Matrix-disorganizing proteases, such as cathepsins or matrix metalloproteinases from macrophages, can destabilize atheromata, followed by plaque rupture to cause thromboembolic stroke or myocardial infarction.1-4 We previously showed that molecular imaging of cathepsin-B (CatB) or matrix metalloproteinase-2/9 protease activity reflected the inflammatory component of atherosclerotic pathology in mice5 and human atheromata.6 We also showed that the protease imaging could quantitatively demonstrate plaque-stabilizing effects of antiatherosclerotic drugs5 and exercise training2 in mice. The principle of photodynamic therapy (PDT) is to kill unwanted cells by using a combination of photosensitizers and light illumination to generate highly reactive oxygen species that locally destroys cells over short diffusion distances.6-10 A recent PDT study using macrophage-targeted photosensitizers preliminarily demonstrated the viability of near-infrared light-activated therapeutic nanoagents in the treatment of atherosclerotic vascular disease by showing preferential destruction of macrophages in vitro and in mouse atheromata.11

Choi et al12 developed a novel protease-mediated photodynamic agent that allowed visualization of targets and therapeutic application of these fluorophores as photosensitizers during photodynamic therapy to attenuate plaque-destabilizing cathepsin-B activity by selectively eliminating macrophages.

Conclusions—This is the first study showing that macrophage-secreted cathepsin-B activity in atheroma could be attenuated by photodynamic therapy using a protease-mediated theranostic agent. (Arterioscler Thromb Vasc Biol. 2013;33:1360-1365.)

Key Words: atherosclerosis ■ cathepsin-B ■ molecular imaging ■ photodynamic therapy ■ photosensitizer ■ protease-mediated theranostic agent
become fluorescent and produce singlet oxygen on protease conversion followed by light therapy. In our recent study, using the RAW 264.7 macrophage cell line, we showed that intracellular uptake of L-SR15 and subsequent activation of the theranostic nanoagent by proteolytic cleavage could induce cell death on light illumination; the macrophage cell death was interfered by commonly prescribed cardiovascular drugs with antioxidant effects: atorvastatin and clopidogrel.13

There is great need for the technologies of PDT and activatable theranostic agents to be applied in a suitable animal model, allowing us to assess whether protease-activated smart probes can be used to target photosensitizers to plaque macrophages and eliminate these cells and their inflammatory enzyme secretions.

In the present study, we hypothesized that (1) intravenously injected L-SR15 could be accumulated and activated in mouse atheromata, allowing for the visualization of inflammatory targets, (2) subsequent light treatment could kill macrophages and (3) consequently decrease CatB protease activity within the vulnerable atheromata. A dual-channel in vivo near-infrared fluorescent (NIRF) imaging was performed to detect Cy5.5 signal from the activated L-SR15 theranostic nanoagents and Cy7 CatB–related signal in atheromatous carotid arteries and aortas of apolipoprotein E (apoE) knock-out (apoE−/−) mice.

Materials and Methods
Materials and Methods are available in the online-only Supplement.

Results
In Vivo Cy5 NIRF Imaging Showed Increased Fluorescent Signal in the Carotids of apoE−/− Mice, Indicating That Intravenously Injected L-SR15 Smart Photosensitizer Was Activated in the Carotid Atheromata
Serial in vivo Cy5.5 NIRF imaging was performed in the same animals 24 hours after the intravenous injection of L-SR15 3x for 2 weeks (days 1, 8, and 15) and 1 week after the last injection (Figure 1). Compared with the saline group, there was a progressive increase in fluorescent signal localized to the carotid arteries (P=0.011, repeated-measures ANOVA) in the L-SR15 group (P<0.001, Dunnett post hoc test), which was not the case in the D-SR16 (chlorin-e6 [Ce6]–conjugated poly(ethylene glycol)–graft-poly(D-lysine) not cleaved by proteases) group (P=0.304; Figure 2A). This reflects more efficient and target-specific cleavage-activation of L-SR15 by CatB and consequently higher reactive oxygen species-generating potential of L-SR15 on light illumination compared with noncleaved D-SR16 and saline controls.

In Vivo CatB Activity Was Significantly Reduced by PDT in Animals Receiving L-SR15
In vivo Cy7 NIRF imaging was performed at days 1 and 22 after the intravenous injection of L-SR15 3x for 2 weeks, each followed by light therapy. As shown in Figure 2B, compared with the saline group, there was a decrease in fluorescent signal in the carotid arteries (P<0.001, repeated-measures ANOVA) not only in the L-SR15 group but also in the D-SR16 group (P=0.001 and 0.017, respectively; Dunnett post hoc tests). Ex vivo Cy7 NIRF imaging (Figure 3) confirmed that CatB-related NIRF signal intensities were significantly lower (P=0.018, ANOVA) in the carotid arteries of L-SR15 group versus saline group (P=0.010, Dunnett post hoc test), which however was not the case in the D-SR16 group (P=0.143). There was no significant intergroup difference in the aortas for which no PDT had been applied.

L-SR15–Based PDT Induced Apoptosis of Macrophages Without Affecting Plaque Size or Smooth Muscle Cell Numbers in Carotid Atheromata of ApoE−/− Mice
As shown in the histological staining of carotid atheroma sections from representative animals that were euthanized after 3 PDTs (Figure 4), terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick end labeling-positive macrophages were more frequently observed

Figure 1. Protease-mediated photodynamic therapy (PDT) in carotid atherosclerosis. Cleavage activation of L-SR15 by cathepsin-B (CatB) releases chlorin-e6 (Ce6) molecules. The dequenched Ce6 fluorophores can be used not only for Cy5.5 near-infrared fluorescent (NIRF) imaging but also for PDT to kill unwanted cells on light illumination by generating highly reactive oxygen species. Thirty-week-old apolipoprotein E knock-out mice fed on a Western diet received intravenous injection of 150 μL L-SR15 theranostic agent, control agent D-SR16, or saline 3x at days 0, 7, and 14. Twenty-four hours after each injection, in vivo Cy5.5 NIRF imaging was performed. After each imaging session, PDT was performed using a continuous wave (CW) diode laser. At days 0 and 22, Cy7 NIRF imaging with a CatB-sensing activatable molecular imaging agent was performed. MPEG indicates methoxypolyethylene glycol.
in the L-SR15 group compared with the D-SR16 group or saline group. Unlike animals in the latter groups, in 2 of the 5 histology-examined animals in the L-SR15 group Mac-3 immunoreactivity was rarely observed (≈0.5% of the total area of the carotid tissue; Figure I in the online-only Data Supplement). Thus, the extent of CatB immunoreactivity was relatively small in the L-SR15 group. Compared with the saline group, quantification of the histological data (Figure 5) did show that Mac-3 and CatB immunoreactivity was less extensive, and that terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick end labeling positivity was more extensive in the L-SR15 group (P=0.009, 0.009, and 0.016, respectively; Kruskal Wallis ANOVA with Mann–Whitney post hoc tests), which was not the case in the D-SR16 group (all P>0.05). In the L-SR15 group, some smooth muscle cells near terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick end labeling-positive macrophages were also terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick end labeling-positive,
unlike other healthy smooth muscle cells located distantly from apoptotic macrophages (Figure II in the online-only Data Supplement). However, no significant intergroup differences were observed in terms of the smooth muscle α-actin positive area as well as the plaque size and the extent of oil-red-O–stained areas (Figure 5 and Figures III and IV in the online-only Data Supplement). Compared with the saline group, the bluish area of Masson-Trichrome–stained collagen was more extensive in the L-SR15 group ($P=0.028$, Mann–Whitney test), which was not the case in the D-SR16 group ($P=0.47$). Fibrous cap thickness did not differ significantly between groups (Figure V in the online-only Data Supplement).

### In Vivo CatB Activity Was Not Attenuated in Carotid Atheromata by One-Time PDT Using L-SR15

Twenty-four hours after L-SR15 and Prosense 750 (D1), in vivo Cy5.5 imaging did not show significantly high NIRF signal in the carotid arteries of the L-SR15 group ($P=0.028$, Mann–Whitney test), which was not the case in the D-SR16 group ($P=0.47$). Fibrous cap thickness did not differ significantly between groups (Figure V in the online-only Data Supplement).

### L-SR15 Did Not Show Systemic/Neurobehavioral Toxicity or Cutaneous Phototoxicity

At 24 hours after the intravenous injection of saline, L-SR15, or free Ce6 and ultraviolet light irradiation (14.9 J/cm$^2$), the median area of erythema attributable to phototoxicity was larger in the Ce6 group than in the L-SR15 group ($P=0.024$; Figure 6). There was no significant difference between the saline group and the L-SR15 group. At lower irradiation intensities, skin phototoxicity did not differ significantly between groups, although there was overall difference at the UVA intensity 11.2 J/cm$^2$ ($P=0.049$, Kruskal-Wallis ANOVA) with a trend of relatively wider photodamage in the Ce6 group compared with the other groups.

### L-SR15 Did Not Show Systemic/Neurobehavioral Toxicity or Cutaneous Phototoxicity

At 24 hours after the intravenous injection of saline, L-SR15, or free Ce6 and ultraviolet light irradiation (14.9 J/cm$^2$), the median area of erythema attributable to phototoxicity was larger in the Ce6 group than in the L-SR15 group ($P=0.024$; Figure 6). There was no significant difference between the saline group and the L-SR15 group. At lower irradiation intensities, skin phototoxicity did not differ significantly between groups, although there was an overall difference at ultraviolet light intensity 11.2 J/cm$^2$ ($P=0.049$) with a trend of relatively wider photodamage in the Ce6 group compared with the other groups.

### Discussion

In this study, we used a protease-mediated theranostic photodynamic agent to show the following: (1) intravenously injected L-SR15 photosensitizers were cleaved in and released photosensitizing fluorophores in the carotid atheromata of 30-week-old apoE$^{-/-}$ mice fed on a Western diet, (2) PDT in the presence of these released fluorophores served to reduce plaque CatB activity, and (3) PDT resulted in relatively selective apoptotic attenuation of macrophages within the atheromata, without significantly affecting plaque size or smooth muscle cell numbers. Free Ce6 induced photodamage in the skin with ultraviolet
exposure, consistent with its systemic photosensitizing nature, but L-SR15 did not cause either cutaneous phototoxicity nor systemic or neurobehavioral toxicity, consistent with controlled local release of photosensitizer in this agent.

Plaque-destabilizing proteases, such as CatB,\textsuperscript{3,5,15} from macrophages, are the same enzymes needed for cleavage activation of L-SR15 photosensitizers,\textsuperscript{12} allowing the selective release of photosensitizer near the CatB-secreting macrophages, their subsequent targeting by PDT to generate reactive oxygen species that are active only over short diffusion distances, and consequently selective destruction of inflammatory cells within the atheromas. This would predict, and we have observed, that smooth muscle cells and collagen fibers in the media would be largely left unscathed by PDT, thereby contributing to plaque stabilization.\textsuperscript{16} The repeated L-SR15–based PDT did not decrease the plaque size; and this kind of functional versus anatomic discrepancy in atherosclerosis pathogenesis is in line with our previous study on antiatherosclerotic effects of exercise:\textsuperscript{2} treadmill exercise could effectively reduce aortic matrix metalloproteinase activity in mice with preexisting atheromata despite the continued high-fat diet and plaque growth. It should also be considered that the relatively short treatment time (3 weeks) may have not allowed us to observe further regression of established plaques that might have occurred at a later time.\textsuperscript{17}

Instead of an intravenous injection of L-SR15 followed by one-time light illumination, repetitive L-SR15–based PDTs were required for a relatively strong activation of the smart photosensitizers and high CatB-reducing antiatherosclerotic effects. It seems to have taken some time for sufficient quantity of L-SR15 to enter the carotid atheromata and there get activated. However, once present, photosensitizing fluorophores did not diffuse out rapidly from the plaque, as evidenced by accumulation after weekly dosing. The fluorescence observed in the plaque is a complex function of the following: (1) delivery of nonactivated L-SR15 to the plaque, (2) cleavage activation by local proteases, and (3) clearance of these fluorescent products from the plaque. Although some of these steps are well understood, the net effect on pharmacokinetics and pharmacodynamics of all these steps acting in synergy is currently unknown. Clearly, our data indicate that the simple model of a small molecule diffusing away and clearing is incorrect. Hypotheses to explain the observation might include the following: (1) increased delivery to plaque over longer times than expected by circulating L-SR15, (2) ongoing protease–mediated activation over a slower time course than anticipated, and (3) retention of the activated fluorescent compounds in the plaque by either biological (phagocytosis by macrophages), chemical (an as yet unknown fixation reaction), or physical (a diffusion barrier out of lipid-filled plaques) means. Modeling of precise drug kinetics would be an important factor to study in the future, to shed light on the dosing and timing of the agent when used in clinic.

It should be noted that atherosclerosis is a progressive and cumulative disease\textsuperscript{18} for which an acute cure might be unrealistic. A repetitive long-term suppression of inflammatory cell populations might be an acceptable way to manage the disease. L-SR15–based PDT has significant translational potential in the atherosclerosis clinic, considering that prolonged administration of effective concentrations of conventional photosensitizers is usually not possible because of dose-limiting systemic phototoxicities.\textsuperscript{9,10,12,19} The combination of intravenous L-SR15 injection and focal light illumination may serve as an effective treatment to avoid unnecessary side effects with reduced phototoxicity, given the quenched state of the native compounds.\textsuperscript{12} In addition, this theranostic approach could be used to tailor treatments,\textsuperscript{20} such as preintervention or presurgical detection and stabilization of inflammatory plaques that are prone to protease-mediated rupture and thromboembolic complications, which could potentially be provoked by the procedure itself.

Our study has limitations. First, we surgically exposed the carotid arteries for imaging and PDT in a mouse atherosclerosis model. Repeated dissection of pericarotid tissues may have resulted in inflammation in the carotid arteries, although between day 0 and day 21, we observed no significant difference in the CatB-related Cy7 NIRF signal intensities in the carotid arteries of control animals, suggesting that pericarotid inflammatory change did not extend to the media. Clinically, a catheter-based system might be able to see and treat vulnerable plaques, where high matrix–disorganizing proteolytic activity is present.\textsuperscript{11} This approach might work in all vessels and might be the only approach in the deeper vasculature, for which surface access by photons is not feasible. However, a catheter-based approach is invasive, and may not allow for repeated treatments. Using longer wavelength photosensitizers (>750 nm) with improved light penetration properties may allow noninvasive external irradiation in superficial vasculatures, such as the carotid artery. If this is successful at stabilizing plaque, and preventing ischemic stroke, a major cause of death and disability worldwide could be treated with a new modality of treatment. CatB molecular imaging based on the diagnostic capability of L-SR15, combined with conventional anatomic imaging, such as ultrasonography and angiography,\textsuperscript{6} could help identify unstable target plaques with high-proteolytic enzyme activity. Second, the control compound D-SR16 also reduced CatB-related NIRF signal intensities in vivo, although the subsequent ex vivo imaging demonstrated that only L-SR15 reduced the CatB activity. As was previously reported,\textsuperscript{12} intact (ie, noncleaved) L-SR15 or D-SR16 conjugates could generate some degree of singlet oxygen molecules on light treatment: \textcircled{=} 13% of the amount of singlet oxygen generated by free Ce6. Thus, D-SR16 could also have produced some low-level phototoxic effects. Third, further studies on bystander apoptotic effects of the protease-mediated PDT are required to confirm whether Ce6 photosensitizers released from macrophage-activated L-SR15 could diffuse out to damage nearby smooth muscle cells on light therapy.Finally, we did not include such control groups as saline with light therapy or L-SR15 without light therapy, because it seemed unlikely to us that these comparisons would teach us much, and would add much to the expense of our study.

In conclusion, we show that plaque-destabilizing CatB activity could be attenuated by PDT using L-SR15, a molecularly targeted protease–mediated photodynamic theranostic agent. This study provides proof-of-principle that molecular targeting of plaque macrophages is possible with a see-and-treat approach.
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Disclosures
None.

References

Significance
This is the first study showing that macrophage-secreted cathepsin-B activity in atheroma could be attenuated by photodynamic therapy using a protease-mediated theranostic agent. We synthesized L-SR15 and demonstrated that the intravenously injected cathepsin-B activatable theranostic agent was cleaved in and released a fluorescent agent (chlorin-e6) in mouse atheroma, allowing both the diagnostic visualization and therapeutic application of these fluorophores as photosensitizers during photodynamic therapy to attenuate plaque-destabilizing cathepsin-B activity by selectively eliminating macrophages. Free chlorin-e6 induced photodamage in the skin with ultraviolet exposure, consistent with its systemic photosensitizing nature, but L-SR15 did not cause either cutaneous phototoxity nor systemic or neurobehavioral toxicity, consistent with controlled local release of photosensitizer in this agent. This study provides proof-of-principle that molecular targeting of plaque macrophages is possible with a see-and-treat approach.
Photodynamic Therapy Using a Protease-Mediated Theranostic Agent Reduces Cathepsin-B Activity in Mouse Atheromata In Vivo
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Supplemental Material

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

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Supplemental Figure I. Absent / scarce Mac-3 (macrophage) and CatB (cathepsin B) immunoreactivity in the carotid atheroma of an animal at day 22 after protease-mediated photodynamic therapy (3 treatments at days 0, 7, and 14) using the L-SR15 theranostic photodynamic agent. Scale bar = 100 μm.
Supplemental Figure II. Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL)-positive smooth muscle cells (red arrows) near TUNEL-positive (Mac-3 positive) macrophages (red *). Please see healthy smooth muscle cells (green arrows) distant from apoptotic macrophages (green *). Black scale bar = 100 μm. Blue scale bar = 50 μm. CatB denotes cathepsin B.
**Supplemental Figure III.** No significant differences are noted in oil-red-o (red) or smooth muscle α-actin immuno-positive lesions (brown) areas, in carotid atheromata by photodynamic therapy using protease-mediated L-SR15 theranostic agent vs. control agent D-SR16 or saline. Scale bar = 100 μm
Supplemental Figure IV. Attenuation of cathepsin-B (CatB) immunoreactivity in macrophages, but not smooth muscle α-actin immuno-positive lesions (brown-colored areas), in carotid atheromata after photodynamic therapy using protease-mediated L-SR15 theranostic agent vs. control agent D-SR16 or saline. Please see that terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL)-immunoreactivity is relatively high in the L-SR15 group compared to the other groups. Scale bar = 100 μm.
Supplemental Figure V. Quantification for fibrous cap thickness and collagen extent on Masson-Trichrome staining. No significant inter-group differences are observed.
Supplemental Figure VI. No attenuation of in vivo cathepsin-B activity in carotid atheromata by one-time photodynamic therapy with the protease-mediated theranostic agent L-SR15. Thirty-week-old Apolipoprotein-E knock-out mice fed on a western diet received intravenous injection of CatB-sensing activatable near-infrared fluorescent (NIRF) molecular imaging agent (Cy7) as well as 150μL L-SR15 theranostic agent, control agent D-SR16, or saline at day 0. At day 1, inter-group differences were not observed in the Cy5.5 NIRF imaging for cleavage activation of theranostic agents and Cy7 NIRF imaging for plaque CatB activities. After the imaging, PDT was performed using a CW diode laser. At day 3 (left column) or 22 (right column), 24 hours after an additional injection of Prosense 750, Cy5.5 and Cy7 NIRF imaging showed no inter-group differences, either. Ex vivo NIRF imaging confirmed this.
### Supplemental Table I. Hematologic Parameters in Saline-, L-SR16-, or Chlorin-e6 (Ce6)-treated C57BL/6 Mice

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Normal Range</th>
<th>Complete Blood Counts and Liver Function Test</th>
<th>Saline (n = 5)</th>
<th>L-SR16 (n = 5)</th>
<th>Ce-6 (n = 5)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>WBC (x10^3 cells/μL)</td>
<td>1.8 ~ 10.7</td>
<td></td>
<td>4.9 ± 0.9</td>
<td>4.7 ± 0.3</td>
<td>3.2 ± 0.9</td>
<td>0.019</td>
</tr>
<tr>
<td>RBC (x10^6 cells/μL)</td>
<td>6.4 ~ 9.4</td>
<td></td>
<td>6.6 ± 0.4</td>
<td>6.7 ± 1.0</td>
<td>10.1 ± 0.8</td>
<td>0.310</td>
</tr>
<tr>
<td>Hemoglobin (g/dL)</td>
<td>11.0 ~ 15.1</td>
<td></td>
<td>10.8 ± 0.4</td>
<td>11 ± 1.0</td>
<td>10.2 ± 0.8</td>
<td>0.273</td>
</tr>
<tr>
<td>Hematocrit(%)</td>
<td>35.1 ~ 45.4</td>
<td></td>
<td>30.1 ± 1.9</td>
<td>32.0 ± 4.9</td>
<td>28.1 ± 3.8</td>
<td>0.690</td>
</tr>
<tr>
<td>MCV (fL)</td>
<td>45.4 ~ 60.3</td>
<td></td>
<td>46.3 ± 0.2</td>
<td>48.0 ± 0.7</td>
<td>47.8 ± 1.1</td>
<td>0.008</td>
</tr>
<tr>
<td>MCH (pg)</td>
<td>14.1 ~ 19.3</td>
<td></td>
<td>17.1 ± 1.1</td>
<td>16.2 ± 0.9</td>
<td>17.5 ± 1.6</td>
<td>0.246</td>
</tr>
<tr>
<td>MCHC (g/dL)</td>
<td>30.2 ~ 34.2</td>
<td></td>
<td>36.9 ± 2.5</td>
<td>28.0 ± 14.0</td>
<td>36.3 ± 2.6</td>
<td>0.137</td>
</tr>
<tr>
<td>RDW (%)</td>
<td>12.4 ~ 27.0</td>
<td></td>
<td>13.2 ± 0.3</td>
<td>13.5 ± 0.5</td>
<td>13.8 ± 0.5</td>
<td>0.274</td>
</tr>
<tr>
<td>HDW (g/dL)</td>
<td>2.2 ~ 2.4</td>
<td></td>
<td>2.3 ± 0.1</td>
<td>2.1 ± 0.06</td>
<td>2.2 ± 0.05</td>
<td>0.032</td>
</tr>
<tr>
<td>MPV (fL)</td>
<td>5.0 ~ 20.0</td>
<td></td>
<td>27.6 ± 1.8</td>
<td>28.1 ± 2.3</td>
<td>30.3 ± 2.0</td>
<td>0.077</td>
</tr>
<tr>
<td>Neutrophil (%)</td>
<td>6.6 ~ 38.9</td>
<td></td>
<td>13.7 ± 1.3</td>
<td>8.3 ± 1.5</td>
<td>29.2 ± 8.7</td>
<td>0.002</td>
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<tr>
<td>Lymphocyte (%)</td>
<td>55.8 ~ 91.6</td>
<td></td>
<td>61.8 ± 6.0</td>
<td>78.2 ± 5.1</td>
<td>56.7 ± 11.4</td>
<td>0.009</td>
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<tr>
<td>Monocyte (%)</td>
<td>≤ 7.5</td>
<td></td>
<td>18.8 ± 4.0</td>
<td>11.7 ± 6.5</td>
<td>13.9 ± 6.2</td>
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<tr>
<td>Eosinophil (%)</td>
<td>≤ 3.9</td>
<td></td>
<td>1.6 ± 0.6</td>
<td>1.1 ± 0.5</td>
<td>0.54 ± 0.4</td>
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<tr>
<td>LUC (%)</td>
<td>0.6 ~ 1.3</td>
<td></td>
<td>1.1 ± 0.5</td>
<td>0.32 ± 0.1</td>
<td>1.0 ± 0.9</td>
<td>0.033</td>
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<tr>
<td>Basophil (%)</td>
<td>≤ 0.2</td>
<td></td>
<td>1.44 ± 0.9</td>
<td>0.42 ± 0.4</td>
<td>1.36 ± 1.8</td>
<td>0.132</td>
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<tr>
<td>Reticulocyte (%)</td>
<td>5.6 ~ 9.9</td>
<td></td>
<td>3.8 ± 0.4</td>
<td>4.0 ± 0.5</td>
<td>3.85 ± 0.3</td>
<td>0.595</td>
</tr>
<tr>
<td>Platelet (x10^3 cells/μL)</td>
<td>592 ~ 2972</td>
<td></td>
<td>1563 ± 410</td>
<td>1224 ± 209</td>
<td>1342 ± 585</td>
<td>0.512</td>
</tr>
<tr>
<td>AST (IU/L)</td>
<td>70 ~ 120</td>
<td></td>
<td>75.0 ± 17.1</td>
<td>78.4 ± 8.7</td>
<td>112.3 ± 26.2</td>
<td>0.030</td>
</tr>
<tr>
<td>ALT (IU/L)</td>
<td>≤ 45</td>
<td></td>
<td>25.5 ± 7.1</td>
<td>25.2 ± 5.5</td>
<td>39.1 ± 13.2</td>
<td>0.057</td>
</tr>
<tr>
<td>ALP (IU/L)</td>
<td>30 ~ 120</td>
<td></td>
<td>21.7 ± 10.1</td>
<td>43.1 ± 10.9</td>
<td>35.1 ± 16.7</td>
<td>0.032</td>
</tr>
<tr>
<td>Total protein (mg/dL)</td>
<td>5.4 ~ 5.8</td>
<td></td>
<td>4.9 ± 0.3</td>
<td>4.6 ± 0.2</td>
<td>4.6 ± 0.3</td>
<td>0.162</td>
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<tr>
<td>Total bilirubin (mg/dL)</td>
<td>0.1 ~ 1.2</td>
<td></td>
<td>0.5 ± 0.2</td>
<td>0.3 ± 0.2</td>
<td>0.2 ± 0.1</td>
<td>0.075</td>
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</tbody>
</table>

Values are mean ± standard error or as frequency (percentage).

*Student’s T-test, Chi-Square test, or Fisher’s exact test

WBC: white blood cell
RBC: red blood cell
MCV: mean corpuscular volume
MCH: mean corpuscular hemoglobin
MCHC: mean corpuscular hemoglobin concentration
CHCM: cellular hemoglobin concentration mean
RDW: red cell distribution width
HDW: hemoglobin distribution width
MPV: mean platelet volume
LUC: large unstained cell
AST: aspartate aminotransferase
ALT: alanine aminotransferase
ALP: alkaline phosphatase
Supplemental Table II. Neurobehavioral Side Effects of L-SR15 (n = 3) and D-SR16 (n = 3) vs. Saline (n = 3)

<table>
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<th>Day 14</th>
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<td>Control</td>
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</tbody>
</table>

Values are numbers of mice exhibiting abnormal neurobehavioral signs.
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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*S-M.S. and Y.C. contributed equally to this work.

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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 150µL 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 150µL 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 reported5,7,8 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**

Protease-Mediated Theranostic Agent를 이용해서 Photodynamic Therapy를 하게되면 Cathepsin B 활성과 죽상경화반의 대식세포를 줄일 수 있다.

한 기훈 교수
서울아산병원 심장내과

Summary

배경
정주로 주입되는 cathepsin-B에 의하여 활성화되는 theranostic agent (L-SR15)가 쥐의 동맥경화반에서 재단되어 형광물질인 chlorin-e6을 방출하고 동맥경화반을 진단할 수 있도록 보이게 할 수 있으며, photodynamic therapy를 시행하였을 때에 이들 fluorophores들이 photosensitizer로서 대식세포를 제거함으로써 폴라크을 안정화시킬 수 있는 치료적인 가치가 있는지를 검증하고자 하였다.

방법 및 결과
30주령의 E knock-out mice (n=15)들이 L-SR15 theranostic agent, control agent D-SR16, 또는 식염수를 총 3회(D0, D7, D14) 정주를 받았다. 각각의 정주 24시간 후에 양 경동맥을 노출시켜 Cy5.5 near-infrared fluorescent imaging을 시행하였다. L-SR15 군에서 죽상경화반에서 침착하는 형광신호들이 포착되어 theranostic agent로부터 유리된 photosensitizer들이 폴라크에 쌓여가고 있음을 보였다. 각각의 imaging 이후에는 photodynamic therapy가 continuous-wave diode-laser의 형식으로 시행되었다. 추가적인 더 긴 파장에의(Cy7) near-infrared fluorescent imaging을 cathepsin-B-sensing activatable molecular imaging agent와 함께 시행하였을 때에 cathepsin-B 신호가 L-SR15 군에서 감소함을 보였다. 조직학적 소견에서 L-SR15–based photodynamic therapy가 대식세포의 고사를 유도하여 그 수를 감소시켰으며 이는 폴라크의 크기 또는 기타 평활근세포의 고사와 동반되지 않았다. 독성실험에서는 (n=24) 심한 피부의 반점(erythemat) 등이 free chlorin-e6와 자외선조사에서는 정주 24시간 이후 관찰되었지만 L-SR15이나 식염수 정주에서는 그 이상의 독성은 없었으며 신경행동이나 기타 전신적인 부작용 독성은 없었다.

결론
대식세포에서 분비되는 cathepsin-B 활성이 protease-mediated theranostic agent를 이용한 photodynamic therapy에 의해 죽상경화반에서 감소될 수 있음을 보여주는 최초의 연구이다.
Commentary

Cathepsin이란?
B형: 쥐의 대식세포에 발현하며 이를 저하시키는 LDL의 분해를 40% 가량 저하시킴.
F형: 인간의 대식세포, 폐활근세포, 내피세포에서 발현하며 apoB를 60% 분해시키며, apoA-1 및 preHDL을 와해함으로써 cholesterol efflux를 50% 감소시킴.
K형: F형과 유사하나 기능이 다소 약함.
S형: F형과 분포 및 기능이 유사함.
C형, S형: 죽상경화와의 연관성은 쥐에서 기술되어 있으나 기전적인 연구는 아직 많지 않음.

본 논문의 접근은?
L-SR15 CatB activatable photosensitizer를 사용하였다. 이는 체내에 전달된 후 photodynamic therapy (PDT)를 시행하면 (대식세포의) cathepsin B에 의하여 분절되고 이때 ROS가 분비되면서 한편으로는 형광성 물질이 노출되기 때문에 ROS에 의한 대식세포의 고사를 유도하며, 대식세포가 많은 부분의 죽상경화반을 형광법으로 포착할 수 있게 만드는 원리를 가지고 있다. 배양세포 수준에서의 대식세포의 고사능력은 검증되었으며, 이러한 효과들을 검증하기 위하여 총 3주의 기간 동안 간헐적인 L-SR15 CatB의 주사와 함께 PDT를 시행하였다.

본 논문의 주요 요점들은?
1. 고지방식이를 시행한 apoE knock-out 쥐에서 L-SR15이 혈관으로 많이 축적되며 이러한 현상은 PDT에 의하여 극적으로 저해된다. 이러한 경향은 3주 후 적출된 동맥조직에서 보았을 때 주로 경동맥에 완연히 나타나고 있다.
2. 이는 아마도 대식세포의 세포고사율을 유도함으로써 나타나는 효과라고 판단한다.
3. 본 논문의 L-SR15 및 치료방법은 PDT에 의하여 야기될 수 있는 피부병변의 위험이 최소화되는 결과를 볼 수 있었다.
 이러한 결과와 함께 discussion에 기술된 부분을 종합하여 볼 때 고려할 수 있는 부분들은 다음과 같다.
첫째, B형 cathepsin은 인체에서 발현이 적으며 활성도가 상대적으로 낮으므로 논문에서 밝혀진 소위 theranostic agent로서 바로 쓰이기에는 제한점이 있을 것으로 생각된다. 이러한 결과를 토대로 다른 아형의 cathepsin을 이용한 접근이 추후 기대된다.
둘째, 저자가 밝혔듯이 대식세포의 부하가 감소하는 소견을 조직학적 관찰에서 보였지만 죽상경화반의 크기는 변화하지 않았다. 특히 동물의 죽상경화모델에서 주로 분포하는 소위 M1형
대식세포의 감소는 죽상경화반의 감소를 기대할 수 있다. 반면 APC, 또는 M2형 대식세포는 그 반대역할을 할 수 있으므로, 실험적으로 L-SR15가 M1형 대식세포의 고사를 선별적으로 유도할 수 있을 것인지를 연구하면 좋을 것이다. 가령 cathepsin B가 M1형 대식세포에 더욱 많이 발현한다면 본 논문의 효과는 더욱 선택적일 것이다.

저자 등은 실험기간이 짧았기 때문에 죽상경화반의 크기는 변화하지 않았으며 충분한 시간 이후에는 죽상경화반의 퇴축이 기대된다고 기술하였다. 그러나 쥐를 이용한 대부분의 죽상경화반 연구는 3주의 연구기간으로 그 변화여부를 알기 자체가 불가능함을 나타내고 있으므로 본 치료로 죽상경화반의 퇴축을 기대하는 것은 추후 증명되어야 한다고 판단된다.

셋째, cathepsin B는 세포내의 lysosome에 존재하므로 본 제재는 대식세포를 안전하게 고사시킬 수 있을 것이다. 그러나 cathepsin은 세포손상을 유도하는 과정에서 세포외 기질로 유입되는 실험적 증거들이 보고된 바 있다. 이럴 때 본 기질에 의하여 발생되는 ROS에 의하여 주위 세포가 영향을 받을 소지가 존재한다. ROS는 일반적으로 죽상경화를 촉진시키는 물질로 인식되고 있다. 저자 등은 catenin B가 대식세포 근방에 분비되며 본 연구의 L-SR15로 인한 ROS의 발현은 그 근방에 한정된다고 기술하지만 이는 추론에 가깝다. 또한 다른 catenin들을 타겟으로 바꾸었을 때는 이들은 대식세포 외 죽상경화의 모든 세포에 발현하기 때문에 그 효용성 및 안정성에서의 평가는 더욱 복잡해진다.

넷째, 저자 등이 대조물질로 사용하였던 소위 D-SR15에 의한 결과는 상대적인 일관성이 다소 낮은 편으로 판단된다. 주사 후 이의 동맥 착착이 적었던 반면 경동맥내의 효과는 LSR15과 유사한 효과를 보인다. 본 물질의 조직/세포 특이성 및 효용에 대한 추가 정리는 필요할 것으로 보인다. Delivery 방법 및 이후의 PDT 방법을 최적화하였을 때의 피부병변 안정성은 높은 정도로 기대된다. 아마도 최적의 시나리오는 (cathepsin을 상대적으로 많이 발현하고 있는 M1형) 대식세포에게 L-SR15의 충분한 전달이 이루어지고 이로 인한 선택적인 세포고사가 일어나고, 이때 발생하게 되는 ROS는 대식세포의 감소에 의하여 자연적으로 소멸하는 것이 아닐까 한다. 이러한 경우 소량대로 포화와 치료가 동시에 가능한 대식세포를 타겟으로 하는 치료의 길이 열리게 될 것이다.

REFERENCE