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ADHERENT PLATELETS AND SURFACE MICROTHROMBI OF THE HUMAN AORTA AND LEFT CORONARY ARTERY: A SCANNING ELECTRON MICROSCOPY FEASIBILITY STUDY

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Abstract

As part of a feasibility phase of an investigator-initiated multicenter NIH supported study on the Pathobiological Determinants of Atherosclerosis in Youth (PDAY), we report observations on microthrombi and adherent platelets on the intima of the aorta and left anterior descending coronary artery. The long-term objective of this cooperative study is to define more precisely the pathogenesis of atherosclerosis during late childhood and early adulthood and to investigate the influence of selected risk factors known to be associated with clinically manifest disease in later life. Scanning electron microscopy (SEM) was utilized to survey broad areas of arterial intima. Of 109 specimens studied from 52 cases, microthrombi composed of a mixture of aggregated platelets and fibrin and measuring approximately 30-70 μm in size were observed in about 10% of the specimens and in about 6% of the cases, while individually adherent platelets were observed in approximately 7% of the specimens and about 10% of the cases. Microthrombi and adherent platelets may be important in atherogenesis by stimulating proliferation of intimal smooth muscle cells through the release of a growth factor from platelets. This feasibility study has shown that SEM is a rapid and effective method for surveying large areas of arterial intima for the study of adherent platelets and microthrombi.

Key Words: Microthrombi, Adherent Platelets, Aorta, Coronary Artery, Human Atherogenesis

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Introduction

A cooperative multicenter feasibility study on the Pathobiological Determinants of Atherosclerosis in Youth (PDAY), supported by the National Institutes of Health, has been conducted over a one year period at fifteen facilities at various locations throughout the United States. The long-term objective of this investigator-initiated multicenter study is to define more precisely the pathogenesis of human atherosclerosis during late childhood and young adulthood. The cooperating scientists hope to determine whether selected risk factors for clinically manifest coronary heart disease in middle age influence the cellular pathobiological and biochemical processes which may be important in stimulating the developing lesions. The PDAY program is designed to provide an opportunity to trace the detailed cellular and biochemical evolution of the human atherosclerotic lesion in arterial specimens obtained at autopsy from young disease-free 15-34 year old Americans who die suddenly, usually from trauma. This age span is important because previous studies indicate that this is the time when raised lesions appear and progress in many individuals of both sexes (McGill, 1968; Sternby, 1968). The forensic autopsy population of sudden unexpected deaths is important because it avoids much of the bias and many of the complications of autopsies on hospital deaths.

At this center, we have undertaken a specialized study to determine the incidence and nature of surface microthrombi on selected areas of the intima of the aorta and the anterior descending branch of the left coronary artery (LAD). Areas of high and low probability for the development of atherosclerosis were selected for the study. The abdominal aorta (Holman et al., 1958; Strong et al., 1958, Mitchell and Schwartz, 1965; Sternby, 1968) and the LAD at the bifurcation of the left circumflex (Enos et al., 1953; Montenegro and Eggen, 1968; Velican and Velican, 1980; Grøttum et al., 1983; Svindland, 1983) were selected as areas of high probability. The thoracic aorta and the distal LAD were selected as areas of low probability. Scanning electron microscopy (SEM) was the technique of choice because it provides an opportunity to

survey rapidly, at the fine structural level, the surface topography of large areas of arterial intima. Transmitted light and electron microscopy were used to further elucidate the relation between microthrombi and the underlying arterial intima.

The presence of surface microthrombi, usually composed of a mixture of aggregated platelets and fibrin, may be important in atherogenesis for two main reasons (Haust, 1978; Woolf, 1978; Chandler, 1987). First, thrombi that are covered by regenerated endothelium and thereby incorporated into the arterial wall often become organized by intimal smooth muscle cells into plaque tissue. Secondly, platelets as well as monocytes, which also occur in thrombi, might release growth factors which stimulate the proliferation of intimal smooth muscle cells (Ross, 1986, Ross et al., 1986), a major component of atherosclerotic plaques (Morgan, 1956; Geer and Haust, 1972; Campbell and Campbell, 1985). Smooth muscle cells take part in early plaque development and are present in fatty streaks (Geer et al., 1961; Geer and Haust, 1972). They may continue to proliferate, produce collagen and progressively enlarge a plaque. In addition, like monocyte/macrophages, they accumulate lipid as part of plaque development (Geer et al., 1961; Geer and Haust, 1972). Proliferation of smooth muscle cells may involve the thrombotic process or other atherogenic stimuli. Establishing the frequency, composition, and relation of microthrombi to a specific site on the human arterial intima should provide insight into their contribution to the atherosclerotic lesion. This paper presents the results of a one year study on the feasibility of using SEM to identify microthrombi and assay their role in atherogenesis.

Materials and Methods

Participating PDAY collection centers at eleven pathology units located throughout the United States contributed the material for this study. The Case Analysis Set for the PDAY feasibility study comprised a total of 237 cases from which the 52 cases in this specialized study were derived, except as otherwise noted. Autopsies performed 12 h or less after death on disease-free 15 through 34 year old Americans who died suddenly, usually from trauma, were included in the SEM study. Two segments of aorta and two segments from the LAD were studied. One segment from each artery was selected from an area of low and high probability for atherosclerosis. Segment number 12 from the thoracic aorta and segment number 17 from the abdominal aorta and segment number 41 from the distal LAD and segment number 35 at the junction of the circumflex and LAD were the areas of low and high probability in each artery, respectively (Figs. 1a & 1b). The coronary artery was fixed on the heart in situ by pressure perfusion, using 4% neutral buffered formaldehyde at 100 mm Hg for 2 h with no prerinse. The aorta was fixed by submersion in 2% buffered glutaraldehyde.

Tissues obtained at the collection centers were forwarded to the distribution centers in New Orleans (Louisiana State University) and Chicago (University of Chicago) for collation and redistribution under the direction of the central statistical center in San Antonio, (University of Texas). The tissues forwarded to the special study centers were obtained from the same area of the aorta and coronary artery in each case according to the PDAY Manual of Operations. Upon arrival at this laboratory each specimen was

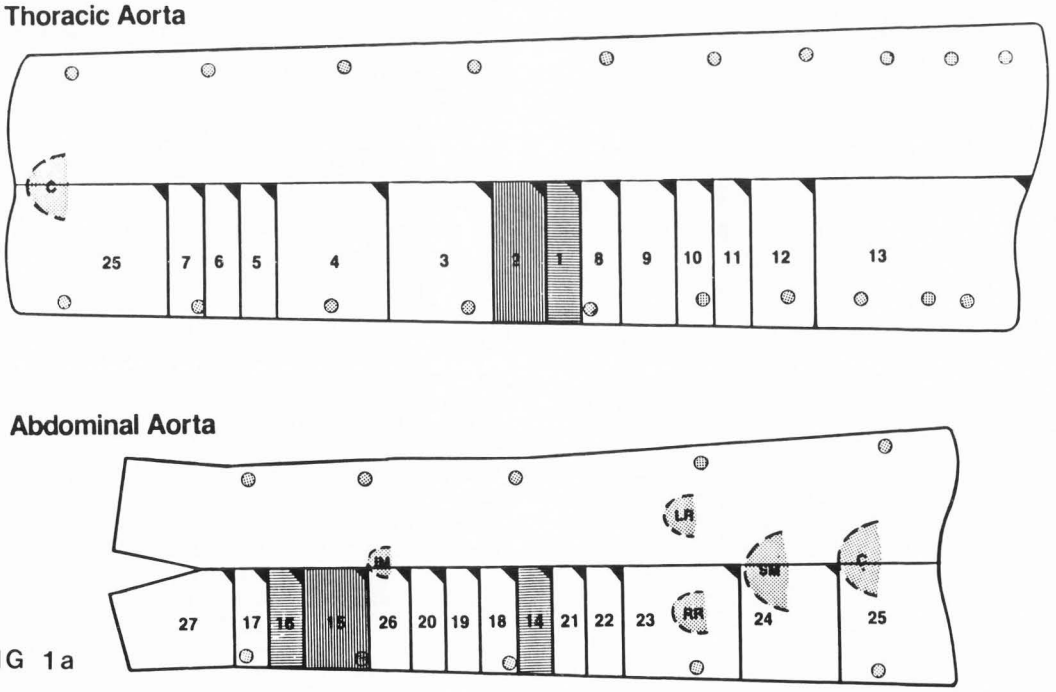

FIG 1a

FIGURE 1a. Diagram depicting thoracic and abdominal aorta by segment number as determined by PDAY Manual of Operations, September 1985.

accessioned and photographed in its transport media. The aortas were then divided into 0.5 cm squares and a small wedge was placed in a corner of the specimen to maintain orientation. A 2 x 5 mm complementary segment was embedded for conventional histology and light microscopy. The samples of perfused coronary arteries, which varied in length from 0.5 cm to 1.5 cm, were opened longitudinally into two halves to expose the endothelial surface, cut into 0.5 cm squares, and then processed for SEM. All tissues were post fixed in 2% buffered osmium tetroxide, dehydrated in graded ethanol, and critical point dried with CO_2. The specimens were mounted endothelial surface up, coated with gold/palladium and viewed and photographed in a JEOL 35U scanning electron microscope at 35 kV. Select areas of the SEM preparation were removed from their mounts and embedded for conventional transmission electron microscopy.

Observations

Table 1 depicts the number of specimens received and selected for this study from the participating collection centers. The number of cases screened and the number of cases selected for inclusion in this study are presented by anatomic site and segment number in the table. Those specimens culled from the study were removed for excessive intervals between time of death and autopsy, the patient's age exceeding 34 years, damage in procurement or transport and for excessive surface contamination; i.e., bacteria and precipitated plasma proteins, which obscured most of the underlying intima. A number of the cases removed from the study for excessive surface contamination, however, exhibited adequate endothelial preservation. Many of the cases selected for study exhibited reasonable endothelial preservation (Fig. 2).

Pure platelet and/or platelet-fibrin microthrombi were observed in approximately 10% of the specimens and 6% of the cases studied (Table 2). One of the specimens obtained from a 28-year-old black female contained four microthrombi in a single 0.5 cm square segment. Figure 3 is representative of one of these microthrombi. Microthrombi containing platelets and fibrin, as well as mononuclear cells (Fig. 4) were observed in one of the two cases collected at this institution as part of the pre-study project and this data is not reflected in cases from the Case Analysis Set given in Tables 1 or 2. Most of the microthrombi identified by SEM were approximately 30-70 μm in size. Platelets contributing to the microthrombi were approximately 2-3 μm in size. They appeared to be adhering to each other in aggregates and to the underlying intimal surface. In a few microthrombi, occasional fine strands of fibrin were seen connecting platelet aggregates and between the microthrombi and intima. Unidentified cells, which were not red blood cells or lymphocytes, were seen in several microthrombi. The endothelium in adequately prepared specimens was intact and consisted of flattened cells with elevated nuclei.

Individually adherent platelets were identified in approximately 7% of the specimens and 10%

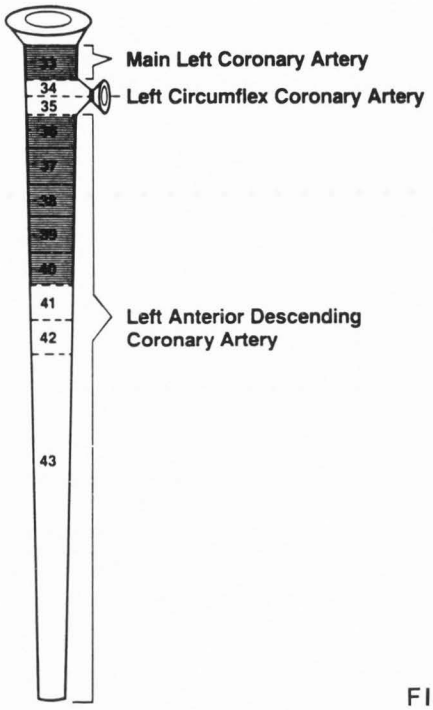

FIG 1b

FIGURE 1b. Diagram depicting the left coronary artery by segment number as determined by PDAY Manual of Operations, September 1985.

TABLE 1. Specimens received and selected for the SEM study.

			ANATOMIC SITE AND SEGMENT NUMBER			
	CASES	SPECIMENS	TA(12)	AA(17)	LAD(35)	LAD(41)
No. Received	88	292	75	72	58	56
No. Screened by SEM	66	196	54	50	47	45
No. Selected for SEM Study	52	109	25	20	36	28

TA(12) Thoracic Aorta
AA(17) Abdominal Aorta
LAD(35) Anterior descending branch of LCA at circumflex
LAD(41) Anterior descending branch of LCA distal to circumflex

TABLE 2. SEM observations on adherent platelets and microthrombi.

		CASE NUMBER OF SEGMENTS WITH	
ANATOMIC SITE & SEGMENT NUMBER	NUMBER OF SEGMENTS	ADHERENT PLATELETS	MICRO-THROMBI
Thoracic Aorta (12)	25	91072 91028	26966(3)
Abdominal Aorta (17)	20	26966 11197 91028	26966(2) 45290(4)
LAD/Circumflex (35)	36	17397 11197	79270
LAD/Distal (41)	28		
TOTAL	109	7	10

of the cases studied (Table 2). Based on their size and shape, they were of two general types. Some adherent platelets were slightly larger, flatter, and exhibited pseudopodia (Fig. 5). Others were more spherical in shape and did not have pseudopods extending to the underlying surface.

Of 19 thoracic aortic segments studied, 2 segments exhibited individually adherent platelets and one had 3 microthrombi. Of 18 abdominal aortic segments analyzed, 3 segments contained adherent platelets and one contained 2 microthrombi. Of 24 segments from the junction of the LAD and circumflex, 2 demonstrated adherent platelets while another segment contained a microthrombus. Of 18 distal segments of the LAD branch, no platelets or microthrombi were identified. An unexpected and unexplained observation was the absence of microthrombi or adherent platelets in the 30 to 34-year-old group.

Transmitted light microscopic (TLM) studies were conducted on specimens immediately adjacent to tissues found to contain microthrombi by SEM. Adjacent sections from each thoracic and abdominal aortic segment and one distal segment of the LAD from the cases submitted for SEM were examined by TLM. One hundred serial sections from each segment were cut at 5 μm and alternately stained with periodic acid-Schiff hematoxylin (PASH) and picro-Mallory trichrome to identify platelets and fibrin. Of 22 thoracic, 20 abdominal aortic, and 9 coronary artery segments studied from 23 cases, no microthrombi were identified and no conclusive adherent platelets were identified. The segments studied included all of those in which microthrombi or adherent platelets were identified by SEM.

Transmission electron microscopy (TEM) studies on select tissue from this SEM study revealed an acceptable endothelium in most of the cases received less than 6 h postmortem. Although no microthrombi or individual platelets were observed, in one case a mononuclear cell adherent to the intima was identified (Fig. 6). One aortic specimen received from the University of Maryland center after completion of the Case Analysis Set and obtained from an immediate autopsy within 1 h of death exhibited excellent endothelial preservation at both the SEM (Fig. 7) and TEM (Fig. 8) level. The observations recorded here have been forwarded to the central statistical center in San Antonio for inclusion and correlation with data accumulated at other centers currently investigating many other factors which may contribute to an understanding of atherogenesis.

Discussion

The study has demonstrated that the three dimensional image and large surface area provided by SEM offers a rapid and effective method for surveying broad areas of arterial intima for the presence of microthrombi and other intimal surface abnormalities.

The microthrombi observed thus far are approximately 30-70 μm in size. Microthrombi were found in about 10% of segments and 6% of cases examined and individually adherent

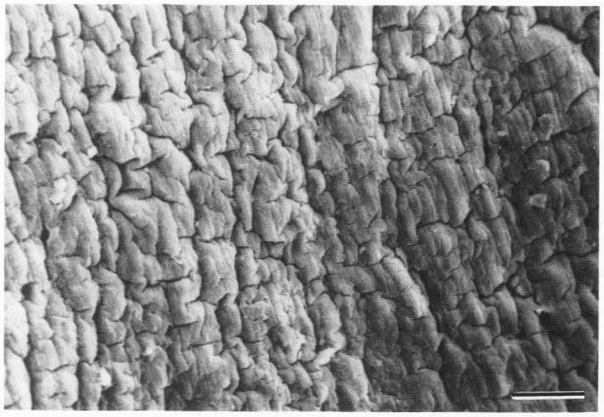

FIGURE 2. Scanning micrograph of an abdominal aorta obtained within 11 h of death (PDAY-A11197-17). The endothelial surface is continuous and reveals the numerous longtitudinal folds characteristic of immersion fixed elastic artery. Bar = 50 μm.

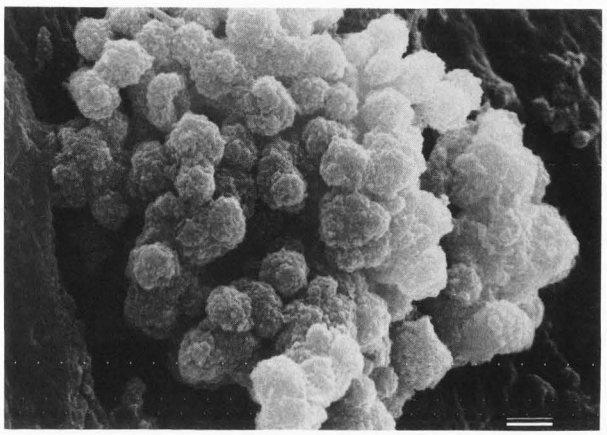

FIGURE 3. Scanning micrograph of an abdominal aorta of a 28 year old black female (PDAY-A45290-17). A pure platelet microthrombus adheres to the endothelial surface. Bar = 2 μm.

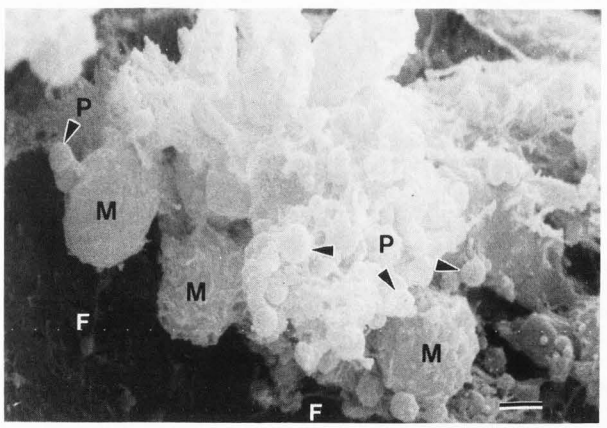

FIGURE 4. Scanning micrograph of a thoracic aorta depicting a microthrombus (UHA-84-155) consisting of platelets (P), fibrin (F), and mononuclear cells (M). Bar = 5 μm.

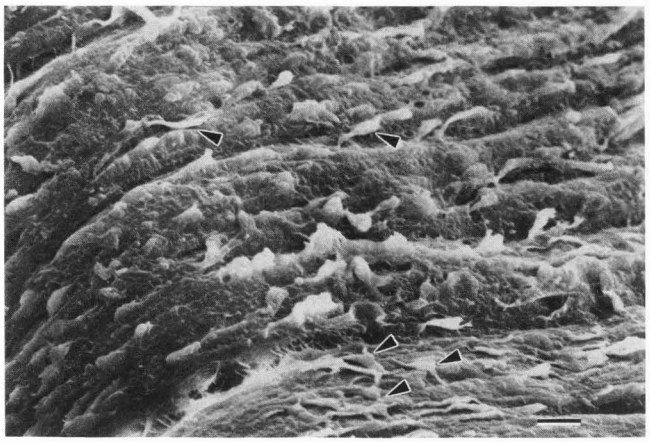

FIGURE 5. Scanning micrograph at the bifurcation of the LAD and circumflex artery of a 16 year old black male (PDAY-A79270-35). The micrograph depicts an intact endothelium with distinct nuclear elevations and attached spread platelets. (arrows). Bar = 5 μm.

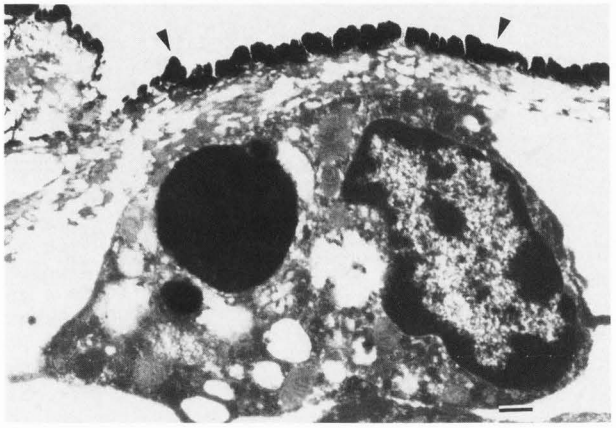

FIGURE 6. Transmission electron micrograph of a mononuclear cell resting on the intima (PDAY-A26966-17). The monocyte contains a nucleus, several mitochondria, a large osmophilic inclusion and several smaller unspecified granules. A metallic coating (arrows) was deposited on the surface for study by SEM prior to transmission electron microscopy. Bar = 1 μm.

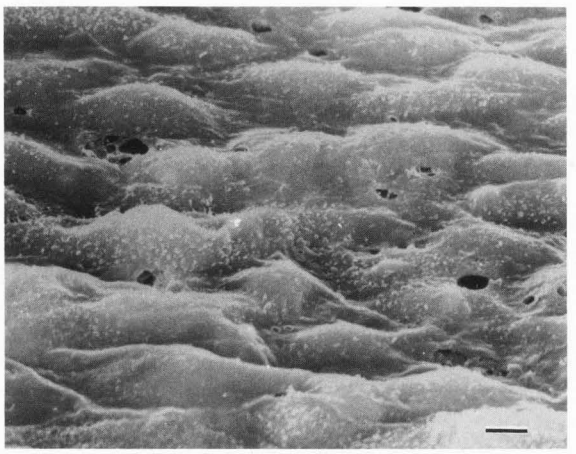

FIGURE 7. Scanning electron micrograph of a segment of aorta removed within 1 h of death (UMIA-5). Excellent endothelial preservation is denoted by clear elevations and numerous small surface microvilli. Bar = 10 μm.

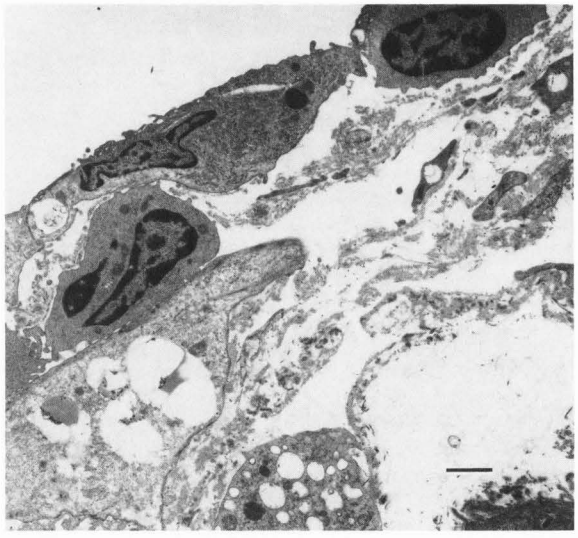

FIGURE 8. A transmission electron micrograph obtained from the same aorta (UMIA-5) as Figure 7. Note the excellent ultrastructural organelle preservation and close proximity of the adjacent endothelial cells. Bar = 2 μm.

platelets in about 7% of segments and 10% of cases, whereas, none of these findings was observed in the correlative TLM studies of serial sections from the same segments of the same cases. It should be noted, however, that each SEM segment studied was a 0.5 cm square, while the TLM study of each segment included 100 serial sections cut at 5 μm over an area of approximately 0.5 x 0.05 cm . It would take approximately 1,000 serial sections cut at 5 μm to cover the area of each 0.5 cm square SEM segment. This observation supports the contention that SEM is an effective method for surveying the intimal surface in relation to blood components.

An unanticipated but related finding was the detection of individual platelets adherent to the endothelial surface both with and without associated microthrombi. Although observed previously in the experimental animal (Sedar et al., 1978), to our knowledge this observation has not previously been recorded in human material. It has been suggested that platelets interacting with an injured arterial wall may release a growth factor that stimulates proliferation of smooth muscle cells and thereby contribute to plaque development (Ross, 1986; Ross et al., 1986).

Collaboration with other centers in this

multicenter project provides the only reasonable mechanism for obtaining the large number of samples required for this specialized study and for correlating data obtained at other centers with the findings in this project. Mean risk factor data obtained through history and postmortem determinations on body tissues and blood from all cases of the Case Analysis Set were compared with the same data on cases studied in the SEM project. Risk factors available on these cases are as follows: age, sex, race, body weight, height, trunk length, panniculus thickness, heart weight, serum chemistry including protein, albumin, cholesterol, HDL cholesterol and thiocyanate, and gross aortic surface involvement. No significant differences were observed in risk factor values between the Case Analysis Set and the cases derived from the set in this specialized study, nor were there any significant differences observed in this study between cases with or without thrombotic lesions. As more cases become available, especially those with a relatively brief postmortem interval and fewer postmortem artifacts, it should be possible to extend the study so that a reasonable number of cases will be available to ascertain meaningful correlations of adherent platelets and surface microthrombi with the results of a number of the other determinations in the PDAY program.

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Discussion with Reviewers

M. Richardson: The authors state that SEM observations provide an effective method for surveying large areas of intima, but do not comment on the value of SEM in the context of immersion-fixed, autopsy material. Do they have any experimental data which relates the appearance of perfusion-fixed material and immersion-fixed material, so that in the interpretation, artifacts associated with postmortem changes can be specifically identified?

Authors: We do not have a perfusion fixed/immersion fixed experimental model of the aorta for this study which encompasses tissues obtained from 1 to 12 h following death. Nor do we know of any reports on such a study in human beings.

M. Richardson: Was there any evidence that the presence of platelets on the intima of autopsy arteries was associated with prior endothelial desquamation?

Authors: The adherent platelets and microthrombi identified in this study are assumed to be at sites of endothelial damage. What remains to be determined is the source and form of endothelial damage.

M. Richardson: Was there any evidence that thrombus formation has occurred in other locations prior to death in the patients who showed thrombus on the intimal surface?

Authors: No. Thrombi in other sites were not searched for at autopsy.

M. Richardson: The structure shown in Fig. 3 is described as platelet microthrombus, is there TEM confirmation of this interpretation? The structures in Fig. 4 labelled (p) are more comparable to platelets seen in other situations.

Authors: The structure in Fig. 3 is interpreted as a cluster of adherent platelets fixed in the round stage which precedes the dendritic stage, as illustrated in Fig. 4. The specimen from which Fig. 3 was obtained was not included in TEM studies.

R. Laschi: You state that a parallel LM and TEM analysis has been performed on the segments which are complementary to those observed by SEM. It should be interesting to know if these specimens were histologically normal or diseased as a result of the atheromatous process.

Authors: Although some early atherosclerotic lesions were noted histologically, no microthrombi were observed on any of the TLM specimens. No such lesions or microthrombi were observed by TEM.

R. Laschi: Have you found any difference between the areas of high and low probability for the development of atherosclerosis?

Authors: No significant differences were observed.

R. Laschi: Fig. 2. The longitudinal folds shown in this figure are mainly due to the immersion fixation procedure (see: M. Richardson et al. "Scanning Electron Microscopy of Normal Rabbit Aorta: Injury or Artifact?" J. Ultrastruc. Res., 1985, 91: 159-173). Why did you not employ a perfusion with an electrolyte solution prior to fixation in the case of aortic specimens? It is well established that the preparation parameters are very critical for the subsequent SEM morphology. For instance, the presence of cellular debris and plasma proteins on the luminal surface of the aorta as well as the occurrence of folding and wrinkling could be minimized by using the aldehydic fixation (under physiological pressure) after proper perfusion with an electrolyte solution (i.e. Eagles medium) for 3-5 min.

Authors: We agree that the longitudinal folds are probably due to immersion fixation as reported by M. Richardson et al. but this study was on human autopsy material. Therefore, the experimental animal literature was not cited. The protocol for this study will encompass approximately 3000 human autopsies from 15 collection centers. It was determined that aortic perfusion was technically not feasible.

R. Laschi: Fig. 6. Is this figure consistent with the text? Indicate the endothelium. Is this over or under the mononuclear cell?

Authors: In the text and caption, we chose to use the term intima, because we were unable to identify the cell type immediately underlying this mononuclear cell. The mononuclear cell is over the intima.