Mechanisms of Hepatocyte Growth Factor Stimulation of Keratinocyte Metalloproteinase Production*

Sarah E. Dunsmore‡§, Jeffrey S. Rubin¶, Stephen O. Kovacs‡, Marcio Chedid¶, William C. Parks‡§, and Howard G. Welgus‡

From the Departments of §Medicine (Dermatology) and ¶Cell Biology and Physiology, Washington University School of Medicine, St. Louis, Missouri 63110 and the ¶Laboratory of Cellular and Molecular Biology, National Cancer Institute, Bethesda, Maryland 20892

Matrix metalloproteinases participate in normal physiologic processes; however, their overproduction has been associated with connective tissue destruction in a variety of pathological states. Migrating basal keratinocytes transiently express collagenase-1 during normal cutaneous reepithelialization. However, the overexpression of collagenase-1 and stromelysin-1 has been associated with the pathogenesis of chronic nonhealing ulcers. aberrant expression of metalloproteinases in inflammation is mediated, at least in part, by soluble factors. Since hepatocyte growth factor/scatter factor (HGF/SF) has been reported to promote keratinocyte migration and proliferation, key events in wound repair, and since HGF/SF is produced by dermal fibroblasts and its c-Met receptor is expressed by basal keratinocytes in wounded skin, we have studied the effects of HGF/SF upon keratinocyte metalloproteinase expression. We have found that HGF/SF can stimulate keratinocyte collagenase-1 and stromelysin-1 production in a dose-dependent and matrix-dependent manner. Expression of 92-kDa gelatinase was not affected by HGF/SF. We determined that HGF/SF regulation of collagenase-1 expression is transcriptionally mediated and requires tyrosine kinase and protein kinase C activations. HGF/SF, hepatocyte growth factor/scatter factor; DMEM, Dulbecco's modified Eagle's medium; ELISA, enzyme-linked immunosorbent assay; CAT, chloramphenicol acetyltransferase; MDCK, Madin-Darby canine kidney.

Although cell:matrix interactions may regulate the normal expression of collagenase-1 in healing wounds, the overproduction of this proteinase, along with the induction of stromelysin-1 in chronic ulcers, suggests that additional factors may contribute to the aberrant production of metalloproteinases by keratinocytes. In the present study, we examined the effects of hepatocyte growth factor/scatter factor (HGF/SF) on the production of collagenase-1 and stromelysin-1 by keratinocytes. Originally characterized as a hepatocyte mitogen (23), HGF/SF stimulates the migration and proliferation of many epithelial cell types, including keratinocytes (24, 25). HGF/SF is produced by dermal fibroblasts (26–28), as well as by other cells of mesenchymal origin. It is secreted as a 90-kDa monomer, which is proteolytically converted to a biologically active heterodimer consisting of a 60-kDa α chain and a 30-kDa β chain. The α chain contains an N-terminal hairpin loop (N) and four kringle domains (K1, K2, K3, and K4); the β chain is a serine proteinase-like structure but lacks proteolytic activity due to 2 amino acid substitutions in the catalytic triad. Binding of HGF/SF to its tyrosine kinase receptor, c-Met (29, 30), appears to be mediated primarily by the N, K1, and possibly K2 domains (31–37). Two truncated isoforms of HGF/SF, extending from the amino terminus through either the first (HGF/NK1) or second (HGF/NK2) kringle domain, retain a high affinity for

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† To whom correspondence should be addressed: Division of Dermatology, Barnes-Jewish Hospital, 216 South Kingshighway Blvd., St. Louis, MO 63110. Tel.: 314-454-7073; Fax: 314-454-8293; E-mail: dunsmore_s@wums.wustl.edu.

The abbreviations used are: MMP, matrix metalloproteinase; HGF/SF, hepatocyte growth factor/scatter factor; DMEM, Dulbecco’s modified Eagle’s medium; ELISA, enzyme-linked immunosorbent assay; CAT, chloramphenicol acetyltransferase; MDCK, Madin-Darby canine kidney.

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c-Met and exhibit agonist or antagonist activity relative to HGF/SF, depending on the assay used (34, 35, 37, 38).

Our results indicate that HGF/SF potently stimulates the production of collagenase-1 and stromelysin-1 by keratinocytes. Because HGF/SF induces keratinocyte matrix metalloproteinase expression and also stimulates keratinocyte migration and proliferation, this epithelial mitogen may have an important role in regulating wound healing responses.

EXPERIMENTAL PROCEDURES

Keratinocyte Isolation and Culture—Human keratinocytes were isolated from healthy adult skin obtained from reduction mammoplasties or skin grafts as described elsewhere (39). Briefly, subcutaneous fat and deep dermis were removed. The remaining tissue was incubated in phosphate-buffered saline containing 0.25% trypsin for 16 h. Following separation of epidermis from dermis, keratinocytes were scraped into Dulbecco’s modified Eagle’s medium (DMEM) containing 5% fetal calf serum.

Unless otherwise indicated, keratinocytes were plated on tissue culture dishes coated with bovine type I collagen (Vitrogen, Celsior, Santa Clara, CA). Previously, we demonstrated that contact with native type I collagen is necessary for induction of collagenase-1 expression in these cells (18, 21). Medium was changed on alternate days until cells reached the appropriate level of confluency. Under these conditions, keratinocytes differentiate and stratify similar to cells in vivo, but are not amendable to passage and cannot be grown on tissue culture plastic that is not coated with extracellular matrix.

Enzyme-linked Immunosorbent Assay (ELISA)—Collagenase-1 content of keratinocyte conditioned medium was measured by indirect competitive ELISA (40). This assay is specific for collagenase-1 (MMP-1), has nanogram sensitivity, and detects both active and inactive forms of the enzyme, as well as collagenase-1 bound to TIMP (tissue inhibitor of metalloproteinases) or bound to substrate. Results were normalized to total protein content of the cell layers as determined by the BCA protein assay (Fierce, Rockford, IL) using bovine serum albumin as a standard.

Metabolic Labeling of Cells—One day post-confluence, medium was replaced, and cells were treated with HGF/SF (R&D Systems, Minneapolis, MN) or other reagents as indicated. Following a 24-h incubation, medium was replaced with methionine-free DMEM containing 5% dialyzed fetal calf serum (to remove free amino acids), 1 mM sodium pyruvate, 2 mM l-glutamine, 0.1 mM each of nonessential amino acids, 50 μCi/ml [35S]methionine, and the identical concentrations of experimental reagents. Conditioned medium was collected 24 h later.

Immunoprecipitation—Specific polyclonal antisera to collagenase-1 (41), stromelysin-1 (42) or 92-kDa gelatinase (43) were used to immunoprecipitate the [35S]-labeled metalloproteinases from keratinocyte-conditioned medium as described previously (44). Samples were preclared with protein A-Sepharose (Zymed, San Francisco, CA) for 20 min at 4°C. Following a brief centrifugation to collect the beads, supernatants were incubated with antibody for 1 h at 37°C and overnight at 4°C. Immune complexes were precipitated with protein A-Sepharose and washed extensively. Radiolabeled proteins were resolved by polyacrylamide gel electrophoresis and visualized by fluorography.

RNA Isolation—RNA was isolated using guanidine thiocyanate and phenol (45). Cells were scraped into a solution of 4 M guanidine thiocyanate and 25 mM sodium citrate which contained 0.1 M 2-mercaptoethanol and 1% Sarkosyl. The following solutions were then sequentially added: 20% Sarkosyl in 4 M guanidine thiocyanate and 25 mM sodium citrate (5% v/v), 2 mM sodium acetate, pH 4.0 (10% v/v), an equal volume of Tris-saturated phenol, and 0.25 volume of a 24:1 mixture of chloroform and isooamyl alcohol. RNA was separated by centrifugation at 8500 rpm for 20 min, alcohol precipitated, and washed.

Northern Blot Analysis—Denatured total RNA was separated on formaldehyde/agarose gels and transferred to Hybond-N+ membranes (Amersham Corp.) by capillary action. Membranes were hybridized with a 2-kilobase human collagenase-1 cDNA (3) which had been radiolabeled with [α-32P]-dCTP by random priming, stringently washed (0.1 × SSC, 0.5% SDS at 55°C), and exposed to x-ray film for an appropriate period of time. Transcripts encoding HGF/SF isoforms were detected with a heavy chain cDNA probe as described previously (37).

Transfections—Keratinocytes were co-transfected with pCL-CAT, an expression construct that contains the chloramphenicol acetyltransferase (CAT) gene downstream of a 2.2-kilobase fragment (-2280 to +341) of the human collagenase-1 promoter (gift of Dr. Stephen Frisch), and pSV-β-galactosidase (Promega, Madison, WI) using LipofectAMINE (Life Technologies, Inc.). Cells were cultured on six well tissue culture plates until the monolayer was 50% confluent. At this time, keratinocytes were incubated with pCL-CAT (2 μg/well), pSV-β-galactosidase (2 μg/well), and Lipofectamine (10 μl/well) in OPTI-MEM I reduced-serum medium (Life Technologies, Inc.) overnight. The next day, the DNA-liposome mixture was removed and replaced with DMEM that contained 5% fetal calf serum and the appropriate concentration of HGF/SF. Following transfection, cells were cultured for 24 h and harvested in 0.25 μl Tris, pH 7.5. Cell lysates were subjected to repeated freeze/thawing prior to determination of β-galactosidase and CAT activity.

β-Galactosidase Assay—Cell lysates were incubated with 1 mM magnesium chloride, 50 mM β-mercaptoethanol, and 5.2 mM o-nitrophenyl-β-D-galactopyranoside in 0.1 M sodium phosphate, pH 7.5, at 37°C for an appropriate period of time. Reactions were stopped by the addition of 1 μl sodium carbonate, and optical densities were determined.

Assessment of CAT Activity—CAT activity was measured in equivalent amounts of cell lysates as determined from the β-galactosidase assay. Cell lysates were incubated overnight at 37°C with 200 μCi of [14C]chloramphenicol and 200 μg of acetyl coenzyme A in 0.25 μl Tris, pH 7.5. Following ethyl acetate partition and evaporation, precursor and acetylated products were resolved by thin layer chromatography on silica gel plates. Chromatograms were exposed to x-ray film overnight. Areas of the chromatograms corresponding to radiolabeled products were excised and quantitated by scintillation counting.

HGF/SF Isoforms—The coding sequences of naturally occurring HGF/NK1 and HGF/NK2, as well as the sequence corresponding to the amino-terminal domain of HGF/SF (residues 32–127) were each subcloned into a plasmid vector containing the CMV promoter and inserted into Escherichia coli. The expressed proteins were isolated, refolded, and purified to homogeneity as described elsewhere (38).

RESULTS

HGF/SF Stimulates Collagenase-1 and Stromelysin-1 Production—To determine whether HGF/SF modulates the production of matrix metalloproteinases by keratinocytes, cells were exposed to the growth factor for increasing periods of time, and the collagenase-1 content in the conditioned medium was quantified (Fig. 1A). Enhanced production of collagenase-1 was observed following a 24-h exposure to HGF/SF; secreted collagenase-1 protein levels continued to increase linearly for at least an additional 24 h. To determine whether HGF/SF stimulation of collagenase-1 production was mediated at the level of new enzyme synthesis, metabolic labeling and immunoprecipitation experiments were performed. When keratinocytes were treated with increasing concentrations of HGF/SF, collagenase-1 biosynthesis increased markedly (Fig. 1B). Specific antiserum to HGF/SF blocked the stimulation of collagenase-1 synthesis.

As shown by immunoprecipitation of the same condition medium, HGF/SF also stimulated stromelysin-1 synthesis but did not stimulate synthesis of the 92-kDa gelatinase (Fig. 1B). Maximal stimulation of collagenase-1 and stromelysin-1 synthesis was observed with 0.06 nM HGF/SF, and higher doses actually caused a decrease in metalloproteinase production (Fig. 2). This biphasic response has been reported for other HGF/SF-mediated effects, such as mitogenesis and cell migration (25). Optimal HGF/SF concentration varied between 0.06 and 0.28 nM and among cells from individual skin donors.

The effect of HGF/SF on collagenase-1 and stromelysin-1 synthesis by keratinocytes was modulated by the extracellular matrix. Although HGF/SF stimulated collagenase-1 and stromelysin-1 synthesis in keratinocytes cultured on type I collagen, gelatin, or Matrigel, the magnitude of enzyme expression was much greater in cells cultured on type I collagen (Fig. 2). Furthermore, cells cultured on gelatin and Matrigel required higher concentrations of HGF/SF for maximal induction of metalloproteinases.
HGF/SF Stimulates Collagenase-1 Transcription—Northern hybridization demonstrated that the steady-state levels for collagenase-1 mRNA (Fig. 3A) increased in parallel to the secreted protein levels indicating pretranslational regulation. Even at maximal doses of HGF/SF, stromelysin-1 mRNA was not detected by Northern hybridization. To determine whether HGF/SF modulated metalloproteinase production by a transcriptional mechanism, collagenase-1 promoter activity was studied. Relative CAT activity increased markedly in the presence of HGF/SF, and maximal effects of HGF/SF were observed at 0.28 nM (Fig. 3B). Thus, the parallel increase of collagenase-1 transcription, steady-state mRNA levels, and secreted protein levels indicates that HGF/SF controls enzyme expression at the level of transcription.

Distinct HGF/SF Isoforms Have Disparate Effects on Collagenase-1 Production—To elucidate structural characteristics of HGF/SF important for its biological functions, we tested the three truncated forms of HGF/SF for their effects on collagenase-1 production. As shown in Fig. 4A, the mature HGF/SF molecule consists of an α chain (463 amino acids) and a β chain (234 amino acids) which are disulfide-linked. The α chain contains an amino-terminal hairpin loop (N) between amino acid
residues 70 and 96, and 4 kringle domains (K1, K2, K3, and K4). Truncated forms of HGF/SF used in these experiments were designated HGF/N, HGF/NK1, and HGF/NK2. HGF/N contains the region from the amino terminus to the beginning of the first kringle, HGF/NK1 contains the amino-terminal hairpin loop plus the first kringle domain, and HGF/NK2 contains the amino-terminal hairpin loop and the first two kringle domains.

Both HGF/NK1 and HGF/NK2 are naturally occurring isoforms of HGF/SF (37, 38). Therefore, we analyzed the expression of HGF/SF, HGF/NK1, and HGF/NK2 in dermal foreskin fibroblasts to determine if these cells have the potential to produce these isoforms. Dermal foreskin fibroblasts were treated with interleukin-1α (20 ng/ml). Transcripts corresponding to HGF/SF and HGF/NK2 are indicated.

Like HGF/SF, HGF/NK1 was also stimulatory to keratinocyte collagenase-1 production, whereas the other two truncated forms had no effect (Fig. 5A). At a concentration of 100 nM, HGF/NK1 was approximately 70% as effective in stimulating collagenase-1 production as HGF/SF at a concentration of 0.28 nM. 50% efficacy was achieved at less than 10 nM HGF/NK1. These same truncated forms were tested for ability to inhibit HGF/SF-induced collagenase-1 production (Fig. 5B). The stimulatory effects of HGF/SF on collagenase-1 production were potently inhibited by HGF/NK2. Approximately 90% of the stimulated collagenase-1 expression produced by 0.28 nM HGF/SF was inhibited by 100 nM HGF/NK2; approximately 50% was inhibited by 1 nM HGF/NK2. HGF/NK1 was also inhibitory but was much less effective than HGF/NK2.

Heparin Inhibits HGF/SF-induced Collagenase-1 Production—HGF/SF is a heparin-binding growth factor. Biological functions of some heparin-binding growth factors are augmented by heparin, whereas heparin inhibits biological activities of other heparin-binding growth factors (46). As shown in Fig. 6, heparin inhibited HGF/SF-mediated collagenase-1 production in a dose-dependent manner.

Tyrosine Kinase and Protein Kinase C Activities Are Necessary for Induction of Collagenase-1 Synthesis by HGF/SF—To investigate potential signal transduction pathways involved in
stimulation of collagenase-1 synthesis by HGF/SF, keratinocytes were exposed to herbamycin A (tyrosine kinase inhibitor), orthovanadate (tyrosine phosphatase inhibitor), and staurosporine (protein kinase C inhibitor). Treatment of keratinocytes with herbamycin A (Fig. 7A) or staurosporine (Fig. 7B) inhibited the stimulation of collagenase-1 synthesis by HGF/SF. HGF/SF stimulation of collagenase-1 synthesis was potentiated by treatment of the cells with orthovanadate (OV), or 0.5 μM staurosporine (Stauro.). All cells (including Control) were cultured in the presence of 1% Me2SO. Results shown in A and B were obtained from separate cell preparations. In these experiments, synthesis of secreted proteins (nanomoles of leucine incorporated/mg of cell protein) were as follows: Control, 2.33 ± 0.49; HGF/SF, 2.45 ± 0.42; Herb A, 1.99 ± 0.46; OV, 2.06 ± 0.17; and Stauro., 3.06 ± 0.69.

In a wound environment, the two matrix metalloproteinases expressed prominently by keratinocytes are interstitial collagenase-1 (MMP-1) and stromelysin-1 (MMP-3) (17, 18, 22). Collagenase-1 is produced by migrating keratinocytes at the front of reepithelialization in both acute and chronic wounds (17, 18). Furthermore, keratinocytes which express collagenase-1 are not in contact with the basement membrane but reside on dermal and provisional matrix (18). Our previous in vitro studies have shown that type I collagen, the most abundant component of the dermal matrix, induces collagenase-1 production in human keratinocytes (18, 21). We report here that HGF/SF stimulation of collagenase-1 production is much greater in keratinocytes cultured on type I collagen than in cells grown on gelatin or Matrigel (Fig. 2). Similarly, matrix components can modulate the morphogenic effects of HGF/SF in other contexts (50). Taken together, these results indicate that cell-matrix interactions are an important regulator of keratinocyte collagenase-1 production and suggest that, in vitro, the effects of HGF/SF and possibly other growth factors on keratinocytes may be modulated by contact with specific extracellular matrix components.

**DISCUSSION**

Keratinocyte function during cutaneous wound repair is probably regulated by cell-cell and cell-matrix (18) interactions and also by exposure to soluble factors. In the present study, we examined the capacity of HGF/SF to modulate keratinocyte metalloproteinase production. Although HGF/SF is known to stimulate keratinocyte migration and proliferation (24, 25) and to promote wound healing in several in vitro models (47–49), this is the first report of its ability to regulate matrix metalloproteinase expression in any cell type in a physiologic setting.
In chronic wounds, stromelysin-1 is expressed by the proliferating population of keratinocytes that are in contact with the underlying basement membrane (22). Since in vitro cell-cell and cell-matrix interactions have not been apparently altered in chronic and normal wounds, we have suggested that a soluble factor likely induces stromelysin-1 expression by these keratinocytes. Interestingly, the c-Met receptor is more prominently expressed by basal keratinocytes in the wound environment than by those in apparently normal skin. Although HGF/SF did stimulate stromelysin-1 production by keratinocytes, its in vitro effects were greater when the cells were cultured on type I collagen than on Matrigel, a substrate of basement membrane components. This result may reflect the following: 1) inability of Matrigel to adequately duplicate the in vivo composition and/or organization of the basement membrane underlying stromelysin-producing keratinocytes, 2) residual growth factors in the Matrigel preparations that inhibit stromelysin-1 production, or 3) limitations of keratinocyte culture in mimicking all aspects of in vivo wound repair.

Specific structural motifs of the HGF/SF molecule are likely to be important in mediating its biological effects. Previous studies suggested that the amino-terminal hairpin loop and the first two kringle domains (K1 and K2) play a significant role in the biological and receptor binding activities of HGF/SF (31–33, 36). In the present study, we investigated the ability of three truncated variants of HGF/SF (HGF/N, HGF/NK1, and HGF/NK2) to modulate keratinocyte collagenase-1 production. Our results demonstrated that HGF/NK1 functioned as a partial agonist/antagonist, HGF/NK2 behaved as a pure agonist, and HGF/N had no significant effects on collagenase-1 production. These data are consistent with the activities of these isoforms in selected mitogenic assays2 (37, 38). In other systems, however, HGF/NK1 functioned as a pure mitogenic antagonist (35), and both HGF/NK1 and HGF/NK2 were partial agonists of MDCK scattering (34, 36, 38). These contrasting results may be due to differences in assay conditions and/or unique features of the signaling pathways associated with particular cellular responses.

Our observations reinforce earlier evidence that the two naturally occurring, truncated HGF/SF isoforms bind to the HGF/SF receptor with a high affinity and are biologically active. Because the essential difference between HGF/NK1 and HGF/NK2 is the presence of the K2 domain in the latter, their distinct effects presumably result from changes in the ligand-receptor interaction attributable to K2. How such changes alter the signaling process leading to the induction of collagenase-1 expression remains to be determined. Nonetheless, given the observation that selected dermal fibroblast preparations exhibit a higher level of HGF/NK2 expression than does HGF/SF and that the ratio of HGF/NK2 to HGF/SF changes upon stimulation (37) (Fig. 4B), in certain instances HGF/NK2 may function in vivo as a negative regulator of keratinocyte collagenase-1 production.

Another extracellular modulator of HGF/SF activity is heparin. Previous work suggested that heparin bound to HGF/SF through interactions with the HGF/N and K2 domains (51), although recent studies indicate that the HGF/N domain itself retains the heparin-binding properties of the full-length molecule.2 Heparin inhibits HGF/SF-induced mitogenesis (52–54), motogenesis (55), and receptor binding (56, 57). In certain assay systems, however, heparin can enhance the mitogenic response to HGF/SF (58). In our experiments, heparin inhibited HGF/SF-induced collagenase-1 production by keratinocytes (Fig. 6). Further investigations are needed to determine if heparin modulates HGF/SF activity by sequestering the growth factor and consequently preventing ligand-receptor interaction, or by altering receptor binding and post-receptor signal transduction through other mechanisms.

The biological effects of HGF/SF are thought to be mediated by c-Met (59) and an array of substrates that couple with this tyrosine kinase receptor (60). In this context, only a few intracellular proteins have been shown to be obligatory for specific cellular responses. For instance, the Ras signaling pathway (61) and phosphatidylinositol 3-kinase appear to be required for scattering of MDCK cells (62). Our results demonstrate that the HGF/SF signal transduction pathway leading to induction of keratinocyte matrix metalloproteinase synthesis is both tyrosine kinase- and protein kinase C-dependent (Fig. 7). These findings are consistent with an earlier report that HGF/SF increases the concentration of intracellular diacylglycerol, an essential cofactor for protein kinase C, through a phospholipase C-dependent pathway (63). Interestingly, collagen induction of collagenase-1 expression is also both tyrosine kinase- and protein kinase C-dependent (21).

HGF/SF stimulation of collagenase production may be important in contexts other than wound healing. Invasion of carcinoma cells into collagen gels is stimulated by HGF/SF, which suggests that this factor might promote tumor metastasis (64). Additionally, NIH/3T3 cells engineered to express an HGF/SF-Met autocrine loop have enhanced invasive and metastatic properties (65). Interestingly, type I and type IV collagenolytic activities secreted by these transfected fibroblasts were increased approximately 5–10-fold. Among transfecants expressing the autocrine loop, there was a significant correlation between the magnitude of collagenolytic activity and invasiveness. These studies suggest that elevated collagenase levels may be necessary, although not sufficient, for invasiveness (65). However, it must be recognized that, physiologically, fibroblasts produce HGF/SF but typically do not respond to this growth factor. Nevertheless, identification of the mechanisms responsible for HGF/SF-dependent collagenase induction and reagents capable of inhibiting this process may prove useful in retarding tumor metastasis.

In summary, the present study provides evidence at the RNA and protein levels that HGF/SF stimulates matrix metalloproteinase expression by keratinocytes. This effect is highly dependent on the matrix in contact with the cells and is blocked by inhibitors of tyrosine kinase and protein kinase C activity. The naturally occurring truncated isoforms HGF/NK1 and HGF/NK2 act as a partial agonist/antagonist and a pure antagonist, respectively, of HGF/SF-induced collagenase-1 production. The diverse biological effects of HGF/SF suggest several possible physiologic and pathologic roles for the growth factor in the regulation of keratinocyte function. For instance, induction of collagenase-1 synthesis and stimulation of keratinocyte migration may be important and even interrelated in normal wound repair. Alternatively, excessive production of collagenase-1 may contribute to the pathogenesis of chronic nonhealing ulcers or play a role in tumor metastasis.

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