Effects of hepatocyte growth factor on differentiation and cMET receptor expression in the promyelocytic HL60 cell line

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Abstract

Aim—To determine the effects of hepatocyte growth factor (HGF) on myeloid cell differentiation and cMET expression using the promyelocytic HL60 cell line.

Methods—HL60 cells cultured with purified recombinant HGF, dimethyl sulphoxide (DMSO), or 12-O tetradecanoylphorbol-13-acetate (TPA) were immunostained for the differentiation markers, human neutrophil elastase (HNE), cathepsin B, MAC387, or the receptor for hepatocyte growth factor (cMET).

Results—HGF treated cells were positive on staining for cathepsin B and MAC387, but were negative for HNE, indicating monocytic differentiation. HGF treated cells had the morphology of monocytes but continued to divide at the same rate as control cells and remained non-adherent. DMSO treated cells were positive for HNE and cell numbers were reduced, confirming myeloid differentiation. TPA treated cells were positive for cathepsin B and MAC387, cell numbers were reduced, and the cells became adherent, confirming terminal monocytic differentiation. Untreated HL60 cells were weakly positive for cMET at the start of the culture period and expression increased after 72 hours. Cells treated with HGF, DMSO, or TPA were also positive for cMET.

Conclusions—These data suggest that HGF induced partial monocytic differentiation in HL60 cells. In addition, expression of cMET by HL60 cells occurs at an early stage in myelomonocytic cells and is maintained after differentiation along either the myeloid or monocytic pathways.

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Hepatocyte growth factor (HGF) (scatter factor) is a potent mitogen for epithelial cells,^{1,2} particularly hepatocytes and biliary epithelial cells.^{3,4} Hepatocyte growth factor was first isolated from rat platelets,⁵ and its human gene homologue was characterised as a mature polypeptide composed of a 60 kilodalton α subunit linked by a single disulphide bridge to a 30 kilodalton β subunit.⁶ The biological effects of HGF are mediated via a 190 kilodalton cell surface tyrosine kinase receptor, the proto-oncogene product cMET.⁷ Besides the mitogenic actions of HGF, other biological properties,

including morphogenesis,⁸ tumour cell cytotoxicity,⁹ neutrophil priming,¹⁰ lymphocyte chemotaxis,¹¹ enhancement of B cell immunoglobulin secretion,¹² metastasis,¹³ and stimulation of growth of haematopoietic progenitor cells of bone marrow¹⁴ have been reported. The effects of HGF on haematopoietic cells and the pathophysiological relevance remain uncertain. However, the broad spectrum of biological effects demonstrated in vitro, coupled with the widespread expression of the cMET receptor on a variety of cell types, suggest that HGF may have significant effects on haematopoietic cell function and differentiation.

In the present study we have used the promyelocytic cell line HL60 as a model to study the effects of HGF on myeloid cell differentiation. We have also attempted to assess expression of cMET throughout differentiation. HL60 cells produce HGF when stimulated with low concentrations (1.6 nM) of 12-O tetradecanoylphorbol-13-acetate (TPA),¹⁵ but the effect of autocrine production of HGF on expression of the cMET receptor remains unknown. Whilst it has been reported that HL60 cells co-cultured with HGF may assume similar morphological characteristics to neutrophils,¹⁶ the effects of HGF on myeloid cell differentiation has not been investigated in greater detail.

To investigate these processes further, we have compared cells treated with purified recombinant HGF with cells treated with the standard differentiating agents dimethyl sulphoxide (DMSO) and TPA. In HL60 cells the latter two agents induce myeloid and monocytic differentiation pathways, respectively. We have assessed, by immunohistochemistry, myeloid differentiation using antibodies to human neutrophil elastase (HNE)¹⁷ and monocytic differentiation with antibodies to cathepsin B¹⁸ and MAC387.¹⁹ Expression of cMET was also determined for cells treated with HGF, DMSO, and TPA.

Methods

HL60 cells were obtained from the European Collection of Animal Cell Cultures (Porton, Wiltshire, UK) as a mycoplasma-free culture. The tissue culture medium was RPMI 1640 basal medium supplemented with 10% v/v heat inactivated fetal calf serum, 2 mM L-glutamine (Flow Laboratories, Herts, UK). Purified recombinant HGF was a gift from Dr T Ishii (Mitsubishi Kasei Corporation, Yokohama, Japan). The concentration and biological activity of the preparation was confirmed by en-

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zyme linked immunosorbent assay (ELISA)²⁰ and a standard hepatocyte proliferation assay.²¹ Dimethyl sulphoxide, TPA, and the alkaline phosphatase substrate (naphthol AS-MX phosphate) were obtained from the Sigma Chemical Company (Poole, Dorset, UK). Antiserum to the human HGF receptor (polyclonal rabbit antihuman cMET) was obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, California, USA). Alkaline phosphatase conjugated swine antirabbit immunoglobulins and mono-

clonal mouse antihuman MAC387 were obtained from Dako Ltd. (High Wycombe, Bucks, UK). Polyclonal sheep anti-HNE and cathepsin B were obtained from The Binding Site (Birmingham, UK). Biotinylated secondary antibodies, non-immune blocking serum, horseradish peroxidase (HRP) labelled streptavidin/biotin complexes, and HRP substrate (diaminobenzidine) were supplied as Vectastain ABC detection kits (Vector Laboratories, Peterborough, UK).

Cells in the logarithmic phase of growth and with viability in excess of 95% (determined by the trypan blue dye exclusion test) were seeded in Linbro 24 well multiwell plates at 2×10^5 /ml in tissue culture medium alone, or medium supplemented with 10% v/v DMSO, 1.6 nM TPA, 16 nM TPA, or HGF (35 ng/ml). At time intervals up to seven days, cells were harvested and viabilities and adherence determined, and cytocentrifuge preparations made for morphological and immunohistochemical analysis.

Briefly, MAC387 was localised as follows: firstly, endogenous peroxidase activity was blocked using 0.6% hydrogen peroxide in methanol, and non-specific binding of immunoglobulin was blocked with non-immune serum (1 in 10 dilution). Cytospins were then incubated with monoclonal mouse antihuman MAC387 (1 in 50 dilution) followed by biotinylated rabbit antimouse immunoglobulins and streptavidin/biotin/HRP complexes. Bound antibody was subsequently visualised with diaminobenzidine substrate. Human neutrophil elastase and cathepsin B were localised using polyclonal sheep anti-HNE or cathepsin B followed by biotinylated goat antisheep immunoglobulins and streptavidin/biotin/HRP complexes, and visualised as described above. Between stages, slides were washed three times with phosphate buffered saline. The cMET receptor was immunolocalised using a double antibody method, rabbit anti-cMET followed by alkaline phosphatase labelled swine antirabbit immunoglobulins. Bound antibody was visualised with naphthol AS-MX phosphate at 0.2 mg/ml in 0.1 M TRIS/HCl buffer containing levamisole (to block non-specific/endogenous alkaline phosphatase activity) at a final concentration of 1 mM. For each immunohistochemical stain, control sections were included where the primary antibody was substituted with non-immune serum. Experiments were performed in triplicate and multiple cytocentrifuge preparations were taken from each culture.

Results

EFFECTS OF HGF ON DIFFERENTIATION MARKERS AND CELL PROLIFERATION

The cells treated with HGF were positive on staining for cathepsin B and MAC387 after 48 hours, and remained negative for HNE, indicating that HGF was able to induce monocytic differentiation (fig 1 and table). Cell proliferation was monitored for HGF treated cells for up to seven days and viability remained in excess of 90%. The difference in cell numbers between HGF treated cells and controls

Figure 1 Immunohistochemical staining of cytospin preparations of HL60 cells treated with 35 ng/ml HGF. A: HNE, B: cathepsin B (cath B), or C: MAC387. Cells in panel A were negative for HNE. Therefore, the preparation has been counterstained with haematoxylin to facilitate visualisation of the cells; n=nucleus. Arrows indicate negatively or positively staining cells. Original magnification $\times 630$.

Summary of immunohistochemical staining of HL60 cells

	cMET		MAC387	Cathepsin B	HNE	Adherence
	0 hours	72 hours	72 hours	72 hours	72 hours	72 hours
HGF (35 ng/ml)	+	++++	++++	++++	Negative	No
DMSO (10%)	+	++	Negative	Negative	++++	No
TPA (16 nM)	+	++++	++++	++++	Negative	Yes
TPA (1.6 nM)	+	++++	++	++	Negative	Yes
Control	+	++	Negative	Negative	Negative	No

Intensity of staining was assessed visually and scored from weak (+) to intense (++++).

did not reach statistical significance, suggesting that HGF had no effect on cell proliferation (fig 2A). After two to three days in culture, the cells treated with DMSO stopped dividing (fig 2B) and became positive for HNE, but remained negative for cathepsin B and MAC387, confirming that DMSO induced differentiation of HL60 cells into myeloid-like cells (table). Cells treated with either 1.6 or 16 nM TPA stopped dividing (fig 2B), became positive for cathepsin B and MAC387, and remained negative for HNE, a pattern consistent with differentiation into monocytes (table). The DMSO and TPA control experiments were not continued for more than three days because of a significant reduction in cell viability. Throughout the ex-

periments, the control cells remained negative for all markers of differentiation (table).

EFFECTS OF HGF AND DIFFERENTIATING AGENTS ON cMET EXPRESSION
Untreated HL60 cells were weakly positive for the cMET receptor (table and fig 3A). After a further three days in culture, the untreated cells showed an increase in intensity of staining (fig 3B), indicating that cMET expression increased spontaneously with time. Cells treated with DMSO for three days also showed immunoreactivity for cMET and remained non-adherent to the culture wells (table). When exposed to low or high concentrations of TPA, strong, evenly distributed cMET expression was observed and the cells became adherent to the culture wells (table). Cells treated with HGF also expressed cMET strongly after 72 hours (table and fig 3C). On visual assessment, cMET expression appeared to increase following culture with either TPA or HGF compared with control cells (table 1 and fig 3C).

Discussion
The present study has compared the effects of HGF on myeloid cell differentiation with those observed with the standard differentiating agents DMSO and TPA. The results show that HGF causes promyelocytic HL60 cells to become committed to monocytic differentiation. We have confirmed this using antibodies that are specific lineage markers for either neutrophils¹⁷ or monocytes.^{18,19} In contrast to TPA, however, the HGF treated cells remained non-adherent and continued to proliferate at a similar rate to control cells. HL60 cells expressed cMET receptors and expression appeared to increase spontaneously with time and was maintained irrespective of myeloid or monocytic differentiation (table). That exogenous HGF did not affect cell proliferation or promote adhesion to culture wells suggested that HGF induced partial monocytic differentiation. Our data do not concur with other findings, which suggested that HGF may induce myeloid differentiation.¹⁶ The reasons for this difference remain uncertain but may relate to the source or the biological activity of the HGF used. We had chosen a HGF concentration of 35 ng/ml to perform our experiments because this was the concentration (of our current HGF preparation) that induced maximum hepatocyte proliferation (the accepted index of biological activity).^{3,4} We performed some initial experiments at concentrations up to 200 ng/ml but demonstrated no further effects (data not shown). In their recent publication Shima *et al*¹⁶ used HGF at a concentration of 200 ng/ml. This is a very high concentration, even in vivo when abnormal or pathological circumstances prevail.²⁰

Extrapolation of our conclusions to normal myeloid differentiation must be approached cautiously as HL60 cells are a cell line and their growth and function may be regulated differently compared with normal myeloid pre-

Figure 2 Effect of HGF and differentiating agents on cell proliferation. A: Cell counts at time intervals up to seven days. B: Cell counts at 72 hours for control cells; cells + 10% v/v DMSO; cells + 1.6 nM TPA; and cells + 16 nM TPA. Values are expressed as mean (SD) (n = 6).

Figure 3 Immunohistochemical staining of cytopsin preparations of HL60 cells for cMET. A: Cells alone at start of culture; B: cells alone after 72 hours; C: cells plus recombinant HGF after 72 hours. Arrows indicate positive cMET staining, ranging from weak (+) to intense (+++). Original magnification $\times 630$.

cursor cells. Nevertheless, our study demonstrates that, in isolated culture systems, exogenous HGF may commit myeloid precursor cells to monocytic differentiation, but its effects are distinct from those elicited by TPA.

It has been shown previously that low concentrations of TPA (1.6 nM) induce production of HGF by this cell line, but higher concentrations (16 nM) do not.¹⁵ We confirmed this in our experiments by monitoring

HGF production after TPA treatment with a specific ELISA assay (data not shown). In HL60 cells HGF synthesis appeared to be induced only by the lower (1.6 nM) concentration, an occurrence which may be related to the dose dependent effects of TPA on protein kinase C isoform activation, as shown in other cell systems.²²⁻²⁴ Thus, our data show that cMET expression does not diminish as a result of treatment with standard differentiating agents (DMSO and TPA), HGF, or when the cells are actively producing HGF themselves.

Hepatocyte growth factor can potentially influence a variety of leucocyte functions.¹⁰⁻¹² It is possible therefore that maintenance of cMET expression is important during leucocyte differentiation, and subsequent migration of cells from the bone marrow compartment. The latter could explain why the HGF treated cells expressed monocytic markers but were not adherent. This would represent a logical stage of maturation for monocytic cells before release into the circulation, with adherence occurring at a later stage of maturation or activation. Similarly, retention of proliferative potential, once cells have left the bone marrow, may also be important. It is known that tissue macrophages can retain proliferative potential.²³ Thus, from the present studies we postulate that TPA treated HL60 cells behave more like terminally differentiated, non-proliferating and possibly activated monocytes. By contrast, HGF treated cells behave more like cells at an earlier stage of differentiation, but committed to the monocytic pathway.

The precise role of HGF in the regulation of leucocyte function is likely to be dependent upon a complex set of variables including the presence of cofactors and the status of the cell population in terms of the proliferative/maturation cycle. Previous work has shown that HGF can act synergistically with interleukin-3 on human bone marrow cultures, to enhance growth of haematopoietic progenitor cells.¹⁴ Hepatocyte growth factor did not affect cellular differentiation in those circumstances. The latter observations serve to highlight that the biological effects of this growth factor may differ, depending on the presence of cofactors and possibly the stage of the cell cycle. The data presented here show that the HL60 cell line will be of value in delineating these pathways.

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