Met Expression and Sarcoma Tumorigenicity

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Abstract

The *met* protooncogene tyrosine kinase receptor (Met) and its ligand, hepatocyte growth factor/scatter factor (HGF/SF), ordinarily constitute a paracrine signaling system in which cells of mesenchymal origin produce the ligand, which binds to the receptor that is predominantly expressed in cells of epithelial origin. However, mouse NIH/3T3 fibroblasts overexpressing Met induce tumor formation in nude mice via an autocrine mechanism (S. Rong et al., Mol. Cell. Biol., 12: 5152-5158, 1992). In this study, we report that human cell lines established from various sarcomas express high levels of activated Met receptor. HGF/SF is also detected in the human sarcoma cell lines but at a reduced level when compared to primary fibroblasts. These properties, high Met expression and reduced ligand levels, are indistinguishable from the properties of NIH/3T3 tumor explant cells overexpressing Met (S. Rong et al., Mol. Cell. Biol., 12: 5152-5158, 1992; S. Rong et al., Cell Growth & Differ., 4: 563-569, 1993). Moreover, paraffin-embedded sections of primary tumors from human osteosarcomas, chondrosarcomas, and leiomyosarcoma stain intensely for Met and/or HGF/SF and display extensive tumor cell heterogeneity with regard to both paracrine and autocrine stimulation. On the basis of these findings, we propose that Met-HGF/SF autocrine signaling may contribute to the tumorigenic process in human sarcomas.

Introduction

Expression of the *met* protooncogene receptor tyrosine kinase (Met) occurs in a majority of adult and embryonic tissues, predominantly in epithelial cells (1-4). The ligand for Met is HGF/SF, a fibroblast-derived mitogen for hepatocytes as well as other cell types (5-7). HGF/SF promotes cell movement and induces epithelial morphogenesis (8-10). Therefore, HGF/SF and Met can constitute a paracrine signaling system in which cells of mesenchymal origin produce HGF/SF and this ligand binds to the receptor predominantly expressed on cells and tissues of epithelial origin (11, 12).

The "met" protooncogene is amplified in most spontaneous transformants of NIH/3T3 cells (13, 14) and we have shown that "met" complementary DNA efficiently transforms NIH/3T3 cells (1). More recently, we have demonstrated that "met"-induced NIH/3T3 cell tumorigenicity is due to an autocrine mechanism (15, 16). The highly tumorigenic behavior of "met"-transformed NIH/3T3 cells led us to examine human primary fibroblast cells and cell lines established from various human sarcomas for Met expression and for evidence of autocrine stimulation. We find that Met is expressed at low levels in fibroblast cells which, in the presence of endogenously expressed HGF/SF, lead to an autocrine interaction. Our studies also show that Met overexpression occurs frequently in human sarcoma cells and tumors and could play an important role in their tumorigenesis.

Materials and Methods

Cell Lines and Antibodies. Cell lines used in this study are listed in Table 1. Most of the cell lines used in this study were obtained from American Type Culture Collection and grown as recommended. A fibrosarcoma cell line, 8387 (grown in Dulbecco's modified Eagle's medium with 10% bovine fetal serum), and a rhabdomyosarcoma cell line, RD-1 (grown in McCoy's 5A with 15% bovine fetal serum), were obtained from Doug Halverson (National Cancer Institute, Frederick, MD). NIH/3T3 cells transfected with neo' and met' were described by Rong et al. (15).

The 19s anti-Met monoclonal antibody was generated against a bacterially expressed p50 form of Met (15, 17). The Met-specific C28 anti-peptide antibody was raised by immunization of rabbits with the 28 amino acid COOH-terminal peptide of human Met (18). A3.1.2 is a monoclonal antibody against human recombinant HGF (15). 23C2 is a monoclonal antibody against human placental SF (19). Anti-rHGF-1 is a rabbit polyclonal antibody against human recombinant HGF (10). Anti-rHGF-2 is purified goat IgG against human recombinant HGF (R&D Systems). 4G10 is a monoclonal phosphotyrosine antibody (anti-P-Tyr) (20).

Immunoprecipitation Analysis. Immunoprecipitation analysis for both Met and HGF/SF was carried out as described previously (15).

Confocal Laser Microscopy and Immunofluorescence Analysis. Immunofluorescence assays were performed as described previously (4, 16).

Western Immunoblot Analysis. Western analysis was done essentially as described previously (15) except that the cells were lysed in RIPA buffer [1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 0.15 M NaCl, 0.02 M NaPO4 (pH 7.2)], containing 1.25 mM phenylmethylsulfonyl fluoride, 2 mM EDTA, 50 mM NaF, 30 mM sodium pyrophosphate, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM sodium ortho-phenanthroline.

Scatter Assay. Scatter assay using MDCK cell line was carried out as described previously (12, 16).

Northern Analysis. Total cellular RNA was isolated using RNAzol as described by the supplier (CINNA/BIOTEX). Twenty μg of total RNA were denatured, electrophoresed on 1% formaldehyde agarose gel, and transferred to nylon membranes (Schleicher and Schuell) as described (1). Hybridizations were carried out for 2 days with 106 cpm/ml of probe (specific activity, 108 cpm/μg) (Random Priming Labeling Kit; Boehringer-Mannheim). Filters were washed twice in 2 x standard saline citrate-0.1% SDS at room temperature for 10 min and then 3 times in 0.2 x standard saline citrate-0.1% SDS at 55°C.

Mitogenic Assay. Three x 104 cells were seeded into 96-well microtiter plates (Costar). After an overnight incubation at 37°C, these cells were starved in serum-free medium for 2 days. Different concentrations of purified HGF' (16) were added in the presence or absence of HGF' neutralizing antibody (anti-rHGF-2; R&D Systems; 1:20 dilution) and incubated overnight. [3H]-Thymidine was added at 1 μCi/well for 4 h and cells were lysed with 0.1 ml of 0.02 M NaOH-0.1% SDS. Aliquots of the lysate were used for scintillation counting.

Results

Met and HGF/SF Expression in Human Fibroblast Cell Cultures and Sarcoma Cell Lines. Total RNA extracted from immortalized human fibroblast cell cultures and from human cell lines established from various human sarcomas was analyzed for met RNA expression using a full length met cDNA probe. The major met mRNA, a 9-kilobase transcript (2), was present in all of the samples tested (Fig. 1A; Table 1). We also examined these cells and cell lines for Met protein expression by immunoblot analysis. Met was precipi-
Table 1 Met and HGF/SF expression in human fibroblast and sarcoma cell lines

<table>
<thead>
<tr>
<th>Met expression</th>
<th>HGF/SF expression</th>
<th>Scatter activity</th>
<th>P-Tyr-Met</th>
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</thead>
<tbody>
<tr>
<td>HEL 299 (fetal lung)</td>
<td>++ ++ +++++ +++++</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Hems (fetal muscle)</td>
<td>++ ++ +++++ +++++</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Hs68 (newborn fibroblast)</td>
<td>++ ++ +++++ +++++</td>
<td>–</td>
<td>–</td>
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<tr>
<td>Malme-3 (skin fibroblast)</td>
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<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Fibrosarcoma</td>
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<td>+++++ +++++ +++++</td>
<td>+</td>
</tr>
<tr>
<td>HT1080</td>
<td>++++ +++++ +++++</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
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<td>+</td>
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<tr>
<td>SW684</td>
<td>+++++ ND +++++</td>
<td>+</td>
<td>+</td>
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<td>Leiomysarcoma</td>
<td>SK-LMS-1</td>
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<td>WM206-4</td>
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a Met protein level was assessed by immunoprecipitation and Western analysis. –, not detected.

b Met gene expression was detected by Northern analysis. ND, not determined.

c HGF/SF protein level was assessed by immunoprecipitation analysis.

d Scatter activity was assayed with MDCK cells.

*P-Tyr-Met was analyzed by Western analysis with anti-P-Tyr.
expression, although we could not exclude the possibility that the difference in steady-state Met levels also reflects ligand-mediated down-modulation of the receptor. The high levels of Met in the sarcoma cell lines and its presence in fibroblast cells represented novel findings, since Met expression was thought to be preferentially present in epithelial cells, while only the ligand was restricted to mesenchymal cells (11, 21).

We determined the levels of immunoprecipitable HGF/SF using growth medium harvested from metabolically labeled fibroblast and sarcoma cells (Fig. 1C). Abundant levels of the HGF/SF p69 α-subunit (p69HGF/SF) were observed in all of the primary fibroblast cell cultures (Fig. 1C, Lanes 1–3; Table 1), but only one sarcoma cell line (Hs913T) secreted high levels (Fig. 1C, Lane 12). The level of HGF/SF was also determined by scatter assays performed on growth medium that was conditioned on confluent cells for 72 h. Comparable to the high levels of p69HGF/SF detected, high levels of scatter activity were detected in conditioned medium from HEL299 and Hems fibroblast cultures (Table 1). However, lower activity was observed in the scatter assays performed with Hs68 and Malme-3 conditioned medium that did not correlate with the high levels of p69HGF/SF detected (Fig. 1C, Lane 3; Table 1). The Hs913T fibrosarcoma cells also express high levels of p69HGF/SF (Fig. 1C, Lane 12), and also exhibit low scatter activity. However, with the exception of Hs913T cells, the levels of HGF/SF and scatter activity were low in cells that expressed high levels of Met (Table 1). A similar marked reduction in the endogenous HGF/SF was observed in NIH/3T3 cells overexpressing Metlox that was presumably due to the depletion of the ligand by the receptor (16). Evidence for the HGF/SF receptor activation was indicated by the high reactivity of Metloxi with anti-P-Tyr antibody (15). We, therefore, performed anti-P-Tyr immunoblot analyses on the Met expressed both in the human fibroblast cell cultures and in the sarcoma cell lines (Fig. 1B,b). These analyses showed that, in general, the overexpressed Met is highly reactive with anti-P-Tyr antibody (Fig. 1B,b, Lanes 2, 4–6, 9, 10; Table 1), similar to Metloxi in NIH/3T3 cells (15). We also found that Metloxi was weakly reactive with anti-P-Tyr antibody in the primary Hems
fibroblasts (Fig. 1B,b, Lane 1), suggesting that the receptor was activated in an autocrine fashion by endogenous HGF/SF.

**Autocrine Interaction of Met and HGF/SF in Primary Fibroblast Cells.** We tested the possibility that an autocrine Met-HGF/SF stimulatory pathway exists in the primary fibroblast cell cultures. HEMS and HEL299 cells expressed low levels of Met and high levels of HGF/SF compared to other cell lines tested (Fig. 1B; data not shown). To test whether the level of Met was being down-modulated by HGF/SF, we added anti-rHGF-1, a neutralizing HGF/SF antibody (10), to the growth medium of HEL299 and HEMS cells. After 48 h, cell lysates were analyzed for levels of Met by immunoblot analysis (Fig. 2). These analyses indicate that there is a significant increase in the amount of p140Met in HEL299 cells (Fig. 2, Lanes 1–4) and HEMS cells (Fig. 2, Lanes 5–6) in the presence of the antibody. These results indicate that Met is down-regulated via extracellular-autocrine activation. The requirement for HGF/SF to be activated by extracellular proteolytic cleavage (22, 23) was consistent with these results.

**Mitogenic Response of Sarcoma Cells to HGF/SF.** Met-HGF/SF signaling has been implicated in both mitogenic and motogenic activities for epithelial cells (21). As a control for HGF/SF mitogenicity, we measured [3H]thymidine incorporation in NIH/3T3 cells overexpressing Methu (15) in response to the addition of exogenous HGF/SFhu (Fig. 3). A 9-fold stimulation of [3H]thymidine incorporation was observed when 5 units/ml of exogenous HGF/SF was added to NIH/3T3 cells overexpressing Methu (Fig. 3B), but not to control NIH/3T3 cells (Fig. 3A). These analyses showed that HGF/SFhu was mitogenic for NIH/3T3 fibroblast cells overexpressing Methu; but, at high levels, the ligand was inhibitory. HGF/SF also stimulated [3H]thymidine incorporation in the human sarcoma cell line SK-LMS-1 (Fig. 3C) and this stimulation was prevented by anti-rHGF-2 neutralizing antibody (Fig. 3D). Curiously, much higher levels of HGF/SFhu (16×) are required for stimulation of the SK-LMS-1 cells compared to NIH/3T3 cells overexpressing Methu. Two other cell sarcoma cell lines, HOS and RD, also showed increased [3H]thymidine incorporation in response to 80–120 units/ml HGF/SF; while SK-UT-1 and U-205 cells did not respond (data not shown) even though they express Methu and HGF/SF was not detected (Table 1). We concluded that HGF/SFhu can elicit a mitogenic signal in sarcoma cells expressing the Met receptor. The low (or lack of) response to HGF/SF in the sarcoma cells suggested the presence of an inhibitor or mutation that interrupts Met signaling.

**Expression of Met and HGF/SF in Human Primary Tumors.** The elevated expression of Met in sarcoma cell lines compared to primary fibroblast cultures and our previous demonstration that NIH/
3T3 cells overexpressing Met are tumorigenic (15) suggested that the high expression of Met may have contributed to the formation of sarcomas in vivo. We therefore examined paraffin-embedded human sarcoma sections stained for Met and HGF/SF by confocal laser scan microscopy. Seven of eight tumors examined were positive for both Met and HGF/SF staining: one leiomyosarcoma (Fig. 4A) and one of two chondrosarcoma examined (the positive tumor is shown in Fig. 4B) expressed both Met and HGF/SF, while three osteosarcomas showed significant Met and HGF/SF staining (two tumors are shown; Fig. 4C and D). For each sample, the Nomarski images are presented in panel 1, whereas panels 2 and 3 show the tumor sections stained with anti-Met and anti-HGF/SF antibody, respectively. In the double-stained overlays (Fig. 4A–D), green corresponds to Met staining, red corresponds to HGF/SF, and yellow represents colocalization of Met and HGF/SF staining. In each tumor, we observed cells which are positive either for both Met and HGF/SF or for Met or HGF/SF alone, suggesting that both autocrine and paracrine modes of stimulation can occur. The differential pattern of Met and HGF/SF expression observed in the sarcoma cells suggests heterogeneity in the population of tumor cells which might reflect the state of cell differentiation and/or tumor progression. These analyses demonstrate that Met overexpression occurs in the primary human sarcomas as well as in sarcoma cell lines (Table 1) and therefore, as in the NIH/3T3 model system (15), may contribute to the formation of these tumors.

Discussion

Cells that synthesize growth factor(s) and express the cognate receptor(s) have the potential for autocrine-mediated growth (24). In an external autocrine loop, receptor binding and signal transduction occurs when a growth factor is secreted and subsequently interacts with its receptor on the surface of the secreting or neighboring cells (25). Met and its ligand, HGF/SF, were first shown to interact in a paracrine fashion (21). The ligand is produced by cells of mesenchymal origin, such as fibroblasts and smooth muscle cells, and this ligand can induce both mitogenic, motogenic, and morphogenic responses in epithelial cells that express the Met receptor (11, 12, 26). In addition to this paracrine model, we have recently demonstrated by overexpressing Methu and HGF/SFhu in NIH/3T3 cells that autocrine-mediated signaling leads to tumorigenesis (15, 16). Here, we provide evidence that endogenous Met-HGF/SF autocrine signaling mediates a mitogenic response in cells of mesenchymal origin. First, nonmortalized human primary fibroblast cultures (HEL299; Hems) which produce abundant HGF/SF express low levels of Met, and Met prepared from one of these cultures (Hems) reacts with anti-P-Tyr. Moreover, Met is increased in HEL299 and Hems cells when HGF/SF neutralizing antibody is added to the medium. This suggests that the Met receptor may be down-modulated by the abundant HGF/SF produced by these cells. Consistent with this interpretation, these cells do not respond mitogenically when exogenous HGF/SF is added (data not shown), while the SK-LMS-1, HOS, and RD human sarcoma cell lines expressing high levels of Methu do. Thus, similar to platelet-derived growth factor, fibroblast growth factor, and insulin-like growth factor I (27), Met-HGF/SF autocrine interaction may be a fundamental property of fibroblast cell mitogenesis in vitro. It has also been suggested that the HGF/SF may play a role in the determination of fibroblast morphology (8, 21). Thus, fibroblasts have extensive pseudopodial extensions and are spontaneously motile, resembling the morphology and motility of epithelial cells exposed to HGF/SF. Furthermore, Met-HGF/SF signaling induces motogenic responses in NIH/3T3 fibroblasts (28), suggesting that this autocrine loop contributes to both mitogenic and motogenic phenotypes of mesenchymal cells. This combination of responses could be causally associated with the development of sarcomas.

Similar to our finding that endogenous HGF/SF expression is dramatically lowered in the NIH/3T3 Metm tumor cells (16), the level of HGF/SF is low in most of the human sarcoma cell lines tested presumably due to the overabundance of Met receptor (16). However, we cannot exclude the fact that other factors are responsible for reduced HGF/SF expression in the sarcoma lines; e.g., expression of HGF/SF in the MRC-5 fibroblast cell line can be positively or negatively affected by conditioned medium from several carcinoma cell lines (29, 30) and HGF/SF expression in MRC-5 cells can be inhibited by transforming growth factor β, epidermal growth factor, and transforming growth factor α (29, 30). We have not tested the human sarcoma cell lines for these factors.

Spontaneous transformants of NIH/3T3 fibroblasts were frequently found to have met protooncogene amplified and overexpressed (13, 14) and Met overexpression in NIH/3T3 fibroblasts induces fibrosarcomas via an autocrine mechanism (1, 15, 16). The results presented here suggest that a Met-HGF/SF autocrine mechanism may also contribute to the tumorigenic process in human sarcomas.

Acknowledgments

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References


* S. Rong et al., manuscript in preparation.


