Expression of LPL in Endothelial-Intact Artery Results in Lipid Deposition and Vascular Cell Adhesion Molecule-1 Upregulation in Both LPL and ApoE-Deficient Mice

Jinyu Wang, Xunde Xian, Wei Huang, Li Chen, Liling Wu, Yi Zhu, Jianglin Fan, Colin Ross, Michael R. Hayden, George Liu

Objective—Overexpression of lipoprotein lipase (LPL) in deendothelialized artery led to profound localized lipid deposition. In this study the role of LPL in atherogenesis in endothelial-intact carotid arteries was assessed in genetically hyperlipidemic LPL- and ApoE-deficient mice.

Methods and Results—Human wild-type LPL (hLPLwt), catalytically inactive LPL (hLPL194), or control alkaline phosphatase (hAP) were expressed in endothelial-intact carotid arteries via adenoviral vectors. Compared with Ad-hAP, lipid deposition in the arterial wall increased 10.0- and 5.1-fold for Ad-hLPLwt and Ad-hLPL194 in LPL-deficient mice, and 10.6- and 6.2-fold in ApoE-deficient mice, respectively. Vascular cell adhesion molecule-1 (VCAM-1) was upregulated in Ad-hLPLwt and Ad-hLPL194 transferred arteries.

Conclusions—Endothelial cell associated LPL, either active or inactive, in the arterial wall is a strong proatherosclerotic factor in both LPL- and ApoE-deficient mice. (Arterioscler Thromb Vasc Biol. 2007;27:197-203.)

Key Words: lipoprotein lipase ■ atherosclerosis ■ vascular cell adhesion molecule-1 ■ mice ■ gene transfer
tion of VCAM-1 causes adhesion of monocytes to endothelium. We therefore evaluated the expression of VCAM-1 in carotid artery walls in both mouse models and studied the adhesion of monocytes to cultured HUVECs expressing either active or inactive LPL in the presence of a high level of CM to mimic the in vivo state of LPL-deficient mice.

Methods

Generation of Recombinant Adenoviral Vectors

Recombinant replication-deficient adenoviruses expressing hLPLwt, hLPL194, and hAP were generated by homologous recombination in Escherichia coli strain BJS183 using the AdEasy vector system (Qbiogene) as previously described.19 The cDNAs encoding these genes were cloned and inserted upstream of the SV40 polyadenylation signal and downstream of the CMV promoter. Clonally derived recombinant viruses were isolated, propagated, purified, and titered as described in the application manual (Qbiogene). The Ad-EGFP was a kind gift from Dr Ruiping Xiao (The Institute of Molecular Medicine, Peking University, Beijing, China).

Animal Procedures

Homozygous LPL-deficient mice were developed in our laboratory.14 ApoE-deficient mice were obtained from the Jackson Laboratory (Bar Harbor, Me.). Male and female mice of 14 to 16 weeks were fed normal chow and water ad libitum. The Principles of Laboratory Animal Care (NIH publication no. 85-23, revised 1996) was followed, and the experimental protocol was approved by the Animal Care Committee, Peking University Health Science Center. Anesthesia was induced by intraperitoneal injection of 1% sodium pentobarbital. A total of 15 LPL-deficient mice and 33 ApoE-deficient mice were used in this study.

After a median incision on the anterior neck, the carotid arteries of the right side were isolated. The external carotid artery was ligated distally, and the proximal end of both the common carotid artery and the internal carotid artery were clamped. A distal incision was made in the external carotid artery. After the blood was removed, a 0.13-mm catheter was advanced gently into the right common carotid artery via the notch of the external carotid artery. After 10 μL of the adenoviral vector-containing solution (1×1010 plaque-forming units/mL) were instilled into the vessel lumen, the arterial segment was isolated with temporary ligature of the external carotid artery for 20 minutes. The vector-containing solution was then withdrawn and the vessel lumen was rinsed again with PBS. The catheter was withdrawn and the proximal end of the external carotid artery was ligated, and the blood flow was re-established after removing the clamps on the arteries. The skin incision was closed and the mouse was allowed to recover. Seven days later, the mice were anesthetized and the manipulated segments of the carotid arteries were excised. Sites of arterial clamping and cannulation were not harvested.

Detection of HLPL Expression by RT-Polymerase Chain Reaction

Recombinant hLPL expression was analyzed by RT-PCR. Five infected arteries of each group were pooled and total RNA was extracted using Tri reagent (Molecular Research Center). The first-strand cDNA was generated by using SuperScript II reverse transcriptase (Invitrogen). cDNA was amplified with hLPL-specific primers (sense, 5'-GAG-ATT-TCT-CTG-TAT-GGC-ACC-3'; antisense, 5'-CTG-CAA-ATG-AGA-CAC-TTC-CTC-3') and the following parameters: 94°C for 45 seconds, 56°C for 45 seconds, and 72°C for 1 minute for 28 cycles. This experiment was only performed in ApoE-deficient mice because of the limited number of LPL-deficient mice available.

Detection of Exogenous Gene Expression and Factor VIII by Immuno/Histological Staining

Segments of infected carotid arteries were embedded in OCT and snap-frozen in liquid N2. Six-micron-thick sections of carotid arteries were incubated with a monoclonal mouse anti human LPL antibody at 1:150 dilution20 for 60 minutes after treatment with blocking reagents (Histomouse Kit, Zymed Co). An SABC kit and DAB substrate were then used to yield a brown reaction product. Staining of AP was performed as previously described.10 To check the endothelial integrity, sections of infected carotid arteries were also incubated with a rabbit anti hVIII related Ag (Santa Cruz) at 1:250 dilution for 60 minutes, then an SABC kit and NBT/BCIP kit (Roche) were used to yield a blue reaction product. Slides were counterstained with eosin, mounted with glycerol gelatin (Sigma) and examined with a light microscope.

Morphometric Analysis of Lipid Deposition

Carotid arteries infected with Ad-hLPLwt, Ad-hLPL194, or Ad-hAP were analyzed for lipid deposition by Oil red O (ORO) staining (n=5 for LPL-deficient mice and n=6 for ApoE-deficient mice). Lipid deposition areas were quantified from 15 sections of LPL-deficient mice and 18 sections of ApoE-deficient mice at intervals of 100 μ m in each of the embedded arteries with a Leica Q550cw graphic analysis system.

Detection of VCAM-1 by Immunofluorescence Staining

Detection of endothelial VCAM-1 expression was carried out by fixation of arterial segments with 4% paraformaldehyde, then permeabilization with 1% Triton X-100 and incubation with primary rabbit anti-mouse VCAM-1 antibody (Santa Cruz). The samples were stained with fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG antibody then with Hoeschest 33258 at 10 μg/mL for 10 minutes. The carotid arteries were opened longitudinally using microsurgical technique, mounted with PBS-glycerol (1:1, vol/vol) and observed with a confocal microscope (Leica).

Cell Culture, CM Preparation, and Monocyte Adhesion Assay

HUVECs were isolated by collagenase digestion and cultured on plates coated with 50 mg/mL collagen. Cells were maintained in M199 medium supplemented with 20 mmol/L HEPES, pH 7.4, 20% FBS, 5 mg/mL recombinant human fibroblast growth factor, antibiotics/antimycotics, and 90 mg/mL heparin (EC medium).21 THP-1, a human monocyte cell line (American Type Culture Collection) was grown in RPMI 1640 containing 10% FBS. CM was isolated from plasma of LPL-deficient mice by ultracentrifugation.

HUVECs were plated at 1.2×105 cells per well on a 12-well plate and cultured to confluence. Triplicate wells were infected with Ad-hLPLwt, Ad-hLPL194, and Ad-hAP, respectively at a multiplicity of infection of 50 in a volume of 400 μL per well. HUVECs were incubated with 300 mg/dL CM for 3 hours, and the medium was collected for measurement of CM hydrolysis. THP-1 cells were infected with Ad-EGFP for 48 hours and then coincubated with the HUVECs for 30 minutes. The nonadhering cells were washed off, and the adherent THP-1 cells were counted under a fluorescence microscope.21

Plasma Lipids Assay and Measurement of Glycerol Release

Blood was obtained after 12 to 14 hours of fasting by puncture of the retro-orbital plexus at the beginning and the end of the experiment. Total cholesterol (TC) and TG in plasma were determined enzymatically using commercial kits (Sigma). HDL-C was measured with the same kit after ApoB-lipoprotein had been precipitated with 20% polyethylene glycol (PEG) solution. Glycerol released into the HUVECs culture medium served as an index of lipolysis of TG-rich CM. The level of glycerol was measured by use of a colorimetric assay (GPO Trinder reaction) from absorption at 490 nm.22 Lipolysis data were expressed as mmol/L glycerol released.
Detection of hLPL messenger in vivo by RT-PCR. Isolated total RNA from carotid artery was reverse transcribed and amplified using specific primers for human LPL and mouse \(\beta\)-actin (internal control). Lane 1 is the positive control (hLPL cDNA in pcDNA3 plasmid); lane 2, a sample from Ad-hAP arteries; lanes 3 and 4, samples from Ad-hLPLwt and Ad-hLPL194, respectively; lane 5, 100-bp DNA marker.

Statistical Analysis

Quantitative data were expressed as mean±SEM. Statistical analysis was performed with one-way ANOVA (Newman-Keuls test) by the PRISM computer program (GraphPad). Differences were considered significant at \(P<0.05\).

Results

Expression of Genes Transferred to Endothelium-Intact Carotid Arteries and Cultured HUVECs

At day 7 after gene transfer, human LPL mRNA was detected in common carotid arteries by RT-PCR. A single 396-bp band specific for human LPL could be easily seen in the infected arteries of both Ad-hLPLwt and Ad-hLPL194 mice (Figure 1, lanes 3 and 4), which was the same size as the positive control using plasmid pcDNA3 containing human LPL cDNA as a template (Figure 1, lane 1). This band was not present in the Ad-hAP control (Figure 1, lane 2). A 500-bp band corresponding to mouse \(\beta\)-actin was present in all of the 3 groups as an internal control.

The expression of delivered exogenous genes was also verified by immuno/histological staining. For the control group, AP activity in Ad-hAP infected arteries was demonstrated by positive blue staining with AP substrate, NBT/BCIP (supplemental Figure I, available online at http://atvb.ahajournals.org). No AP activity could be detected in Ad-hLPLwt and Ad-hLPL194 infected vessels (supplemental Figure I). In both Ad-hLPLwt and Ad-hLPL194 infected arteries, human LPL protein were detected as distinct brown precipitates with an anti-human LPL mAb (Figure 2). There was no reaction with this antibody in the Ad-hAP control arteries (Figure 2). Human LPL protein was also detected in Ad-hLPLwt and Ad-hLPL194 infected HUVECs but not in Ad-hAP infected cells (supplemental Figure II).

For estimation of LPL activity in CM-containing medium from cultured HUVECs, we chose measurement of glycerol concentration instead of a radioisotope method, thus avoiding potential dilution of \(^{3}H\)-triolein emulsion substrate by high concentrations of CM in culture medium. As expected, glycerol release was significantly increased over time in the culture medium from HUVECs infected with Ad-hLPLwt, as compared with those infected with Ad-hLPL194 and Ad-hAP (\(P<0.01\); supplemental Figure III).

Increased Lipid Deposition in Carotid Arteries Expressing Either Active or Inactive LPL

Lipid droplets on the arterial wall are known to be a marker of early atherosclerosis. In both LPL- and ApoE-deficient mice, robust lipid deposition was visible through ORO staining of the carotid arteries infected with Ad-hLPLwt and with Ad-hLPL194 seven days after gene transfer, whereas such staining was barely seen in the arteries infected with Ad-hAP (Figure 3A). Morphometric quantitation of the area of lipid deposition was performed after ORO staining without hematoxylin for background reduction (data not shown). The results demonstrated that lipid-stained areas were significantly larger in both Ad-hLPLwt- and Ad-hLPL194–infected arteries than those in Ad-hAP arteries: 12 244±2103 \(\mu\)m\(^2\) and 6215±1294 \(\mu\)m\(^2\) versus 1222±898 \(\mu\)m\(^2\), \(P<0.01\) and \(P<0.05\), respectively, in LPL-deficient mice; and 11 103±1000 \(\mu\)m\(^2\) and 6510±1049 \(\mu\)m\(^2\) versus 1051±430 \(\mu\)m\(^2\), \(P<0.01\) in ApoE-deficient mice (Figure 3B). The difference between Ad-hLPLwt and Ad-hLPL194 was also significant (\(P<0.05\)) in LPL and ApoE-deficient mice, indicating that active LPL has a stronger effect than inactive LPL in mediating lipid deposition. Lipid deposition in similar pattern was also observed in C57BL/6 mice but it was not as obvious as in LPL- and ApoE-deficient mice (supplemental Figure IV).

Upregulation of VCAM-1 in Carotid Arteries Expressing Either Active or Inactive LPL

Expression of adhesion molecules is a hallmark of activation of endothelial cells and early atherosclerosis. Immunostaining of endface preparations of carotid arteries with a rabbit anti-mouse VCAM-1 antibody was used to detect VCAM-1.
expression. The distinct green precipitates of the VCAM-1 protein were observed in Ad-hLPLwt– and Ad-hLPL194–infected arteries, whereas there were only scattered precipitates in Ad-hAP–infected carotid arteries (Figure 4). These results demonstrated that local expression of LPL in carotid arteries of hyperlipidemic mice could induce the expression of adhesion molecules and promote the formation of early atherosclerosis. In wild-type C57BL/6 mice, VCAM-1 was also upregulated in carotid arteries infected with either Ad-hLPLwt or Ad-hLPL194, respectively (supplemental Figure V).

Enhancement of Monocyte Adhesion to HUVECs by LPL Expression in the Presence of CM
To examine whether upregulation of VCAM-1 in carotid arteries infected with Ad-LPLwt or Ad-hLPL194 could attract monocytes directly, in vitro cultured HUVECs were used in the presence of CM to mimic the environment in vivo. As shown in Figure 5, LPL expression resulted in an approximate 5- and 4-fold increase in monocyte adhesion to HUVECs infected with either Ad-hLPLwt or Ad-hLPL194, respectively, as compared with the Ad-hAP infected cells. However, there was no statistically significant difference in monocyte adhesion between Ad-hLPLwt and Ad-hLPL194.

Integrity of Endothelium, Unchanged Plasma Lipids, and Post-Heparin Plasma LPL Activity
To assess whether endothelium remained intact after gene transfer, we used immunostaining for endothelial cell-specific factor VIII. A thin and continuous layer of distinct blue color at the inner surface of carotid artery was observed, indicating...
the presence of endothelial cells in place, in the representative infected arteries from each group (supplemental Figure VI).

The Table showed that the plasma levels of TG, TC and HDL-C are not statistically different among the 3 groups of Ad-hAP, Ad-hLPLwt, and Ad-hLPL194 in LPL- and ApoE-deficient mice. However, compared with the respective Ad-hAP control, a 20% to 30% reduction in plasma TG and TC levels, respectively, in Ad-hLPLwt group of LPL-deficient mice and TG levels in Ad-hLPLwt group of ApoE-deficient mice can be noted, which may be attributable to relative small numbers of animals in each group. Nevertheless, the quantified areas of lipid deposition in carotid arteries were the highest in Ad-hLPLwt groups in these 2 types of mice. Thus the effects observed regarding lipid deposition and VCAM-1 expression in the infected arteries were most likely due to local expression of LPL in the arterial walls.

Discussion

A previous study from our laboratory demonstrated that LPL expression in balloon injured deendothelized arteries of normolipidemic rabbits led to enhanced lipid deposition. However, LPL exerts its enzymatic role mainly on the vascular endothelial cell surface, and balloon injury of endothelium could generate strong unwanted reactions in the arterial wall which may compromise end results, ie, lipid deposition. Thus, localized delivery of active and inactive LPL genes was tested in endothelial-intact carotid arteries. To minimize interference from local endogenous LPL gene which might be upregulated after procedural manipulation, we chose rescued LPL-deficient hypertriglyceridemic mice as model. At the same time, atherosclerosis-prone ApoE-deficient mice and wild-type C57BL/6 mice were used for parallel comparison. As a result, we found that the presence of either active or inactive LPL in these mice with 3 different genotypes promoted rapid lipid deposit in endothelial-intact carotid arteries.

It is generally accepted that expression of adhesion molecules in endothelial cells of the arterial wall is one of the early inflammatory responses central to the development of atherosclerosis. Among various adhesion molecules, VCAM-1 plays a dominant role in the initiation of atherosclerosis. Nakashima et al found that elevated levels of TG-rich lipoproteins provoked VCAM-1 upregulation along with lipid deposition in arterial walls. In the present study, we also observed VCAM-1 upregulation at comparable levels in endothelial-intact carotid arteries expressing either active or inactive LPL in the mice with different genotypes. Similarly, in vitro adhesion of THP-1 monocytes to cultured HUVECs also showed that cells infected with Ad-hLPLwt or Ad-hLPL194 attracted much more THP-1 than Ad-hAP–infected control cells but there was no difference between the two forms of LPL.

**Figure 5.** Enhanced monocyte adhesion to EC expressing either active or inactive LPL (200×). Monocyte adhesion to EC was detected in the presence of CM. The EC-bound THP-1 cells infected with Ad-EFGP were viewed (A) and counted from 18 fields per treatment for quantitative comparison (B). **P<0.01 for Ad-hLPLwt or Ad-hLPL194 treated cells vs Ad-hAP cells.**

<table>
<thead>
<tr>
<th>Plasma Lipids and Post-Heparin Plasma LPL Activity</th>
<th>TG, mg/dL</th>
<th>TC, mg/dL</th>
<th>HDL-C, mg/dL</th>
<th>LPL, mU</th>
</tr>
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<tr>
<td>LPL−/− Ad-hAP (n=5)</td>
<td>2353±1536</td>
<td>185±116</td>
<td>5.6±1.2</td>
<td>37.03±23.22</td>
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<tr>
<td>Ad-hLPLwt (n=5)</td>
<td>1823±929</td>
<td>130±56</td>
<td>4.8±0.7</td>
<td>38.71±18.83</td>
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<td>Ad-hLPL194 (n=5)</td>
<td>2128±401</td>
<td>162±51</td>
<td>5.4±0.8</td>
<td>17.90±9.45</td>
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<tr>
<td>ApoE−/− Ad-hAP (n=6)</td>
<td>125±26</td>
<td>595±51</td>
<td>19.9±2.1</td>
<td>527.00±60.94</td>
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<tr>
<td>Ad-hLPLwt (n=6)</td>
<td>88±11</td>
<td>623±63</td>
<td>14.5±2.6</td>
<td>632.10±141.40</td>
</tr>
<tr>
<td>Ad-hLPL194 (n=6)</td>
<td>118±12</td>
<td>553±33</td>
<td>18.4±3.9</td>
<td>463.80±46.64</td>
</tr>
</tbody>
</table>

* Data are expressed as mean±SEM. TG indicates triglyceride; TC, total cholesterol; HDL-C, high-density lipoprotein cholesterol.
In contrast to our findings, Ziouzenkova et al. showed that hydrolysis of VLDL-TG by bovine LPL in cultured endothelial cells, which generated fatty acids serving as peroxisome proliferator-activated receptor α (PPARα) ligands, could downregulate VCAM-1 expression, thus limiting the inflammatory responses.26 One main factor which should be considered to explain the discrepancy between our result and theirs was the use of tumor necrosis factor (TNF) α for stimulation of endothelial cells used in their experiment but not in ours. We postulate that under strong stimulation by a molecule such as TNFα, VCAM-1 might be upregulated to the maximal extent, and thus could be sensitive to negative regulators. The PPARα antiinflammation pathway, for example, would be activated easily with the supply of fatty acids generated from hydrolysis of TG-rich lipoproteins. However, at nonstimulated basal expression levels VCAM-1 could be sensitive to positive regulators. In fact, the upregulation of VCAM-1 in our study was not related to fatty acids because expression of active and inactive LPL in both in vivo and in vitro conditions resulted in similar levels of VCAM-1 upregulation. Thus, the observed VCAM-1 regulation in our case is more likely attributable to the well known nonenzymatic effect of LPL, which is to act as a molecular bridge to anchor TG-rich lipoproteins for cellular uptake. Further investigation is thus warranted to delineate mechanisms of the nonenzymatic role of LPL on VCAM-1 upregulation.

Population studies have proposed that LPL deficiency is not associated with the development of atherosclerosis despite severe hypertriglyceridemia.27,28 However, as long as the LPL mass is preserved even in catalytically inactive form, the atherogenic effect of hypertriglyceridemia is obvious.29 This is in accord with our findings that the presence of inactive LPL on arterial walls of LPL-deficient mice resulted in significant lipid deposition. This indicated clearly that LPL protein per se could mediate lipid uptake via its molecular bridging effect. Local hydrolysis of plasma TG-rich lipoproteins by catalytically active LPL in our study further promoted lipid accumulation in arterial walls, possibly by generation of remnant lipoproteins.

Although gene expression mediated by adenovirus is transient, it is a powerful tool and used widely in research on atherosclerosis.30–33 In the present study, we chose day 7 as the time point to study the long-term process of atherosclerosis because adenovirus mediated arterial gene expression reaches a peak level at approximately day 7.34 In addition, our previous work with rabbits was also designed to examine the atherosclerotic effect by local expression of active and inactive LPL at day 7, and similarly gained meaningful results as expected.

In conclusion, our data demonstrate that transient expression of both active and inactive LPL results in rapid lipid deposition and VCAM-1 upregulation in endothelial-intact carotid artery of extreme hypertriglyceridemic and severe hypercholesterolemic mice, which indicates that LPL present at the arterial wall is a strong proatherosclerotic factor. We further demonstrate that the VCAM-1 upregulation of endothelial cells depends on the noncatalytic effect of LPL, because both the active and inactive forms of LPL upregulate VCAM-1 at similar levels.

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Disclosures
None.

References


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Fig. I Detection of hAP gene expression *in vivo* by histological staining (400×).

Arteries infected with Ad-hAP, Ad-hLPLwt or Ad-hLPL194 were stained for AP activity and blue stainings with the AP substrate, NBT/BCIP designated positive reaction (as indicated by arrows). No AP activity could be detected in Ad-hLPLwt and Ad-hLPL194 infected vessels (Fig. I).
Fig. II

Fig.II Detection of hLPL gene expression at protein levels in cultured HUVECs by immuno/histological staining (400×).

In both Ad-hLPLwt and Ad-hLPL194 infected HUVECs, hLPL protein was detected by immunostaining with an anti-hLPL mAb, showing distinct brown precipitates. There was no reaction with this antibody in the Ad-hAP infected cells (Fig. II).
CM hydrolysis in the medium from HUVECs infected with different viral vectors.

CM hydrolysis was assessed by glycerol assay in the culture medium. Glycerol concentration was significantly increased over time only in the medium of HUVECs infected with Ad-hLPLwt, as compared with those of HUVECs infected with Ad-hLPL194 and Ad-hAP (Fig. III, **p<0.01).
Fig. IV Enhanced lipid deposition in carotid arteries over-expressing either active or inactive LPL in wild type C57BL/6 mice (400×).

The areas of lipid deposition in arteries by ORO staining were quantitated by morphometric measurement and compared among Ad-hAP, Ad-hLPLwt and Ad-hLPL194 groups (Fig. IVA, IVB). The bar height represents mean values calculated from areas of lipid deposition in each group; error bars indicate SEM. **p<0.01 for Ad-hLPLwt versus Ad-hAP, *p<0.05 for Ad-hLPL194 versus Ad-hAP.
Fig. V

Up-regulation of VCAM-1 in carotid arteries over-expressing either active or inactive LPL in wild type C57BL/6 mice.

VCAM-1 expression was evaluated at day 7 after gene delivery in Ad-hAP, Ad-hLPLwt or Ad-hLPL194 infected arteries. Distinct green VCAM-1 protein precipitates were observed in Ad-hLPLwt and Ad-hLPL194 infected arteries, while there were only scattered precipitates in Ad-hAP infected arteries.
Fig. VI  

**Integrality of endothelium of the carotid arteries after gene delivery (400×).**

Endothelium integrality was evaluated at day 7 after gene delivery in Ad-hAP, Ad-hLPLwt or Ad-hLPL194 infected arteries. A thin layer of distinct blue color along the inner surface of carotid artery was observed, indicating the presence of endothelial cells in place, in the representative infected arteries from each group.