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Technical Advance

Biotin- or Digoxigenin-Conjugated Nucleotides Bind to Matrix Vesicles in Atherosclerotic Plaques

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The present study analyzes the staining pattern of DNA* in situ *end-labeling techniques of human and rabbit atherosclerotic plaques. Both the terminal deoxynucleotidyl transferase end-labeling and the* in situ *nick translation technique detected, besides apoptotic nuclei, numerous round vesicles with diameters from 0.5 to 5 μm within the atherosclerotic plaques. These vesicles did not contain DNA but contained calcium. A pretreatment with EDTA or citric acid abolished the labeling of the vesicles but did not influence the detection of apoptotic nuclei. Ultrastructurally, the vesicles were of variable diameter and density, and their aspect was compatible with matrix vesicles, which are well known in the epiphyses during bone formation. The larger vesicles contained cell organelles, and the small vesicles were very dense. X-ray microanalysis demonstrated high calcium and phosphorus levels within the most dense vesicles. Different stages of the process were present in the plaques. In this way we could demonstrate that cytoplasmic fragmentation of smooth muscle cells and subsequent formation of matrix vesicles are a frequent finding in atherosclerotic plaques. The association of apoptotic cell death and formation of matrix vesicles could be an interesting pathway in explaining calcification of atherosclerotic plaques. Both the terminal deoxynucleotidyl transferase end-labeling and the* in situ *nick translation technique detected simultaneously apoptotic nuclei and matrix vesicles if calcium is not removed from the sections. (Am J Pathol 1996, 148:1771–1777)

The detection of DNA fragmentation is a marker of apoptotic cell death.[1] Recently, this technique has been used on tissue sections by *in situ* end labeling or *in situ* nick translation.[2,3] The technique has been used to quantitate apoptotic percentages in tumors. We have used this technique on atherosclerotic plaques of human and rabbit arteries and found high numbers of labeled fragments, especially around the necrotic core. Therefore we tested the hypothesis that non-nuclear structures were labeled by this technique. The problem has important consequences for quantitative analysis of apoptotic cell death in atherosclerotic plaques.

Materials and Methods

Atherosclerotic plaques of human carotid endarterectomy specimens (n = 11) were fixed in formalin and paraffin embedded. The specimens were opened along their longitudinal axis. The specimens contained the inner wall of the distal common carotid artery, the proximal part of the external carotid artery, and the carotid sinus. In the same section, advanced atherosclerotic plaques alternate with regions of fibromuscular intimal thickening.

Atherosclerotic plaques of male New Zealand White rabbits were induced by a diet supplemented

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with low-dose cholesterol (0.3%) for 27 weeks (n = 6). For histological examination, the thoracic aorta was dissected and fixed *in situ* in 4% neutral formalin. Five -micron-thick transversal sections of paraffin-embedded tissues were mounted on Starfrost precoated slides.

DNA in Situ *End Labeling*

After deparaffinization and rehydration, tissue sections were incubated with proteinase K (20 μg/ml; Boehringer Mannheim, Mannheim, Germany) for 10 minutes at room temperature. This proteolytic digestion enables enzymatic incorporation of nucleotides. Both the terminal deoxynucleotidyl transferase (Tdt) end-labeling (TUNEL)[2] and the *in situ* nick translation (ISNT)[3] techniques were used.

For the TUNEL technique, the ApopTag kit (Oncor, Gaithersburg, MD) was used with minor modifications.

For the ISNT technique, the sections were rinsed in a buffer (50 mmol/L Tris-HCl, 5 mmol/L $MgCl_2$, 0.0005% bovine serum albumin, pH 7.5) for 10 minutes, dried, and afterwards incubated at 37°C for 1 hour with the same buffer containing 0.01 mmol/L dATP, dCTP, and dGTP (Sigma Chemical Co., St. Louis, MO) and 0.01 mmol/L biotin-16-dUTP (Boehringer Mannheim) with 20 U/ml of the Klenow fragment of DNA polymerase I (Boehringer Mannheim). Incorporated biotin-16-dUTP was demonstrated by incubating the sections with a monoclonal antibody against biotin (Dako, Glostrup, Denmark) at a dilution of 1/40 for 30 minutes. The antibody was visualized by a goat anti-mouse peroxidase (Jackson Immunoresearch Laboratories, West Grove, PA) at a dilution of 1/125 for 45 minutes.

In both the TUNEL and the ISNT technique, the labeled antibody was visualized by 3-amino-9-ethylcarbazole (Sigma). Sections were lightly counterstained with hematoxylin and mounted in glycerin jelly. Negative controls included omission of Tdt or the Klenow fragment from the labeling mixture.

Pretreatment with Citric Acid or EDTA to Remove Calcium Accumulation

To test our hypothesis that other tissue components are labeled by TUNEL or ISNT, we pretreated the sections with EDTA or citric acid to remove small calcium deposits, which are a frequent finding in atherosclerotic plaques. The sections were treated with 3% EDTA (Merck, Darmstadt, Germany) at a pH of 7.2 for 1 or 2 hours. Alternatively, the sections were treated with 3% citric acid for 1 hour before DNA *in situ* end labeling. Calcium was demonstrated in the tissue sections by an alizarin-red stain.[4]

Quantification

The images were analyzed using a color image analysis system (PC Image Color, Foster Findlay Associates, Newcastle upon Tyne, UK). The same region in two succesive serial sections of the atherosclerotic plaques were studied. The first section was stained by ISNT without citric acid pretreatment, the second by ISNT with citric acid pretreatment. The labeling of the same region of the plaque was quantified for each technique. The labeled elements were counted in a standard area of 0.06 mm^2 in regions of the plaque either with or without matrix vesicles. Distinction between both regions was based on the alizarin-red stain. The segmentation of labeled elements was done by first measuring the average brown color of 10 different labeled elements. A minimal diameter of 3 μm was selected.

Statistical Analysis

The number of *in situ* end-labeled elements per 0.06 mm^2 plaque was counted for each technique in regions either with or without matrix vesicles. Statistical evaluation was done by the Wilcoxon matched-pairs signed ranks test). The SPSS package for Windows (SPSS, Chicago, IL) was applied for these purposes. A 5% level of significance was selected.

Transmission Electron Microscopy

The fragments for transmission electron microscopy were fixed for 2 hours in 1% (v/v) glutaraldehyde in 0.1 mol/L sodium cacodylate buffer (pH 7.4). They were postfixed for 30 minutes in 1% (v/v) osmium tetroxide in 0.1 mol/L sodium cacodylate buffer (pH 7.4). After dehydration in an ethanol gradient, they were embedded in LX-112 (Ladd Research Industries, Burlington, VT). Selection of the zones most representative for the lesions was made on 2-μm sections orientated in a transverse plane (perpendicular to the bloodstream) and stained with toluidine blue, and 50-nm-thick sections were cut on an Ultratome Nova (Reichert-Jung, Vienna, Austria). They were stained for 30 minutes at 40°C with uranyl acetate and for 15 minutes at 20°C with lead citrate in an Ultrostainer 2168 (LKB, Bromma, Sweden). The sections were examined in a Jeol-1200 EX transmission electron microscope at 80 kV. Photographs

were made with electron microscopy film 4489 Estar Thick Base (Kodak).

X-Ray Microanalysis

For the study of calcium and phosphorus distribution in the ultrathin sections, a scanning/transmission electron microscope (Jeol 1200 EX) equipped with an EDS detector (Tracor TN 5500) was used to acquire x-ray mappings.

Results

DNA in Situ *End Labeling*

The human carotid endarterectomy specimens showed few labeled nuclei in the regions with a fibromuscular intimal thickening. In the atherosclerotic plaques, numerous fragments were labeled, particularly near or within the lipid-rich core (Figure 1A). These fragments were round and had diameters of 0.5 to 5 μm. The fragments were not stained by the Feulgen stain or propidium iodide. An alizarin-red stain showed a strong staining, which suggests that these fragments contained calcium. The round vesicles could not be distinguished from apoptotic nuclei by either the TUNEL or the ISNT technique.

The atherosclerotic plaques of the cholesterol-fed rabbits showed labeled nuclei. Within the deep fibrous layer of the plaque, numerous small fragments were labeled. These fragments were similar to those described within the human atherosclerotic plaques. Also, these fragments were not stained by a Feulgen or propidium iodide stain. An alizarin-red stain showed a strong staining, which suggests that these fragments contained calcium. The round vesicles could not be distinguished from apoptotic nuclei by either the TUNEL or the ISNT technique.

The concentration and incubation time of proteinase K influenced the staining pattern. Without a proteinase K pretreatment, no labeling could be detected.

The TUNEL and ISNT techniques gave a similar staining pattern. Apoptotic nuclei and the round particles were labeled. Omission of Tdt in the TUNEL or the Klenow fragment in the ISNT enzyme results in a loss of the labeling of the apoptotic nuclei, but the round particles were still labeled by this technique. As different labeled nucleotides were used in the TUNEL and ISNT techniques, an effect of the label itself on this effect is unlikely.

The vesicles did not contain an endogenous peroxidase activity. The vesicles remained strictly negative when the sections were incubated with a streptavidin-biotin complex, followed by the anti-biotin antibody, the goat anti-mouse peroxidase, and visualization with 3-amino-9-ethylcarbazole. This indicates that the biotin-conjugated nucleotides did not bind via their biotin group. This result could be expected as the digoxigenin-conjugated nucleotides gave similar results.

Effect of Pretreatment with Citric Acid or Chelating Agents

When the sections were treated either with citric acid or EDTA, the TUNEL and ISNT techniques demonstrated labeled elements within the plaque of the cholesterol-fed rabbits and in the human carotid endarterectomy specimens, but the percentages were much lower. The labeling of the small vesicles within the deep region of the atherosclerotic plaques of the cholesterol-fed rabbits was completely abolished. The same was true for the labeling of the vesicles near the lipid-rich core region of human atherosclerotic plaques (Figure 1, A and B). However, within this region, labeled nuclei could still be detected, but the percentages were much lower than those without pretreatment with calcium-chelating agents. In the regions with numerous matrix vesicles, the number of ISNT-labeled elements per standard area was significantly decreased after pretreatment with citric acid (Figure 2). In the regions without matrix vesicles, the number of ISNT-labeled elements per standard area was not affected by the citric acid pretreatment. The pretreatment of the tissue sections with citric acid or EDTA resulted in a complete loss of the alizarin-red staining (Figure 1, C and D).

The detection of apoptotic nuclei by the ISNT or the TUNEL technique in well established control samples (hyperplastic lymphoid tissue) was not influenced by pretreatment of the sections with citric acid or EDTA.

Transmission Electron Microscopy

The smooth muscle cells in the regions of the atherosclerotic plaques with matrix vesicles showed cytoplasmic fragmentation. Different stages of smooth muscle cell fragmentation were present in the same region of the plaque. It was possible to detect cells that were completely fragmented in small vesicles. Their smooth muscle cell origin could still be revealed because these vesicles were surrounded by a cage composed of thickened basal laminae (Figure 3). The vesicles had different diameters but fitted

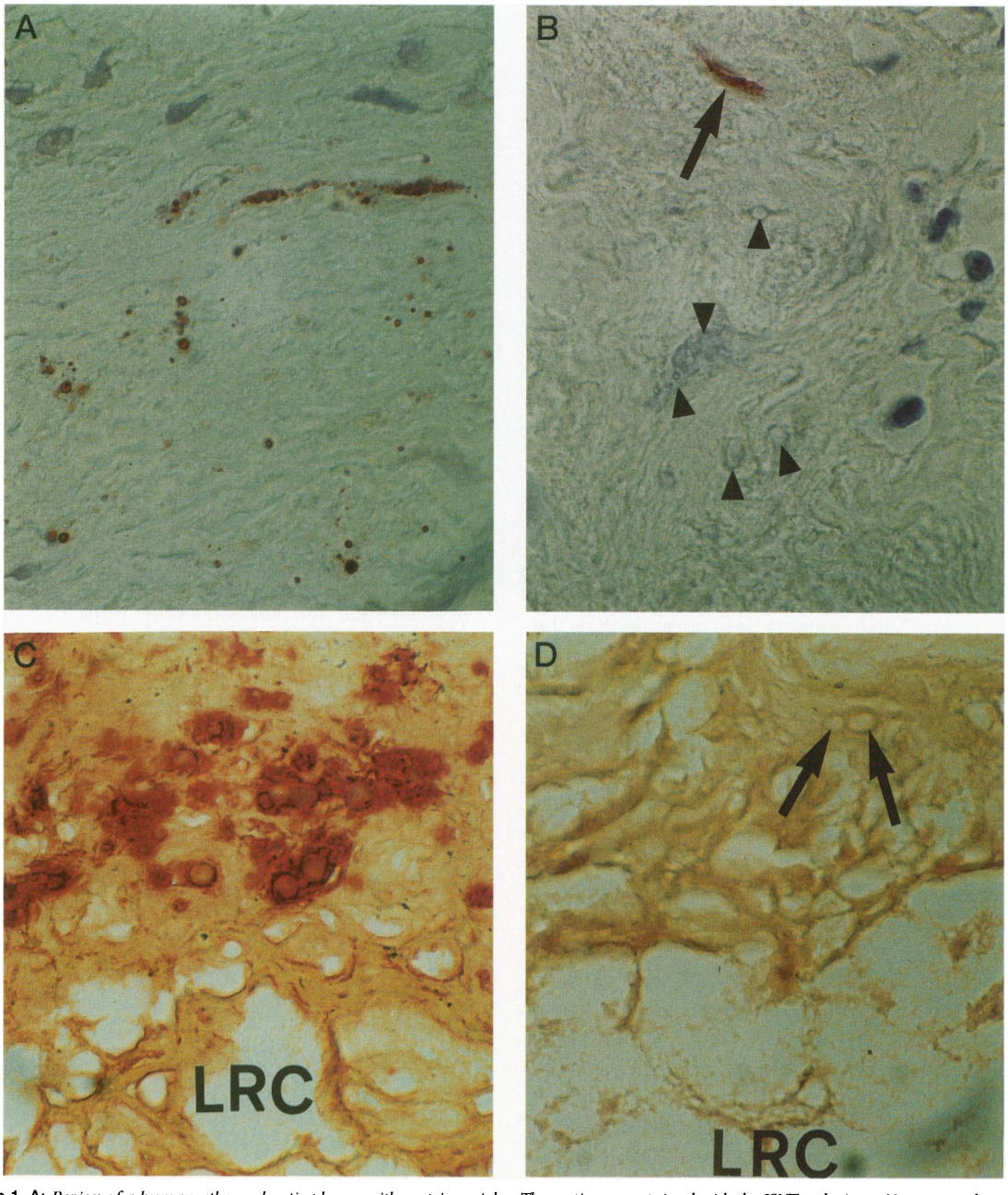

Figure 1. A: *Region of a human atherosclerotic plaque with matrix vesicles. The section was stained with the ISNT technique. Numerous elements are labeled within this region, suggesting high rates of apoptotic cell death.* B: *Section adjacent to* A. *The section was stained with the ISNT technique after citric acid pretreatment. A labeled nucleus* (arrow) *could still be detected within this region. The main fraction of the stained elements of* A *are matrix vesicles that did not stain after EDTA or citric acid pretreatment in the TUNEL or ISNT techniques* (arrowheads). *The diameter of a fraction of these vesicles was comparable to a condensed nucleus and can be confused with apoptotic nuclei. The effect of pretreatment with citric acid on the number of ISNT-labeled elements is quantified in Figure 2.* C: *alizarin-red stain of a human atherosclerotic plaque with matrix vesicles. Numerous particles are stained within the fibrous cap. The high density of vesicles in some regions of the atherosclerotic plaque results in a confluent staining with alizarin-red.* D: *alizarin-red stain of the same region as* C *after citric acid pretreatment. The particles have lost their staining capacity* (arrows). *LRC, lipid-rich core. Magnification,* × *720.*

the ultrastructural characteristics of matrix vesicles. A number of the vesicles showed a dense staining. Between the smooth muscle cell clusters, surrounded by thickened basal laminae, a dense connective tissue matrix of cross-banded collagen fibers was present.

X-Ray Microanalysis

The vesicles detected by transmission electron microscopy were further analyzed. The vesicles with a dense staining (Figure 3) showed a clear calcium and phosphorus signal. Adjacent cell fragments with

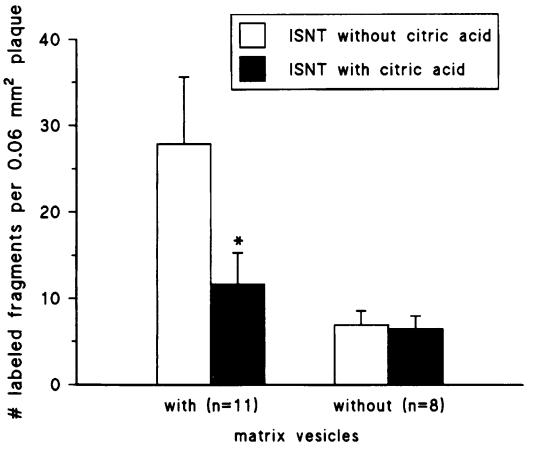

Figure 2. *Quantification of the effect of a pretreatment with citric acid on the number of ISNT-labeled elements per 0.06-mm² plaque area. A clear difference was present in the regions of the plaque with matrix vesicles* (left side), *whereas the value was not different in regions of the plaques without matrix vesicles* (right side). *All data are expressed as means ± SEM.* $P < 0.05$.

normal cytoplasmic density did not show calcium or phosphorus accumulation.

Discussion

The present study analyzes the staining pattern of DNA *in situ* end-labeling techniques (TUNEL or ISNT) of human and rabbit atherosclerotic plaques. When the standard procedure[2,3] was used, numerous round vesicles with diameters of 0.5 to 5 μm were labeled in some regions of the plaques. Especially the deep region of atherosclerotic plaques of cholesterol-fed rabbits, the lipid-rich core, and the fibrous cap of human atherosclerotic plaques contained numerous round vesicles that were strongly labeled by this technique. The vesicles did not contain DNA as demonstrated by propidium iodide and Feulgen stains. This suggests that non-nuclear structures in atherosclerotic plaques are labeled by a standard TUNEL or ISNT technique. The vesicles contained calcium as showed by an alizarin-red stain. The present study analyzes the staining pattern by the ISNT or the TUNEL technique in atherosclerotic plaques after treatment of the sections with calcium-chelating agents or citric acid. Within the atherosclerotic plaques of cholesterol-fed rabbits and in the carotid endarterectomy specimens, labeled nuclei could still be detected. However, the labeling of the round vesicles within or near the lipid-rich core was lost. An alizarin-red stain to detect calcium deposits co-localized with the TUNEL- or ISNT-positive non-nuclear elements found in and near the lipid-rich core. The labeling of these fragments was not influenced by a change in the enzyme Tdt by the Klenow fragment of polymerase I. Surprisingly, when we omitted the Tdt or the Klenow enzyme from the reaction, the fragments were still labeled. This suggests a passive, nonenzymatic but specific binding of the labeled nucleotides to these elements. Both digoxigenin- and biotin-labeled nucleotides showed this effect. A change of the label by biotin did not influence the labeling of these elements. Therefore, the labeled nucleotides bind not by their label (biotin or digoxigenin) but by a region within the nucleotide itself. As calcium removal abolishes this effect, a binding of the phosphate groups of the nucleotide to calcium has to be considered.

Schwartz and Bennet remark that estimates of rates of cell death are difficult to derive from the frequency of TUNEL-positive cells as we have no reliable method to estimate the duration of time a cell undergoing apoptosis will display a positive reaction using Tdt to detect fragmented DNA.[5] Moreover, the reported percentages of TUNEL-positive cells present in atherosclerotic plaques showed remarkable differences. Isner et al[6] found low values in primary atherosclerotic lesions of human coronary arteries. Restenotic lesions, showing high replication rates, demonstrated more apoptotic nuclei. A similar result was found by Bochaton-Piallat[7] in the intimal thickening induced after endothelial denudation of the rat aorta. When apoptosis was present in the human atherectomy specimens, it was typically limited to <2% of the cells. This value is remarkably lower than the percentages of 30 to 40% reported by two other groups[8,9] in some regions of human atherosclerotic plaques. Both authors used ISNT or TUNEL techniques without a pretreatment by calcium-chelating agents or citric acid. Some authors[8] describe labeling of structures that were not stained with methyl green or routine histological techniques. These elements could be the small calcium-containing non-nuclear structures. In the present study we have demonstrated that, in regions of the atherosclerotic plaque with numerous calcium-containing non-nuclear structures, two-thirds of the ISNT-labeled elements were removed after citric acid pretreatment. In regions of the atherosclerotic plaque without these calcium-containing elements, the number of ISNT-labeled elements was not influenced by citric acid pretreatment. This means that, in regions of atherosclerotic plaque without calcium-containing vesicles, the TUNEL or ISNT technique detects DNA fragmentation. High levels of labeling in these regions represent high levels of apoptotic cell death. Han et al[9] found high levels in myxomatous regions of human atherosclerotic plaques, and the level of apoptosis determined by TUNEL and propidium iodide was

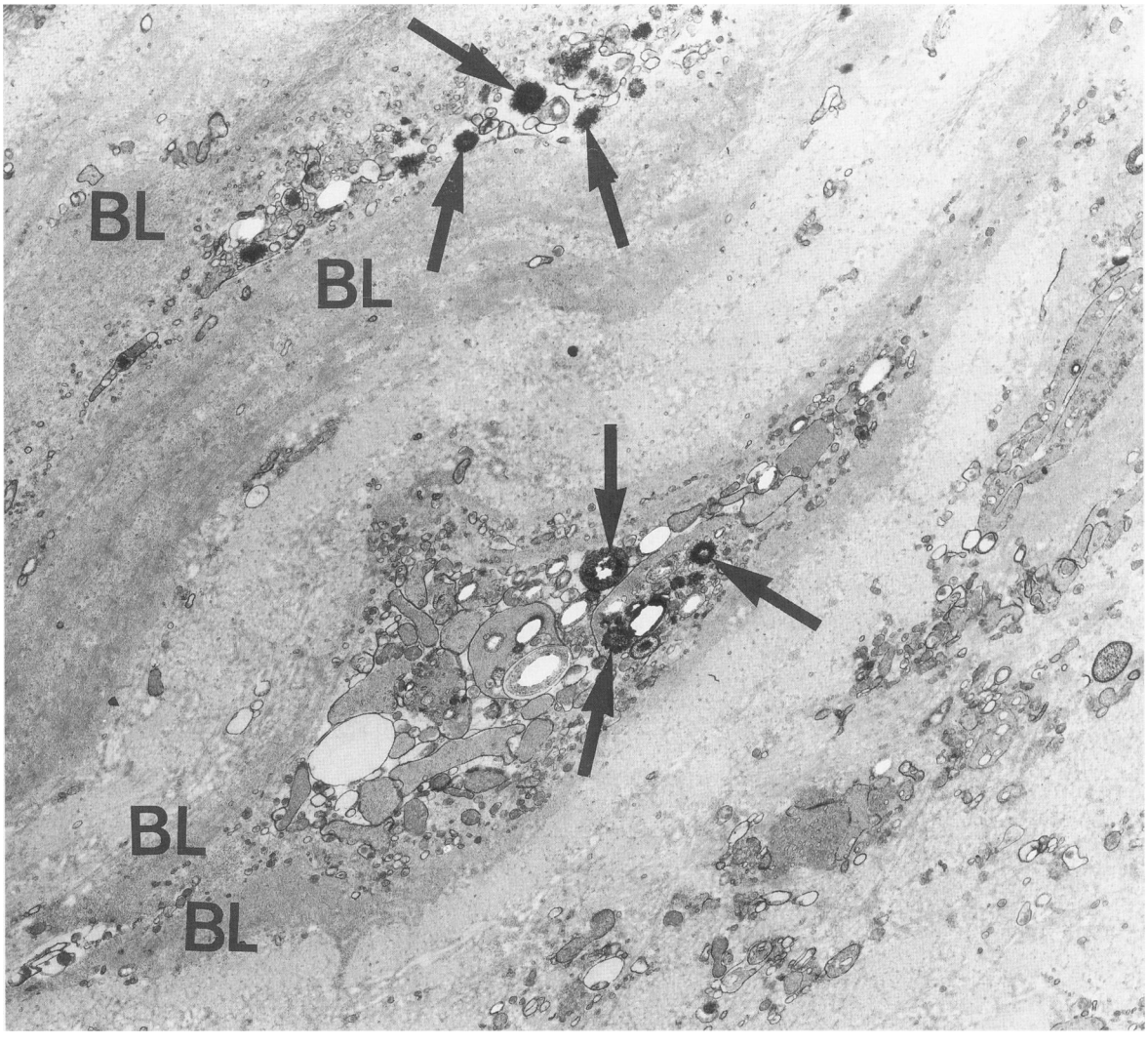

Figure 3. *Transmission electron microscopy of a region of an atherosclerotic plaque with matrix vesicles. The cytoplasm of the smooth muscle cells in this region is often fragmented, and it was possible to detect cells that were completely fragmented in small vesicles. Their smooth muscle cell origin could still be revealed because these vesicles were surrounded by a cage composed of thickened basal laminae (BL). The vesicles had different diameters but fitted the ultrastructural characteristics of matrix vesicles. A number of these vesicles are very dense* (arrows), *and X-ray microanalysis showed a clear calcium and phosphorus signal within these vesicles. Magnification, ×10,000.*

very similar. This suggests that the labeling of the non-nuclear calcium-containing vesicles can only account for some of the differences in the level of apoptosis found by different authors. As the detection of apoptotic nuclei by ISNT or the TUNEL technique in well established control samples was not influenced by pretreatment of the sections with citric acid, we recommend the use of a citric acid pretreatment to omit a possible nonspecific binding without influencing the detection of true apoptosis.

Recently we have quantified apoptotic cell death within atherosclerotic plaques of cholesterol-fed rabbits by DNA *in situ* end-labeling techniques after a pretreatment with citric acid and found apoptotic percentages from 0.2 to 1.7%.[10] Without pretreatment by citric acid, we found numerous labeled fragments in the deep layer of the plaques. In some regions, >50% of the elements were labeled. A large fraction co-localized with small round calcium-containing vesicles with a mean diameter of 2.5 μm. The vesicles did not contain DNA and could not be distinguished from apoptotic nuclear fragments by ISNT or TUNEL techniques.

In an additional stage, we examined the ultrastructure of the calcium-containing non-nuclear structures that were labeled by the standard ISNT and TUNEL techniques. In the regions of the atherosclerotic plaques with numerous calcium-containing non-nuclear ISNT-labeled elements, we found numerous smooth muscle cells with a complete fragmentation of their cytoplasm in numerous vesicles of different

diameters. A similar finding was present in atherosclerotic human saphenous vein grafts.[11] Their smooth muscle cell origin could still be revealed because these vesicles were surrounded by a cage composed of basal laminae. The ultrastructural apects of these vesicles was compatible with matrix vesicles. The vesicles with dense staining showed a clear signal for calcium and phosphorus with x-ray microanalysis. Interestingly, within a fraction of the cell fragments, intact organelles could be detected.

Matrix vesicles are submicroscopic, membrane-invested extracellular particles that were first identified in the matrix of the epiphyseal growth plate cartilage.[12,13] Evidence of matrix vesicle calcification came from ultrastructural studies in growth plate cartilage showing that vesicle distribution in cartilage corresponded closely to that of calcification. Vesicles appear in clusters in the longitudinal septal matrix of the growth plate, and it is this portion of the matrix that selectively calcifies. Matrix vesicles also occur in the walls of blood vessels in man and under various experimental conditions in other animals.[14–17] Most authors think that matrix vesicles serve as the initial locus of calcification in normal and pathological conditions.

It must be stated that the matrix vesicles in the present study did not contain DNA as demonstrated by propidium iodide or Feulgen stains. Therefore, the vesicles can be considered as calcium-bearing remnants of cytoplasmic fragments and not as cells undergoing apoptosis if we use the detection of DNA fragmentation as a marker of apoptotic cell death. However, the association of apoptotic cell death and matrix vesicle formation could be an interesting pathway. Besides nuclear condensation and DNA fragmentation, apoptotic cell death is characterized by cytoplasmic fragmentation. Apoptotic cell death is associated in a cell line with the appearance of phosphatidylserine on the cell surface.[18] The same type of acidic phospholipids are present in matrix vesicles of human chondrocytes.[12] Phosphatidylserine was shown to have a strong affinity for ionic calcium with increased calcium affinity in the presence of phosphate. Thus, phosphatidylserine and other phospholipids could act as a non-energy-requiring mechanism localized at the site of mineralization. The possibility that calcification in atherosclerotic plaques is the consequence of apoptotic cell death and subsequent matrix vesicle formation could be an interesting pathway for pharmacological interventions.

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