Targeted Antiproliferative Drug Delivery to Vascular Smooth Muscle Cells With a Magnetic Resonance Imaging Nanoparticle Contrast Agent

Implications for Rational Therapy of Restenosis

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Background—Restenosis is a serious complication of coronary angioplasty that involves the proliferation and migration of vascular smooth muscle cells (VSMCs) from the media to the intima, synthesis of extracellular matrix, and remodeling. We have previously demonstrated that tissue factor–targeted nanoparticles can penetrate and bind stretch-activated vascular smooth muscles in the media after balloon injury. In the present study, the concept of VSMC-targeted nanoparticles as a drug-delivery platform for the prevention of restenosis after angioplasty is studied.

Methods and Results—Tissue factor–targeted nanoparticles containing doxorubicin or paclitaxel at 0, 0.2, or 2.0 mole% of the outer lipid layer were targeted for 30 minutes to VSMCs and significantly inhibited their proliferation in culture over the next 3 days. Targeting of the nanoparticles to VSMC surface epitopes significantly increased nanoparticle antiproliferative effectiveness, particularly for paclitaxel. In vitro dissolution studies revealed that nanoparticle drug release persisted over one week. Targeted antiproliferative results were dependent on the hydrophobic nature of the drug and noncovalent interactions with other surfactant components. Molecular imaging of nanoparticles adherent to the VSMC was demonstrated with high-resolution T1-weighted MRI at 4.7T. MRI 19F spectroscopy of the nanoparticle core provided a quantifiable approach for noninvasive dosimetry of targeted drug payloads.

Conclusions—These data suggest that targeted paramagnetic nanoparticles may provide a novel, MRI-visualizable, and quantifiable drug delivery system for the prevention of restenosis after angioplasty. (Circulation. 2002;106:2842-2847.)

Key Words: restenosis • drugs • magnetic resonance imaging

Restenosis is a serious complication of coronary angioplasty that involves the proliferation and migration of vascular smooth muscle cells (VSMCs) from the media to the intima, the synthesis of extracellular matrix, and remodeling.1,2 Early local administration of numerous therapeutic agents into injured vessel walls poorly inhibited smooth muscle cell proliferation.3–12 Recently, newer stent-based drug-delivery systems, particularly devices incorporating hydrophobic antiproliferative agents, have been successful in the clinic.13–15 These promising results indicate that local deposition and prolonged release of appropriate antiproliferative agents can effectively ameliorate restenosis. These results highlight an opportunity for drug-delivery systems that achieve similar prolonged release of appropriate therapeutics directly within the balloon-injured vascular wall.

Ligand-directed nanoparticles are a novel, site-specific agent that can be administered locally and can penetrate into the tunica media via microfractures created by balloon overstretch injury. We have demonstrated that tissue factor (TF)–targeted nanoparticles specifically bind with high avidity to smooth muscle cell membranes in vivo16,17 and may be readily detected with intravascular ultrasound. Targeted nanoparticles bound to medial smooth muscle cells could also provide a unique vehicle to deliver antiproliferative chemotherapeutic agents, like those eluting off stents in the lumen, directly within the balloon-injured vascular wall.

In the present study, the potential of site-directed nanoparticles to incorporate and deliver potent lipophilic antiproliferative agents with different water solubilities, ie, doxorubicin (highly water soluble) and paclitaxel (poorly water soluble), is studied. Moreover, the unique potential to utilize T1-weighted MRI to visualize nanoparticle delivery and the

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opportunity to quantify local drug dosimetry with 19F-fluorine spectroscopy are demonstrated. Collectively, these experiments illustrate that targeted therapeutic nanoparticles could provide visualizable and quantifiable therapy to prevent restenosis after percutaneous revascularization.

Methods
Preparation of Perfluorocarbon Nanoparticles
A biontinated perfluorocarbon contrast agent was produced for in vitro studies by incorporating biontinated phosphatidylethanolamine (Avanti Polar Lipids) into the outer lipid monolayer of a perfluoro- carbon microemulsion.18 A 2% lipid surfactant comixture included doxorubicin (DXR) or paclitaxel (Taxol, Sigma Chemical Co) at concentrations of 0.0 mole%, 0.2 mole%, or 2.0 mole% and gadolinium diethylene-triamine-pentaacetic acid-bis-oleate (Gd-DTPA-BOA) at concentrations of 0 mole% or 20 mole% (Gateway Specialty Chemicals). The surfactant comixture of lecithin (Pharma- cia Inc), cholesterol (Sigma Chemical Co), Gd-DTPA-BOA, and biontinated phosphatidylethanolamine (1 mole%) was dissolved in chloroform, evaporated under reduced pressure, dried in a 50°C vacuum oven, and dispersed into water by sonication. The suspen- sion was combined with perfluoroctylbromide (Gateway Specialty Chemicals), safflower oil, and distilled, deionized water and was continuously processed at 20,000 PSI for 4 minutes with an S110 Microfluidics emulsifier (Microfluidics). With the use of a laser light-scattering submicron particle size analyzer (Malvern Instru- ments), particle sizes were determined in triplicate at 37°C to be nominally around 250 nm for the treated and control emulsions.

TF Antibody
A polyclonal antibody to the extracellular domain of recombinant porcine TF (derived from E. coli), residues 1 to 208 (molecular weight, 22.8 kDa), was generated in rabbits (by Eser Tolunay, Monsanto Company, St Louis, Mo). The antibodies were purified by application to a Hi-Trap Protein G column (Pharma- cia Biotech), safflower oil, and distilled, deionized water and was continuously processed at 20,000 PSI for 4 minutes with an S110 Microfluidics emulsifier (Microfluidics). With the use of a laser light-scattering submicron particle size analyzer (Malvern Instruments), particle sizes were determined in triplicate at 37°C to be nominally around 250 nm for the treated and control emulsions.

VSMC Proliferation Assays
In general, porcine aortic smooth muscle cells grown in Clonetics smooth muscle basal media as previously reported17 were incubated for 2 days and then exposed to treatments (below) for 30 minutes. Unbound nanoparticles and unabsorbed drug were washed from wells, and cultures were grown to ~85% confluence (ie, about 3 days). Attached viable cell numbers were counted. In experiment 1, VSMCs (n=9/treatment) were exposed sequentially to excess biontinated anti-TF antibody, avidin (Pierce Chemical Co), and then biontinated DXR nanoparticles that incorporated 0.0 mole%, 0.2 mole% or 2.0 mole% DXR into the outer 2% (wt/vol) surfactant layer. Positive control groups were exposed to equivalent amounts of DXR in buffer.

VSMCs were also grown on glass coverslips and treated with 0.0 mole% or 2.0 mole% DXR nanoparticles for histochemistry. Smooth muscle cells were stained with a routine hematoxylin-eosin procedure or for α-smooth muscle actin with a rhodamine-phalloidin immunofluorescence technique (Molecular Probes Inc).

In experiment 2, VSMCs (n=9 per treatment) were similarly treated with TF-targeted or nontargeted paclitaxel nanoparticles incorporating 0.0 mole%, 0.2 mole%, or 2.0 mole% paclitaxel. Unbound nanoparticles were washed from the VSMC culture wells. Attached viable cell numbers were counted as above and a subset of cultures was then fixed with 2% agarose and stacked for MR imaging.

In experiment 3, VSMCs (n=9/treatment) were similarly treated with TF-targeted or nontargeted DXR nanoparticles incorporating 0.0 mole%, 0.2 mole%, or 2.0 mole% DXR. Unbound nanoparticles were washed from the VSMC culture wells. Attached viable cell numbers were counted as above.

Dissolution Assays
The duration of paclitaxel and DXR release in vitro was estimated from dissolution studies. Duplicate samples of drug-laden nanoparticles containing 2 mole% paclitaxel (400 μg/mL nanoparticles) or 2 mole% DXR (353 μg/mL nanoparticles) were dialyzed with continuous agitation at 37°C using Spectra/Per regenerated cellulose membranes with 60 K MWCO against a sink volume of 0.9% saline (ie, supplemented with 0.2 mg/mL human serum albumin). Free drug concentrations within the media were analyzed in duplicate with reverse-phase high-pressure liquid chromatography (HPLC) methodologies for one week with complete replacement of the media daily to maintain sink conditions.

HPLC Analysis of Paclitaxel and DXR
Paclitaxel was determined by reversed-phase HPLC system using an absorbance detector measuring at 227 nm employing an acetonitrile (ACN) gradient (50% to 73%) to ensure the elution of lipophilic compounds (flow rate of 1 mL/min, column temperature 35°C). DXR was analyzed by reversed-phase HPLC with a Waters 474 fluorescence detector at lex=470/nm=555. A Vydac C18 reversed-phase Multi-Ring, 4.6×250 mm column with a mobile phase consisting of methanol and 0.01 mol/L phosphate buffer (65:35, v/v), pH adjusted to 2.96 with 19 mol/L potassium hydroxide was employed. The flow rate was 1 mL/min at ambient temperature.

MRI of VSMC Culture
MRI of the targeted and untargeted cells was performed at 4.7 T on a Varian INOVA system (Varian Associates). Spin-echo proton images were acquired with the use of a 3-cm custom-designed birdcage coil. Sagittal T1-weighted spin-echo images of the plating wells were acquired. Imaging parameters were: TR, 150 ms; TE, 30 ms; FOV, 4×4 cm; data matrix, 512×256; slice thickness, 1 mm, yielding an in-plane resolution of 156×156 μm. 19F signal was measured at 4.7 T by 19F MRI with the use of a 1.5-cm single-turn solenoid coil, dual-tuned to 1H and 19F. The imaging parameters were: TR/TE/α, 5 s/6.3 ms/90°; FOV 2 cm×2 cm×1 mm; image matrix 256 by 256.

19F-Fluorine Spectroscopy
Paramagnetic nanoparticles containing 0 mole% or 20 mole% Gd-DTPA were diluted serially from 50% to 1%. The 19F signal intensity of each dilution was measured spectroscopically at 0.47T on a Bruker MQ20 Minispec NMR Analyzer (Bruker Optics, Inc) and quantified with regard to reagent grade perfluorooctylbromide.

Statistical Analysis
VSMC proliferation data (ie, cells counts) were analyzed as a completely randomized block design (SAS Inc). Means for specific a priori hypotheses were tested with least significant difference procedure (P<0.05). Fluorine spectroscopy data were analyzed with general linear regression.

Results
TF-Targeted Drug Delivery
In experiment 1 the concept for local drug delivery was established. VSMCs were treated with TF-targeted nanoparticles containing 0.0 mole%, 0.2 mole%, and 2.0 mole% DXR or an equivalent amount of drug in buffer alone. After 30 minutes of exposure, washout, and 3 days further growth, the cellular proliferation of the control group was >85% confluent, and cells exhibited normal morphology and cytoskeletal architecture (Figure 1). Treatment with targeted DXR nanoparticles dramatically decreased the proliferation of smooth
muscle cells and disrupted the α-smooth muscle actin cytoskeleton of many surviving cells (Figure 1).

Three days after the 30-minute exposure, the antiproliferative effects of the targeted DXR and control nanoparticles and free DXR were quantified. As a percent of the control cell counts, DXR impaired cell proliferation in a dose-dependent manner (Figure 2). At the higher DXR dosage, cell counts decreased by more than half in both the free drug and targeted therapeutic nanoparticle groups. At the lower chemotherapeutic dosage, free DXR mildly decreased cell proliferation ($P<0.05$), but the targeted DXR nanoparticles markedly decreased cell counts ($P<0.05$) by $\approx40\%$. Thus, DXR incorporated into targeted nanoparticles manifest enhanced effectiveness as compared with free drug.

In a second series of studies, paclitaxel or DXR, at 0.0 mole%, 0.2 mole%, or 2.0 mole%, was incorporated into the surfactant layer of the nanoparticles (Figure 3). In contradistinction to the first experiment, VSMC cultures were exposed to control or drug-containing nanoparticles that were ligand-targeted to TF or nontargeted (lacking ligands to TF). In each set of experiments, control nanoparticles, whether targeted or nontargeted, exhibited no effect on smooth muscle cell proliferation. TF-targeting to VSMCs of either DXR or paclitaxel nanoparticles increased ($P<0.05$) antiproliferative effectiveness relative to nontargeted formulations. Nontargeted paclitaxel nanoparticles exhibited no effect on cell proliferation, whereas, nontargeted DXR nanoparticles decreased VSMC proliferation but less ($P<0.05$) than their targeted counterparts. The probable basis for the antiproliferative effect of the nontargeted DXR nanoparticles was reflected in the relative dissolution release profiles of the two formulations.

**Dissolution Studies**

The majority ($\approx75\%$) of DXR incorporated into the lipid membrane was noncovalently associated with surfactant components and released very slowly over seven days (Figure 4, top). However, $\approx25\%$ of the DXR payload was poorly retained within the nanoparticle and diffused out rapidly during the first day of dissolution. Paclitaxel did not have marked early release but rather emerged steadily from the nanoparticles at a rate of 9% per day over the seven-day study (Figure 4, bottom).

**High-Resolution MRI**

Unlike other drug-delivery systems, the nanoparticles have inherent acoustic reflectivity when bound to a surface and provide a prominent $T_1$-weighted MR signal when formulated with gadolinium. In the next experiment, TF-targeted or nontargeted paclitaxel nanoparticles were presented to VSMCs in culture as previously described. The incorporation of drug into the surfactant layer did not impact the paramag-
netic payload capacity of the nanoparticles, which easily incorporated more than 50,000 surface Gd-DTPA complexes each. Magnetic resonance images obtained at 4.7T clearly reveal that paramagnetic nanoparticles targeted to a monolayer of cells could be readily detected with T1-weighted spin-echo imaging despite a 100-fold partial volume dilution effect (Figure 5, bottom). Image intensity of the nanoparticle targeted VSMCs increased nearly 2-fold (0.062 ± 0.004) as compared with nontargeted cells (0.032 ± 0.002) (Figure 5, top).

Fluorine Quantification of TF-Targeted Nanoparticles
In addition, the presence of the nanoparticles bound to the cell surface was verified by detection of the fluorine signal with MR spectroscopy (Figure 6, top). This unique feature of targeted perfluorocarbon nanoparticles allows high-resolution MRI detection of the nanoparticles at the target site to be confirmed by independent colocalization of the fluorine signal, potentially eliminating confusion with other tissue components or spurious artifacts. Moreover, the fluorine signal amplitude (0.47T) was unaffected by the presence of surface gadolinium and was linearly correlated to perfluorocarbon concentration, which by direct inference could be related to nanoparticle number (Figure 6, bottom).

Discussion
We have previously reported that TF-targeted nanoparticles can specifically bind to smooth muscle cells in the tunica media of arteries after balloon overstretch injury. Deposition of nanoparticles within the media provides a unique opportunity to locally release hydrophobic antiproliferative agents, such as those successfully employed on eluting stents. In the present study, nanoparticles incorporating DXR, a watersoluble lipophilic compound, or paclitaxel, a highly hydrophobic drug, were targeted to VSMCs in culture and significantly inhibited their proliferation. TF-targeted paclitaxel nanoparticles with large payloads of paramagnetic chelates adhered to smooth muscle membrane surfaces were visualized with T1-weighted 1H MRI and detected with 19F spectroscopy at 4.7T.

Drug Delivery With Therapeutic Nanoparticles
Targeted delivery of nanoparticles significantly enhanced the potency of both DXR and paclitaxel nanoparticles. Nontargeted DXR nanoparticles released drug rapidly during the first day of dissolution and slowly thereafter. Early day 1 release of DXR correlated with antiproliferative effects noted in culture for the nontargeted DXR nanoparticles, but these were not apparent for the paclitaxel formulations. We hypothesize that effectiveness of therapeutic nanoparticles is dependent on their binding to and close apposition with the target membrane surface. Increased collisional interactions facilitated by ligand-directed binding likely promote the concomitant exchange of chemotherapeutic agents and lipids from the encapsulating monolayer to the target cell membranes. The transfer of membrane components between lipid vesicles and cell membranes has been well described.
and mathematically modeled by others, but this mechanism has not been effectively employed for drug delivery. Ordinarily, collision-mediated lipid exchange proceeds as a slow, concentration-dependent, second-order process of little consequence for circulating lipid particle systems. However, the present data suggest that direct binding of nanoparticles to cell surface receptors can increase collision-mediated lipid exchange efficiency by decreasing the activation energy barrier for the desorption of lipid molecules to the nearby cell membrane. In other words, ligand binding of the nanoparticles minimizes the equilibrium separation of surfaces and promotes the formation of collisional complexes with resultant interchange of lipids. The increased frequency and duration of these surface interactions can be expected to substantially enhance the net delivery of drug to the target cell.

A second element of nanoparticle drug-delivery mechanism is diffusion. The effectiveness of this release mechanism is also improved by the close proximity of the nanoparticle to the target cell. The contribution of this facet of the delivery mechanism is dependent on the chemical nature of the incorporated drug. Very hydrophobic drugs like paclitaxel are delivered less effectively by this route than are water-soluble compounds like DXR. Moreover, when hydrophobic compounds, like paclitaxel, diffuse from the nanoparticles into the surrounding extracellular interstitium, they may be better retained within the media and continue to inhibit proliferation or migration of neighboring cells.

**Molecular Imaging and Drug Quantification**

Magnetic resonance molecular imaging and drug quantification are unique advantageous features of targeted therapeutic nanoparticles in comparison with other delivery approaches. We have reported that TF-targeted nanoparticles can be delivered locally after balloon overstretch injury, bound to VSMCs within the tunica media of porcine carotid arteries, and visualized with intravascular ultrasound. The recent rapid developments in interventional MRI for vascular imaging and coronary artery stent placement highlight the opportunities to use targeted paramagnetic nanoparticles to delineate the depth and breadth of vascular balloon injury as well as to confirm and to quantify the delivery of nanoparticle-based drug therapy.

Targeted paramagnetic nanoparticles are easily visualized with MRI even at clinical magnetic field strengths (1.5T). This potent T1-weighted paramagnetic relaxivity stems from the high surface payloads of gadolinium chelates borne by each bound nanoparticle, which overcome the partial volume dilution effects that diminish the effectiveness of other targeted MR contrast agents. In the present study, TF-targeted nanoparticles bound to the surface of smooth muscle cells in culture occupy <1% of the imaging voxel volume yet produce a strong contrast signal. Moreover, by virtue of their perfluorocarbon core, the nanoparticles bound at a site can be detected in vitro, and in the future, they should be quantifiable with 19F MR spectroscopy in vivo.

**Prevention of Restenosis After Percutaneous Revascularization**

Recent successful eluting stent–based drug-delivery systems confirm that local therapy with appropriate antiproliferative agents can prevent restenosis. Moreover, these data suggest that nanoparticle drug-delivery systems, releasing similar agents directly from within the injured tunica media, may be equally effective, particularly for vessels less amenable to stent placement. Moreover, the intramural release of antiproliferative therapy presents additional opportunity to develop new stent formulations designed to augment endothelial growth and accelerate intimal healing. Rapid endothermalization of the lumen lining combined with a dampened medial cellular response to stretch injury may be an effective therapeutic combination.

**Limitations**

The present study presents concepts for using targeted therapeutic nanoparticles in the prevention of restenosis, but further research will be required to demonstrate this technology in animal models. Although nanoparticles were targeted to TF to demonstrate the concept, in clinical application alternative smooth muscle cell or extracellular matrix epitopes present within the tunica media in abundance at the time of balloon injury may be better molecular targets.

**Conclusions**

Ligand-directed nanoparticle technology could provide an alternative avenue for delivery of antiproliferative agents to prevent restenosis after angioplasty. Uniquely, these targeted paramagnetic nanoparticles afford the added potential for high-resolution MR molecular imaging of vascular injury concomitant with local dosimetry of antiproliferative therapy.


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