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Pleiotropic effects of hepatocyte growth factor in proximal tubule involve different signaling pathways

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Pleiotropic effects of hepatocyte growth factor in proximal tubule involve different signaling pathways. Hepatocyte growth factor (HGF) accelerates renal tubule cell regeneration and induces tubulogenic differentiation via the intracellular tyrosine kinase (TK) domain of its receptor, the proto-oncogene *c-Met*. We tested whether different signaling pathways may be involved by examining HGF binding and effects on cell proliferation, migration, scattering, and tubulogenic differentiation in the bipolar differentiating rabbit proximal tubule cell line PT-1 under serum-free conditions in the presence or absence of the protein TK inhibitors (PTKIs) herbimycin-A, genistein, methyl-2,5-dihydroxycinnamate, and geldanamycin. These PTKIs inhibit pp60$^{c\text{-}src}$, a nonreceptor TK involved in cell-growth control. HGF bound to a single high-affinity receptor class, increased microvilli numbers 1.5-fold, enhanced cell proliferation and migration 1.8-fold, and stimulated formation of tubule structures 2.2-fold. PTKI inhibited the mitogenic and motogenic effects of HGF with different potencies and comparable maximal effects but had no specific influence on HGF-induced tubulogenic cell differentiation. These data underline the importance of pp60$^{c\text{-}src}$ in mediating mitogenic and motogenic effects of HGF, whereas stimulation of tubulogenic cell differentiation may be transduced by a pp60$^{c\text{-}src}$–independent pathway.

Acute renal failure is relatively common and potentially life threatening. Because growth factors (GFs) are important in regeneration of damaged renal epithelia, they might be effective for treating renal failure and accelerating renal repair [1]. Hepatocyte GF (HGF) is one important renotropic GF and stimulates renal tubule cell proliferation in the remnant kidney after unilateral nephrectomy [2] and promotes renal tubule cell regeneration in experimental acute renal failure [3]. As a multifunctional cytokine, HGF acts on various cells, including renal proximal tubule cells, via high-affinity binding to the receptor tyrosine kinase (TK) c-Met [4]. *c-Met* autophosphorylates on stimulation by HGF and then binds via a multifunctional docking site to various intracellular proteins containing src-homology-2 domains, such as pp60$^{c\text{-}src}$ and PI_3 kinase [4, 5]. Which pathway mediates which response is, however, generally unknown. We thus investigated binding, signal transduction, and action of HGF in the bipolar differentiating rabbit proximal tubule cell line PT-1 [6].

Key words: tyrosine kinase, protein tyrosine kinase inhibitors, herbimycin-A, genistein, methyl-2,5-dihydroxycinnamate, geldanamycin, pp60$^{c\text{-}src}$.

METHODS

Human recombinant HGF was obtained from R&D Systems (Wiesbaden, Germany). Protein TK inhibitors (PTKI) geldanamycin, genistein, herbimycin A, and the stable erbstatin analog methyl-2,5-dihydroxycinnamate (MDC) were obtained from GIBCO BRL (Eggenstein, Germany) and dissolved in dimethylsulfoxide. PT-1 cells were grown in Dulbecco's minimal essential medium as previously described [6]. To characterize the actions of HGF without influence of serum GFs, cells were grown under defined serum-free conditions and thus showed minimal further proliferation and were viable for five to seven days (trypan blue exclusion). HGF binding was assayed in confluent cell monolayers incubated with ^{125}I-HGF (20,000 cpm) and various concentrations of unlabeled HGF (10 pM-0.1 μM) and number and affinity of HGF receptors were determined [6]. Short-term effects of HGF on cell proliferation were assessed using a nonradioactive cell proliferation assay (CellTiter 96TM, technical bulletin, #TB112; Promega, Madison, WI, USA) with results comparable to the [^{3}H]thymidine incorporation assay. After 48-hours incubation with HGF and PTKI, cell numbers were determined (Coulter Counter, Krefeld, Germany). For all further experiments, PTKI were used in concentrations yielding 50% inhibition of cell growth (EC_{50}). The effect of HGF and PTKI on cell scattering was scored semiquantitatively [7]. Cell migration was studied by microcarrier bead motility assays using cytodex 2 collagen-coated microcarrier beads [8]. Tubule structures were quantified by light microscopy; microvilli formation on the apical cell membrane was assessed using scanning electron microscopy. If not otherwise indicated, data are presented as the

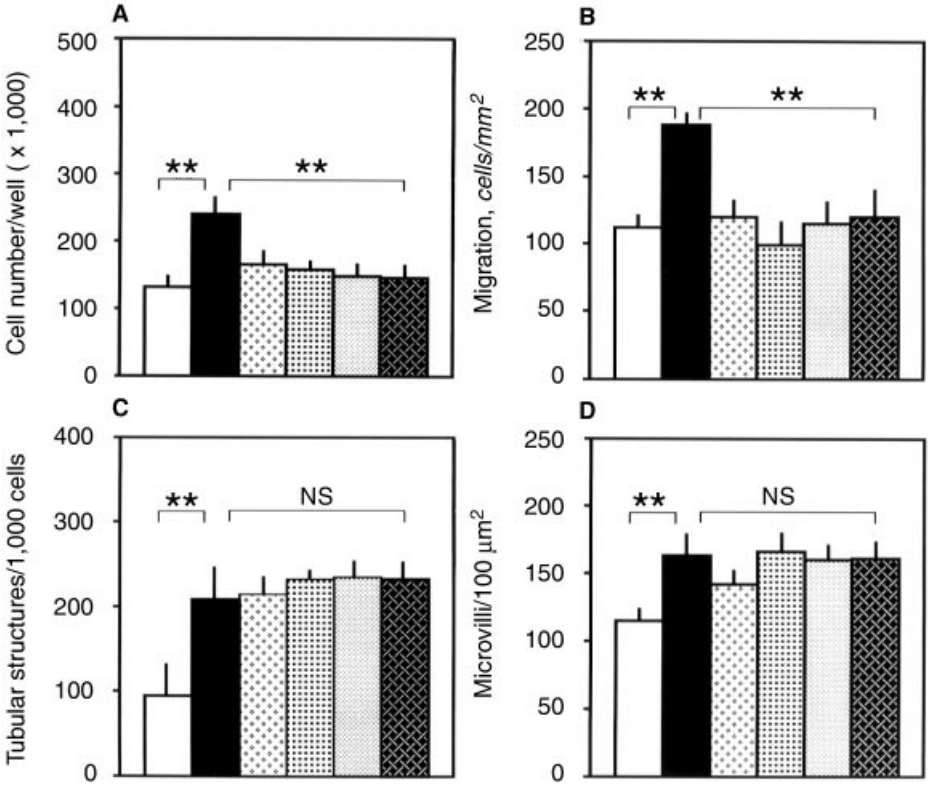

Fig. 1. Effects of hepatocyte growth factor (HGF) on PT-1 cell proliferation and differentiation and modulation by different protein tyrosine kinase inhibitors (PTKI). PT-1 cells were grown for 48 hours under serum-free conditions in the presence of 1 nM HGF alone or in combination with different PTKI at concentrations yielding half-maximal inhibition of cell growth (geldanamycin 0.01 μM, genistein 1 μM, herbimycin A 0.1 μM, and methyl-2,5-dihydroxycinnamate (MDC) 10 μM). (*A*) Cell proliferation. (*B*) Cell migration. (*C*) Formation of tubule structures. (*D*) Formation of microvilli. Data are means ± SEM of six independent experiments. $^*P < 0.05$, $^{**}P < 0.01$ versus serum-free control. Symbols are: (□) control; (■) HGF; (▨) HGF + genistein; (▦) HGF + MDC; (▧) HGF + geldanamycin; (▩) HGF + herbimycin A.

mean ± SEM of 4 to 10 independent experiments. The one-tailed Student's *t*-test for unpaired data was used to test means. $P < 0.05$ was considered significant.

RESULTS AND DISCUSSION

Hepatocyte growth factor bound to a single class of high-affinity sites (68,500 ± 3,000 per cell, K_d 2.5 ± 0.2 nM) in confluent PT-1 cells.

Hepatocyte growth factor stimulated cell growth already at 10 pM and maximally at 1 nM (used for all further experiments). The proliferative response could first be demonstrated after one hour, and cell number increased 1.8-fold after 48 hours ($P < 0.01$ vs. serum-free controls; Fig. 1A). All PTKI inhibited HGF-induced cell growth dose dependently (Table 1). Geldanamycin had the highest potency (EC_{50} 0.01 μM). Herbimycin A, genistein, and MDC had comparable effects at 10-, 100- and 1,000-fold higher concentrations, respectively. HGF enhanced migration of PT-1 cells from 112 ± 11 to 205 ± 15 cells/mm^2 ($P < 0.01$; Fig. 1B). All PTKI inhibited HGF-induced migration (Table 1). Cells cultured in collagen type I gels or under conventional conditions showed scattered phenotypes when incubated with HGF (Fig. 2). All PTKI inhibited the stimulatory effects of HGF on cell scattering with potencies as for cell proliferation and migration.

Table 1. Effects of different protein tyrosine kinase inhibitors on hepatocyte growth factor-induced proliferation, migration, and differentiation of PT-1 cells

Agent	Concentration μM	Biological effect (hepatocyte growth factor control = 100%) Cell growth ($N = 6$)	Cell migration ($N = 3$)
Geldanamycin	0.001	85 ± 15%	91 ± 5%
	0.01	62 ± 17%[a]	56 ± 6%[a]
	0.1	26 ± 8%[b]	26 ± 11%[b]
Herbimycin	0.01	93 ± 18%	71 ± 30%
	0.1	60 ± 21%[a]	58 ± 18%[a]
	1	30 ± 11%	46 ± 27%[a]
Genistein	0.1	99 ± 20%	92 ± 6%
	1	69 ± 12%[a]	58 ± 4%[a]
	10	52 ± 16%[b]	31 ± 14%[b]
Methyl-2.5-dihydroxycinnamate	1	85 ± 9%	79 ± 23%
	10	64 ± 14%[a]	48 ± 14%[a]
	100	37 ± 27%[b]	22 ± 27%[b]

Data are mean ± SD.
[a] $P < 0.05$, [b] $P < 0.01$

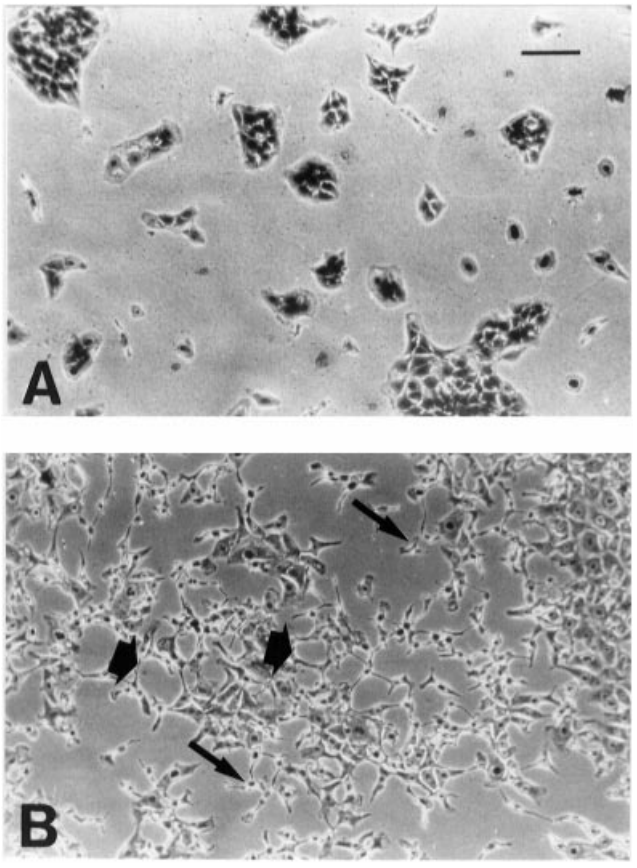

Fig. 2. Morphology of PT-1 cells grown for 48 hours in serum-free medium alone (*A*, bar = 10 μm) or in the presence of 1 nM hepatocyte growth factor (HGF). (*B*) HGF-induced cell scattering (thin arrows) and tubulogenesis (thick arrows).

Hepatocyte growth factor increased the number of tubule structures from 95 ± 14 to 212 ± 35/1,000 cells ($P < 0.01$; Fig. 1C). PTKI reduced the number of tubule structures to the same extent as the total cell counts, indicating that PTKI did not specifically inhibit the formation of tubule structures. In serum-free controls, 115 ± 12 microvilli were counted per 100 μm^2 apical cell membrane area. Incubation with HGF increased this number to 163 ± 32/100 μm^2 ($P < 0.05$; Fig. 1D). This effect of HGF was not inhibited by any PTKI.

Hepatocyte growth factor had multiple effects in PT-1 cells: it bound to a single class of high-affinity binding sites, enhanced cell proliferation and migration, induced a scattered phenotype, and stimulated tubulogenic cell differentiation. Because the complex actions of HGF on different target cells might be mediated by different signal cascades rather than by different receptors [5], we investigated the effects of different tyrosine-specific protein kinase inhibitors. The common feature of the PTKI used in our study is inhibition of $pp60^{c\text{-}src}$ [9, 10]. As a nonreceptor TK, $pp60^{c\text{-}src}$ is involved in cell growth control via the mitogen-activated protein kinase signal transduction pathway, resulting in nuclear transcriptional activation [10]. All PTKI significantly inhibited the effects of HGF on PT-1 cell proliferation, scattering, and migration, but not cell differentiation. Our data thus indicate that $pp60^{c\text{-}src}$ may be essential in mediating the mitogenic response to HGF in PT-1 cells, whereas stimulation of tubulogenic cell differentiation may involve a $pp60^{c\text{-}src}$–independent pathway. Better understanding of the signaling cascades that are crucial for the different actions of HGF in renal proximal tubule cells could provide the basis for future therapeutic use of HGF or substances modulating HGF signal transduction in acute and chronic renal failure.

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