Microbubbles Targeted to Intercellular Adhesion Molecule-1 Bind to Activated Coronary Artery Endothelial Cells

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Background—Preclinical atherosclerosis is associated with increased endothelial cell (EC) expression of leukocyte adhesion molecules (LAMs), which mediate monocyte adhesion during atherogenesis. Identification of cell-surface LAMs may uniquely allow assessment of endothelial function, but there are no in vivo methods for detecting LAMs. We tested a new microbubble designed to bind to and allow specific ultrasound detection of intercellular adhesion molecule-1 (ICAM-1).

Methods and Results—A perfluorobutane gas–filled lipid-derived microsphere with monoclonal antibody to ICAM-1 covalently bound to the bubble shell was synthesized. Bubbles with either nonspecific IgG or no protein on the shell were synthesized as controls. Coverslips of cultured human coronary artery ECs were placed in a parallel-plate perfusion chamber and exposed to 1 of the 3 microbubble species, followed by perfusion with culture medium. Experiments were performed with either normal or interleukin-1β–activated ECs overexpressing ICAM-1, and bubble adherence was quantified with epifluorescent videomicroscopy. There was limited adherence of control bubbles to normal or activated ECs, whereas a 40-fold increase in adhesion occurred when anti–ICAM-1–conjugated bubbles were exposed to activated ECs compared with normal ECs (8.1±3.5 versus 0.21±0.09 bubbles per cell, respectively, P<0.001). Although diminished, this difference persisted even after perfusion at higher wall shear rates.

Conclusions—A gas-filled microbubble with anti–ICAM-1 antibody on its shell specifically binds to activated ECs overexpressing ICAM-1. Diagnostic ultrasound in conjunction with targeted contrast agents has the unique potential to characterize cell phenotype in vivo. (Circulation. 1998;98:1-5.)

Key Words: cells ■ microspheres ■ cell adhesion molecules ■ endothelium

The earliest atherosclerotic lesions involve monocyte adhesion to endothelial cells, which is mediated by abnormal endothelial expression of specific LAMs. Upregulated LAMs on coronary ECs are a specific indicator of incipient endothelial disease, yet there are no methods to identify LAMs on EC surfaces of intact, living organisms. We recently showed that interactions between endothelial surfaces and albumin microbubbles used in the imaging technique of myocardial contrast echocardiography may yield insight into endothelial phenotype. The purpose of this investigation was to develop a method that uses ultrasound to study specific cellular features of coronary endothelium in real time. In the present study, a new ultrasound microbubble contrast agent conjugated to monoclonal antibody to human ICAM-1 is shown to bind specifically to activated human coronary artery ECs overexpressing ICAM-1 in a perfused cell culture model.

Methods

Perfusion Chamber

A previously described parallel-plate perfusion chamber was used to expose ECs to small volumes of microbubbles within a closed system. Coverslips with confluent EC monolayers were mounted in a 200-μm-high perfusion chamber. The chamber entry port was used to introduce microbubbles onto the endothelial surface. The exit port was connected via Silastic tubing to a syringe pump (Harvard Apparatus) set in the withdrawal mode to perfuse the chamber. The chamber was mounted on an inverted epifluorescent microscope (Axiovert 35, Zeiss) connected to a videocamera.

Cell Culture

Human coronary artery ECs (Clonetics Corp) were subcultured at 37°C on glass coverslips and grown to confluence over 4 days in 5% serum culture medium composed of endothelial basal media (Clonetics). To create inflammatory endothelium, 100 U/mL IL-1β (Sigma) was added to the cultures 4.5 hours before the experiment.

Microbubble Preparation

Perfluorocarbon gas–filled microbubbles were prepared with monoclonal antibody on the shell as the ligand for EC binding. Perfluorobutane (PCR Inc) was dispersed by sonication (XL2020 sonicator, Misonix) in aqueous medium containing phosphatidylcholine (Avanti Polar Lipids), a surfactant, a phospholipid derivative containing a carboxyl group, and a fluorescein derivative of
phosphatidylethanolamine (Molecular Probes) in a molar ratio of ~75:15:7:1. The perfluorobutane was encapsulated during sonication by a lipid shell carrying the fluorescent label. The carboxylic groups were exposed to the aqueous environment and used for covalent attachment of antibodies to the microbubbles as follows. First, unbound lipid dispersed in the aqueous phase was separated from the gas-filled microbubbles by flotation. Second, carboxylic groups on the microbubble shell were activated with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (Sigma), and antibody was then covalently attached via its primary amino groups with the formation of amide bonds. Unbound antibody was thereafter removed by flotation. Three different microbubble species were produced, containing either anti–human ICAM-1 IgG monoclonal antibody (Endogen), purified nonspecific mouse IgG (Sigma), or nothing (plain), on the shell. Microbubble diameter ranged from 1 to 10 μm, and concentration was 10^2 to 10^5 bubbles/mL.

**Experimental Protocol**

The EC coverslips were briefly exposed to 10^5 mol/L quinacrine dihydrochloride (Sigma) as a fluorescence label and mounted in the perfusion chamber, and the system was primed with culture medium. A volume of 0.2 mL of microbubbles, equivalent to the volume of the chamber, was injected into the system. Because of buoyancy typical of most gas-filled microbubbles, the chamber was inverted, with the ECs forming the roof of the chamber, to maximize exposure of cells to bubbles. After a 3-minute static exposure time, the chamber was perfused for 3 minutes with bubble-free culture medium (25 s⁻¹ wall shear rate) to wash away unbound bubbles. Experiments were performed separately with either normal or IL-1β-activated endothelium. Each coverslip was exposed to 1 of the 3 preparations: microbubbles with anti–human ICAM-1 antibody, or control microbubbles with either nonspecific IgG or no added protein on the shell (plain).

In a second series of experiments, normal (n=2 coverslips) or activated (n=3 coverslips) ECs were similarly exposed to anti–ICAM-1 microbubbles. Normal ECs were thereafter perfused for 4 minutes at a wall shear rate of 25 s⁻¹, followed by a 2-minute perfusion at 1000 s⁻¹. Activated ECs were perfused for 2 minutes at 100 s⁻¹, succeeded by another 2-minute perfusion at 1000 s⁻¹.

After each perfusion, 20 randomly selected fields (at ×1000) were interrogated with epi-fluorescent videomicroscopy, and the number of bubbles and ECs per field was counted. Selected coverslips were fixed in paraformaldehyde and prepared for multicolor fluorescent microscopy.

**Multicolor Fluorescent Microscopy**

Coverslips were fixed in 2% paraformaldehyde, washed in PBS, and permeabilized with 0.1% Triton X-100 (Sigma) in 2% paraformaldehyde for 5 minutes. To stain for F-actin, the coverslips were incubated with rhodamine-conjugated phalloidin (Molecular Probes) for 30 minutes and washed with PBS. To stain DNA, coverslips were incubated with Hoechst dye (Sigma) for 30 seconds and washed with PBS. Phalloidin binds to F-actin and was used to qualitatively indicate EC activation and define cell boundaries. Hoechst dye was used to identify the nuclei. The slides were mounted in Gelvatol (Monsanto) and placed onto a coverslip for light microscopy (Nikon FXA microscope).

**Selected Abbreviations and Acronyms**

- EC = endothelial cell
- ICAM-1 = intercellular adhesion molecule-1
- IL-1β = interleukin-1β
- LAM = leukocyte adhesion molecule
- PE = phycoerythrin

**Figure 1.** Adherence of 3 microbubble species to normal vs activated ECs. Adherence increased when inflamed ECs were exposed to microbubbles containing anti–ICAM-1. Adherence of other types of microbubbles to normal cells was minimal and comparable to that seen with activated cells.

**Flow Cytometry**

Flow cytometry was used to quantify cell-surface expression of ICAM-1 as previously described. Briefly, EC monolayers were treated with collagenase (0.2 mg/mL) and 2 mmol/L EDTA in PBS. The harvested cells were diluted with Medium 199 (Sigma), centrifuged at 250g for 6 minutes, and the pellet was incubated with PE-conjugated anti–human ICAM-1 murine monoclonal IgG (5 μg/mL, ImmunoTech) or nonspecific PE-conjugated murine IgG (5 μg/mL, Becton Dickinson) for 20 minutes at 4°C. The cells were washed with PBS and fixed in 1% paraformaldehyde in PBS. Samples were analyzed for fluorescence (FacScan, Becton-Dickinson), and 5000 events were captured for each sample (540 nm/L for PE).

**Statistical Analysis**

Results are expressed as mean±SEM. Data were analyzed with a 2-factor ANOVA with 2 levels of activation (unactivated versus activated) in the first factor and 3 levels of bubble type (plain versus nonspecific IgG–labeled versus anti–ICAM-1–labeled) in the second factor, with statistical significance defined as P<0.05. When a significant difference was found, comparisons were made by Student’s t-test, with Bonferroni criteria applied for multiple comparisons and statistical significance defined as P<0.01 (2-tailed).

**Results**

Data from 40 coverslips perfused at a wall shear rate of 25 s⁻¹ are expressed in terms of total number of adherent bubbles normalized to the number of cells observed per coverslip and are summarized in Figure 1. Binding of all microbubble types to normal ECs was minimal. There was a significant interaction between EC activation status and bubble type by ANOVA [F(2,34) = 7.11]. In post hoc analysis it was found that, as predicted, a greater number of anti–ICAM-1–labeled bubbles adhered to activated ECs (8.0±3.5) compared with normal ECs (2.0±0.9), P<0.001. In contrast, there was no difference in the number of “plain” microbubbles adhering to normal (0.04±0.02) versus activated (0.05±0.02) ECs (P=0.42). Similarly, bubbles containing nonspecific IgG had no preference for activated (0.04±0.02) versus normal (0.03±0.01) endothelium.

Figure 2 shows fluorescent micrographs of ECs exposed to nonspecific IgG–labeled bubbles (2A and 2B) or anti–ICAM-1–containing bubbles (2C and 2D) under normal conditions or after endothelial activation with IL-1β. EC nuclei appear blue, rhodamine-labeled F-actin filaments are red, and microbubbles exhibit green fluorescence.
Figure 2 shows no adherence of nonspecific IgG–conjugated bubbles to ECs under basal (2A) or activated (2B) conditions. There is scant adherence of anti–ICAM-1–containing microbubbles to ECs under basal conditions (2C) and extensive adherence of these bubbles to activated ECs (2D). There is dense rhodamine staining of F-actin in the IL-1β–stimulated cells, indicative of activation. Figure 2E shows a single activated EC with multiple adherent anti–ICAM-1 microbubbles.

In the 3 coverslips with activated ECs exposed to anti–ICAM-1 microbubbles and perfused sequentially at 100 and 1000 s⁻¹, the number of adherent microbubbles decreased from 2.6±0.3 to 0.8±0.4, respectively (P=0.04). There was minimal adherence of the targeted microbubbles to normal ECs on the 2 coverslips perfused successively at 25 s⁻¹ (0.2±0.0) and 1000 s⁻¹ (0.1±0.0, P=0.25). Despite the relatively small number of coverslips tested, binding of anti–ICAM-1 bubbles appeared to occur preferentially to activated ECs (0.8±0.4) compared with unstimulated cells (0.1±0.0) even at wall shear rates of 1000 s⁻¹ (P=0.08).

Flow Cytometry

The relative distribution for ICAM-1 binding (number of events versus fluorescent intensity) in normal and IL-1β–stimulated cells is shown in Figure 3, which indicates a shift in the extent of ICAM-1 expression after IL-1β stimulation. The percentage of ECs expressing ICAM-1 above background (nonspecific IgG binding) was increased (P<0.0001) after IL-1β exposure (83±5%, n=4 coverslips) compared with baseline (39±10%, n=5 coverslips).

Discussion

This study demonstrates that a microbubble conjugated to a ligand for a specific molecular epitope selectively adheres to a biological surface expressing this epitope. We show that a novel perfluorocarbon gas–filled microbubble incorporating monoclonal antibody to human ICAM-1 on its outer shell preferentially binds to activated human coronary artery ECs overexpressing ICAM-1. To the best of our knowledge, this is the first demonstration of targeted microbubble attachment to cells expressing a surface protein seen in early atherosclerosis. These data raise the possibility of in vivo ultrasound imaging to identify cell markers that are pathognomonic of disease states and that are not otherwise identifiable antemortem.

Targeted Binding of Microbubbles

A 40-fold increase in the extent of bubble adhesion occurred when activated ECs were exposed to anti–ICAM-1–conjugated microbubbles. The lack of adherence of plain microbubbles to normal or activated endothelium indicates that the components of the lipid shell have no
avidity for ECs. This observation and the fact that nonspecific IgG microbubbles also do not adhere indicate that anti–ICAM-1–conjugated bubble adhesion to activated cells is due to a specific interaction with ICAM-1. This interaction is further supported by flow cytometry data confirming upregulation of ICAM-1 in IL-1β–exposed ECs. Interestingly, there is limited binding of anti–ICAM-1–conjugated microbubbles to ECs under basal conditions, presumably because of low-level constitutive expression of ICAM-1 by normal ECs.6

Our data also suggest that adherence may decrease at the higher wall shear rates (1000 s−1) present in the microvasculature. Nonetheless, the number of targeted microbubbles binding to activated ECs is still greater than that binding to normal cells perfused even at this high shear rate. Thus, despite the high wall shear rates present at the microvascular level, differential adherence of targeted bubbles to activated ECs can occur. Importantly, at the wall shear rate of 100 s−1, which characterizes larger arteries, a substantial number of targeted bubbles continue to adhere to activated ECs.

Comparison With Previous Studies
Published data on site-specific ultrasound contrast agents are limited. Lanza et al7 enhanced the echogenicity of thrombi with a biotinylated particle designed to bind to “pretargeted” thrombus precoated with avidin-biotinylated antibody complexes. This strategy differs from ours, in that we conferred binding specificity by manipulating the ligand on the bubble, rather than the characteristics of the target itself. McCreery et al8 recently developed a microbubble that binds directly to thrombus. Another study described solid liposomes conjugated to antibody to fibrinogen binding to fibrin on glass and atherosclerotic plaque.9 Bloemen et al10 described a non–gas-filled liposome conjugated to antibody to ICAM-1 that bound to detached, suspended umbilical vein endothelial or bronchial epithelial cells. Flow cytometry was used to quantify liposomal attachment, but this approach could not distinguish between surface binding versus uptake of the liposome into the cells.10 A major difference between our study and those of others is that we were able to visualize the specific adherence of a gas-filled, targeted bubble to the surface of activated coronary ECs directly, in situ.

Study Limitations
The goal of this study was to establish proof of principle, and the experiment was thus designed to optimize conditions most favoring bubble adherence. Cultured coronary artery ECs were therefore used as an approximation of the in vivo situation,4 and cells were exposed to bubbles under static conditions and washed with nonpulsatile flow in the absence of a leukocyte-bearing environment. It remains to be determined whether similar binding would occur in vivo and be detected with ultrasound. In this regard, we recently reported that these bubbles (without attached antibody) produce a strong backscatter signal in the left ventricle when injected intravenously in dogs.11 We have also shown that similar, biotinylated bubbles attached to avidin-coated glass surfaces at surface densities of 3% and bubbles contiguous to the wall of saline-filled plastic bags in surface densities <0.8% can be detected by conventional ultrasound systems.11 Studies using both fundamental and harmonic imaging will be necessary to extend these findings to clinical settings.

Potential Future Clinical Applications
The development of an ultrasound imaging agent specific to a molecular marker of early endothelial disease could have implications for the identification of incipient atherosclerosis. Tissue-specific ultrasound contrast imaging could have applications to other disease states: the incorporation of different ligands on the bubble could allow ultrasound localization or characterization of components of atherosclerotic plaque, restenotic lesions, or transplant vasculopathy or could have other noncardiac applications in fields such as oncology.

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