PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 172, 173–177 (1983)

Matrix Vesicles in Atherosclerotic Calcification[1] (41542)

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Abstract. Matrix vesicles, small extracellular membranous structures, are known to be the initial loci of calcification of cartilage, bone, and dentin. Calcification is an important complication of atherosclerosis. Using histologic, ultrastructural, and cytochemical techniques, the present study has demonstrated that matrix vesicle-like structures are involved in the calcification of atherosclerotic lesions, as well as in arterial medial calcification. In aortas from autopsied humans and from rabbits and chickens on atherogenic diets, the matrix vesicles appear to be derived from intimal and medial cellular components, mainly smooth muscle cells.

Atherosclerosis, the vascular intimal thickening which is the major cause of death in the Western world, is commonly complicated by calcification. This important complication contributes to an increased irregularity of the lumen, increased brittleness, and decreased elasticity of vessels. The degree of calcification often corresponds to the severity of the atherosclerotic lesion. Morphological (1–3) and biochemical (4) studies characterizing crystalline calcium deposition in arterial media and intima have been reported. The mechanism of calcification in atherosclerosis, however, is not well understood.

Matrix vesicles, 100- to 700-nm extracellular membrane-invested structures, have been known since 1967 to be the initial loci of calcification in skeletal tissues (5–8). More recently, morphologic studies have suggested the involvement of matrix vesicles in the calcification of aortic valves and aortic media (9–10). The present study, using histologic, ultrastructural and cytochemical techniques, has demonstrated that matrix vesicle-like structures are involved in the calcification of atherosclerotic intimal lesions as well as in arterial medial calcification.

[1] This work was supported in part by grants from Merck, Sharp and Dohme; from the Biomedical Research Support Grant Program (BRSG S07 RR05373), Division of Biomedical Resources, National Institutes of Health; and from NIH, NIDR Research Grant DE05262.

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Materials and Methods. This study utilized aortas from 30 human autopsies, 15 male New Zealand white rabbits, and 15 male white leghorn chickens. The human cases, all men over 45 years of age, included: (i) 20 cases with four portions of thoracic and abdominal aorta sampled for light microscopy, and (ii) 10 cases less than 5 hr postmortem with multiple samples of early atherosclerotic lesions taken for electron microscopy. The rabbits and chickens were each divided into the following groups: (i) three groups of three rabbits and three chickens each, fed an atherogenic diet for 1, 2, or 3 months, and (ii) three groups of two rabbits and two chickens each, fed a normal diet for 1, 2, or 3 months. The normal diet was Purina Rabbit or Chicken Chow and the atherogenic diet was composed of Purina Rabbit or Chicken Chow with 8% peanut oil and 2% cholesterol by weight mixed into the chow. Sacrifice of these animals by intravenous air injection was immediately followed by dissection and processing of their aortas.

Light microscopic study of these human and animal aortas (fixed in 3.7% neutral buffered formaldehyde solution) included the following histological stains on adjacent sections of tissue: hematoxylin and eosin; alizarin red and van Kossa for calcium and phosphate; oil red O for lipid; Verhoeff–van Gieson for elastic fibers; Gomori's trichrome for collagen and smooth muscle; Prussian blue for iron; and alcian blue–PAS for acid mucopolysaccharides. Ultrastructural study of these aortas was performed on a Phillips EM 300 electron microscope and utilized tissue which had been processed in the following ways: (i) routine—

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fixed in 4% glutaraldehyde, followed by 0.1 *M* cacodylate buffer at pH 7.4, postfixation in 1.33% symcollidine buffered osmium tetroxide (pH 7.4), dehydrated in ethanol, embedded in epoxy resin (Epon 812) (11), thin sectioned with a diamond knife on an ultramicrotome, and stained with lead citrate (12) and uranyl acetate (13); (ii) calcium localization by potassium pyroantimonate technique—fixed in potassium pyroantimonate–osmium solution, pH 7.6–7.8, for 4 hr, washed for 30 min in three changes of potassium acetate buffer, pH 7.4, routinely dehydrated and embedded, and thin sections stained only with uranyl acetate (14, 15); (iii) control for calcium localization—processed as in (ii) except that the tissue initially was incubated for 45 min in 1% osmium tetroxide containing (or, for further control, not containing) 10 m*M* ethylene glycol-bis-*N*,*N*-tetraacetic acid (EGTA), a chelating agent that removes calcium (and, to a much lesser extent magnesium, zinc, and cobalt) but not other cations, then fixed in 5% potassium pyroantimonate solution (14, 15); (iv) alkaline phosphatase cytochemical technique—using β-glycerophosphate as substrate, Tris buffer, $MgCl_2$ activator, and $Pb(NO_3)_2$ with lead phosphate as reaction product; controls included 0.2 × 0.2-mm pieces of rabbit kidney incubated and treated similarly (16); and (v) adenosine triphosphatase (ATPase) cytochemical technique—as described elsewhere, using adenosine 5-triphosphate (ATP) as substrate, Tris buffer, $MgSO_4$ activator, and $Pb(NO_3)_2$ with lead phosphate as reaction product; controls included 0.2 × 0.2-mm pieces of rabbit kidney incubated and treated similarly (16, 17).

Results and Discussion. It was found that, although there were some differences in the character of the atherosclerosis in these human, chicken, and rabbit aortas, the morphological findings associated with the presence of early calcification in the atherosclerotic lesions was similar among these three species. Details regarding the morphologic appearance of the atherosclerosis will be reported subsequently (18). Basically, the atherosclerotic lesions in the chicken aortas were more comparable to those in the human, with the initial development of an intimal fibrous plaque, composed largely of modified smooth muscle cells in an interstitium of collagen fibers, ground substance, and a few matrix vesicle-like structures. Spontaneous fibrous plaques were present in the aortas of chickens on a normal diet. Although the number of fibrous plaques did not notably increase, lipid (by light microscopy) and increasing calcium deposition (by light and electron microscopy) occurred in the plaques of all chickens fed the atherogenic diet for 1, 2, or 3 months. The rabbit aorta lesions had similarities to some small human and early chicken lesions but tended to have more the appearance of a fatty streak than a fibrous plaque containing less smooth muscle cells and more lipid-filled foam cells. Light microscopic study of calcification in these atherosclerotic lesions, using von Kossa and alizerin red methods, demonstrated focal prominent calcium deposition in all human aortic intimal lesions studied, and a lesser degree of calcification in the animal lesions. This was absent in controls but increased with increasing duration of atherogenic diet (0, 1+, 2+, and 3+ in chickens and 0, ±, 1+, and 2+ in rabbits, for control, 1, 2, and 3 months of diet, respectively, by independent estimate of two of the investigators).

Electron microscopic study of calcification in these atherosclerotic lesions demonstrated that the association of matrix vesicle-like structures with calcification was striking (Figs. 1, 2). These extracellular vesicles, in routinely processed electron microscopic sections, were generally limited by a single trilaminar membrane, varied in electron density, and occasionally contained crystalline material. They measured 100 to 700 nm in diameter. Occasional larger vesicles, distinct from the matrix vesicles and having a double-limiting membrane similar to mitochondria, were also present, however, suggesting that mitochondria may also be playing a role in the calcification process. The typical matrix vesicles were numerous (3+) in all human atherosclerotic lesions and tended to increase in number in chicken and rabbit aortas with increasing duration of atherogenic diet. Independent estimate by two of the investigators (A.T. and D.M.) indicated the number of vesicles in chickens to be 1+, 2+, 2+, and 3+ and 0, 1+, 2+, and 3+ in rabbits, for control, 1, 2, and 3 months of diet, respectively. The vesicles, particularly in human and chicken aortas, appeared to arise mainly from degen-

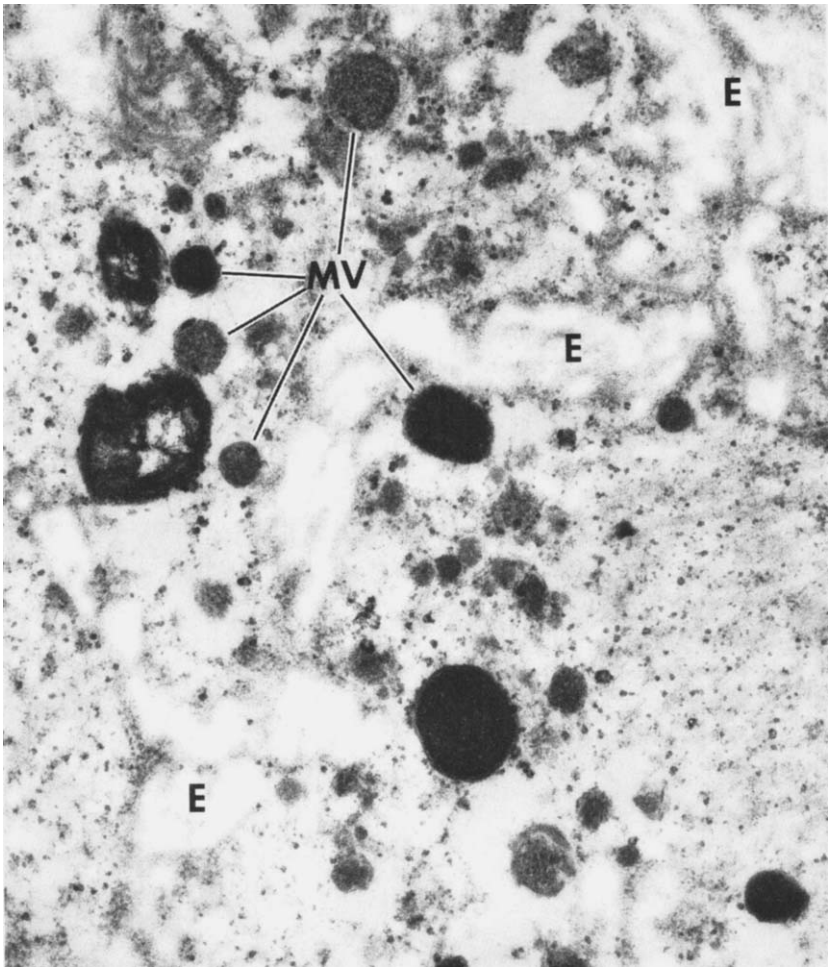

FIG. 1. Electron micrograph of atherosclerotic human aortic intima, stained by potassium pyroantimonate technique. Many membrane-limited matrix vesicles (MV) measuring 100–700 nm in diameter are located mainly in the region of elastic fibers (E). The matrix vesicles are in various stages of calcification. Calcium, indicated by the highly electron-dense pyroantimonate reaction product, is primarily deposited within the matrix vesicles but is also present in the adjacent ground substance in a much less concentrated distribution. Elastic fibers appear to be devoid of calcium deposition. ×43,000.

erated intimal and medial smooth muscle cells. Evidence for this included their location in the region of degenerated-appearing smooth muscle cells, their frequent content of fibrillary and granular material comparable to that of smooth muscle cells, their similarity to vesicles in smooth muscle cells, their occasional display of an outer component of basement membrane-like material similar to that of smooth muscle cells, and their occasionally appearing to bud from the cytoplasmic membrane or to be extruded from the cytoplasm of smooth muscle cells. Of additional interest was the frequent localization of the matrix vesicles adjacent to, or in the region of, intimal elastic fibers (Fig. 1).

Potassium pyroantimonate reaction for calcium demonstrated reaction products mainly within the matrix vesicles, (Figs. 1, 2) although some positivity was also demonstrated in other locations, such as mitochondria of some smooth muscle cells, the

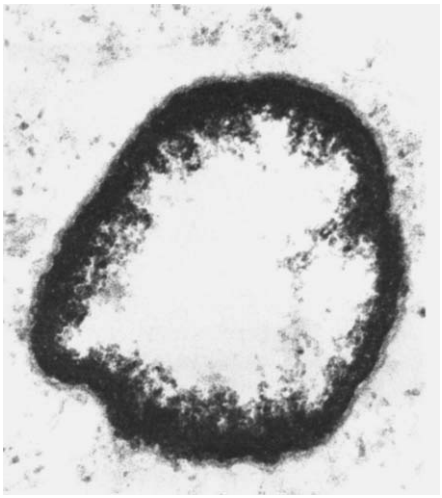

FIG. 2. Electron micrograph of atherosclerotic human aortic intima, stained by potassium pyroantimonate technique. The matrix vesicle has a single trilaminar limiting membrane and is in an intermediate stage of calcium deposition. ×79,200.

occasional extracellular mitochondria-like vesicles, interstitial foci of basement membrane-like material, and dispersed in extracellular ground substance. There was no evidence of pyroantimonate staining within elastic fibers. Although there was some variability among different lesions in the same human or animal, the overall staining reactions among the early human lesions sampled and among animals in the same experimental group were quite similar. With increasing duration of the atherogenic diet, the number of matrix vesicles not only increased but the intensity of potassium pyroantimonate reaction products also increased considerably. Independent estimate by two of the investigators (A.T. and D.M.) indicated the intensity of staining reaction in matrix vesicles to be 1+, 2+, 2+, and 3+ in chickens and ±1, 2+, 2+, and 3+ in rabbits, for control, 1, 2, and 3 months of diet, respectively. In all tissues pretreated with EGTA, essentially no potassium pyroantimonate reaction products were evident, indicating the specificity of this calcium-localization technique. Furthermore, tissues incubated without EGTA showed no decrease of potassium pyroantimonate reaction products.

Regarding the other cytochemical techniques, both ATPase and alkaline phosphatase were positive along the membranes of the matrix vesicle-like structures. In addition, smooth muscle cell mitochondria and intracytoplasmic vesicles were positive by these techniques. The positive extracytoplasmic phosphatase reactions, comparable to matrix vesicles of cartilage, supports the interpretation that the membranous structures in the atherosclerotic plaques represent matrix vesicles. Furthermore, these enzymes may play a dual role in promoting calcification: (i) by providing phosphate for nacent mineral formation, and (ii) by hydrolysis of naturally occurring inhibitors of mineral crystal formation such as pyrophosphate and ATP (19).

Calcification has long been considered an important but poorly understood complication of atherosclerosis. The significance of calcification has recently been reemphasized by reports describing the suppression of diet-induced atherosclerosis by anticalcifying agents (20). The data of this study indicate that, although other mechanisms such as mitochondrial calcification may be involved, a major mechanism in the development of atherosclerotic calcification is the deposition of calcium phosphate (mineral) within matrix vesicles apparently derived from intimal cellular components, predominantly smooth muscle cells. Comparable findings have been previously reported in the clinically less significant arterial medial calcification but to our knowledge not in the highly important process of intimal atherosclerosis. Recognition of this basic pathogenetic mechanism should open the way to several avenues (morphological, biochemical, physiological, and pharmacological) for investigating the etiology, treatment, and prevention of atherosclerotic calcification.

We gratefully acknowledge the technical assistance of William J. Bopp and Marcia L. Degenhardt (electron microscopic processing), the Histology Section of the Laboratory Service, Kansas City VA Medical Center, and the Autopsy Research Histology Section of the Department of Pathology and Oncology, University of Kansas Medical Center (histologic processing), and the Animal Care Unit, University of Kansas Medical Center; the suggestion by Dr. C. Richard Minick to utilize chickens as an animal model; and the advice and support of Dr. Teruyuki Nakashima, Second Department of Pathology, Ku-

rume University School of Medicine, Japan. Electron microscopic studies were performed using the facilities of the Electron Microscopy Unit, Laboratory Service, Kansas City VA Medical Center.

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Received December 17, 1981. P.S.E.B.M. 1983, Vol. 172.