Liposomal Alendronate Inhibits Systemic Innate Immunity and Reduces In-Stent Neointimal Hyperplasia in Rabbits

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Background—Innate immunity is of major importance in vascular repair. The present study evaluated whether systemic and transient depletion of monocytes and macrophages with liposome-encapsulated bisphosphonates inhibits experimental in-stent neointimal formation.

Methods and Results—Rabbits fed on a hypercholesterolemic diet underwent bilateral iliac artery balloon denudation and stent deployment. Liposomal alendronate (3 or 6 mg/kg) was given concurrently with stenting. Monocyte counts were reduced by >90% 24 to 48 hours after a single injection of liposomal alendronate, returning to basal levels at 6 days. This treatment significantly reduced intimal area at 28 days, from 3.88±0.93 to 2.08±0.58 and 2.16±0.62 mm². Lumen area was increased from 2.87±0.44 to 3.57±0.65 and 3.45±0.58 mm², and arterial stenosis was reduced from 58±11% to 37±8% and 38±7% in controls, rabbits treated with 3 mg/kg, and rabbits treated with 6 mg/kg, respectively (mean±SD, n=8 rabbits/group, P<0.01 for all 3 parameters). No drug-related adverse effects were observed. Reduction in neointimal formation was associated with reduced arterial macrophage infiltration and proliferation at 6 days and with an equal reduction in intimal macrophage and smooth muscle cell content at 28 days after injury. Conversely, drug regimens ineffective in reducing monocyte levels did not inhibit neointimal formation.

Conclusions—Systemic transient depletion of monocytes and macrophages, by a single liposomal bisphosphonates injection concurrent with injury, reduces in-stent neointimal formation and arterial stenosis in hypercholesterolemic rabbits. (Circulation. 2003;108:2798-2804.)

Key Words: angioplasty ■ stents ■ restenosis ■ leukocytes ■ inflammation

Innate immunity plays a major role in vascular repair. Monocyte adhesion and infiltration occur immediately after vascular injury.1,2 Macrophage content in atherectomy tissue and activation status of blood monocytes correlate with an increased rate of restenosis in humans.3,4 We recently showed that transient macrophage depletion reduces experimental neointimal formation after balloon injury in rats and rabbits.3 Stent deployment profoundly alters vascular repair, and the role of innate immunity in late restenosis is further stressed. Although recoil and remodeling are negligible, local inflammation and regional neointimal formation are greater than that observed after balloon angioplasty.6,7 Macrophage accumulation is increased in stented arteries compared with balloon-injured vessels,7-9 with tissue macrophage content linearly correlating with neointimal area, suggesting a pivotal role for monocytes and macrophages in stent restenosis.10

Bisphosphonates (BPs), widely used in treating osteoporosis and other bone diseases, are potent inhibitors of osteoclasts. The biological effects of BPs in vivo, characterized by very poor cell membrane permeability, stem from their incorporation into bone, enabling direct interaction through phagocytosis with the monocyte-derived osteoclasts.11,12 The in vitro effects of liposomal BPs (LBPs) are limited to cells with phagocytic capacity (ie, macrophages), with no effect on smooth muscle cells (SMCs) and endothelial cells.5 Depletion of macrophages and circulating monocytes in vivo can be achieved by liposome-mediated intracellular delivery of BPs, which inactivate and kill these cells after effective phagocytosis but are not toxic to nonphagocytic cells.5,13,14

The present study investigated whether systemic transient depletion of monocytes and macrophages by LBPs inhibits in-stent neointimal formation in hypercholesterolemic rabbits and whether inhibition of neointimal SMC growth is as potent as macrophage suppression. A single injection of liposomal alendronate (LA) at the time of endovascular stent implantation suppressed macrophage infiltration and subsequent SMC growth, leading to reduced in-stent neointimal formation.
Methods

Liposomes
Sodium alendronate was encapsulated in liposomes (161 nm in diameter) composed of distearoyl-phosphatidylglycerol, 1,2-distearoyl-sn-glycero-3-phosphocholine, and cholesterol as described previously.5

Rabbit Model
New Zealand White rabbits (Millbrook Farm Breeding Labs, Amherst, Mass) weighing 2.5 to 3.5 kg were used. Animal care and procedures were in accordance with the guidelines of the American Association for the Accreditation of Laboratory Animal Care and the National Institutes of Health. Animals were fed an atherogenic diet of 2% cholesterol and 6% peanut oil (Research Diets) starting 3 weeks before angioplasty and water ad libitum. Aspirin (0.07 mg/mL) was added to drinking water beginning 1 day before surgery. Under xylazine (5 mg/kg) and ketamine (35 mg/kg) anesthesia, both femoral arteries were ligated, an arteriotomy was performed proximally, and the endothelium of the iliac arteries was denuded by 3 passages of a 3-0 Fogarty balloon (Baxter). A 9-mm stent (Nitriflex, Medinol) was then deployed under fluorescein in each common iliac artery to achieve a stent:artery diameter ratio of 1.1:1, and the femoral arteries were ligated proximal to the arteriotomy. Heparin (100 U/kg) was administered before stenting. Animals were randomly assigned to 3 or 6 mg/kg of LA or to saline administered intra-arterially to the right iliac artery after stenting. In separate experiments, different regimens (intravenous injection and single versus double doses) were studied.

Tissue Harvest and Analysis
Angiography of the iliac arteries at 28 days was performed under anesthesia, followed by euthanasia and harvesting of the stented arteries. Arteries for immunostaining were harvested at 6 days. The iliac arteries were perfusion-fixed with 10% neutral buffered formalin at physiological pressure. Stented arteries were oriented longitudinally and embedded with a methacrylate formulation. Multiple sections (5 μm thick) were cut with a tungsten carbide knife (Delaware Diamond Knives) on an automated microtome (Leica, Inc) from the proximal and distal ends and the midpoint of each stented segment. Measurements on Verhoeff’s tissue elastin stained sections were automated microtome (Leica, Inc) from the proximal and distal ends and the midpoint of each stented segment. Measurements on Verhoeff’s tissue elastin–stained sections were performed after digital capturing with Adobe Photoshop 5.0 software. Luminal stenosis was calculated as the ratio between the neointima and the area bound by the internal elastic lamina.

Immunohistochemical identification of rabbit tissue macrophages with a cell-specific antibody for RAM-11, SMCs with antibody for SM α-actin, and proliferating cells with Ki-67 antibody (all from Dako Co) was accomplished by standard immunocytochemical protocols.15 Three sections (see above) of each artery were stained. Immunohistochemical identification of rabbit tissue macrophages with a cell-specific antibody for RAM-11, SMCs with antibody for SM α-actin, and proliferating cells with Ki-67 antibody (all from Dako Co) was accomplished by standard immunocytochemical protocols.15

Blood Count and Flow Cytometry for Monocyte Detection
Blood was drawn at baseline and at 48 hours and anticoagulated with EDTA. Blood count was performed with a Coulter counter. Flow cytometry for CD14+ cells was performed as previously described.4 In short, whole blood (200 μL) was labeled with mouse anti-human R-phycocerythrin–conjugated anti-CD14 (4 μL, Dako), red blood cells were lysed (FACS lysing solution, BD), the cell suspension was washed twice with PBS containing 1% FCS, and the monocyte percentage of total white blood cells (WBCs) was recorded on the basis of relative size, side scattering, and fluorescence. Monocyte number was calculated by multiplying flow cytometry–yielded percentage by Coulter WBC count.

Statistical Analysis
Data are expressed as mean±SD. Comparisons of histological findings between control and treatment group were made by the unpaired Student’s t test. Comparisons of blood monocytes and immunostaining results over time were made by 2-way ANOVA. Differences were termed statistically significant at P<0.05.

Results

Monocyte Depletion
WBC count increased slightly 48 hours after surgery, with no significant difference between controls and the 2 LA dose groups (not shown). Monocyte number was low in all animals after stenting and significantly lower in LA-treated animals. Monocyte numbers at baseline and at 48 hours were 216±62 and 24±20, and 175±50 and 13±3 in control, LA 3 mg/kg–treated, and LA 6 mg/kg–treated animals, respectively (Figure 1). The percentage of WBCs identified as monocytes decreased by 66±4, 91±4, and 95±2 in control, LA 3 mg/kg–treated, and LA 6 mg/kg–treated animals, respectively. WBC and monocyte counts at 6 days after surgery returned to baseline levels.

Effect of LA on Neointimal Formation
Iliac artery stenting resulted in abundant concentric neointimal formation composed of SMCs and foam cells, with both intraluminal and outward neointimal growth (Figure 2, A and B). Intimal area in controls was 3.88±0.93 mm2, luminal area was 2.87±0.44 mm2, and luminal stenosis was 58±11%. LA significantly reduced neointimal formation at 28 days (Figure 2, C–F). There was no significant difference between animals treated with 3 and 6 mg/kg LA. Neointimal area was 2.08±0.58 mm2 and 2.16±0.62 mm2, luminal area 3.57±0.65 mm2 and 3.45±0.58 mm2, and luminal stenosis 37±8% and 38±8% in the 3 mg/kg– and the 6 mg/kg–treated groups, respectively (Figure 2, G and H). Administration of a 1-fold lower dose (0.3 mg/kg) did not suppress monocyte levels and...
did not reduce intimal formation at 28 days (data not shown). The single-injection regimen was as effective as regimens that used 2 injections spaced 4 to 5 days apart (3 mg/kg IV, days 0 and +4 and days -1 and +4, data not shown).

**Early Macrophage Infiltration**
Immunostaining for macrophages at 6 days after stenting revealed abundant arterial infiltration in control rabbits (Figure 3A). LA treatment significantly reduced the area stained positive for macrophages from 68 ± 14% in controls to 21 ± 7% (n=4, P<0.05, Figure 3).

**Cell Proliferation**
Immunostaining for the Ki-67 antigen at 6 days after stenting revealed reduced proliferation in LA-treated rabbits (Figure 4). Mean proliferation was 14 ± 4% of total cells in control rabbits arteries and 6 ± 2% in LA-treated animals (n=4, P<0.05, Figure 4).

**Composition of Lesion**
Dual immunostaining for SMC α-actin and RAM-11 macrophages at 28 days revealed 2 discrete neointimal layers: a deeper layer composed primarily of macrophage foam cells and an inner (luminal) layer composed primarily of SMCs. Abundant extracellular matrix was noted between the layers (Figure 5, A and B). LA treatment significantly reduced the area of both layers (Figure 5, C and D). The macrophage/foam-cell layer was reduced from 1.44 ± 0.58 to 0.81 ± 0.32 mm² (Figure 5E) and composed 37 ± 3% and 34 ± 8% of total neointimal area in control and LA-treated animals, respectively (Figure 5F). The SMC α-actin-positive layer was reduced from 0.75 ± 0.21 to 0.47 ± 0.09 mm² and composed 21 ± 7% and 21 ± 4% of total neointimal area in control and LA-treated animals, respectively (Figure 5, E and F).

**Discussion**
Innate immunity seems to play a central role in vascular injury and repair. Suppression of monocyte number and monocyte stimulatory factors inhibits intimal hyperplasia after balloon injury.\textsuperscript{5,16,17} The impact of monocytes and macrophages on vascular repair of stented blood vessels is considered even more significant. Macrophage content in the vessel wall is markedly higher and prolonged in stented
versus balloon-injured arteries, and it correlates with neointimal formation. Furthermore, the contribution of neointimal formation, which is mediated via innate immunity, to in-stent restenosis outweighs its role in restenosis after balloon injury. The questions that arise are whether it is possible to suppress monocytes/macrophages to such an extent as to inhibit stent hyperplasia and whether inhibition of monocyte/macrophage number inhibits hyperplasia only in concert with reduction in mass of cells or to a greater extent. The results of this study, in which transient depletion of monocytes and macrophages reduces intimal hyperplasia after stenting in excess of the arterial macrophage mass suppression confirms the critical causative role of these cells in in-stent restenosis and the possibility that they may be targeted not solely for their bulk but for their signaling.

In a previous study, multiple doses of LBP begun before surgery reduced monocyte number and inhibited intimal hyperplasia after balloon injury. We now show that a single dose of LA administered at the time of injury was sufficient to inhibit stent hyperplasia and whether inhibition of monocyte/macrophage number inhibits hyperplasia only in concert with reduction in mass of cells or to a greater extent. The large bulk of macrophages in the hypercholesterolemic model raises an important question as to whether the decrease in neointima results primarily in reduction in mass of the targeted macrophages or is shared by SMCs, and to what extent. Dual staining for both RAM-11 macrophages and -actin–positive SMCs at 28 days indicates that the reduction is similar in both layers. Complete suppression of macrophages is thus unnecessary for subsequent suppression of SMC proliferation. Rather, macrophage suppression correlates with its resultant inhibition of SMC growth.

The effect of LBP treatment on proliferating SMCs, which compose the major component of human restenotic lesions, is indirectly mediated via the inhibitory effect on macrophages; in vitro studies showed unequivocally that macrophages are much more sensitive to LBPs than SMCs. The reduction in SMC proliferation and content supports the major role of monocytes in neointimal formation after stent injury and the potential use of macrophage depletion in its modulation.
Clodronate, which was used in our previous balloon-injury studies, is several orders of magnitude less potent than the amino BP alendronate in inhibiting osteoclasts and consequently bone-related disorders such as tumor osteolysis and osteoporosis. The difference in potency stems from the mechanism of action of these BPs on osteoclast inhibition: nonhydrolyzable ATP analogue formation by clodronate and geranyl-geranyl enzyme inhibition in the mevalonate pathway induced by alendronate. The more potent alendronate induces apoptosis at relatively lower concentrations of both osteoclasts and macrophages. Thus, the alendronate dose chosen for this study was lower than the clodronate dose used for reduction of neointima after balloon injury. Furthermore, a single injection regimen was applied in this study, whereas a 2-injection regimen that included pretreatment was used in the balloon-injury model. Inhibition of restenosis even in the highly cellular hypercholesterolemic model was achieved by a single liposomal application of the potent BP alendronate at the time of injury.

The preprocedural activation status of innate immunity correlates with late restenosis, enabling preprocedural risk stratification. Although initial studies with glucocorticoids failed to show reduction in postballoon restenosis, prolonged immunosuppressive therapy with prednisone was found to reduce in-stent restenosis in high-risk high-C-reactive protein patients (IMPRESS study). Thus, systemic immunomodulation effectively suppresses the reparation that leads to neointimal formation in a high-risk “inflammatory” subset of patients. Glucocorticoids suppress both innate and adaptive immunity, with a possible acute stimulatory affect on innate immunity. Specific targeting of monocytes, addressed by LBP therapy, differs from glucocorticoids in specifically targeting innate immunity. Furthermore, it has a favorable pharmacodynamic profile and thus possesses high potential for clinical use.

Monocytes and macrophages are a mainstay of innate immunity, having a major role in phagocytosis and antigen presentation to other immune cells. Thus, inactivation of macrophages carries the theoretical danger of immunosuppression and infection. No infection was recorded in our previous and current experiments. Furthermore, Mac-1–deficient mice and the severely macrophage-deficient OP/H/H11002 mice were not reported to suffer extreme infections. This is at least partly because of other systems that can take over the antigen-presenting capacities of macrophages. Thus, the brief period of macrophage depletion associated with a single dose, coupled with an otherwise intact innate immunity, yields a low likelihood of infection. Nevertheless, further research is warranted to define the risks and adverse effects that are associated with transient systemic macrophage inactivation by LBPs.

Low doses of LA, insufficient to deplete circulating monocytes, did not suppress neointimal formation, further supporting the inflammatory cause for neointimal hyperplasia. Thus, monocyte count after drug administration may be predictive of the late effect of the drug on neointimal formation.

Is there any rationale to examine systemic therapy in the “era of drug-eluting stents”? Most systemic pharmacological therapies have been unsuccessful in preventing arterial restenosis in humans, and the preliminary results of drug-eluting stents are remarkable in reducing in-stent restenosis. Nevertheless, LBPs offer a systemic therapy to a systemic process, regardless of the procedure and the device(s) used. If effective in a clinical setting, it may be an easily adminis-
tered, cost-effective modality that allows flexibility in choosing the type and number of stents to be deployed, may serve as an adjunct therapy in high-risk patients, and may even reduce the need for stenting altogether.33

In conclusion, LBP transiently suppress innate immunity by systemically inactivating and depleting circulating monocytes and tissue macrophages. A single injection of LA concurrent with stenting suffices to suppress in-stent neointimal formation. Low doses that do not suppress blood monocytes are ineffective in reducing neointimal formation, enabling early prediction of drug efficacy in modulating late vascular repair. Innate immunomodulation targets the triggering events in vascular repair that subsequently lead to in-stent restenosis and should be further evaluated in patients undergoing percutaneous coronary interventions.

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Figure 5. Composition of neointima 28 days after stenting. A–D, Low- and high-power photomicrographs of arterial sections from control (A and B) and LA-treated hypercholesterolemic rabbits (C and D), immunostained for RAM-11 (red) and SMC α-actin (brown). E, Bar graph showing reduction in both stained layers areas after treatment. F, Bar graph showing effect of LA on relative percentage of SMCs and macrophages in neointima (n=6, *P*<0.05).
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