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In vivo imaging of tumor apoptosis using histone H1-targeting peptide

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ABSTRACT

In vivo imaging of apoptosis could allow monitoring of tumor response to cancer treatments such as chemotherapy. Using phage display, we identified the CQRPPR peptide, named ApoPep-1(Apoptosis-targeting Peptide-1), that was able to home to apoptotic and necrotic cells in tumor tissue. ApoPep-1 also bound to apoptotic and necrotic cells in culture, while only little binding to live cells was observed. Its binding to apoptotic cells was not dependent on calcium ion and not competed by annexin V. The receptor for ApoPep-1 was identified to be histone H1 that was exposed on the surface of apoptotic cells. In necrotic cells, ApoPep-1 entered the cells and bound to histone H1 in the nucleus. The imaging signals produced during monitoring of tumor apoptosis in response to chemotherapy was enhanced by the homing of a fluorescent dye- or radioisotope-labeled ApoPep-1 to tumor treated with anti-cancer drugs, whereas its uptake of the liver and lung was minimal. These results suggest that ApoPep-1 holds great promise as a probe for *in vivo* imaging of apoptosis, while histone H1 is a unique molecular signature for this purpose.

1. Introduction

Apoptosis plays an important role in the pathogenesis of a variety of diseases, such as cancer, myocardial and cerebral ischemia, and neurodegenerative diseases [1]. Since the induction of apoptosis is an essential event in cancer treatments, such as chemotherapy and irradiation, imaging apoptosis would provide valuable information in regards to monitoring tumor response to different therapies. Annexin V has been most extensively used to image apoptosis in diverse clinical circumstances [2]. Annexin V is a protein with a molecular weight (MW) of 36 kDa and has a high-affinity to phosphatidyserine (PS) that was exposed on the surface of apoptotic cells. It has been reported, however, that the *in vivo* performance of annexin V still needs improvement in terms of signal to background ratio and specificity to apoptotic cells over non-apoptotic cells [3,4].

As an alternative to bulky proteins or antibodies, small peptides may have properties as an ideal imaging probe. They have rapid clearance from blood circulation, efficient tissue penetration due to their small sizes, low immunogenicity, and low production cost [5,6]. Moreover, peptides are relatively easy to be chemically modified for coupling to imaging agents or drugs. Many peptides have already been in clinical use and under investigation in clinical trials. For example, they include the Octreoscan for the diagnosis of neuroendocrine tumors, cyclic RGD peptide for head and neck cancers, and Exendin-4 for insulinoma [6].

Cells in a disease microenvironment put their own molecular signatures on their cell surface. Angiogenic tumor endothelial cells over-express novel proteins such as the αvβ3 integrin. Macrophages in a tumor microenvironment express genes such as mannose receptor C type I, which is distinct from conventional pro-inflammatory macrophages [7]. Such molecular signatures in diverse disease microenvironments have been successfully exploited by screening phage-displayed peptide libraries [8–14].

Given that cells from primary tumor tissues would more closely mimic the cells in a tumor microenvironment than do cultured cell lines, we screened a phage-displayed peptide library in order to select

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peptides that specifically bind to macrophages isolated from primary tumor tissues. Interestingly, we identified a peptide that was able to target apoptotic and necrotic cells in tumor tissue by binding to histone H1 on the surface of apoptotic cells and in the nucleus of necrotic cells. Here, we suggest that the histone H1-targeting peptide is a promising probe for imaging apoptosis *in vivo*.

2. Materials and methods

2.1. Screening of a phage peptide library

A phage library based on T7 415-1b phage vector displaying CX7C (C, cysteine; X, any amino acid residue) random peptides was constructed according to the manufacturer's instruction (Novagen, Madison, WI, USA). The library had a diversity of 5×10^8 plaque forming unit (pfu). Primary human lung squamous carcinoma tissues and adjacent normal tissues were obtained by surgical resection of tumor for the treatment of patients with cancer. Cell suspension was prepared by gently homogenizing tissue using Medimachine (DAKO, Carpinteria, CA, USA). Phages (5.5×10^{10} pfu) were incubated with 10 μl (packed cell volume) of cells from tumor tissue at 4 °C for 1 h. Macrophages were isolated from the cell suspension by using 25 μl (1×10^7 beads) of magnetic beads conjugated with anti-human CD14 antibody (Dynal, Oslo, Norway) at 4 °C for 30 min. Macrophage-bound phages were eluted by lysing the cells with 100 μl of 1% NP-40 and adding a 900 μl culture of BL21 bacteria to the lysates. For subtraction, the eluted phage solution was incubated with cells from normal tissue at 4 °C for 30 min and then the supernatant containing unbound phages was recovered. The recovered phages were propagated in host bacteria and used for the next round of selection. The output titer of phages was determined by counting the number of plaques. After three rounds of screening, phage clones were randomly selected from the second and third round and their DNA inserts were sequenced. Amino acid sequences were analyzed by the Clustal W program.

Peptides were synthesized by Peptron Inc. (Daegeon, Korea) and Anygen Inc. (Gwangju, Korea). Fluorescein isothiocyanate (FITC), biotin, or Cy7.5 dye (GE Healthcare, Piscataway, NJ, USA) was conjugated at *N*-terminal of peptides.

2.2. Immunofluorescence and flow cytometry analysis of peptide binding

A549 and H460 lung tumor cells and MDA-MB-231 breast tumor cells were maintained in RPMI-1640 medium. L132 normal human lung epithelial cells, RAW human macrophage cells, and HeLa human cervical cancer cells were maintained in high-glucose Dulbecco's modified Eagle's Medium. All culture media were supplemented with 10% fetal bovine serum and penicillin/streptomycin.

Apoptosis was induced by incubating cells with 50 μM etoposide for the indicated time periods. Necrosis was induced by incubating cells with glucose-depleted medium for 10–15 h. Apoptotic stages were determined by staining the cells with annexin V and propidium iodide (PI). Live cells exclude cationic dyes such as PI, while dead cells (necrotic or late apoptotic cells) do not.

For immunofluorescence analysis, cells were incubated with 1% bovine serum albumin at 37 °C for 30 min for blocking and then with 10 μM FITC-conjugated peptide at 4 °C for 1 h. Cells were then co-stained with Alexa-594-annexin V (Invitrogen, Carlsbad, CA, USA) for 15 min at room temperature in binding buffer (10 mM HEPES, pH 7.4, 140 mM NaCl, and 2.5 mM $CaCl_2$). For competition with annexin V, cells were pre-incubated with unlabeled annexin V at excess molar concentrations for 30 min at 37 °C and then incubated with Alexa-594-annexin V or FITC-ApoPep-1. For the staining of neuropilin-1 (NRP-1), cells were incubated with a rabbit monoclonal anti-human NRP-1 antibody (1:100 dilution; Abcam, Cambridge, MA, USA). After fixation, cells were incubated with 4′,6′-diamidino-2-phenylindole (DAPI, Sigma-Aldrich, St Louis, MO, USA) for nuclear staining and a mounting solution before observation with a fluorescent microscope (Zeiss, Oberkochen, Germany).

For flow cytometry analysis, cells (1×10^6 cells) in suspension were incubated with FITC-peptide or Alexa-488-annexin V and then subjected to flow cytometry. To see if cells were at late stage of apoptosis or necrosis, cells were co-stained with 1.5 μM PI for 5 min at room temperature.

2.3. Affinity chromatography and mass spectrometry

ApoPep-1 was conjugated with a biotinylated, photoreactive cross-linker, sulfo-N-hydroxysuccinimidyl-2-(6-[biotinamido]-2-(p-azido benzamido)-hexanoamido) ethyl-1,3′-dithioproprionate (sulfo-SBED, Pierce, Rockford, IL), for 60 min at room temperature in the dark. The conjugated ApoPep-1 was purified by removing unincorporated sulfo-SBED using 1 kDa Tube-O-dialyzer (Biosciences, St Louis, MO, USA) for 6 h. The peptide solution was incubated with apoptotic cells (5×10^7 cells) at 37 °C for 60 min in serum-free medium and then exposed to ultraviolet light at 0.04 J for 15 min using Stratalinker (Stratagene, La Jolla, CA, USA) to photoactivate the sulfo-SBED. Cells were lysed and ApoPep-1 (bait) and binding protein (prey) complex was immobilized by incubating the cell lysates with monomeric avidin beads (Pierce) at 4 °C for 1 h. ApoPep-1 was separated by reducing the complex with 50 mM dithiothreitol (DTT). The prey protein was eluted with 2 mM D-biotin followed by treatment with 100 mM glycine, pH 3.0. Eluted proteins were resolved by SDS-PAGE and stained with a silver staining reagent compatible with mass spectrometry. Protein bands were excised from the silver-stained gel and subjected to mass spectrometry.

For in-gel trypsin digestion, an excised gel was cut into small pieces, destained in 30 mM potassium ferricyanide/100 mM sodium thiosulphate, and washed with 100% acetonitrile/100 mM ammonium bicarbonate. The gel pieces were dried by vacuum centrifugation after dehydrated with acetonitrile, reduced with 10 mM DTT at 56 °C for 45 min and alkylated with 55 mM iodoacetamide for 30 min at room temperature. The gel pieces were then dried by vacuum centrifugation, rehydrated with trypsin solution in 50 mM ammonium bicarbonate, and incubated overnight at 37 °C. Peptides were extracted with three steps: 5% formic acid/60% acetonitrile; 5% formic acid/50 mM ammonium bicarbonate; and finally 5% formic acid/100% acetonitrile. The extracts were pooled, dried by vacuum centrifugation and resolved in 10 μl of 0.1% formic acid.

The peptide extracts were separated by a nano-Acquity UPLC system using a Symmetry C18 5 μm, 5-mm×300-μm pre-column and an Atlantis C18 3 μm, 15-cm×75-μm analytical reversed phase column (Waters, Milford, MA, USA). Flow rate was 300 nl/min. Mobile phase A and B are 0.1% formic acid in water and 0.1% formic acid in acetonitrile, respectively. The peptides were separated with a gradient of 3–40% mobile phase B over 60 min at a flow rate of 300 nl/min followed by a 1 min rinse with 90% of mobile phase B. The column was re-equilibrated at initial conditions for 20 min. The column temperature was maintained at 40 °C. Raw data from Q-TOF Premier mass spectrometer (Waters) were processed and identified by Protein Lynx Global Server 2.3 software (Waters) and Mascot v2.0 (Matrix science, Boston, MA) using a IPI Human v3.47 (http://www.ebi.ac.uk/IPI/). For search parameters, fixed modification was carbamidomethyl C and variable modification was oxidation M.

2.4. Analysis of peptide binding to histone H1

Membrane fraction-enriched cell lysates were prepared using a lysis buffer (0.5% Triton X-100, 100 mM NaCl, 1 mM EDTA, 20 mM Tris). Biotin-eluates from affinity chromatography or cell lysates were subjected to 15% SDS-PAGE, transferred to a nitrocellulose membrane, and immunoblotted with a mouse monoclonal anti-human histone H1 (AE-4) antibody (1 μg/ml; Santa Cruz Biotechnology, Santa Cruz, CA,