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Colocalization of Cholesterol and Hydroxyapatite in Human Atherosclerotic Lesions

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Summary. Cholesterol and calcium phosphate, the latter in the form of hydroxyapatite, accumulate in atherosclerotic lesions. In this report, we demonstrate that these organic and inorganic constituents of lesions can accumulate together, closely associated in crystal agglomerates. Using the fluorescent cholesterol probe, filipin, we identified unesterified cholesterol that was associated with calcium granules in tissue sections of lesions. We also have shown that small crystallites of cholesterol can associate with preformed hydroxyapatite crystals *in vitro*. Scanning electron microscopy coupled with energy-dispersive X-ray analysis demonstrated the physical association of many small crystallites of cholesterol with larger crystals of hydroxyapatite. These small crystallites of cholesterol associated with hydroxyapatite stained with filipin. This contrasted with the lack of filipin staining of unassociated larger cholesterol crystals or hydroxyapatite alone. How cholesterol and calcium come to be closely associated in crystal agglomerates within atherosclerotic lesions remains to be determined.

Key words: Atherosclerosis – Calcification – Hydroxyapatite – Cholesterol – Filipin.

Cholesterol accumulates both in its esterified and unesterified forms in human atherosclerotic lesions. The detection of lipids in histological studies of atherosclerotic lesions has been carried out with the use of lipid-soluble Sudan dyes. Lipid-soluble Sudan dyes stain triglycerides, some phospholipids, and the esterified form of cholesterol; they do not, however, stain unesterified cholesterol. Consequently, little was known concerning the location of accumulated unesterified cholesterol within lesions until a suitable dye was found to stain this form of cholesterol. Unesterified cholesterol can be identified in tissue sections and cells with the use of the fluorescent polyene antibiotic, filipin, which specifically binds to 3β-hydroxysterols [1]. The results of a study on the localization of unesterified cholesterol in human atherosclerotic lesions suggest that unesterified cholesterol is sometimes associated with calcium-containing deposits [2].

Calcification is one of the most serious complications of atherosclerotic lesions and is irregularly distributed along the thickened atherosclerotic intima and underlying media [3, 4]. These calcific deposits represent a secondary phenomenon of focal degenerative changes associated with progressive lipid deposition [5]. The predominant form of calcium deposits in atherosclerotic lesions is hydroxyapatite, the most stable calcium phosphate polymorph [6].

The objective of this study was to further examine the histochemical localization of cholesterol and calcium in human atherosclerotic lesions and to examine the filipin staining characteristic of agglomerate crystals of cholesterol and hydroxyapatite produced *in vitro*. In the case of the *in vitro* preparations, the nature of the association between the organic (cholesterol) and the mineral (calcium phosphate) parts was investigated. The comparison between the appearance of filipin-stained samples of calcium hydroxyapatite crystals artificially coated with cholesterol and deposits of calcium hydroxyapatite and cholesterol detected in human atherosclerotic tissues may help elucidate the nature of the close association of cholesterol and calcium hydroxyapatite in atherosclerotic lesions.

Materials and Methods

Preparation of Tissue Sections

Human aortic and coronary artery tissues were obtained at autopsy. Aortic tissue was placed in Dulbecco's phosphate-buffered saline (DPBS) (4°C) and transported to the laboratory. Human coronary arteries were dissected from hearts previously fixed in 10% formalin. After adventitial tissue was removed, vessels were opened longitudinally if this had not been done during autopsy. Blood or loose clot was washed away with DPBS before the vessels were pinned flat onto a dissection board. Atherosclerotic lesions were cut out and pinned flat onto corkboards covered with oilcloth. Corkboards with pinned tissues were floated tissue facing down in sealed plastic boxes containing 10% formalin. Tissues were fixed within 24 hours after death well within the time interval (up to 48 hours after death) in which vascular unesterified and esterified cholesterol levels have been shown to remain unchanged [7].

After the tissue was fixed a minimum of 24 hours, the formalin was discarded and replaced with formalin containing 2% gelatin. The tissue remained in this solution overnight. Next, tissue pieces were elevated along their pins, and corkboards with tissue were submerged in 20% gelatin (37°C) (granular, 100-bloom laboratory grade, Fisher Scientific Co., Fair Lawn, NJ). Gelatin was allowed to solidify over night at 4°C before the pins were removed, and blocks of gelatin, each containing one piece of tissue, were cut out and stored in formalin. Gelatin blocks were hardened in formalin a minimum of 2 days before frozen sections (2.5 and 5.0 μm) were cut (sliding arc CO_2 freezing microtome, E. Leitz, Inc., Rockleigh, NJ) and collected in distilled water. Sections were floated onto gelatin-coated microscope slides and allowed to dry a minimum of 48 hours before staining.

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Demonstration of Cholesterol and Calcium Colocalization in Tissue Sections

Slides with adherent tissue sections were stained with hematoxylin (stains mineral calcium, as discussed in Blumenthal et al. [4], and nuclei, blue) followed by filipin (stains unesterified cholesterol, green), as described below and in [2]. Sections were first immersed in 70% ethanol for 30 minutes; this eliminated all filipin staining except that associated with calcium deposits. Next, sections were stained for 20 minutes in Mayer's hematoxylin and rinsed for 10 minutes in distilled water. Hematoxylin staining was enhanced by immersing slides in Scott's tap water substitute for 2 minutes. After the sections were rinsed for 2 minutes in DPBS, the excess DPBS was removed by careful blotting of the sections with tissue paper. Filipin stain (2.5 mg filipin dissolved in 1 ml dimethylformamide and added to 50 ml DPBS) was layered over tissue sections, which were stained for 30 minutes in a humidified chamber. The sections were rinsed for 10 minutes in distilled water and mounted in glycerol-gelatin containing 1% phenol.

Tissue sections were viewed and photographed using bright-field, polarization, and fluorescence microscopy. Filipin dye was excited with epi-illumination using ultraviolet light (UG 1 filter) from a 100-watt mercury arc lamp. Fluorescence was viewed through a 510-nm barrier filter.

Hydroxyapatite-Induced Cholesterol Deposition

Ethanol solutions were prepared which were very slightly supersaturated with cholesterol, that is, 91 mg of cholesterol per 50 ml of solution composed of 80 parts ethanol:20 parts buffer [8]. The buffer was a 46 mM tris-buffered (pH 7.4) solution of 0.15 ionic strength (5 g NaCl/liter and 4.8 g KCl/liter). These solutions were checked to determine their metastability. Metastability means there was no spontaneous formation of cholesterol crystals on the addition of a defined volume (10 ml) of the tris-buffered solution to 40 ml of ethanolic cholesterol solution [8].

Hydroxyapatite crystals were prepared according to the previously described procedure [9]. Hydroxyapatite crystals (8 mg) were introduced to 50 ml of the metastable alcoholic solution of cholesterol and allowed to remain in the solution for 20 minutes at room temperature. The solid phase was then filtered out and dried in air. This procedure has been shown to result in the nucleation of cholesterol crystal formation upon the hydroxyapatite seeds [8]. In some cases, the immersion time of the seeds was prolonged.

Staining and Microscopic Analysis of In Vitro*-Produced Hydroxyapatite/Cholesterol Crystal Agglomerates*

The hydroxyapatite crystals which nucleated the cholesterol overgrowths were spread on microscope slides, immersed in filipin solution, and viewed by phase, fluorescence, and polarization microscopy.

Some of the unstained artificial hydroxyapatite/cholesterol agglomerate particles were viewed with a scanning electron microscope (Jeol JSM-35) and analyzed using energy-dispersive X-ray analysis (EDAX).

Results

A tissue section of an atherosclerotic lesion stained with filipin and hematoxylin is shown in Figure 1. Hematoxylin stains cell nuclei and calcium particles in blue (Fig. 1a). The blue, spherical calcium-containing particles can be distinguished from the blue, elongated cell nuclei. Many, but not all, calcium-containing particles stain intensely with filipin indicating colocalization of unesterified cholesterol and calcium in these particles (Fig. 1b).

We next carried out experiments to produce hydroxyapatite/cholesterol crystal agglomerates *in vitro*. We stained these *in vitro*-produced crystal agglomerates with filipin and examined them microscopically. Pure hydroxyapatite crystals produced by the procedure outlined by Lerner et al. [9] were also stained with filipin. Not the slightest trace of filipin fluorescence was seen with the pure hydroxyapatite crystals (data not shown). This result shows that hydroxyapatite crystals alone, without cholesterol, do not give rise to fluorescence when stained with filipin.

Next, some hydroxyapatite crystals were immersed in an ethanolic solution of cholesterol to produce a hydroxyapatite/cholesterol crystal agglomerate. Such a crystal, stained with filipin, is shown in Figure 2a. The hydroxyapatite/cholesterol crystal agglomerates demonstrated filipin fluorescence (Fig. 2b). Three of four particles in Figure 2 showed fluorescent replicas of their shapes, suggesting that the cholesterol molecules responsible for the filipin fluorescence were attached to the surfaces of the hydroxyapatite particles.

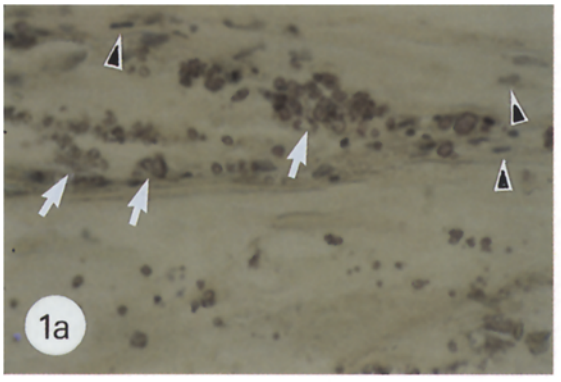
A more varied assortment of particles resulting from a prolonged immersion of hydroxyapatite crystals in an ethanolic solution of cholesterol is shown in Figure 3a. Besides two dark lumps, well-developed translucent plates of cholesterol (with overlying air bubbles) can be observed (Fig. 3a). These cholesterol plates (about 100 μm long) are opaque white in polarized light (i.e., they are birefringent) (Fig. 3c) but do not exhibit filipin fluorescence (Fig. 3b). In contrast to the lack of filipin staining of cholesterol plates, the dark lumps of hydroxyapatite show peripheral filipin fluorescence (Fig. 3b).

It is possible to estimate the size of the cholesterol crystals deposited on the hydroxyapatite seeds immersed in the ethanolic solution by viewing such crystal agglomerates with the scanning electron microscope. Figure 4 shows the structure of such a crystal agglomerate. In Figure 4a, the whole agglomerate crystalline particle is seen. The crystalline particle appears as a dark irregular mass incompletely covered by thin bright crystallites. An analysis of the chemical composition of the two solid phases of different appearance was performed with an EDAX system coupled with the scanning electron microscope. The solid dark background seen in Figure 4b was identified as hydroxyapatite (as shown by the energy diagram in Fig. 5), whereas the bright thin crystallites were identified as organic material. As no other organic material (that is able to crystallize at ambient temperatures) was present in the experimental system employed, it is clear that the crystallites are cholesterol. Although their sizes vary greatly, many of the crystallites ranged between 1 and 5 μm in length.

Discussion

It is apparent that the fluorescence induced by filipin staining of cholesterol in the hydroxyapatite/cholesterol agglomerate crystals produced *in vitro* was much weaker than that of cholesterol in agglomerate crystals observed in tissue sections of human atherosclerotic lesions. This is accounted for by the fact that agglomerate hydroxyapatite/cholesterol crystals produced *in vitro* have cholesterol only on their surface, whereas, the hydroxyapatite/cholesterol crystals formed *in vivo* presumably have cholesterol incorporated throughout the crystal. This interpretation is supported by the fact that the filipin staining associated with the *in vivo* apatite crystals is not eliminated by immersion of lesion sections in organic solvents [2].

Differences between the filipin staining of *in vitro* and *in vivo* agglomerate hydroxyapatite/cholesterol crystals should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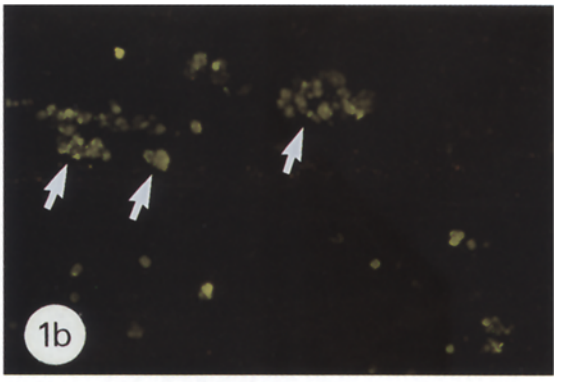
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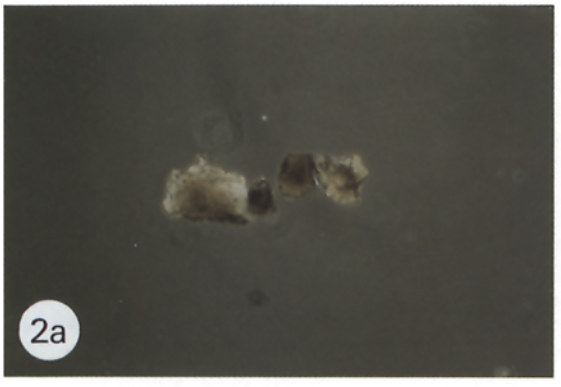
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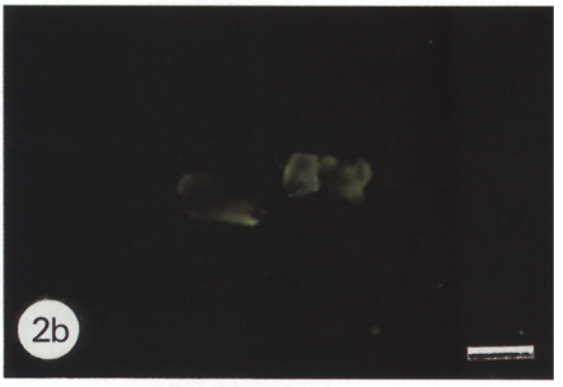
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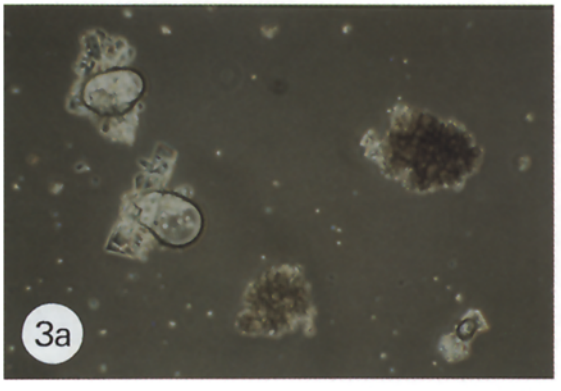
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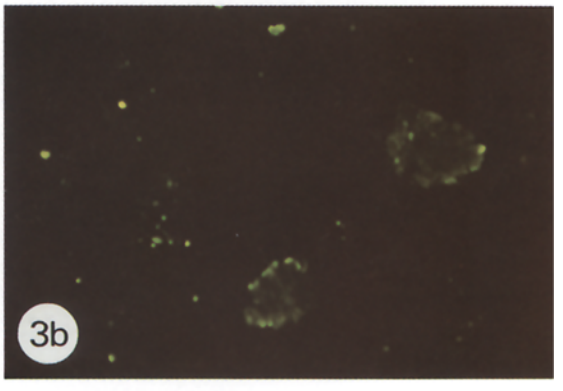
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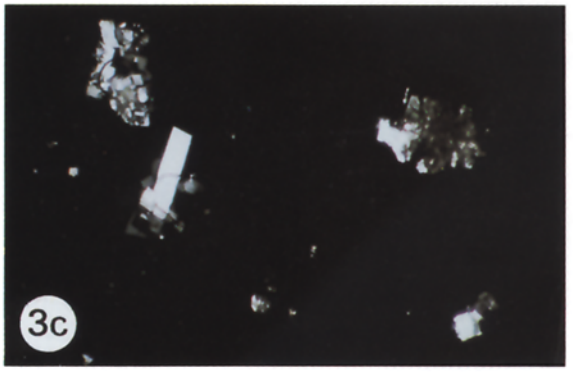
3c

Fig. 1. Tissue section of an advanced human atherosclerotic lesion stained with hematoxylin and filipin. Brightfield (**a**) and fluorescence (**b**) photomicrographs of the same field. Spherical granules of calcium (i.e., hydroxyapatite, indicated by arrows) stain blue with hematoxylin in (**a**) and their associated unesterified cholesterol stains green with filipin in (**b**). Some blue-stained nuclei are indicated with arrowheads.

Fig. 2. Hydroxyapatite crystals produced *in vitro*, immersed for 20 minutes in an ethanolic solution of cholesterol, and then stained with filipin. A phase (**a**) and a fluorescence image (**b**) of the same crystals. Only cholesterol-treated hydroxyapatite showed filipin fluorescence. Hydroxyapatite crystals alone, stained with filipin, showed no fluorescence (data not shown). Bar in (**b**) equals 40 μm for Figures 2 and 3 and equals 100 μm for Figure 1.

Fig. 3. Mixture of hydroxyapatite and cholesterol crystals obtained when hydroxyapatite crystals were kept for approximately 40 minutes in an ethanolic solution of cholesterol and then stained with filipin. Phase (**a**), fluorescence (**b**), and polarization (**c**) image of the crystals. There is only weak filipin staining of the hydroxyapatite/cholesterol crystal agglomerates and almost no staining of well-developed cholesterol crystals. It was only possible to visualize the weak fluorescence by using a longer photographic exposure time than was used to produce the fluorescence photomicrograph in Figure 2b. The cholesterol crystals can be differentiated from the hydroxyapatite crystals by the air bubbles which overlie the cholesterol crystals. Air bubbles were trapped and adhered to the cholesterol crystals during placement of the coverslip. The cholesterol crystals are birefringent (i.e., opaque-white) in (**c**) and do not stain with filipin in (**b**).

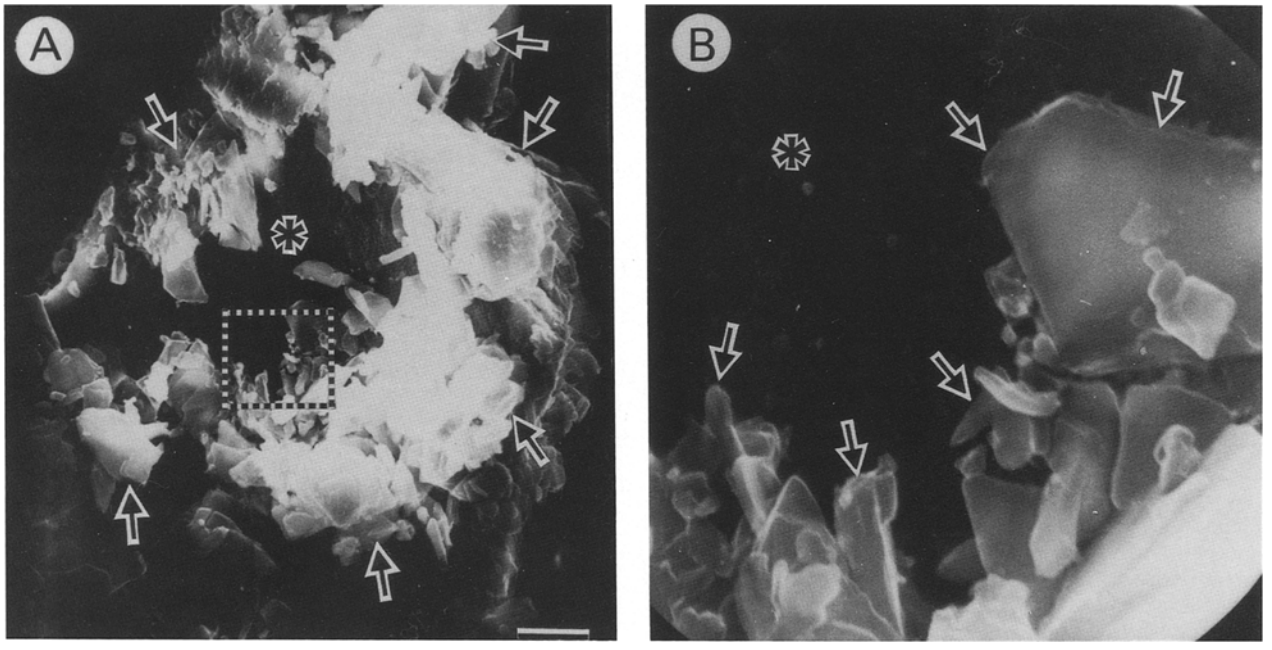

Fig. 4. Scanning electron microscope micrographs of the hydroxyapatite/cholesterol agglomerate particles produced, *in vitro*. Asterisks show regions of hydroxyapatite and arrows show associated cholesterol crystallites. Box in (**A**) shows approximate region shown at higher magnification in (**B**). Bar equals 10 μm for (A) and 2 μm for (B).

not be unexpected. The model *in vitro* agglomerates were prepared by nonspecific, heterogeneous nucleation, which may not be similar to the *in vivo* cholesterol-apatite interaction.

When the hydroxyapatite seeds were immersed in the ethanolic solution of cholesterol for a short period (20 minutes), evidently small cholesterol crystallites were deposited on their surfaces, and these crystallites produced fluorescence when stained with filipin, preserving the contours of the original seeds. A more prolonged immersion (about 40 minutes) resulted in two populations: well-formed, separate, relatively large cholesterol plates which did not yield filipin fluorescence and dark lumps which showed peripheral fluorescence, evidently caused by small cholesterol crystallites.

In order to understand the potential for decreased interaction of filipin with large, well-developed cholesterol crystals, as compared with tiny cholesterol crystallites, the nature of the filipin-cholesterol bond must be considered. It has been demonstrated that filipin possesses a unique property, among the polyene antibiotics, of packing its molecules in a parallel array [10]. The plane of filipin molecules is oriented perpendicular to the array and the arrays then can be combined in parallel to form an extensive aggregate. One side of this planar aggregate is hydrophobic because of the presence of repeating double bonds, whereas the opposite side is hydrophilic because of the presence of repeating hydroxyl groups. The hydrophilic side of one planar aggregate can associate with the hydrophilic side of a second planar aggregate to form a double layer aggregate. However, a possibility also exists for hydrogen bonding between the hydroxyl group of cholesterol and the hydroxyl group of filipin, producing a compound aggregate. The stoichiometry in the compound aggregate is one molecule of filipin per one molecule of cholesterol [10].

A filipin aggregate can adsorb onto a crystallographic face of cholesterol containing hydroxylic groups, thus forming the filipin-cholesterol complex that can be detected by its fluorescence. The intensity of the signal will depend upon the number of such complexes formed. In a well-formed, large cholesterol crystal, the area of the hydroxyl-containing faces will be small, as most of the hydroxyl groups are buried in the interior of the crystal structure, and consequently, the

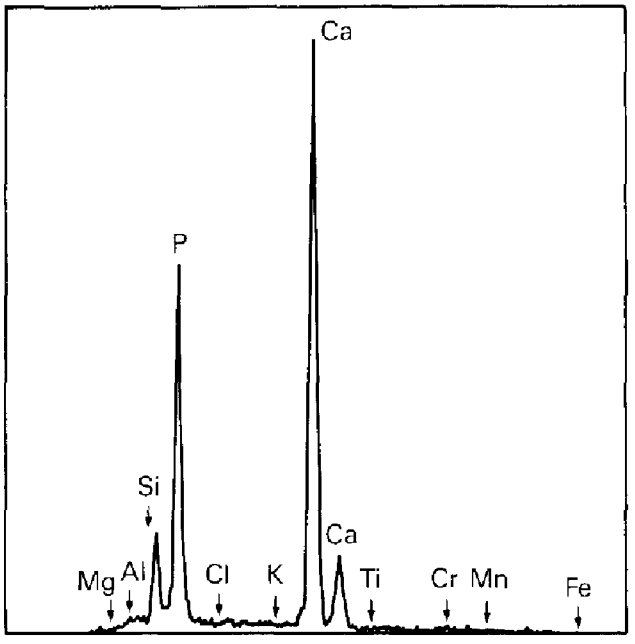

Fig. 5. Energy-dispersive X-ray analysis of the crystal shown in Figure 4. The ratio of Ca and P peaks indicates that the crystal is hydroxyapatite.

fluorescence signal will be negligible. By contrast, in small and imperfect cholesterol crystals, the surface-to-mass ratio is large and significant numbers of complexes will be formed giving rise to a strong fluorescence signal.

The experimental evidence of this study strongly suggests that in human atherosclerotic lesions, unesterified cholesterol and hydroxyapatite appear in close association. The *in vitro* experiments showed that cholesterol tends to crystallize upon hydroxyapatite seeds. Additionally, it has been shown that large, well-formed cholesterol crystals are not effectively stained by filipin, whereas small cholesterol crystallites produce filipin fluorescence when reacted with filipin. We conclude, as indicated by the intense filipin fluorescence observed in samples of human atherosclerotic lesions, that cholesterol associated with hydroxyapatite in lesions exists as large numbers of small cholesterol crystallites intermixed with the hydroxyapatite. This results in a large surface area of cholesterol that can be stained with filipin.

Colocalization of cholesterol and calcium phosphate does not indicate the nature of the association between these crystals. Also, one cannot infer the sequence of events that results in colocalization of some cholesterol and calcium deposits in lesions. Craven [11] has suggested that because of the similarities in crystal structure of hydroxyapatite and cholesterol monohydrate, a microcrystal of either may serve to stimulate epitaxial growth of the other. It remains to be determined whether cholesterol induces the deposition of calcium or whether calcium may induce the deposition of cholesterol; alternatively, both crystallization processes may be independent. Hydroxyapatite and cholesterol crystals may then admix with other lesion constituents to form large crystal agglomerates, eventually leading to massive hardening of the vascular wall.

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