Molecular Imaging

Tumor imaging using technetium-99m bound to pH-sensitive peptides

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Abstract

Solid tumors often display metabolic abnormalities that consistently produce low pH in the extracellular space of poorly perfused tissue. These acidic regions may provide a mechanism for drug targeting. Peptides have been designed in such a manner that they exist in an anionic hydrophilic form at the pH of normal tissues, but then undergo a sharp transition to a non-ionic lipophilic form at reduced pH. Peptides were labeled with fluorescein or technicium-99m (99mTc) and evaluated in vitro and in two murine models of cancer. Our studies suggest that PAP-1, an 18 amino acid pH activated peptide with a pH of transition between hydrophilic and lipophilic forms (pT) of 6.4, will deliver fluorescein and 99mTc to tumors. Activation of PAP-1 by low pH and penetration into the plasma membrane of cells and tumors were confirmed using flow cytometry, fluorescence microscopy, and gamma scintigraphy. These results support our central hypothesis that PAP-1 may enable the selective delivery of macromolecules to tumors. This technology has potential for exploiting a common property of tumors to achieve highly specific medical intervention.

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A major distinction between nearly all malignant solid tumors and their surrounding normal tissue is the abnormal nutritional and metabolic environment within tumors due to rapidly expanding cell populations. One striking example is that malignant tumors generally contain hypoxic regions, and the extracellular pH within such hypoxic areas is significantly lower than the pH in normal tissues under physiological conditions [1-3]. There are many examples of peptides, both naturally occurring and synthetic, that respond to stimuli and perform a variety of mechanical tasks. The idea that engineered peptides could perform tasks at the nanoscale was postulated as early as 1981 by Dr. Eric Drexler, who speculated on the application of myosin protein to produce linear movement [4]. Today, the use of natural physiology, including pH, to drive the movement of macromolecules with specific function is the focus of significant research efforts [5]. Recently, a pH-sensitive polypeptide related to the bacteriorhodopsin C helix (pHLIP) was shown to translocate a drug or an imaging molecule into the cytoplasm of cells in culture [6]. Translating this phenomenon from cell culture to animal models is complex and includes pharmacological variables that must be considered in the design and formulation of the molecule.
We present data on a new class of peptides that self-activate in response to the reduced pH present in hypoxic areas within the extracellular space of tumors \[7\]. These peptides are soluble in plasma at physiological pH; moreover, they can be engineered to self-activate and transition into a lipophilic form in acidic areas within tumors, allowing them to embed into the plasma membrane of tumor cells. The peptides described herein are based on earlier published work with modifications that allow a higher pH of transition to a lipophilic form \[7-9\]. We demonstrate, both in vitro and in vivo, that a properly designed molecule can be engineered to activate at the pH found in tumors. Such molecules preferentially accumulate in tumor tissue and, when not bound to tumor tissue, are excreted rapidly with a half-life on the order of 2-4 hours. Furthermore, we show that, with proper formulation, we can achieve adequate systemic peptide concentrations for scintigraphic imaging of tumors in vivo, thus providing a potential new platform technology for cancer diagnosis and treatment.

Methods

Design and production of pH-activated peptides

Transporter peptides may contain an amino acid sequence ranging in length from about 16 to about 50 amino acids and consist primarily of side chain carboxyls of glutamic acid interspersed with leucines. In addition to core sequence, the C terminus includes lipophilic amino acids to facilitate entry into the cell membrane. These amino acids may consist of leucines, isoleucines, norleucines, or methionines. Preferred sequence motifs contain less than 40% glutamic acid (specifically: 37.5% glutamic acid) and have an axial rotation between acid pairs that is greater than 500 deg (specifically, 550 deg). PAP-1 was chosen as a prototype molecule for peptide transporters that are polyanionic at pH 7.2 and above, and that convert to a lipophilic form effective to transport into cells at a pH below 7.2\[7\]. The utility of this peptide sequence was determined experimentally and is based on particular sequence motifs that often give an isobutyryl-amide moiety at the C terminus. For the Control-1 peptide, cleavage from the resin was with isobutyl amine, to give an isobutyryl-amide moiety at the C terminus. For the Control-1 peptide, cleavage from the resin was with trifluoroacetic acid (TFA), resulting in a carboxylic acid moiety at the C terminus. Protective groups on the amino acid side chains were removed with TFA, plus triisopropylsilane/water to scavenge carbonium ions generated in the deprotection step. All other reagents were molecular biology grade or were purchased through the Oregon State Veterinary Hospital pharmacy as formulations suitable for injection. The unique feature of PAP-1 that is of particular interest in this research is the peptide’s ability to switch between water-soluble and membrane-soluble forms in response to a small change in pH. To assess the pH at which the peptide makes this transition, fluorescein-labeled peptides were partitioned between n-octanol and a series of buffers with pH values ranging from 5.6 to 8.0. The pH values at which the transitions were half complete (pT) are provided in Table 1.

Preparation of fluorescein-labeled peptides

Peptides, while still on the synthesis resin and with side chains still protected, were reacted with the nitrophenyl ester of carboxyfluorescein. Cleavage from the resin and deprotection of the side chains were as described above.

Preparation of technetium-labeled peptides

Peptides, while still on the synthesis resin and with side chains still protected, were reacted with the nitropenyl ester of S-tritylated mercaptoacetylglycine (MAG3). Cleavage from the resin and deprotection of the side chains and the S-tritylated MAG3 were as described above. Technetium was purchased as $^{99m}$Tc (NaTcO$_4$), 4-8 Ci/mL from Cardinal Health, Pharmacy Service Center (Springfield, OR). Labeling and bench-top quality control of the MAG3-peptide conjugates with $^{99m}$Tc was adapted from a previously published work by Rusckowski et al. with minor modifications \[10\]. Briefly, the conjugated peptide was diluted to 4.0 mg/mL in 250 μL of 5% NaHCO$_3$ in normal saline, pH approximately 7.0. The labeling buffer consisted of a tartrate solution prepared by adding 2.1 g sodium bicarbonate, 0.96 g ammonium acetate, and 0.31 g ammonium hydroxide to a solution of 50.0 mg/mL sodium tartrate. Labeling was then performed by adding 30 μL of the tartrate solution to the peptide reaction vial containing 1.0 mg of peptide, followed by 740 MBq (20 mCi) NaTCO$_4$. Finally, 10 μL of 1 mg/mL SnCl$_2$, prepared with 10 mmol HCl was added to reduce the valence of the $^{99m}$Tc pertechnetate to $+5$. The solution was then incubated at room temperature (25-27 °C) for
30 minutes. The final specific activity of the labeled peptide was approximately 740 MBq/mL (20 mCi/mL) for a final injection volume of 50 μL. In later studies 250 μL 5% injectable mannitol were added to the final labeled peptide solution so as to inhibit peptide aggregation. Labeling efficiency was determined using reverse-phase bench-top liquid chromatography and was greater than 95% in these studies. A Sep Pak C18 mini cartridge (Waters Corp., Milford, MA) was prepared by flushing with 10.0 mL ethanol, followed by 10.0 mL sterile H2O and, finally, purged with 5.0 mL air. Approximately 100 μL of labeled peptide was loaded onto the cartridge, which was then eluted with 5.0 mL 1.0 mmol HCl to remove any free99mTcO4– and 99mTc-tartrate, followed by 5-10 mL 5% NaHCO3 to recover the labeled peptide. The elution fractions were then assayed on a Victoreen Cal/Rad dose calibrator (Moedling, Austria) and labeling efficiency calculated based on the assay measurements. Radiolabeling efficiency was equal to the assayed dose in the peptide solution divided by the combined assayed dose in the peptide and in the free 99mTc/99mTc-tartrate solutions. Labeling efficiency was 95% or greater. Doses of approximately 37 MBq (1 mCi)99mTc-MAG3 labeled peptide (Tc-PAP-1) were drawn for each animal.

Murine solid tumor models

Transplantation of lung and mammary carcinomas to mice

The Lewis lung carcinoma (LLC1) and 4T1 mammary carcinoma transplant models were chosen as examples of fast-growing syngeneic murine tumors. These models are well suited to our studies and produce tumors with a pH of approximately 6.8 when measured by insertion of a pH microprobe. All animal protocols were approved by the Institutional Animal Care and Use Committee at Oregon State University. Mice were given food and water ad libitum throughout the experiments. C57BL/6J mice were injected with 50,000 LLC1 Lewis lung cancer cells subcutaneously on the right flank to produce palpable tumors by approximately day 10. Balb-c mice were injected with 50,000 4T1 mouse mammary carcinoma cells directly into the mammary fat pad. These injections produced small nodular tumors by day 12.

Analysis of peptide conjugates in cells

Flow cytometry assay

Data were collected on at least 100,000 freshly stained cells by list-mode acquisition using a Beckman Coulter EPICS XL flow cytometer (Coulter Electronics, Hialeah, FL) and analyzed using WINLIST software (Verity Software House, Topsham, ME).

Confocal microscopy

A Zeiss LSM510 META with Axiovert 200 motorized microscope with version 3.2 LSM software (Carl Zeiss, Thornwood, NY) was used to visualize the fluorescein-labeled PAP-1 (F-PAP-1) on tumor cells. The pinhole size, gain, and photomultiplier tube signal amplification were adjusted manually. Most images were further magnified by electronic zoom. A series of optical x-y slices were collected at 10-μm intervals in the z plane. Confocal images were recorded and stored as Leica files. Individual optical slices were merged to produce extended-focus images using LSM software.

Analysis of peptide conjugates in mice

Mouse imaging using fluorescence microscopy

Small portions of LLC1 and 4T1 tumors were snap frozen at −80°C following excision from right flanks or mammary fat pads, respectively, of tumor-bearing mice. Frozen sections were placed on coated microscope slides and visualized under a 40× objective using a fluorescence microscope.

Mouse imaging using gamma scintigraphy

Mice were anesthetized with levoflurane and injected with 25.9 to 37.0 MBq (0.7 to 1.0 mCi) Tc-PAP-1 or control peptide via the tail vein or jugular vein as indicated. Mice were anesthetized during each procedure and were awake with access to food and water ad libitum between imaging
sessions. All images were acquired on the IS2 single-head gamma camera fitted with a low-energy, high-resolution collimator, peaked at 141 keV for $^{99m}$Tc with a 20% window in a $512 \times 512 \times 16$ bit depth (IS2 Medical Systems, Ottawa, ON, Canada). Static images were performed at 0, 2, 4, 8, and 24 hours after injection. Acquisition time ranged from 2 to 20 minutes depending on time between injection and imaging to compensate for radioactive decay.

Experimental design

Each animal experiment was performed with 8 to 16 animals because of the number of time points and need for anesthesia during scintigraphy and radiography. Imaging was done at 2, 4, 8, 12, and 24 hours with each peptide. A minimum of 4 animals were imaged with each peptide formulation. A total of 8-12 animals were imaged with PAP-1 peptide in complex with $^{99m}$Tc (Tc-PAP-1); further, both administration by jugular vein and tail vein injection in separate experiments were assessed to ensure that the site of injection did not affect peptide distribution. Experiments that used fluorescein-labeled peptides comprised 4 to 5 animals per group. Animals were divided into control and peptide groups based on tumor size rather than randomly assigning animals to groups. Tumor sizes were measured using calipers and varied from a diameter of 0.8 to 1.6 cm. In the last set of experiments, animals with transplanted 4T1 tumors were imaged using gamma scintigraphy while under anesthesia within a Plexiglas tank. Immediately following acquisition of the scintigraphic image, the animals were placed on a radiographic cassette and imaged by radiography, and position was documented by taking a digital photograph of the animals in each group. Animals were not moved between nuclear medicine, radiography, and photographic imaging.

Results

Determining the effects of pH on interactions between peptide and cells in culture

To assess the principal disposition of the peptide in cells in an acidic environment, the peptide was activated in the presence of LLC1 cells suspended in culture. This was done by adding acid (HCl) into the medium to reduce the pH from 7.4 to 6.5 and observing the accumulation of fluorescence associated with the cells using confocal microscopy. Initially, we used water/octanol partitioning to determine the pT (the pH at the midpoint of the transition between hydrophilic and lipophilic forms). These pT values for F-PAP-1 and related control peptides are tabulated in Table 1, and n-octanol partitioning as a function of pH of the partitioning buffer is plotted in Figure 1, A. We also activated PAP-1 at a concentration of approximately 10 M in 2 mL of medium containing 50,000 LLC1 cells. We immediately began observing the progressive accumulation of fluorescent PAP-1 associated with the cells under the confocal microscope. Using this technique we were able to observe the accumulation of PAP-1 on cells. As seen in Figure 1, B, the three-dimensional reconstruction of the confocal images under 40× objective reveals fluorescence on the surface of cells with little or no apparent intracellular accumulation of F-PAP-1. In time series experiments, using a 100× objective, we measured the fluorescence associated with the cell membrane at 10-second intervals for a total of 20.0 minutes (fluorescence images at 2-minute intervals from 6 to 20 minutes are shown in Figure 1, C). Each individual scan was analyzed as a digital image using Scion Image software Beta 4.0.2 (Scion Corp., Frederick, MD), and the results of integrated density analysis of the cell surface are presented in Figure 1, C. Our analysis demonstrates a nearly 5.0 log increase in intensity over an 8-minute period (Figure 1, D). It is important to recognize that this increase does not take into account the substantial bleaching of the fluorescein-derived signal that occurs with each scan of the laser; thus, our analysis may underestimate the increase in signal by several logs.

Determination of tumor cell uptake in vitro and in vivo

Use of F-PAP-1

To determine if PAP-1 is taken up by tumors following systemic administration, we conducted a study using fluorescein-labeled PAP-1. We used fluorescence-activated cell sorting (FACS) of disaggregated LLC1-derived tumor cells to improve the quantification of the pH-dependent translocation of F-PAP-1 in vitro and in vivo. Cells, treated with F-PAP-1 at pH 7.4 and washed with phosphate-buffered saline before fixing in 70% ethanol, showed a very low level of fluorescence identical to control because of limited membrane attachment at pH 7.4 (data not shown); however, following activation at pH 6.8, a population of highly fluorescent cells was observed. The peaks obtained suggested that two populations of cells were present and that all the cells displayed some labeling under these conditions. We then tested the ability of cells within tumors transplanted onto the right flank of mice to take up F-PAP-1 following intravenous administration. Tumor-bearing mice were injected by tail vein with approximately 20 μg of F-PAP-1. Tumors were excised at 4 hours and the cells disaggregated using a Dounce homogenizer (VWR Corporation, West Chester, PA). FACS analysis from the cells harvested following intravenous injection revealed a similar bimodal pattern; however, at this 4-hour time point the two populations consisted of labeled and unlabeled cells. This observation was consistent with the fact that variability in pH and O$_2$ exists in areas within tumors. FACS analysis was confirmed by comparing bright-field microscopy to fluorescence microscopy of the disaggregated cells (Figure 2, A-C, center and right panels, respectively).

Portions of tumor tissue from mice bearing LLC1 transplanted onto the right flank and from mice bearing 4T1 tumors transplanted onto the mammary fat pad were assessed. Tissues were snap frozen, and 5-μm sections were observed by bright-field and fluorescence microscopy. As seen in Figure 2, F-PAP-1–treated mice displayed intense fluorescent staining (Figure 2, E) within the LLC1 tumor as
compared with control (Figure 2, D). Inspection of the LLC1 tumor margin revealed a clear difference between tumor cell staining and normal tissue (Figure 2, F and G). Similar results were obtained with the 4T1 tumor margins (Figure 2, H and I), which suggested that the binding is not related to specific tumor type.
Use of Tc-PAP-1: gamma scintigraphy

PAP-1 and control peptides conjugated to MAG3 and in complex with $^{99m}$Tc were used to test the hypothesis that a pH-activated peptide could direct a radioisotope to tumors. Control peptides included in these studies were similar in sequence to PAP-1 but varied from PAP-1 with respect to

Fig 2. Analysis of tumor cells ex vivo and in situ. A-C, FACS analysis (left panel) along with bright-field (center panel) and fluorescence (right panel) of tumor cells collected from Lewis lung tumor transplants in C57BL/6J mice. A, The background fluorescence of tumor cells from a vehicle-treated animal 2 hours after injection. B, Analysis of the cells from a vehicle-treated animal incubated with 0.004 mg/mL peptide ex vivo in phosphate-buffered saline at pH 6.5. C, Analysis of cells excised from the tumor of a C57BL/6J mouse treated with a single intravenous injection of 0.1 mg (27 nmol) 8-carboxyfluorescein-labeled peptide and collected 2 hours after injection. D, Fluorescence image of a control LLC1-derived tumor from a C57BL/6J mouse. E-G, Images from the treated animal (see description in C) with bright-field (F) image as a reference for the tumor margin (blue line) seen in G. H and I, Similar images from 4T1-derived tumors with normal (lower left) and tumor tissue (upper right) present.
either the entry end or length (see Table 1). In our studies peptides with these variations served as negative controls because of their similarity in composition relative to the active peptide but their lack of optimal properties for being selectively sequestered in acidic areas of tumors. In addition, mice were also injected with free $^{99m}$Tc ($^{99m}$TcO$_4$) to determine the main target organs of unconjugated radioisotope. Tc-Control-2 peptide was included in the studies to determine if a peptide similar to PAP-1 but with a higher pT of 6.9 would retain adequate selectivity for tumors. However, this Tc-Control-2 peptide with the appreciably higher pT value was found to adhere primarily to the site of injection and was not used in subsequent studies. For later studies Tc-Control-1 (see Table 1) was used. This control peptide was similar in structure to the Tc-PAP-1 but had an appreciably lower pT value of 5.9 and remained soluble in solution at the pH present in acidic areas of tumors.

We injected C57BL/6J mice with LLC1 cells subcutaneously onto the right flank. This produced palpable tumors at the injection site within 21 days. PAP-1 and control peptides were administered by jugular vein injection in tumor-bearing mice and imaged at 2, 4, 8, and 24 hours. Peak concentrations in the tumors were observed at 4 hours for Tc-PAP-1, and the resulting images are presented in Figure 3. Tc-Control-2, Tc-Control-1, and Free $^{99m}$Tc (Figure 3) failed to accumulate in the tumors, whereas Tc-PAP-1 demonstrated clear labeling of the tumor. At 4 and 8 hours the gamma emissions were limited to injection site, liver, and tumor. Interestingly, the Tc-PAP-1 intensity had diminished by 8 hours.

Fig 3. Scintigraphic images of representative C57BL/6J mice bearing LLC1 tumors on their right flanks. Each mouse was treated with free $^{99m}$Tc, Tc-PAP-1, Tc-Control-1, or Tc-Control-2 at 4 and 8 hours after jugular vein injection. Two representative mice are shown for the various peptide treatments. An intensity scale is provided in the upper right corner of the figure.
We decided to observe the actions of Tc-PAP-1 in a second tumor type and conducted a study in Balb-c mice bearing 4T1 tumors transplanted into the mammary fat pad. We chose to administer the peptides by tail vein injection and image animals during the study by gamma scintigraphy, conventional radiography, and digital photograph so as to allow image fusion and align images with more ease. This allowed us to more closely match the gamma emissions to the small nodular tumors that result from 4T1 cell injections. Representative animals are presented in Figure 4, which demonstrates accumulation of Tc-PAP-1 within the mammary carcinoma in tumor-bearing mice. Control peptides did not show accumulation at the tumor site consistent with our observations in the LLC1 lung carcinoma model.

Discussion

Previous studies with cancer chemotherapies and contrast agents have established that acidic regions of tumors present a barrier to entry of weakly basic molecules [11-15]. This low pH in hypoxic areas of tumors has been recognized as a potential opportunity to increase therapeutic success in chemotherapy and enhance detection of tumors [16-19]. Our results suggest that activation of pH-sensitive peptides by this low pH in tumors is feasible and novel. Our experimental model focused primarily on the use of this technology for diagnostic purposes, but clearly it also holds promise for therapeutic applications. Gamma scintigraphy proved to be a valuable tool in the identification of lead compounds for tumor targeting in our studies. This technique allowed us to visualize the kinetics of our molecules in real time and provided preliminary indications of drug distribution and half-life of excretion.

The primary sites of peptide accumulation seemed to be the tumor and the liver. Although at this time we do not know if the ⁹⁹ᵐᵐ⁻⁴⁻Tc signal in the liver is due to accumulation of intact molecules or if the liver is a site of metabolism and degradation, nonetheless it seems likely that the peptides remain intact because their unnatural D chirality effectively foils the peptidases and proteases that normally degrade peptides in biological systems. As we develop this technology further, an emphasis will be placed on understanding the fate of these peptides in the liver. The amount of peptides used in these studies was low and, while there was no sign of toxicity from the peptides, dose escalation and toxicity studies are needed to ensure that these molecules are safe.

Much of the current anticancer nanotechnology has focused on mimicking naturally occurring peptides, antibodies, and growth factors for tumor targeting [20]. Other emerging technologies combine synthetic nanoparticles with drugs or contrast agents to enhance tumor uptake [21-24]. Compared to these approaches, our data suggest that peptides can be rationally designed with the proper pharmacological properties to perform a specific task in vivo.

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