Macrophage Depletion by Clodronate-Containing Liposomes Reduces Neointimal Formation After Balloon Injury in Rats and Rabbits

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Background—Inflammation is critical to vascular repair after mechanical injury. Excessive inflammation enhances neointimal formation and restenosis. We examined whether transient systemic inactivation of macrophages at the time of vascular intervention could attenuate the degree of expected restenosis.

Methods and Results—Liposomal clodronate (LC) inhibited the growth of cultured macrophages but had no effect on endothelial or smooth muscle cells and suppressed neointimal hyperplasia in hypercholesterolemic rabbits and rats after intravenous administration of LC, with no adverse effects. LC treatment reduced the number of blood monocytes and decreased macrophage infiltration in the injured arteries as well as smooth muscle cell proliferation, interleukin-1β transcription, and production and matrix metalloproteinase-2 activity.

Conclusions—Macrophages play a pivotal role in vascular repair after mechanical arterial injury. Systemic inactivation and depletion of monocytes and macrophages by LC reduce neointimal hyperplasia and restenosis. (Circulation. 2002;106:599-605.)

Key Words: angioplasty  balloon  restenosis  monocytes  drugs

Inflammatory cells play a major role in vascular repair, recruited immediately after injury.1-2 Macrophage infiltration in atherectomy tissue and the activation status of blood monocytes correlate with an increased rate of restenosis.3,4 The effects of macrophages in restenosis probably are caused by their capacity to express numerous growth factors, cytokines, and enzymes that contribute to the development of restenosis.5 Thus, we hypothesized that systemic inactivation of monocytes and macrophages may lead to attenuation of neointimal formation and that macrophages play a pivotal role in the pathogenesis of restenosis.

Macrophage depletion can be achieved with the systemic injection of liposomes containing clodronate.6 Clodronate belongs to the family of bisphosphonates (BPs), bone-seeking agents that are potent inhibitors of osteoclasts. Like other BPs, clodronate has poor cell membrane permeability.7 Liposomes are readily taken up by cells of the reticuloendothelial system, in particular macrophages. Liposome-mediated delivery of clodronate inactivates and kills macrophages after effective phagocytosis8 but is not toxic to nonphagocytic cells.6

Methods

Liposomes

Clodronate (Sifavitor) and rhodamine RE (Avanti Polar Lipids) were encapsulated in liposomes composed of 50 μmol/L distearoylphosphatidylglycerol (DSPG) (Avanti), 100 μmol/L cholesterol (Sigma Chemicals), and 150 μmol/L of 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC) (Avanti) by reverse-phase evaporation technique, as described elsewhere.9 The average size of liposomes was 190±18 nm, 24.5 mmol/L, and 20 mmol/L, clodronate and lipids, respectively.

Validation of LC biological activity was determined on macrophage-like RAW 264 cells. LC but not free clodronate significantly reduced the number and proliferation of viable cells in a dose-dependent manner and did not affect smooth muscle cells (SMCs) or endothelial cells (EC) viability and proliferation at concentrations up to 500 μmol/L (data not shown), in accordance with the published data.8,10

Rabbit Model

New Zealand White rabbits (Harlan Laboratories, Jerusalem, Israel) weighing 2.5 to 3.5 kg were used in accordance with the guidelines for animal care of the Hebrew University of Jerusalem and National Institutes of Health (USA). Animals were fed an atherogenic diet of 2% cholesterol and 6% peanut oil, starting 30 days before angioplasty. Hypercholesterolemia was ascertainment (plasma cholesterol >1200 mg/dL). Animals were anesthetized with xylazine (7 mg/kg), ketamine (40 mg/kg), Heparin (200 U/kg), atropine (0.05 mg), and norflaxacin nitocinale (70 mg) were given. Balloon injury was performed on the left common carotid artery with a 3-mm angioplasty balloon catheter (Cordis, 2×1-minute inflation at 8 atm). Animals were randomly assigned to intravenous liposomal or free clodronate (15 mg/kg), empty liposomes, or buffer. An investigator blinded to the type of experimental group performed the experi-
ments. After euthanasia with pentothal, arteries were perfusion-fixed in situ with 150 mL of 4% formaldehyde solution (pH 7.4), processed for morphometric analysis, and stained with Verhoeff elastin staining, Mayer hematoxylin and eosin, and modified Movat pentachrome.

Rat Model
Male Sabra rats (Harlan Laboratories), weighing 350 to 420 g, were used. The rat carotid injury model was performed as described previously. Liposomal clodronate (15 mg/kg) was injected on days -1 and +6. Organs were harvested on day 14 and processed as described above.

Morphometric Analysis
Eight to 10 sections in each slide were analyzed by means of computerized morphometric analysis (NIH Image) by an investigator blinded to the type of the experimental group. The section with the greatest luminal narrowing by neointima was analyzed as previously described. The residual lumen, the area bounded by the internal elastic lamina (original lumen), and the area circumscribed by the external elastic lamina (total arterial area) were measured directly. The degree of neointimal thickening was expressed as the ratio between the area of the neointima and the original lumen (% stenosis) and as the ratio between the neointimal area to the area of the media (N/M). The degree of remodeling, constrictive (negative) and expansive (positive), and remodeling ratio (RR) was estimated by comparing the ratio of the total arterial area of the balloon-injured segment with that of an adjacent, noninjured reference segment.

Flow Cytometry
Anticoagulated blood (200 μL) was incubated for 30 minutes (4°C, in the dark) with mouse anti-human RPE-conjugated anti-CD14 (DAKO). FACS lysing solution (1:20 dilution) was added for 15 minutes. The residual cells were washed (×1500 RPM, 5 minutes, 4°C) in FACS medium (PBS, 1% BSA, 0.02% sodium azide) and suspended in 1 mL FACS medium for flow cytometry. Monocytes were identified according to their relative size, side-scattering, and fluorescence.

Distribution of Liposomes
Rabbits were injected with rhodamine-labeled liposomes (0.4 mg/kg) and LC or buffer at day −1 and euthanized at days +1 and +6. Blood monocytes were separated with the use of a Ficoll gradient (Sigma) and centrifugation (×1500 RPM, 5 minutes). Harvested tissues were rinsed in saline; sections were mounted on slides and observed by confocal microscopy (Zeiss LSM 410).

Immunohistochemistry
Explanted specimens were excised after brief saline perfusion and immediately frozen in OCT compound for cryosectioning (Ted Pella, Inc). Slides were deparaffinized, incubated with 1% H₂O₂ in methanol (10 minutes) to block endogenous peroxidase, and then with 10% horse serum PBS (20 minutes). Primary antibodies for rabbit RAM-11 (DAKO) or PCNA (PC10, DAKO) were applied for 1 hour at 37°C. Sections were then washed with PBS followed by biotinylated secondary antibody (horse anti-mouse IgG, Vector Laboratory) and an avidin-biotin-peroxidase complex (ABC Elite kit, Vector Laboratory) for 30 minutes each. Development of color was achieved by 5-minute exposure to 3,3'-diaminobenzidine tetrahydrochloride (DAB peroxidase substrate, Sigma Chemical Co) in the presence of peroxidase substrate (Sigma). Slides were slightly counterstained with Gill No. 3 hematoxylin (Sigma). Positive staining was evaluated under a microscope (Olympus, BX40) at 2×0.25 10×0.25 magnification and digitized video frames. The prevalence of macrophages was assessed as the mean percentage area occupied by positively stained cells in 5 to 6 high-power fields.

Figure 1. Hypercholesterolemic rabbit carotid artery 30 days after balloon injury. Photomicrographs of Movat pentachrome (a through d) and Verhoeff tissue elastin staining (e through h) of full-sized (a, c and e, g) and higher magnification (b, d and f, h) sections from untreated (control) and treated (15 mg/kg liposomal clodronate, days −1 and +6, n=12) rabbits. Control animals were treated with either buffer, free clodronate (equivalent doses), or empty liposomes and grouped as control (n=30). Note reduction in SMCs, foam cells, and extracellular matrix in the treated animal. i, Bar graph showing luminal, medial, intimal (IEL), and total (EEL) arterial areas and neointimal hyperplasia expressed as mean neointima-to-media area ratio (N/M).
IL-1β Production and Transcription

Separate groups of animals were used for these studies. Arteries and livers were homogenized in collagenase buffer and extracted IL-1β was measured using commercial ELISA kits (R&D Systems).

For reverse transcription–polymerase chain reaction (RT-PCR) analysis, RNA from the carotid arteries was extracted with the use of an RNA isolation kit (Life Technologies). Quality, size, and quantity of RNA were examined, and values of the bands were normalized to β-actin mRNA expression.13

Matrix Metalloproteinase-2 Activity

The supernatant of arteries homogenate in collagenase buffer was analyzed for collagenase activity. Samples were separated on gelatin-impregnated (1 mg/mL: Difco) SPS 8% polyacrylamide gels under nonreducing conditions, shaken 30 minutes in 2.5% Triton X-100 (BDH), incubated in collagenase buffer (16 hours, 37°C), and stained with 0.5% Coomassie G 250 (BioRad) in methanol/acetic acid/H2O (30:10:60). Band intensity was determined by computerized densitometry (Molecular Dynamics type 300A).

Statistics

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 cytokines over time were made with 2-way ANOVA analysis. Differences were termed statistically significant at P<0.05.

Results

Prevention of Postangioplasty Hyperplasia

Massive proliferation of SMCs and extracellular matrix formation was seen in control animals after balloon injury...
No significant differences were found between treatment by empty liposomes, saline, or free clodronate in solution (results pooled as controls). N/M ratio was 1.4/0.44 (Figure 1e), and luminal stenosis was 75/8%. LC (15 mg/kg, days 1 and 6) reduced N/M ratio to 0.66/0.2 (Figure 1c, d, and e) and luminal stenosis to 41/8%. Mild expansive remodeling occurred in both control (RR = 1.22/0.24) and LC-treated animals (RR = 1.29/0.25). The medial area, bone morphology, and mineral composition were not affected by LC treatment (data not shown). No overt infection and no detectable systemic side effects were observed.

To ascertain the effect of macrophage depletion in a nonhypercholesterolemic animal model (no foam cells) the rat carotid injury model was used. The marked neointima was suppressed by LC treatment, with no significant change in medial and total arterial area (Table).

**Mechanism of Action**

**Reduction of Blood Monocytes and Tissue Macrophages**

Baseline monocyte level was 2.8 ± 0.5% of white blood cells (WBC). Before surgery, 24 hours after LC injection, monocytes were sharply reduced to <0.2% of total WBC (Figure 2), whereas WBC count remained unchanged. Three days after surgery, blood monocytes were mildly increased to 3.5 ± 0.4% in control animals and 0.7 ± 0.7% in LC-treated animals, returning to baseline levels after 6 days (Figure 2c).

Liver and spleen macrophages were reduced by LC (RAM 11 staining) 6 days after injury (Figure 3). The positively stained area in the liver was significantly reduced from 21.5 ± 4% to 14.7 ± 2.9% and in the spleen from 33.3 ± 1.5% to 11.4 ± 3% in control and LC-treated rabbits, respectively. Similarly, decreased arterial RAM-11 staining for macrophages was observed in LC-treated rabbits 3 and 6 days after injury (Figure 4).

Reduction of monocytes and macrophages was also detected by injection of fluorescent liposomes (FL). Marked reduction of the fluorescent signal was observed in blood monocytes (as well as reduced number) and in the liver and spleen of LC-treated animals (Figure 5). FL were detected in injured but not in intact arteries. FL coadministered with LC significantly reduced the fluorescent signal in the injured arterial wall (Figure 5).

**PCNA, IL-1β, and Matrix Metalloproteinase-2**

The area positively stained for PCNA at 6 days after injury was significantly reduced from 5.6 ± 2.6% in control animals to 1.7 ± 1.3% in LC-treated rabbits (Figure 6, a and b). Neointima was scarcely observed at this early time point in which SMC proliferation is maximal.

Analysis of IL-1β levels in arterial tissue after injury revealed a bell-shaped pattern peaking at 6 days after injury and returning to basal levels after 30 days (Figure 7a), and a significant decrease of IL-1β levels was observed after 3 and 6 days in LC-treated animals. In control animals, IL-1β
mRNA transcription was stronger on day 3 than the weaker expression 1 day after injury, but both were significantly reduced by LC treatment (Figure 7b). IL-1β levels in the liver were also reduced after a single injection of LC on day 1, inclining to basal levels at 30 days (data not shown).

Arterial matrix metalloproteinase (MMP-2) activity increased after injury, exhibiting a bell-shaped pattern peaking at 6 days (292 ± 46) and returning to basal levels at 14 days (Figure 7c). LC significantly alleviated MMP-2 activity, and at day 6 it was only 52 ± 17.

Discussion

This study shows for the first time that inactivation of macrophages by systemic administration of liposome-encapsulated clodronate inhibits luminal loss after balloon injury in both rats and hypercholesterolemic rabbits. These results validate our hypothesis that macrophages play a pivotal role in the pathogenesis of accelerated arteriopathies. The observed increase in luminal area was achieved mainly through reduction in neointimal hyperplasia. A mild increase in expansive remodeling was also demonstrated, but its contribution to the difference in luminal area was minimal.

Clodronate belongs to the family of BPs, drugs used clinically in bone-related disorders including osteoporosis. Being highly hydrophilic and negatively charged, free BPs are almost incapable of crossing cellular membranes.3 BP uptake by bone-resorbing osteoclasts (originating as macrophages from blood monocytes) occurs when the cells engulf drug-coated bone.7 Neither free clodronate nor LC inhibited SMC or EC proliferation or neointimal formation. Effective and selective endocytosis of clodronate in macrophages is achieved by encapsulating clodronate in liposomes.9,10,14 After phagocytosis, lysosomal action disrupts the fatty bilayers of the liposome and free clodronate is released into the cell, causing irreversible functional damage and apoptosis.8,15 LC administration probably aborted the early phase response to injury, which is mediated by macrophage migration in response to MCP-1 expression.16 Injection of LC before injury sharply depleted blood monocyte (Figure 2) and macrophage number and activity in the liver, spleen, and injured arterial wall (Figures 3, 4, and 5). The reduction in monocytes available at the time of injury reduced intimal hyperplasia, perhaps similar to the effects seen with deactivation of blood monocytes by IL-10.17 Blocking monocyte/macrophage migration to the injured vessel from the lumen and/or adventitia (Figure 5) prevented the effects of these cells on SMC migration and proliferation.

IL-1β and MMP-2 levels were reduced in injured arterial segments after LC treatment. These major products of activated macrophages, secreted after arterial injury, contribute to the process of neointimal proliferation.18–20 Reduced SMC proliferation also may contribute to the reduction of IL-1β and MMP-2.21 Nevertheless, since LC do not affect SMCs and ECs and have no effect on fibroblasts,9 the macrophage probably is the primary target for LC. The inhibition of intimal hyperplasia in the rat model, with no foam cells in the artery, further supports systemic depletion of macrophages as the mechanism driving reduced SMC proliferation and neointimal formation. Taken together, this transient systemic immunomodulatory and anti-inflammatory effect reduced SMC migration and proliferation and arterial restenosis.
Inactivation of macrophages carries the danger of immunosuppression and infection. However, as in studies on Mac-1-deficient mice and rats, no overt infection was observed in our study with transient macrophage depletion. Blood monocytes fully recovered in this study 6 days after LC-induced depletion induce no long-term toxic effects. The clinical implications of transient, partial depletion of liver and splenic macrophages with systemic LC should be further examined in human trials.

A large number of reports of pharmacological attempts to inhibit intimal hyperplasia in animals were not translated into subsequent inhibition of restenosis in humans. The current study uses LC as an investigative tool to elucidate the role of inflammation in vascular repair and the possible value of modulating innate immunity in reduction of postinjury intimal hyperplasia. That the benefit was observed in two animal models, the rat and the hypercholesterolemic rabbit, with disparate injury and different degrees of inflammation, supports the major role of monocytes and macrophages in vascular repair and increases the predictive value for inhibition of restenosis in humans.

Macrophage-rich areas are prevalent in atherosclerotic lesions of patients with unstable angina and acute myocardial infarction, and macrophages probably mediate rupture of the atherosclerotic plaque and sudden onset of acute coronary syndromes. Further studies are warranted to examine whether macrophage depletion by liposomal bisphosphonates may confer a strategy for stabilizing other inflammatory-mediated vasculopathies and cardiomyopathies, including acute coronary syndromes.

In conclusion, administration of LC inhibited neointimal proliferation after balloon injury in the rat and in the hypercholesterolemic rabbit models. The suggested mechanism is systemic selective, transient modulation of monocyte/macrophage activity. Macrophages, although relatively meager in neointimal tissue, play a major role in the process of neointimal proliferation. Therefore, early modulation and inactivation of macrophages for 1 week after injury significantly decrease postangioplasty arterial narrowing at a later time.

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