National Cholesterol Awareness Month Article

Plaque Rupture and Thrombosis Are Reduced by Lowering Cholesterol Levels and Crystallization With Ezetimibe and Are Correlated With Fluorodeoxyglucose Positron Emission Tomography

Roshan Patel, Abed Janoudi, Ameeth Vedre, Kusai Aziz, Umesh Tamhane, Jack Rubinstein, Oliver G. Abela, Kevin Berger, George S. Abela

Objective—This study evaluated effects of lipid lowering with ezetimibe on plaque burden and associated cholesterol crystallization and inflammation in a rabbit model of plaque disruption and thrombosis.

Methods and Results—Atherosclerotic rabbits (Group I, n=10 without; Group II, n=12 with ezetimibe, 1 mg/kg per day) were pharmacologically triggered for plaque disruption. Fluorodeoxyglucose positron emission tomography, RAM 11 macrophage staining, and serum inflammatory markers detected arterial inflammation. Serum and aortic wall cholesterol levels were measured, and thrombus area was planimetered. Cholesterol crystal density on aortic surface was scored (0 to +3) by scanning electron microscopy. Serum and aortic wall cholesterol, plaque area, and thrombosis area were significantly lower in Group II versus Group I (83.4±106.4 versus 608±386 mg/dL, P=0.002; 3.12±1.40 versus 9.39±5.60 mg/g, P=0.003; 10.84±1.6 versus 17.48±1.8 mm², P<0.001; and 0.05±0.15 versus 0.72±0.58 mm², P=0.01, respectively). There were significant correlations between crystal density and plaque area (r=0.75, P<0.003) and between crystal density and RAM 11 (r=0.82, P<0.001). Scanning electron microscopy demonstrated that there were fewer crystals in Group II versus Group I (+1.2±0.61 versus +2.4±0.63, P<0.001) and less inflammation detected by fluorodeoxyglucose positron emission tomography and RAM 11 (P<0.004 and P<0.04, respectively).


Key Words: acute coronary syndromes ■ arterial thrombosis ■ atherosclerosis ■ pathology ■ positron emission tomography

Plaque disruption is the primary cause of arterial thrombosis leading to acute cardiovascular events.1-2 Few in vivo models of plaque disruption are available, but an atherosclerotic rabbit model of plaque disruption and thrombosis using a pharmacological trigger has already been validated to induce acute cardiovascular events.3-6 Furthermore, this model was used to demonstrate that increased thrombosis was correlated with elevated arterial wall cholesterol levels and increased inflammation measured by fluorodeoxyglucose positron emission tomography (FDG-PET), as well as serum inflammation markers.5,7 Recently, we demonstrated that as cholesterol crystallizes, it expands in volume, which can cause tearing of surrounding tissues and intima triggering plaque rupture.8,9 Also, an association between plaque rupture and cholesterol crystals was demonstrated in both human coronary plaques post mortem and carotid plaques from patients having carotid endarterectomy.10 Given this background, we proceeded to evaluate the effect of cholesterol lowering in vivo on plaque disruption by inhibiting intestinal absorption of cholesterol with ezetimibe. In addition, we investigated the association of cholesterol crystal formation, inflammation, and cholesterol levels in serum and arterial wall with thrombosis using an atherosclerotic rabbit model of plaque disruption.

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Materials and Methods

Study Groups

Atherosclerosis was induced in 22 male New Zealand White rabbits (2.7 to 3.2 kg) using balloon deendothelialization and feeding a cholesterol-enriched diet (1%) alternating with normal chow every other month for 6 months as previously reported.3 Briefly, all rabbits were anesthetized with ketamine (50 mg/kg IM) and xylazine (20 mg/kg IM) during the right femoral artery cut-down for balloon deendothelialization. Analgesics (buprenorphine, 0.01 mg/kg SC,
After rabbits were euthanized, the aorta and iliofemoral arteries were dissected, and opened by a longitudinal incision exposing the intimal surface for inspection. Adherent thrombi in all aortic segments were identified visually and confirmed by histology. Aortic plaque and thrombus surface areas were measured by planimetry using a custom computerized package.

**Microscopy**

Light microscopy (LM), scanning electron microscopy (SEM), transmission electron microscopy (TEM), and confocal microscopy (CM) were performed. For LM and SEM, aortic segments from all rabbits were fixed overnight in buffered 4% glutaraldehyde.

**LM**

Fixed tissue segments were serially dehydrated with graded ethanol, embedded in paraffin blocks, and cut in 5-μm sections using a microtome. These sections were stained with hematoxylin or cosin, Masson trichrome, or Movat pentachrome for examination under a light microscope (Laborlux12, Leitz, Oberkochen, Germany).

**FDG-PET**

Imaging by PET/computed tomography (CT) was performed 2 hours after intravenous injection of 2 mCi of F-18 FDG. Under general anesthesia as described above, the rabbits were held in a supine position on a plastic board using medical tape to minimize motion artifacts and then placed inside the PET/CT tube for scanning. Images were acquired using a GE Discovery STE (fusion PET/CT scanner). Following a noncontrast CT examination for attenuation correction, PET images were acquired over 30 minutes in a 3-dimensional mode using 5 beds (15.4-cm axial field of view, 6 minutes per bed with 50% overlap) and reconstructed using an iterative algorithm (2 iterations, 28 subsets, 128 matrix). Standardized uptake values (SUVs) were calculated with adjustment for rabbit weight, injected dose, and F-18 decay. Maximal SUV (SUVmax) was measured in vivo over the thoracic and upper and lower abdominal aortas and averaged for each rabbit by a reader blinded to use of ezetimibe. In addition, to compare gross pathology, including thrombus count, with PET scans, a direct measure was obtained of the FDG uptake of the dissected aortas in 5 rabbits from each group. The excised aorta was placed in the PET scanner, and SUVmax measurements were obtained.

**Cholesterol and Serum Markers**

Blood samples were collected to measure serum cholesterol, C-reactive protein (CRP), plasminogen activator inhibitor-1 (PAI-1), and matrix metalloproteinase-9 (MMP-9) at baseline, after atherosclerosis induction (2, 12, and 24 weeks) and following thrombus triggering.

Cholesterol concentrations were determined in the serum and aortic wall using a kit according to the manufacturer’s instructions (Thermo Electron Corp, Louisville, CO). Aortic samples (1 cm long) were obtained from the thoracic, upper abdominal, and lower abdominal aortas from each rabbit, and the total cholesterol content was averaged.

CRP was measured using an ELISA kit from Immunology Consultant Laboratory (Newberg, OR).

MMP-9 was measured in serum samples by indirect ELISA. Test plate wells were coated with serum and then incubated with mouse anti-human-MMP-9 monoclonal antibody LS-C8808 (LifeSpan Biosciences, Seattle, WA). These were then incubated with a goat anti-mouse IgG horseradish peroxidase–conjugated antibody (Abbiotec, San Diego, CA), TMB (horseradish stain) was used to stain the second antibody, and the optical density was measured at 450 nm. Human MMP-9 (Chemicon, Temecula, CA) was used as a standard.

PAI-1 was measured using an ELISA kit (Molecular Innovations, Southfield, MI).

**Gross examination**

After rabbits were euthanized, the aorta and iliofemoral arteries were dissected, removed, and opened by a longitudinal incision exposing the aortic wall using a kit according to the manufacturer’s instructions (Thermo Electron Corp, Louisville, CO). TMB (horseradish stain) was used to stain anti-mouse IgG horseradish peroxidase–conjugated antibody (Abbiotec, San Diego, CA). Tissue samples (3 to 5 mm long) were dehydrated for 12 hours in a vacuum chamber (Speed Vac SC110, Savant Instruments Inc, Farmingdale, NY) evacuated by a pump (VP110, Franklin Electric, Bluffton, IN). Because our earlier studies had demonstrated superior preservation of cholesterol crystals by vacuum dehydration, this was used instead of ethanol in tissue preparation. These tissue segments were then mounted on stubs and gold coated in a sputter coater (EMSCOPE SC500; Emscope, Ashford, UK). The intimal surfaces of aortic lumen were then examined using a Jeol SEM (model JSM-6300F, Jeol Ltd, Tokyo, Japan). We defined plaque disruption by SEM as cholesterol crystals perforating the intimal surface with plaque cap tears and intimal damage with loss of endothelium, often with thrombosis, as modified from the definition for LM. These tissue segments were used to examine the aortic surface and quantify the cholesterol crystals as absent (+0), scattered few (+1), dense in a limited area (+2), or dense and widely distributed (+3).

**Tissue Shrinkage**

Tissue shrinkage was assessed by measurements of fresh aortic samples in the short and long axis after fixing in 4% glutaraldehyde followed by vacuum dehydration and compared with samples from the same artery processed using ethanol dehydration and paraffin embedding.
Fresh-Frozen TEM

Tissues were immersed in a saturated sucrose solution overnight and then processed for cryosectioning.13 These were then stained with osmium tetroxide. This process avoided the use of solvents (ie, uranyl acetate) that can dissolve the cholesterol crystals. Ultrathin sections were then examined using a Jeol 100CX electron microscope.

CM

Fresh tissue segments were incubated for 4 hours at 37°C in Eagle minimum essential medium under O2 and CO2 atmosphere with 10 µg/mL Alexa Fluor 594 acetylated-low density lipoprotein (Molecular Probes, Eugene, OR) specific for endothelium. After incubation, tissue was washed with PBS and fixed with 4% glutaraldehyde.14 These were then counterstained for cholesterol crystals using a green fluorescent dye (cholesteryl Bodipy-C12, Invitrogen, Eugene, OR) at a 1/100 dilution (75% ethanol) in a test tube for 3 minutes.10 Samples were then transferred to a slide incubator chamber filled with PBS and visualized. Adjacent cuts of unstained plaque samples were also examined to detect native tissue fluorescence. CM fluorescence images of the plaque were acquired using a Zeiss Pascal LSM 310 microscope (Carl Zeiss, Inc, Jena, Germany). The green fluorescence was excited with the 488 nm argon laser line, and emission was collected using a 505 to 530 nm band-pass filter. Sequentially, the red fluorescence was excited with the 543 nm helium-neon laser line, and the emission was collected using a 560 nm long-pass filter.

Statistical Analysis

GraphPad Prism (San Diego, CA) was used for statistical analysis. Data are reported as mean±SD. An unpaired 2-sided t test was used to calculate statistical difference in cholesterol crystal density, plaque area, thrombus area, lipid content of aorta, serum inflammation markers, RAM 11–stained macrophages and SUVmax between the 2 rabbit groups with and without ezetimibe. Correlations were obtained between cholesterol crystal density and plaque area, cholesterol content, SUVmax, and RAM 11–stained macrophages as an index of arterial wall inflammation. Also, correlations between thrombus area and serum and arterial wall cholesterol were obtained as well as correlations between RAM 11 staining and cholesterol crystal density and serum inflammation markers (MMP, CRP, PAI).

### Table. Cholesterol-Lowering Effects on Crystallization, Thrombosis, and Inflammation

<table>
<thead>
<tr>
<th>Ezetimibe</th>
<th>Serum Cholesterol (mg/dL)</th>
<th>Aortic Cholesterol (mg/g)</th>
<th>Plaque Area (mm²)</th>
<th>Thrombus Area (mm²)</th>
<th>CC Density (0 to +3)</th>
<th>CRP (µg/mL)*</th>
<th>MMP-9 (ng/mL)*</th>
<th>RAM 11 (Macr Density)</th>
<th>SUVmax (g/mL)†</th>
</tr>
</thead>
<tbody>
<tr>
<td>No (Group I)</td>
<td>608±386</td>
<td>9.4±5.6</td>
<td>17.5±1.8</td>
<td>0.72±0.58</td>
<td>2.4±0.6</td>
<td>62.8±25.2</td>
<td>261±130</td>
<td>0.31±0.22</td>
<td>1.25±0.46</td>
</tr>
<tr>
<td>Yes (Group II)</td>
<td>83±106</td>
<td>3.1±1.4</td>
<td>10.8±1.6</td>
<td>0.05±0.15</td>
<td>1.2±0.6</td>
<td>34.7±21.2</td>
<td>29±35</td>
<td>0.06±0.08</td>
<td>0.96±0.31</td>
</tr>
</tbody>
</table>

*After triggering.
†Mean SUVmax in vivo

CC indicates cholesterol crystal; CRP, C-reactive protein; MMP-9, matrix metalloproteinase 9; Macr, macrophage; SUVmax, maximal standardized uptake value.

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**Figure 1.** Top: Correlations between crystal density and plaque area, aortic wall cholesterol, and RAM 11. Middle: Correlations between thrombus area and plaque area and aortic cholesterol, and between RAM 11 and plaque area. Bottom: Correlations between matrix metalloproteinase-9 (MMP-9) and maximal standardized uptake value (SUVmax), cholesterol crystal density, and RAM 11.
Differences in SUVmax from the subgroup of extracted in vitro aortas and the number of thrombi in ezetimibe-treated and nontreated animals were analyzed using a Mann–Whitney test. A probability value of 0.05 was used to determine statistical significance.

**Results**

**Gross Examination**

The amount of thrombus was significantly reduced in Group II (ezetimibe treated) compared with Group I (atherosclerotic control) (Table). There was a significant correlation between thrombus area and plaque area ($r=0.60$, $P=0.02$) and between thrombus area and arterial wall cholesterol ($r=0.64$, $P=0.01$) (Figure 1). Also, thrombus area was significantly correlated with serum cholesterol ($r=0.65$, $P=0.003$).

**Microscopy**

In Group I, LM demonstrated that there were extensive clusters of cholesterol crystal clefts within the plaque and in macrophages (Figure 2). Also, by LM, extensive cholesterol crystal clefts were noted in the arterial wall below thrombi but not at the intimal surface, whereas by CM and SEM, extensive deposits of cholesterol crystals with tissue injury were readily detected at the surface.

Dual fluorescent imaging by CM demonstrated that there were cholesterol crystals present at the intimal surface of the aorta protruding above the endothelial cell layer (Figure 2). SEM of the aorta demonstrated that there were very few crystals over plaque surfaces in Group II, whereas cholesterol crystals in large clusters were noted over plaque surfaces in

**Figure 2.** Top row: Light microscopy (Movat stain) of Group I aorta demonstrating cholesterol crystals in the subintimal intra- (arrow) and extracellular spaces. Middle row: Group II aorta demonstrating smaller plaques with few cholesterol crystals. Bottom row: Fluorescence images of intimal surface (Group I). Images show cholesterol crystals stained green (left), endothelium stained red (middle), and overlay demonstrating cholesterol crystals over endothelial surface (right).
Group I (Table and Figure 3). Cholesterol crystal density was significantly correlated with plaque area ($r=0.75$, $P=0.003$), cholesterol content ($r=0.72$, $P=0.001$), and RAM 11–stained macrophages ($r=0.82$, $P=0.001$) (Figure 1).

TEM imaging of fresh-frozen arteries demonstrated that there were cholesterol crystals emerging at the intimal surface (Figure 3). Some of these crystals were well below 1 μm in size.

Tissue shrinkage in 18 vacuum dehydrated arteries was 16% in the short axis and 19% in the long axis, whereas standard ethanol dehydration resulted in shrinkage of 9% in the short axis and 12% in the long axis.

Serum and Arterial Wall Cholesterol Levels
Both serum and aortic wall cholesterol content were significantly lower in Group II compared with Group I (Table).

Serum Inflammation Response
CRP and MMP-9 were significantly lower in Group II than in Group I (Table). Although the mean PAI-1 levels appeared to be lower in Group II versus Group I, the difference was not significant (16.6±8.1 versus 33.7±31.1, $P=\text{not significant}$). Serum inflammatory markers increased progressively with development of atherosclerosis and increased further after triggering. However, these markers were consistently higher in Group I versus Group II (Figure 4). There was a strong
A significant correlation was found between SUVmax in the aorta and serum cholesterol. Additionally, there was a significant increase in FDG uptake measured in vitro from excised aortas of Group I versus Group II (Table). By RAM 11, macrophage density was significantly greater in Group I versus Group II aortic specimens (Table and Figure 5). However, no correlation was found between FDG uptake and RAM 11 in vivo or in the excised aortas. This may be due in part to the small sample size.

FDG-PET

The SUVmax measured in vivo over the entire aorta was significantly lower in Group II versus Group I (Table), and a significant correlation was found between SUVmax in the thoracic aorta and serum cholesterol (r=0.71, P=0.004). Also, there was a significant increase in FDG uptake measured in vitro from excised aortas of Group I versus Group II by SUVmax (0.25±0.20 versus 0.04±0.03 g/mL, P=0.01). More plaque and a higher average number of thrombi per artery (2 versus 0.4, P<0.05) were noted when comparing gross pathology with corresponding FDG-PET scans of excised aortas (Figure 5). By RAM 11, macrophage density was significantly greater in Group I versus Group II aortic specimens (Table and Figure 5). However, no correlation was found between FDG uptake and RAM 11 in vivo or in the excised aortas. This may be due in part to the small sample size.

Discussion

Using an atherosclerotic rabbit model, this study demonstrated that lowering cholesterol absorption reduces plaque burden and plaque disruption. The greater plaque burden observed in Group I rabbits was associated with higher arterial wall cholesterol content, cholesterol crystal density, and inflammation leading to more intimal disruption. However, by lowering the plaque burden with ezetimibe, there was a significant reduction in arterial wall and serum inflammation, as reflected by decreased SUVmax, RAM 11, and serum inflammatory markers. This was accompanied by reduced plaque disruption, thrombosis, and cholesterol crystal density. These findings are consistent with earlier reports in humans that a large lipid pool and a large plaque burden are associated with greater incidence of cardiovascular events.15–17

An increased expression of serum inflammation markers has been reported in the setting of plaque disruption in humans.18 Our report showed higher levels of tissue macrophage cell density, SUVmax, and serum inflammatory markers after triggering in rabbits whose arteries had a greater plaque burden than ezetimibe-treated rabbits. In prior studies using the same model, as well as in human carotid arteries, SUVmax elevation has also been shown to be significantly correlated with macrophage cell density in the arterial wall.5,7,19–22 In the current study, we recognize a similar finding of greater macrophage staining in rabbits with more atherosclerosis that is accompanied by a visible increase in FDG uptake as noted in Figure 5, but no significant correlation was found between SUVmax and RAM 11 in this small sample.

Free cholesterol in plaque is derived mainly from intracellular contents of dying macrophages, as well as cholesterol converted from the ester form to free cholesterol by the action of both cytoplasmic and lysosomal lipolytic enzymes.23,24 Moreover, vasa vasorum in atherosclerotic rabbit aorta may be another potential source of free cholesterol from the membranes of red blood cells that may have bled into plaques.25

We recently reported that cholesterol crystals form very early during atherogenesis leading to activation of the NLRP3 inflammasome.26 This suggests that cholesterol crystals can trigger a danger signal within macrophages to initiate an inflammatory response that can eventually lead to macrophage apoptosis. In the current report, we found cholesterol crystals both intra- and extracellularly within plaques at the submicron range using both LM and fresh-frozen TEM techniques. This further supports the observation of an early role of cholesterol crystals in triggering inflammation. Cholesterol oxide (7-ketocholesterol) has been shown to form crystals in macrophages in a dose-dependent fashion, leading to moderate apoptosis.27 Similarly, oxidized low-density lipoprotein has been implicated in the destabilization of arterial plaque leading to macrophage apoptosis via oxidative stress.28 In addition to the known role of oxidized cholesterol, our findings suggest that free cholesterol in crystal form plays a role in plaque rupture. However, additional studies will be required to define the role of cholesterol crystals in foam cell...
apoptosis and triggering of local inflammation as it pertains to plaque rupture.29

The morphological appearance and distribution of cholesterol crystals in this atherosclerotic rabbit model were similar to those reported in human coronary and carotid plaques.30

This was confirmed by various microscopic methods, including CM (Figure 2) and fresh-frozen TEM (Figure 3), that revealed crystals perforating the intimal surfaces of fresh and unprocessed arterial tissues. Both CM and fresh-frozen TEM methods verified the findings of cholesterol crystals perforating the intima by SEM. Although tissue shrinkage was noted in both vacuum and standard ethanol dehydration, the CM and TEM findings in fresh unprocessed tissue suggest that the reported SEM findings are not an artifact of tissue shrinkage. This is similar to the findings noted earlier in human plaques.10

In a prior in vitro study, we demonstrated that increasing cholesterol saturation was a major trigger for cholesterol crystallization.9 Because ezetimibe is not known to have pleiotropic effects, we used it in this model to evaluate the effects of lipid lowering on cholesterol crystals, plaque formation, and inflammation. The data in this study suggest that serum cholesterol lowering by inhibiting intestinal absorption can reduce intramural cholesterol content, as well as cholesterol crystallization. These data are also consistent with clinical findings showing that lower low-density lipoprotein cholesterol levels are associated with lower cardiovascular events regardless of the cholesterol-lowering agent used.30

The SUVmax elevation in Group I compared with Group II indicates that there was greater wall inflammation in Group I that was associated with an increased amount of crystal deposits and thrombosis in the aorta. These data suggest that a larger plaque burden is associated with greater arterial wall inflammation and cholesterol crystallization, leading to more thrombosis and systemic inflammation consistent with severe arterial wall injury. Similar observations were made on individual excised aortas with corresponding FDG uptake by PET. Therefore, these findings lend additional value for the potential use of PET scanning of arteries for screening patients at increased risk for cardiovascular events.5,7,19–22

Conclusions

Our study supports the premise that increased cholesterol burden in the arterial wall leads to a greater inflammatory response and cholesterol crystal formation that is associated with increased wall disruption and thrombosis. This in vivo study and our prior studies support the hypothesis that plaque disruption is enhanced by greater local and systemic inflammation in association with cholesterol crystal formation. Additional studies will be needed to evaluate these effects both at a cellular level and in humans.

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Disclosures

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References


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Ezetimibe에 의한 콜레스테롤 농도와 콜레스테롤 결정 (crystal) 발생의 저하는 죽상반의 파열과 혈전의 발생을 감소시킨다.

Summary

목적
죽상반의 파열과 혈전이 발생하는 토끼 모델에서 ezetimibe에 의한 지질 저하가 죽상반의 양, 콜레스테롤의 결정화(crystallization) 및 염증에 미치는 영향에 대해 조사 하였다.

방법 및 결과
동맥죽상경화 토끼(1군, 10마리, 비치료군; 2군, 12마리, ezetimibe 1mg/kg/일 치료군)의 죽상반 파열을 약물로 유발시켰으며, FDG (Fluorodeoxyglucose) PET (positron emission tomography), RAM 11 대식세포 염색, 혈중 염증 표지자들을 통해 동맥의 염증을 조사하였다. 혈청과 대동맥벽의 콜레스테롤 수치를 측정했으며 혈전의 면적을 측량하였다. 전자현미경을 이용해 대동맥 표면의 콜레스테롤 결정의 농도를 점수화(0~+3점) 하였다. 혈청 및 대동맥벽의 콜레스테롤, 죽상반 면적, 혈전 면적 등이 유의하게 2군이 1군에 비해 낮았다(각각 83.4±106.4 vs 608±386mg/dL, P=0.002; 3.12±1.40 vs 9.39±5.60mg/g, P=0.003; 10.84±1.6 vs 17.48±1.8mm², P<0.001; 0.05±0.15 vs 0.72±0.58mm², P=0.01). 콜레스테롤 결정 농도는 죽상반 면적(r=0.75, P<0.003) 및 RAM 11(r=0.82, P<0.001)과 유의한 상관성이 있었다. 전자현미경 하에서 2군은 1군에 비해 콜레스테롤 결정이 적었고(+1.2±0.61 vs +2.4±0.63, P<0.001), FDG-PET 및 RAM 11 검사상 염증도 유의하게 적었다(각각 P<0.004, P<0.04).

결론
Ezetimibe에 의한 콜레스테롤 저하는 죽상반의 양과 콜레스테롤 결정, 염증을 줄였으며 죽상반의 파열과 혈전의 발생을 억제하였다.
Ezetimibe is a sterol transporter that decreases the absorption of dietary cholesterol. It blocks the NPC1L1 transporter in the intestine, thereby reducing the absorption of dietary cholesterol. Ezetimibe is often used in combination with statins to achieve more effective cholesterol lowering.

**REFERENCE**


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Key Words: acute coronary syndromes ■ arterial thrombosis ■ atherosclerosis ■ pathology ■ positron emission tomography

Plaque disruption is the primary cause of arterial thrombosis leading to acute cardiovascular events.1,2 Few in vivo models of plaque disruption are available, but an atherosclerotic rabbit model of plaque disruption and thrombosis using a pharmacological trigger has already been validated to induce acute cardiovascular events.3–6 Furthermore, this model was used to demonstrate that increased thrombosis was correlated with elevated arterial wall cholesterol levels and increased inflammation measured by fluorodeoxyglucose positron emission tomography (FDG-PET), as well as serum inflammation markers.5,7 Recently, we demonstrated that as cholesterol crystallizes, it expands in volume, which can cause tearing of surrounding tissues and intima triggering plaque rupture.8,9 Also, an association between plaque rupture and cholesterol crystals was demonstrated in both human coronary plaques post mortem and carotid plaques from patients having carotid endarterectomy.10 Given this background, we proceeded to evaluate the effect of cholesterol lowering in vivo on plaque disruption by inhibiting intestinal absorption of cholesterol with ezetimibe. In addition, we investigated the association of cholesterol crystal formation, inflammation, and cholesterol levels in serum and arterial wall with thrombosis using an atherosclerotic rabbit model of plaque disruption.

Materials and Methods

Study Groups
Atherosclerosis was induced in 22 male New Zealand White rabbits (2.7 to 3.2 kg) using balloon deendothelialization and feeding a cholesterol-enriched diet (1%) alternating with normal chow every other month for 6 months as previously reported.3 Briefly, all rabbits were anesthetized with ketamine (50 mg/kg IM) and xylazine (20 mg/kg IM) during the right femoral artery cut-down for balloon deendothelialization. Analgesics (buprenorphine, 0.01 mg/kg SC, }
Committee following National Institutes of Health guidelines. Aldrich, St. Louis, MO) and histamine (0.02 mg/kg IV, Sigma-Aldrich, St. Louis, MO) given at 48 and 24 hours before euthanasia. General anesthesia was used again as described above for FDG-PET, and euthanasia solution (butanalamol 2 mL IV) was administered after scanning. Procedures were performed according to protocols approved by Michigan State University’s Animal Care and Use Committee following National Institutes of Health guidelines.

FDG-PET
Imaging by PET/computed tomography (CT) was performed 2 hours after intravenous injection of 2 mCi of F-18 FDG. Under general anesthesia as described above, the rabbits were held in a supine position on a plastic board using medical tape to minimize motion artifacts and then placed inside the PET/CT tube for scanning. Images were acquired using a GE Discovery STE (fusion PET/CT scanner). Following a noncontrast CT examination for attenuation correction, PET images were acquired over 30 minutes in a 3-dimensional mode using 5 beds (15.4-cm axial field of view, 6 minutes per bed with 50% overlap) and reconstructed using an iterative algorithm (2 iterations, 28 subsets, 128x128 matrix). Standardized uptake values (SUVs) were calculated with adjustment for rabbit weight, injected dose, and F-18 decay. Maximal SUV (SUVmax) was measured in vivo over the thoracic and upper and lower abdominal aortas and averaged for each rabbit by a reader blinded to use of ezetimibe. In addition, to compare gross pathology, including thrombus count, with PET scans, a direct measure was obtained of the FDG uptake of the dissected aortas in 5 rabbits from each group. The excised aorta was placed in the PET scanner, and SUV max measurements were obtained.

Cholesterol and Serum Markers
Blood samples were collected to measure serum cholesterol, C-reactive protein (CRP), plasminogen activator inhibitor-1 (PAI-1), and matrix metalloproteinase-9 (MMP-9) at baseline, after atherosclerosis induction (2, 12, and 24 weeks) and following thrombus triggering.

Cholesterol concentrations were determined in the serum and aortic wall using a kit according to the manufacturer’s instructions (Thermo Electron Corp, Louisville, CO).

Aortic samples (1 cm long) were obtained from the thoracic, upper abdominal, and lower abdominal aorta from each rabbit, and the total cholesterol content was averaged.

CRP was measured using an ELISA kit from Immunology Consultant Laboratory (Newberg, OR).

MMP-9 was measured in serum samples by indirect ELISA. Test plate wells were coated with serum and then incubated with mouse anti-human-MMP-9 monoclonal antibody LS-C8808 (LifeSpan Biosciences, Seattle, WA). These were then incubated with a goat anti-mouse IgG horseradish peroxidase-conjugated antibody (A-biotin, San Diego, CA). TMB (horseradish stain) was used to stain the second antibody, and the optical density was measured at 450 nm. Human MMP-9 (Chemicon, Temecula, CA) was used as a standard.

PAI-1 was measured using an ELISA kit (Molecular Innovations, Southfield, MI).

Gross examination
Ater rabbits were euthanized, the aorta and iliofemoral arteries were dissected, removed, and opened by a longitudinal incision exposing the intimal surface for inspection. Aherent thrombi in all aortic segments were identified visually and confirmed by histology. Aortic plaque and thrombus surface areas were measured by planimetry using a custom computerized package.

Microscopy
Light microscopy (LM), scanning electron microscopy (SEM), transmission electron microscopy (TEM), and confocal microscopy (CM) were performed. For LM and SEM, aortic segments from all rabbits were fixed overnight in buffered 4% glutaraldehyde.

LM
Fixed tissue segments were serially dehydrated with graded ethanol, embedded in paraffin blocks, and cut in 5-μm sections using a microtome. These sections were stained with hematoxylin or eosin, Masson trichrome, or Movat pentachrome for examination under a light microscope (Laborlux12, Leitz, Oberkochen, Germany).

RAM 11
Tissue sections from formaldehyde-fixed segments were cut, mounted on glass slides, and stained with RAM 11 (Laboratory Vision, Milford, MA), the immunohistochemical stain for monoclonal antibodies to rabbit macrophages. To determine macrophage density, tissue sections were examined with a light microscope (microphot FXA, Nikon, Tokyo, Japan) and photographed with a ×20.1 numerical aperture Nikon PlanApo objective. All sections were photographed at ×2 under identical conditions of illumination voltage and condenser settings. Photographs were corrected for uneven illumination and camera sensor response by dividing the tissue image by a blank image taken under identical settings with Image Arithmetic, version 2.5.12 Images were analyzed with ImageJ, version 1.43u (http://rsb.info.nih.gov/jij). The total surface area measured of the arterial wall was based on the trichrome-stained sections. Macrophage density was characterized for each RAM 11-stained section as the total area darker than a predetermined threshold representing relatively strong staining. Thrombus, when present, was excluded from measurements of the RAM 11 images. The percentage of RAM 11-stained tissue within the arterial section under the field of view of the microscope was used to determine the density of the macrophages in that section.

SEM
Tissue samples (3 to 5 mm long) were dehydrated for 12 hours in a vacuum chamber (Speed Vac SC110, Savant Instruments Inc, Farmingdale, NY) evacuated by a pump (VP110, Franklin Electric, Bluffton, IN). Because our earlier studies had demonstrated superior preservation of cholesterol crystals by vacuum dehydration, this was used instead of ethanol in tissue preparation. These tissue segments were then mounted on stubs and gold coated in a sputter coater (EM SCOPE SC500; Emscope, Ashford, UK.). The intimal surfaces of aortic lumen were then examined using a Jeol SEM (model JSM-6300F, Jeol Ltd, Tokyo, Japan). We defined plaque disruption by SEM as cholesterol crystals perforating the intimal surface with plaque cap tears and intimal damage with loss of endothelium, often with thrombosis, as modified from the definition for LM.10 SEM was used to examine the aortic surface and quantify the cholesterol crystals as absent (+0), scattered few (+1), dense in a limited area (+2), or dense and widely distributed (+3).

Tissue Shrinkage
Tissue shrinkage was assessed by measurements of fresh aortic samples in the short and long axis after fixing in 4% glutaraldehyde followed by vacuum dehydration and compared with samples from the same artery processed using ethanol dehydration and paraffin embedding.
Table. Cholesterol-Lowering Effects on Crystallization, Thrombosis, and Inflammation

<table>
<thead>
<tr>
<th>Ezetimibe</th>
<th>Serum Cholesterol (mg/dL)</th>
<th>Aortic Cholesterol (mg/g)</th>
<th>Plaque Area (mm²)</th>
<th>Thrombus Area (mm²)</th>
<th>CC Density (0 to +3)</th>
<th>CRP (μg/mL)*</th>
<th>MMP-9 (ng/mL)*</th>
<th>RAM 11 (Macr. Density)</th>
<th>SUVmax (g/mL)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>No (Group I)</td>
<td>608±386</td>
<td>9.4±5.6</td>
<td>17.5±1.8</td>
<td>0.72±0.58</td>
<td>2.4±0.6</td>
<td>62.8±25.2</td>
<td>261±130</td>
<td>0.31±0.22</td>
<td>1.25±0.46</td>
</tr>
<tr>
<td>Yes (Group II)</td>
<td>83±106</td>
<td>3.1±1.4</td>
<td>10.8±1.6</td>
<td>0.05±0.15</td>
<td>1.2±0.6</td>
<td>34.7±21.2</td>
<td>29±35</td>
<td>0.06±0.08</td>
<td>0.96±0.31</td>
</tr>
</tbody>
</table>

P value | 0.002                         | 0.003                    | 0.001             | 0.01               | 0.001              | 0.001        | 0.001       | 0.04                 | 0.004        |

CC indicates cholesterol crystal; CRP, C-reactive protein; MMP-9, matrix metalloproteinase 9; Macr, macrophage; SUVmax, maximal standardized uptake value.

*After triggering.
†Mean SUVmax in vivo

Fresh-Frozen TEM

Tissues were immersed in a saturated sucrose solution overnight and then processed for cryosectioning. These were then stained with osmium tetroxide. This process avoided the use of solvents (i.e., uranyl acetate) that can dissolve the cholesterol crystals. Ultrathin sections were then examined using a Jeol 100CX electron microscope.

CM

Fresh tissue segments were incubated for 4 hours at 37°C in Eagle minimum essential medium under O2 and CO2 atmosphere with 10% FCS and 1% penicillin-streptomycin. After incubation, tissue was washed with PBS and fixed with 4% glutaraldehyde. These were then counterstained for cholesterol crystals using a green fluorescent dye (cholesteryl BODIPY® 493/503, Invitrogen, Eugene, OR) at a 1/100 dilution (75% ethanol) in a test tube for 3 minutes. Samples were then transferred to a slide incubator chamber filled with PBS and visualized. Adjacent cuts of unstained plaque samples were also examined to detect native tissue fluorescence. CM fluorescence images of the plaque were acquired using a Zeiss Pascal LSM 510 microscope (Carl Zeiss, Inc., Jena, Germany). The green fluorescence was excited with the 488 nm argon laser line, and emission was collected using a 505 to 530 nm band-pass filter. Sequentially, the red fluorescence was excited with the 543 nm helium-neon laser line, and the emission was collected using a 660 nm long-pass filter.

Statistical Analysis

GraphPad Prism (San Diego, CA) was used for statistical analysis. Data are reported as mean±SD. An unpaired 2-sided t test was used to calculate statistical difference in cholesterol crystal density, plaque area, thrombus area, lipid content of aorta, serum inflammation markers, RAM 11-stained macrophages and SUVmax between the 2 rabbit groups with and without ezetimibe. Correlations were obtained between cholesterol crystal density and plaque area, cholesterol content, SUVmax, and RAM 11-stained macrophages as an index of arterial wall inflammation. Also, correlations between thrombus area and serum and arterial wall cholesterol were obtained as well as correlations between RAM 11 staining and cholesterol crystal density and serum inflammation markers (MMP, CRP, PAI).

Figure 1. Top: Correlations between crystal density and plaque area, aortic wall cholesterol, and RAM 11. Middle: Correlations between thrombus area and plaque area and aortic cholesterol, and between RAM 11 and plaque area. Bottom: Correlations between matrix metalloproteinase-9 (MMP-9) and maximal standardized uptake value (SUVmax), cholesterol crystal density, and RAM 11.
Group I (Table and Figure 3). Cholesterol crystal density was significantly correlated with plaque area ($r=0.75$, $P=0.003$), cholesterol content ($r=0.72$, $P=0.001$), and RAM 11–stained macrophages ($r=0.82$, $P=0.001$) (Figure 1).

TEM imaging of fresh-frozen arteries demonstrated that there were cholesterol crystals emerging at the intimal surface (Figure 3). Some of these crystals were well below 1 µm in size.

Tissue shrinkage in 18 vacuum dehydrated arteries was 16% in the short axis and 19% in the long axis, whereas standard ethanol dehydration resulted in shrinkage of 9% in the short axis and 12% in the long axis.

Serum and Arterial Wall Cholesterol Levels
Both serum and aortic wall cholesterol content were significantly lower in Group II compared with Group I (Table).

Serum Inflammation Response
CRP and MMP-9 were significantly lower in Group II than in Group I (Table). Although the mean PAI-1 levels appeared to be lower in Group II versus Group I, the difference was not significant ($16.6\pm8.1$ versus $33.7\pm31.1$, $P=\text{not significant}$).

Serum inflammatory markers increased progressively with development of atherosclerosis and increased further after triggering. However, these markers were consistently higher in Group I versus Group II (Figure 4). There was a strong

Figure 3. Top: Group I aorta with thrombus (arrows) and extensive plaque vs Group II aorta with minimal plaque and no thrombosis. Middle 2 rows: By scanning electron microscopy, cholesterol crystals were seen to be markedly greater in Group I (left column) vs Group II aorta (right column). Bottom row: Transmission electron microscopy demonstrates very small cholesterol crystals at plaque surface (small arrows).

Figure 4. Progression of serum inflammatory markers including C-reactive protein (CRP), matrix metalloproteinase-9 (MMP-9), and plasminogen activator inhibitor-1 (PAI-1) with development of atherosclerosis. *$P=0.05$, **$P=0.01$, ***$P=0.001$; ns indicates not significant.

Results
Gross Examination
The amount of thrombus was significantly reduced in Group II (ezetimibe treated) compared with Group I (atherosclerotic control) (Table). There was a significant correlation between thrombus area and plaque area ($r=0.60$, $P=0.02$) and between thrombus area and arterial wall cholesterol ($r=0.64$, $P=0.01$) (Figure 1). Also, thrombus area was significantly correlated with serum cholesterol ($r=0.65$, $P=0.003$).

Microscopy
In Group I, LM demonstrated that there were extensive clusters of cholesterol crystal clefts within the plaque and in macrophages (Figure 2). Also, by LM, extensive cholesterol crystal clefts were noted in the arterial wall below thrombi but not at the intimal surface, whereas by CM and SEM, extensive deposits of cholesterol crystals with tissue injury were readily detected at the surface.

Dual fluorescent imaging by CM demonstrated that there were cholesterol crystals present at the intimal surface of the aorta protruding above the endothelial cell layer (Figure 2).

SEM of the aorta demonstrated that there were very few crystals over plaque surfaces in Group II, whereas cholesterol crystals in large clusters were noted over plaque surfaces in
Cholesterol crystal density was significantly correlated with plaque area ($r = 0.75, P = 0.003$), cholesterol content ($r = 0.72, P = 0.001$), and RAM 11–stained macrophages ($r = 0.82, P = 0.001$) (Figure 1). TEM imaging of fresh-frozen arteries demonstrated that there were cholesterol crystals emerging at the intimal surface (Figure 3). Some of these crystals were well below 1 µm in size.

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correlation between MMP-9 and SUVmax (r=0.73, P=0.02), cholesterol crystal density (r=0.75, P=0.01), and RAM 11 macrophage staining (r=0.93, P=0.001) (Figure 1). Also, CRP was significantly correlated with RAM 11 (r=0.76, P<0.01).

**FDG-PET**

The SUVmax measured in vivo over the entire aorta was significantly lower in Group II versus Group I (Table), and a significant correlation was found between SUV max in the thoracic aorta and serum cholesterol (r=0.71, P=0.004). Also, there was a significant increase in FDG uptake measured in vitro from excised aortas of Group I versus Group II by SUV max (0.25±0.20 versus 0.04±0.03 g/mL, P=0.001). More plaque and a higher average number of thrombi per artery (2 versus 0.4, P<0.05) were noted when comparing gross pathology with corresponding FDG-PET scans of excised aortas (Figure 5). By RAM 11, macrophage density was significantly greater in Group I versus Group II aortic specimens (Table and Figure 5). However, no correlation was found between FDG uptake and RAM 11 in vivo or in the excised aortas. This may be due in part to the small sample size.

**Discussion**

Using an atherosclerotic rabbit model, this study demonstrated that lowering cholesterol absorption reduces plaque burden and plaque disruption. The greater plaque burden observed in Group I rabbits was associated with higher arterial wall cholesterol content, cholesterol crystal density, and inflammation leading to more intimal disruption. However, by lowering the plaque burden with ezetimibe, there was a significant reduction in arterial wall and serum inflammation, as reflected by decreased SUVmax, RAM 11, and serum inflammatory markers. This was accompanied by reduced plaque disruption, thrombosis, and cholesterol crystal density. These findings are consistent with earlier reports in humans that a large lipid pool and a large plaque burden are associated with greater incidence of cardiovascular events.15-17

An increased expression of serum inflammation markers has been reported in the setting of plaque disruption in humans.18 Our report showed higher levels of tissue macrophage cell density, SUVmax, and serum inflammation markers after triggering in rabbits whose arteries had a greater plaque burden than ezetimibe-treated rabbits. In prior studies using the same model, as well as in human carotid arteries, SUVmax elevation has also been shown to be significantly correlated with macrophage cell density in the arterial wall.5,7,19-22 In the current study, we recognize a similar finding of greater macrophage staining in rabbits with more atherosclerosis that is accompanied by a visible increase in FDG uptake as noted in Figure 5, but no significant correlation was found between SUVmax and RAM 11 in this small sample.

Free cholesterol in plaque is derived mainly from intracellular contents of dying macrophages, as well as cholesterol converted from the ester form to free cholesterol by the action of both cytoplasmic and lysosomal lipolytic enzymes.23,24 Moreover, vascular atherosclerotic rabbit aorta may be another potential source of free cholesterol from the membranes of red blood cells that may have bled into plaques.25

We recently reported that cholesterol crystals form very early during atherogenesis leading to activation of the NLRP3 inammasome.26 This suggests that cholesterol crystals can trigger a danger signal within macrophages to initiate an inflammatory response that can eventually lead to macrophage apoptosis. In the current report, we found cholesterol crystals both intra- and extracellularly within plaques at the submicron range using both LM and fresh-frozen TEM techniques. This further supports the observation of an early role of cholesterol crystals in triggering inflammation. Cholesterol oxide (7-ketocholesterol) has been shown to form crystals in macrophages in a dose-dependent fashion, leading to moderate apoptosis.27 Similarly, oxidized low-density lipoprotein has been implicated in the destabilization of arterial plaque leading to macrophage apoptosis via oxidative stress.28 In addition to the known role of oxidized cholesterol, our findings suggest that free cholesterol in crystal form plays a role in plaque rupture. However, additional studies will be required to define the role of cholesterol crystals in foam cell...
apoptosis and triggering of local inflammation as it pertains to plaque rupture.  

The morphological appearance and distribution of cholesterol crystals in this atherosclerotic rabbit model were similar to those reported in human coronary and carotid plaques.  

This was confirmed by various microscopic methods, including CM (Figure 2) and fresh-frozen TEM (Figure 3), that revealed crystals perforating the intimal surfaces of fresh and unprocessed arterial tissues. Both CM and fresh-frozen TEM methods verified the findings of cholesterol crystals perforating the intima by SEM. Although tissue shrinkage was noted in both vacuum and standard ethanol dehydration, the CM and TEM findings in fresh unprocessed tissue suggest that the reported SEM findings are not an artifact of tissue shrinkage. This is similar to the findings noted earlier in human plaques.  

In a prior in vitro study, we demonstrated that increasing cholesterol saturation was a major trigger for cholesterol crystallization. Because ezetimibe is not known to have pleiotropic effects, we used it in this model to evaluate the effects of lipid lowering on cholesterol crystals, plaque formation, and inflammation. The data in this study suggest that serum cholesterol lowering by inhibiting intestinal absorption can reduce intramural cholesterol content, as well as cholesterol crystallization. These data are also consistent with clinical findings showing that lower low-density lipoprotein cholesterol levels are associated with lower cardiovascular events regardless of the cholesterol-lowering agent used.  

The SUV max elevation in Group I compared with Group II indicates that there was greater wall inflammation in Group I that was associated with an increased amount of crystal deposits and thrombosis in the aorta. These data suggest that a larger plaque burden is associated with greater arterial wall inflammation and cholesterol crystallization, leading to more thrombosis and systemic inflammation consistent with severe arterial wall injury. Similar observations were made on individual excised aortas with corresponding FDG uptake by PET. Therefore, these findings lend additional value for the potential use of PET scanning of arteries for screening patients at increased risk for cardiovascular events.  

Conclusions  

Our study supports the premise that increased cholesterol burden in the arterial wall leads to a greater inflammatory response and cholesterol crystal formation that is associated with increased wall disruption and thrombosis. This in vivo study and our prior studies support the hypothesis that plaque disruption is enhanced by greater local and systemic inflammation in association with cholesterol crystal formation. Additional studies will be needed to evaluate these effects both at a cellular level and in humans.  

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