Endothelial Changes Produced by Ascorbic Acid Deficiency in Guinea Pigs

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CAPILLARY hemorrhage, a manifestation of fragility, is an important clinical feature of scurvy.[1] To account for it, investigators, variously, have inferred alterations in the vascular endothelium, the intercellular cement, or the supporting tissues of the vessel wall.[2] Still, clearly defined morphological changes of the capillary walls have not been reported in ascorbic acid deficiency. On the premise that these might involve the endothelium and might be better visualized in en face preparations[3] than in routine histological sections, the aorta of scorbutic guinea pigs was examined, essentially because the "haütchen" technique was not applicable to microchannels. It seemed reasonable to assume, however, that any change in capillary endothelium produced by a systemic disturbance would be shared by the aortic lining as well. By electron microscopy, Buck has observed that "endothelial cells of capillaries seem to possess no structure not shared by those of arteries."[4] As an index of functional endothelial alterations as well, Todd's procedure[5] for evaluating fibrinolytic activity was used. Finally, since ordinary histology had been so unrevealing, these observations on aortic endothelium were supplemented by electron microscopy.

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Materials and Methods

Using young adult guinea pigs weighing about 400 gm each, scurvy was induced in 13 animals by feeding a diet of autoclaved Purina rabbit chow for periods of one to four weeks. A supplement of vitamin A and D was added twice weekly. A second group of 12 animals was fed a commercial scorbutigenic diet (Nutritional Biochemical Corp.) for three weeks. Four of the animals in the latter group were maintained on the deficient diet for six weeks, but during the third and fourth weeks, liberal quantities of vitamin C were given as a supplement. All animals were weighed twice weekly.

Four of 12 control guinea pigs were fed Purina rabbit chow supplemented with fresh vegetables twice weekly; the remaining eight received the commercial scorbutigenic diet to which a vitamin fortification mixture had been added. Prior to sacrifice by ether inhalation, all animals were heparinized (100 units per kg, I.V.). Blood ascorbic acid levels were measured by titration with the sodium salt 2,6-dichlorophenol-indophenol.[6] Silver staining of the cement lines in the aortic endothelium and fixation in situ[3] were accomplished by perfusion through tubes in the left atrium and the abdominal aorta. En face or "hautchen" preparations of endothelium were prepared from the arch, thoracic, and abdominal segments of the opened aorta. The stains used were: hematoxylin and eosin, periodic acid-Schiff (PAS), and toluidine blue at a pH of 2.5. To ascertain the nature and frequency of cytologic abnormalities, 1,000 cells were examined from widely dispersed, randomly located fields in each slide. For the assay of fibrinolytic activity, endo-

TABLE 1.—*Endothelial Changes in Ascorbic Acid Deficiency*

	Guinea Pigs	Ascorbic Acid Level mg/100 cc	Cement Line Stainability *			Nuclear Abnormalities		
			Arch	Thor	Abd	Pyknosis	Vacuoles	Enlargmt
Control 3 wk	8	0.66	2.0	2.5	2.5	0.16	0.46	0.14
Scurvy 3 wk	4	0.2	1.4	1.3	1.3	0.16	3.11	0.49
4 wk	4	0.2	2.0	1.1	1.1	0.34	2.67	0.75
Chronic scurvy †	4	0.2	0.5	1.3	1.8	0.35	1.07	0.21

* Scored from 1 to 3 plus; a value of 0.5 was assigned to very faint lines.

† Scorbutigenic diet for six weeks interrupted during the third and fourth weeks by the addition of ascorbic acid to the deficient diet.

thelium of fresh aortic segments was isolated by freezing to the surface of a glass slide and removing the external layers. The resultant strips of endothelium were then overlaid with fibrin, formed by covering with a solution of fibrinogen promptly after the addition of thrombin, and incubated in a moist chamber at 37° for four hours. They were then fixed in formaldehyde vapor for one minute, formol saline for one hour, stained with hematoxylin and eosin, and mounted in glycerin jelly.

Tissues for electron microscopy were removed under ether anesthesia, fixed immediately in cold buffered isotonic 1% osmium tetroxide,[7] processed through graded alcohols, and embedded in Epon. Ultra-thin sections were cut on a Porter-Blum microtome, stained with lead and examined with an electron microscope, RCA EMU-3G.

Results

Starting during the third week of a continuous scorbutic diet, progressive weight loss was observed. Blood levels of ascorbic acid which normally exceeded 0.6 mg/100 cc, dropped below 0.2 mg by the fourth week of the deficient diet. Concurrently, hemorrhages were observed regularly in the periarticular tissues of the knee and occasionally in the intestinal mucosa. There were no abnormalities of platelets, fibrinogen, prothrombin or thromboplastin generation.

As observed in the en face preparations of aortic endothelium (Table 1), the uniformly intense silver staining of the cement lines of the control animals was greatly diminished in scorbutic guinea pigs (Fig 1).

It was noted that a small proportion of endothelial cells in normal animals, and a somewhat greater number in C-deficient animals, displayed swollen nuclei more than twice normal size. Occasionally, such giant nuclei were distorted and misshapen, but mitoses were not observed. Intranuclear vacuoles were several times as frequent in scorbutic animals as in controls. At times, the vacuoles, which stained neither for fat, glycogen, nor acid mucopolysaccharides, communicated with the cytoplasm leaving a pyknotic nucleus. Cytoplasmic vacuolization was observed to about the same degree in

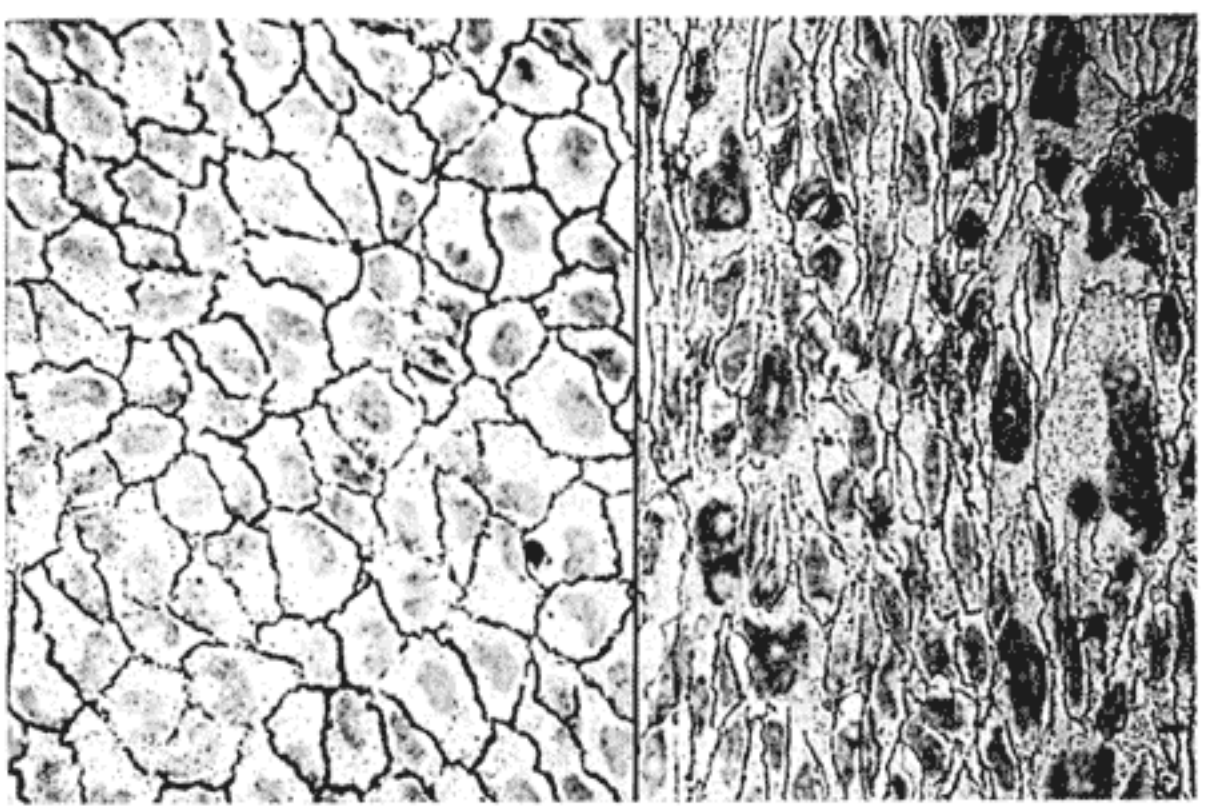

Fig 1.—En face preparations of normal and scorbutic guinea pig aortic endothelium. Observe the uniformly intense silver staining junction lines in the normal on the left. By contrast, the cell junctions stain faintly and irregularly in scurvy. Note also the variability of cell size, giant nuclei, and nuclear vacuoles in the deficient endothelium.

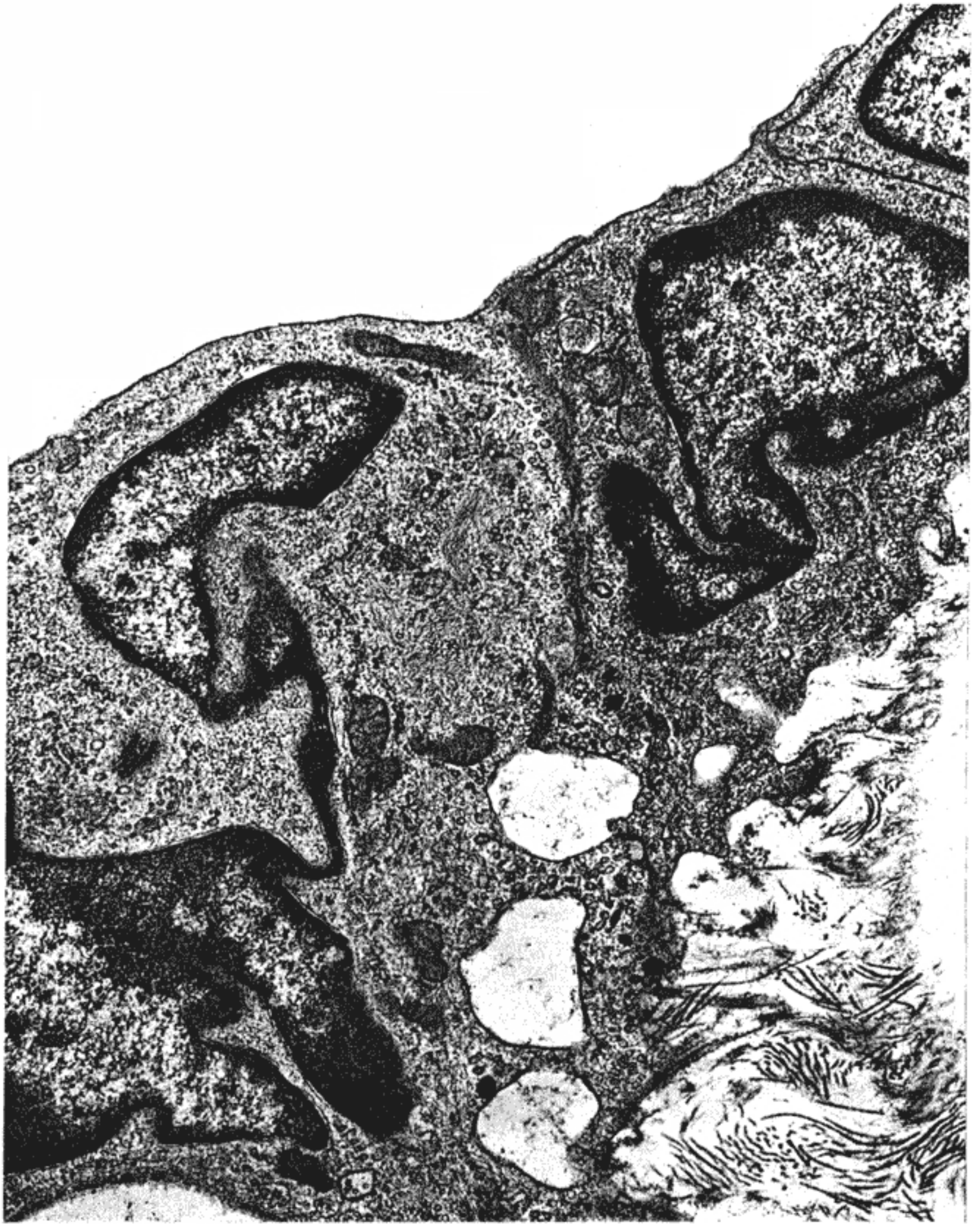

Fig 2.—Electron micrograph of normal guinea pig aortic endothelium. Observe the narrow intercellular junction at the upper right, the finger-like overlapping cell process, and the meshwork of collagen fibers between the endothelium and the unstained internal elastica at the right (× 8,000, reduced about 14%).

TABLE 2.—*Fibrinolytic Activity* of Aortic Endothelium in Scurvy*

			Thoracic			Abdominal		
	Animals	Arch	Prox	Mid	Dist	Prox	Mid	Dist
Control	10	0.4	0.5	1.2	1.5	1.8	1.9	1.9
Scorbutic diet 3 wks	10	0.6	0.7	0.9	1.6	2.0	1.7	1.7

* Graded from 0 to 3 plus; 0.5 representing minimal, and 3 representing prominent and diffuse lysis.

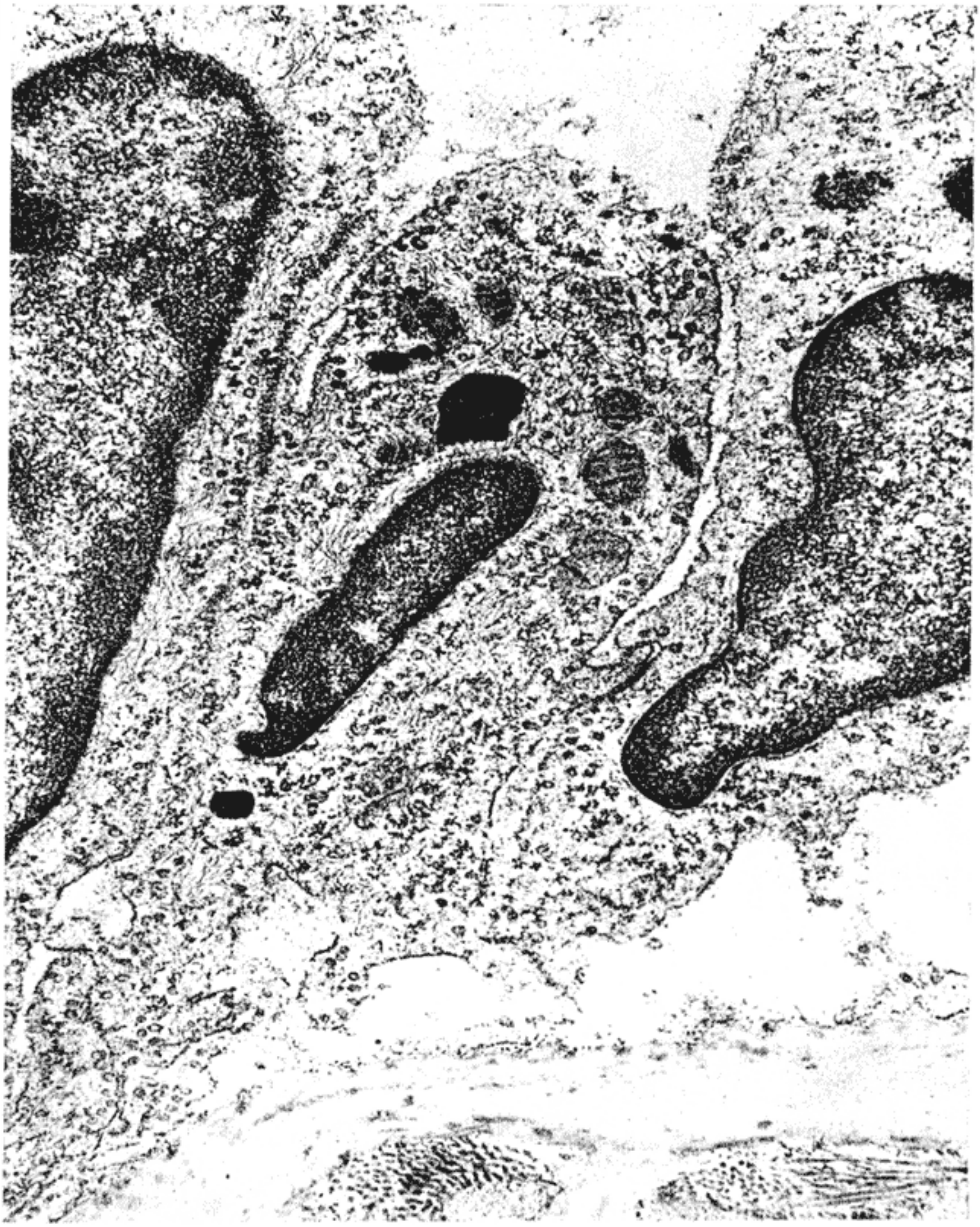

Fig 3.—Electron micrograph of scorbutic guinea pig aortic endothelium. In comparison with Fig 2, note the widened intercellular junctions, the depletion of subendothelial collagen, and the reduction in cytoplasmic organelles (× 12,000, reduced about 19%).

control and deficient animals. Small platelet thrombi occurred haphazardly on the endothelial surface of both control and scorbutic animals; in most instances, the underlying endothelium was intact.

The fibrinolytic activity of aortic endothelium was unaffected by ascorbic acid deficiency. Curiously, in both control and scorbutic pigs, fibrinolytic activity was greater in the abdominal aorta than it was in the thoracic portion (Table 2).

Examined with the electron microscope, the normal aortic endothelium of the guinea pig was characterized by a well-developed

Golgi apparatus and a modest endowment of mitochondria and rough endoplasmic reticulum. Numerous small membrane-limited vesicles were observed in the cytoplasm along the cell margins. Occasionally the cytoplasm also contained clusters of fine filaments. Adjoining endothelial cells were closely apposed; often a short finger-like process originating from the margin of one cell covered the junction and part of the surface of its neighbor. There was not a distinct basement membrane. A rather wide space separating the endothelium from the internal elastic lamina contained small numbers of collagen fibers (Fig 2). In the scorbutic animal, endothelial cells displayed decreased numbers of ribosomes, both free and attached. Nuclear deoxyribonucleic acid (DNA) was visibly reduced in some cells. Loosening and occasional gapping of the intercellular junctions could be seen as well as a distinct depletion of collagen between the endothelium and the internal elastic lamina (Fig 3).

Comment

On the basis that swollen, distorted, or vacuolated nuclei in scorbutic endothelial cells indicate injury, it seems reasonable to suggest that there is an associated reduction of those functions which, normally, provide the silver staining material in the intercellular junctions. The actual junction space as revealed by electron microscopy is widened, and there is also depletion of subendothelial collagen fibers. Since subendothelial collagen is sparse and inconstant in the capillary [8] channels from which scorbutic bleeding originates, endothelial cell disjunction must be the essential structural basis for the occurrence of hemorrhage in scurvy. It is possible, though, that loss of perivascular collagen may contribute to dilatation (and increased porosity) as Lee [9] had observed. Other studies have shown that permeability of microscopic channels to colloidal particles induced by serotonin or histamine,[10] and the emigration of leukocytes and erythrocytes from capillaries in inflammation [11] also result from widening of the intercellular junctions. In scurvy, the attenuation of cytoplasmic organelles suggests that loss of cohesiveness could be a consequence of reduced metabolic capacity of the cell. The prompt and reversible production of the same change by pharmacologic stimuli or by inflammation clearly must have some other explanation.

The endothelial cell junctions and the material within it have been considered the anatomic route of physiological permeability.[12] Its dimensions, estimated by light microscopy, however, are greatly exaggerated. Electron micrographs have shown that cement lines are actually only optical projections of very narrow oblique intercellular spaces.[3] In scurvy, loss of the normal argyrophilia clearly indicates no relation between this stain and the size of the intercellular space. The nature of the contents of the junctions has not been established, though it has been considered either to be acid mucopolysaccharides,[13,14] or a substance that has affinity for them.[15]

The nature of the biochemical lesion in scorbutic endothelium remains to be determined. The ultrastructural changes in them are similar to those described in ascorbic acid deficient fibroblasts (dilated cisternae of the endoplasmic reticulum, depletion of membrane attached ribosomes, and relative increase of free ribosomes)[16-18] for which much more information is available. The failure of wound healing in scurvy results from a lack of collagen formation.[19,20] Despite hypertrophy and hyperplasia, ascorbic acid deficient fibroblasts fail to hydroxylate proline and lysine to form constituents essential to the structure of collagen.[21]

Summary

With scurvy in guinea pigs, the aortic endothelium, examined with en face preparations, displays reduced silver stainability of the cement lines and an increased incidence of such nuclear abnormalities as vacuolization, swelling, distortion, and pyknosis.

The deficient diet is not associated with a change in the fibrinolytic activity of the

endothelium. This displayed a gradient which increased from the arch to the abdominal segment of the aorta.

Electron microscopy displayed separation of endothelial cells in scurvy as the presumptive cause of leakage. There is also a depletion of subendothelial collagen and a reduction of cytoplasmic organelles.

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