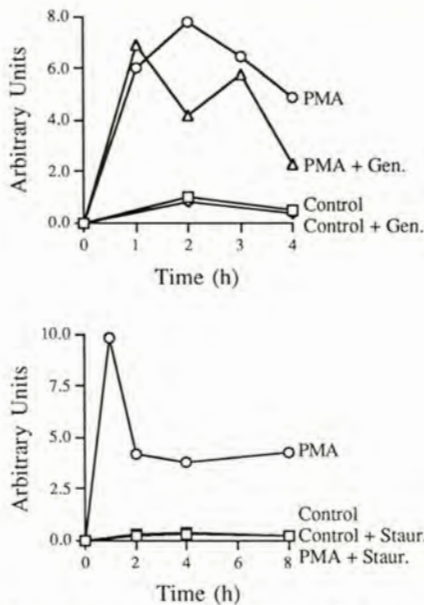


FIG. 9. **TPA induction of *c-fos* is blocked by staurosporine but is not affected by genistein.** Keratinocytes were grown to confluence on gelatin-coated dishes and were pretreated for 30 min with 0.2% Me<sub>2</sub>SO, 40 µM genistein (Gen, upper graph), or 0.5 µM staurosporine (Staur, lower graph). Half of the cultures were stimulated with  $5 \times 10^{-9}$  M TPA (PMA on figure). At the indicated times, total RNA was isolated, and 20 µg/lane was resolved by agarose electrophoresis and probed with a <sup>32</sup>P-labeled human *c-fos* cDNA. Graphs show the intensity of the signal for *c-fos* mRNA as quantified by densitometry.

10). Indeed, we consistently found that collagen-mediated induction of collagenase in keratinocytes is effectively blocked by tyrosine kinase antagonists and augmented by the phosphatase inhibitor orthovanadate (Fig. 2). Since the epidermis is not normally in contact with type I collagen, it is tempting to speculate that the basal production of collagen-binding integrins, besides being involved in cell:cell and basement membrane interactions in the intact skin, keeps keratinocytes primed and ready to respond to injury. Additional experiments are needed, however, to demonstrate that integrins are involved in the control of collagenase production by keratinocytes.

As stated, our results indicate that both tyrosine kinase and protein kinase C activities are involved in collagen-initiated signal transduction; however, this pathway likely bifurcates before the activation of collagenase transcription (Fig. 10). Although genistein and staurosporine both inhibited the collagen- and TPA-mediated induction of keratinocyte collagenase (Figs. 2, 4, and 6), they had different effects on the TPA-induced expression of *c-fos* RNA (Fig. 9). Increased protein kinase C

activity leads to the activation of specific transcription factors such as *c-fos* (65), and, predictably, staurosporine blocked the increase in *c-fos* expression following TPA treatment. In contrast, genistein, at concentrations that inhibited collagenase production, failed to block the TPA-mediated induction of *c-fos* RNA. Thus, the induction of *c-fos* is not tyrosine kinase-dependent, although its ability to act as a transcription factor may be. Since the transcription-inducing activity of c-Fos and c-Jun is dependent upon their phosphorylation state (66–68), genistein may block collagenase production by affecting the processing of AP-1 subunits. Alternatively, genistein may affect the expression or activity of other stimulatory *trans*-acting factors which bind to elements in the collagenase gene promoter, such as PEA-3 or TTCA (41).

Our findings also suggest that the regulation of collagenase by keratinocytes is cell-type specific. PGE<sub>2</sub> is a potent inflammatory mediator that exerts its biological effects through coupling to G-proteins and adenylate cyclase. In a number of cell types, such as fibroblasts and macrophages, increased prostaglandin synthesis and cAMP-dependent protein kinase activity correlates with increased collagenase production (46–48, 69–71). Even though PGE<sub>2</sub> synthesis was predictably increased with TPA (data not shown), our results indicate that the type I collagen-mediated induction of keratinocyte collagenase is not influenced by blocking prostaglandin synthesis or cAMP-dependent protein kinase activity or by the addition of a non-hydrolyzable cAMP analog.

Because interstitial collagenase is prominently and invariantly expressed by keratinocytes during wound healing, we suggest that it serves a critical role in re-epithelialization rather than in dermal remodeling. Types I and III collagen are the major structural components of the dermal matrix and may serve as a substratum for keratinocyte migration. Collagenase catalyzes the rate-limiting step in collagen degradation by making a single site-specific cleavage in the triple-helical region of this abundant matrix molecule. At physiological temperature, these fragments are thermally unstable and denature into their constitutive polypeptide chains, forming gelatin peptides. Since the α2β1 integrin receptor for type I collagen binds native collagen with a higher affinity than it does for gelatin (72), we speculate that the production of interstitial collagenase by migrating keratinocytes aids in dissociating the cell from the dermal matrix and, hence, promotes efficient locomotion over the dermal and provisional matrices.

In conclusion, our data indicate that the induction of interstitial collagenase by native type I collagen and TPA requires both tyrosine kinase and protein kinase C activities, suggesting that the same or part of the same signal transduction pathway is used by multiple agonists to up-regulate the expression of

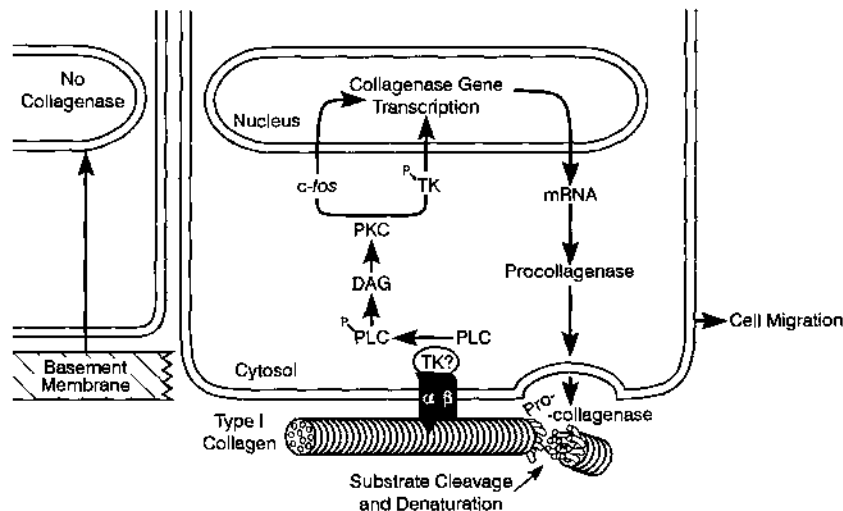


FIG. 10. Model of the signal pathways involved in the collagen-mediated induction of collagenase in keratinocytes during re-epithelialization. After wounding, basal keratinocytes dislodge from the basement membrane and migrate over the dermal and provisional matrices. Although not demonstrated in this report, cell surface receptors ( $\alpha\beta$ ), such as the  $\alpha2\beta1$  integrin, may interact with dermal type I collagen and activate an intrinsic or non-receptor tyrosine kinase (TK). These initial events likely start an intracellular signaling pathway that involves the activation of phospholipase C (PLC), the generation of diacylglycerol (DAG) and inositol trisphosphate, and activation of protein kinase C (PKC). Since genistein did not block TPA-mediated induction of *c-fos* mRNA (Fig. 9) but did inhibit TPA-mediated induction of collagenase (Figs. 6 and 7B), the pathway likely bifurcates leading to activation of collagenase transcription. Secretion and extracellular activation of procollagenase cleave type I collagen, thereby lowering the affinity for attachment and promoting cell migration.

collagenase. Because TPA induces collagenase independently of the matrix substratum, the specificity of the collagen-induced response most likely lies in an early part the tyrosine kinase-mediated portion of the signaling pathway. Understanding the details of these signal transduction events will be necessary to selectively modify the biological expression of this important matrix metalloproteinase.

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