Multiple myeloma cells catalyze hepatocyte growth factor (HGF) activation by secreting the serine protease HGF-activator

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Abstract

Multiple myeloma (MM) is a common hematological neoplasm consisting of malignant plasma cells, which expand in the bone marrow. A potential key signal in the evolution of MM is hepatocyte growth factor (HGF), which acts as a potent para- and/or autocrine growth- and survival factor for MM cells. Proteolytic conversion of HGF into its active form is a critical limiting step in HGF/MET signaling. Here, we show that malignant MM plasma cells convert HGF into its active form and secrete HGF-activator (HGFA), a serine protease specific for HGF activation. By using serine protease inhibitors and neutralizing antibodies, we demonstrate that HGFA produced by the MM cells is responsible for their ability to catalyze HGF activation. We therefore suggest that autocatalyzation of HGF conversion by MM cells is an important step in HGF/MET-induced myeloma growth and survival, which may have implications for the management of this incurable form of cancer.
Introduction

The unrestrained growth of tumor cells is generally attributed to mutations in essential growth control genes, but tumor cells are also influenced by signals from the environment. In MM, the factors and signals coming from the bone marrow (BM) microenvironment are possibly even essential for the growth of the tumor cells. As targets for intervention, these signals may be equally important as mutated oncogenes. Recent studies have identified HGF as a potential key signal in the evolution of MM. HGF has a domain structure and proteolytic mechanism of activation similar to that of the blood serine protease plasminogen, but lacks protease activity. Instead, via its tyrosine kinase receptor MET, HGF induces complex biological responses in target cells, including motility, growth, and morphogenesis. Whereas a functional HGF/MET pathway is indispensable for mammalian development, uncontrolled MET signaling, provoked by MET activating mutations or MET amplification and overexpression, is oncogenic, and has been implicated in the development and progression of a variety of human cancers. In MM, HGF exerts strong proliferative and anti-apoptotic effects via the RAS/MAPK and PI3K/PKB pathways. Within the BM microenvironment, stromal cells present a paracrine source of HGF, however, an autocrine HGF/MET loop has also been reported in myeloma cells. Furthermore, in a recent gene-profiling study HGF was the only significantly overexpressed growth factor in MM, while high serum HGF levels in MM patients predict unfavorable prognosis.

Upon secretion, HGF normally retains its 90-kDa single-chain (sc) precursor form, which is probably cell surface or extracellular matrix associated. For biological function however, proteolytic conversion of scHGF to the heterodimeric active form is essential. Although the role of HGF in tumor progression has attracted much attention, the molecular mechanisms underlying HGF activation in tumor tissue remain largely unexplored. Plasminogen activators, particularly uPA and factor XIIa have been shown to activate scHGF, although at low rates. More recently, hepatocyte growth factor activator (HGFA), a factor XIIa-related serine protease with an efficient HGF-activating activity, was identified. This enzyme is secreted by the liver as an inactive zymogen and has recently also
been shown to be produced by colorectal cancer cells $^{18}$. In this paper, we have studied the mechanism of HGF activation in MM. We show that myeloma cell lines as well as primary myelomas secrete HGFA and in this way are able to autcatalyze HGF activation.
Material and Methods

Antibodies

Monoclonal antibodies were: anti-HGFA, A-1 (IgG1) and P1-4 (IgG1)\textsuperscript{15}; anti-factor XIIa, OT-2 (IgG1) (Sanquin, Amsterdam, The Netherlands); IgG1 control antibody (DAKO, Glostrup, Denmark); anti-hepatocyte growth factor activator inhibitor-1 (HAI-1)\textsuperscript{18}. Polyclonal antibodies used were goat anti-human HGF (R&D Systems, Abington, UK); R-phycoerythrin-conjugated goat anti-mouse (Southern Biotechnology, Birmingham, AL); horseradish peroxidase (HRP)-conjugated rabbit anti-mouse (DAKO); HRP-conjugated goat-anti-rabbit (DAKO).

MM cells, cell cultures, transfectants and conditioned medium

MM cell lines UM1, UM3, UM6, L363, NCI-H929, OMP-1, LME-1, and XG-1 were grown as described previously\textsuperscript{6,7}. COS7 cells were transiently transfected with the mammalian expression vector pClneo-HGFA containing full-length HGFA\textsuperscript{18} using the DEAE-dextran method. Conditioned medium was obtained as described previously\textsuperscript{19}.

Primary myeloma cells (PM) were obtained from the pleural effusion of a 67 year old male patient. FACS analysis showed > 95% CD138\textsuperscript{high}, CD38\textsuperscript{high} cells. Mononuclear cells were harvested by standard Ficoll-Paque density gradient centrifugation (Amersham Pharmacia, Uppsala, Sweden).

Immunoprecipitation and western blot analysis

Immunoprecipitation and western blotting was performed as described\textsuperscript{6}. For the HGF activation assay, serum free cultured cells were lysed in the absence of protease inhibitors since these affect the function of HGFA. For immunoprecipitation of HGFA, the lysates were incubated with the monoclonal antibody A-1 pre-coupled to Protein G-Sepharose beads (Pharmacia Biotech, Uppsala, Sweden). The precipitates were washed three times with lysis buffer and were resolved by sodium dodecylsulfate polyacrylamide gel electrophoresis under reducing conditions. The immunoblots were stained with anti-HGF or anti-HGFA and
detected with HRP-conjugated swine anti-goat and HRP-conjugated rabbit anti-mouse respectively.

**Assay for HGF activation**

HGF activation was assayed as described previously. In brief, single chain HGF (R&D Systems) was incubated with either intact MM cells, with MM conditioned medium or with HGFA immunoprecipitated from MM conditioned medium. To study activation by cells, these were washed thoroughly and incubated serum free overnight. Subsequently, the cells were washed and $10^5$ cells were incubated in 0.1 ml medium containing scHGF (1 µg/ml) for the time indicated in the presence or absence of 4 units/ml thrombin (Sigma Aldrich Chemie GmbH, Germany). For HGF activation, 20 µl conditioned medium or sample containing immunoprecipitated HGFA were pretreated with 1 unit of thrombin and added to 0.1 µg scHGF. Inhibitor studies were done in the presence of aprotinin (2TIU/ml), leupeptin (500 µg/ml), C1-inhibitor (kindly provided by E. Hack, Sanquin, Amsterdam, The Netherlands) or neutralizing antibody P1-4 (40 µg/ml).

**Immunocytochemistry**

HGFA expression in MM cell lines and primary myeloma cells was studied on aceton-fixed cytospins with mAb A-1 using biotin-conjugated rabbit as second step. The reaction was developed with 3,3-amino-9-ethylcarbazole (Sigma) and cytospins were counterstained with Haematoxylin. COS-7 cells transfected with a construct containing HGFA were used as positive control, and appropriate isotype antibodies as negative controls.

**Flow Cytometry**

For the determination of HGFA expression, monoclonal antibody PI-4 and secondary antibody PE-conjugated goat anti mouse Ig (Southern Biotechnology) were used. For intracellular HGFA staining, the MM cell lines were fixed with 2% paraformaldehyde and
permeabilized with saponin. Analysis was carried out on a FACScalibur flow cytometer (Becton Dickinson Biosciences) with CELLQuest TM software (BD).
Results and discussion

Proteolytic activation of HGF in the extracellular milieu is a critical limiting step in HGF/MET signaling. We observed that the MM cell lines NCI-H929, XG-1 and OMP-1 cells were all able to process scHGF to its active form (Figure 1A). The processing of scHGF either required or was greatly enhanced by the addition of thrombin and was completely inhibited by the serine protease inhibitors leupeptin (Figure 1A) and aprotinin (not shown). Since HGFA is a serine protease specifically activated by thrombin\textsuperscript{17,20}, these observations suggested that the HGF processing activity might be due to HGFA. Indeed, RT-PCR analysis demonstrated the presence HGFA mRNA in all MM cells tested (data not shown), while a band of 96-kDa, which corresponds to the molecular mass of the proform of HGFA, was detected by immunoblotting (Figure 1B). This band was also detected in the lysates of the colorectal cancer cell lines DLD-1 and SW480, which express HGFA\textsuperscript{18}, and in the lysates of COS-7 cells transfected with a plasmid containing HGFA, but not in COS-7 cells transfected with empty vector (Figure 1B). Immunocytochemistry showed a distinct granular intracytoplasmic HGFA expression pattern, which was present in all MM cell lines as well as in the HGFA transfected COS-7 cells (Figure 1C and data not shown). The same expression pattern was also found in all (n=8) primary MM samples analyzed as exemplified in figure 1C. FACS analysis confirmed the expression and intracellular localization of HGFA (Figure 1D).

The 34-kDa catalytically active form of HGFA\textsuperscript{16} was not detected in the MM lysates (Figure 1B). By contrast, the conditioned media of the cell lines contained variable amounts of the 34-kDa form of HGFA indicating that MMs secrete and, to a certain extent, activate HGFA (Figure 2A). Indeed, HGFA immunoprecipitated from the MM conditioned media effectively converted scHGF (Figure 2B). Since proteases other than HGFA are, although with low efficiency, capable of activating scHGF \textit{in vitro}\textsuperscript{14,15}, we explored whether the conversion of scHGF by MM cells could be specifically inhibited by interfering with HGFA activity. We observed that the anti-HGFA monoclonal P1-4, which blocks HGFA function\textsuperscript{20} (Figure 2C, left panel), effectively inhibits scHGF conversion by MM cells (Figure 2C, middle + right panel). By contrast, scHGF conversion was not affected by interfering with factor XIIa.
function with either a blocking mAb (OT-2) or with the protease inhibitor C1-inhibitor (Figure 2C). Hence, HGFA is the (major) serine protease responsible for the conversion of scHGF by MM cells. Most MM cell lines, including H929 and XG-1, also expressed HAI-1 (data not shown), but the presence of this HGFA-regulatory protein apparently did not block HGF conversion (Figure 1A). This seemingly contradictory finding may be explained by the complex effects of HAI-1 on HGF conversion. Thus, whereas the soluble Kunitz 1 form of HAI-1 can inhibit HGF conversion, the membrane bound form of HAI-1 is believed to concentrate active HGFA at the cell surface and, upon release, may promote activation of HGF. Hence, it is not surprising that HAI expression per se does not predict inhibitory activity.

Our study identifies expression and secretion of HGFA by MM cells as a potentially important factor in regulating the bioavailability of active HGF in the MM microenvironment, while the activated BM stroma in MM may present an additional source of both HGF and HGFA. Catalyzation of HGF activation by MM cells may directly stimulate HGF/MET signaling in the tumor cells, promoting MM cell growth and survival. In addition, since HGF is a potent angiogenic factor, it may also contribute to tumor angiogenesis which has recently been identified as an important process in the progression and prognosis of MM (Figure 2D). Our study identifies the activation step of HGF as a promising new target in MM therapy.
Reference List


Figure 1

MM cells proteolytically convert HGF into its active form and express the serine protease HGFA. (A) MM cells convert HGF into its active form. MM cell lines NCI-H929, XG-1 and OMP-1 were incubated with scHGF for 6 hours in the presence or absence of thrombin and/or the serine protease inhibitor leupeptin, as indicated. HGF conversion was determined by immunoblotting with anti-HGF. As positive control, HGF conversion by recombinant HGFA is shown (left panel). The right panel shows the time kinetics of scHGF conversion by MM cells (in the presence of thrombin). As positive and negative controls, scHGF conversion by COS-7 cells transfected with either a plasmid containing HGFA or empty vector are shown. (B) Expression of HGFA in MM cell lines. Cell lysates were immunoblotted using a monoclonal anti-HGFA antibody (A-1). COS-7 cells transfected with HGFA and the colorectal carcinoma cell lines DLD-1 and SW480 were used as positive controls. COS-7 cells transfected with empty vector were used as negative controls. β-actin was used as loading control (lower panel). (C) Expression of HGFA in MM cell lines and primary myeloma cells. MM cell line NCI-H929, primary myeloma cells (PM), or COS-7 cells transfected with
either empty vector or a plasmid containing HGFA were immunocytochemically stained with mAb A-1 against HGFA or isotype control. (D) HGFA expression in MM cells is intracellular. The indicated MM cells, either permeabilized (right panel) or not (left panel), were stained with anti-HGFA mAb PI-4 (bold line) or isotype control antibody (grey line). Expression was measured by FACS analysis.
Figure 2

HGFA mediates HGF conversion by MM cells. (A) MM cells secrete HGFA. To detect the active (34 kDa) form of HGFA, MM conditioned medium (CM), either or not pre-treated with thrombin, as indicated, was immunoblotted with anti-HGFA antibody A-1. (B) HGFA from MM conditioned medium converts HGF. HGFA (+) or IgG control (-) immunoprecipitates from MM conditioned medium were analyzed in a HGF conversion assay. (C) HGFA mediates HGF activation by MM cells. The effects of neutralizing antibodies against HGFA (P1-4) and Factor XIIa (OT-2), protease inhibitors aprotinin and leupeptin, and C1-inhibitor on HGF activation by recombinant HGFA (left panel) and conditioned medium of MM cell line NCI-H929 (middle panel) and primary MM cells (right panel) were analyzed by the HGF conversion assay. HC = immunoglobulin heavy chain.

(D) Activation and biological actions of HGF in the myeloma microenvironment.
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