c-Met Ectodomain Shedding Rate Correlates with Malignant Potential

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Abstract Purpose: Many proteins are proteolytically released from the cell surface by a process known as ectodomain shedding. Shedding occurs under normal physiologic conditions and can be increased in certain pathologies. Among the many receptors for which ectodomain shedding has been shown is c-Met, the hepatocyte growth factor (HGF) receptor tyrosine kinase. HGF stimulates mitogenesis, motogenesis, and morphogenesis in a variety of cellular targets during development, homeostasis, and tissue regeneration. Inappropriate HGF signaling resulting in unregulated cell proliferation, motility, and invasion occurs in several human malignancies. This can occur through paracrine signaling, autocrine loop formation, receptor mutation, gene amplification, or gene rearrangement, accompanied frequently with overexpression of ligand and/or receptor proteins. We hypothesized that c-Met overexpression in cancer might result in increased ectodomain shedding, and that its measure could be a useful biomarker of tumor progression.

Experimental Design: We developed a sensitive electrochemiluminescent immunoassay to quantitate c-Met protein in cell lysates, culture supernatants, and biological samples.

Results: A survey of cultured cell models of oncogenic transformation revealed significant direct correlations ($P < 0.001$, $t$ test or ANOVA) between malignant potential and the rate of c-Met ectodomain shedding that was independent of steady-state receptor expression level. Moreover, weekly plasma and urine samples from mice harboring s.c. human tumor xenografts ($n = 4$ per group) displayed soluble human c-Met levels that were measurable before tumors became palpable and that correlated directly with tumor volume ($R^2 > 0.92$, linear regression).

Conclusions: For a variety of human cancers, c-Met ectodomain shedding may provide a reliable and practical indicator of malignant potential and overall tumor burden.

Ectodomain shedding is a process by which many transmembrane proteins are proteolytically released from cell surface. Shedding is an important process during normal development, and shedding defects are known to contribute to certain pathologies. Ligands of the epidermal growth factor receptor, for example, which regulate a variety of developmental processes and are involved in several human cancers, are synthesized as transmembrane proteins that require ectodomain shedding by tumor necrosis factor-α converting enzyme (or ADAM17) for biological activity (1). The failure to shed a cytokine receptor in response to normal stimuli contributes to tumor necrosis factor receptor–associated periodic syndrome, an inherited disease associated with tumor necrosis factor receptor gene mutations that is characterized by recurrent attacks of fever and pain in the joints, abdomen, muscles, skin, and eyes (2). These well-studied cases of ectodomain shedding illustrate its fundamental biological relevance, but much more remains to be learned about shedding and its consequences in other signaling networks.

Among the many receptors for which ectodomain shedding has been shown is c-Met, the hepatocyte growth factor (HGF) receptor tyrosine kinase (3–7). HGF is a pleiotropic heparin-binding protein discovered for its mitogenic activity on hepatocytes and epithelial cells and independently discovered for its ability to stimulate cell motility (scatter factor; refs. 8, 9). HGF is typically produced by cells of mesenchymal origin and acts in a paracrine manner on a variety of cellular targets during embryonic development and throughout adulthood, in normal and pathologic processes (10). HGF is essential for embryonic development, where it is involved in somite migration, limb bud and limb skeletal muscle formation, placenta formation (11, 12), and later in organogenesis (13), neural development (14), and tissue repair and regeneration (15, 16). Although
the role of HGF in adult homeostasis is not yet fully defined, a growing body of evidence suggests that it is an endogenous tissue protective factor for several major organs and has potent antifibrotic activity (17). Consistent with its relationship with HGF, c-Met is widely expressed early in development; deletion of the gene is lethal in mice; and widespread expression persists throughout adulthood (10). Both HGF and c-Met are up-regulated after kidney, liver, or heart injury, suggestive of a general mechanism of protection against tissue damage as well as one of tissue repair and regeneration (15–20).

HGF and c-Met are implicated in a wide variety of human malignancies, including colon, gastric, bladder, breast, kidney, liver, lung, head and neck, thyroid, and prostate cancers as well as in sarcomas, hematologic malignancies, melanoma, and central nervous system tumors (10, 21–23). Through paracrine signaling, overexpression of ligand and/or receptor, autocrine loop formation, and/or receptor mutation and gene rearrangement, this signaling pathway can enhance tumor cell growth, proliferation, survival, motility, and invasion. Inappropriate c-Met signaling in disease can resemble, at least in part, developmental transitions between epithelial and mesenchymal cell types normally regulated by HGF (8, 10). Among the many genes up-regulated in response to activation of this pathway is that of the receptor itself, creating the potential for c-Met overexpression in otherwise normal target cells through persistent ligand stimulation (8, 10). Consistent with this, c-Met overexpression is widely observed in cancers of epithelial origin where paracrine delivery of HGF results in dysregulated signaling. In contrast, tumors or cancer cells of mesenchymal origin that normally express HGF often acquire c-Met expression, and several sarcomas have been shown to have autocrine c-Met signaling (8, 10, 21). Importantly, c-Met signaling activates a program of cell dissociation and motility coupled with increased protease production that has been shown to promote cellular invasion through extracellular matrices, a process that closely resembles tumor metastasis in vivo (23–25). In addition, pathway activation in vascular cells stimulates tumor angiogenesis, facilitating tumor growth for cancers that are growth limited by hypoxia, and promoting tumor metastasis. Hypoxia alone up-regulates c-Met expression and enhances HGF/scatter factor signaling in cultured cells and mouse tumor models (26).

We hypothesized that the overexpression of c-Met characteristic of many malignancies might result in increased ectodomain shedding, and that its measure could be a useful biomarker of tumor progression. We developed a sensitive two-site immunoassay to quantitate c-Met protein in cell lysates, cell culture supernatants, and biological samples. This assay was also adapted to measure the ligand binding capacity of shed c-Met ectodomain fragments. A survey of cultured cell models of oncogenic transformation revealed a direct correlation between malignant potential and the rate of c-Met ectodomain shedding that was, surprisingly, independent of the steady-state level of receptor expression. Moreover, plasma and urine samples from mice harboring s.c. human tumor xenografts displayed soluble human c-Met levels that were measurable before tumors became palpable and that later correlated directly with increasing tumor volume. These results suggest that for a variety of human cancers, c-Met shedding may provide a reliable and practical indicator of malignant potential, tumor progression, and overall tumor burden.

Materials and Methods

Reagents and cell culture. Full-length purified recombinant human HGF protein was obtained from R&D Systems (294-HG, Minneapolis, MN). Antibodies against c-Met were obtained from R&D Systems or Upstate Biotechnology (DO-24 and DL-21 anti-c-Met monoclonal antibodies, Lake Placid, NY) as noted.

The following human normal/tumor and primary tumor/metastasis cell line pairs derived from single individuals were obtained from the American Type Culture Collection (Manassas, VA) and maintained according to the American Type Culture Collection’s recommendations: CRL7636 (normal skin) and CRL7637 (skin melanoma), HTB125 (normal mammary gland) and HTB126 (mammary gland ductal carcinoma), and CCL228 (colorectal adenocarcinoma) and CCL227 (lymph node metastasis of colorectal adenocarcinoma). The human cell line pair UOK124 (renal cell carcinoma) and UOK124 LN (lymph node metastasis of renal cell carcinoma) as well as UOK261 (bladder cancer) were developed at the Urologic Oncology Branch, National Cancer Institute, Bethesda, MD. UOK124, UOK124 LN, UOK261, A431 (epidermoid carcinoma), U-87 MG (glioblastoma), C100 (breast carcinoma), H1.177 (C100 transfected with Nm23), PC3 (prostate carcinoma), PC3M (PC3 derived metastatic variant), and P(C3M mxt(A (PC3M transfected with MxA) cells were maintained in DMEM supplemented with 10% fetal bovine serum, antibiotics, and antimycotics (Bethesda Research Laboratories). Normal human mammary epithelial cells were cultured as previously described (27). The MCF10A-derived breast epithelial cell lines M1-M4 (MCF10A or M1, MCF10AT1k.c12 or M2, MCF10AC1h or M3, and MCF10CA1a.c1 or M4; ref. 28) were obtained from the Barbara Ann Karmanos Cancer Institute (Detroit, MI) and were maintained as described (28).

SDS-PAGE and immunoblotting. Analysis of cellular and soluble c-Met by immunoprecipitation and immunoblotting was done as described previously (27). Samples for soluble c-Met analysis were obtained by harvesting cell culture supernatants; cells and debris were removed by high-speed centrifugation and 0.2-μm filtration. To obtain cellular c-Met samples, intact cells were serum deprived for 16 or 24 hours as noted; lysed in cold buffer containing nonionic detergents, protease, and phosphatase inhibitors; and cleared by high-speed centrifugation. After immunoprecipitation of detergent extracts for 2 hours on ice, immunocomplexes were captured using immobilized protein-G (GammaBind G-Agarose, GE Healthcare Bio-Sciences Corp., Piscataway, NJ), washed, eluted with SDS sample buffer, and subjected to SDS-PAGE and electrophoretic transfer to polyvinylidene difluoride membranes (Immobilon P, Millipore, Inc., Bedford, MA). Immunodetection was done by conventional methods (Enhanced Chemiluminescence, GE Healthcare Bio-Sciences Corp.)

Electrochemicalimmunolucensencenoimmunohas. Streptavidin-coated 96-well plates designed specifically for use in a Meso Scale Discovery (MSD, Gaithersburg, MD) Sector 2400 Imager were first coated with 1-Block solution (300 μg/mL, 1 hour; Applied Biosystems, Foster City, CA). Wells were then washed thrice with PBS (150 μL/well).

For c-Met ectodomain assays, a biotin-tagged, affinity-purified c-Met ectodomain-specific capture antibody [R&D BAF 358 diluted in 0.5% bovine serum albumin (BSA) in PBS] was added to each well (5 μg/mL, 25 μL/well) for 1 hour with shaking. Wells were washed thrice with PBS before adding samples or standards (R&D 358-MT recombinant c-Met ectodomain-IgG-Fc fusion protein in PBS + 0.5% BSA). Standards (100 μL/well) were added to generate a curve from 0.01 ng/mL to 100 ng/mL in semi-log increments for 1 hour with shaking. Samples for soluble c-Met analysis were obtained by harvesting culture supernatants from cells at 80% confluence; cells and debris were removed by high-speed centrifugation and 0.22-μm filtration and stored at –80°C before c-Met quantitation. In some cases, samples were concentrated using Centricon YM-10 microconcentration units (Millipore) before analysis, as described, or subjected to a single round of immunodepletion using the human c-Met ectodomain-specific monoclonal antibody DO24.
(Upstate Biotechnology) followed by antibody capture with protein G-Sepharose. Wells were washed thrice with PBS before adding detection antibody (R&D AF 276 labeled with MSD Sulfotag diluted in 0.5% BSA in PBS) at 1 μg/mL, 25 μL/well for 1 hour with shaking. Wells were then washed four times with PBS before adding MSD Read Buffer T with surfactant (150 μL/well) and then read immediately in a MSD Sector 2400 Imager.

For cellular c-Met assays, intact cells at 80% confluence were serum deprived for 24 hours, washed twice with cold PBS, and then lysed in cold buffer containing nonionic detergents and protease and phosphatase inhibitors. Detergent extracts were clarified by high-speed centrifugation and applied to 96-well plates as described for soluble c-Met ectodomain samples, above.

For HGF immunosassays, I-Block–treated plates were coated as described above with an affinity-purified HGF-specific capture antibody (R&D MAB 694 diluted in 0.5% BSA in PBS) that had been biotin labeled. Wells were washed as described above before adding detection antibody or standards (R&D 294-HG recombinant HGF protein in PBS + 0.5% BSA); standards were added to generate a curve from 0.03 ng/mL to 30 ng/mL in semi-log increments. Wells were washed before adding detection antibody (R&D AF-294 labeled with MSD Sulfotag diluted in 0.5% BSA in PBS) at 1 μg/mL, 25 μL/well. Wells were then washed four times with PBS before adding Read Buffer T and reading in a MSD Sector 2400 Imager.

All samples were measured in quadruplicate unless otherwise noted. Mean values from negative control wells were subtracted from all other raw values, and a standard curve was constructed by plotting signal intensity against 358-MT c-Met Fc fusion protein or recombinant HGF protein standard concentration. A nonlinear regression curve fitting algorithm (Microsoft Excel or GraphPad Prism software) was used to generate an equation from which sample values for c-Met or HGF concentration were derived from mean signal intensity values. Mean values among groups were compared for statistically significant differences using unpaired t test (paired human cell lines) or ANOVA (MCF10A-derived cell lines); R² and P values are presented in the text and figure legends.

Results and Discussion

**c-Met shedding by cultured cells: characterization and assay development.** We first examined c-Met ectodomain shedding in a cultured cell model of breast cancer progression where successive derivatives of the parent cell line show increasing malignancy (28). MCF10A (M1) is a spontaneously immortalized normal breast epithelial cell line that was transfected with activated Hras and xenografted in mice to obtain the premalignant MCF10AT1k.cl2 (M2) cell line. Subsequent passages in mice and single-cell cloning facilitated the isolation of cell lines that produced tumors with the phenotypic characteristics a low-grade carcinoma (MCF10CA1h or M3) and a high-grade metastatic carcinoma (MCF10CA1a.cl1 or M4). In contrast to many carcinoma-derived cell lines where c-Met is overexpressed, cell surface c-Met expression among the four cultured cell lines seemed to decrease with increasingly malignant phenotype (Fig. 1A). Nonetheless, analysis of c-Met shedding over 16 hours by immunoblotting with ectodomain-specific monoclonal antibodies showed progressively higher ectodomain levels from normal cells to those displaying a metastatic phenotype (Fig. 1B). c-Met ectodomain fragments of approximate molecular masses 75, 85, and 100 kDa were the predominant species observed, similar to those observed in the normal human mammary epithelial cell line B5/589 (Fig. 1B) and to the predominant species present in cell culture supernatants and human plasma as reported previously (3–7). These c-Met reactive protein bands were detectable with several antibodies against the c-Met ectodomain but not with polyclonal antiserum raised against a peptide corresponding to the COOH-terminal c-Met sequence (data not shown).

**Fig. 1.** c-Met shedding in a cultured cell model of breast tumor progression. A, reducing SDS-PAGE and immunoblot analysis of the c-Met expression in four mammary cell lines described previously (28); p145 is the intact c-Met β subunit. In this model, M2, M3, and M4 represent derivatives of the normal breast cell line M1 with increasingly malignant phenotype. The normal mammary epithelial cell line B5/589 was used as a positive control for c-Met expression, and lysates were immunoblotted for β-actin to confirm equal sample loading. B, reducing SDS-PAGE and immunoblot analysis of c-Met ectodomain shedding by cell lines M1, M2, M3, and M4. Culture supernatant from B5/589 and a purified recombinant c-Met ectodomain IgG Fc fusion protein (358-MT, R&D Systems) were used as positive controls of c-Met shedding and ectodomain recognition, respectively. Molecular masses of predominant c-Met ectodomain fragments are indicated in kDa (left).
To better characterize c-Met shedding across a range of cell lines and biological samples, we developed a two-site immunoassay to provide greater sensitivity, higher throughput, and more precise quantitation than immunoblotting. Distinct human c-Met-specific antibodies are used for capture and detection; capture antibodies were biotin labeled for use with streptavidin-coated multiwell plates, and the amount of detection antibody bound was measured by electrochemiluminescence generated using ruthenium chelates in the presence of sacrificial redox reagents. The optimized c-Met immunoassay has a threshold of detection of 750 fg for the purified recombinant c-Met ectodomain protein and a dynamic range of four log units (Fig. 2A). Sample quenching or hypersensitivity that could occur in complex biological samples was investigated by adding known amounts of recombinant c-Met ectodomain to cultured cell supernatants from B5/589 mammary epithelial cells (data not shown) or A431 epidermoid carcinoma cells (Fig. 2B), both of which contained shed c-Met ectodomain as determined by immunoblotting. Agreement between expected and observed c-Met concentrations indicated the absence of any interference (Fig. 2B). Ultrafiltration of B5/589 conditioned medium through a membrane with a 10-kDa cutoff removed all of the c-Met signal, consistent with the ectodomain molecular masses observed by immunoblotting (Fig. 2C). The c-Met selectivity of the immunoassay was tested by subjecting B5/589 conditioned medium to immunodepletion using a monoclonal antibody against c-Met ectodomain (DO-24) that was distinct from either capture or detection antibody used in the immunoassay. As shown in Fig. 2C, a single round of immunodepletion resulted in an 80% loss of signal.

To determine whether shed c-Met ectodomain fragments could act as competitive antagonists of ligand binding by cell surface receptors, the two-site c-Met immunoassay was adapted to analyze the HGF binding capacity of c-Met fragments found in cultured cell supernatants. A purified recombinant full-length c-Met ectodomain-IgG Fc fusion protein was used as positive control for HGF binding. The amount of c-Met ectodomain present in B5/589 conditioned medium was determined from a c-Met-Fc standard curve, and assays were designed such that equal amounts of c-Met ectodomain in B5/589 conditioned medium and in the c-Met Fc fusion protein preparation were captured in replicate wells. Purified recombinant HGF was added to two sets of wells at concentrations of 10, 100, and 300 ng/mL; one set of wells was then detected using anti-c-Met and the other with anti-HGF. An HGF standard curve was created using the same HGF detection antibody and an HGF-specific capture antibody. No change in c-Met detection was observed in c-Met-Fc–containing wells that had bound HGF, indicating that bound HGF did not interfere with c-Met capture or detection (data not shown). As shown in Fig. 2D, the c-Met Fc fusion protein bound HGF in direct proportion to the amount added, whereas no HGF binding was detected for the c-Met fragment present in B5/589 conditioned medium. These results, consistent with prior studies (7), show that the predominant c-Met ectodomain fragments found in B5/589 cell culture supernatants have lost meaningful HGF binding capability. In light of the observation that the B5/589 c-Met ectodomain fragments are very similar in size and distribution to those shed by a variety of cultured cell lines...

Fig. 2: Quantitation of c-Met shedding using an electrochemiluminescent two-site immunoassay. A, standard curve of purified recombinant c-Met ectodomain–IgG fusion protein (358-MT). Points, mean from quadruplicate samples; log [soluble c-Met] in ng/mL versus log [signal intensity] in relative units; bars, SD. SDs are smaller than the symbol size. B, analysis of sample quenching in A431 human epidermoid carcinoma cells. Cells were serum deprived for 24 hours, and soluble c-Met ectodomain was measured in the presence and absence of added 358-MT. Columns, mean values of quadruplicate samples from 358-MT (358MT, open column), A431 conditioned medium (A431, dark gray column), and A431 conditioned medium + 358-MT (obs, light gray columns); bars, SD. The expected sum of 358-MT and A431 conditioned medium sample values is also shown (exp, dark gray/open columns). C, soluble c-Met in 24-hour B5/589 conditioned media (B5, open column) was filtered using a 10-kDa cutoff molecular sieve, and soluble c-Met in the filtrate was measured (YM10, dark gray column). Twenty-four-hour B5/589 conditioned medium (open column) was immunodepleted using an ectodomain-specific monoclonal antibody (mAb D024, Upstate Biotechnology; light gray columns). D, HGF binding (ng/mL) was measured in samples containing equivalent amounts of c-Met Fc fusion protein (1 ng/mL).
(refs. 3–7; data not shown) as well as to what has been found in human plasma (7), our data support the conclusion that c-Met shedding does not yield a soluble receptor fragment that could compete efficiently for ligand binding by intact cellular c-Met.

**c-Met shedding correlates with malignancy in cultured cell cancer models.** Quantitative analysis of c-Met shedding by the MCF10A-derived breast cancer cell lines that we had analyzed previously by immunoblotting showed excellent agreement in the trends among the cell lines for both cellular c-Met expression and c-Met ectodomain shedding (Figs. 1B and 3A). Note that the external recombinant protein standard in the immunoassay allows results to be expressed in absolute terms (e.g., receptor concentration or number per total cellular protein or per cell). This enables realistic comparisons between successive experiments, different cell lines, or with other biological samples. Manipulation of the numerical results also offers insight into trends in shedding relevant to molecular mechanism, such as determining shedding rate per cellular receptor (Fig. 3C) or the percentage of cellular receptor shed per time interval (Fig. 3D). In the MCF10A-derived model of breast cancer progression, the steady-state cellular c-Met expression level is progressively and significantly lower at each step of increasingly malignant phenotype ($R^2 = 0.998$ and $P < 0.0001$, ANOVA). In contrast, c-Met shedding is significantly increased with the change from premalignant (M2) to malignant (M3) phenotype (Fig. 3B; $P < 0.001$, t test). This trend is maintained in the metastatic (M4) cell line (Fig. 3A and C), and shedding in M4 is nearly 4-fold higher than its normal counterpart M1 when expressed as a function of available cellular c-Met (Fig. 3D; $R^2 > 0.995$ and $P < 0.0001$, four-group ANOVA). The observed trends in expression and shedding are consistent with significant and progressive increases in proteolytic activity characteristic of advancing breast cancer, and they offer a very different picture of c-Met involvement than what would probably be gleaned using other methods, such as immunohistochemistry.

To investigate the generality of the trends observed in the MCF10A-derived cell lines, we measured cellular and soluble c-Met levels in a series of paired human cell lines, each obtained from a single cancer patient, derived from tumor versus corresponding normal tissue or primary tumor versus distant metastatic lesion. Of the available cell lines pairs, we focused on widespread cancers where the c-Met pathway is known to contribute to tumor progression and metastasis: cancers of the skin, breast, colon, and kidney (Fig. 4). Specifically, the paired cell lines were derived from normal skin (CRL7636) and skin melanoma (CRL7637), normal mammary gland (HTB125) and mammary gland ductal carcinoma (HTB126), colorectal adenocarcinoma (CCL228) and lymph node metastasis of colorectal adenocarcinoma (CCL227), and renal cell carcinoma (UOK124) and lymph node metastasis of renal cell carcinoma (UOK124 LN).

All four cell line pairs exhibited one overall trend similar to that displayed by the MCF10A model: significantly greater c-Met shedding rate with more malignant phenotype (Fig. 4B-C). In contrast, cellular c-Met levels varied widely among the paired lines (Fig. 4A). Relative to the normal skin-derived cell line CRL7636, cellular c-Met was 60% lower ($P < 0.0001$) than in the paired melanoma-derived cell line CRL7637 (Fig. 4A), whereas soluble c-Met concentration was 60% greater ($P < 0.0001$), contributing to a 4-fold greater c-Met shedding rate per cell protein (Fig. 4C; $P < 0.0001$) and per receptor (Fig. 4D; $P < 0.0001$) correlating with the acquisition of malignancy. These findings suggest that, similar to the MCF10A-derived cell lines, significantly increased proteolytic activity was the primary cause of the observed trend. In contrast, in the cell line pair derived from normal mammary gland and ductal carcinoma (HTB125 and HTB126), acquisition of malignancy correlated with 2.5-fold greater cellular c-Met level (Fig. 4A; $P < 0.0001$) and 4-fold greater soluble c-Met level (Fig. 4B; $P < 0.0001$), producing a 2.5-fold greater c-Met shedding rate per cell protein (Fig. 4C; $P < 0.0001$). This trend represented a...
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Fig. 4. Quantitation of c-Met expression and shedding in paired cell line models of cancer progression. Cell line pairs, each derived from a single patient, were compared for levels of cellular (A) and soluble (B) c-Met. These data were used to calculate the amount of soluble receptor shed after 24 hours corrected for cellular receptor (C) and the amount of soluble receptor produced in 24 hours as a percentage of cellular receptor (D). Cell lines derived from normal tissue (open columns) corresponding to the tissue of tumor origin were paired with tumor-derived cell lines (light gray columns) and other tumor derived cell lines (dark gray columns) were paired with cell lines derived from corresponding metastatic lesions (black columns). Individual cell lines are identified in Results and Discussion.

In addition to cultured normal/tumor or tumor/metastasis cell line pairs, we examined cellular and soluble c-Met levels in two genetically engineered models representing reconstitution of metastasis suppressor genes in aggressively malignant prostate- and breast tumor-derived cell lines. The prostate cancer cell line PC3 is tumorigenic in mice but not metastatic; PC3M is a PC3-derived cell line that is aggressively tumorigenic and metastatic (29). This phenotypic difference was exploited to identify genes whose loss could contribute to metastasis in prostate cancer, leading to the identification of the MxA gene as a suppressor of metastasis (30). Upon reconstitution of MxA expression in PC3M, the aggressive metastatic phenotype of cultured cell xenografts in mice is reverted to that of the parental cell line.6 We found that cellular c-Met expression in PC3M was nearly double that of PC3 (P < 0.0001); restoration of MxA expression was associated with an even greater cellular c-Met expression level (Fig. 5A, top). Shedding by PC3M was 50% greater than that of the parental cell line, consistent with the overall trend of increased shedding with increasing malignancy observed in other cell models (Fig. 5A, top middle; P < 0.0001). The absolute level of c-Met shedding in PC3M and PC3M MxA transfectants was not remarkably different (Fig. 5A, top middle). However, when corrected for increased c-Met expression, the rate of c-Met shedding per cellular receptor was reduced in MxA transfectants to PC3 levels or below (Fig. 5A, bottom middle and bottom; P < 0.001). Thus, MxA expression apparently attenuated the shedding mechanism but substantially increased c-Met expression compensated for this completely.

A dramatic effect on cellular c-Met expression was exhibited by the metastasis suppressor gene Nm23 in a genetically engineered cell model of breast cancer progression (Fig. 5B). In this model, the breast cancer cell line C100, which aggressively forms primary tumors and metastases in mouse xenografts, was reverted to a nonmetastatic phenotype upon transfection with Nm23 (H1.177; ref. 31). Correlated with the loss of metastatic phenotype was 70% lower cellular c-Met expression in H1.177 relative to the parental cell line (Fig. 5B, top; P < 0.0001) and 75% lower soluble c-Met production (Fig. 5B, top middle; P < 0.0001). A 25% lower level of c-Met shedding per cellular receptor was also associated with Nm23 expression (Fig. 5B, bottom middle; P < 0.0001). The decreased shedding rate correlated with reduced Nm23 expression, but did not completely compensate for the reduced shedding observed in the primary cell line (P = 0.0001). These results suggest that Nm23 may be involved in the repression of c-Met expression in vivo.

6 Unpublished observations.
volume and plasma soluble c-Met levels at weekly intervals. We observed that cultured tumor cells expressing c-Met tended to shed more c-Met ectodomain than their normal tissue counterparts, independent of changes in overall c-Met expression level, and that this tendency was enhanced with increasingly malignant phenotype. These phenomena could provide the basis for an indirect assay of tumorigenesis, overall tumor burden, and/or metastasis for cancers where c-Met is expressed, particularly if systemic c-Met shedding and were measurable in an animal system. To test this hypothesis, we injected s.c. two different human tumor cell lines with known c-Met shedding rates (data not shown) into immunocompromised (severe combined immunodeficient/Beige) mice and thereafter measured tumor volume and plasma soluble c-Met levels at weekly intervals. The cell line UOK261 was derived from a human bladder carcinoma and displayed a relatively high level of soluble c-Met in culture, whereas the cell line U-87 MG was derived from a human glioblastoma and displayed a lower level of c-Met shedding in culture. c-Met signaling is suspected of playing an important role in the progression of both cancers represented by these models (32–34). Note that the antibodies used in the immunoassay described here do not cross-react with mouse c-Met; thus, the assay was conducted in the absence of any normal soluble murine c-Met background; any c-Met detected originated from the human tumor xenografts. Pooled plasma samples obtained from the same strain of mice was used as a diluent for the recombinant c-Met ectodomain-Fc standard curve so that the absolute soluble c-Met values obtained could be related directly to those obtained from cultured cell experiments and to future animal studies.

Remarkably, soluble human c-Met was easily detected in plasma samples obtained from several mice receiving UOK261 xenografts more than a week before s.c. tumors became palpable (data not shown). Nonpalpable tumors up 5.0 mm³ in volume are difficult to detect radiologically and represent an early but nonetheless clinically relevant stage of tumorigenesis. The soluble c-Met fragments measured in the immunoassay were similar in size to the fragments found in cultured cell conditioned media, as determined by SDS-PAGE and immunoblotting (data not shown). With increasing tumor mass, each mouse showed significant weekly increases in plasma c-Met levels (P < 0.05; data not shown); these data were pooled and plotted as plasma c-Met against tumor volume (Fig. 6, top left). Regression analysis showed a direct linear relationship between circulating soluble c-Met concentration and tumor burden (Fig. 6, top left; R² = 0.944 and n = 4 animals). Consistent with the lower level of c-Met shedding by cultured U-87 MG cells, soluble c-Met was not detected in plasma samples from mice receiving the U-87 MG glioblastoma xenografts before the tumors became palpable. Nonetheless, the smallest measurable tumors were each associated with plasma c-Met levels that were well above the threshold of detection, and each mouse showed significantly increasing c-Met concentrations measured at weekly intervals (P < 0.05; data not shown). Regression analysis of the pooled data plotted as c-Met concentration against tumor volume also supported a direct linear soluble c-Met/tumor burden relationship (Fig. 6, top right; R² = 0.933 and n = 4 animals).

Prior reports of elevated HGF concentrations in the urine of bladder cancer patients (33), overexpression of c-Met in bladder cancer (34), and our own results showing readily detectable levels of circulating c-Met in tumor cell xenografts prompted us to investigate the possibility that a measurable portion of soluble plasma c-Met might be excreted into urine. As shown in Fig. 6 (bottom left), s.c. UOK261 xenografts produced urinary soluble c-Met concentrations that were detectable at very small tumor sizes and that increased linearly with tumor burden over weekly intervals (R² = 0.922, P < 0.01, n = 4 animals). Urinary soluble c-Met concentrations were typically less than 1/1,000th of corresponding plasma c-Met concentrations (Fig. 6, bottom left). Urine samples from mice receiving U-87 MG xenografts also contained soluble c-Met at early stages of tumor growth that increased significantly with time in each mouse.

Fig. 5. Genetically modified cultured cell models of cancer progression. A, the human prostate cancer derived cell line PC3 (open columns), a derived metastatic variant PC3M (black columns), and PC3M transfected with an MxA gene expression plasmid (+MxA, gray columns) were compared for levels of cellular (top) and soluble (top middle) c-Met. These data were used to calculate the amount of soluble receptor shed after 24 hours corrected for cellular receptor (bottom middle) and the amount of soluble receptor produced in 24 hours as a percentage of cellular receptor (bottom). B, the human breast cancer derived cell line C100 (black columns) and a C100 derivative cell line cloned after transfection with an Nm23 gene expression plasmid (H1.177, gray columns) were compared for levels of cellular and soluble c-Met as in (A).
(P < 0.05; data not shown), and group data showed a similar direct linear relationship between c-Met concentration and overall tumor burden (Fig. 6, bottom right; \( R^2 = 0.953 \) and \( n = 4 \) animals).

**Conclusion**

Relatively little is known about c-Met ectodomain shedding specifically, but existing evidence supports at least three mechanisms linking accelerated c-Met shedding with malignant progression. First, enhanced activation of proteolytic networks concomitant with malignancy could increase c-Met shedding (6, 10, 13, 24, 25, 35). Prior studies show activation of shedding through broadly distributed signaling pathways including that of epidermal growth factor, G-protein coupled receptors, and integrins (6) as well as intracellular pathways activated by phorbol myristate acetate (5). In all likelihood, one or more of these pathways are activated in the cancer cell lines analyzed here. In addition to direct control over matrix metalloproteinase activity, aberrant c-Met pathway activation characteristic of many tumor types may complicate this process by induction of matrix metalloproteinase expression (10, 13, 25, 35). Second, suppression of proteinase inhibitors with increasing malignancy may exacerbate this process. Tissue inhibitor of metalloproteinase-3, but not tissue inhibitor of metalloproteinase-1 or tissue inhibitor of metalloproteinase-2, potently inhibited c-Met shedding in a lung cancer cell line (6). Tissue inhibitor of metalloproteinase expression is also HGF regulated (25, 36), and sustained HGF signaling strongly suppressed tissue inhibitor of metalloproteinase-3 expression while stimulating metalloproteinase production by the human leiomyosarcoma cell line SK-LMS-1, which is driven by autocrine HGF signaling (36). Third, the release of an activated and potentially oncogenic cytoplasmic c-Met kinase domain containing fragment as a byproduct of shedding permits a direct positive feedback relationship between sustained HGF pathway activation and c-Met shedding that is vigilantly suppressed in normal cells but may be selected for among transformed cells (6, 37, 38). We too observed a cytoplasmic c-Met fragment using a COOH-terminal specific antibody in the M3 and M4 breast cancer cell lines but not in M1 or M2, consistent with a role in enhanced malignancy (data not shown). Finally, the apparent inability of shed c-Met ectodomain to attenuate HGF signaling through competitive ligand binding fails to provide any negative feedback for shedding. In combination, these processes may explain the accelerated rate of c-Met shedding shown here in multiple models of malignant progression.

Our results also show that soluble c-Met produced by tumor-derived cultured cell xenografts can be detected in plasma at very early stages of tumorigenesis, and that urinary c-Met concentration is a reliable indicator of the soluble c-Met level in plasma. These data further support the overall fidelity of c-Met shedding as an index of malignant phenotype, tumor progression, and tumor burden in c-Met-expressing models of oncogenesis. A preliminary analysis of normal urine samples and samples obtained from human patients with urologic cancers revealed a relatively narrow range of normal values and a trend of substantially higher values in cancer patient samples. An extended study with the goal of correlating results with disease type, stage and outcome is in progress. The simplicity and sensitivity of the assay described here make it amenable to high throughput screening should diagnostic and/or prognostic value be shown for patients with urologic or other malignancies.

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References


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