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Original Articles

Cellular Pathology of the Rat Aorta

Pseudo-Vacuoles and Myo-Endothelial Herniae

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Summary. The aortic intima of adult rats, studied by electron microscopy, showed several changes indicative of spontaneous cellular pathology. These changes occurred almost exclusively at the level of fenestrae in the internal elastic membrane. The initial event was the formation of club-shaped cell processes arising from the smooth muscle cells and protruding into the fenestrae; this phenomenon gave rise to four types of images: (a) short *pseudopodia* reaching through the fenestrae; (b) long pseudopodia that pushed their way into the body of the overlying endothelial cells, giving rise to *myo-endothelial herniae* (reminiscent of the cell-to-cell herniae previously described in small, normal muscular arteries); (c) membrane-bound cellular parts apparently lying free beneath the endothelium, for which the current term *ghost bodies* in convenient; and (d) intraendothelial structures lined by two membranes, clearly arising through the mechanism of herniation, and best referred to as *pseudo-vacuoles*. Some of the myo-endothelial herniae become very large and stretch the endothelium to such an extent that it could easily burst, especially during tissue processing. This mechanism should account for many of the endothelial bulges and "craters" often seen by scanning electron microscopy. The formation of such craters (arising from the collapse of myo-endothelial herniae as well as of endothelial blebs) offers a plausible explanation for the "stomata" and "stigmata" that have been described in silver nitrate preparations of the endothelium for over a century.

Key words: Endothelium – Aorta – Intima – Smooth muscle – Herniae, cellular – Vacuoles – Stomata – Stigmata – Atherosclerosis.

In an electron microscopic study of the rat aorta, as affected by various drugs, we were impressed by certain abnormal features of the untreated controls.

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Changes indicative of cellular pathology occurred at all levels of the aortic wall; especially intriguing were large, clear "vacuoles", lined by two membranes, within endothelial cells. The purpose of this report is to examine the pathogenesis and significance of these structures.

Materials and Methods

We used 40 male Wistar rats weighing 180–280 g (Charles River Breeding Laboratories, Wilmington, MA). Since the original aim of this study was to duplicate the results of Robertson and Khairallah (1973), who had claimed that small doses of vasoactive agents caused gaps to appear in the aortic endothelium of the rat, we used the same technique as these authors. Thus the rats were divided into two groups: control (8) and experimental (32). The control rats received only an intracardiac injection of colloidal carbon black in order to label any intercellular gap (Majno and Palade, 1961); we used filtered Pelikan "biological ink" (batch C11/1431a, Morilla Co., Long Island City, New York) in the dose of 0.1 cc added to 0.4 cc of Ringer solution. The experimental rats received intracardially, together with carbon black, one of the following drugs: angiotensin II (Hypertensin Ciba; 10, 1 or 0.1 ng); histamine diphosphate (Sigma; 100, 10 or 1 ng); bradykinin triacetate (Sigma; 10 ng); or norepinephrine (Levophed bitartrate, Winthrop; 100 ng). For each injection the drug was dissolved in 0.5 cc of Ringer solution mixed with carbon black as mentioned above. All injections were delivered into the left ventricle as follows. Under ether anesthesia, the rat was intubated and connected to a rodent respirator (Model 680 of Harvard Apparatus; stroke-vol. 5 cc, 110 strokes per min). The heart was exposed by left thoracotomy, and the 0.5 cc bolus was injected into the left ventricle from a tuberculin syringe. Three min later the left ventricle was re-entered with an 18G needle connected via intramedic polyethylene tubing to a bottle containing 3% glutaraldehyde in 0.1 M cacodylate buffer for perfusion. The perfusing fluid was delivered by gravity at room temperature and at a pressure of 110–130 mm Hg. The femoral vessels were opened for outflow.

After 20 min of perfusion (Joris and Majno, 1974) the heart and aorta were dissected out, and 1 mm long aortic rings were taken at 6 levels from the ascending aorta to just above the bifurcation. The rings were immersed in fixative at room temperature. Total fixation time was 5 h. The tissues were left overnight in 0.1 M cacodylate buffer at 4° C, post-fixed in 2% Oso_4 in 0.2 M collidine buffer at 4° C for 2 h, and embedded in Epon 812 (Ladd Research, Burlington, VT). One half of the tissues were embedded so as to be longitudinally oriented, the other half were oriented transversally. One-micron thick sections were cut with glass knives and stained with toluidine blue for light microscopy. Thin sections were cut with a diamond knife on a LKB Ultrotome III and mounted on 200 or 300 mesh uncoated copper grids, stained with uranyl acetate and lead citrate, carbon-coated and examined with a Philips 301 electron microscope. Ultrathin serial sections were also cut and mounted on carbon-coated formvar films on single-hole grids, stained and examined as above.

Results

Intima. With the exceptions listed below, the general characteristics of the aortic endothelium were the same as described in previous studies (Gerrity and Cliff, 1972; Schwartz and Benditt, 1972a, b; Majno and Joris, 1978). The endothelial gaps described by Robertson and Khairallah (1973) were not found; no difference at all was found between the injected and the control rats. However, pathologic changes did occur – to the same extent – in both groups of animals. These changes were of two kinds: (a) the presence of peculiar "vacuoles" within endothelial cells, and of membrane-bound structures beneath them; and (b) mo-

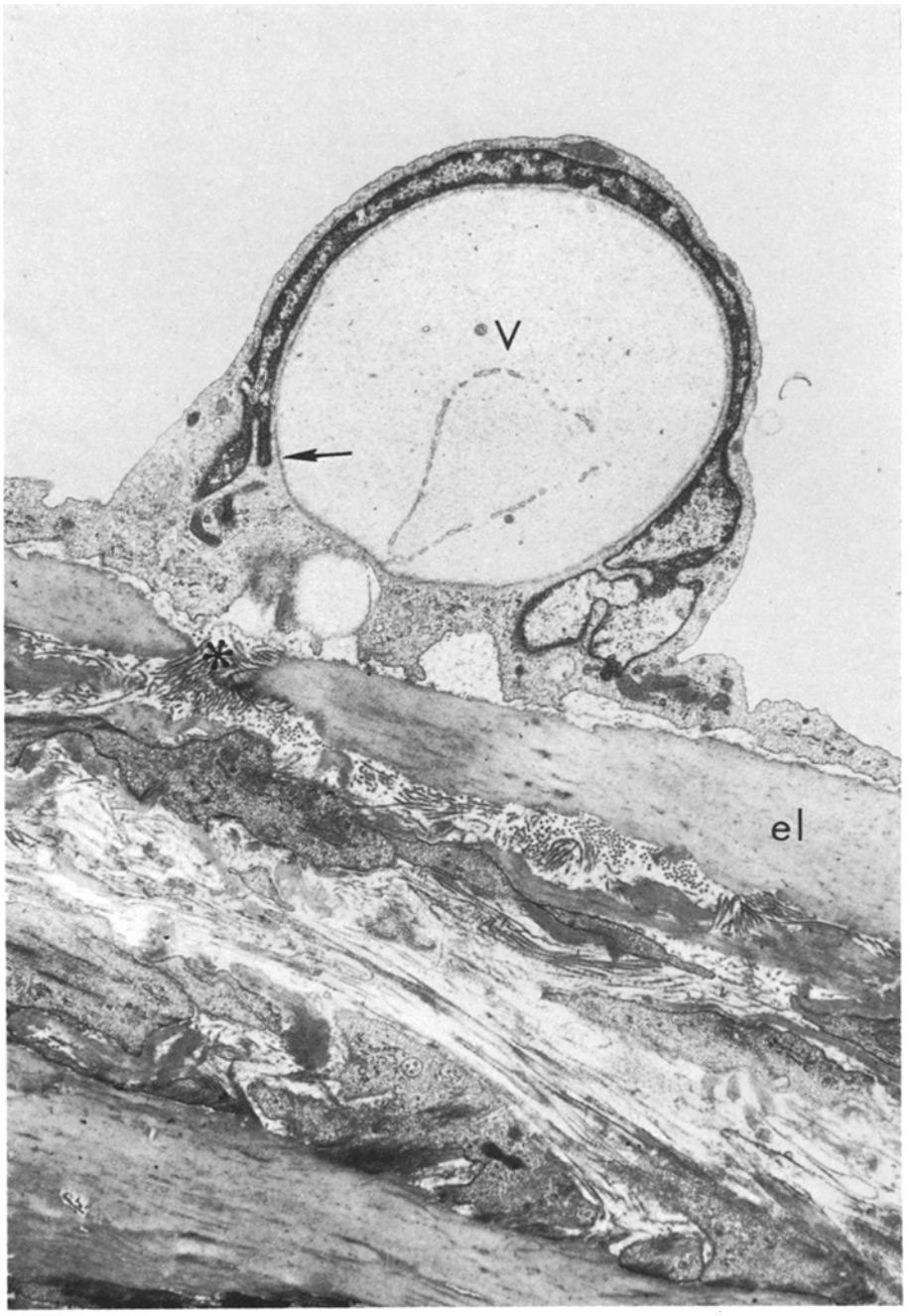

Fig. 1. Typical pseudo-vacuole (*V*) causing an endothelial cell to stretch and bulge into the lumen. It is lined by a double membrane, barely visible at this power (*arrow*). Note that this pseudo-vacuole lies just above a fenestra (*) in the elastica interna (*el*). (Angiotensin II, 0.1 ng) (×9,000)

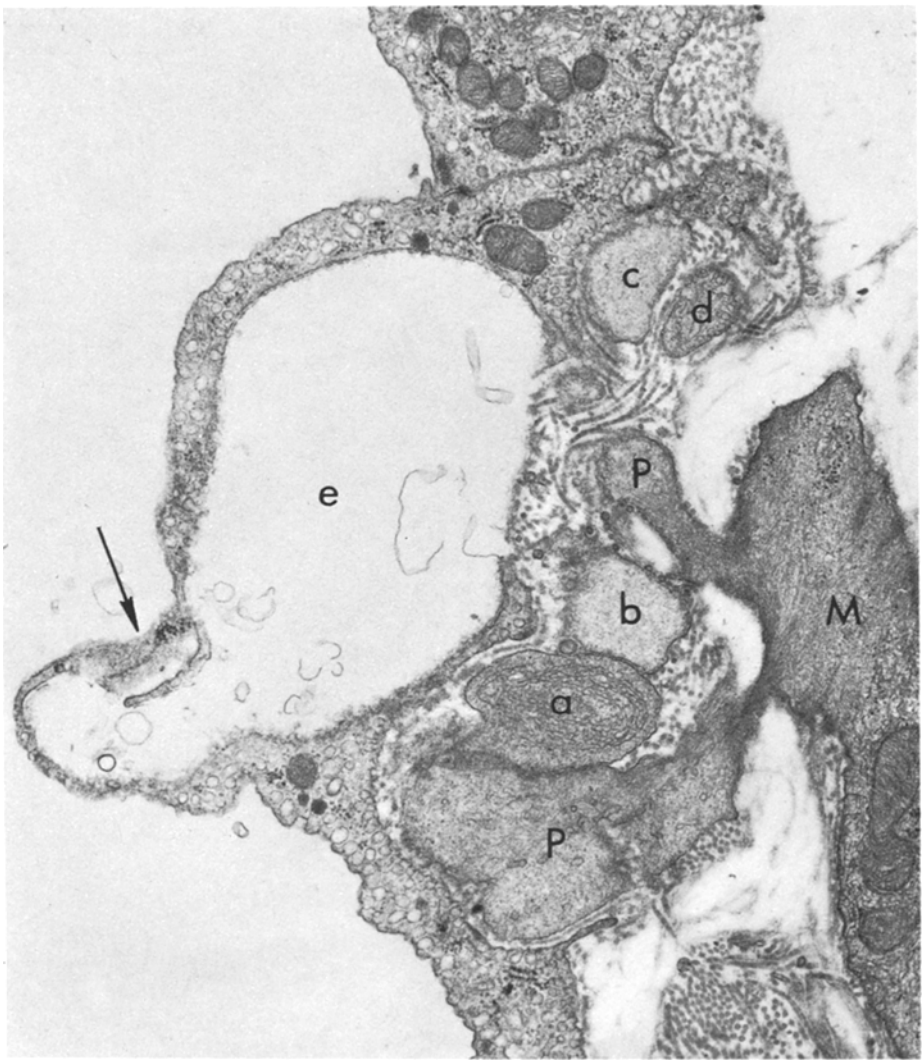

Fig. 2. A group of pseudopodia (*P*) and "ghost bodies" (*a, b, c, d*) presumably all arising from the smooth muscle cell (*M*). When these structures are swollen like (*e*) and stretch the endothelium as shown here (*arrow*), bursting of the membrane-bound structure is likely, either in vivo or during tissue processing. (Control) (× 17,750)

nonuclear cells, both lymphocytes and monocytes lodged beneath the endothelium and occasionally migrating across it; this finding will be reported later.

The endothelial "vacuoles" were limited by two distinct, closely apposed membranes visible along most of the periphery (Fig. 1); their content was usually electron-lucent and included myelin figures, vesicles, fibrils and clumps of amorphous material. Some appeared very close to bursting (Fig. 2). *They occurred far more frequently in those endothelial cells that lay above a fenestra in the internal elastic membrane.* Pathologic cellular events were common at this level

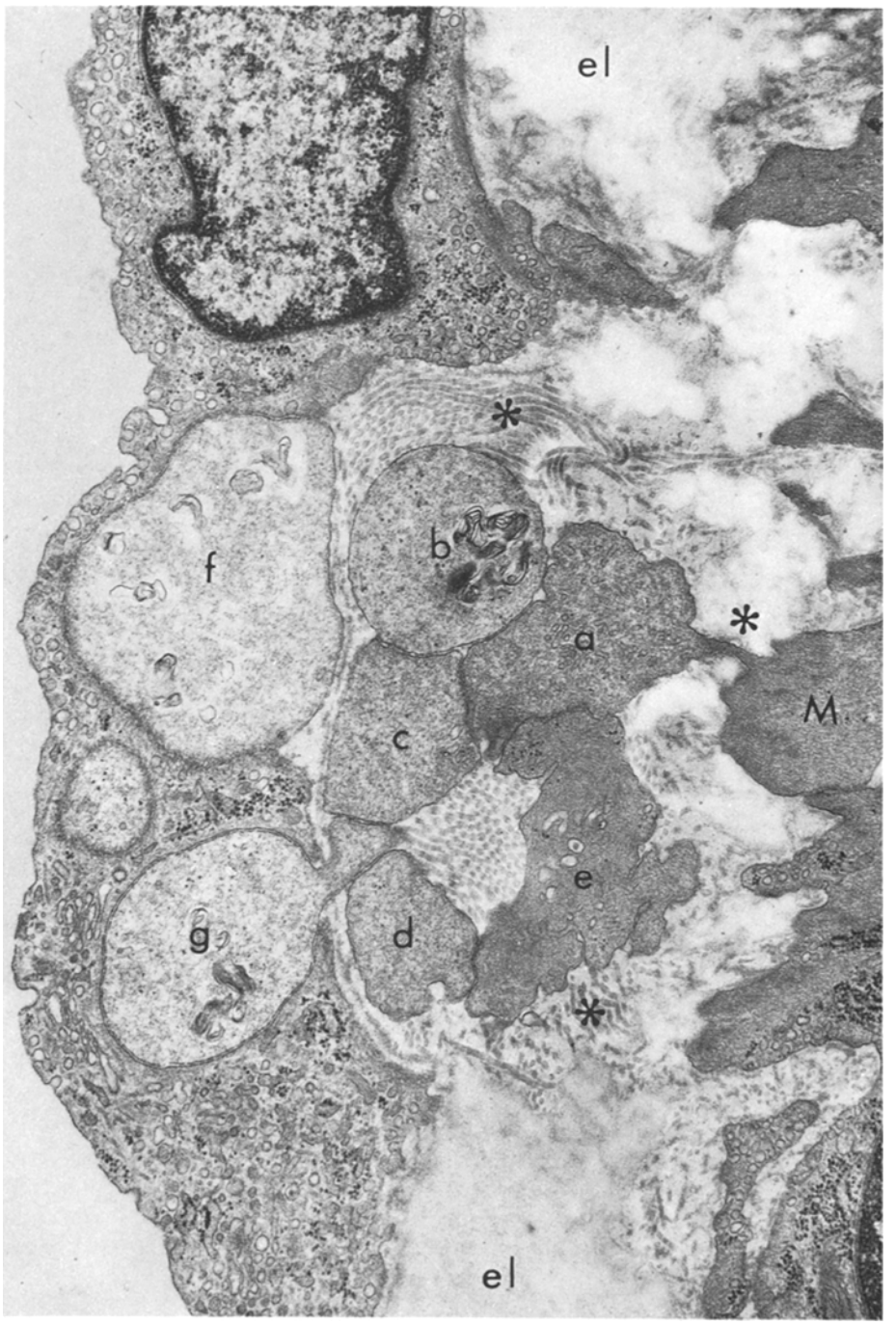

Fig. 3. Higher magnification of a fenestra (*) in the internal elastic membrane (*el*). The fenestra is partially closed by clumps of elastin; through it the smooth muscle cell (*M*) has sent a number of protrusions: (*a*) is visibly attached, (*b, c, d, e*) appear detached, (*f*) bulges beneath the endothelium, (*g*) forms a typical herniation through the basement membrane and into the endothelium. Note myelin figures (*b, f, g*). Despite these events in the fenestra, the endothelium over it is almost flat. (Angiotensin II, 10 ng) (×20,000)

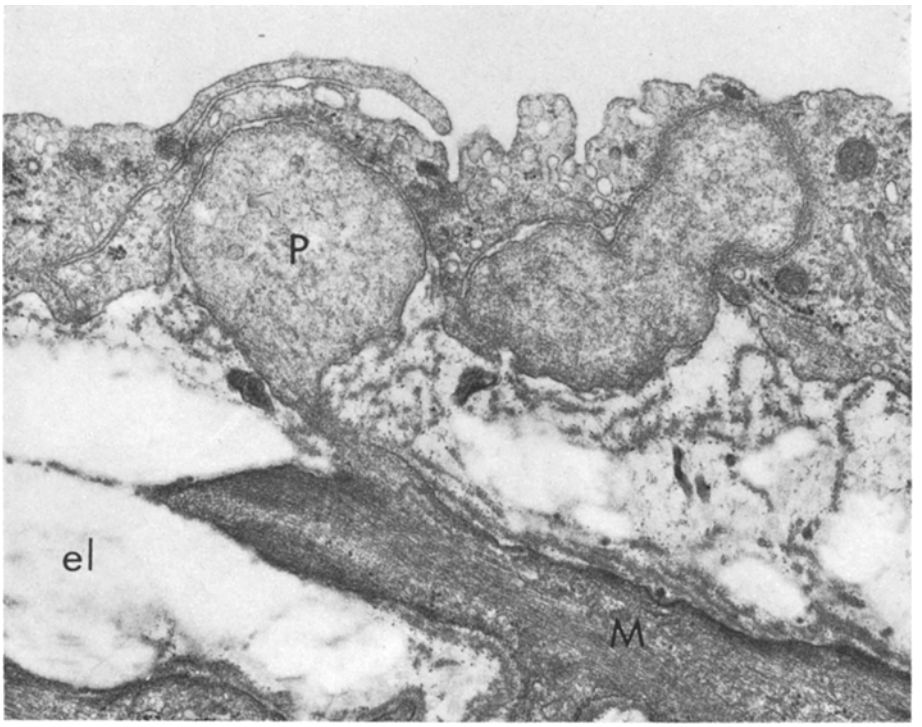

Fig. 4. A subendothelial protrusion (*P*) arising from a smooth muscle cell (*M*) lying just beneath the internal elastic membrane (*el*). The connection between M and P has the same diameter as an endothelial vesicle, i.e. about 700 Å; thus, if cylindrical, it could be seen only in one or two ultrathin sections. (Control) (×27,500)

(Figs. 2 and 3). The fenestrae were often occupied by rounded or irregular membrane-bound structures which never included a nucleus; their content ranged from electron-lucent to electron-dense (Fig. 3). We will refer to them as *ghost bodies* (see Discussion). Some of these were deep in the intima, others made contact with the overlying endothelial cell and even herniated into it (Fig. 3).

On most electron micrographs the endothelial "vacuoles" and the ghost bodies appeared to be isolated; in some cases, however, they were connected to a medial smooth muscle cell by a thin cytoplasmic stalk passing through a fenestra, and could therefore be defined as *herniations* of smooth muscle cells. These connecting stalks were very thin, 0.1 μ or less in diameter (Figs. 4 and 5). On rare occasions a smooth muscle cell process poked through the endothelial layer and became exposed to the aortic lumen (Fig. 6).

Media. Herniations of smooth muscle cells were also found within the depth of the media, occurring through fenestrae in the deeper elastic membranes (Fig. 7). In the outer layer of smooth muscle cells, herniation occurred outward,

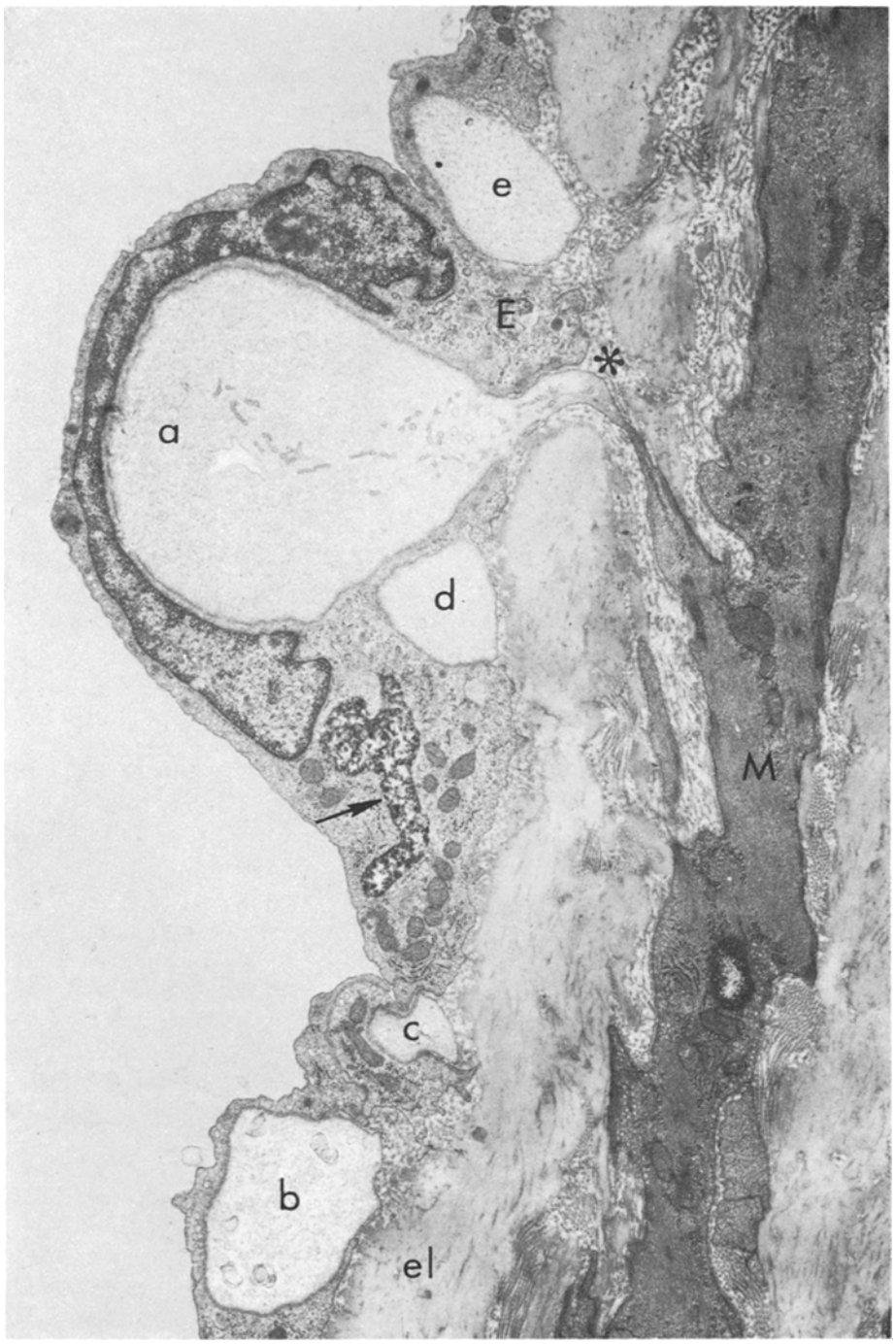

Fig. 5. The endothelial cell (*E*) is filled with a vacuole-like structure (*a*), actually a pseudopod which arises from the smooth muscle cell (*M*), and passes through a narrow fenestra (*) in the internal elastic membrane (*el*). The connection, at its thinnest point, is about 1,000 Å thick. *Arrow*: A residual body containing material in advanced state of degeneration; it presumably arose from a hernia such as (*a*), which broke off from (*M*) and was subsequently treated by the endothelial cell as foreign material to be digested. Note (*b, c, d, e*) with no visible connections. (Angiotensin II, 1 ng) (×12,500)

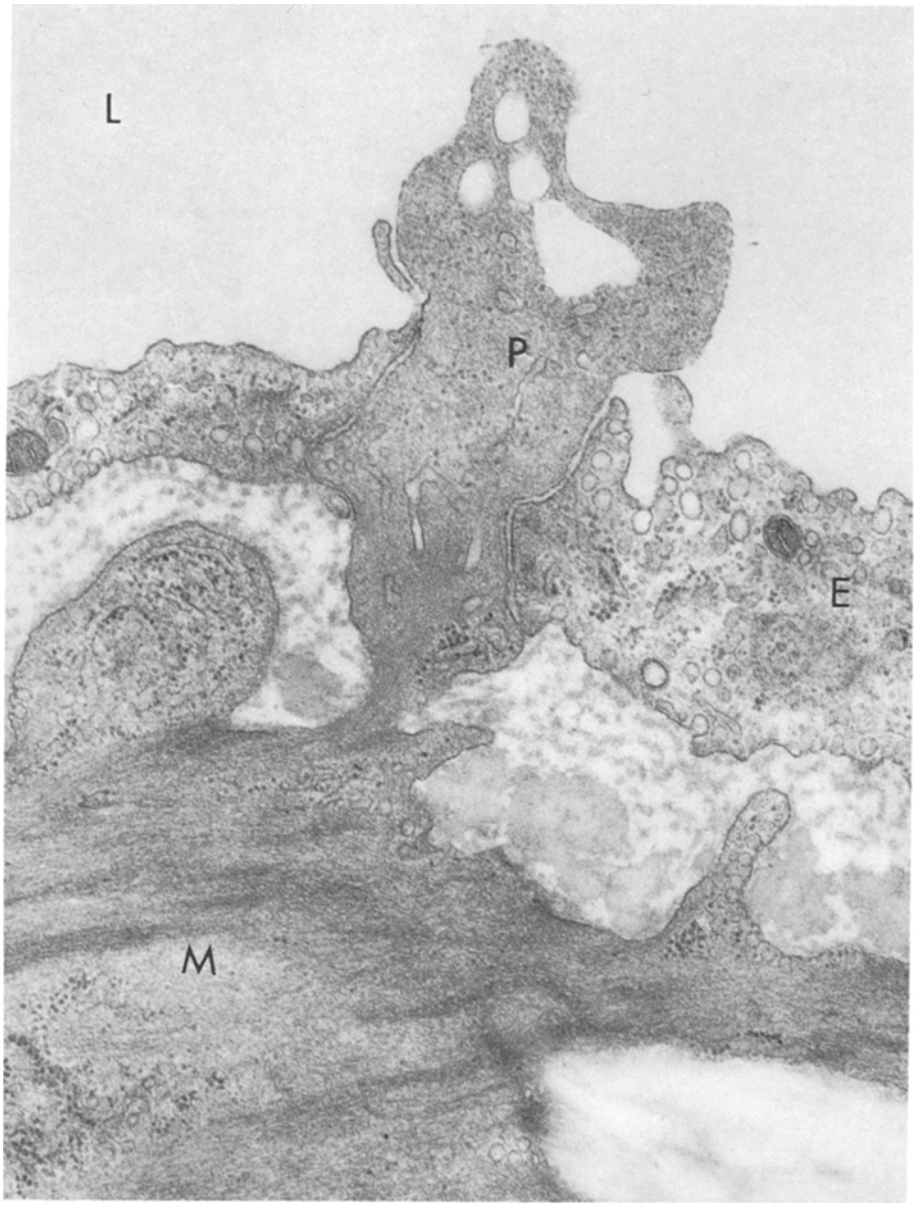

Fig. 6. A smooth muscle cell (*M*) sends a pseudopod (*P*) through the endothelium (*E*) into the lumen (*L*) of the aorta. It is not possible to decide whether (*P*) passed between two endothelial cells or rather through the cytoplasm of one cell. (Histamine, 1 ng) (×37,500)

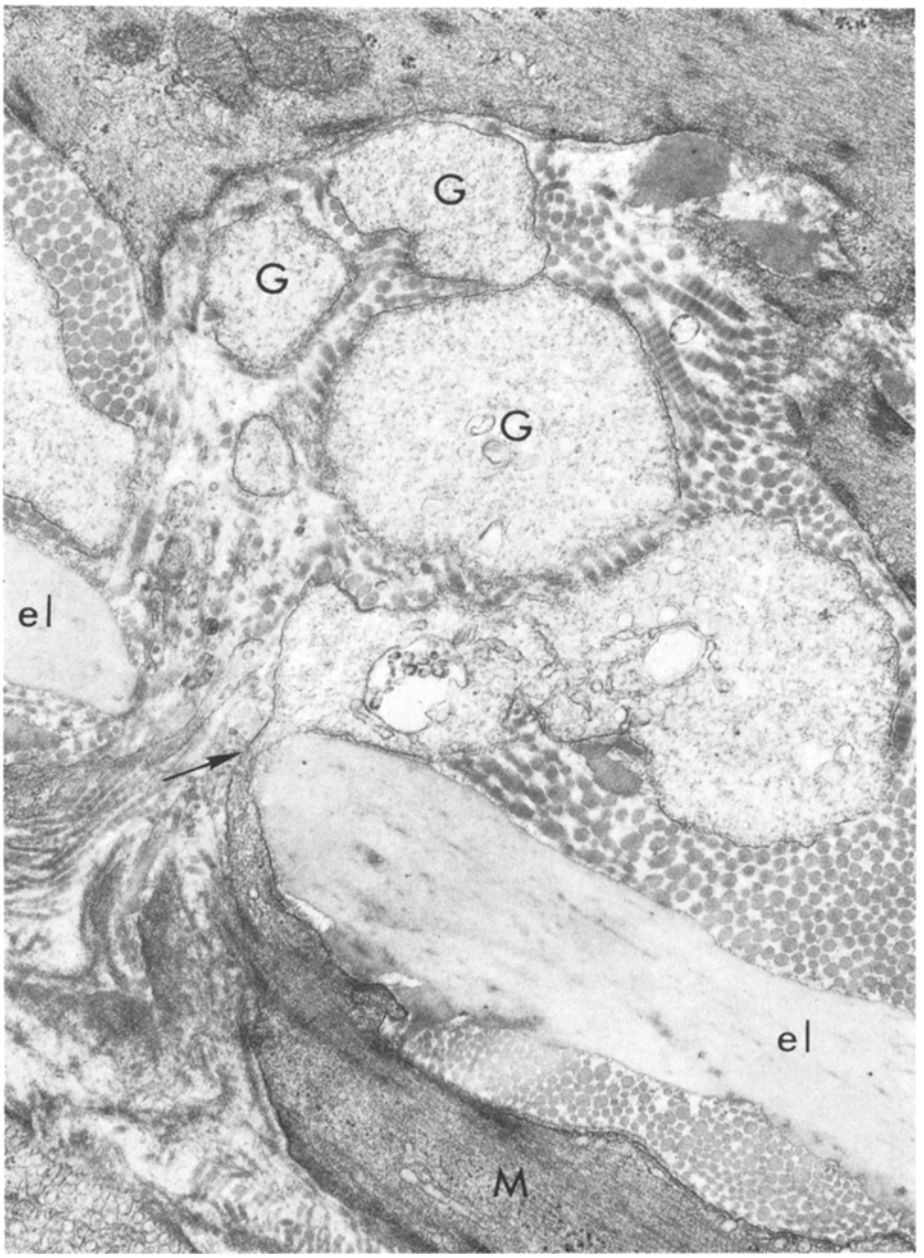

Fig. 7. Aortic media. A smooth muscle cell (*M*) sends a pseudopod (*arrow*) through a gap in an elastic lamella (*el*). The pseudopod then expands and shows signs of degenerations. Note "ghost bodies" (*G*) nearby, presumably related to the same cell (*M*). (Bradykinin, 10 ng) (×20,000)

usually forming clear vacuoles that bulged against the "veil cells" (Joris and Majno, 1974).

Discussion

We report in this paper a series of pathologic changes that occur spontaneously in the aorta of the rat at the level of fenestrae in the elastica interna. The key event is the formation of cell processes which arise from smooth muscle cells and extend through the fenestrae. We conclude that four different types of electron microscopic images can occur by this phenomenon: (a) a *pseudopodium* arising from a smooth muscle cell and protruding into the fenestra, often with an expanded, club-shaped head; (b) a *myo-endothelial hernia,* which occurs when a long pseudopodium bulges into the body of an endothelial cell; (c) a *ghost body,* i.e. an isolated, membrane-bound mass of cytoplasm, lying apparently free beneath the endothelium; and (d) a *pseudo-vacuole:* a structure lined by two concentric membranes and contained within an endothelial cell. The question then arises whether (c) and (d) are truly free structures which broke off a smooth muscle cell, or simply smooth muscle cell pseudopodia incompletely shown on the section. A decision could be made only on examining serial sections; however, there is indirect evidence that the heads of the pseudopodia can break off: some endothelial cells contained, besides a recent pseudo-vacuole, a residual body (Fig. 5). The presence of these two inclusions side by side suggests that an earlier hernia broke off its stalk and was subsequently treated by the cell as foreign material to be digested.

Critique of Our Findings. Myo-endothelial herniae were not mentioned in two recent descriptions of the aortic intima of the normal rat (Gerrity and Cliff, 1972; Schwartz and Benditt, 1972b). We ascribe this discrepancy primarily to a sampling problem, because these changes are focal. Schwartz and Benditt (1972b) did observe parts of necrotic cells beneath the endothelium of adult rats; in speculating on their possible significance, they favored the notion that "intimal cells" had somehow become obsolete and died. It is far more likely that the parts of necrotic cytoplasm illustrated represented herniated cytoplasm from medial smooth muscle cells.

It could also be objected that cytoplasmic blebbing and similar phenomena can be caused by improper fixation, and therefore we – as well as previous authors – may have merely described the effects of inadequate fixation. It has been reported that glutaraldehyde or osmium alone (used on mouse peritoneal cells) are reliable fixatives, but that osmium used after glutaraldehyde induces blebs as long as 5 μ (Shelton and Mowczko, 1977). On the other hand, it has also been reported (in a study on corneal fibroblasts) that glutaraldehyde alone does cause bleb formation, whereas a mixture of glutaraldehyde and osmium *prevents* their development (Hasty and Hay, 1978). Presumably different cellular models yield different results. As regards the rat aorta, the study of Pease and Paule (1960), in which smooth muscle blebbing was reported as frequent, osmium alone was used, a reliable fixative according to both studies just mentioned. To sum up, it is most unlikely that we are dealing with a

fixation artefact, because sometimes the clear, balloon-like structures, which could presumably have time to develop during fixation, are accompanied by signs of cellular pathology which must have existed before fixation (Fig. 5).

A third objection is that some of our rats had been injected intravenously with small doses of vasoactive agents 3 min before fixation, and therefore any cellular change found in these animals could be the effect of drugs. However, identical herniae were found in our controls. Furthermore, "vacuoles" were found in the normal rat aorta by others by light (Richardson and Settles, 1977) as well as by electron microscopy (Gutstein et al., 1978).

Earlier Observations in Normal Vessels. Myo-endothelial herniae in normal vessels have not been previously described. However, the tendency of smooth muscle cells to insinuate *pseudopodia* into fenestrae of the internal elastic membrane has been observed in the aorta of the rat (Pease and Paule, 1960) as well as in arteries of the dog (Moore and Ruska, 1957), rabbit (Parker, 1960), pig (French, 1966) and Chinese hamster (Soret et al., 1976). The *"vacuoles"* observed recently in the normal rat aorta by Gutstein et al. (1978) are identical to the pseudo-vacuole described by us; their association with fenestrae in the internal elastica was also noticed (but a connection with smooth muscle cells was not seen). Thus it appears that these cellular events are common in the arterial system of mammals.

As regards the apparently isolated structures that we have labelled *ghost bodies*, we chose this term because it is already in use, although rather loosely. It was introduced by Scott et al., in 1967, in a study of atherosclerotic lesions in Rhesus monkeys; the authors applied it to membrane-bound masses, intra- or extracellular, which had "an amorphous, flocculent background with shadowy figures within it. These shadowy figures were often strongly suggestive of degenerating organelles". When found inside smooth muscle cells, they were interpreted as areas of cytoplasmic degeneration (see also Daoud et al., 1968); today, there is no doubt that they represent cell-to-cell herniae (Joris and Majno, 1977). The free ones were recognized as arising from smooth muscle by Veltmann et al. (1975). It should be noted that the term ghost body was used rather differently by Gerrity and Cliff (1972): in a study of the aortic media in aging rats, they applied it to necrotic masses having "the general shape of cells and often associated with cell debris".

Myo-Endothelial Herniae in Pathologic Vessels. The first clearcut description of smooth muscle cell herniations into the endothelium was given by Hoff and Gottlob (1967), who studied rabbit aortas perfused in situ with various noxious agents. They referred to them as giant vacuoles; Hoff (1970) described them again later as pseudopodia and vacuoles. Thereafter similar changes were seen in arteries of hypoxic cattle (Jaenke and Alexander, 1973), of hypoxic rabbits (Kjeldsen and Thomsen, 1975) and of hypertensive rats (Wiener and Giacomelli, 1973). Smith and Heath (1977) saw endothelial vacuoles with double membranes in hypoxic rats, but appear to have misinterpreted them as dilated mitochondria. In summary, the herniation observed by us in normal rats is a well-established event under pathologic conditions.

Significance of Smooth Muscle Herniation. The smooth muscle cells of the media are prone to herniate into each other even in normal arteries (Joris and Majno, 1977). Studies on isolated smooth muscle cells have shown that they tend to form cytoplasmic protrusions when they contract (Fay and Delise, 1973). Why the herniated buds tend to swell is not clear. Takeuchi et al. (1973) stated that herniation vacuoles observed in spastic renal arteries contain an increased amount of potassium histochemically demonstrable, but the evidence is not compelling.

The penetration of smooth muscle cells *through* the endothelium has at least two analogies in endothelial biology. It was demonstrated by scanning as well as by transmission electron microscopy that white and red cells of the bone marrow penetrate into the lumen of the sinusoids by punching a hole through the body of the endothelial cell (De Bruyn et al., 1971; Becker and De Bruyn, 1976). When the kidney is perfused for electron microscopy, glomeruli that are not well reached by the fixative develop herniations of mesangial cells into the capillary lumen (Johnston et al., 1973); similar changes occur during autolysis (Cook et al., 1965), and therefore presumably also in vivo, under pathologic conditions.

Relationship Between Myo-Endothelial Herniae, "Craters", Stomata and Stigmata. When Julius Arnold used silver nitrate to demonstrate the interendothelial junctions in 1873, he was intrigued by small black dots or rings (mostly occurring along the intercellular junctions) which he interpreted as microscopic openings; he called *stigmata* the smaller, *stomata* the larger ones. Much debate ensued as to the significance of these structures; some considered them artefacts (see Altschul, 1954). In recent times, the artefact theory seems to have been abandoned. Caplan et al. (1974) illustrated stomata and stigmata in the aorta of the pig. Silkworth et al. (1975) described an "increase" in stomata and stigmata in hypercholesterolemic rabbits. Björkerud et al. (1972) interpreted stomata and stigmata as subcellular valves with the function of regulating the transfer of material from the blood stream to the artery.

By transmission electron microscopy corresponding openings in the endothelium have not been detected; yet it is inconceivable that such large openings could have escaped notice. We propose a new interpretation: *both stomata and stigmata represent the outlines of myo-endothelial herniae, endothelial blebs or similar fluid-filled structures, which exploded during the preparation of the tissue.* When a hernia stretches the endothelium to the extent shown in Fig. 2 it is conceivable that the entire structure could burst: the result would be a smooth muscle cell process exposed to the lumen, as in Fig. 6.

If our suggestion is correct, scanning electron microscopy of arterial endothelium should detect structures compatible with the notion of "exploded fluid-filled pockets." The *craters* described by many authors fulfill this requirement; balloons and craters are mentioned in almost every publication of SEM on the endothelium (Edanaga, 1974; Gertz et al., 1975; Gregorius and Rand, 1975).

Our interpretation fits well also with the findings of Björkerud et al. (1972). They noticed that the stomata and stigmata were usually surrounded by a raised rim (which concurs with the "crater" notion); they also found that the opening led into a "preformed, microscopic, tubular space extending below

the intimal elastic membrane." This description applies almost literally to the channel followed by a smooth muscle pseudopodium extending upward through a fenestra in the internal elastic membrane.

Thus the debate as to the nature of stomata and stigmata – artefacts or real structures – appears to end with the conclusion that both sides were right. According to our view, the ring-shaped structure is in most cases an artefact; but it does correspond to an event of cellular pathology, which may occur either in vivo or during the manipulation of the tissue.

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