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Hepatocyte Growth Factor is a Potent Stimulator of Human Melanocyte DNA Synthesis and Growth

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SUMMARY: Hepatocyte growth factor (HGF) is a potent mitogen for adult rat hepatocytes in primary culture. HGF stimulates growth and DNA synthesis of normal human epidermal melanocytes in culture. The maximal stimulation of DNA synthesis by 4.0-fold occurred with 10 ng/ml HGF. This stimulatory effect was additive with both acidic and basic fibroblast growth factors, while it was inhibited by transforming growth factor-β1. Melanocytes expressed a single class of specific, high-affinity receptors for HGF with a Kd of 22 pM and approximately 120 receptors/cell. Thus, HGF is a potent mitogen for normal human epidermal melanocytes.

Hepatocyte growth factor (HGF) first identified in the serum of partially hepatectomized rats (1) was purified to apparent homogeneity from rat platelets (2, 3). Thereafter, HGF in human plasma (4) and rabbit serum (5) was purified. The purified rat HGF is a 82kD polypeptide growth factor composed of the 69kD α-subunit and the 34kD β-subunit (3). Recently, we cloned HGF cDNAs from human (6) and rat (7), and determined their primary structures. Human HGF cDNA was also cloned independently by another group (8). The predicted amino acid sequence revealed that HGF is derived from a single chain precursor of 728 amino acids and proteolytically processed to form a two-chain mature HGF. Among known peptide growth factors, HGF has the highest potential to promote the growth of mature hepatocytes in primary culture. HGF activity and HGF mRNA levels were markedly increased in the liver following the development of hepatitis (9, 10). *In situ* hybridization (11) and cell fractionation (10) revealed that HGF-producing cells in the rat liver are non-parenchymal liver cells, presumably

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Kupffer cells and sinusoidal endothelial cells. Thus, HGF seems to be a hepatotropic factor which acts as a trigger for the liver regeneration.

We obtained evidence that HGF mRNA is present in various rat tissues other than the liver (7, 11), and we recently found that HGF stimulated DNA synthesis of normal rabbit renal epithelial cells in secondary cultures (12). We have also identified a high affinity specific receptor for HGF on rat hepatocytes and a wide tissue distribution of the HGF receptor became apparent (submitted for publication). Therefore, HGF is not restricted to targeting only mature hepatocytes.

Epidermal melanocytes have a important role in producing melanin. Elucidation of growth factor requirement by melanocytes is important to determine how melanocytes are regulated in normal and pathological conditions and in epidermal tissue repair. As melanocytes only rarely undergo mitosis *in vivo* (13) and constitute a minor population in epidermal cells, cultivation is difficult. Melanocytes in culture require the presence of phorbol-12-myristate-13-acetate (PMA) for growth (14-17) and basic fibroblast growth factor (bFGF) is a potent mitogen for human melanocytes (16, 17). We now report evidence that HGF is a potent mitogen for normal human epidermal melanocytes and that the cells express high-affinity receptors for HGF.

MATERIALS AND METHODS

Cell Culture: Normal human epidermal melanocytes were obtained from Kurabo Co. (Osaka) and cultured as described (17). Briefly, these melanocytes were cultured in the nutrient medium MCDB153 supplemented with ethanolamine (0.1 mM), phosphoethanolamine (0.1 mM), hydrocortisone (0.5 mM), insulin (5 mg/ml), PMA (10 ng/ml), and bovine pituitary extract (BPE, 25 μg protein/ml). In some experiments, BPE was deleted from the medium. Fourth or fifth passage cells were used.

Measurement of Cell Growth and DNA Synthesis: Melanocytes were plated at a density of 10^4 cells/well on a 12-well plate (Corning), and next day the medium was changed to fresh medium without BPE. An appropriate amount of growth factor was added to each culture and the cells were cultured for 15 days. Cells were treated with trypsin and the number of cells was counted using a hematocytometer.

DNA synthesis was determined by measuring the incorporation of [^{125}I]-deoxyuridine into the nuclei. Cells were plated at a density of 4 x 10^4 cells/well on a 24-well plate. The next day, the medium was changed to

fresh medium without BPE and an appropriate amount of growth factor was added. Cells were cultured for 24 h and pulse-labeled with 1 μCi of [^{125}I]-deoxyuridine (2200 Ci/mmol, New England Nuclear) for 4 h. These cultured cells were washed with PBS and then with ice-cold 10% (w/v) TCA. The cells were solubilized with 1 M NaOH and the radioactivity was counted in a gamma counter. Protein concentration was measured using the Micro BCA protein assay system (Pierce Chemical) with bovine serum albumin as the standard.

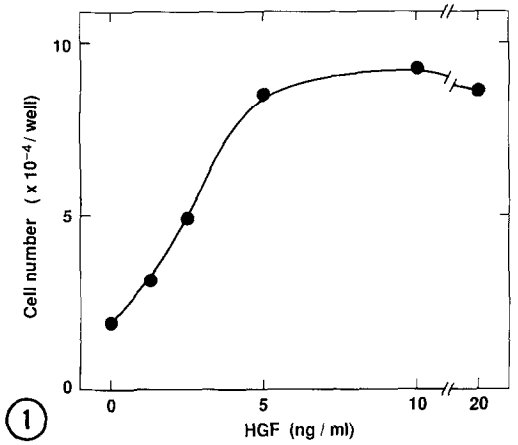
Assay for [^{125}I]-HGF Binding: Human recombinant HGF was radioiodinated by the chloramine-T method. Details on the methods for the radioiodination and the binding of HGF to cells should be published elsewhere. Briefly, 1.5 M sodium phosphate buffer, pH 7.0 (10 μl), 0.5 μg HGF (17 μl), and 0.5 mCi Na[^{125}I] (14 Ci/mg I, IMS 30, Amersham) were added to a siliconized tube and the reaction was started by adding of 5 μl of chloramine-T (100 μg/ml), four times at 30 sec intervals. The reaction was stopped by adding 20 μl N-acetyl-L-tyrosine (50 mM), 200 μl KI (60 mM) and 200 μl urea solution (1.2 g/ml in 1 M acetic acid). [^{125}I]-HGF was separated by molecular sieve chromatography on a Sephadex G-25 column (Pharmacia) equilibrated with 4 mM HCl, 75 mM NaCl, and one mg/ml bovine serum albumin. [^{125}I]-HGF thus prepared had a specific activity of 70-160 mCi/mg.

Cells were cultured in 24-well plates and when subconfluency was reached, the cultures were refed with fresh medium without BPE. After a 24 h culture, the cells were washed once with binding buffer (Hanks' solution containing 20 mM HEPES, 2 mg/ml bovine serum albumin, pH 7.0) and equilibrated in the buffer for 30 min at 10°C. Ice-cold binding buffer containing appropriate concentrations of unlabeled HGF was added and the cells were incubated at 10°C for 1 h. Cultures were washed three times with the ice-cold binding buffer and [^{125}I]-HGF specifically bound to cells was measured in a gamma counter after detaching the cells with trypsin-treatment.

Growth Factors: Human recombinant HGF was purified from culture medium of the COS-1 cells transfected with plasmid containing human HGF cDNA (6). Human recombinant bFGF and human recombinant acidic fibroblast growth factor (aFGF) were obtained from TOYOBO (Osaka). Transforming growth factor-β1 (TGF-β1) was purified from human platelets, as described previously (18).

RESULTS

We examined the effect of HGF on the growth of normal human melanocytes in the presence of PMA, since the stimulatory effect of growth factors on the DNA synthesis of melanocytes is not usually evident in the absence of PMA. Fig.1 shows that HGF stimulated the growth of melanocytes, in a dose-dependent manner. Effects became apparent with 1.25 ng/ml and the cell growth reached the maximum with 10 ng/ml HGF. In addition, HGF stimulated DNA synthesis with the same dose-dependency evident in the cell growth promotion by HGF, as shown in Fig. 2. The maximal response was reached with about 10 ng/ml, a 4.0-fold stimulation compared to finding



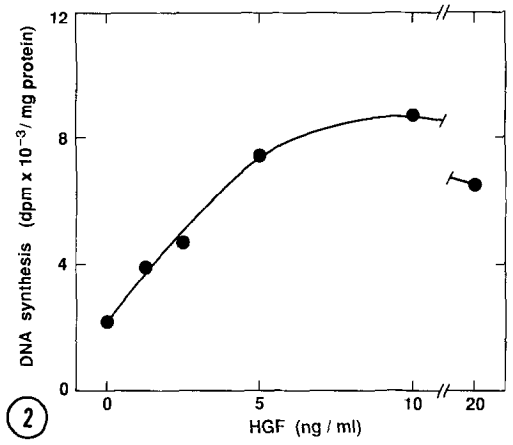


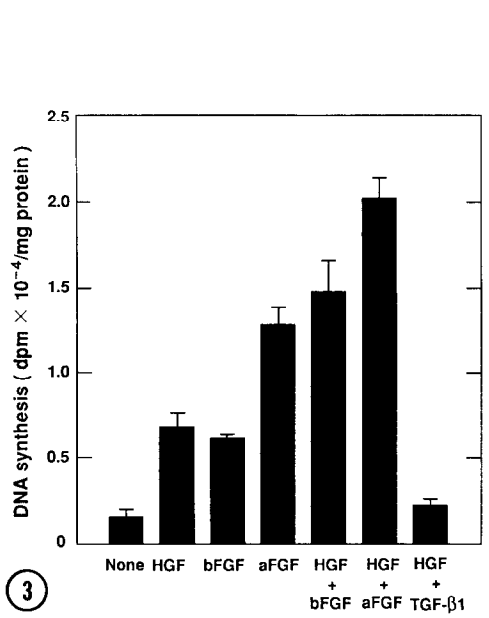
Fig. 1. Effect of HGF on the growth of normal human epidermal melanocytes. Cells were plated at a density of 10^4 cells/well on a 12-well plate. The next day, the medium was changed to fresh medium without BPE, and HGF was added at the various concentrations. Cultures were refed on days 6 and 12 with the same medium and the culture was stopped on day 15. The average of duplicate measurements is shown.

Fig. 2. Effect of HGF on DNA synthesis of normal human epidermal melanocytes. Cells were plated at a density of 4 x 10^4 cells/well on a 24-well plate. The next day, the medium was changed to fresh medium without BPE, and HGF was added at the various concentrations. Cells were cultured for 24 h and pulse-labeled with 1 μCi of [^{125}I]-deoxyuridine for 4 h. The average of triplicate measurements is shown.

in the absence of HGF. The half-maximal stimulation was seen with 3 ng/ml HGF.

Fig. 3 shows the effects of HGF, aFGF, bFGF and the combinations of HGF and other growth factors on the DNA synthesis of melanocytes. bFGF (10 ng/ml) and aFGF (10 ng/ml) stimulated DNA synthesis by 3.8-fold and 8.0-fold, respectively. HGF (10 ng/ml) alone stimulated it with almost the same potency as seen with bFGF. The effect of HGF was additive with those of bFGF and aFGF. TGF-β1 can inhibit the growth of various epithelial cells, including normal human epidermal keratinocytes (19, 20). TGF-β1 (10 ng/ml) strongly inhibited DNA synthesis of the human melanocytes stimulated by HGF.

To further confirm that human melanocytes are target cells of HGF, we searched for a specific receptor for HGF on human melanocytes. Fig. 4 shows that [^{125}I]-radiolabeled HGF specifically bound to the cells in a saturable manner. Scatchard analysis of the specific binding showed that human melanocytes express approximately 120 receptors/cell with a Kd of



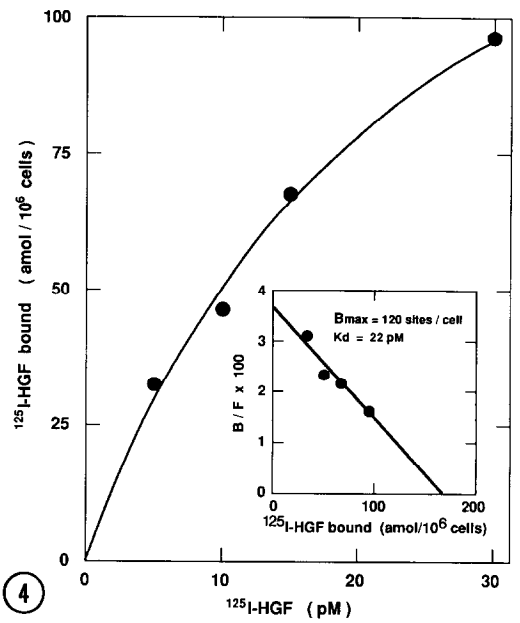


Fig. 3. Effect of HGF, aFGF, bFGF, and combinations of HGF plus other growth factors on DNA synthesis of normal human epidermal melanocytes. Cells were plated at a density of 4 x 10^4 cells/well on 24-well plates. The next day, the medium was changed to fresh medium without BPE, and growth factors (10 ng/ml for each) were added. Cells were cultured for 24 h and pulse-labeled with 1 μCi of [^{125}I]-deoxyuridine for 4 h. The average of triplicate measurements (± SD) is shown.

Fig. 4. Analysis of [^{125}I]-HGF binding to normal human epidermal melanocytes. Binding of [^{125}I]-HGF was performed as described in the text. Each point shows the amount of [^{125}I]-HGF specifically bound to melanocytes exposed to increasing concentrations of HGF. Inset: Scatchard analysis of the data on binding.

22 pM. The very low Kd value is fairly consistent with the half-maximal dose for stimulation of both growth and DNA synthesis of 3 ng/ml HGF (35 pM HGF).

DISCUSSION

Evidence that HGF acts as a potent growth factor for normal human epidermal melanocytes is as follows: 1) HGF strongly enhanced both DNA synthesis and cell growth of human melanocytes; 2) human melanocytes carried high affinity receptors for HGF. The effect of HGF was additive with those of bFGF and aFGF, hence HGF probably acts through a specific receptor for HGF, distinct from those for other growth factors. In fact, Scatchard

analysis revealed that the character of the HGF receptor on melanocytes seems to differ from that for bFGF (17). The Kd value (22 pM) and the number of receptors (120 sites/cell) in HGF-binding to human melanocytes are similar to findings with adult rat hepatocytes in primary culture. We have identified specific, high affinity receptor for HGF on rat hepatocytes and plasma membranes from rat liver. Hepatocytes have approximately 500-600 receptors/cell with a Kd value of 20-30 pM (Higuchi and Nakamura, submitted for publication).

The human epidermal melanocytes derived from the neural crest are located at the epidermal-dermal junction of the skin and at the matrix of hair folicles. Since melanocytes only rarely undergo mitosis *in vivo* (13), the role HGF may play *in vivo* for epidermal melanocytes has remained elusive. HGF may function as a mitogen for melanocytes under certain pathological conditions, wound-healing process, and in response to selected stimuli such as ultraviolet radiation (21). HGF producing cells are found among those of mesoderm-origin: Kupffer cells and endothelial cells in the liver (11), blood monocytes and macrophages, and embryonic lung fibroblasts (unpublished data). Therefore, if HGF is produced by dermal fibroblasts, endothelial cells, or infiltrating monocytes and macrophages under pathological conditions and in wounded tissue, a certain quantity may diffuse to the basal layer of the epidermis to stimulate the growth of melanocytes.

A scatter factor which stimulates the motility of epithelial cells was found to be identical or highly related to HGF (22). In addition, we found that HGF stimulates the growth of renal tubular epithelial cells in secondary culture (12). There is a wide distribution of the HGF receptor in epithelial cells including epidermal keratinocytes (to be published). Thus, HGF may well be a mediator for epithelial-mesenchymal interactions and would play an important role in development, morphogenesis, wound-healing, tissue regeneration, and carcinogenesis.

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