Constitutive c-Met Signaling through a Nonautocrine Mechanism Promotes Metastasis in a Transgenic Transplantation Model

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ABSTRACT

Normal cells are dependent on exogenous, receptor-mediated growth stimulation for cell cycle entry and progression, providing a critical homeostatic mechanism regulating cellular proliferation. In contrast, tumor cells acquire some degree of growth signal autonomy, often through their ability to produce growth factors as well as their receptors (autocrine signaling). Recently, data have begun to emerge implicating heterotypic signaling between diverse cell types within a tumor in the genesis and progression of cancer; however, current experimental approaches in vivo have not adequately addressed this critical relationship. Here we used transgenic mice overexpressing hepatocyte growth factor/scatter factor (HGF/SF), or its growth antagonist NK2, as genetically modified hosts for transplantation of tumor cells expressing their receptor, c-Met, to directly assess the contribution of heterotypic signaling to metastatic colonization. We demonstrate that metastatic potential under nonautocrine signaling conditions (i.e., where tumor cells expressing c-Met are transplanted into transgenic hosts producing HGF/SF) rivaled that observed under conditions of autocrine signaling (i.e., where tumor cells expressing both HGF/SF and c-Met are transplanted into wild-type hosts). HGF/SF and NK2 were not functionally equivalent in vivo. Attenuation of NK2-associated growth inhibition by the presence of an HGF/SF-Met autocrine loop uncovered a shift in metastatic site preference from lung to liver only in NK2-transgenic hosts, a qualitative behavioral alteration likely detectable only through the genetic approach used here. Our data demonstrate that growth factors not intrinsic to malignant cells can have profound effects on metastatic efficiency in vivo and provide experimental support of a role for heterotypic signaling in tumor progression.

INTRODUCTION

Growth of normal cells is highly dependent on extracellular growth stimulation. A cell carrying a cell-surface receptor can respond to specific growth factors secreted by a neighboring cell (paracrine signaling) or a distant organ (endocrine signaling). This heterotypic signaling configuration provides an important homeostatic mechanism by which normal cellular proliferation is regulated. In contrast, tumor cells acquire some degree of growth factor independence, often through an ability to generate their own growth signals (1). The term "autocrine signaling" describes the situation in which a tumor cell gains the ability to express a growth factor as well as its corresponding receptor, generating a positive feedback loop (2). Human sarcomas and carcinomas, for example, have been shown to possess autocrine signaling involving a number of cell-surface receptor tyrosine kinases, including c-MET (reviewed in Refs. 3 and 4).

c-MET mediates a broad range of normal cellular activities, including growth, motility, and morphogenetic branching by binding its ligand, HGF/SF.7 HGF/SF induces autophosphorylation of c-MET, generating phosphotyrosine docking sites able to engage an array of SH2-containing signal transducers, which in turn activate appropriate signaling pathways (5). Under normal conditions, c-MET is expressed on epithelial cells and activated in a paracrine fashion by mesenchymally derived HGF/SF (6–8). HGF/SF-Met signaling is required for normal development of skeletal muscle, liver, and placenta (9–11). HGF/SF has 38% overall sequence similarity with plasminogen (12) and contains an α chain with an N-domain and four kringle domains and a β chain with an enzymatically inactive serine protease domain. HGF/SF RNA can undergo alternative splicing to create truncated isoforms able to bind c-Met and either mimic or antagonize HGF/SF, depending on the cell type and environmental context (13, 14). One natural variant possessing the N-domain and the first two kringle domains, NK2, has been shown to inhibit growth but stimulate motility, invasiveness, and tubulogenesis in cultured cells (15–21). The ability of NK2 to antagonize HGF/SF-induced phenotypes in vivo was demonstrated in studies with HGF/SF-NK2 bitransgenic mice (22), c-MET is also a proto-oncogene (23), involved in the development of human solid tumors, including melanoma (24, 25). Constitutive c-MET activation can stimulate angiogenesis, extracellular matrix dissolution, invasiveness and metastasis (18, 26) and promotes broad tumorigenesis in HGF/SF-overexpressing transgenic mice (27, 28).

In human cancer, aberrant c-MET stimulation can be achieved through activating c-MET mutations, c-MET amplification/overexpression, and acquisition of autonomous growth control through creation of autocrine signaling loops (5, 29). HGF/SF-Met autocrine loops have been detected in human primary and metastatic tumors, including melanoma, glioblastoma, osteosarcoma, and breast cancer (30–33). Experimental recreation of autocrine loops through forced coexpression of c-Met and HGF/SF in immortalized cultured cells can confer a metastatic phenotype (34, 35). Recently, data have begun to emerge implicating heterotypic signaling pathways in tumor development and progression, originating from fibroblasts, endothelial cells, and inflammatory cells (1, 36). However, analysis of the contribution of organ microenvironment to metastasis has been limited to experimental manipulation of tumor cells in vitro and assessment of their behavior when transplanted into immunodeficient or syngeneic wild type mice (37). We recently reported that mice harboring transgenes encoding ligands for c-Met strongly support metastasis of transplanted melanoma cells characterized by a highly potent HGF/SF-Met autocrine loop. Here we directly assess the in vivo efficacy of nonautocrine c-Met signaling with respect to metastatic colonization using HGF/SF and NK2 transgenic mice as transplantation recipients.

MATERIALS AND METHODS

Cell Culture, Transfection, and Reagents. B16F1 murine melanoma cells were grown in DMEM supplemented with 10% FBS (Life Technologies, Inc.). The 37-7 cell line was derived from a neoplasm arising in HGF/SF transgenic mouse line MH37 (28) and maintained in DMEM plus 15% FBS, 5 ng/ml epidermal growth factor, and 5 μg/ml insulin (Upstate Biotechnology). B16F1 and 37-7 parent cell lines were transfected with the following expression vectors: TRE-HGF/SF plus CMV-tetracycline transactivator (ptTA3; CloneTech); MT-NK2; CMV-c-Met; CMV-c-Met plus TRE-HGF/SF plus ptTA3; or MT-NK2 plus TRE-HGF/SF plus ptTA3. Although available, the tetracycline-inducible feature was not exploited in these studies. Different promoters were used to minimize competitive depletion of common transcription factors in

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2 The abbreviations used are: HGF/SF, hepatocyte growth factor/scatter factor; CMV, cytomegalovirus; FBS, fetal bovine serum; MT, metallothionein; TRE, tetracycline responsive element.
cells carrying more than one expression vector. Stable clones expressing HGF/SF, NK2, c-Met, HGF/SF plus c-Met, and NK2 plus HGF/SF were established by selection for G418 (Life Technologies, Inc.) resistance. For each construct, two or three individual clones were subjected to in vitro and in vivo testing (described below) and shown to behave in a similar fashion. Phosphotyrosine monoclonal antibody (α-PY20) was from Transduction Laboratories, and HGF(N17) polyclonal antibody and anti-in-Met (SP260) polyclonal antibody were from Santa Cruz Biotechnology.

**Transgenic Mice.** HGF/SF and NK2 transgenic mice were generated on an albino FVB background, using the expression constructs MT-HGF/SF and MT-NK2, respectively (22, 38). Host animals immunologically compatible with B16F1 melanoma cells were generated through a single cross of either MT-HGF/SF or MT-NK2 mice with C57BL/6 mice. Transgene expression profiles in both MT-promoter driven HGF/SF and NK2 transgenic mice were characterized by relatively strong expression in most tissues, including the liver and lung (Refs. 22 and 38; data not shown). Hepatic HGF/SF and NK2 transgene expression levels diverged by <3-fold based on measurement of RNA and protein (Ref. 22; data not shown). Positive HGF/SF and NK2 transgenic mice were identified by PCR using the following primer sets: for HGF/SF transgenic mice, MT-S (5′-ACTCGTCCAACGACTATA-3′) and HGF292 (5′-CTGAGAACTGCTACAGACTTG-3′); and for NK2 transgenic mice, MT-S and YYL7 (5′-GCACTGAGTGGCAAATCC-3′). For histopathological analysis, mouse tissues were fixed in 10% buffered formalin, embedded in paraffin, sectioned at 5 μm, and stained with H&E. All mouse work was performed in accordance with guidelines established by the NIH.

**Experimental Metastasis Analysis.** Either 1 × 10^5 or 10^6 cells in 0.2 ml of PBS were i.v. injected via the tail vein into 5–6-week-old male hosts: wild-type FVB, HGF/SF transgenic, or NK2 transgenic mice. Conclusions were essentially the same whether 10^5 or 10^6 melanoma cells were injected. Tumor numbers were obtained by visual inspection in mice euthanized 21 days after transplantation. Representative tissues were subjected to histopathological analysis. Nude mouse hosts confirmed metastasis patterns observed in wild-type mice (data not shown). Statistical analyses were performed using the paired Student t test.

**Cell Growth Analysis.** Proliferation rates of cell lines were assessed by [3H]thymidine incorporation (39). Briefly, cells were plated in DMEM plus 10% FBS at 2 × 10^4 cells/well in 96-well plates in quadruplicate; after 24 h, cells were labeled for 4 h with [3H]thymidine (1 μCi/ml; NEN). Cells were then washed with ice-cold PBS and 5% trichloroacetic acid and solubilization in 0.25 N NaOH. After solubilization, lysates were neutralized with 3 N HCl and counted by liquid scintillation (Beckman).

**Motility and Invasion Assays.** Motility and invasion assays were performed in 8-μm-pore transwells (6.5 mm; Costar) in quadruplicate (40). Cells were detached by trypsin, and 5 × 10^4 cells were plated in 200 μl of DMEM plus 10% FBS in the upper wells. Bottom wells contained 500 μl of the same medium. Cells were incubated at 37°C in humidified 5% CO₂ for 24 h. Cells on the transwells were rinsed in PBS, fixed with 1% glutaraldehyde in PBS, and stained with 0.1% crystal violet in water for 30 min. After destaining in water, nonmigrating cells on top of the filter were removed with a cotton swab. Migrating cells on the bottom of the filter were solubilized in 500 μl 0.2% Triton X-100, and the absorbance measured at 590 nm. For invasion assays, transwell filters were layered with 100 μl of Matrigel (Collaborative Research) diluted 1:20 in PBS. After rinsing with PBS, cells were plated as above. Seventy-two h later, cells migrating to the bottom of the filter were evaluated, after removal of material from the upper side of the filter, by 0.1% crystal violet staining and measurement of solubilized dye at A590. Results using Matrigel and growth factor-reduced Matrigel were the same and were combined for statistical analysis.

**Biochemical Analysis.** Immunoblots were performed on lysates obtained from cells grown in cell culture or tissues, solubilized in RIPA buffer [50 mM Tris (pH 7.4), 50 mM NaCl, 1% Triton X-100, 5 mM EDTA, plus protease inhibitor mixture (Boehringer Mannheim)]. Equivalent amounts of lysates were incubated with α-c-Met antibody (Santa Cruz Biotechnology) for 1 h, and then protein A/G agarose was added for 1 h and washed in RIPA buffer four times. Samples were fractionated by SDS 4–20% PAGE and transferred to nitrocellulose. Membranes were stripped using Re-Blot (Chemicon International) and reprobed as described.

**RESULTS**

**HGF/SF-Met Signaling and Tumor Cell Behavior in Vitro.** C57BL/6-derived B16F1 melanoma cells, which contain very low levels of c-Met (40), were used as the parent cell line to generate stable transfectants that overexpress cDNAs encoding c-Met, HGF/SF, or both. Clonal cell lines of each type were selected that demonstrated readily detectable and comparable expression levels (Fig. 1A and B). The same constructs were also used to make stable transfectants of the FVB-derived 37-7 tumor cell line (data not shown), which also makes very low levels of c-Met (28). Each selected cell line was characterized with respect to c-Met phosphorylation. c-Met phosphorylation was undetectable in either vector-transfected B16F1 control cells or in transfectants ectopically overexpressing c-Met alone (Fig. 1B). In contrast, c-Met activity was detectable in cells expressing HGF/SF alone, and more pronounced in cells overexpressing both HGF/SF and ectopic c-Met (Fig. 1B). These B16F1 cell lines were subjected to a series of in vitro assays for growth, motility, and invasiveness. Fig. 2 shows that the consequence of forced expression of c-Met and/or HGF/SF was similar for all three activities. In the absence of ligand, ectopic c-Met expression had a minor effect on growth, motility, or invasiveness. In contrast, forced expression of HGF/SF stimulated growth, motility, and invasiveness, an effect that...
Signaling Promotes Metastasis

Autocrine and Nonautocrine c-Met Signaling in Metastasis.

The stable cell lines described above were introduced into the tail vein of FVB wild-type, HGF/SF transgenic, or NK2 transgenic mice. Fig. 3 shows that endogenous levels of c-Met present in control B16F1 and 37-7 cells were sufficient to permit a modest but significant increase in lung metastasis in the ligand-rich environment provided by the HGF/SF transgenic, but not NK2 transgenic, host mice. Consistent with in vitro data (Fig. 2), genetic enhancement of c-Met expression alone did not significantly elevate the efficiency of pulmonary metastasis of B16F1 or 37-7 cells in FVB hosts (Fig. 3). In contrast, c-Met-overexpressing B16F1 and 37-7 cells demonstrated robust metastasis to the lung when introduced into HGF/SF transgenic or NK2 transgenic hosts (Fig. 3). These data show that the requirement for ligand exposure exhibited by tumor cells in vitro can be satisfied cell nonautonomously in vivo, and that nonautocrine stimulation of c-Met can promote metastasis.

We next directly compared the ability of autocrine and nonautocrine c-Met signaling to support metastasis of B16F1 cell lines. In these experiments, we used immunologically compatible transgenic hosts, the F1 product of cross-breeding between HGF/SF transgenic mice and C57BL/6 inbred mice. As before, ectopic c-Met expression alone did not affect metastatic efficiency to wild-type lungs, whereas forced HGF/SF expression significantly stimulated pulmonary metastasis, an effect that was marginally enhanced by ectopic c-Met coexpression (Fig. 4A). These data indicate that ectopic cellular expression of HGF/SF, but not ectopic c-Met, is required for efficient B16F1 cell metastasis. To compare these results with nonautocrine stimulation, the same B16F1 lines were injected into HGF/SF transgenic mice. Fig. 4A shows that the efficiency of pulmonary metastasis of B16F1 cells expressing low endogenous levels of c-Met was modestly but significantly (P < 0.01) heightened in HGF/SF transgenic mice relative to wild-type animals. However, host expression of the HGF/SF transgene had a dramatic effect on metastatic colonization of the c-Met-overexpressing B16F1 cell line (Fig. 4A), a transfectant variant that did not metastasize efficiently in wild-type hosts. This differential, which could only be accounted for by a paracrine or endocrine mechanism, indicates that given ample receptor, constitutive c-Met activation through heterotypic stimulation can be as potent as autocrine signaling in promoting metastatic colonization. Fig. 4 shows representative sections of lung metastases induced through enhanced autocrine (Fig. 4D) or nonautocrine (Fig. 4E) signaling conditions. c-Met phosphorylation levels in tumor cells growing at metastatic sites in HGF/SF transgenic host mice rivaled those achieved through addition of HGF/SF in vitro (Fig. 1C); in contrast, c-Met phosphorylation in tumor cell metastases from wild-type host mice was modest.

Qualitative Difference between HGF/SF- and NK2-mediated Signaling in Tumor Cell Behavior. The i.v. introduction of various B16F1 lines into NK2 transgenic mice produced a pattern of pulmo-
nary metastasis similar to HGF/SF transgenic hosts (Fig. 4A). As in HGF/SF transgenic mice, B16F1 cells with ectopic c-Met expression showed a significant increase in metastatic potential to the lungs of NK2 hosts, and c-Met kinase activity was evident within metastases arising in this NK2-rich environment (Fig. 1C). Notably, B16F1 cells expressing HGF/SF also demonstrated a dramatic shift in target organ preference to the liver, but only in NK2 transgenic hosts (Fig. 4, B and F). This shift in organotropism was most dramatic in cells ectopically expressing both HGF/SF and c-Met, creating a potent autocrine loop (Fig. 1B). To elucidate the effects of NK2 on B16F1 behavior, in vitro assays were performed. Fig. 2 shows that although forced expression of NK2 alone in B16F1 cells stimulated motility and invasiveness, it strongly inhibited growth. When coexpressed with NK2, HGF/SF rescued the B16F1 cells from NK2-induced growth inhibition (Fig. 2A). A similar pattern was observed when these B16F1 lines, or similarly constructed 37-7 lines, were i.v. injected into wild-type mice (Fig. 5). Whereas HGF/SF was stimulatory, expression of NK2 alone either failed to stimulate, or actually inhibited, pulmonary metastasis. However, coexpression of HGF/SF and NK2 produced a metastatic response that was significantly elevated relative to HGF/SF alone (Fig. 5).

DISCUSSION

It is evident that cell autonomous growth signals are not sufficient to explain the development or behavior of tumor cells. The tissue microenvironment at both primary and metastatic tumor sites can supply critical signaling factors for numerous functions, including cellular growth, motility, invasiveness, and survival, thought to be provided through heterotypic signaling (1, 36, 37). Recent data suggest that aspiring tumor cells “learn” to co-opt the aid of surrounding
fibroblasts, endothelial cells, and immune cells (41–45). However, little experimental in vivo evidence exists directly demonstrating the efficacy of such nonautonomous signaling in tumor progression. Here we describe a genetically tractable experimental system capable of evaluating the potential contribution of heterotypic receptor tyrosine kinase signaling in vivo. Specifically, we show that pulmonary metastasis of c-Met-overexpressing tumor cells is stimulated when introduced into transgenic mice overexpressing either HGF/SF or its variant NK2, and that the metastatic potential of the resulting heterotypic c-Met signaling was equivalent to that of the HGF/SF-Met autocrine signaling loop.

c-MET has been associated with metastatic progression in human and other tumors (reviewed in Refs. 4 and 46). B16F1 melanoma cells harbor very low levels of c-Met and as used here were weakly metastatic to the lungs of wild-type mice. This basal level of c-Met was nevertheless sufficient to support enhanced in vitro growth, motility, and invasiveness of B16F1 cells ectopically expressing HGF/SF. Similarly, B16F1 metastasis could be stimulated by exposure to high levels of HGF/SF, whether originating directly from the cell (autocrine stimulation) or from host tissue (paracrine and/or endocrine stimulation). However, greater c-Met levels were required to achieve efficient metastasis under conditions of nonautocrine stimulation, perhaps indicating that higher local concentrations of HGF/SF are reached through the autocrine mechanism. This observation is consistent with reports correlating tumor progression with c-MET overexpression/amplification (4, 46).

We are currently unable to discern the relative contributions of paracrine and endocrine c-Met stimulation to metastasis in our mouse models. HGF/SF and NK2 levels are elevated in both the target tissues and sera of HGF/SF transgenic and NK2 transgenic mice, respectively (22, 38). However, both paracrine and endocrine signaling represent highly relevant, and perhaps overlapping, mechanisms in human cancer. Elevated HGF/SF serum levels have been significantly associated with histological invasiveness, progressive metastasis, and prognosis in patients with breast, gastric, colorectal, and lung cancer (47–50). Surgical resection of breast and gastric neoplasms significantly reduced serum HGF/SF levels (47, 51), suggesting that the source of the elevated HGF/SF was the tumor itself; however, the specific cellular origin within the tumor has not been clarified. Paracrine regulatory loops have been described in which human tumor cells coax neighboring stromal cells to secrete HGF/SF, in turn promoting expression of angiogenic factors by, and invasiveness of, the tumor cells (52–56). Furge et al. (57) have proposed that paracrine release of HGF/SF from host stromal tissue is essential for metastasis of Ras-transformed human carcinoma cells, which typically do not produce HGF/SF.

It has been suggested that c-Met overexpression and constitutive activation can favor the liver as a metastatic target of both mouse and human tumor cells (25, 40, 58). Here we show that HGF/SF transgene overexpression, either by tumor cells or transplantation hosts, did not by itself alter the preference of B16F1 or 37-7 tumor cells for metastasis to the lungs. These data are consistent with Lin et al. (59), who concluded that although c-Met activation could enhance the metastatic potency of B16 cells, it did not overly influence organotropism. We were therefore surprised to discover that B16F1 cells possessing an HGF/SF-Met autocrine loop were highly metastatic to the liver in immunologically compatible NK2 transgenic hosts. These results were reminiscent of our earlier observation that host-derived NK2 facilitated hepatic metastasis of 37-32 melanoma cells, which also highly express both HGF/SF and c-Met (28), when transplanted into these same NK2 transgenic mice (22). Although in vitro growth and pulmonary metastasis of tumor cells were inhibited by expression of NK2 alone, consistent with previous reports of NK2 as an HGF/SF growth antagonist (15, 16, 22), this block was relieved by forced coexpression of HGF/SF (Fig. 2). One attractive hypothesis is that overcoming NK2 growth antagonism through HGF/SF overexpression uncovers NK2-initiated prometastatic pathways, such as phosphatidylinositol 3′-kinase/Akt activation. Although an explanation for the preference for hepatic metastasis in the NK2 transgenic host is currently obscure, our data clearly show that non-cell autonomous effects are critical in determining metastatic behavior.

In this report, we demonstrate the feasibility of using a genetically tractable transplantation system consisting of transgenic recipient mice and transplanted tumor cells to directly assess the contribution of host factors to metastasis. As a test model, we compared the role of autocrine and nonautocrine c-Met signaling in experimental metastasis of B16F1 melanoma cells. We show that the metastatic potential of heterotypic signaling, initiated by growth factors originating from host tissue and not intrinsic to the tumor cell, can reach levels achieved through autocrine signaling. By using emerging techniques to achieve specific targeting and inducibility of transgene expression, we anticipate that this approach can be greatly refined to uniquely address questions about the relationship between malignant cells and their microenvironment in tumorigenesis.

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