

Modulation of Intracellular Calcium Levels Inhibits Secretion of Collagenase 1 by Migrating Keratinocytes

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Calcium concentration influences keratinocyte differentiation, and, following injury, keratinocytes move through an environment of changing calcium levels. Because these migrating cells in wounds invariably express collagenase 1, we assessed if modulation of calcium levels regulates collagenase 1 production by primary human keratinocytes. Accurately reflecting the confined spatial pattern of enzyme production seen in vivo, collagenase 1 mRNA was expressed only by keratinocytes migrating from foci of differentiated cells. Treatment with calcium ionophores A23187 or thapsigargin markedly inhibited the basal and phorbol 12-myristate 13-acetate- (PMA) stimulated accumulation of keratinocyte collagenase 1 in the medium but did not affect collagenase 1 production by control or PMA-treated fibroblasts. A23187-mediated inhibition of collagenase 1 protein was not associated with a decrease in mRNA levels but rather was controlled by a selective and reversible block of enzyme secretion. This block in secretion was likely not due to altered protein folding as the proenzyme within A23187-treated cells remained capable of autolytic activation upon treatment with *p*-aminophenylmercuric acetate. In contrast, 92-kDa gelatinase mRNA and secreted protein levels were coordinately reduced by A23187. Keratin 14 expression, a basal keratinocyte marker, was reduced with PMA treatment, but A23187 did not affect keratin 14 expression. In human wounds, both basal and suprabasal keratinocytes at the migrating front of epidermis stained for keratin 14, but only the basal cells expressed collagenase 1. These data suggest that collagenase 1 production is not necessarily linked with expression of basal cell markers and that modulation of intracellular calcium levels can block secretion of collagenase 1 by keratinocytes which have moved away from the stratum basalis and from their natural substrate.

INTRODUCTION

Degradation and remodeling of the extracellular matrix is associated with many normal biological events, including wound repair, tissue involution, and cell migration, as well as with various disease processes, such as arthritis, ulceration, and tumor metastasis (Werb, 1989; Matrisian *et al.*, 1991; Parks and Sires,

1996). Matrix metalloproteinases (MMPs) constitute a subfamily of metalloenzymes with the collective potential of degrading essentially all structural components in the extracellular environment. The MMPs with known matrix-degrading ability include three collagenases, two gelatinases, two stromelysins, a metalloelastase, matrilysin, and membrane-type matrix metalloproteinase 1 (Birkedal-Hansen *et al.*, 1993; Pei and Weiss, 1996). Because matrix remodeling needs to be accurately controlled both spatially and temporally, the expression and activity of MMPs is highly regulated, with essentially no basal production of most proteinases in intact tissues. [The constitutive expres-

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sion of matrilysin by epithelium is a notable exception (Saarialho-Kere *et al.*, 1995; Wilson *et al.*, 1995).] Thus, agents and processes associated with tissue remodeling, particularly cytokines and altered cell-matrix and cell-cell interactions, modulate MMP production.

Collagenase 1, along with the other two metallocollagenases, can cleave fibrillar collagens (types I, II, and III) within their triple helical domain at a neutral pH. Because type I collagen is the most abundant protein in the body, collagenases serve an essential role in initiating efficient matrix turnover. We reported that collagenase 1 is prominently, invariably, and selectively expressed by migrating basal keratinocytes involved in reepithelialization in human cutaneous wounds (Saarialho-Kere *et al.*, 1992, 1993a; Parks, 1995). Furthermore, collagenase 1-positive cells are not associated with an intact basement membrane but rather are migrating over the dermal and provisional matrices. These findings indicate that the new cell-matrix interactions which are established as keratinocytes migrate from their basement membrane onto the dermal matrix are critical determinants in regulating collagenase 1 expression. Indeed, we demonstrated that collagenase 1 transcription and secretion are induced in primary human keratinocytes upon contact with native type I collagen but not in cells grown on a mixture of basement membrane components (Saarialho-Kere *et al.*, 1993a; Sudbeck *et al.*, 1994). As reepithelialization proceeds, active expression of collagenase 1, as determined by *in situ* hybridization for its mRNA, is rapidly shut off in basal keratinocytes in contact with the restored basement membrane, although staining for the protein remains evident in some suprabasal cells (Saarialho-Kere *et al.*, 1993a; Inoue *et al.*, 1995). Thus, in addition to regulation of gene expression by cell-matrix interactions, the release or activation of collagenase 1 protein may be regulated by other mechanisms in newly healed areas.

During epidermal differentiation, keratinocytes move from a low calcium environment to layers containing progressively higher concentrations of calcium (Malmqvist *et al.*, 1984; Menon *et al.*, 1985). This spatial change in ion levels is associated with irreversible changes in cell phenotype characterized by alterations in cytokeratin expression, growth arrest, and cornification (Fuchs, 1990). Cell culture studies have demonstrated that keratinocyte differentiation can be induced by raising extracellular calcium levels (Yuspa *et al.*, 1989) and that the intracellular concentration of calcium ions increases concomitantly with keratinocyte differentiation (Sharpe *et al.*, 1993). Thus, because a natural calcium gradient may regulate the growth and phenotype of keratinocytes during differentiation in intact skin, a similar change in the extracellular microenvironment may influence cell behavior during epidermal wound repair.

In response to wounding, basal keratinocytes begin to migrate and acquire many characteristics associated with cell movement, such as changes in cytoskeleton structure (Kubler and Watt, 1993) and in the expression of new integrins (Hertle *et al.*, 1992; Juhasz *et al.*, 1993) and proteinases (Mignatti *et al.*, 1996). During reepithelialization, keratinocytes migrate through a low calcium environment and, as healing nears completion, enter a progressively higher calcium environment (Grzesiak and Pierschbacher, 1995). Because collagenase 1 is selectively expressed and released by basal keratinocytes during periods of active reepithelialization, we assessed if modulation of intracellular calcium stores influence keratinocyte collagenase 1 production. In this study, we show that mobilization of intracellular calcium stores inhibits keratinocyte collagenase 1 secretion without affecting collagenase 1 mRNA levels. This mechanism may be important in blocking the unwanted release of proteinase at the cessation of epidermal repair.

MATERIALS AND METHODS

Cell Culture

Primary keratinocytes were isolated from full-thickness skin obtained from reductive mammoplasty or lateral abdominoplasty and were grown under high Ca^{2+} conditions as described (Pentland and Needleman, 1986). Briefly, s.c. fat and deep dermis were removed, and the remaining tissue was incubated in 0.25% trypsin in phosphate-buffered saline for 16 h. The epidermis was then removed from the dermis, and keratinocytes were suspended in DMEM supplemented with 5% fetal calf serum, 1% HEPES, and penicillin/streptomycin. This cell suspension was then plated in 1.75-cm² tissue culture dishes coated with a 1 mg/ml solution of monomeric native-type I collagen (Vitrogen, Collagen Corp., Palo Alto, CA), and the adherent cells were grown for 3 to 5 d when they were about 70% confluent. A23187, thapsigargin (Calbiochem, San Diego, CA), and phorbol 12-myristate 13-acetate (PMA, Sigma Chemical Co., St. Louis, MO) were dissolved in dimethyl sulfoxide. Control cultures received an equal concentration (0.2%) of solvent. In some experiments, keratinocytes were treated with 5×10^{-9} M PMA. All experiments were done with freshly isolated keratinocytes.

The human keratinocyte cell line HaCaT (Boukamp *et al.*, 1988) and adult human fibroblasts isolated from healthy skin were grown to confluence in DMEM containing 10% fetal calf serum, 1% HEPES, and penicillin/streptomycin. As a positive inducer of collagenase 1 production, HaCaT cultures were stimulated with 30 ng/ml epidermal growth factor (Sudbeck *et al.*, 1994), and 1905 fibroblasts were stimulated with 10^{-8} M PMA.

In Situ Hybridization

Primary keratinocytes were plated on collagen-coated Lab-Tek chamber slides (Nunc, Naperville, IL) and processed for *in situ* hybridization as described (Prosser *et al.*, 1989; Saarialho-Kere *et al.*, 1993b). Briefly, control and 24-h PMA-treated cells were fixed for 15 min in buffered formalin and processed for *in situ* hybridization as described in detail (Prosser *et al.*, 1989), except that proteinase K pretreatment was omitted. Cells were hybridized with 2.5×10^4 cpm/ μl of ³⁵S-labeled antisense RNA overnight at 57°C. Samples of pyogenic granuloma (n = 12) were obtained from Dr. Neil Penneys (St. Louis University, St. Louis, MO), and 5- μm sections were hybridized for collagenase 1 mRNA (Saarialho-Kere *et al.*, 1992). After hybridization, slides were washed under stringent conditions, in-

cluding treatment with RNase A, and were processed for autoradiography. After development of the photographic emulsion, slides were stained with hematoxylin-eosin. Parallel controls were processed with ³⁵S-labeled sense RNA. The specificity of the antisense RNA probe for collagenase 1 and the complete lack of reactivity with the sense probe have been demonstrated in previous studies (Saarialho-Kere *et al.*, 1992, 1993a,b).

Zymography

Gelatin and casein zymography were performed as described (Heron *et al.*, 1986). Serum-free conditioned medium samples were collected, and total cellular protein content was determined with the bicinchoninic acid microassay (Pierce, Rockford, IL) using bovine serum albumin as a standard. Medium samples, equivalent to 150 µg of protein, were resolved at 4°C through 10% nonreducing SDS-polyacrylamide gels impregnated with 1 mg/ml gelatin or casein. Gels were washed twice in 2.5% Triton X-100 and once in 50 mM Tris (pH 7.5) containing 10 mM CaCl₂ and 1 mM ZnCl₂, and were then incubated overnight at 37°C in the same buffer. Gelatinolytic and caseinolytic proteins were identified as clear bands on a dark background after staining with 1% Coomassie blue. In parallel gels, 25 mM EDTA or 1 mM phenylmethylsulfonyl fluoride (PMSF, Sigma) were added during the zymogram incubation to verify the presence of metalloproteinase activity. Molecular weight standards (Pharmacia, Uppsala, Sweden) were used to estimate the weight of protein samples.

Immunoassays

Collagenase 1 protein was quantified using a competitive enzyme-linked immunosorbent assay (ELISA; Saarialho-Kere *et al.*, 1993b). This assay has nanogram sensitivity, is specific for human collagenase 1, and measures total enzyme present whether free or bound to inhibitor or substrate or whether in an inactive or active form. Other than dilution, media samples were assayed without modification. Standard curves using purified human collagenase 1 were included in each assay. Results were normalized to total cellular protein.

Total Protein Synthesis

Keratinocytes were treated with 0.1–5 µM A23187 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 [³H]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 (Sudbeck *et al.*, 1994).

Immunoprecipitation

Keratinocytes were pretreated for 30 min with 1 µM A23187 or 0.1% dimethyl sulfoxide, then one-half of the cultures were stimulated for 4 h with 5 × 10⁻⁹ M PMA. Cultures were washed twice with methionine-free DMEM and pulsed with 50 µCi/ml [³⁵S]methionine in methionine-free DMEM with additives. After 4 h, the conditioned medium was removed, and the monolayers were rinsed with phosphate-buffered saline and 20 mM EDTA before being scraped and sonicated in 500 µl of solubilization buffer (0.25% Nonidet P-40, 10 mM EDTA, 50 mM Tris, pH 8, leupeptin, pepstatin A, soybean trypsin inhibitor, and pefabloc, Boehringer Mannheim, Indianapolis, IN). Procollagenase was activated by treating medium and cell layer samples with 1 mM *p*-aminophenylmercuric acetate (APMA, Sigma) in 0.1 M Tris, pH 8.0, for 1 h at 37°C before immunoprecipitation. For APMA activation studies, cell layers were solubilized in 0.1 M Tris, pH 8.0, containing 1 mM PMSF but no EDTA. Extraction buffer (100 µl: 1% SDS, 5% Triton X-100, 50 mM EDTA, 200 mM Tris, pH 7.4, and proteinase inhibitors) was added to each sample, and the samples were incubated overnight with

collagenase 1 polyclonal antiserum. Antibody-enzyme complexes were precipitated from solution with protein A-Sepharose (Sigma) and were resolved by electrophoreses through 10% SDS-polyacrylamide gels. Radiolabeled proteins were detected by fluorography, and autoradiographic bands were quantified with laser densitometry.

mRNA Analysis

RNA isolation and Northern blot hybridization were done as described previously (Parks *et al.*, 1988). Probes were prepared by labeling cDNAs for human collagenase 1, human 92-kDa gelatinase, human keratin 14, or glyceraldehyde -3-phosphate dehydrogenase (GAPDH) with [³²P]dCTP by random priming. Collagenase 1 mRNA was also detected using a reverse transcription-polymerase chain reaction (RT-PCR) assay modified from an established quantitative procedure (Swee *et al.*, 1995). To remove any contaminating DNA, total RNA was treated with RQ1 RNase-free DNase and RNasin (both from Promega, Madison, WI) as described (Swee *et al.*, 1995). DNase-treated RNA (10 ng) 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). For each sample, a parallel reaction was run with no reverse transcriptase. The 3' antisense primer was complementary to bases 1496–1518 in the 3' untranslated region (5'-GGACTCACACCATGTGTTTCC-3'), and the 5' sense primer was defined by bases 1114–1135 of the hemopexin-like domain (5'-GGGCTGTTCAGGGACAGAATGT-3'). These primers are to adjacent exons, and thus, the 405-bp DNA produced from collagenase 1 mRNA would be easily distinguished from products amplified from contaminating DNA. In addition, the resultant cDNA contains a single *Bam*HI restriction site, which, along with microsequencing, was used to verify product identity. PCR was done for 21 cycles, and the resultant products were detected by Southern blot hybridization using a ³²P-labeled collagenase 1 cDNA. RT-PCR for GAPDH mRNA was done as described (Swee *et al.*, 1995).

Immunoblotting

After exposure to 1 µM A23187 and/or 10⁻⁸ M PMA, keratinocytes were scraped and boiled in 500 µl of denaturing electrophoresis sample buffer. Equal aliquots (25 µl) were resolved through 10% SDS-polyacrylamide gels and were transferred to an Immobilon-P membrane (polyvinylidene difluoride, Millipore, Bedford, MA). Blots were stained with anticytokeratin 14 as described (Busiek *et al.*, 1995), and bound secondary antibodies were detected by enhanced F (Amersham, Buckinghamshire, England).

Immunohistochemistry

Antibodies against cytokeratins 10 and 14 were purchased from Sigma. Deparaffinized 5-µm sections of pyogenic granuloma were processed for immunohistochemistry as described (Saarialho-Kere *et al.*, 1993a). Endogenous peroxidase activity was blocked by incubation in 0.3% H₂O₂ for 30 min at room temperature. Antibodies were diluted 1:100, and bound antibody was detected using 3,3'-diaminobenzidine and a Vectastain ABC Elite kit (Vector Laboratories, Inc., Burlingame, CA) following the manufacturer's instructions. Sections were counterstained with Harris hematoxylin. For negative controls, we processed serial sections with nonimmune serum at a comparable dilution.

RESULTS

In Vitro Pattern of Collagenase 1 Expression

To determine whether our keratinocyte culture model reflects the spatially confined expression of collagenase 1 to migrating keratinocytes as seen in actual

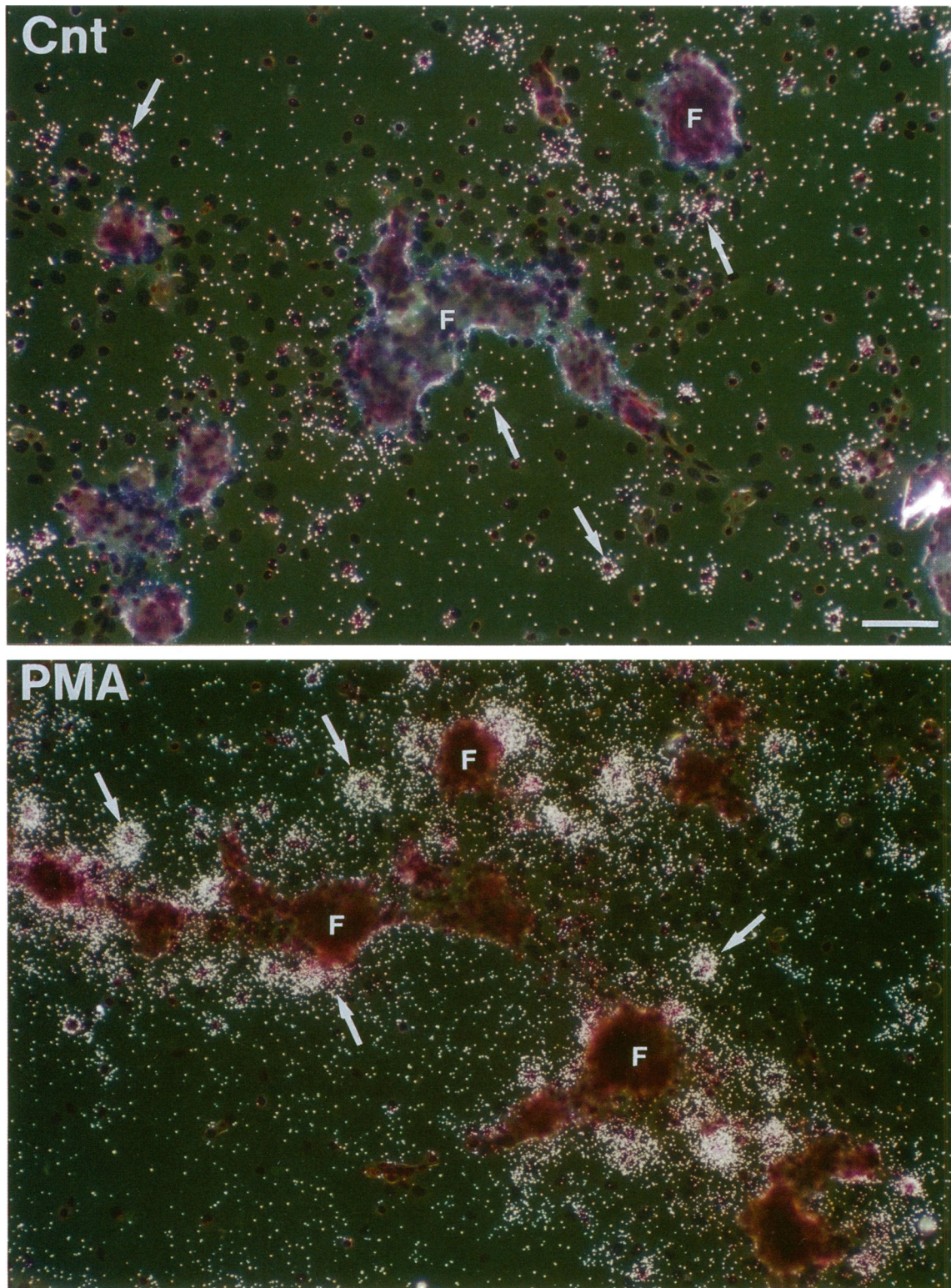


Figure 1. Collagenase 1 is expressed by migrating keratinocytes in culture. Primary human keratinocytes were plated on type I collagen-coated glass slides, and one-half of the cultures were treated with 5×10^{-9} M PMA. Twenty-four hours later, the slides were processed for in situ hybridization as described in MATERIALS AND METHODS. As seen in these dark-field photomicrographs, primary keratinocytes grown in high-calcium medium form foci (F) of differentiating cells which are surrounded by a monolayer of hyperproliferative cells

wounds, we used in situ hybridization to assess which cells express collagenase 1. Similar to wounded epidermis, cultured keratinocytes grown under high calcium (1.8 mM) conditions form subpopulations of migrating, proliferating, and differentiating cells (Hennings *et al.*, 1980; Pentland and Needleman, 1986). Differentiating keratinocytes are seen as blurred foci and are surrounded by a monolayer apron of hyperproliferative cells. Bordering the hyperproliferative cells, and often detached from them, are migrating keratinocytes (Figure 1, arrows). Reflecting the phenotype of basal cells involved in reepithelialization in vivo, collagenase 1 mRNA was detected only in keratinocytes migrating from the colonies of proliferating and differentiating cells (Figure 1, arrows). Treatment with PMA, a potent stimulator of MMP production in keratinocytes (Sudbeck *et al.*, 1994), markedly increased collagenase 1 expression. Paralleling the pattern seen in non-PMA-treated cells, signal for collagenase 1 mRNA was seen only in migrating keratinocytes (Figure 1). The morphology and organization of PMA-treated cultures was not different from those features of keratinocytes on collagen alone, except that the differentiating foci were more dense (Figure 1). Thus, our primary human keratinocyte model duplicates the restricted expression of collagenase 1 to migrating basal keratinocytes as seen in vivo. These observations also indicate that PMA can be used to enhance collagenase 1 expression, and hence, its detectability, without affecting its limited production by a functionally defined subpopulations of keratinocytes.

A23187 Inhibits Keratinocyte Collagenase 1 Accumulation

To determine the effect of increased intracellular Ca^{2+} levels on MMP production by keratinocytes, primary human cells were treated with 1 μ M A23187. (Primary keratinocytes grown in high calcium medium do not attach to tissue culture plastic and, hence, require an exogenous substratum.) After 16 h, conditioned medium was collected and analyzed by zymography to assess proteinase secretion. A number of proteolytic bands were detected by gelatin zymography in control medium (Figure 2), and these likely represent 92-kDa progelatinase, its activated form at about 82-kDa, 72-

Figure 1 (cont). (Pentland and Needleman, 1986). In the control cultures (Cnt), contact with collagen induced collagenase 1 expression, but signal for the mRNA (arrows) was seen only in cells at the periphery or removed from the differentiating foci. The same subpopulations were seen in PMA-treated keratinocyte cultures. Although expression of collagenase 1 mRNA was up-regulated in response to PMA, signal was still seen only in migrating cells (arrows). Autoradiography was for 14 d. Bar, 40 μ m.

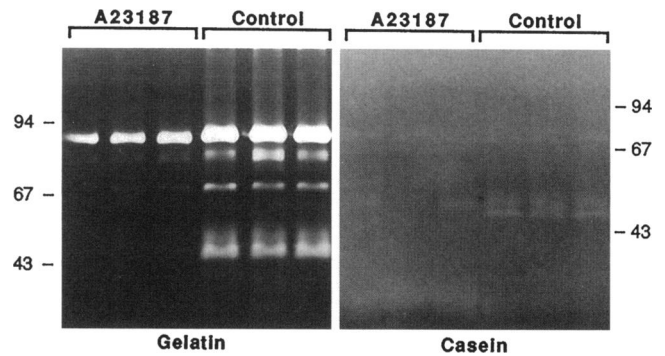


Figure 2. Decreased accumulation of metalloproteinase in A23187-treated keratinocyte-conditioned medium. Primary human keratinocytes were grown in serum-free medium on type I collagen alone (Control) or on collagen plus 1 μ M A23187. Medium samples (equivalent to 150 μ g of protein) from three separate dishes were resolved at 4°C through 10% nonreducing SDS-polyacrylamide gels impregnated with 1 mg/ml gelatin or casein. After electrophoresis, the gels were washed to remove detergents and incubated overnight at 37°C. Gelatinolytic and caseinolytic proteins were identified as clear bands on a dark background after staining with 1% Coomassie blue. Molecular weight standards were used to approximate the molecular weight of protein samples.

kDa progelatinase, and procollagenase 1, which resolves as a doublet at 55–57 kDa. The collagenase 1 bands were also detected by casein zymography, which is a much less sensitive assay than gelatin zymography but more selective for collagenase activity. Gelatinase levels were greatly reduced, and collagenase 1 was eliminated in medium from keratinocytes treated with A23187 (Figure 2). In both gel systems, all proteolytic activity was inhibited by EDTA but not by PMSF, a serine proteinase inhibitor, indicating that the bands detected were MMPs.

To better define the effect of A23187 on keratinocyte MMP production, we quantified the accumulation of collagenase 1 protein in the medium in response to various doses of this ionophore. A23187 mediated a dose-dependent decrease in collagenase 1 accumulation, and at 5 μ M, secreted enzyme was not detected (Figure 3). PMA stimulated collagen-mediated collagenase 1 production about 10-fold, but these enhanced levels were markedly reduced by A23187 (Figure 3). To determine whether inhibition of collagenase 1 accumulation was specific to A23187, we tested another calcium ionophore. Thapsigargin, which increases intracellular calcium levels by specifically inhibiting endoplasmic reticulum Ca^{2+} -ATPase (Takemura *et al.*, 1989; Thastrup *et al.*, 1990), markedly reduced the release of collagenase 1 from control keratinocytes but had only a minor inhibitory effect on PMA-stimulated cells (Figure 3). This weak inhibition in PMA-treated keratinocytes suggests that thapsigargin and A23187 block collagenase 1 accumulation by distinct mechanisms. In agreement with gelatin zymography (Figure 2), A23187 reduced secreted levels of 92-kDa gelati-

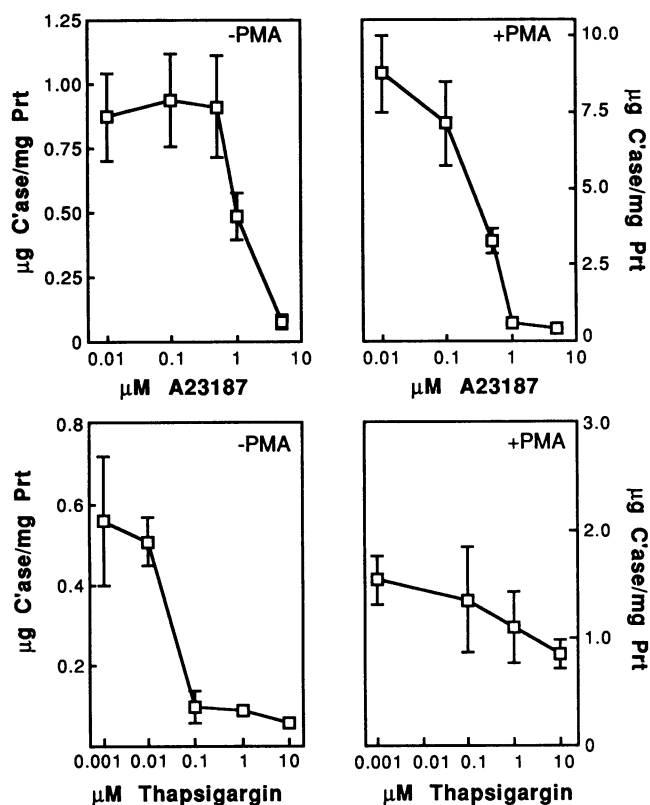


Figure 3. Calcium ionophores inhibit basal and PMA-stimulated accumulation of keratinocyte collagenase 1. Keratinocytes were grown on collagen-coated dishes and were treated with the indicated concentrations of A23187 or thapsigargin. One-half of the cultures were also treated with 5×10^{-9} M PMA. After 48 h, collagenase 1 accumulation in the medium was determined by ELISA and normalized to total protein. The data presented are the mean \pm SD of values from three separate plates. The magnitude of collagen-induced collagenase 1 expression and PMA-mediated stimulation varies among cells isolated from different individuals (Sudbeck *et al.*, 1994), thus accounting for the different scales of the y-axes. (Note: collagenase 1 levels in control cultures, i.e., cells on collagen alone, were not different from the levels detected in cells treated with the lowest dose of thapsigargin or A23187.)

nase about threefold in both control and PMA-treated keratinocytes (Figure 4). Thus, these data indicate that calcium mobilization is involved in the regulation of MMP production by keratinocytes.

Because fluctuations of intracellular Ca^{2+} levels influence numerous cellular events, we measured total protein synthesis to determine whether the production and release of new proteins was modulated by A23187. With ionophore concentrations of ≤ 1 μM , cell-associated radioactivity was not significantly affected, but secreted radiolabeled protein, which accounted for about 3% of total radioactivity, was reduced to about 60% of control levels (Table 1). With 5 μM A23187, cell-associated and secreted protein were reduced about twofold. Therefore, for subsequent ex-

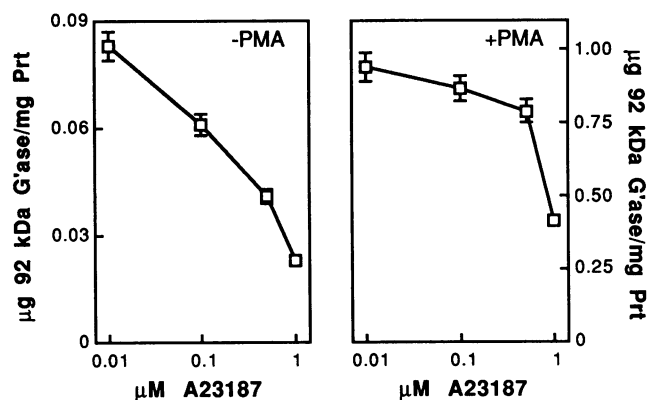


Figure 4. A23187 inhibits basal and PMA-stimulated accumulation of keratinocyte 92-kDa gelatinase. Keratinocytes were grown on collagen-coated dishes and were treated with the indicated concentrations of A23187. One-half of the cultures were also treated with 5×10^{-9} M PMA. After 48 h, the 92-kDa gelatinase accumulation in the medium was determined by ELISA and normalized to total protein. The data presented are the mean \pm SD of values from three separate plates.

periments we used 1 μM A23187, a concentration which reduced collagenase 1 levels without affecting total protein synthesis.

A23187 Selectively Inhibits Collagenase 1 Production in Keratinocytes

HaCaT cells, a human keratinocyte cell line, do not basally produce collagenase 1 or in response to PMA or to contact with type I collagen but do express collagenase 1 upon treatment with epidermal growth factor (EGF). Similar to primary keratinocytes, A23187 caused a dose-dependent decrease in the EGF-stimulated accumulation of HaCaT collagenase 1 protein (Figure 5, HaCaT). A23187 increased collagenase 1 production in synovial and dermal fibroblasts (Unemori and Werb, 1988; Lohi *et al.*, 1994). In our studies, however, A23187 did not affect the low basal secretion of collagenase 1 by human skin fibroblasts (Figure 5, HSFb). In addition, PMA-stimulated levels of collagenase 1 in fibroblasts were not significantly affected with low doses of the ionophore but were slightly increased with 1 μM A23187 (Figure 5, HSFb). These data indicate that cell-specific mechanisms regulate collagenase 1 expression in keratinocytes.

A23187 Does Not Inhibit Collagenase 1 mRNA Accumulation

In most experimental models, the concentration of secreted collagenase 1 correlates with the steady-state levels of its mRNA, but posttranscriptional mechanisms can also contribute to the regulation of enzyme expression (Delany and Brinckerhoff, 1992; Vincenti *et al.*, 1994). To determine the mechanism controlling

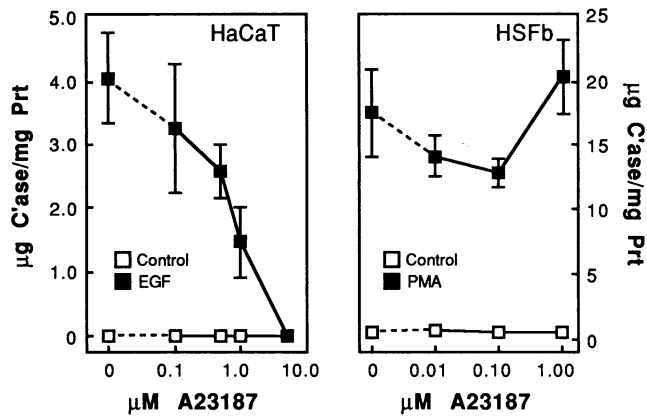


Figure 5. A23187 selectively inhibits collagenase 1 accumulation in keratinocytes. HaCaT keratinocytes and 1905 human skin fibroblasts (HSFb) were grown on uncoated tissue culture plastic plates. Collagenase 1 production was induced in HaCaT cells with 30 ng/ml epidermal growth factor (EGF) and was stimulated in fibroblasts with 10^{-8} M PMA. Both cell types were treated with the indicated concentrations of A23187. After 48 h, collagenase 1 accumulation in the medium was determined by ELISA and normalized to total protein. The data presented are the mean \pm SD of values from three separate plates.

ionophore-mediated inhibition of collagenase 1 accumulation, we assessed enzyme mRNA levels by Northern blot hybridization of total RNA from keratinocytes grown on collagen and treated with A23187 and PMA. Although contact with type I collagen induces collagenase 1 protein accumulation in primary keratinocytes (Saarialho-Kere *et al.*, 1993a; Sudbeck *et al.*, 1994), we could not detect its mRNA by Northern blot hybridization (Figure 6). However, because only a subset of keratinocytes, namely, the migrating cells, express collagenase 1 in response to collagen (Figure 1), its mRNA would be markedly diluted in extracts of total RNA. Collagenase 1 mRNA was detected by RT-PCR, and the relative levels were not affected by increasing concentrations of A23187 (Figure 6). In agreement with the ELISA (Figure 3) and *in situ* hybridization (Figure 1) data, PMA markedly increased collagenase 1 mRNA levels (Figure 6). Cotreatment with A23187, however, did not affect the PMA-mediated stimulation of collagenase 1 mRNA levels (Figure 5), even though the ionophore significantly reduced secreted protein levels (Figures 2 and 3). These data suggest that A23187 inhibits accumulation of collagenase 1 in both control and PMA-stimulated keratinocytes by a posttranscriptional mechanism.

Unlike collagenase 1, levels of 92-kDa gelatinase mRNA in PMA-stimulated keratinocytes dropped about threefold to fivefold in response to A23187 (Figure 6), similar in magnitude to the drop in secreted protein levels (Figure 4). These data indicate that A23187 regulates production of different MMPs in keratinocytes by distinct mechanisms.

Table 1. Total protein synthesis

Treatment	Protein synthesis (cpm/ 10^6 cells)	
	Cell Layer	Medium
Control	17,446 \pm 3,229	712 \pm 120
0.1	23,458 \pm 3,590	478 \pm 59
0.5	18,512 \pm 1,558	408 \pm 43
1.0	14,097 \pm 1,845	413 \pm 68
5.0	8,788 \pm 321	291 \pm 53

Primary human keratinocytes were plated on collagen-coated dishes and treated with the indicated concentration of A23187 for 24 h before the addition of [3 H]leucine as described in MATERIALS AND METHODS. Radioactivity incorporated into trichloroacetic acid-precipitable material was determined in the culture medium and the isolated cells. The data presented are the mean \pm SD of triplicate determinations from three separate plates per treatment.

A23187 Inhibits Collagenase 1 Secretion

Because collagenase 1 mRNA levels were unaffected by A23187 treatment, we assessed at which step collagenase 1 accumulation was blocked. Two possible mechanisms are that translation of collagenase 1 mRNA is inhibited or that the protein is not secreted. To assess these possibilities, we immunoprecipitated collagenase 1 from [35 S]methionine-labeled conditioned medium and keratinocyte cell layer following treatment with A23187 and/or PMA. In agreement with other parameters, the levels of secreted and cell-associated collagenase 1 were markedly increased in PMA-treated cultures compared with those seen in control cultures (Figure 7A). Reflecting the ELISA results (Figure 3), cotreatment with A23187 inhibited collagenase 1 accumulation in the medium; however, A23187 led to an accumulation of intracellular collagenase 1 (Figure 7A). In agreement with our Northern blot data (Figure 6), the sum of the densitometric values for the immunoprecipitated bands of the medium and cell layer indicated that nearly equivalent levels of collagenase 1 were produced in PMA-treated cultures with or without A23187 (Figure 7A). The principle quantitative difference was the distribution of the protein in response to ionophore, suggesting that modulation of intracellular calcium stores regulate collagenase 1 secretion and not translation. In another experiment, A23187 mediated a dose-dependent and marked sequestration of intracellular collagenase 1 in basal (*i.e.*, not PMA-treated) keratinocytes (Figure 7B). Procollagenase 1 typically resolves as a doublet protein of 54 and 52 kDa, separated by approximately 2 kDa, due to differences in glycosylation of the precursor protein. In both experiments, we detected no difference in the migration and pattern of immunoprecipitated procollagenase 1 (Figure 7), suggesting that A23187 treatment did not influence post-translational modification of the enzyme and that the

block in secretion occurred distal to the Golgi apparatus.

Alteration of intracellular calcium levels can hinder protein secretion by affecting protein folding (Lodish and Kong, 1990; Suzuki *et al.*, 1991). To determine whether treatment with A23187 affected the procollagenase 1 structure, we assessed the ability of the proenzyme to cleave itself upon activation with APMA. Treatment with this organomercurial disrupts a conserved cys-his bond which maintains the enzyme in an inactive state. Upon disruption of this bond, the catalytic domain becomes active and cleaves the N-

terminal prodomain, reducing the mass of the enzyme by about 10 kDa. With the addition of APMA, both secreted and cell-associated procollagenase 1, whether from control or A23187-treated keratinocytes, was converted to the lower molecular weight, active form of the enzyme (Figure 7B). The higher molecular weight bands seen above procollagenase 1 in the APMA-treated samples likely represent complexes that form with activated MMPs (Goldberg *et al.*, 1992). The ability of procollagenase 1 to be activated and to cleave itself indicates that A23187 did not cause a change in enzyme conformation.

A23187 Inhibition of Collagenase 1 Accumulation Is Reversible

One explanation for the ionophore effect on collagenase 1 secretion is that as the keratinocytes differentiate and become cornified, they create a physical barrier to secretion. Cornification is irreversible, and cells stimulated to differentiate develop a cornified envelope within a short period of time (Hennings *et al.*, 1980). To assess whether cornification impedes collagenase 1 accumulation, cells were treated with 1 μ M A23187, and 12 h later, one-half of the cultures were washed and incubated in ionophore-free medium for 4 h more. Again, PMA stimulation caused an increase in collagenase 1 accumulation that was blocked by cotreatment with A23187 (Figure 8). However, the ability to secrete collagenase 1 was restored in cultures that were washed and subsequently incubated in A23187-free medium (Figure 8). These results indicate that the effect of A23187 on the ability of keratinocytes to secrete collagenase 1 is reversible upon removal of the ionophore.

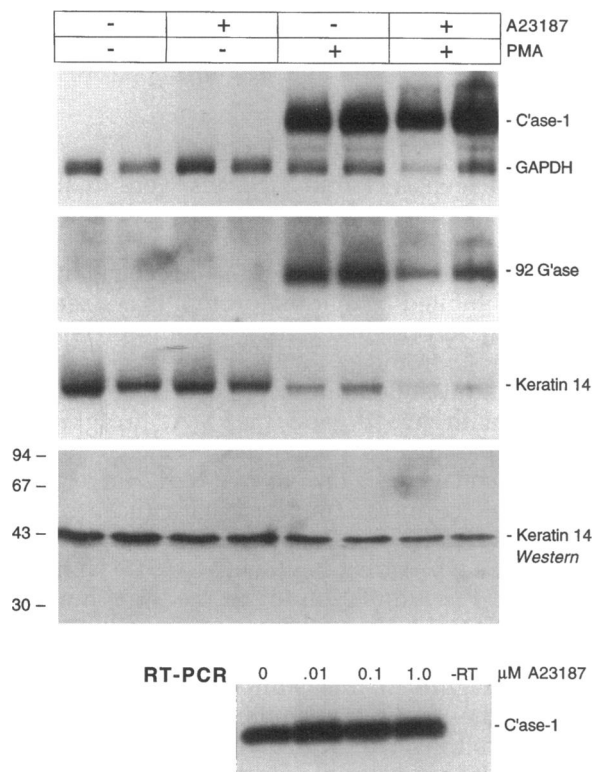
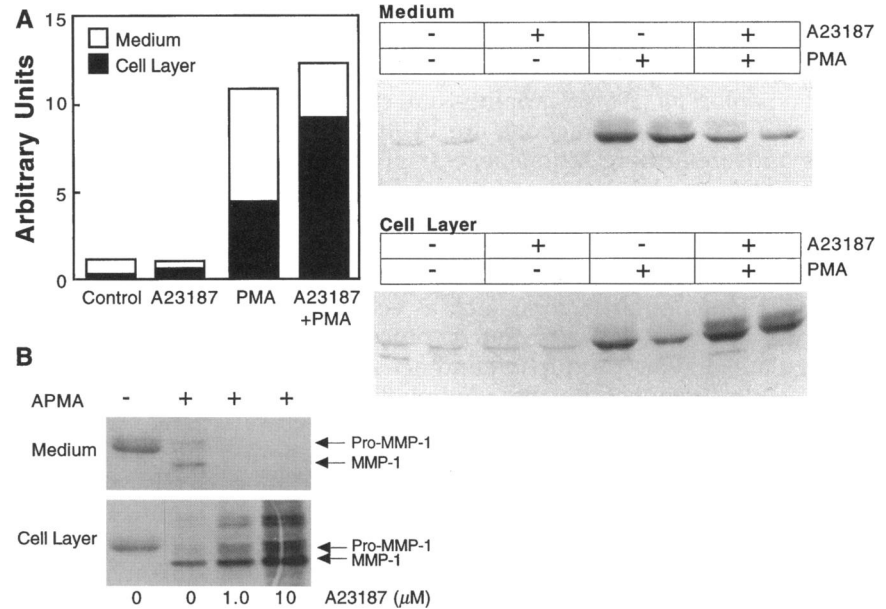


Figure 6. A23187 does not inhibit collagenase 1 mRNA levels. Primary keratinocytes were grown on collagen-coated dishes. As indicated, some cells were treated with 1 μ M A23187 with or without 5×10^{-9} M PMA. Cell layers were harvested 24 h later, and total RNA was isolated and resolved (10 μ g/lane) by agarose-gel electrophoresis. Steady-state levels of collagenase 1, 92-kDa gelatinase, GAPDH, and keratin 14 mRNAs were assessed by Northern blot hybridization with 32 P-labeled cDNAs. Autoradiographic exposure was 24 h. Western blot: a portion of the cell layer was extracted and processed for immunoblotting using an antikeratin 14 antibody. Numbers on the left indicate the migration of molecular weight standards. Bottom panel, keratinocytes were treated with indicated concentrations of A23187, and total RNA was isolated 24 h later. Collagenase 1 mRNA was detected by RT-PCR and Southern blot hybridization. No autoradiographic signal was seen in samples processed without reverse transcriptase (-RT), indicating that the signal seen in the other lanes was RNA dependent. RT-PCR of GAPDH mRNA produced equivalent signal for all samples (data not shown). Autoradiographic exposure was 2 h.

Keratinocyte Differentiation

During wound healing *in vivo*, collagenase 1 is produced only by basal keratinocytes; enzyme expression is not detected in suprabasal cells (Saarialho-Kere *et al.*, 1993a). To determine whether inhibition of collagenase 1 secretion by A23187 correlated with a change in keratinocyte differentiation, we assessed the expression of keratin 14, which is selectively expressed by basal keratinocytes in intact skin. Treatment with A23187 alone did not significantly affect the level of keratin 14 mRNA or protein in control cells (Figure 6). PMA treatment decreased the expression of keratin 14 mRNA (Figure 6), consistent with its differentiation-inducing effects (Hawley-Nelson *et al.*, 1982), and cotreatment with ionophore caused a further decrease in keratin 14 mRNA (Figure 6). The PMA-mediated decrease in keratin 14 protein was accompanied by a slight decrease in protein levels over the 18-h treatment period (Figure 6, Western blot). Because PMA decreased keratin 14 expression while inducing collagenase 1 accumulation, these results suggest that the

Figure 7. A23187 inhibits secretion of collagenase 1 from keratinocytes. (A) Primary keratinocytes were treated for 4 h with 1 μ M A23187, 5 $\times 10^{-9}$ M PMA, or with both compounds, and were then washed with methionine-free medium. Proteins were radiolabeled by pulsing with 50 μ Ci/ml [35 S]methionine in methionine-free DMEM plus additives. After 4 h, the conditioned medium and monolayers were processed for collagenase 1 immunoprecipitation. Radiolabeled proteins were detected by fluorography and were quantified by laser densitometry and expressed as arbitrary units. The duplicate lanes represent data from keratinocytes derived from two separate patients. (B) Keratinocytes from another donor were treated with 1 or 10 μ M A23187 (but no PMA) and metabolically labeled as described above. Before immunoprecipitation, conditioned medium and cell extracts were treated with APMA for 1 h at 37°C. Autoactivated collagenase-1 (MMP-1) migrates faster than the inactive zymogen (Pro-MMP-1).



effect of the ionophore on collagenase 1 secretion is not strictly due to cellular differentiation.

To assess the *in vivo* correlation between collagenase 1 expression and basal cell markers, we stained sections of pyogenic granulomas with antibodies for keratin 14 (basal cells) and keratin 10 (suprabasal cells). Pyogenic granulomas are small, highly vascular lesions with many small ulcerations of the epidermis. As we have reported, pyogenic granulomas display many of the epidermal features seen in acute wounds (Saarialho-Kere *et al.*, 1992, 1993a). In intact skin, keratin 14 was detected only in basal keratinocytes, and keratin 10 was seen in suprabasal cells (Figure 9, top panels). At the wound edge, however, staining for keratin 10 was not detected at the migrating front of the epidermis, even though stratification was evident in this area (Figure 9, middle panels). In contrast, all keratinocytes, whether basal or suprabasal, stained for keratin 14 at the ulcer edge (Figure 9, middle panels). No signal was seen in sections incubated in nonimmune serum. Collagenase 1-expressing keratinocytes were confined to the basal layer at the migrating front and were not seen in any suprabasal layer (Figure 9, bottom panels). These findings, which were consistently seen in 12 specimens, suggest that the migrating front of epidermis is made of keratinocytes with a basal or hyperproliferative phenotype.

DISCUSSION

In the intact epidermis, keratinocytes maintain a natural calcium gradient, with the basal proliferative cells in a low calcium environment and suprabasal cells in

a gradually increasing higher calcium environment (Malmqvist *et al.*, 1984; Menon *et al.*, 1985). Apparently, external calcium plays a role in the development and maintenance of a healthy epithelium. Following wounding and during reepithelialization, distortion of the normal calcium gradient may modify the local environment of the basal keratinocyte and modulate cell phenotype (Grzesiak and Pierschbacher, 1995). Soon after injury, basal keratinocytes at the wound margin change their morphology and behavior from that of a progenitor cell to one appropriate for their new role as a migratory cell. Our *in vivo* studies on a variety of chronic and normally healing wounds indicate that collagenase 1 is always expressed by migrating basal keratinocytes at the wound edge (Saarialho-Kere *et al.*, 1992, 1993a, 1994). The invariant expression of collagenase 1 in wounded skin indicates that this enzyme serves a critical role in epidermal repair. Indeed, as we recently reported (Pilcher *et al.*, 1997), the catalytic activity of collagenase 1 is required for the migration of keratinocytes over a collagen matrix.

At the onset and during epidermal migration, keratinocytes contact extracellular matrix components of the dermal and provisional matrices, and these altered cell-matrix interactions may provide early and sustained signals informing keratinocytes that they are in an injured environment. In addition, expression of collagenase 1 mRNA is rapidly shut off once reepithelialization is completed and the basement is restored (Saarialho-Kere *et al.*, 1993a; Inoue *et al.*, 1995). Although a compelling argument can be made for the role of cell-matrix interactions in initiating collagenase 1 expression and activating basal keratinocytes in

wounds, other factors likely contribute to regulating proteinase production. For example, collagenase 1 is overexpressed in chronic ulcers compared with acute wounds (Sank *et al.*, 1989; Saarialho-Kere *et al.*, 1993a). Because cell-matrix interactions are likely similar in healing and nonhealing lesions, excess production of collagenase 1 may be stimulated by soluble factors in the wound environment. In addition, our *in vitro* data demonstrate that only migrating keratinocytes express collagenase 1, although all cells are grown on collagen (Figure 1). Thus, other factors, such as cell-cell contacts and differentiation-associated processes, may also regulate enzyme production and activity.

Our results demonstrate that ionophore-mediated mobilization of intracellular calcium stores blocked secretion of collagenase 1 by keratinocytes without affecting the steady-state levels of its mRNA. Furthermore, chloramphenicol acetyltransferase transcription under control of a 2.2-kb fragment of human collagenase 1 promoter was not affected by ionophore treatment (data not shown but provided to reviewers), indicating that collagenase 1 gene transcription was not altered by modulation of intracellular calcium levels. Previously, we demonstrated that expression from this construct parallels modulation of endogenous collagenase 1 production in keratinocytes (Sudbeck *et al.*, 1994). In contrast to keratinocytes, calcium ionophores stimulate collagenase expression by fibroblasts (Unemori and Werb, 1988), although our data showed only a minimal, if any increase (Figure 5). Thus, inhibition of enzyme secretion by increased intracellular calcium concentration appears to be a keratinocyte-

specific mechanism to control collagenase 1 activity. Analogously, transforming growth factor- β 1 represses collagenase 1 expression in fibroblasts but stimulates production in keratinocytes (Mauviel *et al.*, 1996), indicating further that this metalloproteinase is regulated by cell-type-specific mechanisms. In contrast, A23187 inhibits expression of the 92-kDa gelatinase enzyme by keratinocytes (Figures 2 and 4) and fibrosarcoma cells (Lohi and Keski-Oja, 1995). Thus, our findings support the idea that different MMPs are regulated differently in different cell types.

In agreement with earlier findings (Kallioinen *et al.*, 1995), all keratinocytes of the migrating front of epidermis stained for keratin 14, a basal cell marker, but none stained with antibody for keratin 10, a suprabasal-specific protein (Figure 9). Because suprabasal cells at the migrating front do not express collagenase 1 mRNA, these *in vivo* observations support the idea that induction and maintenance of gene expression of this metalloproteinase is regulated more by the location of the producing cell rather than its state of differentiation. As stated, interaction with type I collagen induces collagenase 1 gene expression in keratinocytes (Sudbeck *et al.*, 1994), and loss of contact with the matrix may down-regulate transcription without concomitantly reducing mRNA stability or inhibiting enzyme translation. Indeed, we have observed collagenase 1 protein in suprabasal cells in many wounds (Saarialho-Kere *et al.*, 1993a). If secreted and activated, collagenase 1 may degrade potential substrates in the epidermis. Although fibrillar collagens are the preferred substrates for collagenase 1, the enzyme can degrade other molecules, such as proteoglycans and serpins (Desrochers *et al.*, 1991; Halpert *et al.*, 1996). Extracellular inhibitors are probably not plentiful in the extracellular space surrounding suprabasal keratinocytes. For example, tissue inhibitor of metalloproteinase 1 is expressed by basal keratinocytes in wounded skin but only transiently during the initial phase of reepithelialization (Stricklin *et al.*, 1993). Thus, blocking secretion of collagenase 1 from keratinocytes may provide an important mechanism to rapidly decrease the release of this enzyme which is no longer needed once reepithelialization is completed and stratification resumes.

Modulation of calcium levels may provide a means to block secretion of more than just collagenase 1. After all, secretion of many cell products is affected by calcium concentration, and indeed, the slight decrease we detected in soluble radioactive protein (Table 1) suggests that secretion of proteins, in addition to collagenase 1, was also inhibited by A23187. Inhibition of the secretion of multiple proteins, however, does not diminish the relevance of our findings. Although collagenase 1 production and release are reliable markers of activated basal keratinocytes, these cells make other proteins as well, such as integrins, basement mem-

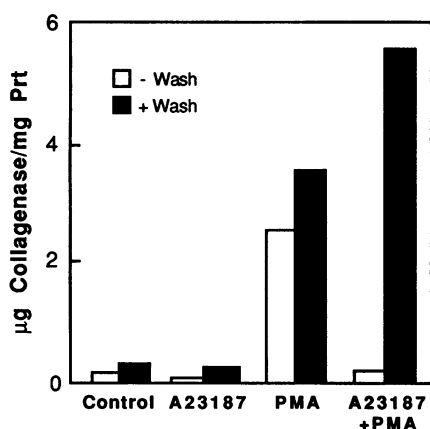


Figure 8. Reversibility of A23187 inhibition of collagenase 1 secretion. Primary keratinocytes were grown on collagen-coated dishes. As indicated, some cultures were treated with 1 μ M A23187, with 5×10^{-9} M PMA, with both compounds, or neither (Control). At 12 h, the treatment media were removed from one-half of the cultures (+Wash) and replaced with fresh medium with or without PMA and lacking ionophore. The medium in the other dishes (-Wash) was not changed. After 16 h, levels of secreted collagenase 1 were determined by ELISA and normalized to total protein. The data presented are the mean values of three separate plates.

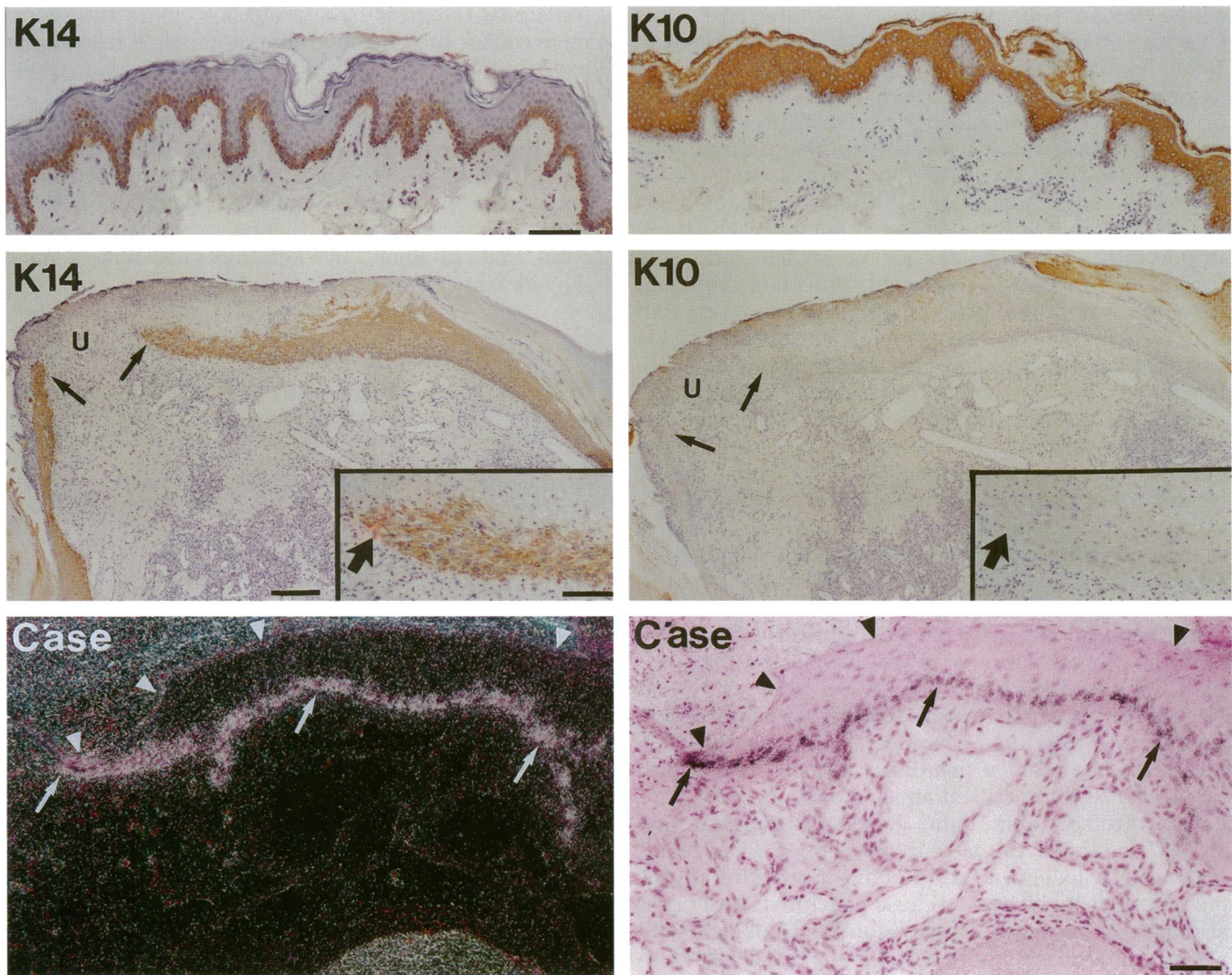


Figure 9. Keratin expression in the migrating front of keratinocytes in wounds. Sections of pyogenic granuloma ($n = 12$) were stained with antibodies for keratin 14 (K14) or keratin 10 (K10). The top micrographs show an area of intact skin within the pyogenic granuloma biopsy and the predictable presence of keratin 14 in the basal layer of the epidermis and keratin 10 in the suprabasal layers. Bar, $100 \mu\text{m}$ for top panels. In the middle micrographs, an ulceration (U) bordered by two migrating tongues of epidermis (arrows). The inset shows a higher magnification of the forward extent of the horizontal migrating front. At the wound edge, staining for keratin 14 was seen in both basal and suprabasal keratinocytes, but keratin 10 was not detected. Bars, $240 \mu\text{m}$ for middle panels and $50 \mu\text{m}$ for insets, respectively. The bottom panels show dark-field and bright-field illuminated views of a section hybridized for collagenase 1 mRNA. Autoradiographic signal is seen only in basal keratinocytes (arrows) at the ulcer edge and not in any suprabasal cell. The upper extent of the epidermis is marked by arrowheads. Autoradiographic exposure was for 14 d. Bar, $50 \mu\text{m}$ for bottom panels.

brane proteins, cytokines, and other proteinases (Røomer *et al.*, 1991; Saarialho-Kere *et al.*, 1993a, 1994; Fini *et al.*, 1996). Modulation of calcium levels may contribute to regulating the site-specific expression of many, if not all, of these markers.

Depletion of calcium from the endoplasmic reticulum can inhibit protein secretion by affecting protein folding (Lodish and Kong, 1990; Suzuki *et al.*, 1991), but we do not believe that a change in enzyme conformation contributed to the inefficient release of collagenase 1 from keratinocytes exposed to A23187. Col-

lagenase 1, as for all MMPs, has an N-terminal prodomain of 8 kDa that is responsible for maintaining the enzyme in an inactive or zymogen state, a catalytic domain of about 21 kDa that binds the active site Zn^{2+} , and other domains that contribute to defining substrate specificity. The zinc-coordinating region contains three conserved histidine residues which represent three of four zinc-interactive ligands (Goldberg *et al.*, 1986). The propeptide is characterized by the invariant sequence PRCGVPD, with the cysteine residue representing the fourth interactive ligand of the

active site Zn²⁺. Enzyme latency is conferred by this Cys–Zn interaction (Van Wart and Birkedal-Hansen, 1990). For MMP zymogens to be activated, the Cys–Zn association must be disrupted, which is thought to occur *in vivo* by cleavage of the prodomain by another protease. However, any mechanism that disrupts this bond, including nonproteolytic processes, such as treatment with chaotropes, sulfhydryl chelators, and reactive oxygen species, leads to enzyme activation. Importantly, and relevant to our article, once the Cys–Zn interaction is disrupted, procollagenase will cleave its N-terminal prodomain, producing a protein that resolves in a polyacrylamide gel about 10 kDa smaller than the zymogen. Our data in Figure 7B show that in response to APMA intracellular and secreted procollagenase 1, in both control and A23187-treated keratinocytes, was converted to the active form of the enzyme. Because improper folding would likely hinder autoactivation, which requires the proper function of the zinc-binding and more C-terminal domains, we conclude that inhibition of collagenase 1 secretion by calcium ionophore is not caused by improper protein folding.

All metalloproteinases have two conserved calcium-binding domains located near the zinc-binding domain (Birkedal-Hansen *et al.*, 1993). Thus, if altered calcium levels caused misfolding of procollagenase 1 and, in turn, hindered secretion of the enzyme, then release of the 92-kDa gelatinase, which shares high homology in these critical areas, would likely be similarly affected. Our data, however, suggest that A23187-mediated down-regulation of 92-kDa gelatinase mRNA levels and did not affect secretion of this enzyme. Furthermore, because calcium ionophores stimulate collagenase 1 expression by fibroblasts with a concomitant increase in soluble enzyme levels (Unemori and Werb, 1988), the block in collagenase 1 secretion from keratinocytes is seemingly a cell-specific response to modulation of intracellular stores and is not a common effect of treatment with ionophores.

The potential importance of this proposed mechanism is highlighted by observations in transgenic mice which demonstrated that forced expression of collagenase 1 in suprabasal keratinocytes leads to severe epidermal defects such as markedly thickened living skin (acanthosis) and cornified layers (hyperkeratosis; D'Armiento *et al.*, 1995). Similarly, functional expression of other basal cell-specific proteins can cause epidermal anomalies. At the completion of reepithelialization, integrins which are normally confined to the basal keratinocytes are seen for a few days posthealing in the newly formed suprabasal cells (Hertle *et al.*, 1992; Juhasz *et al.*, 1993). Similar to what we propose here for preventing collagenase 1 secretion, posttranscriptional mechanisms inhibit the transport of these matrix receptors to the surface of suprabasal cells (Hotchin and Watt, 1992). However, expression of

functional integrins by suprabasal cells in transgenic mice causes hyperproliferative anomalies resembling psoriasis (Carroll *et al.*, 1995). In addition, overexpression of keratin 16, which is normally expressed by postmitotic cells at the wound edge, causes acanthosis and hyperkeratosis (Takahashi *et al.*, 1994). Thus, altered calcium levels may provide a mechanism to prevent the potentially deleterious secretion of collagenase 1, as well as production or activity of other basal cell proteins, by keratinocytes which have moved away from the stratum basalis.

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REFERENCES

- Birkedal-Hansen, H., Moore, W.G.I., Bodden, M.K., Windsor, L.J., Birkedal-Hansen, B., DeCarlo, A., and Engler, J.A. (1993). Matrix metalloproteinases: a review. *Crit. Rev. Oral Biol. Med.* 4, 197–250.
- Boukamp, P., Petrussevska, R.T., Breitkreutz, D., Hornung, J., Markham, A., and Fusenig, N.E. (1988). Normal keratinization in a spontaneously immortalized aneuploid human keratinocyte cell line. *J. Cell Biol.* 106, 761–771.
- Busiek, D.F., Baragi, V., Nehring, L.C., Parks, W.C., and Welgus, H.G. (1995). Matrilysin expression by human mononuclear phagocytes and its regulation by cytokines and hormones. *J. Immunol.* 154, 6484–6491.
- Carroll, J.M., Romero, M.R., and Watt, F.M. (1995). Suprabasal integrin expression in the epidermis of transgenic mice results in developmental defects and phenotype resembling psoriasis. *Cell* 83, 957–968.
- D'Armiento, J.D., DiColandrea, T., Dalal, S.S., Okada, Y., Huang, M.-T., Conney, A.H., and Chada, K. (1995). Collagenase expression in transgenic mouse skin causes hyperkeratosis and acanthosis and increases susceptibility to tumorigenesis. *Mol. Cell. Biol.* 15, 5732–5739.
- Delany, A.M., and Brinckerhoff, C.E. (1992). Post-transcriptional regulation of collagenase and stromelysin gene expression by epidermal growth factor and dexamethasone in cultured human fibroblasts. *J. Cell. Biochem.* 50, 400–410.
- Desrochers, P.E., Jeffrey, J.J., and Weiss, S.J. (1991). Interstitial collagenase (matrix metalloproteinase-1) expresses serpinase activity. *J. Clin. Invest.* 87, 2258–2265.
- Fini, M.E., *et al.* (1996). Role of matrix metalloproteinases in failure to re-epithelialize following corneal injury. *Am. J. Pathol.* 149, 1287–1302.
- Fuchs, E. (1990). Epidermal differentiation: the bare essentials. *J. Cell Biol.* 111, 2897–2814.
- Goldberg, G.I., Strongin, A., Collier, I.E., Genrich, L.T., and Marmer, B.L. (1992). Interaction of 92-kDa type IV collagenase with the tissue inhibitor of metalloproteinases prevents dimerization, complex formation with interstitial collagenase, and activation of the proenzyme with stromelysin. *J. Biol. Chem.* 267, 4583–4591.

- Goldberg, G.I., Wilhelm, S.M., Kronberger, A., Bauer, E.A., Grant, G.A., and Eisen, A.Z. (1986). Human fibroblast collagenase. Complete primary structure and homology to an oncogene transformation-induced rat protein. *J. Biol. Chem.* 261, 6600–6605.
- Grzesiak, J.J., and Pierschbacher, M.D. (1995). Shifts in the concentrations of magnesium and calcium in early porcine and rat wound fluids activate the cell migratory response. *J. Clin. Invest.* 95, 227–233.
- Halpert, I., Roby, J.D., Sires, U.I., Potter-Perigo, S., Wight, T.N., Welgus, H.G., Shapiro, S.D., Wickline, S.A., and Parks, W.C. (1996). Matrilysin is expressed by lipid-laden macrophages at sites of potential rupture in atherosclerotic lesions and localizes to areas of versican deposition, a proteoglycan substrate for the enzyme. *Proc. Natl. Acad. Sci. USA* 93, 9748–9753.
- Hawley-Nelson, P., Stanley, J.R., Schmidt, J., Gullino, M., and Yuspa, S.H. (1982). The tumor promoter, 12-O-tetradecanoylphorbol-13-acetate accelerates keratinocyte differentiation and stimulates growth of an unidentified cell type in cultured human epidermis. *Exp. Cell Res.* 137, 155–167.
- Hennings, H., Michael, D., Cheng, C., Steinert, P., Holbrook, K., and Yuspa, S.H. (1980). Calcium regulation of growth and differentiation of mouse epidermal cells in culture. *Cell* 19, 245–254.
- Herron, G.S., Banda, M.J., Clark, E.J., Gavrilovic, J., and Werb, Z. (1986). Secretion of metalloproteinases by stimulated capillary endothelial cells. *J. Biol. Chem.* 261, 2814–2818.
- Hertle, M.D., Kubler, M.-D., Leigh, I.M., and Watt, F.M. (1992). Aberrant integrin expression during epidermal wound healing and in psoriatic epidermis. *J. Clin. Invest.* 89, 1892–1901.
- Hotchin, N.A., and Watt, F.M. (1992). Transcriptional and post-translational regulation of beta 1 integrin expression during keratinocyte terminal differentiation. *J. Biol. Chem.* 267, 14852–14858.
- Inoue, M., Kratz, G., Haegerstrand, A., and Stähle-Bäckdahl, M. (1995). Collagenase expression is rapidly induced in wound-edge keratinocytes after acute injury in human skin, persists during healing, and stops at re-epithelialization. *J. Invest. Dermatol.* 104, 479–483.
- Juhász, I., Murphey, G.F., Yan, H.-C., Herlyn, M., and Albelda, S.M. (1993). Regulation of extracellular matrix proteins and integrin cell substratum adhesion receptors on epithelium during cutaneous wound healing in vivo. *Am. J. Pathol.* 143, 1458–1469.
- Kallioinen, M., Koivukangas, V., Jarvinen, M., and Oikarinen, A. (1995). Expression of cytokeratins in regenerating human epidermis. *Br. J. Dermatol.* 133, 830–835.
- Kubler, M.D., and Watt, F.M. (1993). Changes in the distribution of actin-associated proteins during epidermal wound healing. *J. Invest. Dermatol.* 100, 785–789.
- Lodish, H.F., and Kong, N. (1990). Perturbation of cellular calcium blocks exit of secretory proteins from the rough endoplasmic reticulum. *J. Biol. Chem.* 265, 10893–10899.
- Lohi, J., Kähäri, V.M., and Keski-Oja, J. (1994). Cyclosporin A enhances cytokine and phorbol ester-induced fibroblast collagenase expression. *J. Invest. Dermatol.* 102, 938–944.
- Lohi, J., and Keski-Oja, J. (1995). Calcium ionophores decrease pericellular gelatinolytic activity via inhibition of 92-kDa gelatinase expression and decrease of 72-kDa gelatinase activation. *J. Biol. Chem.* 270, 17602–17609.
- Malmqvist, K.G., Carlsson, L.-E., Forslind, B., Roomans, G.M., and Akselsson, K.R. (1984). Proton and electron microprobe analysis of human skin. *Nucl. Instr. Methods Physics Res. B* 3, 611–617.
- Matrisian, L.M., McDonnell, S., Miller, D.B., Navre, M., Sefter, E.A., and Hendrix, M.J.C. (1991). The role of the matrix metalloproteinase stromelysin in the progression of squamous cell carcinomas. *Am. J. Med. Sci.* 302, 157–162.
- Mauviel, A., Chung, K.Y., Agarwal, A., Tamai, K., and Uitto, J. (1996). Cell-specific induction of distinct oncogenes of the jun family is responsible for differential regulation of collagenase gene expression by transforming growth factor-beta in fibroblasts and keratinocytes. *J. Biol. Chem.* 271, 10917–10923.
- Menon, G.K., Grayson, S., and Elias, P.M. (1985). Ionic calcium reservoirs in mammalian epidermis: ultrastructural localization by ion-capture cytochemistry. *J. Invest. Dermatol.* 84, 508–512.
- Mignatti, P., Rifkin, D.B., Welgus, H.G., and Parks, W.C. (1996). Proteinases and tissue remodeling. In: *The Molecular and Cellular Biology of Wound Repair*, ed. R.A.F. Clark, 2nd ed., New York: Plenum Press, 427–474.
- Parks, W.C. (1995). The production, role, and regulation of matrix metalloproteinases in the healing epidermis. *Wounds* 7, 23A–37A.
- Parks, W.C., Secrist, H., Wu, L.C., and Mecham, R.P. (1988). Developmental regulation of tropoelastin isoforms. *J. Biol. Chem.* 263, 4416–4423.
- Parks, W.C., and Sires, U.I. (1996). Matrix metalloproteinases and skin biology. *Curr. Opin. Dermatol.* 3, 240–247.
- Pei, D., and Weiss, S.J. (1996). Transmembrane-deletion mutants of the membrane-type matrix metalloproteinase-1 process progelatinase A and express intrinsic matrix-degrading activity. *J. Biol. Chem.* 271, 9135–9140.
- Pentland, A.P., and Needleman, P. (1986). Modulation of keratinocyte proliferation in vitro by endogenous prostaglandin synthesis. *J. Clin. Invest.* 77, 246–251.
- Pilcher, B.K., Dumin, J.A., Sudbeck, B.D., Krane, S.M., Welgus, H.G., and Parks, W.C. (1997). The activity of collagenase I is required for keratinocyte migration on a type I collagen matrix. *J. Cell Biol.* (*in press*).
- Prosser, I.W., Stenmark, K.R., Suthar, M., Crouch, E.C., Mecham, R.P., and Parks, W.C. (1989). Regional heterogeneity of elastin and collagen gene expression in intralobar arteries in response to hypoxic pulmonary hypertension as demonstrated by in situ hybridization. *Am. J. Pathol.* 135, 1073–1088.
- Rømer, J., Lund, L.R., Eriksen, J., Ralfkier, E., Zeheb, R., Gelehrter, T.D., Danø, K., and Kristensen, P. (1991). Differential expression of urokinase-type plasminogen activator and its type-1 inhibitor during healing of mouse skin wounds. *J. Invest. Dermatol.* 97, 803–811.
- Saarialho-Kere, U.K., Chang, E.S., Welgus, H.G., and Parks, W.C. (1992). Distinct localization of collagenase and TIMP expression in wound healing associated with ulcerative pyogenic granuloma. *J. Clin. Invest.* 90, 1952–1957.
- Saarialho-Kere, U.K., Crouch, E.C., and Parks, W.C. (1995). Matrix metalloproteinase matrilysin is constitutively expressed in human exocrine epithelium. *J. Invest. Dermatol.* 105, 190–196.
- Saarialho-Kere, U.K., Kovacs, S.O., Pentland, A.P., Olerud, J., Welgus, H.G., and Parks, W.C. (1993a). Cell-matrix interactions modulate interstitial collagenase expression by human keratinocytes actively involved in wound healing. *J. Clin. Invest.* 92, 2858–2866.
- Saarialho-Kere, U.K., Kovacs, S.O., Pentland, A.P., Parks, W.C., and Welgus, H.G. (1994). Distinct populations of keratinocytes express stromelysin-1 and -2 in chronic wounds. *J. Clin. Invest.* 94, 79–88.
- Saarialho-Kere, U.K., Welgus, H.G., and Parks, W.C. (1993b). Divergent mechanisms regulate interstitial collagenase and 92 kDa gelatinase expression in human monocytic-like cells exposed to bacterial endotoxin. *J. Biol. Chem.* 268, 17354–17361.
- Sank, A., Chi, M., Shima, T., Reich, R., and Martin, G.R. (1989). Increased calcium levels alter cellular and molecular events in wound healing. *Surgery* 106, 1141–1147.

- Sharpe, G.R., Fisher, C., Gillespie, J.I., and Greenwell, J.R. (1993). Growth and differentiation stimuli induce different and distinct increases in intracellular free calcium in human keratinocytes. *Arch. Dermatol. Res.* 284, 445–450.
- Stricklin, G.P., Li, L., Jancic, V., Wenczak, B.A., and Nanney, L.B. (1993). Localization of mRNAs representing collagenase and TIMP in sections of healing human burn wounds. *Am. J. Pathol.* 143, 1657–1666.
- Sudbeck, B.D., Parks, W.C., Welgus, H.G., and Pentland, A.P. (1994). Collagen-mediated induction of keratinocyte collagenase is mediated by tyrosine kinase and protein kinase C activities. *J. Biol. Chem.* 269, 30022–30029.
- Suzuki, C.K., Bonifacino, J.S., Lin, A.Y., Davis, M.M., and Klausner, R.D. (1991). Regulating the retention of T-cell receptor alpha chain variants within the endoplasmic reticulum: Ca(2+)-dependent association with BiP. *J. Cell Biol.* 114, 189–205.
- Swee, M.H., Parks, W.C., and Pierce, R.A. (1995). Developmental regulation of elastin production. Expression of tropoelastin pre-mRNA persists after downregulation of steady-state mRNA levels. *J. Biol. Chem.* 270, 14899–14906.
- Takahashi, K., Folmer, J., and Coulombe, P.A. (1994). Increased expression of keratin 16 causes anomalies in the cytoarchitecture and keratinization in transgenic mouse skin. *J. Cell Biol.* 127, 505–520.
- Takemura, H., Hughes, A.R., T, O., and Putney, J.W. (1989). Activation of calcium entry by the tumor promoter thapsigargin in parotid acinar cells. Evidence that an intracellular calcium pool and not an inositol phosphate regulates calcium fluxes at the plasma membrane. *J. Biol. Chem.* 264, 12266–12271.
- Thastrup, O., Cullen, P.J., Drøbak, B.K., Hanley, M.R., and Dawson, A.P. (1990). Thapsigargin, a tumor promoter, discharges intracellular Ca²⁺ stores by specific inhibition of the endoplasmic reticulum Ca²⁺(+)-ATPase. *Proc. Natl. Acad. Sci. USA* 87, 2466–2470.
- Unemori, E.N., and Werb, Z. (1988). Collagenase expression and endogenous activation in rabbit synovial fibroblasts stimulated with the calcium ionophore A23187. *J. Biol. Chem.* 263, 16252–16259.
- Van Wart, H.E., and Birkedal-Hansen, H. (1990). The cysteine switch: A principle of regulation of metalloproteinase activity with potential applicability to the entire matrix metalloproteinase gene family. *Proc. Natl. Acad. Sci. USA* 87, 5578–5582.
- Vincenti, M.P., Coon, C.I., Lee, O., and Brinckerhoff, C.E. (1994). Regulation of collagenase gene expression by IL-1 beta requires transcriptional and post-transcriptional mechanisms. *Nucleic Acids Res.* 22, 4818–4827.
- Werb, Z. (1989). Proteinases and matrix degradation. In: *Textbook of Rheumatology*. ed. W.N. Kelley, E.D. Harris, Jr., S. Ruddy, and C.B. Sledge, Philadelphia: W.B. Saunders, 300–326.
- Wilson, C.L., Heppner, K.J., Rudolph, L.A., and Matrisian, L.M. (1995). The metalloproteinase matrilysin is preferentially expressed by epithelial cells in a tissue-restricted pattern in the mouse. *Mol. Biol. Cell* 6, 851–869.
- Yuspa, S.H., Kilkenny, A.E., Steinert, P.M., and Roop, D.R. (1989). Expression of murine epidermal differentiation markers is tightly regulated by restricted extracellular calcium concentrations in vitro. *J. Cell Biol.* 109, 1207–1217.