Abstract

Objective: To determine whether serum complement C3 mediates adherence of albumin-encapsulated microbubbles to vascular endothelium in the development of atherosclerotic plaques.

Methods: Adherence of microbubbles to aortic endothelium was examined with scanning electron microscopy following intravenous injection of 20% intralipid in wild-type mice, genetic complement-deficient mice (C3−/−), and in pharmacologic C3-depleted wild-type mice. In a second experimental model, atherosclerotic plaque was induced in apolipoprotein E-deficient mice (apoE−/−), and adherence of microbubbles to atherosclerotic plaques was evaluated using fluorescent microscopy of fluorescein isothiocyanate-conjugated microbubbles. Finally, imaging of aortas was performed in eight rats (four JCR:LA-cp atherosclerosis-prone rats on high cholesterol diets; four controls) following intravenous albumin microbubble injections (PESDA) to determine whether microbubble adherence to the endothelium could be detected with low mechanical index pulse sequence schemes.

Results: Scanning electron microscopy confirmed the adherence of microbubbles to the endothelial cells of the aorta in wild-type mice following induction of hypertriglyceridemia but not in C3-depleted mice. Microbubble adherence to the endothelial surface of atherosclerotic plaque was confirmed in all apoE−/− mice (median 172 microbubbles/field; compared to a median of 3 microbubbles/field in cobra venom factor-treated apoE−/− mice; p<0.001). Low mechanical index ultrasound imaging detected microbubble adherence in all JCR atherosclerosis prone rats even in the absence of vasomotor or phenotypical evidence of endothelial dysfunction. The numbers of adherent microbubbles correlated with serum triglyceride levels, and were seen in conjunction with increased endothelial nitric oxide synthase activity.

Conclusions: Complement C3 binds to albumin-encapsulated microbubbles and mediates microbubble adherence to vascular endothelium both early and late in the atherosclerotic process.

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Keywords: Atherosclerosis; Complement activation; Echocardiography; Endothelial function; Ultrasound

1. Introduction

Inflammation has been shown to play a critical role in the pathogenesis of atherosclerosis. Atheromas have been characterized as chronic inflammatory lesions in which some components of the immune system are active [1]. In-vivo methods for assessing the degree of inflammation that is occurring within atherosclerotic plaques are currently limited. It has recently been demonstrated that albumin-encapsulated microbubbles adhere to arterial endothelium in the setting of endothelial dysfunction, and can be visualized with ultrasound using newly developed low mechanical index pulse sequence schemes [2]. One potential mechanism for microbubble binding to dysfunctional endothelium may be due to complement activation. Several studies have provided evidence that complement (C3) is an important mediator in both endothelial dysfunction and atherogenesis. Components of the
Terminal complement pathway are frequently found in human atheromas [3,4]. Furthermore, in advanced atherosclerotic plaque development, complement activity is increased, while complement inhibitor activity is unchanged [5].

We hypothesized that complement mediates the adhesion of microbubbles to atherosclerotic plaques. To test our hypothesis, we sought to characterize the in vitro interaction between C3 and albumin-encapsulated microbubbles using flow cytometry. The role C3 plays in the adherence of microbubbles to arterial endothelium was then determined in two different experimental models: the first one included induction of early endothelial dysfunction by transient hypertriglyceridemia in wild-type and in complement C3 deficient (C3−/−) mice. In the second animal model, the adhesion of microbubbles to established atherosclerotic plaque was evaluated in apolipoprotein E deficient mice (apoE−/−), in the presence and absence of selective C3 depletion. Finally, in a third animal model, low mechanical index imaging of aortas in rats predisposed to early atherosclerosis was performed following intravenous albumin microbubble injections, and correlated with endothelium-dependent vasodilator responses, triglyceride levels, as well as endothelial nitric oxide synthase activity.

2. Methods

2.1. Albumin-encapsulated microbubbles formulation

Perfluorocarbon-Exposed Sonicated Dextrose and Albumin (PESDA) microbubbles were prepared in our laboratory as previously described [6]. PESDA microbubble size by hemocytometry is 4.6±0.4 μm and mean concentration measured by a Coulter counter is 1.4×10^9 bubbles/ml. Fluorescein isothiocyanate (FITC)-labeled microbubbles were prepared as previously described [7]. A 10-ml sample of PESDA was allowed to stand for 3 h until a foamy bubble layer was formed at the top, and separated from the clear lower layer. Then, four ml of the lower layer was removed and mixed with 3 mg of 5-[(4,6-dichlorotriazin-2-γi amino)-fluorescein (Sigma-Aldrich, St. Louis, Missouri). The solution was returned to the vial, mixed with the foamy layer for 15 min, and allowed to stand for 2 h until separation into two discrete layers. This mixture was washed with an equivalent volume of unlabeled 5% human albumin to remove unbound FITC. For in vivo imaging studies in rats, the PESDA was diluted 10 fold with 0.9% Sodium Chloride and given as a 0.1 ml bolus injection.

2.2. Part I. In vitro determination of complement binding to PESDA microbubbles

The binding of complement to PESDA microbubbles was determined in vitro by flow cytometry. For this, 0.7×10^9 PESDA microbubbles were combined with 0.5 ml of mice serum, incubated at 37 °C for 15 min and washed twice with phosphate buffered saline (PBS). The serum-exposed microbubbles were then combined with goat anti-C3 monoclonal antibody (Gamma Biologicals, Inc., Houston, Texas) optimized at 1:400 dilution for 30 min, washed twice, and subsequently combined with an R-phycoerythrin conjugated secondary antibody (Becton Dickinson Pharmingen, San Diego, California) at 1:1000 dilution for 30 min. Aliquots of

Fig. 1. Example of low mechanical index imaging criteria for a retained microbubble. The reflective microbubbles at the vascular border on the longitudinally scanned image were present after clearance of the blood pool and stayed in the same location for at least four cardiac cycles (panel C and D) on the low mechanical index image (arrows).
0.7 \times 10^9 \text{ PESDA microbubbles were also incubated with serum and subsequently with R-phycoerythrin conjugated secondary antibody at dilutions of 1:1000 for 30 min. Control samples included the replacement of serum by phosphate buffered saline. Microbubbles were then analyzed on a FACSCalibur (Becton Dickinson, San Jose, California) which counted 50,000 microbubbles per sample to generate graphs of red fluorescent intensity.

2.3. Part II. PESDA binding to aortic endothelium in the setting of hypertriglyceridemia

All animal procedures were approved by the Institutional Animal Care and Use Committee at the University at Nebraska Medical Center and conformed with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). Six female wild-type mice (C57BL/6J; mean weight 28 \pm 2 g) and three female C3\(^{-/-}\) mice B6.12954-C3tm1Crr/J (Jackson Laboratory, Bar Harbor, Maine; mean weight 21 \pm 2 g) were compared. The mice received standard rodent chow and tap water ad libitum. In three of the wild-type mice, depletion of serum complement C3 was induced by intraperitoneal injections of 4 units of cobra venom factor (CVF, from Quidel Corporation, San Diego, California) diluted in 1.0 ml of 0.9% saline twice a day, starting 24 h before and in the morning of the procedure [8]. The other three wild-type mice received intraperitoneal injections of the same volume of 0.9% saline.

On the subsequent day, the animals were anesthetized by intraperitoneal injection of ketamine (80 mg/Kg) and xylazine (8 mg/Kg). Hypertriglyceridemia was induced by an intravenous injection of 20% intralipid (Fresenius Kabi Clayton, L.P., Clayton, North Carolina). Eighty \(\mu\)l of intralipid were diluted in 80 \(\mu\)l of saline and injected into the tail vein. This is the equivalent dose previously described to cause endothelial dysfunction, confirmed by impaired arterial vasoreactivity to acetylcholine [2]. After 10 min of the intralipid infusion, 100 \(\mu\)l of 10% PESDA microbubbles were bolus injected by tail vein. Samples of blood were withdrawn for measurement of the serum complement C3 levels, at a median time of 3 h after the last intraperitoneal injection of CVF. The mice were euthanized a minimum of 10 min after the intravenous PESDA injection to ensure that microbubbles had cleared from the blood pool. The adherence of microbubbles to aortic endothelium in the control wild-type, CVF-treated, and C3\(^{-/-}\) mice were evaluated by scanning electron microscopy (SEM).

2.4. Part III. Microbubble adherence to atherosclerotic plaque

The adherence of PESDA microbubbles to atherosclerotic plaque was evaluated in ten female apolipoprotein E deficient (apoE\(^{-/-}\)) mice (B6.129P2-Apoetm1Unc, Jackson Laboratories, Bar Harbor, Maine) [9]. To accelerate the atherosclerotic
process, these mice at a mean age of 13±1 weeks underwent surgical procedures to create endoluminal injury to the aorta using a 0.25-mm diameter angioplasty guidewire (Guidant; Advanced Cardiovascular Systems, Temecula, California), as previously described[10] All mice were continued on a 1.25% high-cholesterol diet following the injury procedure. After 15 weeks, elective depletion of serum complement C3 was induced in three of the apoE−/− mice with intraperitoneal CVF as described above. All mice were then anesthetized and 100 μl of 10% fluorescein-labeled PESDA microbubbles (FITC-PESDA) were injected into the tail vein in four apoE−/− mice, and in three apoE−/− mice pre-treated with CVF. Similar to the acute aortic injury, samples of blood were withdrawn for measurement of the serum complement C3 levels, at a median time of 3 h after the last intraperitoneal injection of CVF. Mice were then euthanized a minimum of 10 min after FITC-PESDA injections to ensure that microbubbles had cleared from the blood pool. Three apoE−/− mice did not receive microbubbles and served as controls. The adherence of microbubbles to aortic endothelium was evaluated by laser fluorescent microscopy.

2.5. Part IV. Low mechanical index ultrasonic imaging of atherosclerosis prone rats using intravenous PESDA

Four JCR: LA-cp rats were obtained (Charles Rivers Laboratories, Inc., Wilmington, MA). This rat is leptin receptor negative, and has been shown to develop obesity, insulin resistance, and hypertriglyceridemia, resulting in the eventual spontaneous development of atherosclerosis [11]. The JCR rats were received at 10 weeks of age, fed a 1.0% high-cholesterol diet and were 15 to 18 weeks of age at the time of the experiment. The adherence of PESDA microbubbles to aortas was evaluated in four JCR rats at this age and four control Sprague–Dawley (SD) rats (SASCO laboratories, Portage, MI). SD rats were received and fed normal rat chow. Serum triglyceride levels were measured in all rats at the time of study. Rats were anesthetized with a subcutaneous injection of ketamine (80 mg/kg) and xylazine (10 mg/kg) and the airway supported via a cutdown tracheotomy. For intravenous (IV) access, a 22 gauge catheter was placed into the left femoral vein via a cut down procedure. Via an open peritoneal procedure the proximal abdominal aorta was isolated and a 22 gauge angiocath placed proximal to the imaging location for intra-aortic infusion of incremental acetylcholine (ACH) at 10−8, 10−7, and 10−6 molar concentrations. A high frequency ultrasound imaging probe (7 MHz; Siemens Acuson Sequoia) was placed in a water bath at a standoff of three centimeters to measure aortic longitudinal diameter and pulsed Doppler flow velocities pre- and post-ACH infusion in both JCR and SD rats. Microbubble adherence to endothelium was determined utilizing a high frequency low mechanical index pulse sequence scheme (Contrast Pulse Sequencing; Siemens 15L8, Siemens Acuson). This sequence scheme is able to detect very small quantities of retained microbubbles without destruction when imaging at <0.2 mechanical index and at frame rates of once every four cardiac cycles (1:4 triggered imaging). Longitudinal images of the abdominal aorta were taken at baseline and at 3 to 6 min following an IV injection of PESDA (0.1 ml of a 1:10 dilution) after clearance of microbubbles from the blood pool. Retained microbubbles were defined by an independent experienced reviewer (F.X.) as small circular reflectors adherent to the aortic wall which

<table>
<thead>
<tr>
<th>Number of microbubbles</th>
<th>Wild-type non CVF-treated</th>
<th>Wild-type CVF-treated</th>
<th>C3 deficient</th>
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<tr>
<td>Wild-type non CVF-treated</td>
<td>19±10⁎</td>
<td>2±2</td>
<td>1±1</td>
</tr>
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Data are expressed as mean and standard deviation. CVF = cobra venom factor. *p<0.0001 compared to control group.
persisted in the same location along the vessel wall for at least four cardiac cycles (Fig. 1). After imaging was completed, the same dose of PESDA was again injected without imaging. At a minimum of 10 min following this last injection, the animals were sacrificed. The aorta was harvested and processed for SEM analysis of retained microbubbles. Sections of the aorta were also obtained for light microscopy and immunohistochemistry as described below. Heart, lung, spleen and liver were also harvested for histological evaluation.

2.6. Processing and analysis of aortic tissue

After euthanasia, the abdominal aorta was immediately excised, washed in 0.9% saline and fixed in 2% formalin for a minimum of 30 min. The vessel was immediately cut along its long axis under surgical microscopy. For SEM, the samples were dehydrated through a series of ethanol concentrations increasing from 50 to 100%, immersed in Freon 113, critical point dried, mounted on aluminum stubs and sputter-coated with gold (Polaron E5100, Polaron Inc., Hertfordshire, United Kingdom). Specimens were viewed under a JEOL scanning electron microscope (Japanese Electronic Optical Systems, Tokyo, Japan). Adherent microbubbles were characterized at scanning electron microscopy as a less electron dense structure, with sizes ranging from 1 to 5 μm, spherically shaped (differentiating them from the biconcave-shaped erythrocytes), and not exhibiting any of the surface characteristics of hematopoetic cells, such as microvilli or microridges [2]. The number of microbubbles adherent to aortic endothelium of each animal (control wild-type, CVF-treated, and C3−/− groups in the setting of hypertriglyceridemia, and JCR and SD rats) were quantified by an independent reviewer at 500× magnification. The entire extent of aorta was analyzed in each animal, and bubbles were counted in multiple fields. The results were expressed as the maximum number of microbubbles/500× field.

In the atherosclerotic plaque mouse model, the number of FITC-PESDA microbubbles adherent to aortic endothelium was determined in one focal plane using a standard confocal laser scanning microscope (Zeiss LSM 410, Goettinger, Germany), starting from the surface going through the endothelium and superficial layers of the vessel. To reconstruct the vessel endothelium, images at all vessel depths were combined using three-dimensional projection software. For each aortic sample, adherent microbubbles were counted using a 40x magnification in multiple fields. The results were expressed as the median of five fields that presented the higher number of bubbles. All measurements were performed by individuals who were blinded as to what injections the animals received. Scanning electron microscopy (using the protocol described above) was also performed to confirm the presence of microbubbles adherent to atherosclerotic plaques. Masson Trichrome or hematoxylin eosin staining was performed to confirm the presence of an endothelial layer over the surface of the atheroma.

Paraffin embedded JCR and SD rat tissues (aorta, heart, lung, spleen and liver) were sectioned and processed for eNOS and ICAM immunohistochemistry (IHC) staining. IHC for eNOS was performed with a rabbit anti-rat polyclonal antibody at 1:100 dilution (ABCAM, Cambridge, MA). IHC for ICAM-1 (CD54) was performed with a mouse anti-rat monoclonal at 1:100 dilution (BD Pharmingen, San Jose, CA). IHC sections were blindly scored for the

Table 2

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<tr>
<th>Complement (C3) levels before and after treatment with cobra venom factor in wild type and atherosclerotic apoprotein E deficient mice</th>
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<tr>
<td>Non CVF-treated</td>
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<tr>
<td>Wild-type</td>
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<td>Apolipoprotein E deficient</td>
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Data are expressed as mean and standard deviation. CVF = cobra venom factor. *p<0.05 compared to wild-type. †p<0.05 compared to non CVF-treated.
expression and quantization of eNOS and ICAM by an independent pathologist (S.J.R.) using a score of 1 = low intensity staining involving <50% of the vascular area examined; 2 = low intensity staining involving >50% of the aortic area examined; 3 = high intensity staining involving <50% of the aortic area examined; and 4 = high intensity staining involving >50% of the area examined.

2.7. Statistical analysis

Continuous data are expressed as mean ± standard deviation (S.D.). The Mann Whitney test was used for comparison of complement C3 levels between non-CVF treated and CVF-treated groups, as well as number of retained aortic microbubbles by ultrasound and SEM. The number of microbubbles/field detected in SEM in each group was compared by analysis of variance (ANOVA). Comparison of microbubbles detected by fluorescent microscopy in non CVF-treated and in CVF-treated apoE−/− mice was performed by Student’s t-test. A p value <0.05 was considered statistically significant. Endothelial responses to acetylcholine infusion were compared using paired t-testing. Linear regression was used to correlate the number of retained microbubbles by ultrasound or SEM with triglyceride levels and eNOS or ICAM up regulation.

3. Results

3.1. Complement binding to microbubbles

Serum complement binding to PESDA microbubbles was evaluated by flow cytometry. Fig. 2 illustrates histograms of red fluorescent intensity for PESDA microbubbles alone, and PESDA with serum (with and without anti C3) after mixing with secondary antibody. The data confirmed that in the presence of serum there was attachment of C3 to PESDA microbubbles.

Confocal Microscopy of Apolipoprotein E Deficient Mice

Fig. 5. Example of laser fluorescent microscopy image obtained in an apolipoprotein E deficient control mouse which did not receive FITC-PESDA microbubbles to establish the pattern of autofluorescence of aortic endothelium (A), in an apolipoprotein E deficient mouse pre-treated with cobra venom factor (CVF) (B) and an apolipoprotein E deficient mouse non CVF-treated (C). Note the absence of microbubbles in the control and in the CVF-treated mouse, while in the non CVF-treated there are numerous microbubbles forming a heterogenous pattern along with the aortic endothelium.

3.1. Scanning electron microscopy

Fig. 6. Scanning electron microscopy images showing atherosclerotic plaques in apolipoprotein E deficient mice which did or did not receive cobra venom factor (CVF) to selectively deplete complement C3. Note the presence of microbubbles adherent to atherosclerotic plaques in apolipoprotein E deficient mouse not treated with CVF (A) but not in the pre-treated with CVF (B).
This was reflected by a shift of the mean fluorescent intensity curve to the right when exposing PESDA microbubbles to mice serum. This binding did not occur in the absence of serum or with secondary antibody only.

3.2. Part II. PESDA microbubble adherence to aortic endothelium in hypertriglyceridemic CVF-treated mice

CVF injections resulted in significant reductions in serum C3 levels in all wild-type mice pre-treated with CVF (Table 1). SEM showed normal wavy appearance of endothelial cells in the aorta of all mice. Following induction of hypertriglyceridemia, SEM confirmed the adherence of microbubbles to the endothelial cells of the aorta in all wild-type mice not pre-treated with CVF. Fig. 3 illustrates an example of this demonstrating numerous microbubbles measuring from one to five microns attached to the endothelium. In contrast, only rare adherent microbubbles were detected in CVF-treated mice (Fig. 4, panel B) or in the C3−/− mice during hypertriglyceridemia (Fig. 4, panel C). Table 1 demonstrates the values of adherent microbubbles during hypertriglyceridemia in the wild-type mice (with and without CVF pre-treatment) when compared to the C3−/− mice.

3.3. Part III. PESDA microbubble adherence to atherosclerotic plaque

Serum complement levels were significantly higher in apoE−/− mice than in wild-type mice (Table 2). CVF injections resulted in a significant decrease in serum C3 levels in these mice, but the levels were still higher than wild-type mice before CVF injections. Using fluorescent laser microscopy, no microbubbles were identified in the three apoE−/− mice which did not receive intravenous FITC-PESDA (Fig. 5A). On the other hand, adherent microbubbles were observed in the atheromatous aortas of apoE−/− mice. Microbubbles were distinguished from the autofluorescence pattern of the aortic tissue as rounded structures, and having higher fluorescent intensity. The number of microbubbles found in the different fields was variable, reflecting a heterogeneous pattern of atherosclerosis (Fig. 5C). There were significantly fewer FITC-PESDA microbubbles adherent to the aortic endothelium of apoE−/− mice pre-treated with CVF (Fig. 5B), though a greater range of adherent microbubbles were seen due to the variable reductions in serum C3 levels by CVF. By quantitative analysis, the number of microbubbles in the injured aorta of non CVF-treated mice (median 172 bubbles/field, range 87 to 312) was significantly higher than in the CVF-treated mice (median 3 bubbles/field, range 0 to 92; \( p<0.001 \) between groups).

By SEM, atherosclerotic plaques were identified in different regions of aortic endothelium of all apoE−/− mice. PESDA microbubbles were identified adherent to the atherosclerotic plaques in apoE−/− mice not treated with CVF (4.3±7.4 bubbles/field), with fewer numbers in CVF-treated apoE−/− mice (0.6±1.3 bubbles/field) (Fig. 6). Microbubbles were adherent directly to the endothelial plaque surface, while no microbubbles adherent to leukocytes or other inflammatory cells were identified.

By histological analysis, atherosclerotic plaques were identified in the aorta of all apoE−/− mice (Fig. 7). The presence of an endothelial layer over the surface of the atherosclerotic plaques was confirmed in all apoE−/− mice by hematoxylin eosin staining at 400× magnification.

3.4. Part IV. Imaging of retained microbubbles in pre-atherosclerotic aorta

Histology demonstrated no atherosclerotic lesions in the JCR rats, and vasodilatory responses to acetylcholine were still observed. The percent vasodilation in response to acetylcholine was not significantly different from the SD rats (11.5% vs. 10.7%, \( P>0.05 \)). However, all four of the JCR rats exhibited increased eNOS activity, compared to only one of four controls. Retained microbubbles were observed by low MI ultrasound imaging in all four JCR rats, and in two control rats. The number of retained microbubbles was significantly higher in the JCR rats (Table 3). Counts observed by SEM correlated with the counts by ultrasound (\( r=0.69; P<0.05 \)), but the number of retained microbubbles observed with SEM was systematically higher than ultrasound (Fig. 8). Upregulated eNOS staining in the endothelium was seen in five of the

![Image](https://academic.oup.com/cardiovascres/article-abstract/73/3/597/369107)
six rats where retained microbubbles could be visualized with ultrasound. The number of adherent microbubbles by ultrasound imaging (CPS) and by SEM also correlated with serum triglyceride levels ($r = 0.82$ for SEM; $r = 0.77$ for CPS; $p < 0.01$). ICAM receptor staining was evident in only one of the four JCR rats, and none of the controls.

### 4. Discussion

In this study, we investigated the mechanisms responsible for the adherence of intravenously injected PESDA microbubbles to vascular endothelium, both early and late in the atherosclerotic process. First, we demonstrated that serum C3 binds to albumin-encapsulated microbubbles. Then, we confirmed, in vivo, that C3 plays an important role in the adherence of these microbubbles to aortic endothelium both early and late in the atherosclerotic process.

It has been well established that microbubbles exhibit microvascular rheology similar to that of red blood cells and pass freely through the large and small vessels [14]. However, both Villanueva et al. and Lindner et al. have demonstrated that microbubbles are retained in the microvasculature when endothelial dysfunction exists [7,12–14]. Microvascular attachment of albumin and lipid microbubbles in the setting of ischemia/reperfusion injury and inflammation induced by cytokines has been linked to $\beta_2$ integrin- and complement-mediated binding to activated leukocytes adherent to the venular walls. The extent of microbubble attachment has correlated with the degree of inflammation.

Endothelial dysfunction in larger arteries is defined as a broad alteration in the endothelial phenotype [15], and is manifested initially by both abnormal vasomotor responses and expression of inflammatory markers. We have recently reported that albumin-encapsulated microbubbles bind to the endothelium of carotid arteries which exhibited abnormal vasomotor responses to acetylcholine following balloon injury or induction of hypertriglyceridemia. These adherent microbubbles could be visualized by sensitive high-frequency low mechanical index pulse sequence schemes [2]. Microbubble binding to endothelium has consistently correlated with increased endothelial expression of inflammatory markers [7,12,16]. This inflammatory process appears to play a role in the down regulation of nitric oxide synthase and augmentation of endothelium-derived vasoconstrictor production [17]. Our hypothesis that C3 mediates PESDA microbubble binding to endothelium was based, in part, on our previous observations that commercially available lipid microbubbles do not exhibit this property. Although these microbubbles also take up complement within serum, they contain a polyethylene glycol spacer which prevents complement-mediated binding to the endothelium [18].

Atherosclerotic plaques have been characterized as chronic inflammatory lesions in which many components of immune system are active [1]. Several studies have provided evidence that complement activation is involved in atherogenesis [5,19,20]. Components of the terminal complement pathway are frequently found in human atheromas and local complement activation may induce cell lysis, generating the findings found in the necrotic core of advanced lesions [3,20]. The progression of atheromas from foam cell and lipid-rich lesions to more advanced plaques depends on the presence of an intact complement system [19]. Yasojima et al. demonstrated that all components of classical complement pathway...
are produced within arterial tissue, and are upregulated in atherosclerotic plaques [21]. On the other hand, complement inhibitors are not upregulated to defend against classical complement activation [5].

This study is the first to demonstrate direct adherence of albumin-encapsulated microbubbles to atherosclerotic plaques, as well as to otherwise histologically normal aortas which are prone to atherosclerosis. First, we used a transgenic mouse model in which mechanical injury to aortic endothelium was induced with several passages of a guidewire into the aorta. The apoE−/− mice are a useful model for the development of atherosclerosis, since they express hypercholesterolemia and high levels of autoantibodies against oxidized low density lipoprotein, and thus are known to develop atherosclerotic lesions [9,22]. However, transgenic models of atherosclerosis in mice do not model all the possible factors implicated in human atherosclerosis. Compared to wild-type mice, apoE−/− have unaltered ability to regenerate endothelium. For this reason, mechanical injury to the aortic endothelium in apoE−/− mice results in the development of atherosclerotic lesions with a predominance of foam cells of smooth muscle origin.

Secondly, we demonstrated albumin microbubble adherence to the aortic endothelium to histologically normal aortas that are prone to develop atherosclerosis. Binding of PESDA in atherosclerosis-prone JCR rats was detectable with ultrasound and SEM, even before vasomotor or other phenotypical evidence of endothelial dysfunction. We demonstrated that these retained microbubbles could be detected with sensitive low mechanical index pulse sequence schemes. Unfortunately, the sequence scheme in this study (contrast pulse sequencing) appeared to underestimate the actual number of retained microbubbles by SEM. Improvements in this sensitive imaging technology may permit the detection of early plaque or atherosclerosis-prone regions within arterial vessels, as well as the degree of inflammatory activity within plaque. Imaging of microbubbles by ultrasound would have some advantages over other imaging techniques because it is a bedside technique and no ionizing radiation is required.

4.1. Study limitations

Although we observed microbubbles binding to the aortic endothelium of mice during acute hypertriglyceridemia, the presence of endothelial dysfunction was not confirmed in the mice with complementary methods. Previous studies have shown that transient hypertriglyceridemia induced by an intravenous or oral fat load resulted in endothelial dysfunction [23,24]. Furthermore, we have already demonstrated in experimental animal model that the equivalent dose of intravenous intralipid caused endothelial dysfunction confirmed by a vasoconstrictive response to intraarterial infusion of acetylcholine [2]. In our subsequent rat model, the binding of microbubbles to endothelium was observed in the absence of vasomotor evidence of endothelial dysfunction, and before up regulation of ICAM receptors. This would indicate that complement-mediated microbubble binding to the endothelium occurs prior to the onset of these early traditional markers of endothelial dysfunction.

Previous studies have suggested that microbubbles targeted to upregulated receptors on the endothelial surface are required before one can actually image inflammation [13,14]. Recent studies have suggested that newer low mechanical index pulse sequence schemes may be able to detect these smaller number of complement-mediated adherent “non-targeted” microbubbles in larger carotid vessels [2]. It remains to be determined, however, whether this approach will detect atherosclerotic plaque in coronary vessels, where ultrasound beam attenuation and resolution of adherent microbubbles within smaller vessels will become a factor. In addition, a putative complement receptor on the endothelium was not investigated and further study will be necessary to clarify this issue.

4.2. Clinical implications

Our findings indicate that albumin microbubbles adhere to the vascular endothelium both early in the atherosclerotic process and to established plaque. These retained microbubbles are detectable with sensitive low mechanical index ultrasound detection schemes. This complement-mediated phenomena may have important clinical implications. First, as stated previously, the acoustic reflectivity of retained microbubbles may be a potential technique to image plaque if there is some degree of inflammatory activity at the plaque surface [2,25]. Secondly, since PESDA microbubbles have been shown to bind therapeutic agents [26], its additional ability to bind selectively to plaque may be method of focusing drug delivery. Further studies are still necessary to determine if albumin-encapsulated microbubbles like PESDA (i.e.; not containing a polyethylene glycol spacer) on the surface of atherosclerotic plaques within vessels can be imaged with microbubble-sensitive ultrasound imaging techniques in larger animal models, and can be used for drug delivery.

References


