Inhibition of Apoptosis Through Localized Delivery of Rapamycin-Loaded Nanoparticles Prevented Neointimal Hyperplasia and Reendothelialized Injured Artery

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Background—A significant fraction of vascular smooth muscle cells (VSMCs) undergo rapid apoptosis after balloon angioplasty. In this study, we tested the hypothesis that protecting VSMCs from undergoing apoptosis prevents the cascade of events that lead to intimal hyperplasia.

Methods and Results—Rapamycin-loaded gel-like nanoparticles (mean diameter, 54 ± 5 nm) were infused locally in a rat carotid artery model of vascular injury. The drug has both antiapoptotic and antiproliferative effects on VSMCs and hence was selected for the current study. Localized delivery of nanoparticles sustained the drug level in the target artery for >2 weeks; demonstrated significant inhibition of hyperplasia (intima/media ratio, 1.5 ± 0.02 versus 2.7 ± 0.6; P < 0.01); and most importantly, reendothelialized the injured artery (endothelium coverage: treated 82% versus control 28%). We also demonstrated inhibition of activation of caspase-3/7 enzymes in the treated artery, preventing VSMCs from undergoing apoptosis and subsequent infiltration of macrophages.

Conclusions—It may be postulated that the localized delivery of rapamycin inhibited apoptosis of VSMCs, minimizing the inflammatory response to the injury and, thus, creating conditions conducive to vascular repair (reendothelialization). Unlike stenting, which can lead to thrombosis and increased risk for in-stent restenosis, our approach could eliminate or minimize long-term complications because the injured artery undergoes a natural process of reendothelialization. (Circ Cardiovasc Intervent. 2008;1:209-216.)

Key Words: catheterization ● endothelium ● restenosis ● apoptosis ● nanoparticles

Balloon angioplasty is routinely performed to relieve arterial obstruction; however, a significant number of patients redevelop obstructions, a process known as restenosis. Although drug-eluting stents have considerably reduced the incidence of in-stent restenosis when compared with bare-metal stents in the short term, their long-term efficacy remains an unresolved issue. Reactions to drugs used to coat stents and inflammatory responses to the stent material itself often delay or prevent reendothelialization of the injured artery, thus rendering the surface of stents prothrombotic. It has also been reported that patients with stents develop fatal late thrombosis, the incidence of which was found to be more prevalent with drug-eluting stents than with bare-metal stents. This concern prompted many hospitals to review the use of drug-eluting stents, which is considered the main factor contributing to their delayed use in recent years. Furthermore, the risk of rebound of hyperplasia persists once the drug coating the stent becomes depleted.

It has been shown that a significant number of vascular smooth muscle cells (VSMCs) undergo apoptosis (in humans, 20% to 30%; in a rat carotid model, 60% to 70%) in the injured artery within 1 hour after angioplasty. We hypothesized that protecting VSMCs from undergoing apoptosis would prevent the cascade of events that lead to inflammation, cytokine response, and proliferation of VSMCs at the site of injury. This view is contrary to the general therapeutic strategy through which cytotoxic drug/gene therapy is used to counterbalance the proliferation of VSMCs that follows the vascular injury. However, further loss of VSMCs attributable to the use of cytotoxic drugs, in addition to the loss of cells that occurred because of angioplasty and apoptosis, leads to significant elastic recoiling of the artery; this results in reduced lumen diameter and sometimes causes collapse of the artery or an aneurysm subject to arterial rupture and bleeding. Moreover, greater loss of VSMCs could provoke a heightened body response to the injury (eg, accumulation of platelets and secretion of growth factors), resulting in exacerbated hyperplasia. In contrast, inhibiting apoptosis of VSMCs with rapamycin prevented the inflammatory response to the injury, resulting in inhibited proliferation of VSMCs and reendothelialization of the injured artery. This novel approach to vascular injury using local delivery of rapamycin may provide a new therapeutic strategy to prevent in-stent restenosis and complications associated with stent placement.
VSMCs to begin with could prevent this cascade of events, including the inflammatory response. This would not only inhibit hyperplasia but also may create conditions suitable for reendothelialization, a key factor for the long-term patency of the injured artery after an intervention.

We chose rapamycin to test our hypothesis because this drug is known to inhibit proliferation of VSMCs, primarily by cell-cycle arrest. More important, the drug has been shown to downregulate the genes responsible for induction of apoptosis in VSMCs. Rapamycin, however, is not water soluble and thus cannot be delivered intraluminally as a solution. We therefore developed water-dispersible gel-like nanoparticles (NPs) that were easily loaded with rapamycin. These loaded NPs were infused locally in the arterial wall immediately after balloon angioplasty using an infusion catheter. Because of their smaller size and gel-like flexible structure, our NPs were expected to result in efficient arterial catheter. Because of their smaller size and gel-like flexible structure, our NPs were expected to result in efficient arterial

Rapamycin-Loaded NPs

Cross-linked gel-like polymeric NPs were synthesized through random free-radical polymerization of N-isopropylacrylamide, N-vinyl pyrrolidone, and pegylated maleic polymers, as previously described. For rapamycin loading, 20 mg of lyophilized NPs was dispersed in 2 mL of distilled water by vortexing for 2 minutes, to (60% to 70%) after angioplasty. The results demonstrated hypothesis in a rat carotid artery model of vascular injury because this model shows significant apoptosis of VSMCs (60% to 70%) after angioplasty. The results demonstrated significant inhibition of intimal hyperplasia and increased reendothelialization of the injured artery.

Methods

Synthesis, Formulation, and Characterization of Rapamycin-Loaded NPs

Cross-linked gel-like polymeric NPs were synthesized through random free-radical polymerization of N-isopropylacrylamide, N-vinyl pyrrolidone, and pegylated maleic polymers, as previously described. For rapamycin loading, 20 mg of lyophilized NPs was dispersed in 2 mL of distilled water by vortexing for 2 minutes, to which 250 µL of methanolic solution of rapamycin (Sigma, 4 mg/mL) was added with constant stirring on a magnetic stir plate for 2 hours to allow drug entrapment into NPs. The unentrapped rapamycin was separated by overnight dialysis of the dispersion of NPs against 1 L of distilled water using a Spectrapor (Spectra/por Inc) dialysis bag (MW cutoff size, 12 kDa). Drug-loaded NPs were then lyophilized for 48 hours. Particle size distribution and zeta potential of NPs were determined using a ZetaPlus particle size and zeta analyzer (Brookhaven Instruments Corp). NPs were also viewed for size using a transmission electron microscope (Philips/FEI Inc) after negative staining with 2% (wt/vol) uranyl acetate solution. Drug release from NPs in vitro was determined in phosphate-buffered saline (154 mM/L, pH 7.4) containing 0.1% (wt/vol) Tween-80 at 37°C to maintain a sink condition, as described in our previous study. For rapamycin loading, 20 mg of lyophilized NPs was dispersed in 2 mL of distilled water by vortexing for 2 minutes, to which 250 µL of methanolic solution of rapamycin (Sigma, 4 mg/mL) was added with constant stirring on a magnetic stir plate for 2 hours to allow drug entrapment into NPs. The unentrapped rapamycin was separated by overnight dialysis of the dispersion of NPs against 1 L of distilled water using a Spectrapor (Spectra/por Inc) dialysis bag (MW cutoff size, 12 kDa). Drug-loaded NPs were then lyophilized for 48 hours. Particle size distribution and zeta potential of NPs were determined using a ZetaPlus particle size and zeta analyzer (Brookhaven Instruments Corp). NPs were also viewed for size using a transmission electron microscope (Philips/FEI Inc) after negative staining with 2% (wt/vol) uranyl acetate solution. Drug release from NPs in vitro was determined in phosphate-buffered saline (154 mM/L, pH 7.4) containing 0.1% (wt/vol) Tween-80 at 37°C to maintain a sink condition, as described in our previous study. For rapamycin loading, 20 mg of lyophilized NPs was dispersed in 2 mL of distilled water by vortexing for 2 minutes, to which 250 µL of methanolic solution of rapamycin (Sigma, 4 mg/mL) was added with constant stirring on a magnetic stir plate for 2 hours to allow drug entrapment into NPs. The unentrapped rapamycin was separated by overnight dialysis of the dispersion of NPs against 1 L of distilled water using a Spectrapor (Spectra/por Inc) dialysis bag (MW cutoff size, 12 kDa). Drug-loaded NPs were then lyophilized for 48 hours. Particle size distribution and zeta potential of NPs were determined using a ZetaPlus particle size and zeta analyzer (Brookhaven Instruments Corp). NPs were also viewed for size using a transmission electron microscope (Philips/FEI Inc) after negative staining with 2% (wt/vol) uranyl acetate solution. Drug release from NPs in vitro was determined in phosphate-buffered saline (154 mM/L, pH 7.4) containing 0.1% (wt/vol) Tween-80 at 37°C to maintain a sink condition, as described in our previous study.

Cell Culture and Antiproliferative Effect of Rapamycin-Loaded NPs

Human VSMCs (Cascade Biologics) at passage 3 to 4 were seeded in 96-well plates (4000 cells per well); cells were allowed to attach for 2 hours and were then treated with rapamycin. Medium in the well was changed on day 2 and every alternate day thereafter with no further addition of drug. Inhibition of cell proliferation was determined on day 8 using an MTS assay (CellTiter 96 AQ ueous, Promega). For cell-cycle analysis, cells cultured in T-75 culture flasks were incubated with a dispersion of rapamycin-loaded NPs in the growth medium (dose of rapamycin, 50 ng/mL). Two days after treatment, the cell monolayers were washed with phosphate-buffered saline, trypsinized, and resuspended in 1 mL of Telford reagent. The cellular DNA content was analyzed by a fluorescence-activated-cell sorter FACStarPlus flow cytometer operating under Lysis II (Becton Dickinson). For rapamycin loading, 20 mg of lyophilized NPs was dispersed in 2 mL of distilled water by vortexing for 2 minutes, to which 250 µL of methanolic solution of rapamycin (Sigma, 4 mg/mL) was added with constant stirring on a magnetic stir plate for 2 hours to allow drug entrapment into NPs. The unentrapped rapamycin was separated by overnight dialysis of the dispersion of NPs against 1 L of distilled water using a Spectrapor (Spectra/por Inc) dialysis bag (MW cutoff size, 12 kDa). Drug-loaded NPs were then lyophilized for 48 hours. Particle size distribution and zeta potential of NPs were determined using a ZetaPlus particle size and zeta analyzer (Brookhaven Instruments Corp). NPs were also viewed for size using a transmission electron microscope (Philips/FEI Inc) after negative staining with 2% (wt/vol) uranyl acetate solution. Drug release from NPs in vitro was determined in phosphate-buffered saline (154 mM/L, pH 7.4) containing 0.1% (wt/vol) Tween-80 at 37°C to maintain a sink condition, as described in our previous study.

Rat Carotid Artery Model of Vascular Injury and Morphometric Analysis of Artery for Inhibition of Hyperplasia

Male Sprague-Dawley rats (240 to 260 g; Charles River Laboratories, Wilmington, Mass) were anesthetized with an intraperitoneal injection of a ketamine (100 mg/kg)/xylazine (10 mg/kg) mixture, then underwent carotid artery angioplasty using a 2F Fogarty balloon catheter (Edwards Life Sciences). The balloon was inflated in the carotid artery sufficiently to generate slight resistance and was withdrawn 3 times consistently to produce endothelial denudation of the entire length of the left common carotid artery. On removal of the balloon catheter, a PE-10 catheter was inserted into the left common carotid artery to infuse a suspension of NPs (210 µL containing 60 µg of rapamycin-equivalent NPs) for >5 minutes at 2 atm of pressure (3 infusions of 70 µL each, with a 1-minute period between infusions). The distal and proximal ends of the common carotid artery and internal carotid artery were tied at the time of NP infusion. Five minutes after infusion, the ties were removed and blood flow was restored.

Three weeks after injury, the rats were euthanized and transcardially perfused through the left ventricle with 100 mL of heparinized saline followed by 4% paraformaldehyde, and arteries were collected in 10% formalin. After 24 hours, arteries were cut into 3 pieces, each piece cut every 2 mm from the proximal to the distal end. These pieces of arteries were embedded in paraffin for sectioning (5-µm thickness), and duplicate slides were stained with hematoxylin and eosin. The stained sections were used for morphometric analysis of medial, intimal, and luminal areas using computer-assisted image analysis (Image-Pro Plus 6.1, Media Cybernetics Inc).

Quantification of Rapamycin Levels in Arterial Tissue

To determine arterial drug uptake and retention, carotid arteries from both sides (injured/treated and uninjured contralateral) were removed at different time points after administration of NPs. Arterial samples were rinsed with saline, blotted dry with absorbent paper, and measured for wet weight. Next, the arteries were finely cut into small pieces and homogenized in 2 mL of distilled water using a tissue homogenizer (BioSpec Products Inc) at 1000 rpm for 2 minutes. The homogenates were then lyophilized for 48 hours. Drug from each dry tissue was extracted by shaking samples with 1 mL of methanol at 37°C for 48 hours at 150 rpm using an Environ orbital shaker (Fisher Scientific). The samples were centrifuged at 14 000 rpm for 10 minutes (Eppendorf, microcentrifuge, model 5417R, Brinkmann Instruments) to remove cellular debris. The supernatants were analyzed by high-performance liquid chromatography for rapamycin content. A standard plot was prepared using arteries collected from animals that did not receive rapamycin treatment to determine the recovery efficiency of the drug.

Immunohistochemistry

Animals were euthanized and arteries were collected postangioplasty at 1 hour for investigation of apoptosis and caspase activity, at 24 hours for recruitment of macrophages, and at 3 weeks for evaluation of reendothelialization. Frozen sections of 5-µm thickness were incubated with I-VIEW inhibitor to block endogenous peroxidase activity, washed with phosphate-buffered saline, and incubated with primary antibody for 1 hour at room temperature. The following primary antibodies were used: monoclonal mouse CD31 antibody (1:100 dilution; DAKO, Carpenter, Calif), anticeaved Caspase-3/7 (1:200 dilution; Cell Signaling Technology, Beverly, Mass), and monoclonal CD68 antibody (1:50 dilution; Clone proaglandin-M1, DAKO). Sections were then incubated with I-VIEW biotin and I-VIEW streptavidin-horseradish peroxidase. Sections were visualized using 3,3’ diaminobenzidine chromeogen and were counterstained using I-VIEW copper.

Reendothelialization was calculated as the percentage of luminal surface covered by CD31-positive cells 3 weeks after the angioplasty. In addition, in a separate experiment, the extent of Evans blue dye leakage (blue staining) was used as an index for endothelial cell lining integrity. The dye (0.5 mL of 0.5% solution, Sigma) was injected intravenously 30 minutes before euthanasia 3 weeks after
angioplasty, and carotid arteries were harvested after transpericardial perfusion with heparinized saline (20 mL). The arteries were fixed in methanol, carefully cut open vertically, and imaged using a flatbed scanner (HP Scanjet 3970, Hewlett-Packard Company).

For apoptotic cells, the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) method was used according to the manufacturer’s specifications (In Situ Cell Death Detection Kit, Fluorescein, Roche Applied Sciences). Nuclei were counterstained with propidium iodide. Sections were photographed on a Diaphot microscope (Nikon Instruments Inc) equipped with a phase-contrast and epifluorescence optics (×100) lens. The percent-ages of apoptotic nuclei were calculated by determining the number of propidium iodide-stained nuclei that were also positive for TUNEL staining (n = 9 sections per artery; n = 5 animals per treatment). Approximately 100 nuclei were counted for each section.

Caspase-3/7 Assay
Caspase-3/7 activity was determined in the tissue homogenates of arteries treated with rapamycin-loaded NPs versus control arteries (those infused with NPs that carried no drug, called “void” NPs). The animals were euthanized 1 hour after angioplasty and arteries were homogenized in a hypotonic cell lysis buffer. Caspase activity was measured by the Apo-ONE Homogeneous Caspase-3/7 Assay System (Promega). The intensity of emitted fluorescence (relative fluorescence units) was determined at a wavelength of 521 nm using a FL×800 Microplate Fluorescence Reader (BioTek Instruments Inc).

Statistical Analysis
All the data are presented as mean±SE of means. The statistical significance of differences among treatment groups was determined by 1-way analysis of variance. Differences were considered significant when P<0.05.

The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

Results
Characterization of NPs
Gel-like NPs were almost spherical in shape, typically ranging in size from 46 to 60 nm with a mean diameter of 54±5 nm (Figure 1A) and zeta potential (surface charge) of −8.45 mV at pH 7. The drug loading in NPs was 4.2% (w/w) (ie, 100 mg of formulation contained 4.2 mg of rapamycin) with an entrapment efficiency of 84% (ie, 84% of the added drug was trapped in NPs). The release profile of rapamycin from NPs under in vitro conditions demonstrated relatively rapid release during the initial stages (≈20% cumulative release in the first 24 hours) with more gradual release thereafter (>80% cumulative release in 28 days; Figure 1B).
Inhibition of VSMC Proliferation in Vitro

Rapamycin-loaded NPs demonstrated greater inhibition of VSMC proliferation than did rapamycin in solution during a dose-response study conducted 8 days after treatment (Figure 1C). The inhibitory effect of rapamycin was evident at as low as 1 ng/mL concentration, which was the lowest dosage evaluated in our study. Cells treated with void NPs showed almost similar cell growth as medium control, indicating their cytocompatibility (data not shown). Flow cytometry data of cells treated with rapamycin-loaded NPs revealed that their antiproliferative effect arose primarily because of inhibition of cell cycle progression at the G1 checkpoint. Approximately 75% of cells treated with rapamycin-loaded NPs were arrested in G0/G1 phase, when compared with 63% in void NPs (Figure 1D).

Arterial Drug Levels

Quantitative analysis of the arterial segments for total drug levels demonstrated an initial drop during the first 24 hours after infusion, but levels were maintained thereafter for 2 weeks before a slow decline (Figure 2). The drug was not detectable in the contralateral carotid artery, indicating localized delivery of the drug.

Inhibition of Hyperplasia and Reendothelialization

Morphometric analysis of the arterial sections from the animals treated with drug-loaded NPs demonstrated significantly reduced hyperplasia than in control animals that received void NPs (intima/media ratio, 1.5±0.02 versus 2.7±0.6; P<0.01; Figure 3A through 3C). The intraperitoneal administration of the same dose of drug-loaded NPs demonstrated no effect on inhibition of hyperplasia, suggesting that the localized arterial drug delivery was necessary to achieve the therapeutic dose of the drug in the target artery (data not shown). Inhibition of hyperplasia in the treated group also resulted in a significant increase in arterial lumen area than in the control group (Figure 3D). Saline control and void NPs demonstrated a comparable extent of hyperplasia, indicating that NPs themselves do not cause any change in proliferative response (data not shown). Therefore, all subsequent control experiments were carried out using void NPs. The group treated with rapamycin-NPs demonstrated significantly greater reendothelialization of the injured artery than control arteries (82% versus 28%; Figure 4A). This was further evident from reduced extravasation of Evans blue dye in rapamycin-treated arteries when compared with control arteries (Figure 4B and 4C).

Protection of VSMCs From Apoptosis

Histochemical analysis of arterial sections using the TUNEL assay demonstrated a significant reduction in the number of apoptotic VSMCs (treated 24% versus control 70%) in the medial layer of the artery at 1 hour posttreatment with rapamycin-loaded NPs (Figure 5A and 5B). In addition, a significant (P<0.01) reduction in the levels of active caspases was observed in the treated arteries when compared with control arteries, which was further confirmed by immunohistochemical analysis of the arterial sections from the 2 groups (Figure 5C and 5D). Together, these results indicate that rapamycin inhibited caspase activity in the injured artery and thus prevented VSMCs from undergoing apoptosis.

Inhibition of Infiltration of Macrophages

Analysis of the artery sections 24 hours after the angioplasty procedure indicates that rapamycin-loaded NPs reduced the number of macrophages infiltrating into the adventitial layers of the injured artery when compared with control (void) NPs (Figure 6). These results suggest that preventing apoptosis of VSMCs also reduces the inflammatory reaction of the body in response to vascular injury, but this reduction in macrophages could also be attributed to the antiinflammatory effect of the rapamycin in the injured artery itself.10

Figure 2. Tissue concentration of rapamycin in injured artery at different time points (1 hour, 1 day, 3 days, 7 days, 14 days, and 21 days) after localized delivery of drug-loaded NPs. Results are expressed as mean±SEM (n=3).
Discussion

Drug-eluting stents and catheter-based drug delivery are the 2 main strategies used to inhibit the excessive VSMC migration and proliferation that contribute to restenosis after balloon angioplasty.\textsuperscript{11,12} The advantage of drug-eluting stents is that they can provide localized drug therapy as well as a support structure for the injured artery to prevent the elastic recoiling that often occurs within 24 hours after balloon angioplasty.\textsuperscript{13} However, several recent studies have reported that patients with drug-eluting stents are vulnerable to developing late (up to 18 months) stent thrombosis, aneurysm formation, extensive inflammatory reaction (attributed to hypersensitivity to the coated polymer),\textsuperscript{14} and are at risk for restenosis once the drug coating is depleted.\textsuperscript{15} These results have thus renewed concerns about the long-term efficacy of drug-eluting stents.\textsuperscript{2} The underlying cause for such unintended effects is attributed to the inhibition or delay in reendothelialization of the stented artery.\textsuperscript{2} This delay could result from the drug used to coat the stent, which has direct contact with the endothelium, from the continued inflammatory response to the stent material itself, or both.\textsuperscript{2}

To avoid long-term complications after angioplasty, a therapeutic strategy that is focused on preventing hyperplasia, as well as promoting reendothelialization of the injured artery, would be more effective. Toward this goal, we have...
been investigating colloidal drug carrier systems that can be administered intraluminally in the target artery using an infusion catheter. The success of colloidal-based drug carrier systems depends on their ability to maintain a therapeutic drug concentration in the target artery to block proliferation of VSMCs. It has been reported that particulate drug carriers used in the coronary model have demonstrated that the drug level of 95% of the tissue weight of carotid artery segment 3.5 mg, which is 4-fold higher than that reported previously for other colloidal drug delivery systems. This greater efficacy of drug uptake with our gel-like NPs could be attributed to their size (smaller than those used previously; 54 nm versus 160 nm) and gel-like flexible structure that could penetrate the arterial wall more effectively than rigid and solid NPs. The smaller size of NPs is also important because particles smaller than ~100 nm can deposit in the vessel wall, whereas larger-sized particles accumulate primarily at the luminal surface of the artery.

The localization of NPs could also influence drug retention in the target artery. In our study, the rapamycin level in the target artery was 1.5 μg/mg at 1 hour postinfusion, and the level declined during the first 24 hours after infusion of NPs but remained sustained thereafter for >2 weeks. It is possible that the NPs localized in the intimal layer were washed away with blood flow after the initial localization, resulting in the initial decline in drug level. However, the NPs localized in the arterial wall were retained and able to maintain the drug level. In addition to direct localization of NPs into the arterial wall through the disrupted endothelium, a fraction of these NPs could have been deposited in the arterial wall through their transport through the vasa vasorum. These are small capillaries originating from the lumen of the artery with a network spreading to adventitia and media. Because our NPs are small and flexible, they could have passed through these capillaries and be deposited in the media and adventitia. This prolonged arterial drug retention could result from the sustained-release property of NPs in addition to the binding of rapamycin to certain cellular proteins, thus slowing its rate of diffusion. We could not determine the drug retention without NPs because of the insoluble nature of rapamycin, but others have shown that localized infusion of drug in solution results, within 24 hours, in rapid decline and complete loss of drug from the arterial wall.

The studies with rapamycin-coated stents in a porcine coronary model have demonstrated that the drug level of 95 ng/mg of artery weight, observed at 3 days after the stent deployment, was effective in inhibiting restenosis. The drug levels observed in our studies were above target level and hence can be considered as a local therapeutic dose to inhibit proliferation of VSMCs. It has been reported that particulate systems infused intraluminally migrate away from the intimal layer toward the medial layer over time because of blood pressure. Similarly, our NPs might have migrated, which is advantageous because the drug would not be in direct contact with the intimal layer to adversely influence the process of reendothelialization of the injured artery. This fact is important because rapamycin has been reported to suppress the adhesion of endothelial progenitor cells and their differentiation into endothelium. Because we observed significant reendothelialization of the injured artery, the drug localized in the artery was probably not interfering in the process, allowing vascular repair to occur by the natural mechanism.

One of the main reasons that stented arteries do not undergo reendothelialization very well is because of the direct contact with the intimal layer of the drug used to coat the stent, consequently either inhibiting the proliferation of endothelial cells or preventing adhesion and differentiation of circulating endothelial progenitor cells to the injured endothelium.

**Figure 6.** Immunohistochemical staining of arterial sections to assess the extent of infiltration of macrophages. Data are representative arterial sections from contralateral arteries (i and ii) and the injured arteries treated either with void NPs (iii and iv) or rapamycin-loaded NPs (v and vi). i, iii, and v represent macrophages stained with fluoresceinisothiocyanate-conjugated antibody; ii, iv, and vi represent counterstaining with propidium iodide to visualize nuclei. Counterstaining was used to confirm the presence of macrophages in the adventitial layer. Magnification ×200. Ad indicates adventitia; L, lumen.
In addition to the influence of drug carrier systems, the other important determinant is the mechanism of drug action. Most therapies are focused toward administering cytotoxic drugs or genes to combat the proliferative response; however, this approach has raised many concerns, including the long-term patency of the injured artery. Our approach was to protect VSMCs from undergoing apoptosis shortly after vascular injury and to prevent their proliferation. Thus, rapamycin seems to be the right choice of drug, not only because of its anti proliferative and anti inflammatory activities but also because it has been shown to downregulate the proapoptotic genes in VSMCs. Immunohistochemical analysis of the arterial sections collected at 1 hour after infusion of rapamycin-loaded NPs demonstrated inhibition of apoptosis in the treated artery (Figures 5A and 5B), thus supporting the above mechanism of rapamycin. This antiapoptotic effect of rapamycin has been found to be caused by inhibition of activation of Caspase-3/7 in the treated artery, which is a key mediator of apoptosis of mammalian cells (Figure 5C and 5D). Further, this reduction in the apoptotic cell population in the treated artery also successfully inhibited the infiltration of macrophages at the injured site (Figure 6), which is one of the well-known pharmacological effects of rapamycin. The cell-cycle analysis of the rapamycin-loaded NP-treated cells in vitro demonstrated inhibition of cell proliferation primarily by cell-cycle arrest in the G0/G1 phase (Figure 1D).

Other pathways, which we have not investigated in this study, could be critical to the inhibition of hyperplasia. These include the angiogenic effect of rapamycin or its effects on the mammalian target of rapamycin, which directly or indirectly controls several cellular events. Inhibition of cell proliferation with rapamycin is known to be mediated through its binding to the cytosolic receptor FKBP12, and the resulting complex inhibits the protein kinase mammalian target of rapamycin, which is a critical regulator of VSMC migration and proliferation. NPs could have facilitated the intracellular delivery of rapamycin, thus allowing its interaction with the FKBP12 receptor and resulting in inhibition of cell proliferation.

It has been shown in a porcine coronary model of restenosis that proliferation of VSMCs occurs primarily during the first 7 to 10 days after angioplasty. Therefore, it has been suggested that suppressing this proliferative phase is critical to the inhibition of restenosis. The prevention of hyperplasia observed in our studies could have been caused by the ability of NPs to maintain a therapeutic dose of the drug in the target artery to suppress this proliferative phase. Because of the complexity of the disease, it is a debatable question as to which events are critical to inhibition of hyperplasia—initial events such as platelet deposition and thrombosis, late events such as VSMC migration and proliferation, or both. Perhaps our NPs worked early by protecting VSMCs from undergoing apoptosis, which minimized the body’s response to injury (infiltration of macrophages) and later by inhibiting the proliferation of VSMCs, which reduced intimal hyperplasia. By protecting VSMCs from undergoing apoptosis, the therapy diminished hostile conditions, such as the inflammatory response and platelet aggregation, at the site of the arterial injury, possibly creating favorable conditions under which reendothelialization could occur.

Recently, Cyrus et al have demonstrated the efficacy of rapamycin-loaded \(\alpha,\beta\)-targeted paramagnetic perfluorocarbon NPs in inhibiting vascular stenosis in an atherosclerotic rabbit femoral artery injury model, suggesting the utility of the NP-based delivery system in a clinically relevant vascular condition. The polymer system used in our NP formulation has been extensively investigated for drug delivery applications, with a commercial product introduced recently for the delivery of paclitaxel in cancer treatment (Nanoxel, Dabur Pharma Ltd). The NPs used in our study are not biodegradable but could be made so easily by introducing disulfide linkages in the polymer chains such that the smaller polymer fragments formed by degradation would be eliminated. In addition to coronary and carotid artery restenosis, our approach could be effectively used to prevent the hyperplasia that is induced after interventions involving renal or femoral arteries.

In conclusion, our study demonstrates that the apoptosis of VSMCs that follows immediately after intra arterial balloon injury is intricately involved in vascular hyperplasia and that the inhibition of this apoptotic process with locally delivered rapamycin can abrogate the deleterious cascade of events, preventing neointima formation. Our system could also be used in other vascular conditions in which apoptosis of VSMCs is implicated, such as in aneurysms, cerebrovascular occlusive diseases, and atherosclerosis.

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Disclosures

V.L., M.R., and S.K. are coinventors on a pending U.S. patent application (20060134209 A1) that discloses the application of the composition of nanoparticles for vascular delivery of drugs. The technology has been licensed to a company by UneMed Corporation of the University of Nebraska Medical Center, Omaha. V.L., M.R., and S.K. have financially benefited from the licensing agreement. However, the work described herein was not supported by the company and has not influenced the results of the study.

References


**CLINICAL PERSPECTIVE**

Vascular interventions, such as balloon angioplasty or stenting, to relieve arterial obstruction trigger a cascade of events that lead to neointima formation and restenosis. One such early event is thought to be apoptosis of vascular smooth muscle cells (VSMCs) at the site of vascular injury. To test the hypothesis that inhibiting apoptosis of VSMCs mitigates the inflammatory response and hyperplasia, we infused rapamycin-loaded gel-like nanoparticles locally in a rat carotid artery model of vascular injury. Rapamycin is known to down-regulate the genes responsible for induction of apoptosis in VSMCs. Our results demonstrated significant inhibition of hyperplasia, and most important, we observed reendothelialization of the injured artery. We showed inhibition of activation of Caspase-3/7 enzymes in the treated artery that could have prevented VSMCs from undergoing apoptosis, and thus inhibited the subsequent infiltration of macrophages. This result suggests that apoptosis of VSMCs is intricately involved in vascular hyperplasia and that inhibition of apoptosis with locally delivered rapamycin can abrogate the deleterious cascade of events, preventing neointima formation. In the clinical scenario, one could infuse rapamycin-loaded nanoparticles locally immediately after balloon angioplasty using an infusion catheter or could precondition patients with such a protective therapy before intervention. The treatment could also minimize the elastic recoil of the injured artery that usually follows within 24 hours after angioplasty, caused by the loss of VSMCs. Unlike stenting, which can lead to thrombosis and increased risk for in-stent restenosis, our approach could eliminate or minimize such complications because of reendothelialization of the injured artery.
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