In Vivo Molecular Imaging of Met Tyrosine Kinase Growth Factor Receptor Activity in Normal Organs and Breast Tumors

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ABSTRACT

Molecular imaging techniques allow visualization of specific gene products and their physiological processes in living tissues. In this study, we present a new approach for molecular imaging of endogenous tyrosine kinase receptor activity, Met and its ligand hepatocyte growth factor scatter factor (HGF/SF), which mediate mitogenesis, tumorigenicity, and angiogenesis, were used as a model. HGF/SF and Met play a significant role in the pathogenesis and biology of a wide variety of human epithelial cancers and, therefore, may serve as potential targets for cancer prognosis and therapy. We have shown previously that in vitro activation of Met by HGF/SF increases oxygen consumption. In this study, we demonstrate that Met activation in vivo by HGF/SF alters the hemodynamics of normal and malignant Met-expressing tissues. Tumor-bearing BALB/C mice were i.v. injected with HGF/SF and imaged using magnetic resonance imaging (MRI) and Doppler ultrasound. Organs and tumors expressing high levels of Met showed the most substantial alteration in blood oxygenation levels as measured by blood oxygenation level dependent (BOLD)-MRI. No significant alteration was observed in tumors or organs that does not express Met. In the liver, which expresses high levels of Met, MRI signal alteration of about 60% was observed. In the kidneys, signal alteration was approximately 30%, and no change was observed in muscles. The extent of MRI signal alteration was also in correlation with HGF/SF doses. Injection of 7 and 170 ng/g body weight resulted in signal alteration of 5% and 30%, respectively, in tumors. Doppler ultrasound measurements demonstrated that these MRI changes are at least partially attributable to altered blood flow. These hemodynamic alterations, measured by MRI and Doppler ultrasound, were used in this study for the molecular imaging of Met activity in vivo. This novel molecular imaging technique may be used for in vivo diagnosis, prognosis, and therapy of Met-expressing tumors.

INTRODUCTION

Imaging techniques have become an essential aspect of cancer diagnosis and treatment monitoring. Using imaging methods (computed tomography, ultrasound, and MRI),^{3} it is possible to obtain important structural and anatomical information regarding tumors and metastases. New emerging techniques of molecular and functional imaging allow visualization of the physiology and cellular or molecular processes as they take place in living cells and tissues (1). A variety of imaging technologies is being investigated as tools for studying gene expression in living subjects (2). Single photon emission computed tomography and positron emission tomography are molecular imaging techniques that use radiolabeled molecules to image molecular interactions in biological processes in vivo. These techniques provide relatively high sensitivity and quantitative capability (3). Gambhir et al. (3) recently described several radiolabeled probes that can be used to image exogenous gene products using reporter gene (HSV1-1k) both for single photon emission computed tomography and positron emission tomography imaging. Nonradioactive techniques such as fMRI were developed for the analysis of brain activity (4, 5). This method enables detection of spatial and temporal changes in blood flow, volume, and oxygenation. fMRI measures BOLD contrast derived from the existence of inherent paramagnetic contrast material (deoxyhemoglobin in the tissue using T2* weighted GRE sequence. BOLD sensitive MRI was applied previously in cancer research for monitoring tumor response to vaso-markers (6), analysis of tumor vessel functionality (7), vessel maturation, and angiogenesis (8, 9).

Met (10) and its ligand HGF/SF (11, 12) have multifunctional effects on mammalian cells. These include stimulation or inhibition of cellular proliferation, promotion of cell movement, invasion into extracellular matrix, and induction of glandular/tubular morphogenesis by epithelial cells (13–15). HGF/SF and Met play a significant role in the pathogenesis and biology for a wide variety of human cancers (breast, prostate, colorectal, ovary, and brain tumors) and may serve as potential targets for cancer prognosis and therapy (16, 17). Recently (18, 19), HGF/SF-Met signaling was found to have an important role in both mammary development and carcinogenesis. It was shown previously (20) that HGF/SF has hemodynamic effects in rats. HGF/SF reduced mean arterial pressure and increased heart rate. We have shown recently (21) that HGF/SF treatment induces significant metabolic changes including increased glucose consumption and glycogenolysis, concomitant with a 20% increase in oxygen consumption in a murine mammary adenocarcinoma cell line, DA3.

In this study, we report the development of a novel molecular imaging technique of endogenous tyrosine kinase receptors using the met proto-oncogene product as a model. We demonstrate here that injection of HGF/SF induces hemodynamic alteration in normal and tumor Met-expressing tissues, enabling noninvasive in vivo molecular imaging of Met activity using fMRI and CDI.

MATERIALS AND METHODS

Cell Line and Animals. D1-DMBA-3 is a cell line derived from a poorly differentiated mammary adenocarcinoma induced in BALB/C mice by di-methylbenzanthracene. Limiting dilution cloning produced the cell line designated DA3 (22). DA3 cells were grown in DMEM (Life Technologies, Inc.) supplemented with 10% heat-inactivated FCS (Life Technologies, Inc.). To induce tumors, 10⁶ DA3 cells were injected into the mammary gland of BALB/C mice, leading to the formation of tumors expressing high levels of Met (23). For MRI experiments, mice were anesthetized with ketamine (120 mg/kg; Ketaset, Fort Dodge, IA) and xylazine (6 mg/kg; Vitamed, Benyamina, Israel) given i.p. Animal experiments were approved by the Animal Committee of the Tel Aviv University.

Immunoblotting. Tissues were harvested, frozen in liquid nitrogen, homogenized in 1 ml of lysis buffer [20 mM Tris-HCl (pH 7.8), 100 mM NaCl, 50 mM NaF, 1% NP40, 0.1% SDS, 2 mM EDTA, and 1% glycerol], and clarified by centrifugation. Cell lysate protein (1 mg) was immunoprecipitated with SP260 anti-Met antibody (Santa Cruz Biotechnology) and subjected to Western blot analysis using anti-pTyr monoclonal antibody or anti-SP260 rabbit polyclonal antibody (Santa Cruz Biotechnology). Visualization was
achieved using horseradish peroxidase-conjugated antimouse IgG antibody (1:25,000) or horseradish peroxidase-conjugated antirabbit IgG antibody (1:50,000), enhanced chemiluminescence reaction, and exposure to X-ray film.

MRI. Measurements were performed on a 1.9-T whole body MRI system (2T Prestige; Elscint) using small shoulder surface coil (14 cm in diameter). Coronal and axial T2 weighted spinecho images were acquired using TR/TE = 4000/90 ms; FOV 8 × 8 cm; 256 × 256 matrix size. Axial T2* weighted GRE images were acquired using time to repeat/time to echo = 100/11 ms; flip angle of 45° field of view 4 × 4 cm; 128 × 128 matrix size.

BOLD Contrast Alterations. A mouse cursor was used to outline organs and tumor ROI on the T2 weighted image based on structural criteria. Alteration of BOLD contrast was calculated from a series of GRE images using Matlab 5.2 (Math Works, Inc.) and NIH Image (NIH Image; ppc 1.62 software) in three steps: (a) pixel by pixel analysis: signal percentage alteration was calculated by comparing baseline intensity (seven images before HGF/SF injection) with maximum signal change (seven images taken 30 min after HGF/SF injection) for each pixel; (b) BOLD alteration maps: positive and negative alteration maps were generated by separating pixels with increased BOLD contrast (pixel alteration higher than 0.05%) from those with decreased BOLD contrast (pixel alteration less than −0.05%). The defined ROIs outlined on the T2 weighted image were overlaid onto the appropriate positive and negative maps; (c) calculation of mean and SD alteration: mean positive or negative percentage alterations were calculated as the average (±SD) of signal change from all of the increased or decreased pixels in the ROI, respectively.

Ultrasound. Measurements were performed on awakened mice using ACUSON 128XP Computer Sonography System with Art 7.5 mHz in 2–3-min intervals. Mice were held face up, and their abdomen and tumor region was coated with ultrasound-coupling gel. Measurements started 10 min before HGF/SF i.v. injection and continued for 30 min after injection.

RESULTS

Alteration of BOLD Contrast in Met-expressing Tissues. To study the in vivo hemodynamic effects of HGF/SF in Met-expressing organs and tumors, BALB/C mice received i.v. injections with saline (150 μl) and 15 min later with varying doses of purified HGF/SF (150 μl; Ref. 24). Organ morphology was initially imaged using T2 weighted spin echo sequence (Fig. 1A). Axial GRE images of different tissues were acquired with higher resolution (localizer marked on Fig. 1A). Changes in signal intensity were evaluated from GRE images acquired at 1-min intervals for 40 min. Alteration of signal intensity in each pixel was measured by comparing baseline intensity (an average from seven images taken after saline injection) with the maximal effect (an average from seven images taken 30 min after HGF/SF injection). The liver, which expresses very high levels of Met,
Fig. 2. HGF/SF-induced alteration of BOLD contrast in Met-expressing tumors. A, axial GRE images of DA3 tumor with high levels of Met (a–b and d–e) and of DA3-tumors with low levels of Met (f–g). Representative images acquired before (a) and after HGF/SF injection (b). Color maps of the percentage of BOLD signal alteration were calculated from sets of 34 GRE images taken before and after HGF/SF injection (see Fig. 1; c, e, and g). Color scales in C. Bl, bladder; Tu, tumor. B, time course of changes in signal intensity for the marked ROI inside the tumor (green ROI in Fig. 2A, a) and in big vessel at the border of the tumor (red ROI in Fig. 2A, a). Results are the calculated mean from all of the pixels of these ROIs.
exhibited the most substantial change in signal intensity (60% maximum difference of either increase or decrease after injection of HGF/SF at 70 ng/g body weight; Fig. 1, B and C). Organs expressing low levels of Met, such as muscles, exhibited no detectable changes in signal intensity subsequent to HGF/SF injection (Fig. 1, B and C). In the kidneys, which express moderate levels of Met, a maximal change of ±30% was detected after injection of 70ng/g HGF/SF (Fig. 1, D and E).

**Correlation of BOLD Contrast Alteration with Met Activation.** To demonstrate that the intensity of BOLD contrast correlates with Met activation, tumor-bearing BALB/C mice received injections with 70ng/g HGF/SF. Mice that received injections with saline served as control. Met phosphorylation was examined by Western blot analysis in liver, kidneys, and tumor tissues collected 10 min after injection. A dramatic phosphorylation of a M_r 140,000 band, corresponding to Met, was observed in the liver and kidney tissue extracts (Fig. 1F, a). A significant increase in Met phosphorylation was detected in tumor tissue after exposure to HGF/SF, although the basal level of phosphorylated Met was already substantially higher than levels observed in the normal tissues from the same animal (Fig. 1F). Met expression levels exhibited no detectable variation resulting from the short exposure to HGF/SF (Fig. 1F, b). These results demonstrate that induction of BOLD contrast alteration by HGF/SF correlates with Met phosphorylation.

**Alteration of BOLD Contrast in Met-expressing Tumors.** To examine the plausibility of Met molecular imaging in tumors, DA3 cells were injected into the mammary gland of BALB/C mice. This leads to the localized formation of tumors expressing high levels of endogenous Met (Fig. 1F; Ref. 23). Three weeks later, mice were imaged using fMRI. HGF/SF induced heterogenic alteration in signal intensity in areas within and around the tumor (n = 40). Areas within the tumor exhibited increased signal intensity, whereas the tumor periphery exhibited a dramatic decrease in MRI signal (Fig. 2A, c). The red ROI depicted in Fig. 2A, a shows a single blood vessel exhibiting significant constriction. To further corroborate that the BOLD contrast alteration is specific to Met activation, we generated DA3 clones that express very low levels of Met, as well as a dominant negative form of the receptor (23). Tumors derived from these clones grew substantially slower and exhibited scarcely detectable BOLD contrast alteration after HGF/SF injection compared with tumors expressing high levels of Met (Fig. 2A, g and e, respectively). Nevertheless, the hemodynamic effects in the liver and kidneys in these mice were similar to the results described above (data not shown).

**HGF/SF Dose Dependency.** To study the correlation between BOLD alteration and HGF/SF concentrations, different doses of HGF/SF were injected into several mice, and the average and SD of BOLD alteration were calculated. The average increase in BOLD signal within the tumor after injection of 7ng/g and 170ng/g HGF/SF was determined to be 5% ± 2% and 30% ± 4%, respectively. The average decrease of MRI signal in the tumor periphery ranged from 5% ± 1.4% up to more than 15% ± 10% (Fig. 3A). In a small number of pixels at the tumor periphery, the local negative change in several specific peripheral regions may be up to 50% (Fig. 2B). The hemodynamic effects in the liver and kidneys were also dose-dependent (Fig. 3B). The increase in signal intensity ranged from 14% ± 7% to 70% ± 4% in the liver and from 9% ± 2% to 25% ± 15% in the kidney after injection of 7ng/g and 170ng/g HGF/SF, respectively.

**Alteration of Hemodynamics in Met-expressing Tumors.** We analyzed changes in tumor blood flow after HGF/SF injection using CDI in 10 mice (Fig. 4). Before HGF/SF injection, a large surface vessel was clearly visible, whereas within the tumor, blood vessels were sparsely detected (Fig. 4A). Five min after HGF/SF injection (Fig. 4, B–F), the surface blood vessel nearly disappeared, whereas the tumor blood flow increased significantly. Mice with tumors induced by the low Met DA3 cells showed no detectable response to HGF/SF injection (data not shown). These results show that Met signal transduction activation increased tumor blood flow, whereas blood flow in the tumor periphery is markedly reduced.

**DISCUSSION**

Improvements in MRI and ultrasound techniques have increased the sensitivity of tumor and metastasis imaging. However, specificity is still a major problem in identifying malignant tissue. Using molecular-functional imaging methods, along with structural imaging of tumors, could dramatically increase tumor-imaging specificity.

We demonstrate in this study the activation of the Met-HGF/SF signal transduction pathway in a murine mammary cancer model. Met-HGF/SF signal transduction plays an essential role in both the developmental and the malignant processes of epithelial tissues. We show here that the activation of this signal transduction pathway dramatically changes the hemodynamics in Met-expressing epithelial organs and tumors. By detecting changes in blood oxygenation and flow using fMRI and in blood flow using Doppler ultrasound, we can use these hemodynamic changes to enhance imaging specificity.

The specificity to Met-HGF/SF signal transduction is evident from the fact that BOLD contrast alterations in tumors and different epithelial organs correlate with Met expression levels. The correlation with Met activation was further corroborated by immunoblotting analyses and by determining this effect to be HGF/SF dose-dependent. Although Met expression has been found to be heterogenic in the DA3 tumors using indirect immunofluorescence and confocal analysis (data not shown), we observed dramatic BOLD MRI alteration. It has
been shown by others and us (25) that in breast cancer Met expression is typically found to be higher at the tumor margins. Therefore, this distribution may actually enhance tumor edge detection by this methodology. Preliminary results show an intense alteration of BOLD activity in the tumor margins. However, higher resolution analysis is required to ascertain these results.

The alterations of signal intensity in GRE images can indicate modification in blood oxygenation, blood flow, or both. In defined regions of the tumor, the signal intensity increased by up to 60%. We also observed that in areas surrounding the tumors, the BOLD contrast significantly decreased. These dramatic changes cannot be explained solely by alteration in oxygenation level, suggesting that modifications in blood flow also occurred in response to Met signaling. To verify this hypothesis, we analyzed changes in tumor blood flow after HGF/SF injection using Doppler ultrasound technique. We found significant hemodynamic changes in tumors expressing high levels of Met. We show here that by activating Met signal transduction, we can detect blood vessels in the tumor that were undetectable before Met activation, thus increasing detection sensitivity.

On the basis of the results obtained from both in vitro cell culture and in vivo experiments, we hypothesize the following molecular mechanism: HGF/SF-induced Met activation leads to elevated oxygen consumption and regional hypoxia, which in turn induces hemodynamic changes, i.e., diminished arterial vessels in some areas and vasodilation in others, to compensate for the lack of oxygen. HGF/SF may act directly on Met-expressing endothelial cells in blood vessels (26, 27) or indirectly by induction of hemodynamic mediators such as nitric oxide (20).

The current technologies for measurement of endogenous oncogene expression are performed ex vivo using immunohistochemistry. HGF/SF-
induced hemodynamic changes can be used to map Met activity in vivo, enabling molecular imaging of this important oncogene. Molecular imaging of Met may assist in understanding its involvement in development and carcinogenesis. It may also serve as an important tool for diagnosis and prognosis of tumors in which Met is involved. Moreover, administration of HGF/SF with chemotherapeutic agents or radiation (28) may enhance treatment efficiency by facilitating drug delivery and by improving tumor oxygenation in Met-expressing tumors.

Met-HGF/SF-induced alteration of hemodynamics could have important clinical implications in tumor and metastasis detection. Met was shown previously (29, 30) to have prognostic value for epithelial cancers, including breast cancer. Met levels are increased in metastatic cells of the lymph node, in distal metastases originating from breast tumors (31), and in metastatic cells from melanoma (32) and ovary tumors (33). Because we found a direct correlation between Met expression and alteration of BOLD signal, we assume that this methodology can be applied for metastasis detection.

The endogenous level of Met in different organs could affect the feasibility of detecting alterations in MRI and ultrasound signals in response to HGF/SF. Imaging of liver metastases through Met activation may produce high background signal, but most other metastasis sites such as bones and lymph nodes express low levels of Met and, therefore, would exhibit low background signal. Therefore, the molecular imaging technique based on Met expression and activity may offer a specific and sensitive imaging modality of both primary tumors and metastases. Met activation induces signaling through a pathway typical of many other tyrosine kinase receptors. Therefore, we assume that the methodology described here can be implemented for molecular imaging of other tyrosine kinase receptors (e.g., erb2 and vascular endothelial growth factor) in breast cancer, as well as in a wide range of other malignancies.

The results presented here suggest an additional role for tyrosine kinase signal transduction in tumorigenicity, not only induction of angiogenesis but also alteration of tumor hemodynamics. Therefore, the therapeutic potential of tyrosine kinase receptor inhibitors (e.g., Herceptin) may also lie in their ability to reduce blood flow to the tumor, thus depriving the tumor of essential nutrients.

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