Comparative Study of Hepatocyte Growth Factor/ Scatter Factor and Keratinocyte Growth Factor Effects on Human Keratinocytes

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Hepatocyte growth factor/scatter factor (HGF/SF) and keratinocyte growth factor (KGF, also designated FGF-7) are paracrine growth factors secreted by mesenchymal cells and active on a variety of epithelial cell types. In this study, the biologic responses of keratinocytes to these paracrine growth factors were compared. Stimulation of mitogenesis, migration, plasminogen activator (PA) activity, and fibronectin production were examined using human foreskin keratinocytes cultured in serum-free MCDB 153 medium. Although the two factors stimulated a similar level of proliferation when cells were maintained for 5 d in 1.8 mM Ca^{++}, the peak effect of KGF, observed at 10 ng/ml, was approximately threefold higher than that of HGF/SF when cells were in medium containing 0.15 mM Ca^{++}. Both agents promoted the migration of cells in low-calcium medium (0.08 mM Ca^{++}). However, the magnitude of the response was approximately twofold greater for HGF/SF at 10 ng/ml than KGF at the same concentration. None of the matrix proteins such as type I collagen, type IV collagen, laminin, or fibronectin either stimulated or suppressed HGF/SF- or KGF-stimulated keratinocyte migration. Both factors stimulated PA activity of the cell extracts, especially urokinase-type, with similar potencies. Promoted PA activity was maximal with the addition of 10 ng/ml of either factor. Neither factor increased the production of fibronectin under conditions in which transforming growth factor-β1 was active. These results indicate that HGF/SF and KGF, both recognized as paracrine growth factors, elicit distinctive patterns of response by keratinocytes, implying that they have different roles in epidermal physiology. ***Key words: migration/fibronectin/plasminogen activator. J Invest Dermatol 104:958–963, 1995***

Communication between epithelium and mesenchyme is crucial for skin development and regeneration. The cellular interactions are thought to involve both direct contact of keratinocytes and fibroblasts and release of soluble mediators that function in either an autocrine or paracrine manner. Two fibroblast-derived growth factors with a variety of activities on keratinocytes are hepatocyte growth factor/scatter factor (HGF/SF) and keratinocyte growth factor (KGF).

HGF/SF is a heterodimeric polypeptide originally identified as a mitogen for hepatocytes ([1], for review see [2,3]), but subsequently found to promote the growth of other epithelial cells [4–9], as well as melanocytes [4,5,10,11] and endothelial cells [4,12,13]. It also stimulates the motility of selected epithelial [14,15] and vascular endothelial cells [13,16]. In the appropriate setting, it promotes tubule formation by representatives of both cell types [17,18] and stimulates hair-follicle growth [19]. Molecular cloning revealed that HGF/SF is synthesized as a monomeric molecule, which is proteolytically processed to the biologically active two-chain form [1–3]. HGF/SF is structurally related to plasminogen, although it lacks intrinsic proteolytic activity [1–3]. The *c-met* proto-oncogene, a membrane-spanning tyrosine kinase, was identified as a high-affinity receptor for HGF/SF [20,21].

KGF is a heparin-binding, stromally derived polypeptide specifically mitogenic for a variety of epithelial cells [22]. Molecular cloning revealed that KGF is a member of the fibroblast growth factor (FGF) family [23], and the KGF receptor is an alternatively spliced isoform of the FGF receptor-2 gene [24]. KGF, which is produced by fibroblasts from skin and a variety of other sources [23], was shown to stimulate both the proliferation [25] and migration [26] of human keratinocytes, and did not disrupt calcium-induced keratinocyte differentiation [25]. Recent studies also indicate that exogenous KGF can promote re-epithelialization *in vivo* following skin injury [27,28].

Although both of these molecules are recognized as paracrine factors expressed in skin [4,23,29], their multiple biologic effects on keratinocytes have not been directly compared. In this study, we analyzed the relative activities of HGF/SF and KGF in assays that involve parameters relating to wound healing (proliferation, migration, plasminogen activator activity, and synthesis of fibronectin). This comparison was performed to identify any differences in activity that might exist that would suggest distinct roles for these two factors in epidermal development and regeneration.

Manuscript received September 9, 1993; final revision received January 30, 1995; accepted for publication February 20, 1995.

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Abbreviations: HGF/SF, hepatocyte growth factor/scatter factor; KBM, keratinocyte basal medium; KGM, keratinocyte growth medium; KGF, keratinocyte growth factor; PA, plasminogen activator.

0022-202X/95/$09.50 • SSDI0022-202X(95)00082-V •

MATERIALS AND METHODS

Growth Factors and Antibodies Recombinant human HGF/SF (isoform encoded by cDNA with 15 bp deletion [4,30]) and KGF [31] were prepared with baculovirus and bacterial expression vector systems, respectively, and purified by heparin chromatography. Transforming growth factor-β1 (TGF-β1) was purchased from R&D systems (Minneapolis, MN). A rabbit polyclonal neutralizing antiserum against HGF/SF was raised as previously described [4].

Cell Culture Normal human keratinocytes from infant foreskins were cultured as previously described [26]. Keratinocytes were grown in keratinocyte growth medium (KGM, Kurabo Co., Osaka, Japan) containing epidermal growth factor (10 ng/ml), insulin (5 μg/ml), hydrocortisone (0.5 μg/ml), ethanolamine (0.1 mM), phosphoethanolamine (0.1 mM), $CaCl_2$ (0.15 mM), and brain pituitary extract (0.4% v/v) in modified MCDB 153 medium. All experiments were carried out using third-passage keratinocytes seeded on uncoated plastic plates. The effect of growth factor was examined after switching to keratinocyte basal medium (KBM) that contained only ethanolamine and phosphoethanolamine in modified MCDB 153 medium.

Cell Growth Assay Keratinocytes in KGM were plated in 35-mm dishes at a density of 1×10^4 cells/well. After 36 h incubation, the medium was replaced with KBM (0.15 or 1.8 mM Ca^{++}) and growth factors were added (0 d). The cells were incubated for 5 d without change of medium and harvested by treatment with 0.125% trypsin–0.01% ethylenediaminetetraacetic acid (EDTA) for 15 min at room temperature. Cell counts were determined with a hemacytometer.

Migration Assay Cell migration was measured as previously described [26]. Keratinocytes were seeded on 12-well plates at 2.3×10^5 cells/well and grown in KGM (0.08 mM Ca^{++}) to achieve confluency quickly (within 2 d). After 48 h, the medium was switched to KBM (0.08 mM Ca^{++}) and the cells were incubated for an additional 6 h. From each well, half of the confluent monolayer was removed using a razor blade and cell scraper. The remaining keratinocytes were incubated in KBM with 0.08 mM Ca^{++} for 16 h in the absence or presence of varying concentrations of HGF/SF or KGF. After incubation, the cells were fixed with absolute methanol and counted at 100× magnification using a phase-contrast microscope with an ocular grid. Migration was quantitated by counting the number of cells that migrated into separate fields (each field was 1.25 mm × 1.25 mm). Subsequently, the effect of matrix proteins on migration was examined after pretreatment of the plates with bovine type I collagen, bovine type IV collagen, mouse laminin, or bovine plasma fibronectin (Koken Ltd., Tokyo, Japan). Collagen (1 mg/ml in phosphate buffered saline), laminin [0.45 mg/ml in 0.15 M NaCl–0.05 M Tris-HCl buffer (pH 7.2)] or fibronectin (1 mg/ml in PBS) were applied to give approximately 15 μg of protein in each plate and kept at 37°C for 1 h. The cells were seeded after washing the plates with PBS, and then wounded after 48 h. In another set of experiments, matrix proteins were applied only to the cell-free area of each plate after wounding.

Plasminogen Activator (PA) Assay Subconfluent keratinocytes in 24-well plates were incubated for 24 h in KBM in the absence or presence of varying concentrations of HGF/SF or KGF. After washing twice with PBS, the cells were extracted with 0.5% Triton X-100 in 0.1 M Tris-HCl buffer (pH 8.1). PA activities were assayed with a synthetic fluorogenic substrate as previously described [26]. Briefly, the substrate solution was comprised of 0.25 mM t-butyloxycarbonyl-valyl-leucyl-lysine 4-methyl-coumaryl-7-amide (Peptide Institute, Inc., Osaka, Japan) in 0.1 M Tris-HCl buffer (pH 7.5) and 0.1% Tween 80. Plasminogen (Sigma P5661, St Louis, MO) solution was prepared as 0.25 U/ml in the same buffer. A 50-μl sample was reacted with 100 μl of substrate solution in the presence or absence of 50 μl of plasminogen solution at 37°C for 1 h. PA activity was expressed in units of fluorescence intensity (FI)/h/mg protein. To determine the type of PA produced by the keratinocytes, anti-urokinase or tissue type PA IgG (10 μg/tube) was pre-incubated with the sample solution before the reaction.

Immunoblotting of Fibronectin Subconfluent keratinocytes in 12-well plates were incubated for 48 h in KBM (0.15 mM Ca^{++}) with indicated growth factors. After washing with PBS the cells were immediately scraped into 0.5% sodium dodecylsulfate (SDS)/PBS, boiled 2 min, and sonicated. Ten micrograms of cell extract and the corresponding conditioned medium were analyzed in parallel by SDS–polyacrylamide gel electrophoresis using a 4% stacking gel and 5% resolving gel. After electrophoresis, the samples were transferred to polyvinylidene difluoride membranes (Immobilon, Millipore Co., Bedford, MA). The blots were probed with 200× diluted polyclonal anti-fibronectin antibody (Collaborative Research, Inc., Bedford, MA) for 1 h at room temperature, and visualized with an alkaline-phosphatase kit (Promega, Madison, WI) according to the manufacturer's instructions.

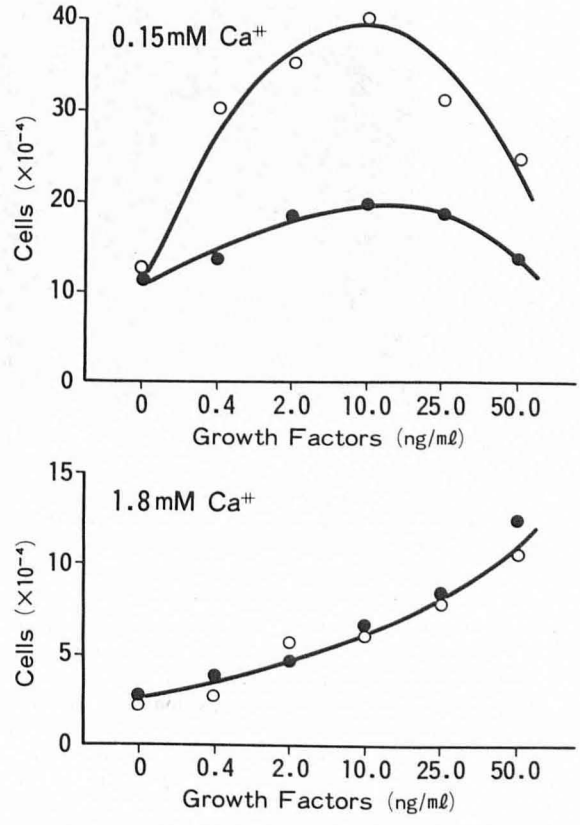

Figure 1. KGF had a stronger effect on keratinocyte growth than HGF/SF. Cells were incubated in 35-mm culture dishes with varying concentrations of HGF/SF or KGF in KBM for 5 d without change of medium. The data are expressed as the mean values of duplicate measurements from a representative experiment; variation from the mean was less than 10% for every data point. The experiment was performed five times. ●, HGF/SF; ○, KGF.

Statistical Analysis The data were compared with those in the control group, and analyzed by Student unpaired t test. Results were expressed as means ± SD.

RESULTS

KGF Had a Stronger Effect on Keratinocyte Growth than HGF/SF Initially, the biologic effects of HGF/SF and KGF on keratinocytes were compared in terms of their activity on keratinocyte growth. As shown in **Fig 1**, HGF/SF and KGF both stimulated the proliferation of normal human keratinocytes in KBM. However, the pattern of activity induced by the factors differed in media containing low versus high calcium concentrations. At 0.15 mM Ca^{++}, the maximal effect of both factors was observed with 10 ng/ml and decreased at higher concentrations. The peak mitogenic effect of KGF was consistently about threefold greater than that of HGF/SF in repeated experiments using keratinocytes from different individuals. In contrast, at 1.8 mM Ca^{++}, cell proliferation rose with increasing growth-factor concentrations throughout the range surveyed (up to 50 ng/ml), and there was no significant difference in the magnitude of response to HGF/SF or KGF. The growth rate of keratinocytes was slower in 1.8 mM Ca^{++}-containing medium compared to that observed in 0.15 mM Ca^{++} (with KGF at 10 ng/ml, doubling time was 75 h in high- versus 28 h in low-Ca^{++} medium). The mitogenic effect induced by HGF/SF and KGF was inhibited with specific neutralizing antibodies against HGF and KGF, respectively (data not shown).

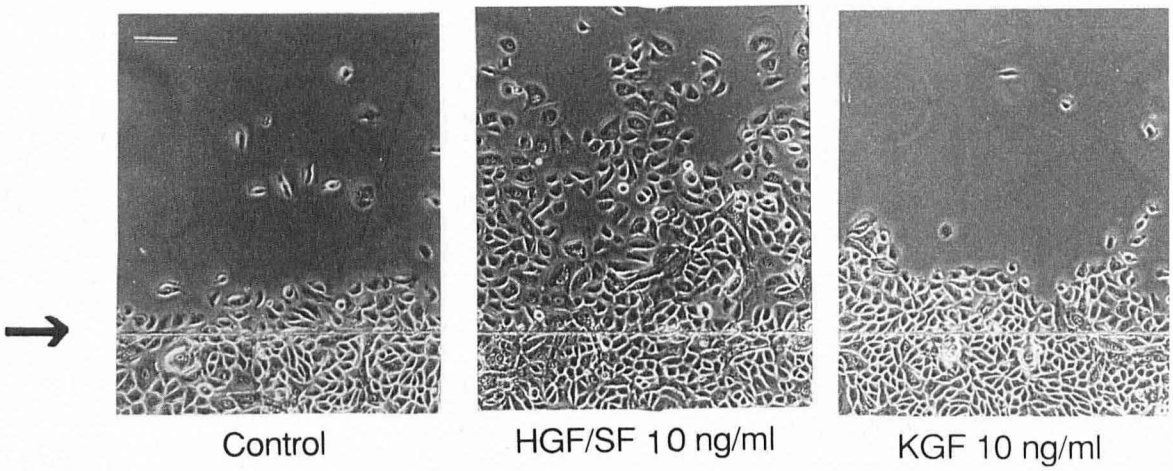

Figure 2. HGF/SF and KGF stimulated keratinocyte migration. Half of the confluent keratinocyte monolayer was removed using a razor blade and cell scraper. The remaining keratinocytes were incubated in KBM (0.08 mM Ca^{++}) for 16 h in the absence or presence of growth factor. *Arrow*, original wound edge. *Bar*, 100 μm.

HGF/SF Had a Stronger Effect on Keratinocyte Migration than KGF Subsequently, the effect of the two factors on migration was assessed by a randomized migration assay. HGF/SF and KGF at 10 ng/ml each stimulated keratinocyte migration compared with control in the randomized migration assay **(Fig 2)**. At optimal concentrations, the distance and number of migrated cells were larger for HGF/SF- than KGF-treated keratinocytes. However, morphologic appearance of HGF/SF-treated cells did not differ microscopically from that of the KGF-treated or control cells. The effect of HGF/SF was dose dependent, and was approximately twofold greater than that observed with KGF **(Fig 3)**. These observations were confirmed in repeated experiments using keratinocytes from different individuals. Anti-HGF/SF and KGF antibodies blocked the HGF/SF- and KGF-dependent migration, but did not suppress the basal movement of keratinocytes in the absence of growth factor (data not shown). At a high-calcium concentration (1.8 mM Ca^{++}), a slight out-growth, but not migration, of the keratinocytes from the edge was observed without significant difference between the two growth factors (data not shown). Next, because matrix proteins are generally known to influence migration, their effect on migration was examined. As shown in **Fig 4**, unexpectedly, none of the matrix proteins tested, i.e., type I collagen, type IV collagen, laminin, and fibronectin stimulated or suppressed migration when applied to the cell-free area of each plate after wounding. HGF/SF- or KGF-dependent migration as well as basal migration were not affected by the matrix proteins in this assay. Slightly decreased migration was observed when plates were coated with laminin or type IV collagen before seeding of the keratinocytes, but this difference was not statistically significant (data not shown).

Both HGF/SF and KGF Stimulated PA Activity of Keratinocytes with Similar Potencies As PA activity has been reported to correlate with tissue remodeling, we examined such

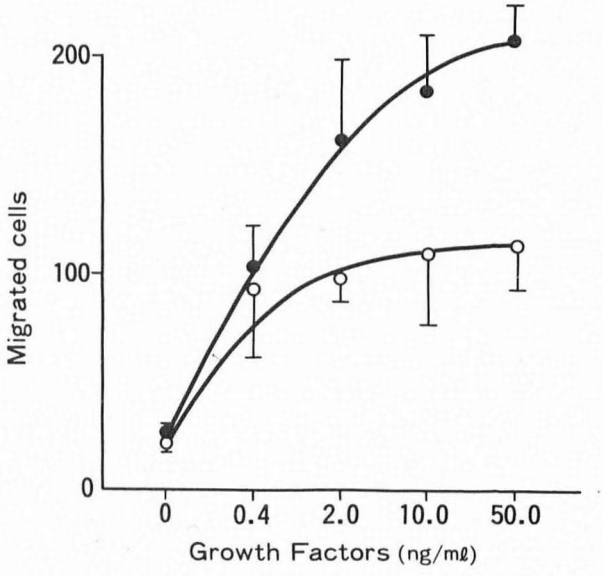

Figure 3. HGF/SF had a stronger effect on keratinocyte migration than KGF. Summary of quantitative results in keratinocyte migration assay is shown. Cell migration was analyzed as described in *Materials and Methods*. Data are expressed as the mean values ± SD of measurements in six different microscopic fields in a representative experiment. The experiment was performed five times. ●, HGF/SF; ○, KGF.

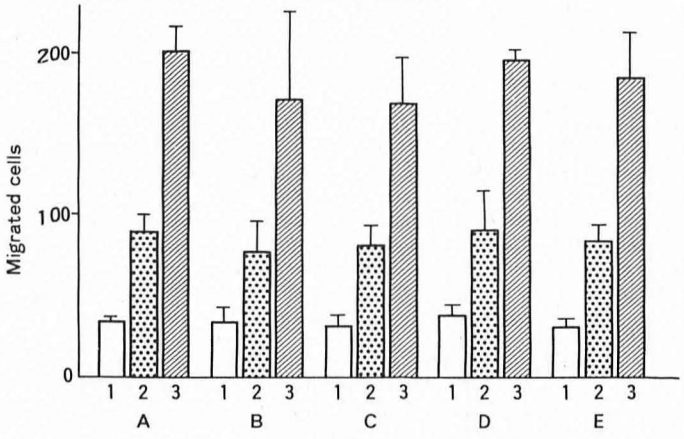

Figure 4. Matrix proteins did not affect HGF/SF- or KGF-dependent migration. Type I collagen, type IV collagen, laminin, or fibronectin were applied to the cell-free area of each plate after wounding, and the cells were incubated in KBM (0.08 mM Ca^{++}) for 16 h. Migration was analyzed as described in **Fig 3**. Data are expressed as the mean values ± SD of measurements in six different microscopic fields in a representative experiment. The experiment was performed four times. 1, non-treated cells; 2, KGF (10 ng/ml); 3, HGF/SF (10 ng/ml). *A*, non-coated plates; *B*, laminin-coated plates; *C*, fibronectin-coated plates; *D*, type I collagen–coated plates; *E*, type IV collagen–coated plates.

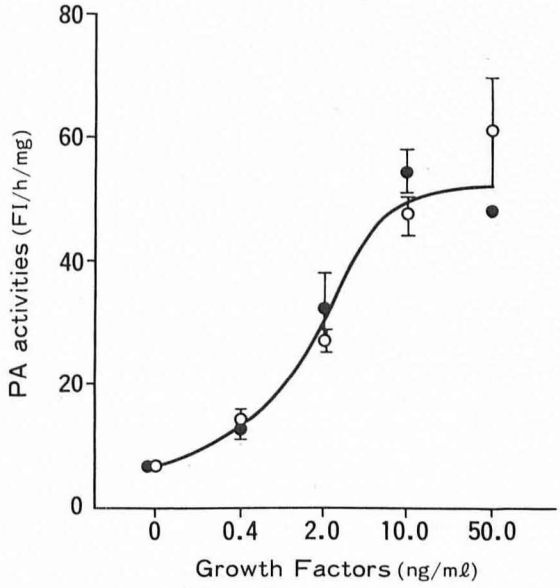

Figure 5. Both HGF/SF and KGF stimulated PA activity of keratinocytes with similar potencies. Subconfluent keratinocytes in 24-well plates were incubated for 24 h in KBM in the absence or presence of varying concentrations of HGF/SF or KGF. PA activity of cell extracts was expressed as fluorescence intensity/h/mg protein. Data are expressed as the mean values ± SD of duplicate measurements in a representative experiment. The experiment was performed three times. ●, HGF/SF; ○, KGF.

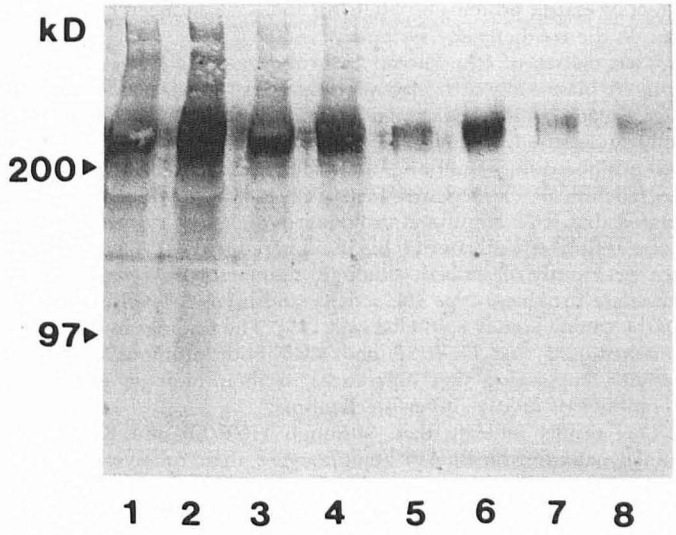

Figure 6. Neither HGF/SF nor KGF stimulated fibronectin production of keratinocytes. Immunoblotting of fibronectin was performed. Ten micrograms of cell extract and the corresponding conditioned medium after 48 h incubation with or without growth factors were loaded on a 5% SDS–polyacrylamide gel, electrophoresed and transferred onto a membrane. The blots were probed with 200× diluted anti-fibronectin antibody and visualized with an alkaline-phosphatase kit. *Lanes 1–4*, 10 μg of cell extracts; *lanes 5–8*, corresponding conditioned media; *lanes 1, 5*, non-treated cells; *lanes 2, 6*, TGF-β1; *lanes 3, 7*, KGF; *lanes 4, 8*, HGF/SF. Concentration of all growth factors was 10 ng/ml.

activity in keratinocytes. As shown in **Fig 5**, both HGF/SF and KGF stimulated PA activity of the cell extracts with similar potencies. Maximal stimulation of PA activity was obtained with 10 ng/ml of either factor. Similar dose-response curves were observed in three different experiments. A comparable increase in activity also was detected in conditioned media from keratinocytes treated with the growth factors (data not shown). HGF/SF- and KGF-stimulated PA activities were completely inhibited by the addition of antibody against urokinase-type PA but not by the addition of antibody against tissue-type PA (data not shown).

Neither HGF/SF Nor KGF Influenced Fibronectin Production of Keratinocytes Finally, the effect of the two factors on the matrix protein production of keratinocytes was examined using fibronectin as an example. By immunoblotting, an immunoreactive band corresponding to fibronectin was observed at 220 kD both in keratinocyte extract and conditioned medium **(Fig 6)**. Although TGF-β1 significantly increased immunoreactive fibronectin, neither HGF/SF nor KGF stimulated fibronectin production at the concentrations tested. Time-course experiments extending from 12 to 72 h, with doses ranging from 2 to 30 ng/ml did not show any stimulation or inhibition of fibronectin expression in cells treated either with HGF/SF or KGF (data not shown).

DISCUSSION

HGF/SF and KGF are multifunctional polypeptides with mitogenic [4,5,7,22,25] and motogenic [7,26] activity on keratinocytes. In the present study, we observed that KGF was a more potent mitogen than HGF/SF when cells were maintained in low calcium, whereas their stimulatory effects were indistinguishable in medium having a physiologic calcium concentration. In contrast, HGF/SF was more active than KGF in the migration assay in low-calcium medium. Both growth factors stimulated cell-associated PA activities with similar potencies. Neither factor promoted the synthesis of fibronectin by keratinocytes under conditions in which TGF-β1 had a positive effect, suggesting that modulation of this extracellular matrix component is not controlled by these factors.

Our demonstration of HGF/SF mitogenic activity for human keratinocytes incubated in low-calcium medium was consistent with earlier studies using mouse keratinocytes [4,5], but contrary to the findings of Matsumoto *et al*, who claimed that the full-length form of HGF/SF inhibited the proliferation of human keratinocytes cultured in low-calcium medium [7]. The basis for this discrepancy is uncertain; perhaps it is due to subtle differences in culture conditions or the fact that different isoforms of HGF/SF were utilized. A recent report [32] and our present result suggest that the deleted form of HGF/SF (lacking a five–amino acid segment from kringle one) has somewhat stronger mitogenic activity on epithelial cells than the full-length form of HGF/SF.

The striking effect of HGF/SF on human keratinocyte migration is in agreement with the data of Matsumoto *et al* [7] and an extensive body of evidence that this protein is a scattering factor for a wide variety of epithelial cells (reviewed in [33,34]). Recent work established that both the scattering and mitogenic effects of HGF/SF are mediated by the same cell-surface receptor [35], the *c-met* proto-oncogene product, which has been detected in skin [36]. Although information is accumulating regarding downstream events in HGF/SF/Met signal transduction (for a review, see [3]), the mechanisms responsible for migration as opposed to mitogenesis have not yet been elucidated. Thus, it remains to be determined why some cells respond to HGF/SF by scattering whereas others proliferate or, like human keratinocytes described in this report, exhibit both effects.

Keratinocyte motility has been reported to be stimulated by fibronectin [37–40], type I collagen [38,41], and type IV collagen [41], and to be inhibited by laminin [37,41]. However, in this study none of the matrix proteins tested, i.e., type I collagen, type IV collagen, laminin, or fibronectin, were found to stimulate or suppress either basal, HGF/SF-dependent, or KGF-dependent migrations. The explanation for this discrepancy may be that most of the previous studies [38–43] used a phagokinetic assay, which traced keratinocyte movement on colloidal gold-coated coverslips. Tracing of cell locomotion may be more sensitive or involve other phenomena than those monitored in simply counting cell numbers in a randomized migration assay. It would be of interest to study the

effect of matrix proteins on HGF/SF and KGF-induced cell migration in these other assay systems.

Two classes of PA, known as urokinase- and tissue-type PA, convert plasminogen to plasmin, which in turn has the ability to digest many proteins. Although their physiologic functions are not fully understood, PA activity has been correlated with enhanced cell proliferation, migration, and tissue remodeling associated with degradation of extracellular matrix [44]. We previously demonstrated that KGF stimulates urokinase-type PA of cultured keratinocytes [26]. An effect of HGF/SF on keratinocyte PA activity was not previously described although the factor was reported to stimulate urokinase-type PA activity and mRNA level in Madin-Darby canine kidney epithelial cells [45]. The findings in this study demonstrated that HGF/SF and KGF both promote urokinase activity, suggesting that differences in their biologic effects on keratinocytes involve other mechanisms.

Our results indicate that, although HGF/SF and KGF have similar activities on human keratinocytes, their relative potencies vary. This implies that they may have overlapping, yet distinct, functions in the skin. HGF/SF [46,47] and KGF [48] are both induced by interleukin 1, implying that each could be recruited for participation in post-inflammatory repair processes. Future efforts will focus on their expression and activities in other experimental systems to better define their respective roles in normal and disease states, and determine whether they act cooperatively or independently as paracrine mediators of cell-cell interaction.

We thank Drs. Andrew Chan and Roy Jensen for development of the HGF/SF baculovirus expression system, and Dr. Stuart Aaronson for his support and encouragement.

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