Materials and Methods

Photodynamic Therapy Using a Protease-Mediated Theranostic Agent Reduces Cathepsin-B Activity in Mouse Atheromata *In Vivo*

Shon et al. Protease-Mediated PDT in Atherosclerosis

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Materials and Methods

This study was performed in accordance with National Institutes of Health guidelines for the care and use of laboratory animals. The animal protocol was approved by the Institutional Animal Care and Use Committee. Animals were anesthetized with 2% isoflurane in a mixture of 30% oxygen and 70% nitrogen.

Theranostic Agents

The L-SR15 cathepsin-B (CatB)-activatable photosensitizer was synthesized as described previously. A biodegradable poly-L-lysine backbone had multiple chlorin e6 (Ce6) molecules conjugated to epsilon-amine groups sufficiently closely spaced to self-quench, with polyethylene glycol units added to reduce aggregation and improve solubility. Conjugation of Ce6 (Frontier Scientific, Logan, UT) to lysine residues on the backbone was performed using a coupling reagent 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide HCl. The conjugates were purified using gel-filtration chromatography using Bio-Gel P-10 gel (Bio-Rad, Hercules, CA). We previously demonstrated efficient cellular uptake and proteolytic cleavage of the L-SR15 in RAW 264.7 murine macrophages known to abundantly express CatB; our pilot experiments re-confirmed this and additionally verified that irradiation of RAW cells after incubation with L-SR15 induced the production of singlet oxygen (data not shown), the main reactive oxygen species that is generated by photodynamic therapy (PDT). Thus, L-SR15 is a long term theranostic agent designed to be cleaved by CatB in inflamed atheromata, and release Ce6 close to macrophages; and, the dequenched Ce6 fluorophores can be used for diagnostic or therapeutic purposes. In addition to L-SR15, Ce6-conjugated poly(ethylene glycol)-graft-poly(D-lysine), not cleaved by proteases and named as D-SR16 previously, was synthesized and used as a control agent.

Animal Experiments

Seven-week-old Apolipoprotein-E (ApoE) knock-out (ApoE−/−) mice were purchased (Japan-SLC, Shizuoka, Japan) and maintained in a controlled environment of 20 °C and 40–50 % humidity, with 12 h of light per 24 h period. The diet and water were available ad libitum. Food and water intake and bodyweight were monitored weekly. After one week of adaptation, mice were fed a western diet for 22 weeks. Thereafter, mice (thirty-week-old) were randomly assigned to receive intravenous injection of 150 µL L-SR15 photosensitizer (n = 5), D-SR16 (n = 5), or saline (n = 5) three times at days 0, 7, and 14.

Twenty four hours after each injection, the bilateral carotid arteries were exposed and PDT was done by illumination with a continuous wave diode laser at 670 nm (light spot diameter = 1 cm, irradiation dose rate = 72.6 mW/cm², irradiation dose = 4.4 J/cm²). Just before the PDT, the carotid arteries were imaged using a fluorescent small animal imager (IVIS Lumina II, Caliper Life Sciences, Alameda, CA; Cy5.5 channel, ex 615 – 665 nm, em 695-770 nm) for the visualization of Cy5.5 fluorescent signal that was emitted from intra-plaque L-SR15 photosensitizers (vs. D-SR16 or saline) after cleavage-activation by macrophage CatB to dequench Ce6 molecules (Figure 1).

At days 0 and 21, intravenous injection of a 2nM CatB-activatable Cy7 probe (Prosense 750, Visen Medical, Woburn, MA) in 150 µL saline was performed to additionally visualize in vivo CatB activity at days 1 and 22. Prosense 750 was used
as a short term diagnostic agent to probe for CatB activity at a different fluorescent wavelength than L-SR15 (Cy7 vs Cy5) to allow for CatB activity to be measured independent of the absence or presence of theranostic fluorophors.

In a second set of experiments, thirty-week-old ApoE−/− mice (n = 24) fed on a western diet received intravenous injection of Prosense 750 as well as 150µL L-SR15 theranostic agent (n = 8), control agent D-SR16 (n = 8), or saline (n = 8). Twenty four hours later (day 1), in vivo Cy5.5 and Cy7 NIRF imaging was performed. At either day 3 (n = 9) or 22 (n = 15), twenty four hours after an additional injection of Prosense 750, the final in vivo Cy5.5 and Cy7 NIRF imaging was performed.

In the two sets of experiments, the animals were euthanized after the final in vivo imaging, and the carotid arteries and aorta were carefully excised and washed with saline three times. After Cy7 NIRF imaging ex vivo, the tissues were snap frozen in liquid nitrogen and stored at -70°C for histology. Quantification of in vivo and ex vivo NIRF signal intensities of entire carotid arteries, aorta, or aortic subdivisions were measured as previously reported5 using the Living Image 4.0 software (Caliper Life Sciences, Alameda, CA).

In a third set of experiments, cutaneous phototoxicity was compared between L-SR15, free Ce6, and saline in C57/BL6 mice (n = 15). After intravenous injection (150µL) of the photosensitizer / control substance, ultraviolet light (UVA, 315 ~ 400nm) irradiation was applied to four 1cm²-areas on the shaved backs of each animal. The dose of UVA light was varied with 20, 40, 60, or 80 min (= 3.7, 7.4, 11.2, or 14.9 J/cm², respectively) of exposure for each square (each animal received 4 UVA doses to 4 separate areas). We photographed the irradiated squares using controlled lighting and distance at 24 hours after exposure. The photographs were analyzed in Adobe Photoshop CS-3 Extended (Adobe Systems, San Jose, CA) by measuring the erythematous lesion area with the color range function and histogram function for each of the four irradiated quadrants. We also assessed for systemic toxicity by means of the following blood assays: complete blood count with differential and liver function test. In nine additional C57/BL6 mice, neurotoxicity of L-SR15 or D-SR16 vs. saline was assessed for three weeks by using a battery of neurobehavioral tests6 with some modifications.

**Histology**

Axial sections (10μm thickness, 6mm spacing) were used for each of the following histological stains: oil-red-o, Masson-Trichrome, smooth muscle cell, macrophage, and terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) staining. Immunohistochemistry was performed using the avidin-biotin-peroxidase method as previously reported.5,7,8 Briefly, sections treated with 0.3% of hydrogen peroxide were incubated for 60 minutes with primary antibodies, followed by biotinylated secondary antibodies (Abcam, Cambridge, UK). The reaction was visualized with DAB substrate (DAKO, Glostrup, Denmark) and counterstained with Harris hematoxylin solution. Cells were identified with anti-Mac3 antibody (1:75; BD Bioscience, San Jose, CA) and anti-α-actin antibody (1:200; Abcam, Cambridge, UK) for macrophages and smooth muscle cells, respectively. TUNEL staining was performed to detect PDT-mediated apoptosis using the ApopTag kit (Chemicon, Temecula, CA) according to the manufacturer’s protocol. The extent of positively-stained (red-colored in the oil-red-o staining, blue-colored in the Masson-Trichrome
staining, and brown-colored in the other staining; n = 5 animals / group; average of 5 sections / animal / staining) areas were segmented and quantified as previously reported\textsuperscript{5, 8} with the color range function and the histogram function of Adobe Photoshop CS-3 Extended. Histological quantification was performed in a strictly blind manner to avoid potential bias. Values are reported as a percentage of the lesion area divided by the total area of the carotid tissue.

**Statistical analysis.**
Repeated measures one-way analysis of variance (ANOVA) with Dunnett’s post-hoc tests or ANOVA with Dunnett’s post-hoc tests were performed using a statistical software package (SPSS 18.0, Chicago, IL). When a nonparametric test was required, Kruskal-Wallis ANOVA and post-hoc Mann Whitney tests with Bonferroni correction were used. A probability value less than 0.05 was considered statistically significant.

**References**