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Plaque-penetrating peptide inhibits development of hypoxic atherosclerotic plaque

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ABSTRACT

Atherosclerosis treatments are generally aimed at altering systemic lipid metabolism such that atherogenesis, the formation of plaque, is curtailed. The plaques themselves offer some potential therapeutic targets. For example, selective depletion of macrophages, which play a key role in atherogenesis, inhibits plaque formation. However, it has not been possible to take advantage of these targets because the drugs that have been tested have not been sufficiently selective. We have developed a peptide, LyP-1, which specifically targets atherosclerotic plaques, penetrates into plaque interior, and accumulates in plaque macrophages. In tumors, LyP-1 can cause apoptosis in cells that take up the peptide. Here we show, using three different atherosclerosis models in ApoE null mice that prolonged systemic treatment with LyP-1 triggers apoptosis of plaque macrophages and reduces plaque in advanced hypoxic plaques, and that it does so without increasing necrotic core of plaques or causing detectable side effects. We also show that LyP-1 recognizes human plaque. These findings suggest that LyP-1 could serve as a lead compound for the development of a new class of anti-atherosclerosis drugs.

1. Introduction

Atherosclerotic cardiovascular diseases, such as acute myocardial infarction and stroke, are major causes of death and disability worldwide [1]. The hallmark of the disease is the development of atherosclerotic plaques, accumulations of multiple cellular types, extra cellular matrix, lipids, and debris in the arterial wall [1]. Hypoxia exists in advanced atherosclerotic lesions and plays a key role in their progression by promoting lipid accumulation, increased inflammation, ATP depletion, and angiogenesis [2,3]. Activated macrophages are abundant within plaques and have been implicated in all stages of atherogenesis [4]. They phagocytize and accumulate lipids, cholesterol in particular, becoming foam cells, which initiate and sustain plaque development [5]. Plaque macrophages also produce pro-inflammatory, cytotoxic and chemotactic molecules that accelerate plaque formation and secrete proteases that can destabilize plaques and trigger their rupture by degrading extracellular matrix [5]. Suppressing inflammation and depletion of monocytes/macrophages in atherosclerotic plaques inhibits the development of atherosclerosis [6–8]. Hence, pharmacological depletion of macrophages is a promising strategy for atherosclerosis treatment [6]. Ideally, macrophage depletion should be highly selective to plaques, and not affect macrophages elsewhere or other cell types in the plaque. For example, depletion of endothelial coverage on the surface of plaque may trigger blood clotting [9,10], while smooth muscle cells depletion may weaken the fibrotic cap of the plaque, or even the arterial wall, which could cause plaque rupture or give rise to an aneurysm [11]. Unfortunately, no practical methods are currently available that would selectively eliminate the plaque macrophages that are critical to plaque formation.

LyP-1 (CGNKRTRGC) is a cyclic nonapeptide that specifically recognizes lymphatic vessels, tumor cells and macrophages in certain tumors. It shows preferentially affinity for hypoxic areas in tumors and accumulates in tumor-associated macrophages [12–14]. The primary receptor for the LyP-1 peptide is p32/p33/gC1qR/HABP1 (p32) [14]. The main location of p32 is in mitochondria [15], but other subcellular locations have been reported, including the cell surface [16]. Cell surface p32 expression is an exclusive characteristic of highly activated cells, such as tumor cells, as well as resident macrophages/myeloid cells and endothelial cells in tumors [14]. In mitochondria, p32 regulates oxidative phosphorylation [17,18]. A link to autophagy has also been proposed [19]. The function of cell surface p32 is not known, but it can act as a

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receptor for drugs carried by p32-binding peptides (or anti-p32 antibodies) into tumors [20,21]. LyP-1 is highly effective in ferrying drugs into tumors because it belongs to a class of tumor-penetrating peptides [22–24]. These peptides home to tumors because they bind to a tumor-specific receptor, p32 in the case of LyP-1, and are then proteolytically converted into a fragment that binds to neuropilin-1 (NRP-1). The NRP-1 binding triggers an endocytic trans-tissue transport pathway that carries through the blood vessel wall and deep into extravascular tumor tissue [22,25].

Various cell types in atherosclerotic plaques also express cell surface p32, and LyP-1 is highly effective in homing to plaques, where it penetrates into the interior of plaques, accumulating primarily in plaque macrophages [26,27]. This trait has been taken advantage of in plaque imaging in mouse models [27]. LyP-1 also possesses an inherent pharmacological activity independent of any coupled drug: it causes depletion of its target cells in tumors [13]. We surmised that the homing of LyP-1 to plaques and its ability to eliminate its target cells (mostly macrophages in plaques) might have a beneficial effect on the development of atherosclerosis. Our initial attempts to use LyP-1 reduce plaques in the standard ApoE null/high fat diet atherosclerosis model failed to show a definitive effect. Given the tendency of LyP-1 to accumulate in hypoxic tissue [13], we hypothesized that LyP-1 might be more effective in an atherosclerosis model that produces hypoxic plaques. Here we test that hypothesis.

2. Materials and methods

2.1. Peptides

Peptides were synthesized on a microwave-assisted automated peptide synthesizer (Liberty; CEM, Matthews, NC) following Fmoc/tertiary butyl strategy on rink amide resin with HBTU (*N*,*N*,*N*′,*N*′-Tetramethyl-O-(1H-benzotriazol-1-yl) uronium hexafluorophosphate) activator, (or alternatively, O-(Benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate), collidine activator base and 5% piperazine for deprotection. When included, 5 (6) carboxy-fluorescein (FAM) was incorporated during the synthesis at the N-terminus of the sequence. Cleavage using a 95% trifluoro acetic acid followed by purification gave peptides with >95% purity. The peptides were analyzed by mass spectrometry as previously described [28].

2.2. Mice, diet, and in vivo administration of peptides

Breeder mice homozygous for the Apoetm1Unc mutation (ApoE-null mice) were from The Jackson Laboratory. Male ApoE null mice were used in all treatment studies, except for one study, which included females. Mice were switched to high-fat diet (HFD [29,30] at the age of 8 weeks to induce atherosclerosis. The diet was continued until the end of every treatment procedure. The mice were housed in animal facilities of University of California, Santa Barbara and Sanford Burnham Prebys Medical Discovery Institute. All procedures received prior approval from the Institutional Animal Care and Use Committees. Peptides were dissolved at 2 mg/mL in PBS, passed through 0.22 μm filter for sterilization, and administered at 200 μg/mouse/day/injection. Tail vein and intraperitoneal injections were given without anesthesia. Retro-orbital injections were given under isoflurane inhalation (isoflurane 2–3% vol/vol + 2 L/min O_2) anesthesia. Mice were euthanized by perfusing successively with ice-cold PBS and 4% paraformaldehyde in PBS following bleeding from the retro-orbital vein (no blood collected from mice in treatment #1 on standard ApoE null atherosclerosis model) after treatments. Aorta and carotid arteries were collected for later analysis.

2.3. Standard ApoE null atherosclerosis model

Mice were fed HFD for 16 or 20 weeks before the initiation of treatment. Three treatment protocols were used: (1) intravenous (i.v.) injections administered into the retro-orbital venous plexus 5 days a week with two recovery days (study #1), (2) daily intraperitoneal (i.p.) injections (study #2), and (3) intravenous (into the tail vein) and i.p. injections, given for alternating 4-day and 3-day periods. The treatment was continued for 8 or 10 weeks. Protocol details are given in Supplemental Fig. S1.

2.4. Accelerated ApoE null atherosclerosis

Accelerated carotid artery atherosclerotic plaque was induced at the age of 8 weeks by either completely ligating the left common carotid artery right before the bifurcation (complete ligation without cuff placement) [31] or by ligating the three main branches of the carotid artery with the smallest branch, superior thyroid artery left open (partial ligation) [32]. Treatment was started immediately after the procedures by daily retro-orbital i.v. injections and continues for 2 weeks.

2.5. Evaluation of atherosclerosis severity

Carotid artery plaque was quantified by calculating the ratio of plaque area relative to vessel area using image Pro Plus software in arteries sections stained with hematoxylin/eosin (H&E). The ratio from five (complete ligation) or three (partial ligation) sections cut at 100 μm interval was averaged to reflect the total plaque of each animal. In the standard atherosclerosis model, plaque occupation was quantified in the whole aorta tree *en face* with the aorta cut open longitudinally, followed by calculation of the area of plaques at the aorta root level [33,34].

2.6. Blood chemistry

Treated mice were fasted for 16 h and then bled from the orbital plexus into lithium heparin collection tubes. Plasma was collected and stored at −80 °C. Thawed plasma was loaded into VetTest Chemistry Analyzer (IDEXX Laboratories) for cholesterol and triglyceride analysis or into Comprehensive Diagnostic Profile Rotor #500-0038 followed by analysis with the VetScan VS2 for liver and kidney function.

2.7. Plaque macrophage analysis by flow cytometry

Atherosclerotic plaques were isolated from male ApoE null mice that had been fed HFD for 20–24 weeks and treated with LyP-1 or control peptide (ARA) for four weeks. Cells were released by incubating the freshly excised plaque tissue in a tissue digestion solution containing 450 U/mL collagenase type 1 (Worthington Biochemical), 1 mg/mL soybean trypsin inhibitor (Worthington Biochemical), 4.7 U/mL elastase (Worthington Biochemical), 1 mg/mL DNase 1 (Sigma Aldrich), and 0.5% FCS for 2 h at 37 °C. CD11b PE-Cy5 (rat monoclonal; eBioscience) and p32 antibody (polyclonal; Millipore) were used to identify monocytes/macrophages. Cell populations were quantified on GUAVA FACS instrument (Millipore) and analyzed using FCS Express Version 3 (De Novo Software).

2.8. Hypoxia analysis

Partial ligation surgery as described above was performed on mice with aortic and carotid plaque. The hypoxia probe (Pim; Hypoxyprobe ™-1 kit; Hpi, Inc.; 60 mg/kg) was intravenously injected and the aortic arch and the operated carotid artery were collected 90 min later and snap frozen for future histological analysis. The frozen tissues were fixed with 1% PFA for 2 h and dehydrated in 20% sucrose overnight before being embed into OTC for tissue sectioning (10 μm). Pim and blood vessels (CD31, BD Science) co-staining was performed according to the instruction of the manufacturer. The intensity of Pim and areas of CD31 staining inside the plaque were quantified using Image pro plus software after confocal imaging (LSM 710 NLO Zeiss Multiphoton) of