Interleukin-1β and Transforming Growth Factor-α/Epidermal Growth Factor Induce Expression of M, 95,000 Type IV Collagenase/Gelatinase and Interstitial Fibroblast-type Collagenase by Rat Mucosal Keratinocytes*

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Rat mucosal keratinocytes serially propagated under permanently serum-free conditions responded to interleukin (IL-1β)/IL-α and to transforming growth factor (TGF-α)/epidermal growth factor (EGF) (as well as to 12-O-tetradecanoylphorbol-13-acetate (TPA)) by up-regulation of M, 95,000 gelatinase (MMP-9) (M, 95K GL) and fibroblast-type collagenase (MMP-1) (FIB-CL), whereas control cells expressed barely detectable levels of either of these enzymes. The cells secreted 8–10 µg/10⁶ cells/day (M, 95K GL) and 2–3 µg/10⁶ cells/day (FIB-CL) of enzyme protein for at least 24 h when maximally induced. This level was attained only after a 24-h lag period, and the earliest emergence of enzyme protein in the culture medium required 10–14 h. IL-1β was by far the most potent cytokine with maximal effect already at 10⁻⁷ M, whereas IL-1α, TGF-α, and EGF required 20–100-fold higher concentrations. Pre-treatment of the cells with TPA (10⁻⁷ M) abolished the subsequent response to IL-1β, TGF-α, and EGF and at the same time resulted in >90% reduction of cytosolic protein kinase C activity. Surprisingly, staurosorpine, a potent kinase inhibitor, not only failed to block growth factor/cytokine responses but itself stimulated expression of the enzymes at a magnitude comparable to TPA. The inducing effect of TGF-α/EGF was down-regulated by 70–85% by 10⁻⁷ M dexamethasone. Dexamethasone was less effective in ablating the IL-1β response yielding 60% reduction M, 95K GL and little or no reduction of FIB-CL. Dexamethasone also failed to block the TPA response.

A body of evidence suggests that remodeling of the extracellular matrix is mediated, at least in part, by the collective action of matrix metalloproteinases, a family of nine or more growth factor- and cytokine-regulated, Zn²⁺-dependent endopeptidases that are capable of cleaving most, if not all, matrix macromolecules (1). Stromal remodeling at the epithelial/mesenchymal interface is an integral part of wound healing and of invasive epithelial growth whether associated with programmed developmental migration into stromal domains during morpho- and organogenesis or with pathologic invasive or expansive growth of carcinomas and cysts. Although the specific communications between epithelial and stromal cells that initiate these events are incompletely understood, both stromal and epithelial cells potentially are capable of driving the process either through inductive influences or through endogenous expression of degradative enzymes. Epithelial cells elaborate several defined as well as unidentified growth factors and cytokines capable of inducing expression of tissue-degrading enzymes by stromal cells (2–6). The recent discovery of keratinocyte growth factor, a member of the fibroblast growth factor family expressed by stromal cells, which acts only on keratinocytes, suggests that the communication may well proceed also in the other direction (7, 8). The role of epithelial cells in remodeling of stromal/epithelial interfaces was originally thought of as primarily inductive, mediated by release of one or more growth factors and cytokines (2, 9, 10). A growing body of evidence, however, suggests that keratinocytes (as well as other cells of epithelial lineage) express several members of the matrix metalloproteinase gene family either constitutively or in response to growth factors/ cytokines or to phorbol esters (11–15). We have observed that rat epithelial cells (mucosal keratinocytes and mammary carcinoma cells) constitutively express an interstitial, fibroblast-type collagenase (MMP-1) (11) and a M, 95,000 gelatinase/Type IV collagenase (MMP-9) in culture (12). Together these two proteinases are capable of degrading a wide range of matrix proteins responsible for the stromal architecture of basement membranes and underlying connective tissue. In this study we have studied growth factors and cytokines that regulate the expression of these two collagen-cleaving proteinases by rat mucosal keratinocytes. Our findings show that the proinflamatory cytokine, IL-1β, and the two epithelial

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1 The abbreviations used are: IL, interleukin; ABTS, diammonium 2,2'-azinodi-(3-ethylbenzthiazoline-6-sulfonate); DMEM, Dulbecco's modified Eagle's medium; EGF, epidermal growth factor; FIB-CL, fibroblast-type collagenase, MMP-1; IFN, interferon; mAb, monoclonal antibody; mAbCoBC1-IID1 (12); mAbCoBC1-IID1 previously identified as mAb GeBC1-ID4 (12); mAb52K, mAb 52K previously identified as mAb CoBC1-IID1 (12); MMP, matrix metalloproteinase; M, 72K GL, M, 72,000 gelatinase, MMP-2; M, 95K GL, M, 95,000 gelatinase, MMP-9; PBS, phosphate-buffered saline; TGF, transforming growth factor; TNF, tumor necrosis factor; TPA, 12-O-tetradecanoylphorbol-13-acetate; ELISA, enzyme-linked immunosorosent assay.
growth factors TGF-α and EGF, are potent inducers of M, 95K GL and FIB-CL in keratinocytes.

EXPERIMENTAL PROCEDURES

Materials—Culture media were from Flow (McCLean, VA) or MediaTech (Washington, D.C.). Heps was from Research Organics (Cleveland, OH). SDS-polyacrylamide gel electrophoresis M, markers, hepkeri-Septharose, and CNBr-Sepharose 4B were from Pharmacia LKB Biotechnology Inc. Labtek chamber slides were from Millipore. Thioglycollate and sodium succinate were from Fisher Scientific. Brilliant Blue G-250 was from Kodak. Electrophoresis chemicals were from Bio-Rad. Immunological reagents were from Southern Biotechnology (Birmingham, AL). Recombinant human interleukin-1α (IL-1α) and -1β (IL-1β), recombinant human interferon-γ (IFN-γ), and recombinant human tumor necrosis factor-α (TNF-α) were from Genzyme (Boston, MA). Natural murine epidermal growth factor (EGF) was from Collaborative Research (Bedford, MA). Recombinant human platelet-derived growth factor, recombinant human insulin-like growth factor-I and -II, recombinant human basic fibroblast growth factor, and recombinant human transforming growth factor-α (TGF-α) were from Bachem (Torrance, CA). Recombinant human transforming growth factor-β1 (TGF-β1) was from R & D Systems Inc. (Minneapolis, MN). T-Triton X-100, Tween 20, dexamethasone, bovine serum albumin, 12-0-tetradecanoyl-phorbol acetate (TPA), EGTA, EDTA, phenylmethylsulfonyl fluoride, leupeptin, staurosporine, and diamidesulfonate [3-ethyldihydroxyacetylsulfonate (ABTS)] were from Sigma. All other reagents were highest available grade from Sigma.

Cell Culture—A cloned rat keratinocyte cell line (CCL-4) was established from normal rat tongue mucosa as described previously (11, 16) and cultured under defined, permanently serum-free conditions without antibiotics. The medium consisted of a 1:1 mixture of Ham's F-12 and Dulbecco's modified Eagle's medium (DMEM) supplemented with insulin (5 μg/ml), transferrin (1 μg/ml), bovine serum albumin (5 mg/ml), and Hepes (10 mM) (17). In growth factor/cytokine experiments, the cells were seeded at a density of 25,000 cells/cm² in 24-well plates (Corning) or (100) well/96-well plates (Bedford, MA) in culture for attachment and growth to confluence. One-hundred μl of medium was then replaced with fresh medium containing the factor of interest, TPA, staurosporine, and dexamethasone were added to the medium from 10⁻⁴ and 10⁻⁵ stock solutions in dimethyl sulfoxide. Concentrations of dimethyl sulfoxide up to 0.1% had no effect on production of M, 95K GL or FIB-CL or on cell morphology.

Early passage rat mucosal keratinocytes were initially established from tongue explants in DMEM, 20% fetal bovine serum as described previously (11, 16) and then subcultured into Life Technology, Inc. low Ca⁺⁺ (0.02 mM) keratinocyte medium supplemented with EGF (6 μg/ml) and bovine pituitary extract (35-50 μg/ml). When grown to conflu­ence, second passage cells were subcultured into chamber slides, exposed to control or stimulating agents in keratinocyte growth medium as described below, and processed for immunofluorescence.

Isolation of M, 95K GL and FIB-CL—M, 95K GL and FIB-CL were obtained from two murine carcinoma cell lines (BC-1) as described previously (12, 16). Conditioned media obtained from serum-free cultures was first passed over a heparin-Sepharose column. The enzyme was retained on this column and eluted with a factor of interest. TPA, staurosporine, and dexamethasone were added to the medium from 10⁻⁴ and 10⁻⁵ stock solutions in dimethyl sulfoxide. Concentrations of dimethyl sulfoxide up to 0.1% had no effect on production of M, 95K GL or FIB-CL or on cell morphology.

Results of two-site ELISAs were performed in 96-well microtiter plates. In preliminary experiments a range of catcher/detector combinations for two-site ELISAs were tested as were blocking procedures (non-fat powdered milk or bovine serum albumin supplemented with either Tween 20 or Triton X-100) and varying incubation times and temperatures. Most experiments were performed as described below. For quantification of rat 95K GL, wells were coated with mAbMr 4C6v11D2 for 6 h at 22 °C with 100 μl of a 3 μg/ml solution of purified IgG in sodium borate buffer, pH 8.5, then blocked with bovine serum albumin containing 3% bovine serum albumin and 2% Tween 20 and washed twice. The coated wells were incubated overnight at 22 °C with 50 μl of appropriate dilutions of culture medium samples or standards in the same buffer followed by three buffer washes. The wells were then incubated with biotinylated mAbMr 4C6v11D4 (100 μl of 10 μg/ml in bovine saline buffer) for 4 h at 22 °C and washed three times. Biotinylation was with N-hydroxy-succinimidyl biotin in 0.1 M sodium borate buffer, pH 8.8, using 25 μl of a 4 mg/ml solution of pure biotin in borate buffer, pH 8.8, essential (18) essentially as described (21). Incubation with the detector antibody was followed by incubation with 50 μl of 1 μg/ml streptavidin-borseradish peroxidase in bovine saline buffer for 2 h at 22 °C. The plates were then washed twice with PBS and once with citrate buffer, pH 4.6, and developed by incubation at room temperature for 10 min, with 0.03% H₂O₂ in citrate buffer containing 0.3 mg/ml ABTS. For quantification of rat FIB-CL, plates were coated with 3 μg/ml mAbMr 4C6v11D2 for 6 h at 22 °C. Detector antibody was 10 μg/ml protein A purified, biotinylated rabbit IgG raised against rat FIB-CL. Incubation was for 4 h at 22 °C.

Other Methods—Cells grown in plastic chamber slides were fixed in 3:1 methanol-acetone for 20 min with 0.03% H₂O₂ in citrate buffer containing 0.3 mg/ml ABTS. For quantification of rat FIB-CL, plates were coated with 3 μg/ml mAbMr 4C6v11D2 for 6 h at 22 °C. Detector antibody was 10 μg/ml protein A purified, biotinylated rabbit IgG raised against rat FIB-CL. Incubation was for 4 h at 22 °C.

Development of Two-site ELISAs for Rat M, 95K GL and FIB-CL—Development of metalloproteinase gene expression involves both transcriptional and posttranscriptional events. Since any biologic effects are mediated by the secreted, functional product, we decided to study growth factor and cytokine regulation of keratinocyte metalloproteinases at the terminal level, i.e. by the emergence of enzyme protein in the extracellular milieu. To this end, the rat homologues of the human M, 92K GL (M, 95K in the rat (12)) and FIB-CL were isolated using as source a rat mammary carcinoma cell line (BC-1) which maintains high level constitutive expression.
of both enzymes (12, 18) (Fig. 1, inset). To permit quantitation of enzyme protein, mAbs were raised against both enzymes and two-site ELISAs developed (Fig. 1). Rat M, 95K GL was quantified by a mAb/mAb catcher/detector combination (mAbMr, 95K GL/VIC2/biotin-mAbMr, 95K GL) with a useful range of 3–300 ng/ml and a lower detection limit of $\approx$ 300 pg of enzyme protein. For quantification of rat FIB-CL a catcher mAb (mAbMr, 95K GL/ID2) was used with biotinylated rabbit-anti-rat FIB-CL IgG. This assay had a useful range of 1–100 ng/ml and a lower detection limit of $\approx$ 100 pg of enzyme protein. At enzyme concentrations up to 3 $\mu$g/ml no cross-reactivity between the two proteins was detected (Fig. 1).

**Rat Mucosal Keratinocytes Express M, 95K GL and FIB-CL in Response to IL-1$\beta$, IL-1$\alpha$, TGF-$\alpha$, and EGF**—We have shown previously that clonal derivatives (CCL-4) of rat mucosal keratinocytes retain typical keratinocyte traits and are capable of orderly passage through a characteristic terminal differentiation repertoire (cornified envelope formation, keratin filament assembly, and degradation of nuclear and cytoplasmic structures) (11, 16). When cycled between serum-free and serum-supplemented periods these cells maintain significant "basal" expression of the M, 95K GL and FIB-CL, as measured by secretion of enzymes to the culture medium (11, 13). Since several matrix metalloproteinase genes, and the transcription factor genes that regulate their expression (c-fos), contain serum response elements (23, 24) we sought to reduce basal expression of the enzymes by serially propagating the cells in a defined, serum-free medium supplemented with insulin and transferrin (17). This medium permitted infinite serial propagation of rat mucosal keratinocytes under high Ca$^{2+}$ conditions and lowered basal expression of both enzymes to near the detection limit of the assays.

To monitor the response of rat mucosal keratinocytes to growth factors and cytokines, cells were seeded at a density of 25,000/cm², allowed to attach and grow for 48 h, and then exposed to growth factors/cytokines for 24 h. Under these conditions, EGF (5 x 10⁻⁶ M), TGF-$\alpha$ (5 x 10⁻⁶ M), and IL-1$\beta$ (10⁻¹⁰ M), as well as TPA (10⁻⁷ M), greatly stimulated expression of M, 95K GL and FIB-CL. M, 95K GL, identified by staining with mAbMr, 95K GL/ID4 (12), emerged in the culture medium in both monomer and dimer forms, and both were recognized by the antibody (Fig. 2B). The dimer did not dissociate in 1% SDS under nondenaturing conditions but was converted to catalytically active form by exposure to this detergent (Fig. 2C). FIB-CL identified by staining with mAbMr, 95K GL/ID1 (12) showed less tendency to dimerize and emerged predominantly in monomeric form (Fig. 2C).

To compare the potency of various growth factors and cytokines, cells were exposed to a broad concentration range of stimulants for 20 h and medium concentrations of M, 95K GL and FIB-CL determined by two-site ELISAs. IL-1$\beta$ was the most potent cytokine with maximal effect at $\approx$ 10⁻¹⁰ M and detectable effect down into the 10⁻¹¹–10⁻¹² M range (Fig. 3, upper panels). IL-1$\alpha$ required approximately 100-fold higher concentration. Under the same conditions, TNF-$\alpha$, which induces high level expression of FIB-CL in human skin
Depletion of Cytosolic Protein Kinase C by TPA Abrogates Growth Factor Responses—The promoter regions of the human FIB-CL gene contain one (30, 31), and the Mr 92K GL gene two (28), TPA-responsive, AP-1 binding sequences. The AP-1 site is a necessary albeit not sufficient requirement for TPA induction of metalloproteinase gene expression (28, 30-32). This regulatory element appears to be functionally conserved in the rat genes as well since TPA dramatically stimulated expression of M, 95K GL and FIB-CL in the 10^-8 M-10^-6 M range (Fig. 2 and Fig. 3, bottom panels). A body of evidence suggests that the AP-1 site is also a necessary requirement for the transcriptional effects of IL-1β and EGF on metalloproteinase gene expression (27, 33, 34). Since the intracellular signaling elicited by these growth factors and cytokines is mediated, at least in part, through protein kinase C-dependent pathways (27, 32, 35-37) and since protein kinase C is activated directly by phorbol esters (38, 39), we examined the effect on metalloproteinase gene expression of pretreatment with TPA which depletes cytosolic protein kinase C and leaves the cells refractory to stimulation through protein kinase C-dependent pathway(s) (40). Exposure to 10^-7 M TPA for 24 h resulted in 90% reduction of cytosolic protein kinase C activity (Fig. 5A) and in 70-95% down-regulation of TGF-α/EGF, IL-1β, and TPA responses for both enzymes (Fig. 5, B and C). Attempts to block growth factor induction by staurosporine, a potent protein kinase inhibitor (41, 42), were not successful since staurosporine itself stimulated expression of the enzymes, particularly M, 95K GL, in a relatively narrow concentration range around 10^-6 M (Fig. 2 and Fig. 3, bottom panels). Concentrations of staurosporine which blocked metalloproteinase induction (≥5 × 10^-6 M) invariably led to cell detachment and cell death. Fig. 5, B and C, also shows that, in contrast to the growth factor and cytokine responses which continued unabated for at least 52 h, the inductive effect of TPA itself was transient, relatively short-lived, and all but played out within the first 24 h.

Dexamethasone Inhibits Growth Factor/Cytokine Induction of Keratinocyte M, 95K GL and FIB-CL—Glucocorticoids repress matrix metalloproteinase gene expression and are thought to mediate anti-inflammatory activity, in part, by down-regulation of these enzymes. In rabbit and human synovial and skin fibroblasts, glucocorticoids reduced IL-1 and EGF responses measured by stromelysin-1 and FIB-CL transcript or medium protein levels by as much as 80-90% (26, 43-45). A sequence partially or completely overlapping the TPA-responsive, AP-1 binding sequences. The M, 95K GL gene contains one (30, 31), and the Mr 92K GL gene two (28), TPA-responsive, AP-1 binding sequences. The AP-1 site is a necessary although not sufficient requirement for TPA induction of metalloproteinase gene expression (28, 30-32). This regulatory element appears to be functionally conserved in the rat genes as well since TPA dramatically stimulated expression of M, 95K GL and FIB-CL in the 10^-8 M-10^-6 M range (Fig. 2 and Fig. 3, bottom panels). A body of evidence suggests that the AP-1 site is also a necessary requirement for the transcriptional effects of IL-1β and EGF on metalloproteinase gene expression (27, 33, 34). Since the intracellular signaling elicited by these growth factors and cytokines is mediated, at least in part, through protein kinase C-dependent pathways (27, 32, 35-37) and since protein kinase C is activated directly by phorbol esters (38, 39), we examined the effect on metalloproteinase gene expression of pretreatment with TPA which depletes cytosolic protein kinase C and leaves the cells refractory to stimulation through protein kinase C-dependent pathway(s) (40). Exposure to 10^-7 M TPA for 24 h resulted in 90% reduction of cytosolic protein kinase C activity (Fig. 5A) and in 70-95% down-regulation of TGF-α/EGF, IL-1β, and TPA responses for both enzymes (Fig. 5, B and C). Attempts to block growth factor induction by staurosporine, a potent protein kinase inhibitor (41, 42), were not successful since staurosporine itself stimulated expression of the enzymes, particularly M, 95K GL, in a relatively narrow concentration range around 10^-6 M (Fig. 2 and Fig. 3, bottom panels). Concentrations of staurosporine which blocked metalloproteinase induction (≥5 × 10^-6 M) invariably led to cell detachment and cell death. Fig. 5, B and C, also shows that, in contrast to the growth factor and cytokine responses which continued unabated for at least 52 h, the inductive effect of TPA itself was transient, relatively short-lived, and all but played out within the first 24 h.

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Regulation of Keratinocyte Matrix Metalloproteinase Expression

Fig. 4. Kinetics of Mr 95K GL and FIB-CL secretion in response to growth factors and cytokines. Keratinocytes were seeded as described in Fig. 2 and then exposed to IL-1β (10^{-9} M), TGF-α (5 × 10^{-8} M), EGF (5 × 10^{-8} M), or to medium alone (CON). Media aliquots were withdrawn at increasing time intervals from 2 to 52 h after the addition of stimulant and the concentration of Mr 95K GL and FIB-CL in quadruplicate wells determined by two-site ELISA as described in Fig. 1. Insets show the emergence of Mr 95K GL or FIB-CL during the first 20 h.

Fig. 5. Effect of TPA pretreatment on growth factor/cytokine responses. Subconfluent keratinocyte cultures prepared as described in Fig. 2 were pretreated for 27 h with 10^{-7} M TPA and then washed to remove traces of phorbol ester and incubated for another 24 h with medium (DMEM/F-12 containing with insulin, transferrin) supplemented with EGF (5 × 10^{-9} M), TGF-α (5 × 10^{-9} M), IL-1β (10^{-10} M), or TPA (10^{-7} M). Panel A, the cytosolic protein kinase C activity of untreated (CON) and TPA-pretreated cultures (TPA) was determined as described by Franklin et al. (22). Panel B, Mr 95K GL concentrations were determined by two-site ELISA as described in Fig. 1. Panel C, FIB-CL concentrations were determined by two-site ELISA as described in Fig. 1.

Induction of Mr 95K GL and FIB-CL Is Associated with Morphologic Changes—Induction of metalloproteinase genes in stromal cells is often associated with more extensive shifts in gene expression which result in dramatic changes in cell shape (49). IL-1β, TGF-α, EGF (as well as TPA) had similar, striking effects on the morphology of mucosal keratinocytes (Fig. 7). Unstimulated cells were fully spread with typical epitheloid morphology (Fig. 7A). Stimulated cells developed more stellate or spindle-shaped appearances with long, thin (stringy) projections across the surface (Fig. 7, C-K) and, particularly in the case of TPA, retraction and cell rounding (Fig. 7F). It is of interest to note that staurosporine mimicked the effect of IL-1β and TGF-α/EGF also on cell morphology (Fig. 7K). Changes of cell shape were accompanied by the emergence of cytoplasmic enzyme protein. Few isolated cells (<5%) expressed the enzymes in unstimulated cultures (Fig. 7B), but each of the stimulatory agents increased the fraction of immunoreactive cells by >10-fold and, in most cases, visibly augmented the level of immunoreactivity in individual cells (Fig. 7). Because of the clonal origin of the cell line described above and because of its ability to propagate serially under permanently serum-free conditions, we asked the question whether the growth factor and cytokine responses of these cells were significantly altered as compared with freshly isolated rat mucosal keratinocytes. Since early passage keratinocytes have...
Regulation of Keratinocyte Matrix Metalloproteinase Expression

an absolute requirement for growth factor or serum supplementation, it is not possible to establish completely comparable conditions. To address the question, rat mucosal keratinocytes obtained by the explant method using DMEM, 20% fetal bovine serum were subcultured into a low Ca\textsuperscript{2+} keratinocyte growth medium supplemented with EGF and bovine pituitary extract and then exposed to IL-1\beta (10\textsuperscript{-8} M), TGF-\alpha (5 \times 10\textsuperscript{-8} M), IL-1\beta (10\textsuperscript{-10} M), or TPA (10\textsuperscript{-7} M) and with increasing concentrations of dexamethasone. Incubation was for 20 h. Since the various stimulants induce different levels of enzyme secretion, the results are shown relative to induced, dexamethasone-free controls after subtraction of basal expression of uninduced cultures.

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\frac{[E]_{\text{induced, dexameth.-}} - [E]_{\text{uninduced}}}{[E]_{\text{basal, dexameth.-}} - [E]_{\text{uninduced}}} \quad \text{(Eq. 1)}
\]

Mean and standard deviation of quadruplicate wells are shown.

FIG. 6. Repression of cytokine responses by dexamethasone. Panel A, M, 95K GL. Panel B, FIB-CL. Rat mucosal keratinocytes seeded as described in Fig. 2 were supplemented with EGF (6 \times 10\textsuperscript{-8} M), TGF-\alpha (5 \times 10\textsuperscript{-8} M), IL-1\beta (10\textsuperscript{-10} M), or TPA (10\textsuperscript{-7} M) and with increasing concentrations of dexamethasone. Incubation was for 20 h. Since the various stimulants induce different levels of enzyme secretion, the results are shown relative to induced, dexamethasone-free controls after subtraction of basal expression of uninduced cultures.

DISCUSSION

The findings of this study show that rat mucosal keratinocytes in culture respond to the proinflammatory cytokines IL-1\beta/IL-1\alpha and to the epithelial growth factors EGF and TGF-\alpha by expression of M, 95K GL and FIB-CL as measured by emergence of enzyme of protein kinase in the extracellular milieu. IL-1\beta was by far the most potent stimulant with maximal induction already at 10\textsuperscript{-10} M. In this capacity it was at least 100-fold more potent than TPA. IL-1\alpha and TGF-\alpha/EGF also induced expression of the enzymes but required 20-100-fold higher concentrations (10\textsuperscript{-8}-10\textsuperscript{-9} M). Since IL-1 concentrations in inflammatory exudates such as rheumatoid synovial fluid and gingival crevicular fluid are frequently in the 10\textsuperscript{-9}-10\textsuperscript{-11} M range (50-53) our findings suggest that keratinocytes respond to growth factors and cytokines in a physiologically meaningful concentration range. It is of note that emergence of M, 95K GL and FIB-CL in the culture medium is preceded by a considerable lag period. The earliest response was detected after 10-14 h, which is considerably longer than the 3-8 h required for appearance of transcripts (26-28), and fully 24 h were required for attainment of maximal steady-state secretion rates. During the linear phase, cells induced by IL-1\beta and TGF-\alpha/EGF secreted 2-10 \mu g/10\textsuperscript{6} cells/day of M, 95K GL and FIB-CL, i.e. levels that are comparable to, and perhaps exceed, those attained by stromal cells. By contrast, the effect of TPA (as well as that of staurosporine; data not shown) was transient and rather short lived.

Several studies have suggested that the transcriptional effects of IL-1\beta and EGF (and TGF-\alpha) on metalloproteinase gene expression are mediated by signaling pathways involving protein kinase C (27, 36). TPA pretreatment of rat mucosal keratinocytes lowered cytosolic protein kinase C activity by greater than 90% and all but abolished subsequent responses to IL-1\beta and TGF-\alpha/EGF (as well as to TPA itself). These findings support the notion that these growth factors regulate matrix metalloproteinase expression by rat mucosal keratinocytes through protein kinase C-dependent mechanisms. Somewhat surprisingly, however, staurosporine, a broad spectrum protein kinase inhibitor with particular potency against protein kinase C, also induced expression of the enzymes, particularly M, 95K GL. This observation is apparent at variance with findings by Case et al. (36) that 2.5-5.0 \times 10\textsuperscript{-8} M staurosporine reduces stromelysin-1 transcript levels in synovial fibroblasts in response to IL-1\beta. A number of factors may possibly account for this discrepancy, as the two studies differ with respect to cell type (keratinocytes versus fibroblasts), method of analysis (medium enzyme concentration versus steady-state mRNA levels), and gene products analyzed (FIB-CL and M, 95K GL versus stromelysin-1). Our observation is of particular interest in view of the finding by others that staurosporine possesses tumor promoting activity in mouse skin keratinocytes (54, 55), induces expression of urokinase-type plasminogen activator in LLC-PK, porcine kidney epithelial cells (56), and mimics the effect of nerve growth factor in inducing neurite outgrowth in PC12 pheochromocytoma cells (57). The induction of degradative enzymes such as matrix metalloproteinases and urokinase-type plasminogen activator are likely to impart aggressiveness to developing tumors and therefore may be directly linked to the tumor promoting activity. It is possible that the staurosporine response is biphasic with inductive effects prevailing at lower concentrations and inhibitory effects at higher, as suggested by other studies (58). Although the staurosporine dose response (Fig. 3) might suggest the existence of such a biphasic response because of the precipitous drop beyond 10\textsuperscript{-8} M, the inherent cytotoxicity of staurosporine, noted previously by others (41), in the case of rat mucosal keratinocytes precluded meaningful analysis at concentrations beyond 10\textsuperscript{-7} M, which coincide with the reported inhibitory range on protein kinases.

The observation that keratinocytes express two matrix metalloproteinases (M, 95 K GL and FIB-CL) with action against stromal and basement membrane components in response to IL-1\beta/IL-1\alpha and TGF-\alpha/EGF is of considerable interest in the context of keratinocyte function in development and in wound healing. Wound healing sites contain several potential sources of IL-1\beta and TGF-\alpha. Wound macrophages express each of these cytokines (59), and stimulated keratinocytes are capable of expressing a wide range of growth factors and cytokines including IL-1 (4) and TGF-\alpha (5, 60, 61). A body of evidence suggests that keratinocytes also express the appropriate receptors: basal keratinocytes express EGF receptors in vitro (62, 63), and stimulated epithelial cells in culture express receptors for IL-1 as well (64). Studies by Barrandon and Green (65) have shown that human keratinocyte colonies respond to TGF-\alpha by centripetal migration and have suggested that this effect is responsible for epithelial sheet expansion. It is therefore likely that epithelial cell functions in wound healing such as proliferation, migration,
FIG. 7. Growth factors/cytokines induce morphologic changes and stimulate expression of M, 95K GL. Rat mucosal keratinocytes were seeded in chamber slides and then stimulated for 24 h (TPA, 10 h). The cells were fixed with 3.7% formaldehyde in PBS and permeabilized with 1% Triton X-100. After the blocking of nonspecific binding sites with an irrelevant 100 μg/ml IgG, mAb, the cells were stained with 50 μg/ml biotinylated mAb IVIC2 in PBS containing 1% bovine serum albumin. Staining was developed with 1 μg/ml Texas Red-conjugated streptavidin. Left panels, phase-contrast microscopy; right panels, immunolocalization of M, 95K GL. Panels A and B, control; panels C and D, IL-1β (10^{-10} M); panels E and F, TGF-α (5 × 10^{-9} M); panels G and H, EGF (5 × 10^{-9} M); panels I and J, TPA (10^{-7} M); and panels K and L, staurosporine (10^{-8} M). Arrowheads point to identical cells in phase-contrast and immunofluorescence microscopy. Magnification, × 200.

and sheet expansion are regulated by TGF-α. We speculate that epithelial migration at wound healing sites to cover the denuded connective tissue surface may well be intimately linked to expression of degradative enzymes with activity against stromal macromolecules. Other inflammation-related epithelial proliferative conditions that may require expression of metalloproteinases include the expansive growth of epithelial cysts (for instance radicular cysts surrounding infected teeth) and the “invasive” epithelial apical migration in human periodontal diseases.

It is equally likely that epithelial invasion into mesenchymal domains is dependent on expression of one or more matrix metalloproteinases and that this process is regulated by growth factors and cytokines. A substantial body of evidence suggests that invasiveness of both normal and malignant cells is associated with elevated expression of metalloproteinases and/or diminished expression of metalloproteinase inhibitors (66-71). Moreover, Turkson et al. (14) recently showed that human keratinocyte raft cultures grown on collagen lattice/fibroblast matrix respond to TGF-α by induction of FIB-CL throughout the epithelial layer, and of M, 72K GL in the basal layer, and that emergence of these enzymes precedes or coincides with expression of an invasive phenotype. It is often not appreciated that epithelial invasion, although important to tumor progression, is not a tumor-specific trait. Highly invasive but nonmalignant epithelial movements occur during embryonic organo- and morphogenesis and later in life during hair and tooth development, during remodeling of epithelium/connective tissue interfaces (rete peg formation) and during pocket deepening in progressive human periodontal disease. The observation that normal epithelial cells in culture express FIB-CL (11, 15), M, 72K GL (13), M, 95K GL (13), and
to disrupt the normal organization of the cytoskeleton. This finding was consistent with previous observations that TPA-induced expression of keratin is controlled by a regulatory mechanism that is sensitive to glucocorticoids.

2. Several studies have suggested that TPA-induced expression of keratin may be mediated by a glucocorticoid-responsive element (GRE). These studies have shown that TPA-induced expression of keratin is down-regulated by dexamethasone, a glucocorticoid that inhibits the expression of several other genes.

3. The mechanism by which TPA induces keratin expression is not fully understood. It is possible that TPA directly activates the expression of keratin genes, or that TPA induces the expression of a transcription factor that stimulates keratin expression.

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Regulation of Keratinocyte Matrix Metalloproteinase Expression