Identification of a Met-Binding Peptide from a Phage Display Library

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Abstract

Purpose: Aberrant c-Met expression has been implicated in most types of human cancer. We are developing Met-directed imaging and therapeutic agents.

Experimental Design: To seek peptides that bind specifically to receptor Met, the Met-expressing cell lines ST14 and SK-LMS-1 were used for biopanning with a random peptide phage display library. Competition ELISA, fluorescence-activated cell sorting analysis, an internalization assay, and a cell proliferation assay were used to characterize a Met-binding peptide in vitro. To evaluate the utility of the peptide as a diagnostic agent in vivo,125I-labeled peptide was injected i.v. into nude mice bearing s.c. xenografts of the Met-expressing and hepatocyte growth factor (HGF)/scatter factor expressing SK-LMS-1/HGF, and total body scintigrams were obtained between 1 and 24 h postinjection.

Results: One Met-binding peptide (YLFSVHWPPLKA), designated Met-pep1, reacts with Met on the cell surface and competes with HGF/scatter factor binding to Met in a dose-dependent manner. Met-pep1 is internalized by Met-expressing cells after receptor binding. Met-pep1 inhibits human leiomyosarcoma SK-LMS-1 cell proliferation in vitro. In SK-LMS-1 mouse xenografts, tumor-associated activity was imaged as early as 1 h postinjection and remained visible in some animals as late as 24 h postinjection.

Conclusions: Met-pep1 specifically interacts with Met: it is internalized by Met-expressing cells and inhibits tumor cell proliferation in vitro; it is a potential diagnostic agent for tumor imaging.

Since the discovery of the c-Met proto-oncogene more than 20 years ago, c-Met and its gene product Met have been extensively studied in terms of their relevance to cancer biology (1, 2). Activation of Met via autocrine, paracrine, or mutational mechanisms can lead to tumorigenesis and metastasis (3–9). Numerous studies have linked inappropriate expression of this ligand-receptor pair to most types of human solid tumors, including those of brain (10), breast (11, 12), ovary (13), thyroid (14), pancreas (15), stomach (16), prostate (17), and nasopharyngeal carcinoma (18).

Neutralizing mouse monoclonal antibodies (mAb) (19) and human mAbs (20) against human hepatocyte growth factor (HGF)/scatter factor (SF) have been generated and have shown high affinities to HGF/SF in inhibiting HGF-mediated cell proliferation, survival, and invasion in vitro and tumor growth in animal models. Mouse anti-Met mAbs were developed as nuclear-imaging agents in xenograft mouse models (21, 22). Monoclonal antibodies have become the most rapidly expanding class of pharmaceuticals for treating a wide variety of human cancers, but poor tumor penetration of antibodies due to the size of molecules, as well as liver/bone marrow toxicity by nonspecific uptake of the antibodies, has limited applications (23–25). Over the past decade, antibody engineering has made remarkable progress for tumor diagnosis and therapy. Antibody fragments and peptidic-targeting agents have been successfully developed by using combinatorial libraries displayed on microorganisms (26–32). Compared with antibodies and the fragments, peptides (1-2 kDa) are considerably smaller, generally do not bind to the reticuloendothelial system, and should not elicit an immune response upon repeated administration (33, 34). Therefore, peptides are promising molecules for delivering radionuclides or therapeutic drugs into tumors.

Peptidic agonists have been selected by screening peptide libraries against purified cell surface molecules (35–38). However, functionally folded soluble extracellular domains of some target membrane proteins have been difficult to obtain. In addition, because cell surface proteins are frequently posttranslationally modified, peptides selected against purified recombinant protein may not be able to access their targets on living cells (39). Therefore, some specific ligands for cell surface targets have been discovered by biopanning on whole intact cells (40–43).

The aim of this study was to seek a Met-binding peptide that could be used as a diagnostic agent and possibly as a therapeutics carrier. A random peptide phage display library was used to identify a Met-binding peptide by a subtractive...
panning approach on intact cells. Specificity and affinity of this peptide were examined by a number of in vitro assays. Nuclear imaging of the radiolabeled peptide in mouse xenograft model exhibited tumor-associated activity, recommending this peptide as a promising candidate for future clinical applications.

**Materials and Methods**

**Reagents and cell lines.** The Ph.D.-12 Phage Display Peptide Library kit was purchased from New England Biolabs. NaI [480-630 MBq (13-17 mCi)]/µL was purchased from Amersham Corp. LEDA for 5 min at 37 °C was eluted by incubation with 0.5 mL PBS containing 0.05% trypsin and were spun down and washed thrice with PBS. Cell-bound phage were used for the subsequent rounds at RT with gentle agitation. The cells were blocked and incubated with Met pep-1-biotin or nonavid peptide-biotin (50 µg/mL) and a specific anti-Met murine monoclonal antibody, Met4 (20 µg/mL), for 1.5 h at RT. After washing, fluorescein (DTAF)- conjugated streptavidin (Jackson ImmunoResearch) and antitau murine IgG-TRITC (Jackson ImmunoResearch) were added to cells and incubated for 1.5 h at RT. Nuclei were stained with 4',6-diamidino-2-phenylindole.

**Affinity measurement.** The binding affinity of Met pep-1 to Met was measured by KinExA 3000. Serially diluted SK-LMS-1 cells (1:4 dilutions, starting from 16 × 10^5 cells per mL for curve 1 and 4 × 10^6 cells per mL for curve 2) were incubated with constant Met pep-1-FTIC at 500 nmol/L for curve 1 or 100 nmol/L for curve 2 overnight until equilibrium was reached. Cells were removed by centrifugation. Unbound Met pep-1-FTIC was retrieved by passing the supernatant fractions through Met-coated beads. Multiple curve analysis was done to determine optimal values for the equilibrium dissociation constant K_d by using the KinExA Pro program.

**Internalization.** SK-LMS-1 cells were plated in eight-channel slides. Met pep-1-biotin or nonavid peptide-biotin was added to the medium to a final concentration of 25 µmol/L for 1 or 16 h. The cells were then washed and fixed with acetone/methanol (50:50) for 10 min. After blocking, the slides were incubated with Met4 at 20 µg/mL for 1.5 h at 37 °C. After washing with PBS twice, the cells were incubated with a mixture of either antimouse FITC conjugate and streptavidin–rhodamine red conjugate or DTAF-streptavidin and antitau-Mouse TRITC for 1.5 h at RT. 4',6-Diamidino-2-phenylindole was used to stain nuclei. The slides were examined by confocal laser scanning microscopy.

**Cell proliferation assay.** SK-LMS-1 cells were seeded in 96-well plates (2,000 cells per well) and incubated for 24 h. After 12 h of serum starvation, Met pep-1 or nonavid control peptide (100 µmol/L) was added to the medium to a final concentration of 25 µmol/L for 1 or 16 h. The cells were then washed and fixed with acetone/methanol (50:50) for 10 min. After blocking, the slides were incubated with Met4 at 20 µg/mL for 1.5 h at 37 °C. After washing with PBS twice, the cells were incubated with a mixture of either antimouse FITC conjugate and streptavidin–rhodamine red conjugate or DTAF-streptavidin and antitau-Mouse TRITC for 1.5 h at RT. 4',6-Diamidino-2-phenylindole was used to stain nuclei. The slides were examined by confocal laser scanning microscopy.

**DNA sequencing and peptide synthesis.** Single-stranded phage DNA was prepared from ELISA-positive clones as described in the phage display peptide library manual. DNA sequencing was carried out in a 3700 DNA Analyzer (Applied Biosystems) following the manufacturer’s instructions.

Peptides representing the sequence of positive clone 30 (YLFSVHWPPLKA) and nonavid peptide (ASYPFILVKLWHP) and their FITC and biotin conjugates were synthesized by Genemed Synthesis, Inc. DNA sequencing and peptide synthesis. Random peptide phage library

| Pre-absorption on NIH3T3 cells
| Biopanning on S114 and SK-LMS-1 cells alternately
| Single phage clone recovery and ELISA screening
| Sequence analysis
| Peptide synthesis
| Phage and Peptide competition ELISA
| FACS analysis and fluorescence imaging
| Internalization assay
| Cell proliferation assay
| Radionuclear imaging in Xenograft mouse

Fig. 1. Schematic representation of the experimental design.
added at different concentrations, with or without HGF/SF (100 ng/mL), and incubated for 12 h. Finally, [3H]thymidine was added for 4 h (0.5 mCi per well; PerkinElmer). Thymidine incorporation was measured using a scintillation counter (MicroBeta TriLux, PerkinElmer).

Identification of a Met-Binding Peptide

Results

Identification of peptide binders against human Met using phage display. A schematic representation of the experimental design is shown in Fig. 1. A 12-mer random peptide phage display library was screened with Met-expressing S114 and SK-LMS-1/HGF cells alternately to seek Met-binding peptides. NIH 3T3 cells were used for negative selection to remove nonspecific binders. After four rounds of biopanning, 30 phage clones were picked randomly for screening, all of which were found reactive against Met recombinant protein. They were then tested by competitive ELISA with or without HGF for Met binding. Three clones with the ability to compete with HGF in a dose-dependent manner for Met binding were sequenced (Fig. 2A; Table 1). Clone 30 (YLFSVHWPPLKA) displayed the highest competitive ability (59.3% inhibition as summarized in Table 1). The synthetic peptide of clone 30 (designated Met-pep1) also showed the competition ability to HGF for Met-binding in a dose-dependent fashion (Fig. 2B). At the concentration of 100 and 500 μmol/L, Met-pep1 competed 30% and 50% of HGF at 25 μg/mL for Met-binding. The nonavid peptide showed no competitive effect on HGF/Met binding (Fig. 2B).

Met-pep1 binds Met on a live or fixed cell surface. The specific binding activity of Met-pep1 to Met on live Met-expressing cells was determined by fluorescence-activated cell sorting analysis. FITC-conjugated Met-pep1 specifically binds Met-expressing DU145, MKN45, and PC3 cells (Fig. 3A). Very low level of binding was observed in NIH 3T3 cells. FITC-labeled nonavid peptide did not show any binding in all cells.

Since MKN45 and NIH 3T3 cells display different morphology in culture flasks, we mixed these two cell lines when plating them in eight-chamber slides. The colocalization of Met-pep1 and an anti-Met-specific monoclonal antibody Met4 in fluorescent staining indicated that Met-pep1 binds specifically to Met on formalin-fixed MKN45 cells, but not on NIH 3T3 cells (Fig. 3B).

To determine the affinity of Met-pep1, serially diluted SK-LMS-1 cells (1:4 dilution, starting from 16 x 10^5 cells per mL or 4 x 10^9 cells per mL) were incubated with constant Met-pep1-FITC (500 nmol/L, diamonds; 100 nmol/L closed circles) until equilibrium was reached (overnight; Fig. 3C). Cells were removed by centrifugation. The percentage of Met-pep1-FITC free in the supernatant was measured by passing the supernatant through Met-coated beads. Multiple curve analysis was done to determine optimal values for the equilibrium dissociation constant Kd, which was 64.2 nmol/L, calculated by n-curve analysis using the KinExA Pro program.

Met-pep1 internalizes Met-expressing cells via receptor binding. We examined the internalization of Met-pep1 by S114 cells with and without HGF. 30% and 50% of HGF at 25 μg/mL for Met-binding. The

![Fig. 2. Identification of Met-binding peptides. A, phage competitive ELISA. HGF samples (25 μg/mL) mixed with or without phage clones were incubated on the Met-coated plates and detected by a rabbit anti-HGF polyclonal antibody. Bars, mean of duplicate samples; bars, SD.](image)
through endocytosis (Fig. 4). After incubation of S114 cells and NIH 3T3 cells with biotin-conjugated Met-pep1 for 1 and 16 h, cells were fixed and stained with fluorescein-conjugated or rhodamine red-conjugated streptavidin. Anti-Met mAb Met4 was used as a positive control for immunostaining. The green and red colors were found colocalized in S114 cells, suggesting that Met-pep1 was internalized by S114 cells via receptor binding. Internalization of Met-pep1 into S114 cells could be detected as early as 1 h, remained visible at 16 h. No internalization of Met-pep1 was found in NIH 3T3 cells. No internalization of the nonavid control peptide on both cell lines was detected (data not shown).

**Met-pep1 specifically inhibits the proliferation of human leiomyosarcoma cells.** The exposure of Met-expressing cells to HGF/SF can elicit a variety of cellular responses including proliferation. We tested the inhibitory activity of Met-pep1 in HGF-mediated cell proliferation assay using SK-LMS cells. The cells were stimulated with HGF for 12 h before [3H]thymidine was added; then [3H]thymidine incorporation was measured. The results showed that 100 μmol/L of Met-pep1 clearly inhibited cell proliferation when the cells were treated with 100 ng/mL of HGF (Fig. 5). The P value was 0.017, calculated from Student’s t test, indicating that the differences were significant.

**In vivo nuclear imaging with Met-pep1.** As an additional functional test of Met-pep1 and as the first step in evaluating it as a potential in vivo diagnostic agent, we have conducted preliminary nuclear imaging experiments with radiiodinated Met-pep1 in nude mice bearing xenografts of the autocrine Met-HGF/SF–expressing human leiomyosarcoma cell line SK-LMS-1/HGF (Fig. 6). Female athymic nude mice bearing s.c. xenografts of human leiomyosarcoma SK-LMS-1/HGF in the posterior aspect of the right thigh were each injected i.v. with 77 μCi 125I-Met-pep1, whereas an additional five xenograft-bearing mice were each injected i.v. with 124 μCi 125I-labeled nonavid control peptide. Sequential posterior total body nuclear images of each group of mice were obtained 1 to 24 h postinjection (Fig. 6A). Tumor-associated activity was imaged as early as 1 h after injection of 125I-Met-pep1 and remained visible in some animals as late as 24 h postinjection. Activity was highest in the gastric region (g) at all imaging time points.

![Graphs showing characterization of Met-pep1](image)
points, whereas thyroid (t) activity increased over time. Activity was also evident in the nasopharyngeal (n) region and in the urinary bladder (b). The activity in the lower midline at 4 and 7 h postinjection represents the urinary bladder (b). By quantitative region-of-interest analysis (Fig. 6B), mean tumor-associated radioactivity accounted for 4% to 5% of the estimated injected activity of $^{125}$I-Met-pep1 at 1 and 4 h and declined to ~3% by 7 h postinjection. In contrast, mean tumor-associated radioactivity for host mice injected with $^{125}$I-labeled nonavid control peptide was only about one-third of the respective Met-pep1 values at all postinjection time points.

**Discussion**

There has been an exponential growth in the development of radiolabeled peptides for diagnostic and therapeutic applications in oncology. Peptide ligands, which have the advantages of low immunogenicity, easy incorporation into certain delivery vectors, and being readily diffusible, are being pursued as targeting moieties for the selective delivery of radionuclides,
cytokines, chemical drugs, or therapeutic genes to tumors (47). Faster blood clearance, rapid tissue penetration, and higher target-to-background ratios have been attained for peptides relative to macromolecular compounds in nuclear imaging studies. Radiolabeled receptor-binding peptides have become an accepted new class of radiopharmaceuticals (48, 49).

In this paper, we have shown through multiple approaches that Met-pep1 binds specifically to the extracellular domain of human Met, as expressed on the surface of human cancer cells in vitro and in vivo, and our data indicate that it must bind near the HGF/SF ligand binding site. Met-pep1 is internalized after its binding to Met, as evidenced both by direct imaging in vitro and by the liberation of free iodide in vivo.

To begin evaluating the diagnostic potential of Met-pep1, we conducted preliminary nuclear imaging experiments with radiiodinated Met-pep1 in nude mice bearing xenografts of the autocrine Met-HGF/SF-expressing human leiomyosarcoma cell line SK-LMS-1/HGF. We previously used this model system to evaluate full-length murine anti-Met mAbs (Met3 and Met5) as potential radiopharmaceuticals (21, 22, 46), as well as a human Fab fragment that specifically recognizes Met (27). Consistent with the general observation that peptides display a lower affinity for a given antigen, the tumor-associated radioactivity values are lower than we reported for the radiolabeled full-length murine anti-Met mAbs and human Fab (21, 22, 27, 46), but nonetheless are higher than the levels we found for either an irrelevant full-length mAb in the same model (21) or for a nonavid control dodecameric peptide (Fig. 6B).

In conclusion, due to its specific binding to Met-expressing tumor cells in vitro and in vivo together with internalization, Met-pep1 represents a promising candidate useful for clinical Met-directed diagnostic and therapeutic applications. Coupling cytotoxic drugs to macromolecular carriers has been shown to be a promising approach for efficient drug targeting. To further develop the use of this peptide in drug delivery, conjugation of toxins to Met-pep1 and their activities in in vitro assays are now under way in our laboratory.

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References

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