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.1 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, removed, 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 eosin, Masson trichrome, or Movat pentachrome for examination under a light microscope (Laborlux12, Leitz, Oberkochen, Germany).

**SEM**

Tissue samples (3 to 5 mm long) were dehydrated for 12 hours in a vacuum chamber (Speed Vac SC10, 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.10 These tissue segments were then mounted on stubs and gold coated in a sputter coater (EMSCOPE SC500; Emscope, Ashford, UK), the immunohistochemical stain for mononuclear 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 ×20×0.1 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 J, version 2.5.12 Images were analyzed with ImageJ, version 1.43t (http://rsb.info.nih.gov/ij/). 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. Thrombosis, 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.
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 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></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>
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<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>
<tr>
<td>P value</td>
<td>0.002</td>
<td>0.003</td>
<td>0.001</td>
<td>0.01</td>
<td>1.000</td>
<td>0.02</td>
<td>0.001</td>
<td>1.000</td>
<td>0.04</td>
</tr>
</tbody>
</table>

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

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.
1). 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).
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
significant correlation was found between SUVmax in the significantly lower in Group II versus Group I (Table), and a The SUVmax measured in vivo over the entire aorta was also, 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, 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

**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

Discussion
apoptosis and triggering of local inflammation as it pertains to plaque rupture.20

The morphological appearance and distribution of cholesterol crystals in this atherosclerotic rabbit model were similar to those reported in human coronary and carotid plaques.8–10 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.10

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.5,8–19,22

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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**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 표지자를 통해 동맥의 염증을 조사하였다. 혈청과 대동맥벽의 콜레스테롤 수치를 측정했으며 혈전의 면적도 측량하였다. 전자현미경을 이용해 대동맥 표면의 콜레스테롤 결정의 농도를 점수화(0~+3점) 하였다. 혈청 및 대동맥벽의 콜레스테롤, 죽상반 면적이 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 농도와 콜레스테롤 결정(crystal) 발생의 저하는 죽상반의 파열과 혈전의 발생을 억제하였다.
Commentary

Ezetimibe는 소장에서 Niemann-Pick C1 Like 1 (NPC1L1) sterol transporter의 활성을 막아 식이 성 및 담즙성 콜레스테롤의 흡수를 막는 약제로서 혈중 콜레스테롤을 강하게 효과는 이미 입증되어 있다. 따라서 ezetimibe는 비교적 순수하게 혈중 콜레스테롤의 저하와 동맥중경화에 미치는 영향에 대해 평가할 수 있다. 동맥중경화 과정의 초기부터 형성된다고 알려진 콜레스테롤의 결정(crystallization)은 그 체적의 평가에 의해서 뿐 아니라 화학적으로도 염증 반응을 유도할 수 있다. 특히, 결정이 커지면서 주위 조직과 혈관내막에 이상과 혈관내막을 초래하며, 동맥경화성 질환에 미치는 영향에 대해 평가할 수 있다. 따라서 ezetimibe를 통해 혈중 콜레스테롤의 저하에 비례하여 동맥벽의 콜레스테롤 함량 및 콜레스테롤의 결정화와 염증반응이 감소되고, 죽상반의 형성 및 파열과 혈전이 억제될을 보인 본 연구는 ‘동맥중경화병의 가장 결정적인 원인인 인자는 콜레스테롤’이라는 사실을 재확인하기 때문이다. 그 외에도, 아직 사람에서의 임상연구 결과가 충분한 ezetimibe의 동맥중경화병 절환에 대한 효능을 기대할 수 있는 또 하나의 긍정적인 임상적 결과라는 데 의의가 있다. 동맥중경화병의 과정은 여러가지가 약소화되어 있지만, ezetimibe는 동맥경화성 질환에 대한 임상적 효과가 입증되면서도 사람에서의 임상연구 결과가 충분한 ezetimibe의 동맥중경화병 절환에 대한 효능을 기대할 수 있는 또 하나의 긍정적인 임상적 결과라는 데 의의가 있다. 2013년 종료되는 IMPROVE-IT 연구는 급성심혈관사건 10일 이내에 ezetimibe/ simvastatin 10/40mg 대 simvastatin 40mg의 주요 심혈관사건에 대한 효과를 18,000명에서 직접 비교하는 연구로써 스타턴 단독 치료와 비교해 ezetimibe의 추가적인 임상적 효과를 가늠할 수 있는 연구이다. 본 연구와 같은 동물실험의 결과들이 임상적으로도 입증될 수 있을지 흥미롭게 지켜볼 것이다.

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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 macrographie 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.

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, 0.05 mg/kg SC every 12 hours) were administered postoperatively.

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Committee following National Institutes of Health guidelines. Although the dose of ezetimibe used was higher than previously reported for rabbits (0.6 mg/kg per day), it was given for a short period (3 months). A fter 6 months, plaque disruption and thrombosis was induced by Russell’s viper venom (0.15 mg/kg IP, Sigma-Aldrich, St. Louis, MO) and histamine (0.02 mg/kg IV, Sigma-Aldrich) given at 48 and 24 hours before euthanasia. General anesthesia was used again as described above for FDG-PET, and eu- thanasization (buthanol 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, 128×128 matrix). Standardized uptake values (SUVs) were calculated with adjustment for rabbit weight, injected dose, and F-18 decay. Maximal SUV (SUV max) 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 (Ab- sciences, Seattle, WA). These were then incubated with a goat anti-human-MMP-9 monoclonal antibody (LS-C8808, LifeSpan Biosciences, Seattle, WA) and then washed. A solution of tetramethylbenzidine was added to the plate and incubated for 15 minutes. Absorbance 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

A fter rabbits were euthanized, the aorta and iliofemoral arteries were dissected, removed, 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% gluteraldehyde.

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 monocyto- nal antibodies to rabbit macrophages. To determine macrophage density, tissue sections were examined with a light microscope (Microphot FXX, 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 2.5.1.12 Images were analyzed with Imagej, version 1.43u (http://rsb.info.nih.gov/ij). 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 preservation. 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 and SEM 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% gluteraldehyde followed by vacuum dehydration and compared with samples from the same artery processed using ethanol dehydration and paraffin embedding.
scanning. Procedures were performed according to protocols an-
esthesia was used again as described above for FDG-PET, and 
was induced by Russell’s viper venom (0.15 mg/kg IP, Sigma- 
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After rabbits were euthanized, the aorta and iliofemoral arteries were 
Gross examination 

Cholesterol concentrations were determined in the serum and 
Matrix metalloproteinase-9 (MMP-9) at baseline, after athero-

Aortic samples (1 cm long) were obtained from the thoracic, upper 
Cholesterol-Lowering Effects on Crystallization, Thrombosis, and Inflammation

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 0.02 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.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. A djacent 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 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 650 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 SUV max between the 2 rabbit groups with and without ezetimibe. Correlations were obtained between cholesterol crystal density and plaque area, cholesterol content, SUV max, 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 ± 8.1 versus 33.7 ± 31.1, $P$ 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).

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...
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 \(\mu\)m 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.

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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 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 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, 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.\textsuperscript{28}

The morphological appearance and distribution of cholesterol crystals in this atherosclerotic rabbit model were similar to those reported in human coronary and carotid plaques.\textsuperscript{8-10} 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.\textsuperscript{10}

In a prior in vitro study, we demonstrated that increasing cholesterol saturation was a major trigger for cholesterol crystalization.\textsuperscript{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 crystalization. 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.\textsuperscript{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 crystalization, 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.\textsuperscript{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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