Macrophage Depletion by Clodronate Liposomes Suppresses Neointimal Formation After Carotid Artery Injury in Apolipoprotein E-Deficient Mice

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ABSTRACT

Background and Objectives: Clodronate liposomes deplete phagocytic cells, thereby suppressing inflammation after vascular injury. We compared the effect of clodronate liposomes on macrophage depletion and neointimal formation in apolipoprotein E-deficient mice (ApoE (-) mice). Materials and Methods: ApoE (-) mice were randomly assigned to the clodronate liposomes group (Clodronate Group, n=7) and the vehicle liposomes group (Control Group, n=7). Clodronate (0.1 mL/10 g) was injected via the tail vein starting 2 days (d-2) before left common carotid artery injury. Results: The percentage of blood monocytes was subsequently decreased after clodronate injection (14.0 ± 7.4% at baseline, 6.8 ± 4.9% at 24 hours and 0.7 ± 0.3% at 1 week after the clodronate liposome injection). The percentage of macrophages in the plaque area was significantly lower in the clodronate group at week 2 (32.0 ± 6.5 vs. 68.7 ± 7.6%, respectively, p < 0.05) and at week 4 (37.3 ± 8.5 vs. 62.6 ± 9.4%, respectively, p < 0.05). The interleukin (IL)-6 and tumor necrosis factor (TNF)-α concentrations were significantly decreased in the clodronate group at week 4 (12.3 ± 2.5 vs. 22.9 ± 3.5 pg/mL, respectively, p < 0.05 for IL-6 and 16.6 ± 2.2 vs. 43.6 ± 6.1 pg/mL, respectively, p < 0.05 for TNF-α). The plaque volume was significantly greater in the control group at week 2 (0.345 ± 0.063 vs. 0.153 ± 0.053 mm², respectively, p < 0.05) and at week 4 (0.320 ± 0.027 vs. 0.167 ± 0.070 mm², respectively, p < 0.05). Conclusion: Intravenous administration of clodronate liposomes depleted monocytes and macrophages, and so this reduced the inflammatory markers and neointimal formation in ApoE (-) mice. (Korean Circ J 2008;38:244-249)

KEY WORDS: Clodronate; Macrophages; Inflammation.

Introduction

Inflammation plays a crucial role in the process of atherosclerosis, and macrophages play a pivotal role in the pathogenesis of atherosclerosis. Selective depletion of phagocytic cells such as macrophages from the peripheral circulation in vivo can be achieved with the systemic injection of clodronate-containing liposomes. Clodronate is one of the bisphosphonates used for treating osteolytic bone diseases and it is one of the potent inhibitors of osteoclasts. Clodronate is not a toxic drug in itself, and free clodronate can not easily pass the phospholipid bilayers of cell membranes. The highly hydrophilic nature of free clodronate prevents its passage through the cell lipid bilayer; therefore, its cellular uptake requires that it be incorporated into liposomes. Liposomes are artificially prepared spheres that consist of concentric phospholipids bilayers separated by aqueous compartments. Liposomes are readily taken up by cells such as monocytes and macrophages. Once clodronate is taken into phagocytic cells such as monocytes and macrophages with using liposomes as vehicles, it accumulates within the cell.

After exceeding a threshold concentration, the cell is irreversibly damaged and then it dies by apoptosis. We have chosen Apolipoprotein E-deficient mice [ApoE (-) mice] for our study since they are hyperlipidemic and they are well known for spontaneously developing atherosclerosis in the arterial wall such as carotid arteries. Clodronate liposomes deplete phagocytic cells such as monocytes and macrophages, thereby suppressing in-
flammation after mechanical carotid artery injury in ApoE (-) mice. Thus, we hypothesized that clodronate liposomes attenuate the neointimal formation through depleting macrophages in an ApoE (-) mouse model.

Materials and Methods

Clodronate liposomes

Clodronate, when dissolved with phospholipids, can be encapsulated within liposomes, thereby forming clodronate liposomes. The detailed description for preparing clodronate liposomes is as follows. A stock solution of phosphatidylcholine (egg lecithin) in chloroform (100 mg/mL) is prepared, and 8 mg cholesterol is dissolved in 10 mL chloroform. The phospholipid layer is isolated in 10 mL of aqueous solution by mixing the solution at 180 rpm rotation after adding 0.86 mL of the phosphatidylcholine stock solution. 10 mL of a 0.6 M clodronate is then added to the empty liposomes. The suspension is sonicated in a waterbath sonicator for 3 minutes. During the preparation of the clodronate liposomes, about 1% of clodronate is actually encapsulated. Moreover, approximately 20 mg of clodronate is encapsulated in 4 mL of the clodronate liposome suspension. The prepared clodronate liposomes are kept at a temperature of 4°C.

Apolipoprotein E-deficient mice

The ApoE (-) mice were obtained from Jackson Laboratories (Bar Harbor, ME), and the mice were sacrificed at 2 and 4 weeks after starting the clodronate liposome injections. Blood samples (1.5 mL) from the sacrificed mice were drawn into heparinized tubes. Within 1 h of collection, the peripheral blood mononuclear cells (PBMCs) were immediately isolated by density gradient centrifugation with using Ficoll-Paque plus® (Amersham Biosciences Corporation, NJ). For flow cytometric analysis, The PBMCs were fixed in 2% paraformaldehyde and then stained with anti-CD14 (Becton Dickinson, San Jose, CA) and anti-CD45 (Becton Dickinson) monoclonal antibodies for 20 min at 4°C. The cells were then stained with isotype-matched phycoerythrin (PE)-conjugated secondary antibody (BD Biosciences). A negative control was incubated with buffer for 20 min at 4°C, but no primary antibody was used to identify the non-specific binding from the secondary antibody. The 10,000 cells/sample were analyzed on a FACS Vantage SE flow sorter (Becton Dickinson). Dead cells and debris were gated out using the scatter properties of the cells. The data were analyzed using CellQuest Pro software (Becton Dickinson). The CD45 (leukocyte common antigen) positive and CD14 (monocyte marker) positive cells were counted as monocytes during the FACS analysis.

Blood samples were collected by cardiac puncture from the ApoE (-) mice after they were sacrificed. The serum separator tubes (Microtainer, Becton Dickinson, NJ) were centrifuged at 1,000 rpm for 10 minutes, and the serum was stored at -80°C. Tumor necrosis factor (TNF)-α was measured by a sandwich Enzyme Linked Immuno Sorbent Assay (ELISA) with a minimum detectable level of 0.5 pg/mL (ALPCO Diagnostics, Salem, NH). High-sensitivity interleukin (IL)-6 was also measured by a sandwich ELISA with a minimum detectable level of 0.16 pg/mL (ALPCO Diagnostics, Salem, NH).

The high sensitivity C-reactive protein (hsCRP) concentrations were quantified using a latex nephelometer II (Dade Behring Inc., Newark, DE). The total cholesterol levels were determined by enzymatic methods using standard biochemical procedures on a B.M. Hitachi automated clinical chemistry analyzer (Hitachi, Tokyo, Japan).

Microscopic examination of the carotid arteries

Vessel harvesting was performed 2 and 4 weeks after
the left common carotid artery injury. Macrophage infiltrations at the injured left common carotid artery were compared between the two groups by performing light-microscopy between the groups (Hematoxylin and Eosin stain, elastic stain, Masson Trichrome stain and CD68 macrophage antibody stain) after vessel harvesting. The left carotid artery was excised after in situ perfusion-fixation with 4% paraformaldehyde at 100 mmHg and then it was paraffin-embedded. After paraffin fixation, serial tissue sections (5 μm) are obtained from the left common carotid arteries, starting at the bifurcation. Ten sections (50 μm apart) are stained for immunohistochemical analysis. The original lumen (residual lumen), which is the area bounded by the internal elastic lamina, and the total arterial area, which is the area circumscribed by the external elastic lamina, were measured by Imagepro Plus (Media Cybernetics, Inc. MD, USA). The data for each cell type (macrophages and foam cells) and collagen were expressed as the percentage of the immunostained area per the total neointimal area.

Statistical analysis
Data for the continuous variables is expressed as means ± SEs and this data was compared with the Mann-Whitney test, and the comparisons between before and after treatment were analyzed by the Wilcoxon signed rank test. A p value of less than 0.05 was considered significant. Statistical Package for the Social Sciences (SPSS) software (version 10.0) was used for analyses (SPSS Inc., Chicago, Illinois, USA).

Fluorescence-activated cell sorter analysis
FACS analysis showed that more than 50% of the peripheral monocytes were depleted 24 hours after intravenous injection of clodronate liposomes (14.0 ± 7.4% at baseline and 6.8 ± 4.9% at 24 hours after the clodronate liposome injection, p < 0.01), and 0.7 ± 0.3% of the peripheral monocytes remained at one week after the clodronate liposome injections. However, there were no significant changes in the total white blood cell counts (9.5 ± 3.5/10⁶ μL at baseline, 10.4 ± 4.0/10⁶ μL at 24 hours and 10.8 ± 4.5/10⁶ μL at 1 week after the clodronate liposome injection). After the control liposome injections, 14.0 ± 7.4% of the peripheral monocytes remained one week after the repeated clodronate liposome injections. The control liposome injections, 14.0 ± 7.4% of the peripheral monocytes remained at baseline, 13.4 ± 6.7% remained at 24 hours and 13.6 ± 5.9% remained at one week after the control liposome injection.

Inflammatory markers and cholesterol levels
High total cholesterol levels were observed in both the control and the clodronate groups at 2 and 4 weeks after the injection, and no significant differences were observed between the 2 groups (Table 1). Inflammatory markers such as hsCRP, IL-6 and TNF-α were significantly lower in the clodronate group compared with the control group 2 weeks and 4 weeks after the injection (Table 1).

Microscopic analysis of the carotid arteries
Microscopic analysis of the Formalin-fixed common carotid arteries in the Control Group demonstrated a large amount of neointimal formation 4 weeks after the guidewire injury (Fig. 2A, C, E and G). However, a small amount of neointimal formation was observed 4 weeks after guidewire injury in the clodronate group (Fig. 2B, D, F and H). The percentage of macrophages in the plaque area was significantly lower in the clodronate group at week 2 (32.0 ± 6.5 vs. 68.7 ± 7.6%, p < 0.05) and at week 4 (37.3 ± 8.5 vs. 62.6 ± 9.4%, p < 0.05). The plaque volume was similar at baseline between the 2 groups, but it was significantly greater in the control group at week 2 (0.345 ± 0.063 vs. 0.153 ± 0.053 mm², p < 0.05) and at week 4 (0.765 ± 0.137 vs. 0.153 ± 0.053 mm², p < 0.05).

Table 1. Comparison of inflammatory markers and total cholesterol levels between the control group and the clodronate group after left common carotid artery injury

<table>
<thead>
<tr>
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<th>Control</th>
<th>Clodronate</th>
<th>p</th>
<th>Control</th>
<th>Clodronate</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cholesterol (mg/dL)</td>
<td>424 ± 55</td>
<td>467 ± 46</td>
<td>0.08</td>
<td>447 ± 67</td>
<td>452 ± 50</td>
<td>0.85</td>
</tr>
<tr>
<td>hsCRP (mg/L)</td>
<td>0.203 ± 0.057</td>
<td>0.115 ± 0.013</td>
<td>&lt;0.05</td>
<td>0.190 ± 0.045</td>
<td>0.114 ± 0.018</td>
<td>&lt;0.05</td>
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<tr>
<td>IL-6 (pg/mL)</td>
<td>24.9 ± 6.3</td>
<td>12.4 ± 2.8</td>
<td>&lt;0.05</td>
<td>22.9 ± 3.5</td>
<td>12.3 ± 2.5</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>TNF-α (pg/mL)</td>
<td>45.0 ± 9.0</td>
<td>16.8 ± 4.0</td>
<td>&lt;0.05</td>
<td>43.6 ± 6.1</td>
<td>16.6 ± 2.2</td>
<td>&lt;0.05</td>
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hsCRP: high-sensitivity C-reactive protein, IL-6: interleukin, TNF-α: tumor necrosis factor-alpha
Fig. 2. Cross sections of the carotid arteries of the ApoE (−) mice. Representative cross sections of the ApoE (−) mice common carotid arteries taken 4 weeks after guidewire injury demonstrated decreased neointimal formation in the clodronate group (B, D, F and H). Hematoxylin and eosin-staining (A and B), Masson’s Trichrome staining (C and D), CD 68 monoclonal Ab staining (E and F) and elastin staining (G and H) are shown with a bar (250 μm).
Inflammation plays a key role in neointimal formation after mechanical vascular injury. The ubiquitous monocytes are the precursor of macrophages, and these are scavenging and antigen-presenting cells. Macrophages and macrophage-derived foam cells significantly contribute to the neointimal tissue after vessel injury and they further aggravate the local inflammatory response and neointimal growth by secreting cytokines, chemokines, metalloproteinases and growth factors. Continued inflammation results in increased numbers of macrophages and foam cells, which emigrate from the blood and multiply within the injured common carotid arteries. Macrophage colony-stimulating factors partly contribute to the continuing entry, survival and replication of macrophages in the injured common carotid arteries. In our animal study, we had a significantly decreased inflammatory response after common carotid artery injury by almost completely depleting the peripheral monocytes and macrophages, thereby decreasing the release of various cytokines, chemokines and growth factors. The circulating IL-6, TNF-α and hs CRP were significantly decreased in only in the clodronate group 2 weeks and 4 weeks after repeated clodronate liposome injections. The decreased inflammatory environment might have contributed to the decreased neointimal formation in the common carotid arteries of the ApoE (-) mice.

We chose ApoE (-) mice, which suffer with hypercholesterolemia and prominent progression of atherosclerosis, as our animal model since the changes in reactive neointimal formation could be well distinguished between the 2 groups. Apolipoprotein E is an important mediator for the hepatic metabolic clearance of circulating cholesterol. When apolipoprotein E is absent, severe hyperlipidemia occurs in mice and so they develop hypercholesterolemia and atherosclerosis even when given a normal chow diet. Moreover, they show the progression of atherosclerosis in various arteries, including the common carotid artery. Paclitaxel, everolimus and anti-vascular endothelial growth factor peptides are known to suppress neointimal formation after vessel injuries. However, one should be cautious when interpreting those previous studies since a large number of the studies that pharmacologically attempted to inhibit neointimal formation in animals did not translate into subsequent inhibition of neointimal formation in humans. We have chosen clodronate liposomes since the monocyte and macrophages, which are important in the processes of neointimal formation after vessel injury, can be selectively depleted. Clodronate liposomes are readily ingested by macrophages via endocytosis, and after the macrophages fuse with the lysosomes that contain phospholipases, disruption of the bilayers of the clodronate liposomes occurs. The clodronate released within the cytoplasm accumulates to a level that kills the macrophages through apoptosis. Clodronate liposomes are unable to cross vascular barriers, and different administration routes can be considered for the depletion of various macrophage populations. In our study, intravenous administration of clodronate liposomes was chosen since the circulating monocytes and macrophage cells could be depleted after administration of the liposomes. We started the left common carotid artery injury 2 days after the injection of clodronate liposomes since the circulating monocytes were almost completely annihilated 2 days after the initiation of clodronate liposomes injection via a tail vein (Fig. 1). We administered vehicle liposomes in the control ApoE (-) mice in order to identify the effect of clodronate itself. The major limitation of long-term clodronate liposome injection could be disruption of the immune system since circulating monocytes play a major role in immunity. In our study, we kept our ApoE (-) mice in a chamber with a constant temperature and humidity and we fed the ApoE (-) mice sterile water and chow. Although inactivation of macrophages carries the danger of immunosuppression and infection, no overt infection was observed during the 4 weeks of our study after the transient macrophage depletion; however, our experience has shown that the ApoE (-) mice that underwent intravenous injection for longer than 8 weeks may have problems with infection and necrosis. Although the circulating monocytes were depleted after intravenous clodronate liposomes injections and the structure of immunity might have been partially affected by depletion of the monocytes and macrophages, the circulating neutrophils were not affected by clodronate liposomes injections in vivo and in vitro and this might have helped in reducing the chances of infection during our short-term follow-up.

Accelerated neointima formation after percutaneous transluminal coronary angioplasty in patients with coronary atherosclerosis causes restenosis, and endless efforts and experiments have been carried out in attempts to...
reduce this restenosis. Modified smooth muscle cells, macrophages and macrophage-derived foam cells significantly contribute to the neointimal tissue and they further aggravate the local inflammatory response and neointimal growth, especially in the context of hypercholesterolemia.16-21 Although intravenous injections of clodronate liposomes decrease inflammation by reducing the monocytes and macrophages, intravenous injection of clodronate liposomes cannot simply be extrapolated to humans since there are long-term safety issues that have to be solved and oral administration is not yet available. In conclusion, clodronate liposome injections reduced the number of circulating blood monocytes and it decreased macrophage infiltration in the injured left common carotid arteries, as well as reducing the smooth muscle cell proliferation and the IL-6, TNF-α and C-reactive protein production. We have demonstrated that depleting the circulating monocytes and macrophages by clodronate liposome injections could effectively reduce inflammation and thereby reduce the proliferative response to vascular injury in ApoE (-) mice.

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