Plaque disruption (PD) causes most acute cardiovascular events. Although cholesterol crystals (CCs) have been observed in plaques, their role in PD was unknown. However, cholesterol expands with crystallization tearing and perforating fibrous tissues. This study tested the hypothesis that CCs can damage plaques and intima, triggering PD, as observed in tissues prepared without ethanol solvents that dissolve CCs. Coronary arteries of patients who died of acute coronary syndrome (n = 19) and non–acute coronary syndrome causes (n = 12) and carotid plaques from patients with (n = 51) and without (n = 19) neurologic symptoms were studied. Samples were examined for CCs perforating the intima using light and scanning electron microscopy (SEM) with ethanol or vacuum dehydration. In addition, fresh unfixed carotid plaques were examined at 37°C using confocal microscopy. Crystal content using SEM was scored from 0 to +3. SEM using vacuum dehydration had significantly higher crystal content compared with SEM using ethanol dehydration (+2.5 ± 0.53 vs +0.25 ± 0.46; p < 0.0003), with enhanced detection of CC perforations. The presence of CCs using SEM and confocal microscopy was similar, suggesting that CC perforation can occur in vivo at 37°C. All patients with acute coronary syndrome had perforating CCs, but none was present in patients without acute coronary syndrome (p = 0.0001). For all plaques, there were strong associations of CCs with PD, thrombus, symptoms (p < 0.0001), and plaque size (p < 0.02). Crystal content was an independent predictor of thrombus and symptoms. In conclusion, by avoiding ethanol in tissue preparation, CCs perforating the intima were shown to be associated with PD. Crystal content was significantly associated with clinical events, suggesting that cholesterol crystallization may have a role in PD. © 2009 Elsevier Inc. All rights reserved. (Am J Cardiol 2009;103:959–968)

Although cholesterol crystals (CCs) are often seen abundantly within atheromatous plaques and at sites of plaque disruption (PD), their potential role in PD has not been defined.1–3 Using standard histologic study with paraffin-embedded tissues and routine hematoxylin and eosin staining, CCs appeared as needle-shaped clefts that indicated sites where crystals were originally present, but were now dissolved during tissue processing with lipid solvents.4 Therefore, the tissue-preparation process can greatly influence histologic findings. In preliminary work, we showed improved CC preservation using air instead of ethanol dehydration.5 However, because of the shorter preparation time, in this study, we avoided ethanol dehydration by evaluating a novel approach using vacuum dehydration to better visualize the full effect of CCs in PD. Given the recently shown volume expansion observed during cholesterol crystallization in vitro5–7 and the known presence of liquid cholesterol within plaques,8 we investigated the potential role of CCs in patients with PD. The present study used vacuum dehydration of tissues to test the hypothesis that CCs are associated with PD and their presence is associated with cardiovascular syndromes attributable to PD.

Methods

A case-control study design was used by selecting autopsies of patients who died of acute coronary syndrome or non–acute coronary syndrome causes (December 2005 to August 2008). Acute coronary syndrome was considered present in patients who died with symptoms of cardiac ischemia and those found dead with no evidence other than PD and/or ventricular rupture. Patients without acute coronary syndrome died of no cardiovascular cause. Medical history and demographics were obtained from the pathologist.

Carotid plaques were collected consecutively (October 2006 to October 2008) from a cohort of patients who underwent carotid endarterectomy for stenosis ≥70% with or without neurologic symptoms. These included transient weakness, numbness, vision loss, speech deficit, headaches, dizzy spells, and stroke. Patient demographics, symptoms, and angiographic data were obtained without identifiers from the surgeon’s office. Tissues and data were obtained in compliance with the institutional review board guidelines of Michigan State University, East Lansing, Michigan, and Sparrow Hospital, Lansing, Michigan.
At autopsy, coronary arteries were serially sectioned at 2- to 3-mm intervals up to the arterial occlusion. This segment of artery was then excised intact en bloc from suspected culprit arteries. In addition, atherosclerotic and normal segments from other coronary arteries were sampled similarly for comparison. All specimens were examined using scanning electron (SEM) and light microscopy (LM). Carotid plaques removed at endarterectomy were examined grossly, and surface area was calculated as the product of the short and long axes. Half the specimen was used for this study, and the remaining half was submitted to pathology. Several (2 to 3) transverse sections (2 mm thick) were then obtained from sites of ulceration when present and examined using confocal microscopy (CM). All remaining tissues were processed for SEM and LM. Thus, the entire specimen was examined using both SEM and LM.

For SEM, arterial samples were fixed in buffered 4% glutaraldehyde and cut into two 4- to 5-mm long segments. The segments were cut longitudinally and transversely. To show the effects of ethanol on CC preservation, we conducted a substudy comparing ethanol with vacuum dehydration during tissue preparation for SEM. Arterial segments from 7 patients with acute cardiovascular events (5 coronary segments and 2 carotid plaques) were cut into halves. One-half of the same arterial segment was treated using standard ethanol dehydration (ethanol 25%, 50%, 75%, and 95% each for 30 minutes, then 100% for 45 minutes) followed by carbon dioxide critical point drying, and the

Figure 1. SEM of PD from a right coronary artery cut longitudinally shows frayed plaque cap edges (long white arrows). Cap thickness was 131 μm (dual black arrows). The plaque basin (small white arrows) and arterial lumen are filled with thrombus. A color-coded image at low magnification defines thrombus (red); ruptured plaque (blue), and sites of crystals perforating the intima (green-yellow). Examples of CCs perforating the intimal surface at the plaque shoulders: (1) (bar = 20 μm), (2) (bar = 10 μm), (3) (bar = 10 μm), and *.* Additional examples of crystals perforating the intima without and with thrombus ([A] bar = 5 μm, [B] bar = 10 μm) and normal endothelium ([C] bar = 25 μm). Case was a 57-year-old woman who died of acute coronary syndrome.
remaining half was dehydrated in a vacuum chamber for 12 hours (Speed Vac SC110; Savant Instruments Inc., Farmingdale, New York) and evacuated using a pump (VP110; Franklin Electric, Bluffton, Indiana). Tissue shrinkage using vacuum dehydration was calculated by subtracting the width and/or length of gross fresh artery and plaques from those at low-power scanning electron microscopic images of the same specimens divided by the corresponding gross fresh artery or plaque dimensions.

Because of markedly superior preservation of CCs, tissues for SEM from all remaining patients (n = 94) were prepared using vacuum dehydration. All tissue segments, including the substudy, were then mounted on stubs and gold coated in a sputter coater (EMSCOPE SC500, EmScope, Ashford, United Kingdom). The arterial lumen was then examined using a Jeol scanning electron microscope (model JSM-6300F; Jeol Ltd., Tokyo, Japan). Two investigators independently read the scanning electron microscopic images and were blinded to the patient’s clinical status. Using SEM, PD was defined as disruption of the intimal surface with plaque cap tears and intimal damage with loss of endothelium, often with thrombosis, as adapted from the definition for LM.9 Cap thickness on scanning electron microscopic images was measured at the thinnest part of the cap using the AnalySIS imaging software (Soft Imaging System GmbH, Münster, Germany) in the Jeol microscope.

Crystal content on SEM was defined as extensive (+3), reflecting sheets of CCs covering the whole intimal surface; moderate (+2), reflecting extensive CCs covering isolated sites; mild (+1), reflecting some scattered CCs; or no CCs (0).

To confirm that CCs were present at body temperature, CM was used to image CCs at the surface of fresh unfixed carotid plaques. Plaques were collected in the operating room from

Figure 2. SEM of PD from the left anterior descending artery cut longitudinally ([A] bar = 1 mm) and transversely ([B] bar = 1 mm) shows a torn plaque cap and thrombus (A, B; arrows). A color-coded image defines the thrombus (red); ruptured plaque (blue), and sites of crystals perforating the intima (green-yellow). Higher magnification shows crystal clusters: (1a) (bar = 10 μm) and (2a) (bar = 20 μm) with thrombosis and (1b, 2b, 1c, 2c) at the intimal surface (bar = 10μm) with (2c) crystal perforation over the endothelial surface (bar = 20 μm). Case was a 35-year-old man who died of acute coronary syndrome.
Kodak Co., Rochester, New York) camera mounted on a stained sections using a Kodak DCS Pro14n (Eastman verified at the thinnest site from digital light micrographs of eosin or trichrome stains. Plaque cap thickness was measured using a Zeiss Pascal LSM microscope (Carl Zeiss, Inc., Jena, Germany). Fluorescence was collected sequentially using the argon 488-nm laser line for excitation of Bodipy and collecting green fluorescence with a 560-nm long pass filter. Selective staining of CCs by Bodipy was shown by incubating CCs in Bodipy and acetylated low-density lipoprotein or eosin independently or in combination at 37°C. Plaque samples were then transferred to a slide incubator chamber filled with phosphate-buffered saline and visualized. Unstained plaque samples were also examined to detect native tissue fluorescence. Confocal microscopic fluorescence images of plaque were acquired using a Zeiss Pascal LSM microscope (Carl Zeiss, Inc., Jena, Germany). Fluorescence was collected sequentially using the argon 488-nm laser line for excitation of Bodipy and collecting green fluorescence with a 560-nm long pass filter. Selective staining of CCs by Bodipy was shown by incubating CCs in Bodipy and acetylated low-density lipoprotein or eosin independently or in combination at 37°C. Tissue sections for LM were obtained from all patients (n = 101) and cut transversely from the suspected PD sites of culprit arteries and stenotic sites of nonculprit arteries. Additional sections from disruption sites were obtained from examined scanning electron microscopic specimens by rehydrating and then processing for LM. These specimens were sectioned by stepping through the segment to capture PD sites. Tissue sections for LM were prepared in the standard fashion by fixing in 10% buffered formaldehyde, dehydrated using graded ethanol (50% to 100%), and embedded in paraffin. Serial tissue sections (5 μm) were mounted on glass slides and stained with hematoxylin and eosin or trichrome stains. Plaque cap thickness was measured at the thinnest site from digital light micrographs of stained sections using a Kodak DCS Pro14n (Eastman Kodak Co., Rochester, New York) camera mounted on a Nikon Microphot FX (Nikon, Corp., Melville, New York) microscope (Tokyo, Japan) and analyzed using ImageJ, version 1.37 (http://rsb.info.nih.gov/ij/). Necrotic core size was measured using planimetry from photographed images of the atheromatous gruel area enclosed by the inner layer of fibrous cap and arterial wall.

GraphPad Prism 5 (GraphPad Software, San Diego, California) and Statistical Analysis Software (version 9.1; SAS Institute, Cary, North Carolina) were used for statistical analysis. Fisher’s exact test or McNemar’s test was used for testing associations between any 2 categorical variables; the presence of CCs perforating the intima with and without thrombus and associated clinical events, between CCs and PD, thrombus and clinical events, and the presence of CCs detected using SEM versus LM and SEM versus CM, respectively. For continuous variables, a 2-sample t test and its parametric equivalent was used for 2-group comparisons. Simple linear regression was used to estimate the increase in plaque size for each unit increase in CC content. Logistic regression was performed to evaluate the effect of increasing content of CCs (+1, +2, and +3 vs 0) on risk of thrombus and symptoms. A 2-sided p value <0.05 was used as the criterion for statistical significance.

Patients undergoing carotid endarterectomy and immediately placed in phosphate-buffered saline at 37°C. Samples were then transported to the laboratory, where they were stained for CCs using fluorescent dye (cholesteryl Bodipy-C12; Invitrogen, Eugene, Oregon). To show the relation of CCs to the intimal surface, specific counterstaining of endothelium was performed by incubating with acetylated low-density lipoprotein or eosin independently or in combination at 37°C. Plaque samples were then transferred to a slide incubator chamber filled with phosphate-buffered saline and visualized. Unstained plaque samples were also examined to detect native tissue fluorescence. Confocal microscopic fluorescence images of plaque were acquired using a Zeiss Pascal LSM microscope (Carl Zeiss, Inc., Jena, Germany). Fluorescence was collected sequentially using the argon 488-nm laser line for excitation of Bodipy and collecting green fluorescence with a 560-nm long pass filter. Selective staining of CCs by Bodipy was shown by incubating CCs in Bodipy and acetylated low-density lipoprotein or eosin independently or in combination at 37°C.

Only a few crystals are noted in the core (arrows), but none perforating the cap (Masson’s trichrome stain; bar = 500 μm; insert bar = 200 μm).

Results

Coronary arteries from the hearts of 31 patients (23 men, 8 women; age 50 ± 15 years) who had died of acute coronary syndrome and non–acute coronary syndrome causes were examined within 24 hours after death. Coronary artery disease as the primary cause of death was reported in 19 patients. By history, 8 patients had symptoms of myocardial ischemia before death, 2 had left ventricular rupture, and 9 were found with PD as the only cause of death. Patients without acute coronary syndrome (n = 12) had no myocardial ischemia symptoms and the cause of death included motor vehicle accidents (n = 4), drug overdose (n = 3), trauma (n = 3), pulmonary embolism (n = 1), and sepsis (n = 1).

Fresh carotid plaques from 70 patients (32 men, 38 women; age 73 ± 9 years) with and without neurologic
symptoms (n = 51 and n = 19, respectively) were obtained at endarterectomy. Average lesion stenosis was $80 \pm 12\%$ using computed tomographic angiography (n = 52), duplex ultrasonography (n = 6) and/or angiography (n = 9), and magnetic resonance angiography (n = 3).

Average coronary artery segment length was $1.0 \pm 0.9$ cm from the suspected culprit and control arterial segments. Surface area for all carotid plaques was $2.3 \pm 1.2$ cm$^2$.

Extensive CCs were visible perforating the intima in fresh unfixed plaques using CM and in fixed plaques that were vacuum dehydrated for SEM, but nearly absent with ethanol-dehydrated plaques using either SEM or LM.

Coronary artery segments (n = 79) from 31 patients were processed for SEM examination. This showed PD surrounded with extensive pointed tipped CCs perforating the intima and plaque surfaces (Figures 1 and 2). Individual CCs measured 1 to 3 $\mu$m in diameter at the tips, 50 to 150 $\mu$m long, and up to 20 $\mu$m wide at the base. However, CC clusters were thicker and longer. Although LM showed PD in the same plaques, there was little evidence of CCs perforating the intima or cap (Figure 3). CCs perforating the plaque seen using SEM were similar to in vitro observations of CCs penetrating fibrous tissues during cholesterol crystallization. Nearly every quadrant of artery adjacent to PD sites had CCs protruding through the intima.

Using SEM, plaque cap thickness at PD sites measured $109 \pm 49.8$ $\mu$m (range 47 to 182) versus $326 \pm 141.7$ (range 189 to 531) at nondisrupted sites ($p < 0.001$). Moreover, no intimal crystal perforations were noted when
Plaque cap thickness was >200 μm. The 2 independent readers agreed on all microscopic examination results.

In the substudy comparing ethanol versus vacuum tissue dehydration, only 3 of 7 specimens dehydrated using ethanol had rare identifiable CCs, whereas extensive CCs were present in all 7 vacuum-dehydrated specimens (+0.25 ± 0.46 vs +2.5 ± 0.53; p <0.0003). The few CC arterial segments prepared with ethanol appeared eroded, with thinned and distorted shafts (Figure 4).

The percentage of average shrinkage noted using vacuum dehydration was measured to be 27% in width for 35 specimens evaluated. Length was decreased by 17%, measured in 4 specimens.

In 157 light microscopic sections of coronary arteries with and without PD, fibrous caps from disrupted plaques were significantly thinner than caps measured from nondisrupted plaques (109 ± 45.4 vs 344 ± 213.36 μm; p <0.0002). Plaque cap thickness measurements using LM were not significantly different from those using SEM. Mean necrotic core area in PD was 2.6 ± 1.7 mm², consistent with reports for large cores.12

Plaques from 70 carotid endarterectomy specimens were evaluated using SEM, LM, and CM. Using SEM, sheets of CCs were noted penetrating the intima, especially beneath thrombi (Figure 5). However, very few were detected using LM (Figure 6).

CM of fresh unprocessed plaques at 37°C showed wide distribution of green-stained fluorescent CCs. There was no native tissue fluorescence at the same voltages. Using dual staining of carotid plaques, CCs fluoresced green and appeared to be perforating the intimal surface that fluoresced red (Figure 7). Staining CCs alone in vitro showed only green fluorescence using Biodipy and no fluorescence using acetylated low-density lipoprotein or eosin. In addition, using low-power imaging under a dissecting microscope, it was possible to see extensive CCs at the intimal surface in both fresh unfixed and fixed carotid segments (Figure 7).

Comparing SEM with CM in 28 samples, all had CCs identified using at least SEM or CM, and there was complete agreement in 24 of 28 (p = NS). However, comparing LM with SEM in the exact same samples (coronary, n = 18; carotid, n = 56), only 12 of 74 had CCs perforating the intimal surface using LM, and 68 of 74, using SEM (p <0.0001).

Using SEM, PD was noted in all 19 patients with acute coronary syndrome, whereas using LM, only 17 of 19 patients with acute coronary syndrome had evidence of PD. However, none of the 12 patients who died of non–acute coronary syndrome causes had evidence of PD or CCs in the intima (p = 0.0001; Table 1). Eight of these patients without acute coronary syndrome had moderate (n = 3; ≥50%
to ≤75% area stenosis) or severe (n = 5; ≥75% area stenosis) coronary atherosclerosis without evidence of perforating CCs. In 5 patients with acute coronary syndrome, CCs were noted emerging from the arterial intima not only in the culprit artery, but also remotely in 6 plaques of nonculprit arteries. Using SEM, the relation between coro-
nary thrombus (n = 15) and the presence of CCs (n = 19) by patient was statistically significant (p = 0.0001).

SEM showed perforating CCs in 38 of 79 of coronary segments received (Table 2). All those segments were from patients with acute coronary syndrome, and thrombi were present in 25. In the remaining segments, there were 41 without CCs or thrombi and 13 with CCs and PD without thrombi. In atherosclerotic segments with PD, the relation between CCs and thrombi was significant (p = 0.0001). Of note, CCs were occasionally seen deep in the arterial wall of patients without acute coronary syndrome with atherosclerotic plaques without PD. No CCs were present in normal arteries.

Carotid segments were examined from 70 patients (Table 3). Thrombus (>2 times) and plaque surface area were significantly greater in patients with neurologic symptoms. In addition, higher crystal content (+3) was more frequent in plaques from patients with neurologic symptoms (p = 0.01). For all carotid plaques, using simple linear regression, a significant increase in plaque size (0.35 cm²) per unit increase in crystal content was observed (β = 0.35, p < 0.02; Figure 8).

In all plaques from coronary and carotid arteries, a significant association was observed for CCs with PD, thrombus, and clinical symptoms (p < 0.001). To assess the dose-response of crystal content on risk of thrombus, logistic regression was performed. Relative to crystal content of 0, contents of 1, 2, and 3 had odds ratios for thrombi of 3.3 (p = 0.10), 7.0 (p < 0.01), and 22.5 (p < 0.001), respectively. This indicated a significant increase in risk of thrombus with increasing crystal content (Figure 9). Chi-square test for linear trend was significant at p < 0.001. Assessment of the association between CC content and symptoms (100 patients) also showed a significant increase in the proportion of patients who reported symptoms with increasing CC content (p < 0.001; Figure 9).

**Discussion**

The ability to detect the extensive CC distribution and elucidate their potential role in PD was possible by processing tissues without an ethanol solvent. This study showed that arterial tissues and plaque prepared using vacuum dehydration for SEM markedly improved the detection of CCs.
disrupting plaque and penetrating the intimal surface. In addition, these observations using SEM were shown to reflect in vivo events by being concurrent with the presence of CCs in unprocessed plaque at body temperature using CM. Use of ethanol as a tissue-dehydrating agent for histologic preparation was reported by Baker in 1743 and has persisted until the present. However, as shown, ethanol dehydration almost totally eliminated CCs. Thus, compared with SEM without ethanol dehydration, LM greatly underestimated the role of crystal perforations of plaque and intima. Furthermore, LM was highly dependent on the sampling site, and one 5-mm long arterial segment examined for surface morphologic characteristics using SEM would be equivalent to 1,000 sections for LM, each 5 μm thick. Also, visualization using SEM was 3 dimensional and had higher resolution and magnification than LM.

By making this technological change, we were able to support our hypothesis relating CCs perforating the intima and PD, the underlying cause of thrombus formation causing clinical syndromes. Using logistic regression analysis, crystal content was associated with a graded risk of thrombosis, suggesting that more intimal damage by CCs led to more thrombosis. Finally, in all plaques, CC content was an independent predictor for thrombus, and there was a significant association between increasing crystal content and increasing clinical symptoms. Although nondisrupted plaques occasionally had CCs, those were not seen perforating the intima.

Our earlier reports of volume expansion during cholesterol crystallization in vitro and the present observations in human plaques suggested that CCs expanded and cut through fibrous tissue similar to plaque caps causing PD. Moreover, dual fluorescence staining with CM at 37°C showed that CCs had the potential to disrupt and perforate the plaque cap emerging onto the intimal surface in vivo. The same was also noted using low-power microscopy of the whole tissue surface. Morphologically, these were identical to the pointed CCs perforating fibrous tissue during dynamic volume expansion with crystallization reported in vitro. In addition, in those reports, we showed that the amount of cholesterol directly correlated with the rate and volume expansion. In this study, an increasing amount of crystal content was significantly associated with greater plaque size. Thus, a large plaque burden, manifested by large necrotic cores, appeared to be a risk factor for PD and cardiovascular events, as previously reported.

In our study, we also confirmed that disrupted plaques had significantly thinner caps compared with nondisrupted plaques. Although triggers of cholesterol crystallization have yet to be elucidated, large extracellular lipid deposits may constitute a critical amount of cholesterol saturation needed to initiate crystallization in plaques. This could explain why the severity of atherosclerosis and arterial wall cholesterol content directly correlated with the extent of thrombosis.

The main limitation was that this study was conducted in post mortem and ex vivo specimens. However, to our knowledge, there was no current technology that could detect CCs in vivo. Despite this limitation, we were able to show that fresh ex vivo unfixed plaques at 37°C had abundant CCs perforating the intima, suggesting that the process could occur in vivo. Another limitation was that with the exception of the culprit arteries, not all coronary arteries were evaluated in every patient. However, the primary goal of our study was to evaluate the association of CCs with PD. In addition, there was the possibility of cutting-induced artifacts during tissue preparation for SEM, but those would not be much different from those using LM. Most areas examined using SEM were remote from the cut edges. Also, stable plaques that had CCs below the surface did not have evidence of CCs on the intimal surface, suggesting no spillover by cutting. With regard to possible artifact from vacuum dehydration causing tissue shrinkage, we showed the presence of crystals penetrating the plaque surface in fresh unfixed and not vacuum-treated tissue. Arterial tissue shrinkage is known to occur by 25% using standard tissue preparation, but less using glutaraldehyde fixation as we had used. Vacuum dehydration did not markedly increase tissue shrinkage compared with standard ethanol dehydration (27% vs 25%, respectively), possibly because the tissue was already fixed. Furthermore, the tissue morphologic characteristics seen using SEM with the vacuum technique were well preserved.

Figure 9. Graph of percentages of (A) thrombus risk for each CC content level and (B) patients with symptoms and their CC content levels.
In conclusion, the significance of this work was that when preserving CCs by avoiding ethanol in tissue preparation, we were able to show an association between CCs and PD using SEM. This association may provide a useful marker for early PD. The clinical implications would be to develop strategies that detect CCs perforating the intima, followed by targeted pharmacologic and/or mechanical interventions to prevent or reverse cholesterol crystallization with consequent PD.

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