Virchows Arch. Abt. A Path. Anat. 361, 263—282 (1973)

Cortisone Influences Developing Collaterals

1. A Morphologic Study

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Received December 4, 1972

Summary. Light microscopic and ultrastructural changes in developing coronary collateral vessels were investigated after treatment with hydrocortisone (20 mg/kg/day orally) in the dog. The expected inflammatory reaction in the early stage of development (3 weeks) was absent, signs of injury to the vascular wall were lacking. At 3 and 8 weeks proliferation of rough and smooth endoplasmic reticulum and of the Golgi field was prominent indicating changes in the metabolism of the cells. The amount of extracellular MPS, collagen and new elastic material was decreased. Intimal hyperplasia occurred at the same rate in the treated as in the untreated animals. At 3 months the vascular architecture was restored, cellular hyperactivity had decreased.

It is concluded from these results that:

1. Only collaterals in the acute stage of growth are influenced by treatment with hydrocortisone.

2. When collateral growth had come to a standstill, an influence of hydrocortisone was no longer demonstrable.

3. Normal non-growing coronary arteries in normal hearts and in those with coronary occlusion are not influenced by hydrocortisone.

Introduction

In the canine heart the transformation of small pre-existing arteriolar collateral vessels into larger arteries is characterized by 2 successive phases:

1) By injury of the vascular wall, accompanied by an inflammatory reaction.

2) By regeneration and proliferation of the vascular wall, i.e. by repair processes.

Hydrocortisone (HC), among other glucocorticoids, is known to have an influence on both inflammation and regeneration of tissues (Mills, 1965; Sarett and Patchett, 1963; Schlagel, 1965; Whipple, 1967). This study describes the specific effects of hydrocortisone on the structure of developing coronary collateral vessels at the light microscopical and ultrastructural level. A few remarks concerning functional data will be mentioned, because they may add to the understanding of the morphological changes.

Material and Methods

Twenty one pure bred beagle dogs of either sex with an average body weight of 14 kg were used in this study.

They were subdivided in several groups as shown in Table 1.

In order to achieve stenosis and occlusion of one major coronary artery, in 17 dogs an ameroid constrictor (Litvak *et al.*, 1957) was placed around the circumflex branch of the left coronary artery (Schaper, 1967). Four dogs without constrictor served as controls, one for each experimental group. In the 4-weeks group which was investigated first, the dosage of 10 mg/kg/day orally apparently was not effective: The vessels of this group were identical at the light microscopic as well as at the ultrastructural level with those of the untreated 3-weeks group. Therefore the dosage of hydrocortisone was increased in all other groups to 20 mg/kg/day orally. Treatment was started 5 days after the operation in order not to disturb wound healing. After various time intervals (3 weeks, 4 weeks, 8 weeks and 3 months after the operation), the animals were sacrificed and the hearts were prepared for light and electron microscopy by perfusion fixation in the anesthetized dog with open thorax. Via one carotid artery a catheter was placed in the thoracic aorta and after clamping of the aorta distal to the catheter and of the brachiocephalic truncus, perfusion was initiated. The right auricular appendage was opened for removal of blood and perfusion fluid. After perfusion of the whole coronary system with oxygenated tyrode solution, the still beating heart was perfused with a 2% solution of freshly distilled glutaraldehyde (Fahimi and Drochmans, 1965) at a pressure of 100 mm Hg.

Perfusion time was about 3–5 minutes during which the heart stopped beating and gained a yellow-brownish colour. The heart was then removed from the chest cavity and small pieces of epicardial collaterals and normal blood vessels were cut out and put immediately in cold 3% cacodylate buffered glutaraldehyde. In the dog heart, the collateral vessels are situated on the epicardial surface, they are clearly visible and their course can be followed over the entire length. The stem is that part of the collateral at its origin, the reentry connects with the recipient vessel, the midzone is located in between reentry and stem. This terminology corresponds to Longland's (1953) definition.

The tissue samples were fixed by immersion for 2 hours, then rinsed for 2 hours in cacodylate buffer and thereafter postfixed in OsO_4, dehydrated in graded series of ethanol, treated with propylenoxide and embedded in epon.

For light microscopy, sections 1 or 2 μm thick were made from the epon blocks and stained with a trichrome stain (Sevier and Munger, 1968). Thin sections were cut on a LKB III ultrotome, mounted on uncoated copper grids, stained with uranyl acetate and lead citrate, and viewed in a Siemens Elmiskop IA or a Hitachi HS 8-1 electron microscope.

Results

I. Functional Aspects

Since the major aim of this publication is the detailed description of the influence of hydrocortisone on the morphology of growing collateral blood vessels, observations regarding functional aspects will only be briefly summarized.

All dogs appeared to be healthy and showed normal behavior. The large thoracotomy incisions healed without complications, and they were hardly visible in the 3-month dogs. The peripheral coronary pressure (= PCP, measured distally of the constrictor), which is known to be a good indicator of collateral enlargement (Schaper, 1967), was determined in all animals. In the hydrocortisone treated group without constrictor PCP was very low because, in the absence of a stenosis or occlusion of a large coronary artery, no collateral vessels had developed. There was no significant difference with respect to PCP's between the 2 groups with coronary constrictors, regardless of treatment. From this and from other pathophysiological observations (such as rate of occurrence of infarction and mortality rate) we concluded that, functionally, treatment with hydrocortisone had not influenced the development of coronary collaterals.

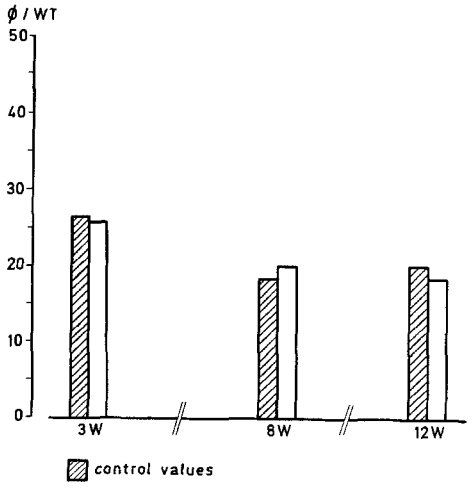
Table 1. Control and experimental groups

Time after operation	Animals *with* coronary constrictor	Animals *without* coronary constrictor	Dose and duration of treatment
3 weeks	3	1	20 mg/kg/day hydrocortisone orally for 16 days
	2		without treatment
4 weeks	2	1	10 mg/kg/day hydrocortisone orally for 23 days
	2		without treatment
8 weeks	4	1	20 mg/kg/day hydrocortisone orally for 37 days
	3		without treatment
12 weeks	3	1	20 mg/kg/day hydrocortisone orally for 85 days
	2		without treatment

II. Light Microscopy

The most characteristic alterations occur in the so-called "midzone" (Longland, 1953) of the collateral vessel. This is the middle part of the growing arteriole which has the smallest diameter and the thinnest wall at the onset of development. Stem and reentry, on the other hand, show alterations of varying degrees. Light microscopic observations in this study served for measurements and calculations, whereas cellular details were studied intensively at the ultrastructural level (section III).

In the population which had no constrictor but received treatment with hydrocortisone only few collateral vessels were present. The macroscopic picture was not different from that of a normal heart possessing some preexisting small collateral connections. In the light microscope these arterioles were completely normal. Therefore this group was omitted from all statistical calculations. In the following chapters the term control group is used exclusively for the untreated constrictor groups. In order to evaluate quantitatively vascular development modified by hydrocortisone treatment, wall thickness (intima plus media) and internal diameter of the vessels of all groups were measured. The graph represents the values obtained for both groups. It is evident from these data that there is no quantitative difference between the control group and the HC-treated group. The frequency of occurrence of normal vascular segments, of injury of the vessel wall, diapedesis, disturbances of the internal elastic membrane, and of the presence of a zone of subintimal proliferation was determined and evaluated statistically for all vessels from control and treated dogs. Table 2 shows the results



Diameter to Wall Thickness Ratios of Collaterals. There is no statistically significant difference of diameter/wall thickness ratio between control and HC-treated groups. ø/WT is significantly higher for both groups at 3 weeks than at 8 or 12 weeks

Table 2

	I	IIa	IIb	IIc	IIIa	IIIb	IIIc	IVa	IVb	IVc	V	*n* total
A	41%	42%	53%	5%	29%	22%	49%	0%	31%	69%	0%	59
B	61%	78%	17%	4%	0%	4%	0%	8%	22%	70%	13%	23
C	41%	39%	51%	13%	25%	25%	53%	2%	27%	71%	4%	51
D	46%	52%	39%	9%	34%	14%	55%	0%	15%	85%	0%	56
E	2%	15%	73%	12%	24%	20%	54%	12%	66%	22%	39%	41
F	18%	27%	47%	25%	33%	31%	27%	11%	24%	65%	27%	51
G	52%	54%	33%	13%	35%	12%	53%	0%	8%	92%	0%	47

A — 3 weeks HC 20 mg/kg/day
B — 4 weeks HC 10 mg/kg/day
C — 8 weeks HC 20 mg/kg/day
D — 3 months HC 20 mg/kg/day
} with constrictor

E — 3 weeks without HC
F — 8 weeks without HC
G — 3 months without HC
} with constrictor

I number of normal vessels
II IEL, normal, interrupted, absent
III subintimal proliferation, concentric, focal, absent
IV injury, strong, moderate, absent
V diapedesis

Levels of significance (x^2)
A I — E I $p<0.01$
C I — F I $p<0.05$

A II — B II $p<0.05$
A II — E II $p<0.01$
A IV — E IV $p<0.01$
A V — E V $p<0.01$
C V — E V $p<0.01$

Since II, III and IV are composed of several subgroups, the X^2-test only allows the statement: The changes of II as a whole in group A are significantly different from those in group B.
The same holds for III and IV.

of these calculations. The main effect of hydrocortisone is the inhibition of inflammation at 3 weeks after operation which is a common feature in the control group. This is evidenced by the absence of diapedesis at 3 weeks-HC and at 8 weeks-HC (Table 2) and by the diminished number of injured vascular segments (Table 2) or, vice versa, the increased frequency of normal vascular segments at 3 weeks-HC (Table 2) and 8 weeks-HC. The occurrence of more normal elastic membranes at 3 weeks-HC is another indication of the absence of injury usually due to inflammation.

There was no statistically significant difference between the number of subintimal proliferations at 8 weeks in the control and in the HC-treated group.

III. Ultrastructure

The vessels of the group without constrictor but treated with hydrocortisone appeared normal at the subcellular level.

Since the normal pattern of vascular growth with its ultrastructural details has already been described (Schaper, 1972) only observations will be mentioned which are specifically due to HC-treatment.

1. 3 Weeks-Group

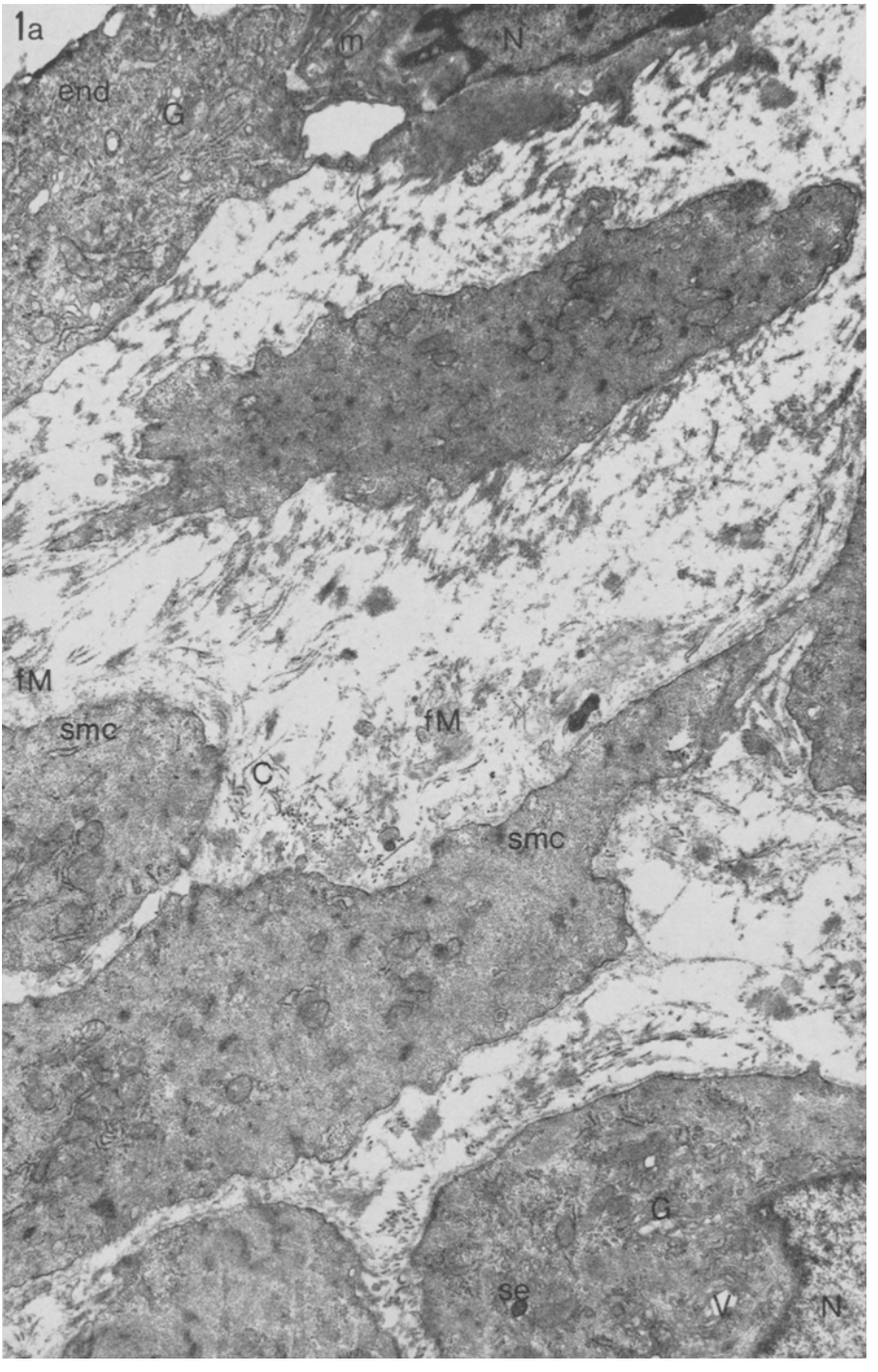
a) Endothelium. The cytoplasm is characterized by an increased amount of rough and smooth endoplasmic reticulum, enlarged Golgi apparatus and many free ribosomes. The cells often contain an increased number of mitochondria, a few multivesicular bodies and lysosomes. Frequently the basement membrane was absent.

b) Subintimal Layer. Under the endothelium mainly smooth muscle cells and some fibroblast-like cells were observed. Many of the cells contained an increased amount of cell organelles, especially of smooth and rough surfaced endoplasmic reticulum, free ribosomes and a large Golgi field. In these cells, dense bodies and many vacuoles containing membranous or granular material were found (Fig. 1).

As "fibroblast-like" we define cells completely lacking contractile filaments, pinocytotic vesicles and a basement membrane and showing a nucleus with very dense chromatin. These cells also exhibit abundant rough and smooth endoplasmic reticulum and an extensive Golgi field. They show many vacuoles which are empty or filled with membranous material (Fig. 2).

c) Medial Cells. Many of the medial smooth muscle cells showed only irregularities in shape, size or position whereas the cytoplasm contained mostly contractile filaments. Other smooth muscle cells contained an abundance of rough and smooth endoplasmic reticulum and free ribosomes, a very prominent Golgi complex and phagocytic vacuoles.

d) Adventitial Cells. The fibroblasts are long with slender processes and contain an increased amount of rough and smooth ER and a very pronounced Golgi apparatus. Intact lysosomes as well as phagocytic vacuoles, dense bodies, or empty vacuoles are present (Fig. 3).



Figs. 1—3 are taken from 3 week-HC-samples

Fig. 1a. Endothelium (*end*) and subintimal smooth muscle cells (*smc*). Endothelial cell and *smc* in lower right show proliferation of cell organelles, mainly of the Golgi apparatus and smooth elements (*se*). *r* free ribosomes, *pv* pinocytic vesicles, *m* mitochondria, *N* nucleus. Fibrillar material (*fM*) in the extracellular space. Magnification 10600

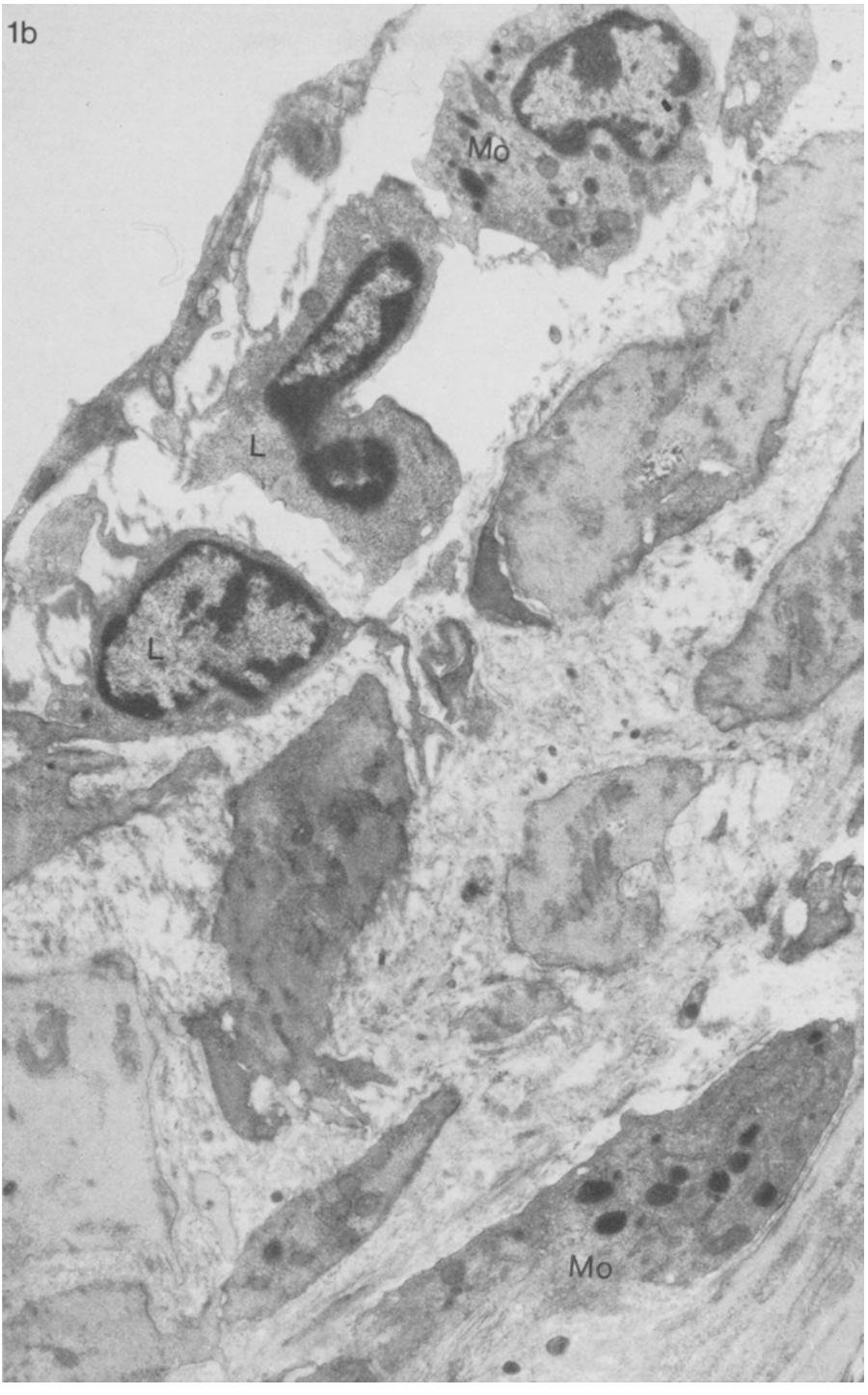


Fig. 1b. Illustration of the typical inflammatory reaction as observed in 3 weeks without treatment with hydrocortisone. *L* immigrated lymphocytes, *Mo* immigrated monocytes. Note the extracellular edema. Magnification 7945

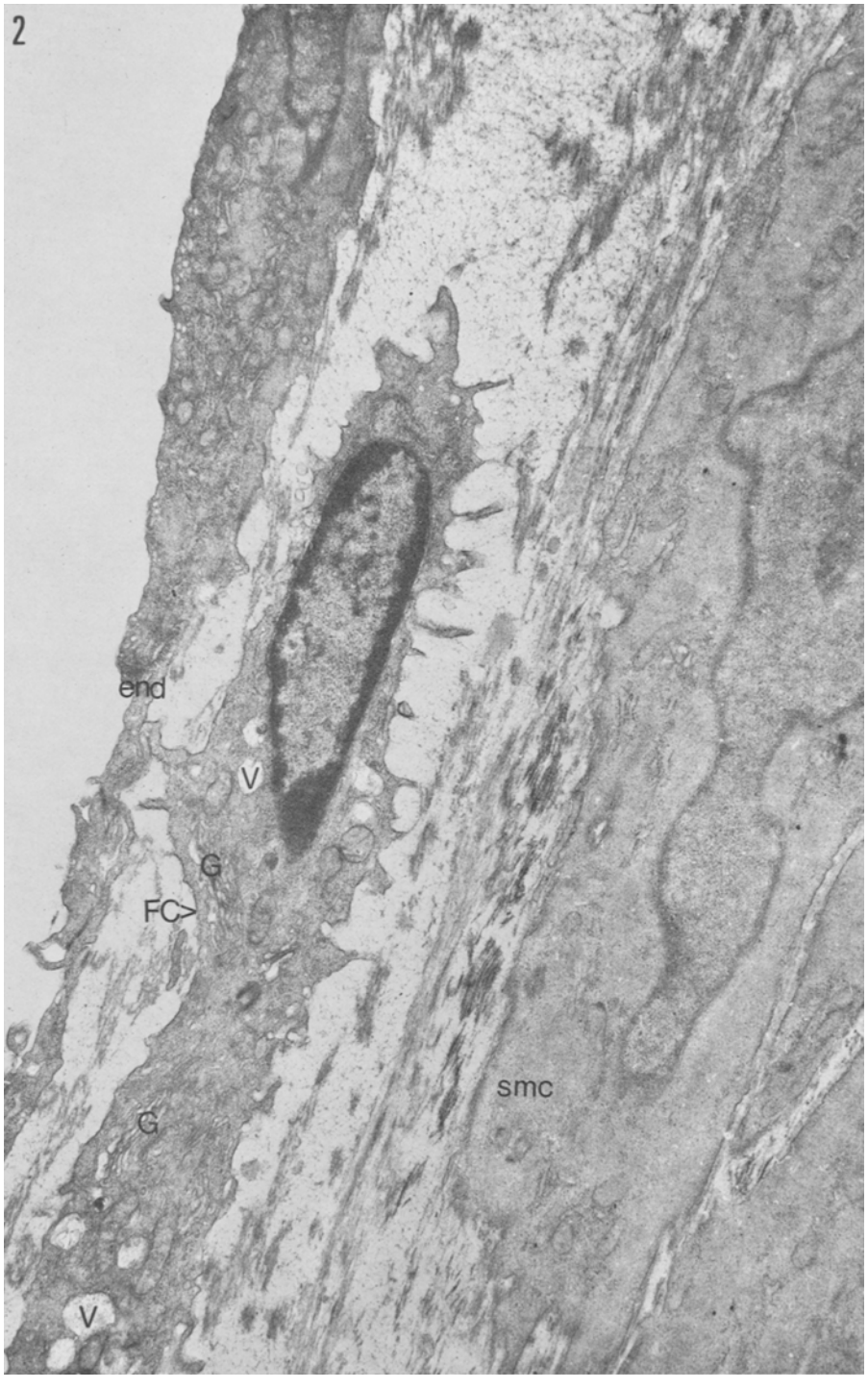


Fig. 2. Shows a fibroblast-like cell (*FC*) between endothelium (*end*) and medial smooth muscle cells (*smc*). Notice the large Golgi field (*G*) of this cell and the numerous vacuoles. Magnification 10600

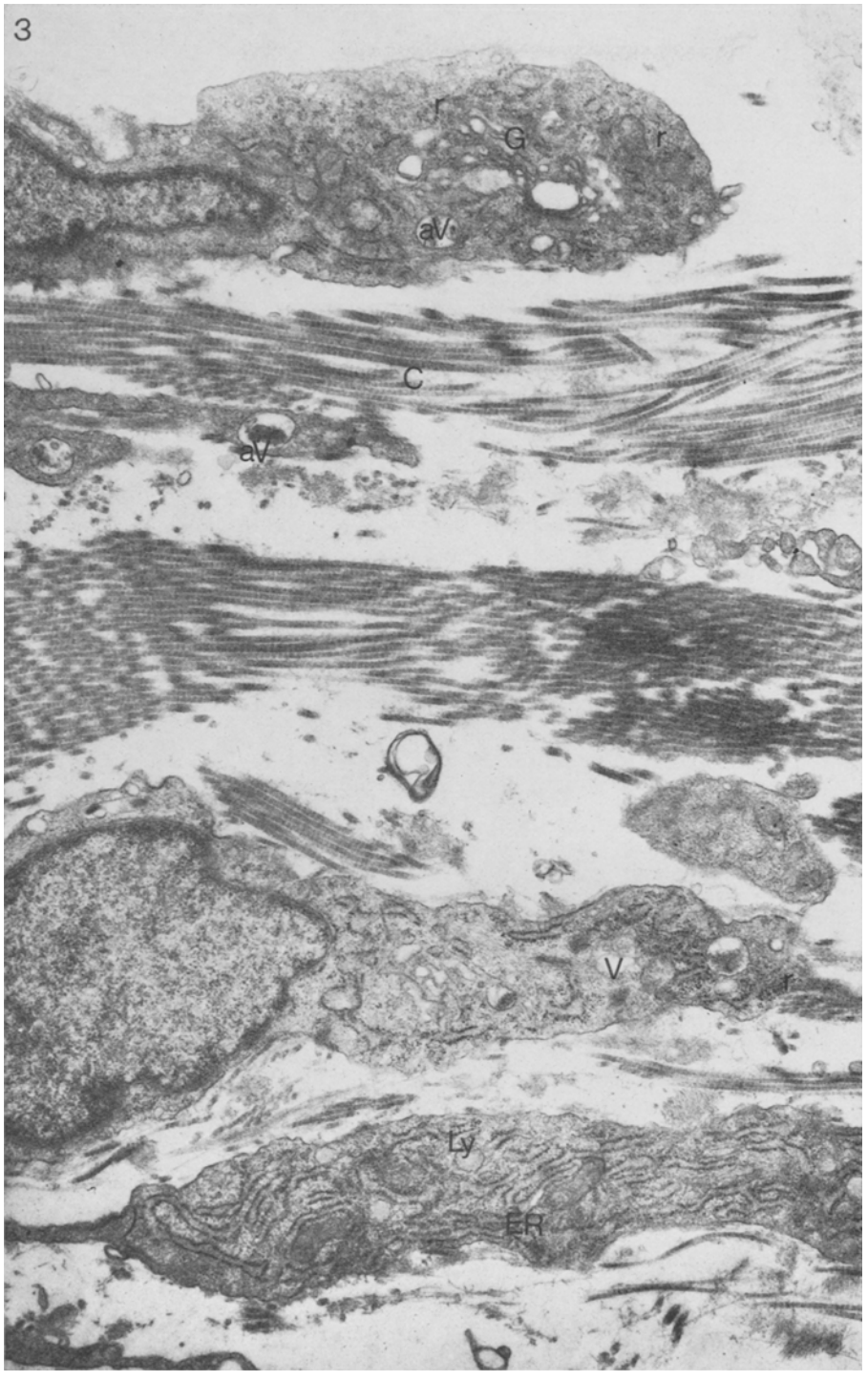


Fig. 3. Adventitial fibroblasts. Prominent Golgi field (*G*), many lysosomes (*Ly*) and vacuoles (*V*) empty or filled with granular material, *aV* autophagic vacuoles. *ER* rough endoplasmic reticulum, *r* free ribosomes, *C* collagen fibers. Magnification 12250

e) Extracellular Space. Frequently the original internal elastic membrane had almost completely disappeared and there was only very little new elastic material present. Ground substance, collagen fibrils and basement membranes were reduced in amount and the empty spaces were filled with fine reticular fibrils (Figs. 1 and 2).

Summarizing, it can be said that mainly the endothelial cells, the smooth muscle cells of the subintimal thickening and the adventitial fibroblasts showed signs of increased activity, whereas the medial smooth muscle cells remained rather unchanged. All components of the extracellular space were reduced.

2. 8 Weeks-Group

a) Endothelium. The endothelial cells contain an increased amount of smooth endoplasmic reticulum, a large Golgi field and many mitochondria. Dense osmiophilic bodies and vacuoles are present (Fig. 4a).

b) Subintimal Layer. The smooth muscle cells of this layer show an increased amount of smooth endoplasmic reticulum and a large dilated Golgi field (Fig. 5). Occasionally fibroblast-like cells occur.

c) Medial Cells. The media consists of regularly arranged smooth muscle cells of normal appearance except for the occurrence of glycogen, which was never observed in smooth muscle cells of normal canine vessels (Figs. 6 and 9). Some cells show increased excretion of myelin membranes (Figs. 5 and 9).

d) Adventitia. The adventitia shows collagen bundles intermingled with elastic material.

The fibroblasts exhibit an extended rough endoplasmic reticulum and numerous smooth structures. They show a high phagocytic activity (Figs. 7 and 8).

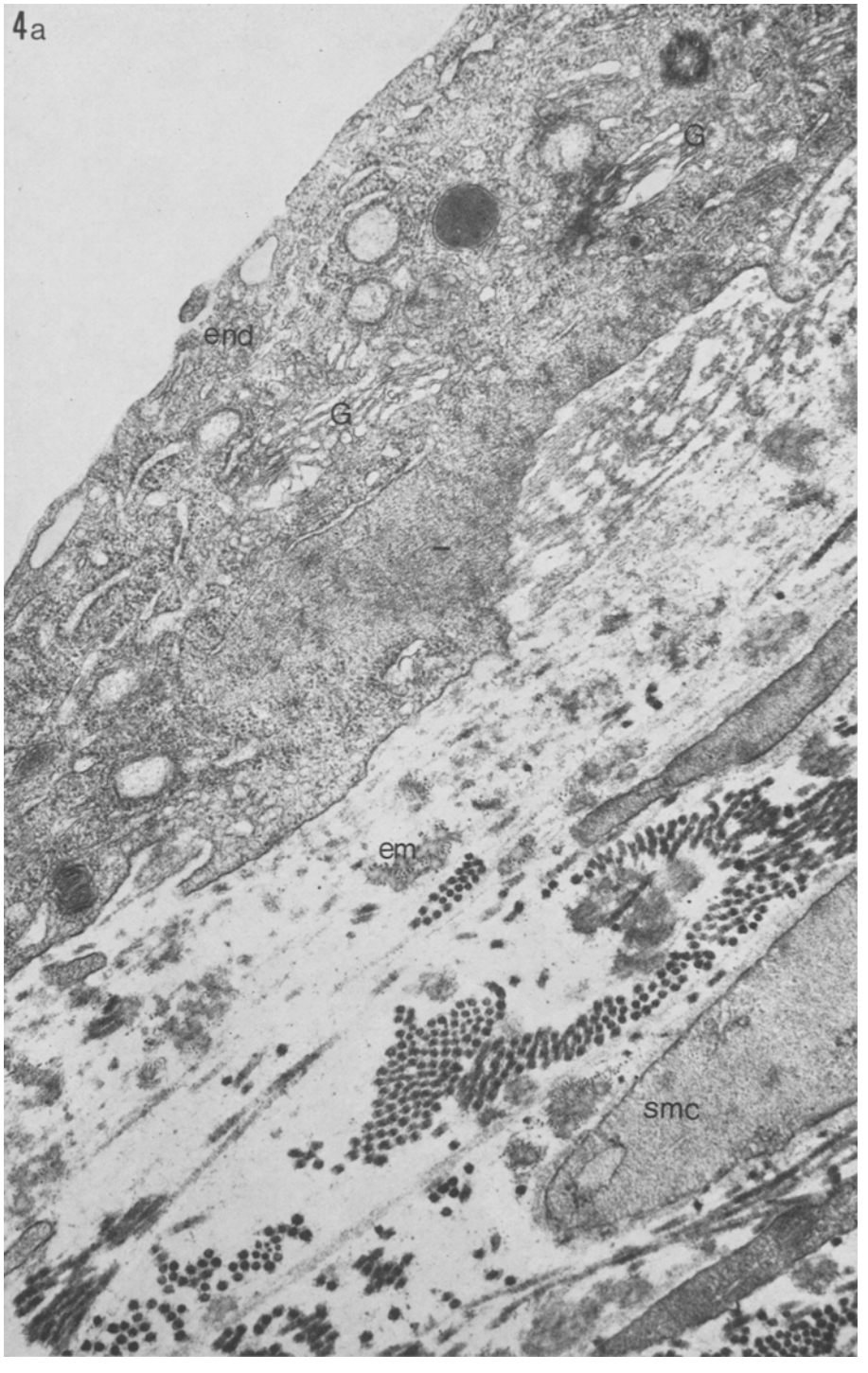
e) Extracellular Space. Frequently the original internal elastic membrane is absent. The extracellular space of all 3 layers is filled with numerous vesicles, myelin structures, collagen fibrils, ground substance, and occasionally with new elastic material (Figs. 4a and 9). The total amount of collagen, however, seems still to be reduced in comparison with the 8 weeks-control-group (Fig. 4).

Conclusion. The normal vascular architecture is not yet restored, all 3 cell types show proliferation and dilatation of the rough smooth endoplasmic reticulum and the Golgi complex, but also partial necrosis of the cell. The fibroblasts of the adventitia show high phagocytic activity.

3. 12 Weeks-Group

a) Endothelium. The endothelial cells have a normal aspect, the nuclei are rather large in proportion to the cell body, there is a small number of mitochondria, and only a small amount of rough and smooth ER, a small Golgi field and many pinocytic vesicles (Fig. 10).

b) Subintimal Thickening. This zone contains mainly normal smooth muscle cells, oriented longitudinally or helically, with contractile filaments and only a few cell organelles (Fig. 10). Some smooth muscle cells still show an increased amount of cell organelles. Occasionally a fibroblast-like cell occurs (Fig. 11).



Figs. 4—9 were taken from 8 week-HC-samples

Fig. 4a. Proliferating endothelial cell (*end*) with a large Golgi field (*G*), *smc* smooth muscle cell, *em* immature elastic material in the extracellular space. Magnification 31800

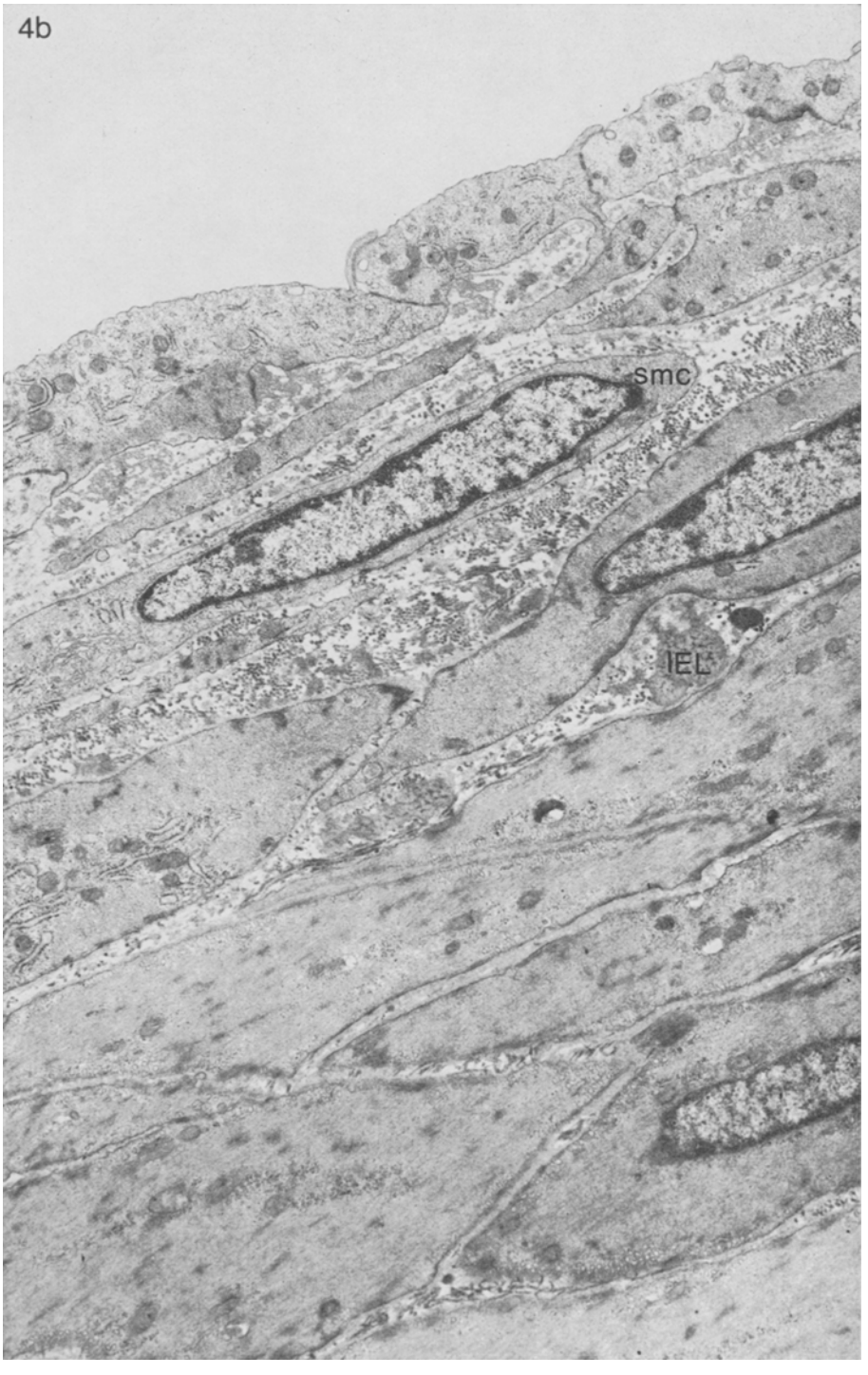


Fig. 4b. Illustration of a vessel at 8 weeks without treatment with hydrocortisone. Normal amount of cell organelles, zone of intimal thickening with smooth muscle cells (*smc*), collagen and ground substance, closely packed smooth muscle cells in the media. *IEL* original internal elastic membrane. Magnification 14000

Fig. 5. Proliferating subintimal smooth muscle cell (*smc*), large dilated Golgi field with numerous smooth elements (*se*), extracellular myelin structures (*ms*). Magnification 36600

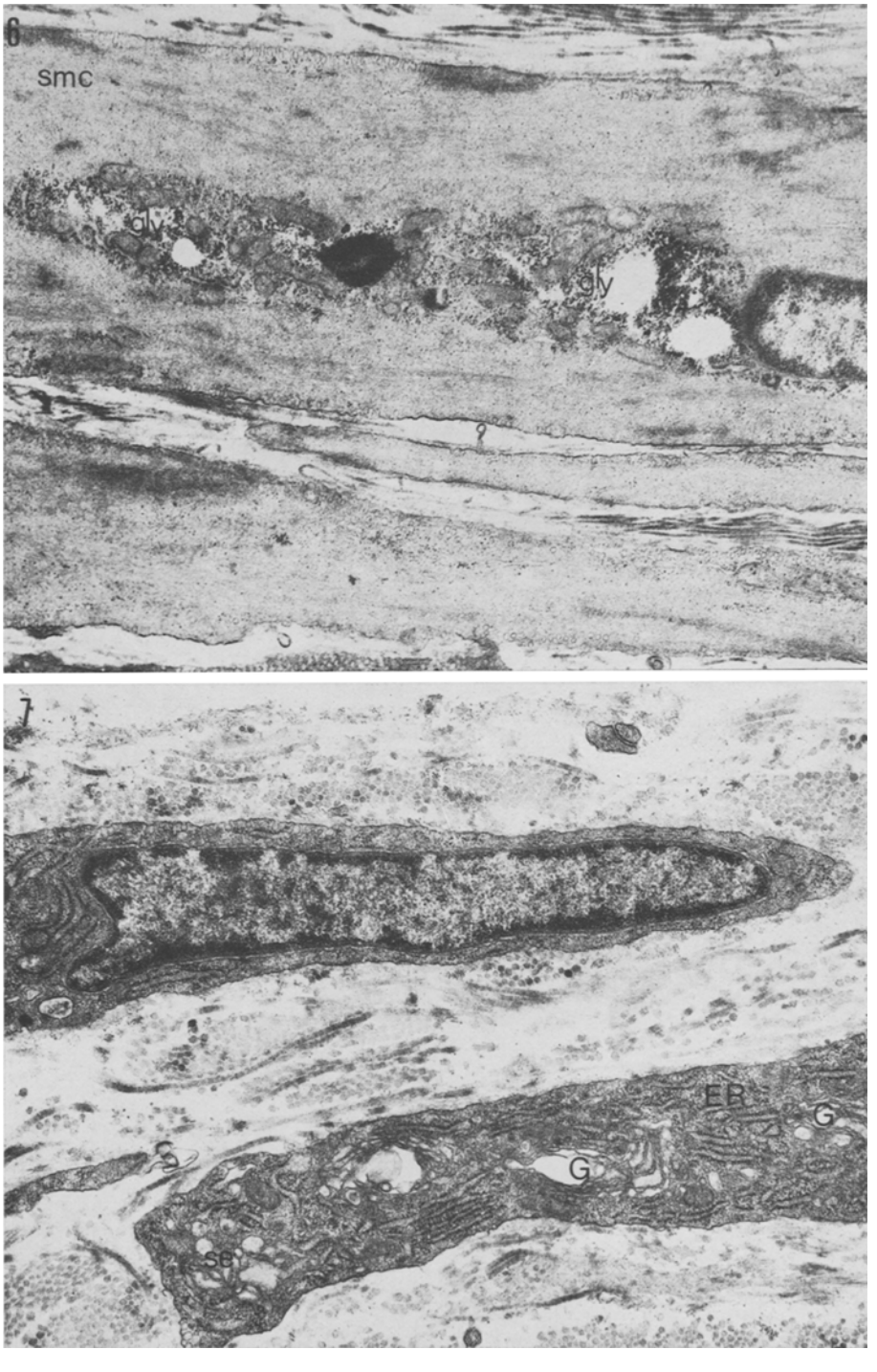


Fig. 6. Medial smooth muscle cell (*smc*) containing glycogen (*gly*). Magnification 15250

Fig. 7. Proliferating adventitial fibroblasts, large dilated Golgi field (*G*) and smooth elements (*se*), *ER* rough endoplasmic reticulum. Magnification 20000

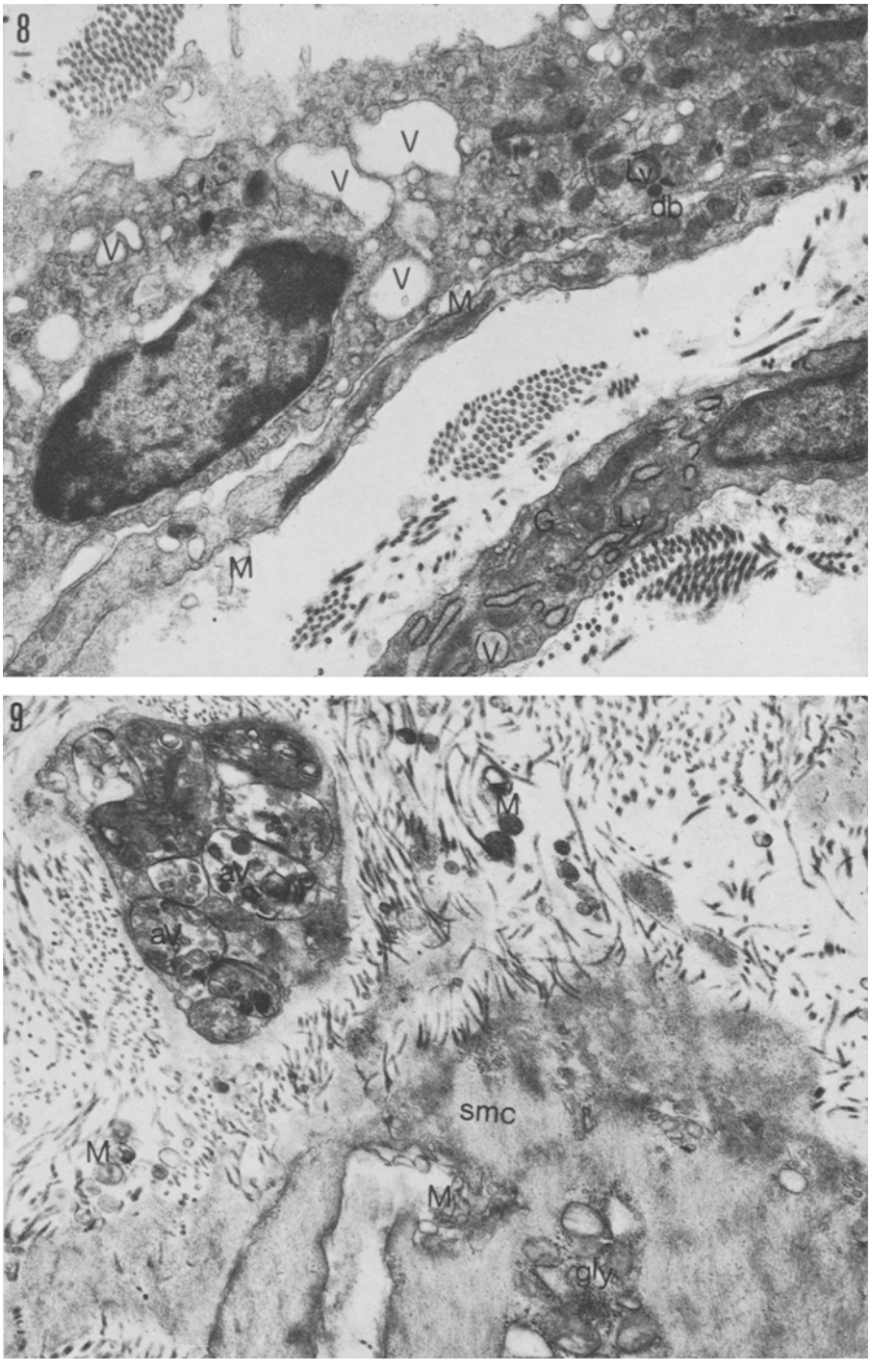
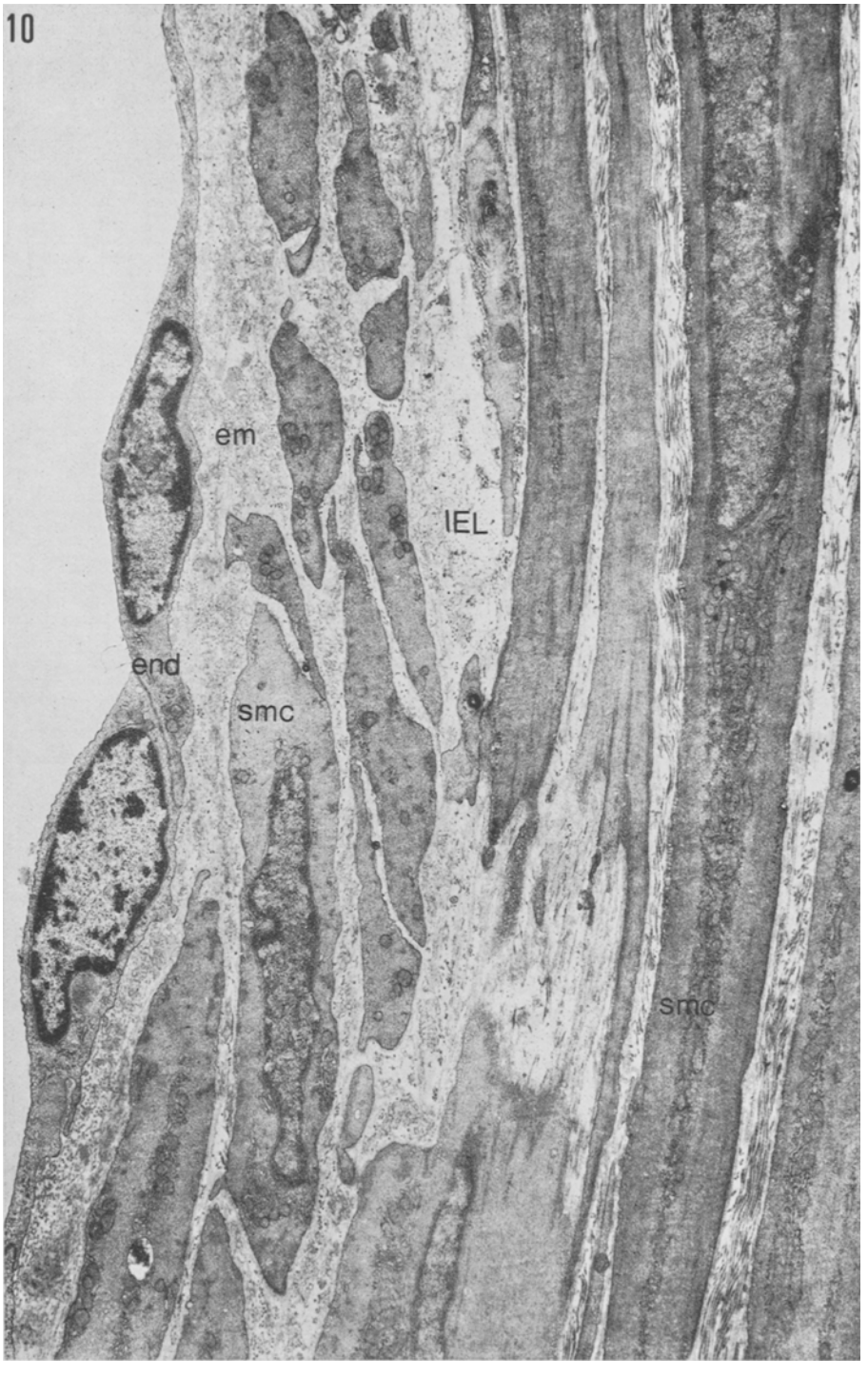


Fig. 8. Adventitial proliferating cells, *V* vacuoles, *db* dense bodies, *Ly* lysosomes, *G* Golgi apparatus, *M* extracellular membranes. Magnification 18500

Fig. 9. Medial smooth muscle cell (*smc*) with glycogen (*gly*). *M* extracellular membranes, some of them filled with dense material, above left: remnant of smooth muscle cell with large autophagic vacuoles (*aV*) containing the same material as seen in the extracellular space. Magnification 16280



Figs. 10 and 11 are from 12 week-HC-samples

Fig. 10. Shows the reorganization of the vascular wall structure, *end* endothelial cells, *smc* smooth muscle cells, *IEL* remnants of the original elastic membrane, *em* new elastic material. Magnification 10000

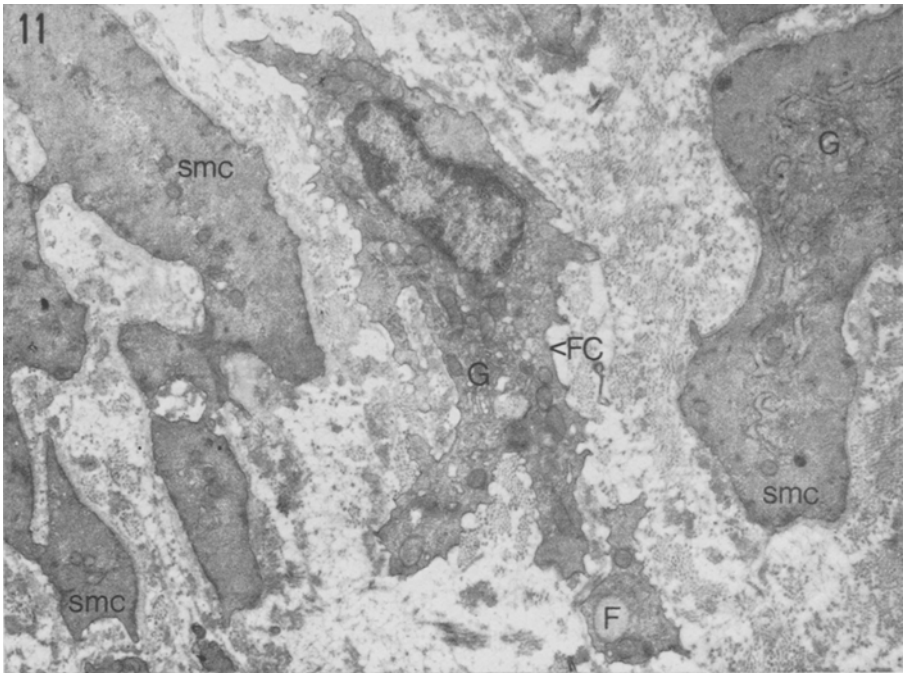


Fig. 11. Smooth muscle cell with increased amount of cell organelles on the right side of the micrograph. *FC* fibroblast-like cell with large Golgi field (*G*), *F* fat vacuole. On the left are normal smooth muscle cells (*smc*). Magnification 10030

c) Medial Cells. The tunica media consists of several regularly arranged layers of smooth muscle cells possessing the normal content of contractile filaments and cell organelles (Fig. 10). Some cytolysomes and dense bodies were seen in the smooth muscle cells of the media and of the subintimal thickening.

d) Adventitia. This layer consists of normally appearing fibroblasts, collagen fibers and elastic material.

e) Extracellular Space between Endothelium and Adventitia. Collagen fibers and new elastic material (elastic microfibrils and amorphous elastin) are present at various degrees (Fig. 10). There is an increased amount of granular material, presumably ground substance.

Conclusion. At 3 months, the spatial arrangement of the different layers of the vessel wall has markedly normalized. The ultrastructural signs of cellular hyperactivity, i.e. proliferated and dilated rough and smooth endoplasmic reticulum have disappeared as has the partial cell necrosis.

Discussion

Hydrocortisone treatment at 20 mg/kg/day orally does not influence the development of collateral blood vessels quantitatively as can be judged from pathophysiological data and from the calculation of diameter/wall thickness ratios. It does, however, modify the pattern of development quantitatively.

Meffert and Liebow in 1966 described the inhibitory action of cortisone acetate administered intramuscularly in rats on pulmonary collateral development. A comparison of their results which are at variance with our own is rendered difficult by the fact that different species have been used and that the mode of administration was not the same.

Furthermore it is impossible to estimate the concentrations of effective substance in blood and tissue, since cortisone is converted into hydrocortisone in the tissue (Asboe-Hansen, 1963a). It is evident from our results in the group treated with 10 mg/kg/day that the hormonal influence on blood vessel growth is a dose dependent process.

At 3 weeks after operation the most prominent difference between treated and control group was the absence of an inflammatory reaction in the vessel wall. Glucocorticoids are known to decrease capillary permeability in an area of tissue injury (Menkin, 1942; Spain *et al.*, 1952), and to inhibit polymorphonuclear cells in their approach to the inflamed zone (Asboe-Hansen, 1963b).

This would explain the absence of edema and of leucocytic infiltration on the adventitial side of the vessel wall, and accounts also for the lack of damage to medial cells characterized by cellular necrosis and massive blood cell infiltration (Borgers *et al.*, 1970; Schaper and Schaper, 1971). Therefore, perivascular inflammation does not seem to be a necessary requirement for the initiation of vascular growth.

Cellular degeneration at 3 and 8 weeks, i.e. membranous structures inside and outside the cells and the presence of digestive vacuoles in various stages of maturation in the cells, could be a specific effect of hydrocortisone since this substance evokes damage and death of vascular and myocardial cells at a high dose level (Ashburn and Williams, 1966; Clarke *et al.*, 1968).

Proliferation of cell organelles occurs mainly in the endothelial cells, the fibroblast-like cells and the modified smooth muscle cells of the subendothelial thickening, and in the fibroblasts of the adventitia. In the 8 weeks-group proliferation of cell organelles occurs also in some of the smooth muscle cells of the media. This indicates a different susceptibility of the various cell types towards the influence of hydrocortisone depending upon the maturation degree of the cells involved. This phenomenon has almost completely vanished at 3 months. Most probably this is due to a decreasing reactivity of the cells because of their progressing maturation.

The relationship between diameter and wall thickness which remains unaltered between 8 weeks and 3 months is also indicative of the fact that the phase of vascular growth characterized by a fast cell turnover progressively declines. At 3 months the vessel wall shows as little responsiveness towards hydrocortisone as normal coronary arteries.

In 3 weeks and especially in specimens from the 8 weeks-group, a marked diminution of ground substance, i.e. mucopolysaccharides (MPS), and of collagen and new elastic material was observed. Inhibition of MPS-synthesis (Whitehouse and Boström, 1961; Lorenzen, 1966; Lorenzen, 1969; Karzel *et al.*, 1969) and increased breakdown of MPS (Lorenzen, 1966) and extracellular microfibrils (Carlson and Low, 1971) caused by glucocorticoids in vitro and in vivo has been described. Recently Ross (1971) demonstrated experimentally that new elastic

microfibrils and amorphous elastic material are produced by smooth muscle cells. The absence of new elastic material at 3 and 8 weeks indicates an inhibition of this specific function of the smooth muscle cells by treatment with hydrocortisone. In 3 months, immature elastic material reappears, there is also an increasing amount of MPS and collagen, indicating a decreasing sensitivity of smooth muscle cells and fibroblasts towards hydrocortisone.

In the 8 weeks-control and in the HC-treated group an intimal thickening occurs of the same frequency and degree. This finding seems of special interest, because an inhibition of intimal hyperplasia in experimentally induced atherosclerosis by corticosteroid treatment has been demonstrated (Oppenheim and Bruger, 1952; Friedman *et al.*, 1964; Bailey and Butler, 1966; Friedman, 1969). This, most probably, is due to the inherent differences in the experimental models used. An atherosclerotic hyperplasia leads to damage and occlusion of an artery, whereas, after regression of the intimal thickening, in the case of a growing collateral a fully functional vessel evolves. These are two distinct processes which could explain the different results obtained by the writers cited above and our own. This explanation, however, is not entirely satisfying and comparative investigations of the mechanisms underlying both kinds of intimal hyperplasia are necessary.

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