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Molecular Imaging of Stretch-Induced Tissue Factor Expression in Carotid Arteries with Intravascular Ultrasound

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Molecular imaging with targeted contrast agents enables tissues to be distinguished by detecting specific cell-surface receptors. In the present study, a ligand-targeted acoustic nanoparticle system is used to identify angioplasty-induced expression of tissue factor by smooth muscle cell within carotid arteries. Pig carotid arteries were overstretched with balloon catheters, treated with tissue factor-targeted or a control nanoparticle system, and imaged with intravascular ultrasound before and after treatment. Tissue factor-targeted emulsion bound and increased the echogenicity and gray-scale levels of overstretched smooth muscle cell within the tunica media, versus no change in contralateral control arteries. Expression of stretch-induced tissue factor in carotid artery media was confirmed by immunohistochemistry. The potential for abnormal thrombogenicity of balloon-injured arteries, as reflected by smooth muscle expression of tissue factor, was imaged using a novel, targeted, nanoparticulate ultrasonic contrast agent.

Key Words: Tissue factor, Angioplasty, Ultrasound, Molecular imaging

INTRODUCTION

In vivo noninvasive molecular imaging of dynamic cellular processes presents a new opportunity for diagnostic medicine. Novel *in vivo* biochemical imaging methods have emerged that allow visualization of intracellular chemical interactions (Tsein et al., 1998) detection of oligonucleotides (Tavitian et al., 1998) and, in this report, high-resolution acoustic localization of tissue factor expressed in carotid tunica media after balloon injury.

Tissue factor is a 43 kDa, transmembrane glycoprotein responsible for initiating the coagulation cascade (Broze et al., 1985; Scarpati et al., 1987). It plays important roles in embryogenesis (Carmeliet et al., 1996), angiogenesis (Ruf et al., 1996), and tumor cell metastasis (Mueller et al., 1998) and has been targeted as a pharmacological approach for destroying tumors *in vivo* by stimulating occlusion of tumor vasculature (Huang et al., 1997). Recently, I (St Pierre et al., 1999) and others (Marmur et al., 1993), have demonstrated that tissue factor protein is increased in the tunica media of arteries in response to overstretch injury and that specific inhibition of tissue factormediated coagulation attenuates neointimal thickening (Oltrona et al., 1997), which may be responsible, in part, for restenosis after balloon angioplasty. I have previously reported the development of a unique, multifunctional, site-targeted, emulsion-based contrast agent used to detect a broad range of intravascular molecular epitopes (Lanza et al., 1996). The agent is a perfluorocarbon emulsion (≈ 250 nm in mean diameter) that incorporates a biotinylated phospholipid into the outer surfactant layer. In the initial three-step process, systemic molecular epitopes were pretargeted with a biotinylated ligand and cross-linked through avidin/biotin interactions to the emulsion particle. Because perfluorocarbon emulsion nanoparticles have inherently poor acoustic reflectivity (Satterfield et al., 1993), except when bound and concentrated on specific targets, I have observed a strong increase in the acoustic signals from desired targets without increasing the background signal. Thus, I have reported large increased in the signal-to-noise ratio for acoustic imaging of arterial thrombi in dogs with the use of intravenous injection of the agent and binding of the three-step, fibrin-targeted contrast system.

Accordingly, the three objectives of this study were to develop a tissue factor-specific, targeted perfluorocarbon

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emulsion suitable for convenient, one-step administration; to determine whether the improved ligand-targeted acoustic nanoparticle system could infiltrate an injured carotid artery wall and afford sensitive ultrasonic detection of balloon overstretch-induced tissue factor; and to assess whether the targeted agent improves the differentiation of overstretch-injured arterial walls from uninjured vessels using routine intravascular ultrasound.

MATERIALS AND METHODS

1. Preparation of single-step prefluorocarbon emulsion

The perfluorocarbon nanoparticle contrast agent was produced by incorporating 1,2-dipalmitoy1-sn-glycero-3-phospjoethanolamine-N-4-(p-maleimidophenyl)butyramide (MPB-PE) into the outer lipid monolayer of the emulsion. The emulsion comprised perfluorodichlorooctane (40% wt/vol), safflower oil (2% wt/vol), a surfactant comixture (2% wt/vol), glycerin (1.2% wt/vol), and water (54.8% wt/vol). The surfactant comixture included lecithin (67.9 mol%), cholesterol (30 mol%), dipalmitoylphosphatidylethanolamine (2 mol%), and MPB-PE (0.1 mol%) and was dissolved in chloroform. The chloroform and lipid mixture was evaporated under reduced pressure, dried in a 50℃ vacuum oven overnight, and dispersed into water by sonication. The suspension was transferred into a blender cup with perfluorodichlorooctane, safflower oil, and distilled, deionized water and emulsified for 30 to 60 seconds. The emulsified mixture was transferred to a microfluidizer and continuously processed at 10,000 psi for 3 minutes. The completed emulsion was placed in a vial, blanketed with nitrogen, and sealed with a stopper crimp seal until use. A control emulsion was prepared identically, except a nonderivatized phatidylethanolamine was substituted into the surfactant comixture. Particle sizes were determined in triplicate at 37℃ with a laser light-scattering submicron particle size analyzer (Malvern Zetasizer 4, Malvern Instruments Ltd., Southborough, MA).

2. Development of tissue factor antibody

A polyclonal antibody to the extracellular domain of recombinant porcine tissue factor (derived from *E. coli*), residues 1 to 208 (Mw 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 (Pharmacia Biotech, Piscataway, NJ), equilibrated and washed with 20 mmol/L sodium phosphate at pH 7.0. The antibody was eluted with 0.1 mol/L glycine-HCL at pH 2.7 and neutralized with 1 mol/L Tris buffer at pH 9.0.

3. Preparation and isolaton of F(ab)' fragments

Anti-tissue factor F(ab)' fragments were generated and isolated using an immunopure F(ab)' preparation kit (pierce Rockford, IL). Briefly, anti-tissue factor IgG was dialyzed into 20 mmol/L, and digested by immobilized papain. Solubilized F(ab)' was purified from Fc fragments and undigested IgG protein using a protein A column. F(ab)' fragment were purified from excess cysteine using a G25 to 150 column and deoxygenated phosphate buffer (pH 6.7). Fraction identity was confirmed by routine sodium dodecyl sulfate-polyacrylamide gel electrophoresis procedures. An analogous emulsion using a nonspecific porcine IgG (Sigma, St. Louis, MO) was used to prepare control ligands with random specificities.

4. Conjugation of F(ab)' with MPB-PE derivatized emulsion

F(ab)' fractions were pooled and combined with the MPB-PE derivatized emulsion (1~2 mg F(ab)'/ml emulsion). The mixture was adjusted to pH 6.7, sealed under nitrogen, and allowed to react overnight at ambient temperatures with gentle, continuous mixing. The mixture was subsequently dialyzed with a 300,000 molecular weight-cutoff Spectra/Por DispoDialyzer (Laguna Hills, CA) against 10 mmol/L phosphate buffer (pH 7.2) to remove unconjugated F(ab)' fragments. The final emulsion was placed in a vial under nitrogen and stored at 4℃ until use. A nonspecific control emulsion was prepared using the control IgG F(ab)' fragments in the above protocol.

5. Preparation and targeting of tissue factor expressed on smooth muscle cells

Porcine aortic smooth muscle cell were grown in Clonetics smooth muscle basal medium with 5% fetal bovine serum at 37℃ in 5% CO_2 in 12 mm glass coverslips to near confluence. Smooth muscle cell identity was confirmed by a positive horseradish peroxidase reaction using antismooth muscle α-actin antibody as the primary ligand. The presence ofμtissue factor epitopes residing on the cell

surface was confirmed by immunohistochemistry. Smooth muscle cells on coverslips were incubated in triplicate with phosphate-buffered salind (PBS); 200 μl of control, unconjugated perfluorocarbon emulsion; 200 μl of tissue factor-targeted emulsion; or 250 μg of anti-tissue factor antibody for 1hour, followed by 200 μl of tissue factor-targeted emulsion.

6. Scanning electron microscopy

Smooth muscle cells on coverslips (n=12; 3 per treatment group) were fixed in 2% glutaraldehyde in PBS for 1 hour, rinsed briefly in PBS, and postfixed in 2% osmium tetroxide for 1 hour. The fixed and washed smooth muscle cells were dehydrated in ascending concentrations of ethyl alcohol, 10 minutes/step: 50%, 70%, 90%, and 3 ×100%, followed by three 15 minute washes in hexamethyldisilazane (Electron Microscopy Sciences, Fort Worth, PA). Scanning electron microscope (Phillips, Wilmington, DE). Five regions per smooth muscle sample were subjectively evaluated from ×2,500 to ×12,000 magnification and compared for nanoparticulate binding. Representative scanning electron micrographs from each treatment group were obtained.

7. Animal studies

Animal protocols were approved by the Animal studies Committee at Washington University. Briefly, pigs (n=5) weighing approximately 20 kg were anesthetized with telazol cocktail (1 ml/23 kg given intramuscularly), followed by 1% to 2% isoflurane in oxygen. Both common carotids were exposed aseptically, and the distal portion of each, proximal to the bifurcation of the internal carotid artery, was cannulated and connected to a Touhy adapter. The right femoral artery was exposed and an 8F sheath was inserted, through which a guide catheter and an 8×20 mm balloon catheter were directed to each carotid using an exchange wire. The balloon was inflated five times to 6 atm for 30 seconds, with 1 minute between inflations. Before the fifth balloon deflation, heparinized (10 units/ml) saline was infused through the distal Touhy access. As the catheter was slowly deflated for the final time and withdrawn from the common carotid, the residual blood within was flushed from the artery. When the catheter was free of the carotid artery, a proximal snare was placed to isolate the carotid segment containing buffer. The anti-tissue factor-targeted emulsion (0.5 ml) was infused into the right carotid artery and was allowed to incubate for 2 hours, the interval associated with increased tissue factor expression on injured smooth muscle cells. The artery was washed repeatedly with heparinized saline before intravascular imaging. The left carotid artery was exposed to the nonspecific control perfluorocarbon emulsion.

8. Intravascular ultrasonic imaging

Two-dimensional ultrasonic images were obtained using an HP Intravascular Ultrasound system (Andover, MA) and 30 MHz, 3.2F, 10° "forward-looking" Boston Scientific Sonicath CV catheters (Natick, MA). Images were obtained using nongated continuous acquisition with fixed gain and compression settings at a pulse repetition frequency of 30 MHz. Intravascular images were produced with 240 line lateral resolution and recorded onto sVHS tape for further analysis. The mechanical index was an unknown, fixed setting inherent to the HP Intravascular Ultrasound system. Still-frame images were obtained in triplicate for each animal, from both the control and targeted carotid arteries, before and after balloon overstretch injury. Still-frame images were captured with Adobe Premiere 4.2 (Adobe, San Jose, CA), and gray-scale levels within selected regions of interest were analyzed with NIH Image 1.61. Gray-scale levels obtained from NIH Image were subtracted from 256 to yield values for pure white of 255 and pure black of 0. Regions of interest of approximately one third of the arterial wall circumference were sampled for gray-scale analysis from each artery.

9. Immunohistochemistry

Ten vessel segments, five targeted with immunoemulsion and five control, were fixed in Molecular Biology Fixative (Streck Laboratories, Omaha, NE), embedded in paraffin, cut at 5 μm, and mounted on gelatin-coated slides. A slide from each vessel was stained with Verhoeff's-van Gieson's elastin stain to verify rupture of the internal elastic lamina as an index of deep vascular injury. For immunohistochemistry, tissue sections were deparaffinized with xylene hydrated with a descending ethanol series, and rinsed with PBS. Endogenous peroxidase activity was blocked by incubation for 30 minutes with hydrogen peroxide in methanol. Nonspecific binding of antibody was blocked by incubation for 45 minutes in PBS containing 1% bovine

serum albumin, 0.3% Triton X-100, 10% normal goat serum, and 1% normal pig serum. The sections were incubated overnight at 4℃ with the rabbit anti-pig tissue factor antibody diluted 1:100 in the blocking solution. After rinsing the slides with PBS, the sections were incubated for 45 minutes at room temperature with biotinylated goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA) diluted 1:200 with blocking buffer. The sections were stained with ABC (Histostain Plus, Zymed, South San Francisco, CA) and counterstained with hematoxylin.

10. Statistics

Gray-scale data were analyzed using analysis of variance or *t* tests as appropriate with the Statistical Analysis System (SAS, Cary, NC).

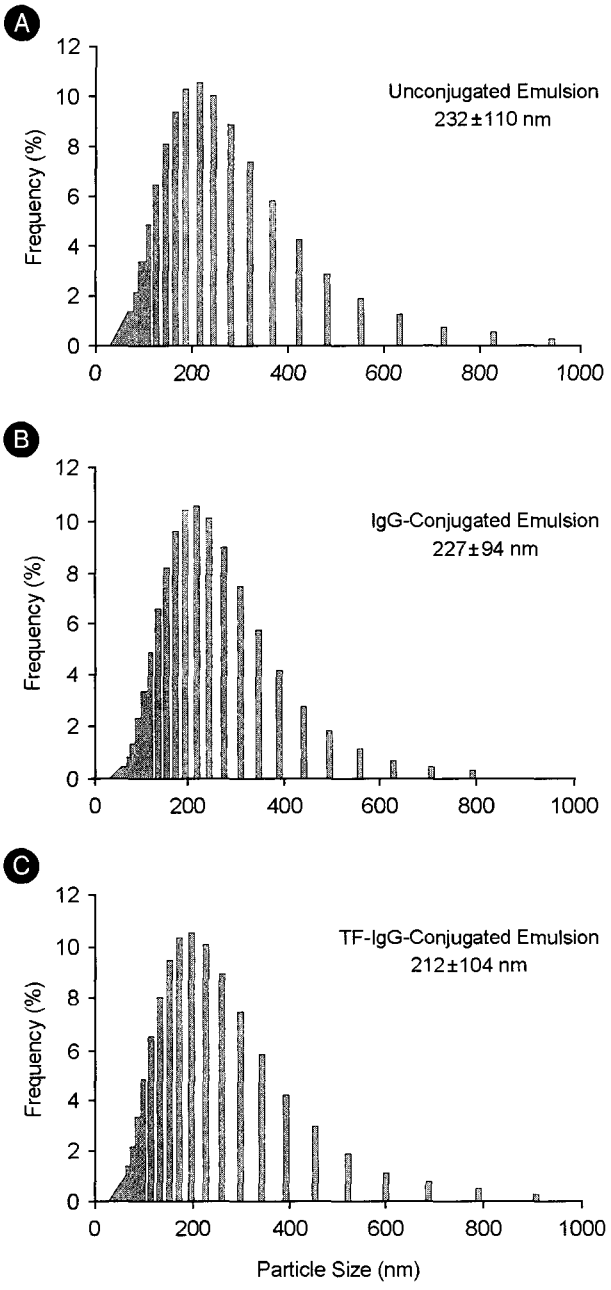

Fig. 1. Particle size distributions of emulsions. Unconjugated control (**A**). F(ab)' conjugated control (**B**). Tissue factor targeted (**C**).

RESULTS

A single-step, targeted perfluorocarbon emulsion (immunoemulsion) was produced by conjugating F(ab)' fragments to the emulsion layer through a thioether bond. The nominal particle size and distribution of the unconjugated control emulsion (232±110 nm) were similar to the nontargeting F(ab)' control (227±94 nm) and tissue factor-targeted emulsions (212±104 nm) (Fig. 1). The efficacy and specificity of the targeted nanoparticles were evaluated *in vitro* against the tissue factor cell-surface epitopes constitutively overex-

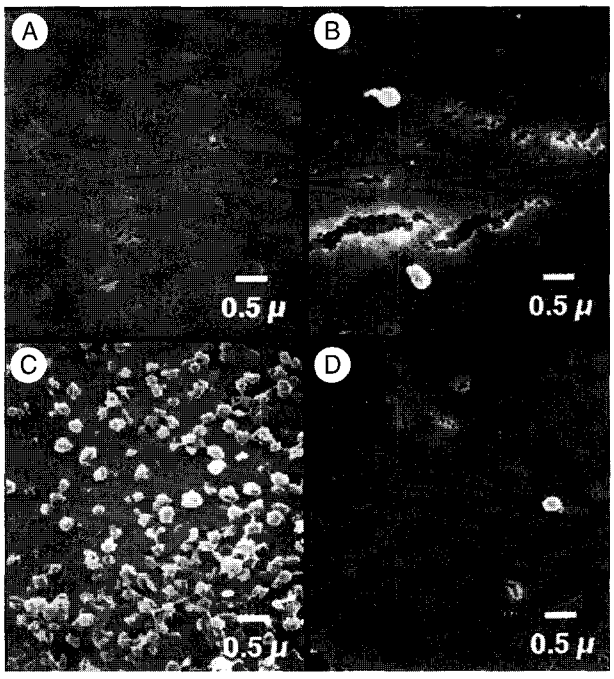

Fig. 2. Scanning electron micrographs (×2,000) of porcine aortic smooth muscle cells known to overexpress cell surface tissue factor in culture. Cells exposed to phosphate buffer as a control (**A**). Cells exposed to unconjugated control emulsion (**B**). Cells exposed to tissue factor-targeted immunoemulsion (**C**). Cells were treated with anti-tissue factor antibody, and subsequently exposed to tissue factor-targeted immunoemulsion (**D**).

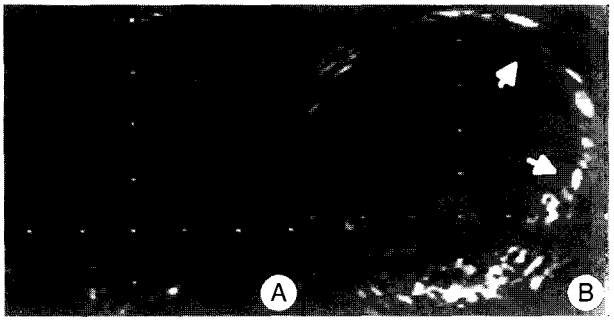

Fig. 3. High-frequency intravascular ultrasonic images of carotid arteries exposed to tissue factor-targeted or control emulsion nanoparticles after angioplasty. Control emulsion showing the lack of acoustic enhancement in the vessel **(A).** Tissue factor targeted. Bright acoustic enhancement in a portion of the vessel wall is consistent with nonuniform overstretch injury with the balloon **(B).**

pressed by porcine arterial smooth muscle cells in culture (Fig. 2). Subjective evaluations of the three replicate coverslip samples for each treatment group were highly concordant and unambiguous. Scanning electron microscopy of smooth muscle cells incubated with buffer alone (Fig. 2A) or a nontargeted perfluorocarbon emulsion revealed rare nonspecific binding of control nanoparticles (Fig. 2B). In contrast, the tissue factor-targeted agent bound abundantly to the peripheral surfaces of the smooth muscle cells (Fig. 2C), and this binding was specifically blocked by pretreatment with anti-tissue factor antibody (Fig. 2D). These electron micrographs reveal that the F(ab)' fragments coupled to the nanoparticles remained biologically active during processing. Moreover, the inununoemulsion bound to the cellsurface tissue factor epitopes as individual particles rather than as clusters of precipitate or aggregate. Immunohistochemistry can finned the identity of the smooth muscle cells and revealed the presence of tissue factor on the cell surfaces.

The immunoemulsion was used to detect the expression of tissue factor within the tunica media after balloon overstretch injury in pig carotid arteries. At baseline, before treatment with either tissue factor-targeted or nonspecifically targeted control emulsions, no differences in acoustic reflectivity within the walls of the balloon-injured arteries were apparent. In each animal, one overstretched carotid artery segment received a tissue factor-targeted perfluorocarbon nanoparticulate emulsion, and the contralateral vessel was exposed to the control, nonspecificaliy targeted perftuorocarbon agent. Injured carotid arteries treated with tissue factor-targeted nanoparticles and imaged with highfrequency intravascular ultrasound revealed a marked increase in acoustic backscatter from the smooth muscle cells of the tunica media (Fig. 3). The contralateral vessels given the control agent had no acoustic enhancement within the tunica media. Moreover, the control arterial walls remained unchanged from the baseline images. Acoustic enhancement within the tunica media was not circumferentially distributed, but was concentrated over one half to two thirds of the vessel wall, presumably in locations where noncircumferential force was applied by the angioplasty balloon. The marked acoustic enhancement noted in Fig. 3B was observed in each experimental animal.

Table 1. Average Gray-scale levels of stretched arterial walls before and after treatment with control or tissue factor-targeted perfluorocarbon emulsion contrast

Treatment	Gray-scale level	
	Preemulsion	Postemulsion
Control	70±5	65±4
Targeted	63±8	134±22

Gray-scale 0 = black; 255 = white.
Significantly different compared with either targeted preemulsion or control postemulsion ($P<0.05$)

Gray-scale analysis of still-frame intravascular images confirmed the visual impressions (Table 1). The targeted emulsion increased the arterial wall gray-scale levels (134 ±22; $P<0.05$) relative to pretreatment (63±8) and the control vessel wall gray-scale levels (preemulsion 70±5, postemulsion 65±4).

Immunohistochemistry of the targeted and control carotid arteries revealed a pattern of localization of tissue factor in all balloon-injured vessels in the media, the site of acoustic contrast enhancement, as well as in the adventitia, where tissue factor is normally expressed (Fig. 4A). Exclusion of the primary, anti-tissue factor antibody eliminated staining (Fig. 4B). No obvious dissections of the arterial walls were observed microscopically, although mild fenestration of the internal elastic lamina was noted.

DISCUSSION

These results indicate that the targeted agent maintained a small particle size after conjugation with F(ab)' fragment. The immunoemulsion bouud specifically to tissue factor epitopes that are expressed by porcine arterial smooth muscle cells *in vitro*. When administered within balloon-

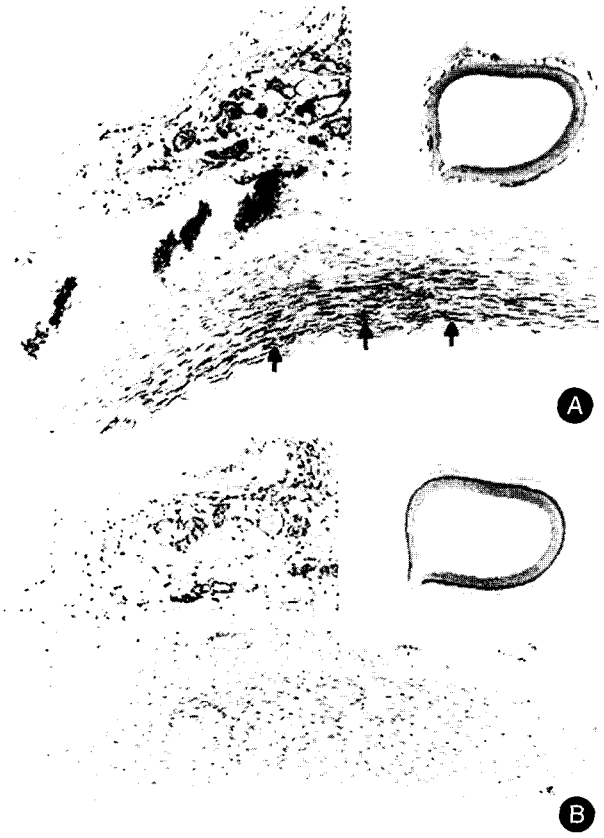

Fig. 4. Light micrographs showing immunohistochemistry for tissue factor in the excised, nanoparticle-targeted carotid artery shown in Fig. 3B. Staining for tissue factor is observed associated with smooth muscle cells in the tunica media (arrows) as well as around vessels of the vaso vasorum in the adventitia (**A**). In the absence of primary antibody, tissue factor staining is not observed (**B**).

injured carotid arteries, the targeted nanoparticles infiltrated the tunica media through the damaged endothelium and internal elastic lamina and bound to tissue factor epitopes expressed on myocyte membranes in response to the overstretch stimulus. Bound contrast within the tunica media markedly enhanced the acoustic reflectivity of the injured arterial walls. Although the control immunoemulsion could also penetrate the same barriers, the lack of a binding and concentrating mechanism within the tunica media allowed the unbound nanoparticles to be washed away or retained at an ineffective concentration for significant acoustic enhancement.

Targeted acoustic contrast agents, a burgeoning area of research and development, are expected to extend the diagnostic and therapeutic horizons of ultrasound imaging greatly. In 1996, our laboratory first reported *in vitro* and *in vivo* ligand-based targeting of an acoustic contrast agent using a perfluorocarbon emulsion nanoparticle targeted to fibrin in arterial thrombi (Lanza et al., 1996). The system markedly enhanced the acoustic reflectivity of clots imaged with routine clinical transducers (3.5~7.5 MHz) and dramatically increased the sensitivity and specificity of thrombosis diagnosis by independent image interpreters. Subsequently, I (Lanza et al., 1997), and others have demonstrated and characterized targeted acoustic contrast systems, including microbubble and nonmicrobubble-based systems. In addition, the expected dual use of these targeted contrast agents as targeted drug delivery systems has been conceptually demonstrated (Wu et al., 1998).

A major advance of the present experiment was that it involved a single-step process to deliver the emulsion to isolated arterial segments. Direct conjugation of the F(ab)' fragment did not increase particle size, did not reduce ligand bioactivity, and in pharmacokinetic studies did not markedly influence circulating half-life. The clear advantage of the one-step approach is its ease of administration in both the present setting and in a clinical setting. Future research will focus on the use of infusion catheters for local arterial wall delivery from a standard femoral approach, with shorter immunoemulsion incubation times. Coupling of a singlestep, tissue factor-targeted nanoemulsion technology with antiproliferative chemotherapeutic agents or antisense DNA may provide a clinically useful mechanism to address the issue of arterial restenosis after angioplasty. In a more general sense, combining imaging and drug delivery technologies could provide unique benefits by qualitatively ensuring adequate location and quantity of drug delivery, which for targeted drug delivery systems remains a fundamental therapeutic concern.

In summary, I have demonstrated the concept of molecular imaging with the use of a unique, one-step, ligandtargeted nanoparticle contrast system to allow the *in vivo* imaging of complex biochemical changes. As proof of concept, I have used the expression of overstretch-induced tissue factor within pig carotid arteries to demonstrate that our novel nanoemulsion can infiltrate into arterial walls after balloon injury, cross the internal elastic lamina, and bind and localize molecular epitopes in intramural tissue. Retention of the targeted nanoemulsion particles creates "acoustic staining" by increasing the ultrasonic backscatter at regions of tissue factor expression. Targeted molecular imaging has

the potential to elucidate other *in vivo*, complex physiologic or pathological processes.

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