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LUMINAL SURFACE OF DISTENDED ARTERIES BY SCANNING ELECTRON MICROSCOPY: ELIMINATING CONFIGURATIONAL AND TECHNICAL ARTEFACTS

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Summary.—Perfusion fixation at physiological pressures, careful tissue handling, adequate drying and reduced beam exposure time eliminated many of the intimal surface projections, ridges and bridges which have been taken for normal structures on scanning electron microscopy. In the normal distended vessel, ovoid endothelial nuclei bulged into the lumen with their major axes aligned in the direction of flow; adjacent cell margins overlapped, consistently in the direction of flow, with each cell overlapping the edge of its downstream neighbour. Regular longitudinal furrows associated with undulations of the internal elastin lamina were entirely eliminated from elastic arteries when distending pressures exceeded diastolic levels during fixation.

REPORTS concerning the configuration of the arterial endothelial surface as revealed by scanning electron microscopy (SEM) include descriptions of several types of folds and excrescences. Smith *et al.* (1971), for example, reported a variety of projections, the significance of which was questioned by Wolinsky (1972) on the grounds that the authors had not distended their vessels during fixation. Stil land Dennison (1974) have shown villous projections at the edges and over the surfaces of endothelial cells and Shimamoto *et al.* (1971) have described intercellular bridges and subcellular channels. All of these investigators have asserted or implied that the intimal surface is normally arranged in longitudinal furrows or ridges. We have now studied aortas, pulmonary arteries and coronary arteries of rats, rabbits and pigs, having taken precautions to fix the vessels by perfusion under controlled pressure. We have also studied the effects of various conditions of specimen fixation, drying, coating and exposure to the electron beam. Details concerning the effects of osmium tetroxide post-fixation on structural details in glutaraldehyde fixed tissues and the relative effects of alcohol, acetone or amyl acetate as intermediate processing fluids will be provided in other reports. It is the purpose of this communication to demonstrate that intimal furrows, cytoplasmic bridges and many of the endothelial projections and villi described in recent papers do not correspond to normal configurations at physiological pressures and to emphasize the need to minimize these and other artefacts by preparing and examining material under carefully controlled conditions. Three dimensional reconstructions of arterial wall architecture as illuminated by combined SEM and transmission electron microscopy (TEM) under such controlled conditions will be presented elsewhere

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MATERIALS AND METHODS

The vessels illustrated in this report were fixed by controlled pressure perfusion using 2·5% glutaraldehyde in 0·067 mol/l phosphate buffer at pH 7·2. Specimens perfused or post-fixed with osmium tetroxide and critical point drying after amyl acetate substitution were identical to those prepared by glutaraldehyde fixation and alcohol substitution alone. Exposure to osmium tetroxide and the additional solvent often increased the incidence of crystalline deposits on scanned surfaces. Perfusions were carried out for 15–60 min in animals anaesthetized with sodium pentobarbitone and alive at the onset of the perfusion. Some vessels were perfused with buffer alone to remove blood before beginning controlled pressure fixation; others were flushed with fixative by syringe without control or measurement of pressure and removed for further fixation by immersion. Additional vessel segments were removed without either prior flushing or perfusion and fixed by immersion.

After fixation, pieces of vessel wall were excised and dehydrated by passage through increasing concentrations of ethyl alcohol in steps of 10%. Alcohol was washed out by flushing 7 times over a period of 40 min with liquid CO_2 at 900 psig at 15°. After critical point drying, specimens were mounted, intimal side up, on aluminum discs, coated with gold–palladium in an Edwards vacuum evaporator and examined in a Hitachi, Model HFS2, scanning electron microscope. Similarly fixed arterial samples were post-fixed in osmium tetroxide and processed for TEM by alcohol dehydration, propylene oxide substitution and Epon embedding. We were satisfied that a vessel was fixed in a nearly physiological configuration when it did not change shape visibly as pressure was removed and when the elastin lamella nearest the lumen was not wrinkled or wavy in thick sections examined by light microscopy. Fixation with formalin solution often resulted in post-fixation recoil of the elastin; this did not occur after glutaraldehyde fixation.

RESULTS AND COMMENTS

Previously observed ovoid bulging of endothelial nuclei with alignment of their major axes in the direction of blood flow (Altschul, 1954) persisted on distended specimens; nuclei tended to be less elongated, closer together and more elevated in neonatal vessels than in those of adult animals. Intimal surface folds and ridges were, however, almost entirely absent from specimens fixed *at or above* distending pressures of 80 mmHg in adults or above normal diastolic pressures for newborn and growing animals (Fig. 1a). Coarse furrows and ridges identical to those demonstrated by others (Still and Dennison, 1974; Shimamoto *et al.*, 1971; Garbarsch and Christensen, 1970) were always present in specimens fixed at pressures below normal diastolic levels or in vessels fixed directly by immersion (Fig. 1b, c). Christensen and Garbarsch (1972) correctly interpreted the regular longitudinal furrows in their specimens of rabbit aorta to be a consequence of undulations of the underlying internal elastin lamina but did not relate this effect to inadequate distension during fixation. Although undulations of the internal elastin lamina and associated intimal folds were eliminated entirely from large elastic arteries when these were fixed at or above diastolic pressures, they were not always completely eliminated from similarly distended muscular arteries. In these vessels undulations persisted focally in relation to agonal medial contractions which were seen to occur during perfusion. Luminal ridges and folds could also be produced by gentle crimping or indentation from the adventitial side of partially or totally dehydrated arterial walls by means of forceps or the tip of a blunt probe (Fig. 1d). Critical point drying, known to cause specimen shrinkage of up to 15% (Boyd, 1971), resulted in slight curling at the edges of samples with associated longitudinal wrinkling of endothelial cell surfaces.

In vessel specimens fixed by perfusion at physiological distending pressures, edges of endothelial cells formed thin flaps extending to overlap the edges of adjacent "downstream" endothelial cells, *i.e.*, endothelial cells immediately distal with regard to the direction of blood flow. This gave the intimal surface of a normally distended artery the appearance of being shingled by its lining endothelial cells. The overlapping extensions could not be

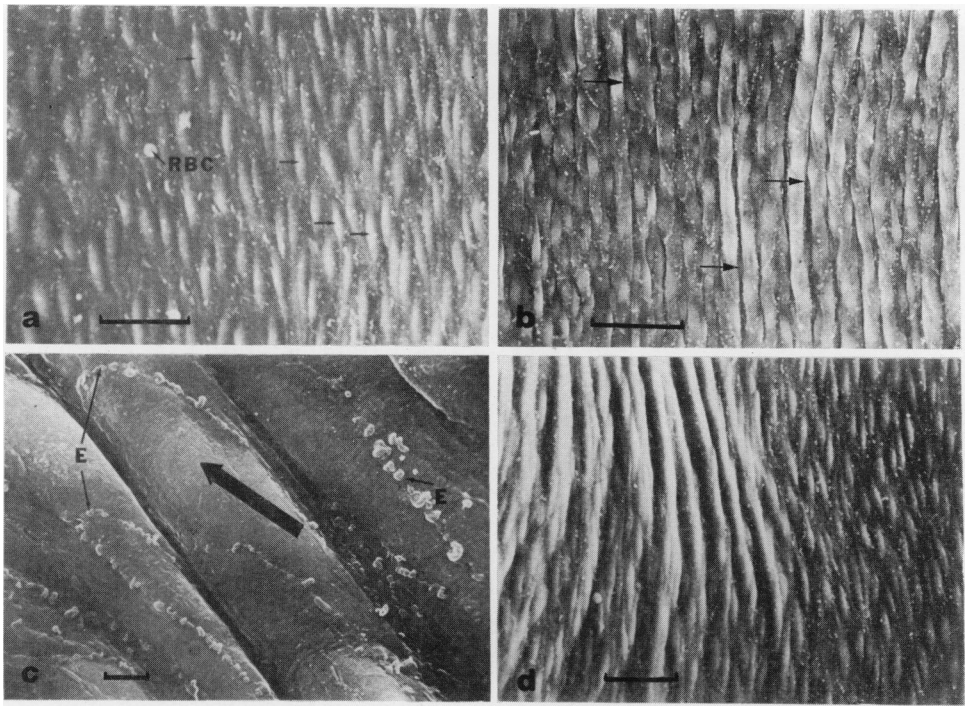

FIG. 1.—Scanning electron micrographs of the luminal surfaces of rabbit thoracic aortas fixed by controlled pressure perfusion. (a) Aorta fixed at 100 mmHg; arrows indicate raised outlines of endothelial cell nuclei bulging from an otherwise smooth and regular surface. Projection in 2 dimensions of the normal curvature of the vessel wall gives rise to the crowded appearance of the nuclei on the right side of the photograph. The fine particles are precipitated protein; occasional erythrocytes (RBC) can be seen. Scale line = 50 μm. (b) Aorta fixed at 50 mm Hg; the surface is thrown into the coarse folds which characterized specimens fixed at pressures below diastolic; arrows indicate longitudinal furrows. Nuclei are not as clearly delineated or oriented as in fully distended specimens. Scale line = 50 μm. (c) Higher magnification of aorta fixed at 50 mm Hg distending pressure; at cell edges (e), buckled membranes form villous projections not usually seen at physiological distending pressures. Scale line = 5 μm. Large arrow indicates direction of blood flow. (d) Aorta fixed at 100 mm Hg. The furrows and wrinkles to the left of this otherwise well distended specimen were caused by the forceps used to grasp the specimen during trimming. Scale line = 50 μm.

appreciated readily in adequately distended vessels, for they were closely applied to adjacent cell surfaces and did not extend as villi or ridges into the lumen if distending pressures were greater than 80 mm Hg. The overlaps could, however, be made to curl up, even in distended vessels, by protracted exposure to the electron beam (Fig. 2b). They sometimes appeared as elevated flaps on transmission electron micrographs of specimens fixed at distending pressures over 80 mm Hg but rarely projected at angles with the surface greater than 30°. No other cytoplasmic surface projections could be found on scanning or transmission electron microscopic sections of perfused material, regardless of the combinations of methods used for fixation or processing.

Endothelial detachments and displacements which could be construed as "intercellular bridges" or subcellular "tunnels" (Fig. 2a) occurred only when specimens were manipulated before perfusion, opened before fixation, allowed to collapse before fixation or inadequately cleared of volatile agents before coating. These changes were generally present in proportion to the degree and extent of

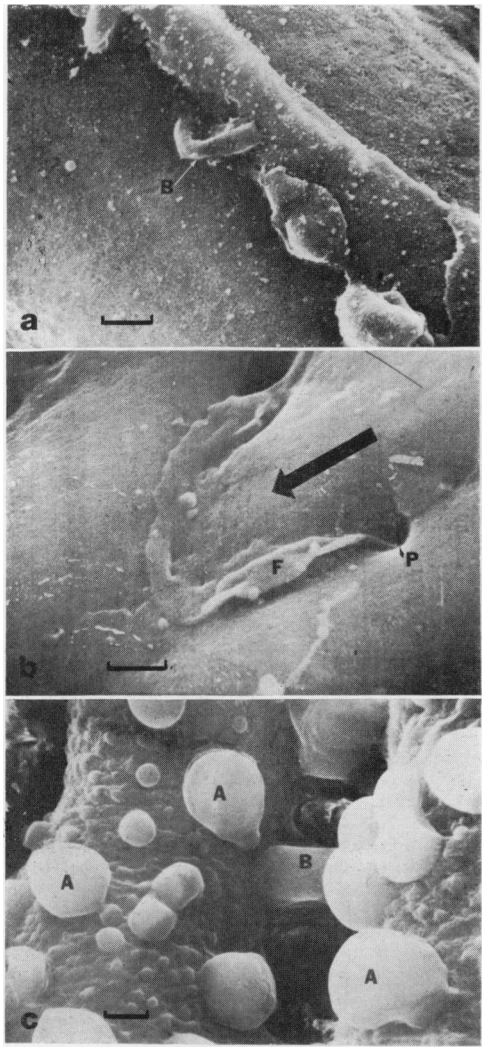

FIG. 2.—Scanning electron micrographs of endothelial cells. (a) Rabbit thoracic aorta fixed at 50 mmHg distending pressure. Loose and distorted membranes at the cell margins take a variety of forms; a small " bridge-like " detachment (B) extends between 2 cells. Scale line = 1 μm (b) Pig aorta fixed at 100 mm Hg. After 2 min of viewing, exposure to the electron beam has resulted in artefactual raising of the normally overlapping and tightly apposed endothelial marginal flap (F) and the formation of a pitted deformity (P); neither of these distortions were present during the first minute of viewing. Large arrow indicates direction of blood flow. Scale line = 2 μm. (c) Pig aorta fixed at 100 mm Hg. The various sized smooth surfaced spherical projections are artefacts which arose during the metal coating process as bubbles of volatile solvents evaporating from the specimen became coated before they could escape. The large spheres (A) may be confused with cell structures but are rare in material prepared in experienced laboratories. The small spheres are seen occasionally in almost all preparations which are coated in high vacuum and are difficult to avoid entirely. One artefactual projection has a " bridge-like " appearance (B). Scale line = 2 μm.

tissue handling before fixation. We believe that the raised cell borders noted in scanning micrographs of endothelium treated with silver nitrate (Garbarsch and Christensen, 1970), probably result to some degree from the deposition of silver beneath elevated marginal overlaps.

Knobs, villous projections or other excrescences either at endothelial cell edges or over portions of the cell surface away from intercellular junctions were rare in vessels fixed while distended at physiological pressures and infrequent in other perfusion fixed preparations *except* if the tissues were introduced into the coating chamber while still containing volatile processing reagents which had not been completely expunged during the critical point drying procedure (Fig. 2c). These spherical, bubble-like processes were similar to many of those shown in the report of Smith *et al.* (1971) and were rare when our material was adequately flushed. Furthermore, the cellular projections in the transmission electron micrographs furnished in the report of Smith *et al.* (1971) do not correspond convincingly to those on their accompanying scanning electron micrographs; the stated magnifications indicate that the projections on the scanning photographs do not have the same dimensions or frequency of occurrence as those in their transmission photographs. We therefore consider many of these and similar excrescences to be bubbles of vapour trapped and coated at the surface as they escape from the specimens during the high vacuum coating process. For other purposes we have perfused lungs by introducing glutaraldehyde under pressure into the

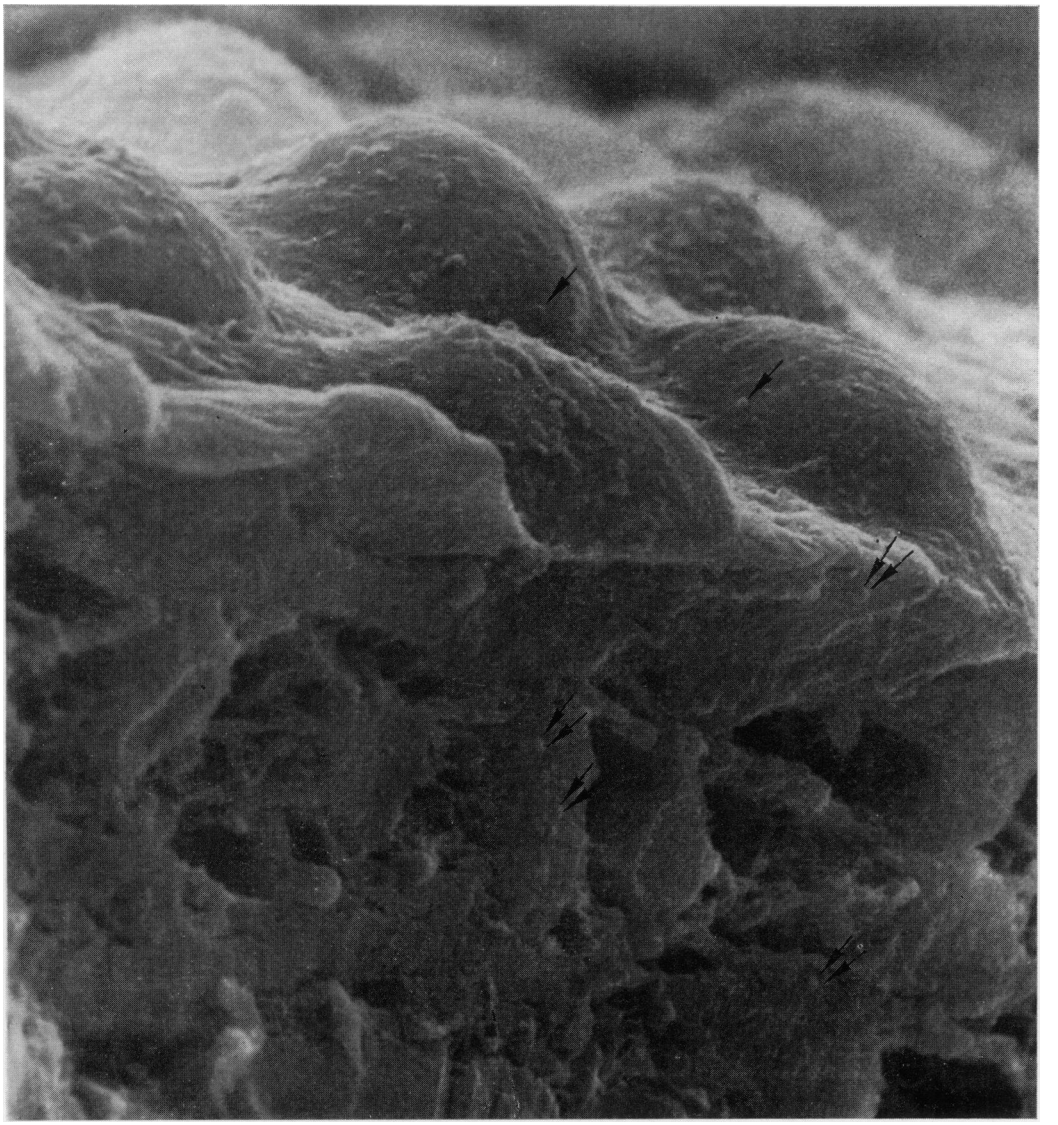

FIG. 3.—Scanning electron micrograph of the ascending aorta of a 2-week old rabbit fixed by perfusion at physiological distending pressure (60 mm Hg) for this age. Upper portion shows intimal surface with endothelial nuclei bulging into the lumen and oriented in the direction of blood flow (obliquely, to the right and toward the viewer); lower portion shows a fracture plane through the inner media. There are none of the furrows, ridges or bridges which are present in undistended material. Some of the endothelial surface projections correspond to true details of the cell surface or to circulating materials attached to the cell membrane, while many of the small luminal spherical excrescences (arrows) are identical with those on the fractured cross-section (double arrows) and must be considered to be technical artefacts of incomplete evaporation until proved otherwise by transmission electron microscopy. × 5145.

trachea and/or the pulmonary artery trunk and processed the tissue as we process arteries. These procedures always preserved the pseudopodia of Type II cells and microvilli on the cilia of bronchial epithelium, indicating that our methods of perfusion and processing do not destroy the usual minute surface structures.

CONCLUSIONS

Overall, our findings tend to support the contention that, although endothelial surface projections may occur, most of those reported in recent papers are artefacts both of inadequate distension during fixation and of inadequate tissue processing. Though these processing artefacts can be minimized, they are not completely avoidable with currently available techniques, even in experienced laboratories. It is therefore useful to compare endothelial surface details with those seen on cross sectional edges of the same specimen (Fig. 3). Comparison of excrescences in both locations will often help to distinguish artefacts from real structural features.

It should also be noted that the surface morphology of living endothelial cells is influenced by circulating levels of endogenous amines (Majno, Shea and Leventhal, 1969) and by metabolic conditions (Ashton and Pedler, 1962). Although we have not addressed ourselves to these factors in the present report, it is evident that these possibilities must also be considered in interpreting endothelial surface configurations even if the vessels have been adequately fixed and prepared.

Previous light and transmission electron microscopic studies in our laboratory indicated that some details of arterial medial architecture seen in cytologically well-fixed but undistended specimens were incorrectly considered to be normal; controlled pressure fixation preserved more nearly physiological relationships and revealed new and significant information concerning the relation of structure to function (Wolinsky and Glagov, 1964). Increasingly accurate data with regard to the configuration of arterial endothelial surfaces may be expected to accrue with the extended application of SEM, for this technique permits direct examination of intact, large surfaces at high resolution. Previous impressions gained from low resolution, *en face* examinations of Hautschen preparations by light microscopy (Florey, Poole and Meed, 1959), and from reconstructions based on high resolution TEM examinations of small cross-sectional areas, are likely to require revision. Current studies utilizing SEM should therefore attempt to minimize departures from *in vivo* haemodynamic conditions during fixation and seek to identify distortions introduced by drying, coating and examining procedures. Our findings emphasize that such precautions are particularly important during the current period of methodological development in order to help prevent futile debates concerning the physiological and pathological significance of technical artefacts.

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REFERENCES

ALTSCHUL, R. (1954) *Endothelium*. New York: The MacMillan Co.

ASHTON, N. & PEDLER, C. (1962) Studies on Developing Retinal Vessels. IX. Reaction of Endothelial Cells to Oxygen. *Br. J. Ophthal.*, **46**, 5.

BOYD, A. (1971) A Review of Problems of Interpretation of the Scanning Electron Microscope Image with Special Regard to Methods of Specimen Preparation. In *Scanning Electron Microscopy*. Ed. O. Johari and I. Corvin. Chicago: IIT Research Institute.

CHRISTENSEN, B. C. & GARBARSCH, C. (1972) A Scanning Electron Microscopic (SEM) Study on the Endothelium of the Normal Rabbit Aorta. *Angiologica*, **9**, 15.

FLOREY, H. W., POOLE, J. C. F. & MEED, G. A. (1959) Endothelial Cells and "Cement" Lines. *J. Path. Bact.*, **77**, 2.

GARBARSCH, C. & CHRISTENSEN, B. C. (1970) Scanning Electron Microscopy of Aortic Endothelial Cell Boundaries After Staining with Silver Nitrate. *Angiologica*, **7**, 365.

MAJNO, G., SHEA, S. M. & LEVENTHAL, M. (1969) Endothelial Contraction Induced by Histamine-type Mediators. *J. Cell Biol.*, **42**, 3.

SHIMAMOTO, T., YAMASHITA, Y., NUMANO, F. & SUNAGA, T. (1971) Scanning and Transmission Electron Microscopic Observation of Endothelial Cells in the Normal Condition and in Initial Stages of Atherosclerosis. *Acta path. jap.*, **21**, 1.

SMITH, U., RYAN, J. W., MICHIE, D. D. & SMITH, D. S. (1971) Endothelial Projections as Revealed by Scanning Electron Microscopy. *Science, N.Y.*, **173**, 925.

STILL, W. J. S. & DENNISON, S. (1974) The Arterial Endothelium of the Hypertensive Rat. *Archs Path.*, **97**, 337.

WOLINSKY, H. (1972) Endothelial Projections. *Science, N.Y.*, **176**, 1151.

WOLINSKY, H. & GLAGOV, S. (1964) Structural Basis for the Static Mechanical Properties of the Aortic Media. *Circ. Res.*, **14**, 400.