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High-affinity peptide against MT1-MMP for *in vivo* tumor imaging

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ABSTRACT

Membrane type-1 matrix metalloproteinase (MT1-MMP) is a key member of the matrix metalloproteinase (MMP) family. It participates in pericellular proteolysis of extracellular matrix (ECM) macromolecules and is essential for many biological and pathological processes, such as tumor development, angiogenesis and metastasis. A ligand that specifically binds to MT1-MMP may facilitate the labeling of this molecule, allow imaging at the cellular and organism levels, and provide a means for targeted drug delivery specific to MT1-MMP. A non-substrate MT1-MMP binding peptide was identified by screening a Ph.D.-12™ phage display peptide library and conjugated with near-infrared fluorescent (NIRF) dye Cy5.5 for tumor imaging. Peptide HWKHLHNTKTFL (denoted as MT1-AF7p) showed high MT1-MMP binding affinity. Computer modeling verified that MT1-AF7p binds to the MT-loop region of MT1-MMP and interacts with MT1-MMP through hydrogen bonding and hydrophobic interactions. MDA-MB-435 xenografts with high MT1-MMP expression had significantly higher tumor accumulation and better tumor contrast than the low MT1-MMP expressing A549 xenografts after intravenous injection of Cy5.5-MT1-AF7p. Using NIRF imaging, we have demonstrated specific targeting of MT1-AF7p to MT1-MMP-expressing tumors. Thus, MT1-AF7p is an important tool for noninvasive monitoring of MT1-MMP expression in tumors, and it shows great potential as an imaging agent for MT1-MMP-positive tumors.

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1. Introduction

The matrix metalloproteinases (MMPs) are a family of zinc-dependent endopeptidases that consist of 24 human MMPs, six of which are membrane bound [1]. The membrane type MMPs (MT-MMP) display the common structural domains of the MMP family, including a propeptide, a catalytic domain containing the Zn^{2+}-binding region, a hinge or linker region, and a hemopexin domain [2]. Four members of the MT-MMPs, MT1-, 2-, 3-, and 5-MMP (MMP-14, -15, -16, and -24), possess a type I transmembrane domain, while MT4-, and 6-MMP (MMP-17 and -25) anchor to the membrane with a glycosylphosphatidylinositol (GPI) linkage. Membrane type-1 matrix metalloproteinase (MT1-MMP) has been shown to be a key member of the MMP family. It is also the most prominent member of the membrane type MMPs with much biological and pathological significance. MT1-MMP is intrinsically associated with the plasma membrane of normal and tumor cells and remodels the extracellular matrix (ECM) [3]. MT1-MMP displays a broad spectrum of activity against ECM components, such as type I and II collagens, fibronectin, vitronectin, laminin, fibrin and proteoglycan. MT1-MMP also activates pro-MMP-2 and pro-MMP-13 (pro-collagenase 3). This process requires the tissue metallopeptidase inhibitor 2 (TIMP 2), which acts as an adaptor molecule mediating pro-MMP2 binding to MT1-MMP [4,5]. In addition, MT1-MMP cleaves ECM proteins and controls the functionality of a number of cell adhesion and signaling receptors [6,7]. It is directly involved in the cleavage of cell surface receptors including tissue transglutaminase, CD44, pro-αv integrin, syndecan-1, low-density lipoprotein (LDL) receptor-related protein and L-glycan [8,9]. MT1-MMP knockout mice are dwarfs and most die from wasting by the time of early adulthood [10]. The mice also develop skeletal dysplasia, arthritis, severe osteopenia, and generalized soft tissue

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disorders. MT1-MMP-dependent proteolysis of both extracellular matrix components and cell surface adhesion receptors plays an essential role in physiological processes (wound healing; adipocyte differentiation; bone growth and remodeling) and pathological processes (arthritis; tumorigenesis; tumor growth; invasion; metastasis and angiogenesis) [9,11].

The MT1-MMP is highly expressed in different cancers. For example, Takahashi et al. found that expression of MT1-MMP was elevated in 22 of 24 colon carcinomas that they examined [12]. It mediates pericellular proteolytic events that modulate cell attachment and motility [8], and its overexpression promotes migration, invasion and metastasis of cancer cells *in vitro* as well as *in vivo* [13,14]. The enzyme has been shown to be essential for angiogenesis [15,16]. Expression of MT1-MMP is crucial for cancer cell growth in a 3D collagen-based matrix [17], suggesting that MT1-MMP has important roles not only in cancer invasion but also in overall tumor progression [9].

Molecules capable of tracing MT1-MMP *in vivo* would be crucial to a detailed understanding of the nature of its expression, distribution, and its many biological and pathological functions. Furthermore, molecules that specifically target MT1-MMP would provide the potential for earlier detection and characterization of disease [18], targeted drug delivery [19,20], and evaluation of treatment in diseases where MT1-MMP is overexpressed.

Phage display technology has been widely used to indentify cell surface receptor binding peptides by screening with immobilized, purified targets, intact cells or by *in vivo* selection [21–25]. A series of protease substrates of MT1-MMP have already been well documented [26,27]. Ohkubo et al. searched for amino acid sequences cleaved by this protease using a hexamer substrate phage library and found that the consensus substrate sequences for MT1-MMP appeared to be P-X-G/P-L at the P3–P1′ site of the substrate [27]. Kridel et al. also identified a panel of optimal peptide substrates for MT1-MMP using substrate phage display. They divided the peptide substrates into different groups based on degree of selectivity for MT1-MMP. They showed that highly selective substrates lacked the characteristic Pro at the P3 position and instead contained an Arg at the P4 position, which is essential for efficient hydrolysis and selectivity for MT1-MMP [26].

Despite the success of identifying MT1-MMP substrates, no high affinity binding peptide for this protease has been reported. In order to find a peptide that can bind to MT1-MMP with high specificity, we used a recombinant form of MT1-MMP as a target to screen a random 12-mer phage-displayed library. We obtained a number of peptides with similar amino acid motifs as previously described, by using a substrate phage display [27]. To avoid repeat discovery of MT1-MMP substrates as affinity ligands, we abandoned the traditional approach of using recombinant protein as the screening target but used a peptide sequence that is specific to MT1-MMP as the target to screen the phage display peptide library. There are several advantages of using peptide as a phage display target: First, peptide libraries are readily available and the purity of the target peptide is higher than that of the other potential targets such as proteins or cells, therefore screening can be more effective. Second, the exact recognition site between the target and ligand is known, which facilitate the understanding of the ligand-target binding mechanism. Third, using a unique peptide from certain protein as target is more prone to get specific ligands to the protein.

In the present study, we screened for peptides that bind to a unique peptide sequence on the surface of MT1-MMP by *in vitro* panning of a phage display library and found a 12-mer peptide HWKHLHNTKTFL (MT1-AF7p) that binds MT1-MMP with high affinity and specificity. We further showed that the peptide can be used to image MT1-MMP expression *in vivo*. To the best of our knowledge, this is the first time that a non-substrate MT1-MMP affinity peptide has been found. We believe that it has good potential for use as a ligand in applications targeting this protease.

2. Materials and methods

2.1. In vitro panning of MT1-MMP binding peptides

The panning procedure was done as described in Ph.D.-12™ phage display peptide library kit manual. Briefly, the synthetic MT1-MMP sequence of amino acid 160–174 (MT1–160p) was dissolved in 0.1 M $NaHCO_3$ (pH 8.6) at a concentration of 100 μg/ml and coated onto a 35 mm polystyrene dish in a humidified incubator, stored at 4 °C overnight. After being blocked by blocking buffer (0.1 M $NaHCO_3$, pH 8.6, 5 mg/ml BSA, 0.02% NaN_3) for 1 h at 37 °C, 10 μl phage display peptide library (4×10^{10} phages) were diluted in 1 ml TBST (TBS containing 0.1% [v/v] Tween-20) and exposed for 1 h to the dish. After that, unbound phages were washed off with TBST ten times. The bound phages were collected by adding 2 ml of 0.2 M Glycine–HCl (pH 2.2) containing 1 mg/ml BSA to the plate for 10 min and neutralized with 150 μl of 1 M Tris–HCl (pH 9.1). One μl of collected phages was picked for phage tittering. For the amplification of selected phage clones to be used in the next round of panning, the remaining phages were mixed with 20 ml of ER2738 culture (at early log stage) and incubated at 37 °C with vigorous shaking for 4.5 h. The culture was then centrifuged for 10 min at 10,000 rpm at 4 °C. Then, the upper 80% of the supernatant was pipetted to a fresh tube and added to 1/6 volume of NaCl/PEG (2.5 M NaCl with 20% [w/v] PEG-8000). The phages were allowed to precipitate overnight at 4 °C. After centrifugation for 10 min at 10,000 rpm, 4 °C, the amplified phages were collected and dissolved in 200 μl of TBS buffer (50 mM Tris and 150 mM NaCl, pH 7.5) and the titer was determined on LB/IPTG/Xgal plates. This panning protocol was repeated two more times. In the fourth round of panning, the panning protocol was a little modified. Briefly, a 35 mm polystyrene dish was coated with 100 μg/ml MT1–160p. After being blocked by blocking buffer, the amplified phages in the third round were added to co-incubate with MT1–160p for 1 h at room temperature. Unbound phages were washed off by TBST for ten times. The bounded phages were washed off competitively by incubating with TBS containing MT1–160p at a concentration of 100 μg/ml for 1 h and were collected for amplification and titer determination. At the end of the fourth round of panning, the phage clones were analyzed by ELISA, and clones that displayed high binding ability to MT1–160p were amplified and, after in *vitro* replication, the appropriate DNA regions were sequenced, using −96 gIII sequencing primer, to determine the corresponding peptide sequences.

2.2. Phage capture ELISA

MT1–160p was diluted in bicarbonate/carbonate coating buffer (100 mM, pH 9.6) to a final concentration of 100 μg/ml. Aliquots (100 μl) of this solution were added to the 96-well plate and incubated at 4 °C for overnight. After being washed with 250 μl phosphate buffered saline (PBS, 2.32 g/L Na_2HPO_4, 0.2 g/L KCl, 0.2 g/L K_3PO_4, 8.0 g/L NaCl, pH 7.4), each well was filled with 200 μl of PBS containing 1% BSA to block the nonspecific sites. Then 100 μl of selected phages were added into each well, and incubation was carried out at 37 °C for 2 h to allow the phages to bind to MT1–160p. Then the plate was washed 3× by PBST (PBS containing 0.05% [v/v] Tween-20), and each well was filled with 80 μl of mouse anti-M13 phage antibody (1:1000 diluted by PBS) and incubated at 37 °C for 1 h. After three washes with PBST, 100 μl of horseradish peroxidase-conjugated goat anti-mouse antibody (1:2500 diluted by PBS) was added to each well. As negative controls, three wells were coated by MT1–160p and blocked with 200 μl PBS containing 1% BSA without adding phages. Finally, 200 μl HRP substrate, 3, 3′, 5, 5′-tetramethylbenzidine (TMB) solution, was added to each well and kept in the dark at 37 °C for 30 min. The reaction was stopped by adding 50 μl 2 M H_2SO_4 before the absorbance was measured by microtiter plate reader (Thermo Labsystems) at 450 nm.