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ULTRASTRUCTURAL ASSESSMENT OF LESION DEVELOPMENT IN THE COLLARED RABBIT CAROTID ARTERY MODEL

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Abstract

Cellular reactions associated with the formation of lesions generated in the carotid artery of rabbits fed either normal or high cholesterol diets by the placement of a flexible, silastic collar around the artery, were studied by electron microscopy.

Endothelial cells remained as a monolayer throughout lesion development. The endothelial cell surface in both experimental and sham operated carotids, 4 hours and 8 hours after the initiation of the experiments, were covered with platelets and leukocytes. Neutrophils were present until 7 days in the arteries from within the collar of animals maintained on a normal diet, but only to 1 day in the cholesterol-fed animals. Neutrophils were observed within the medial layer. Few monocytes were identified. An intimal lesion had formed after 7 days in both groups of animals. Macrophage-like cells and foam cells were identified in the cholesterol-fed animals. The size of the lesion increased up to 56 days in animals maintained on a high cholesterol diet, but regression occurred after the 14-days sample in those animals on a normal diet. Concurrently a proportion of the smooth muscle cells changed from contractile to synthetic phenotype within the intimal and medial region of the collared artery of both high cholesterol and normocholesterolaemic animals. Lesions did not form in the contralateral, sham operated arteries.

Key Words: Atherosclerosis, rabbit, ultrastructure, model lesions.

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Introduction

Model systems in which intimal thickenings are induced in blood vessel walls following sheathing by silastic or polyethylene tubes (Meessen *et al.*, 1975, Huth *et al.*, 1975, Gebrane *et al.*, 1982) have contributed to our knowledge of cellular changes occurring during the formation of arterial lesions. We have recently described the induction of a focal proliferative lesion in the carotid arteries of rabbits fed either a high cholesterol or a normal laboratory diet within 7 days of the placement of a soft, silastic collar around the artery (Booth *et al.*, 1989). In this report we describe in detail the cellular changes during the formation of the lesion in this model with particular regard to the endothelial cells, platelets, neutrophils, monocytes, macrophages, foam cells and smooth muscle cells to further the understanding of the collar model.

Materials and Methods

Animals

New Zealand White male rabbits (weighing 2.0-3.0 kg) were treated in groups of six (Booth *et al.*, 1989). One group was fed a normal laboratory diet (0.004% cholesterol, w/w) supplemented with 1.5% cholesterol (w/w), whilst the other group was fed only the normal laboratory diet. Our previous experiments (Booth *et al.*, 1989) have shown that the cholesteryl ester increases within the lesions thus formed.

At the beginning of the experiment the rabbits were anaesthetised using fentanyl/fluanisone (0.3 ml kg^{-1} intramuscular, i.m.) and Midazalam (1.5 mg kg^{-1} intravenous, i.v.) and both carotids were exposed surgically. One carotid artery was enclosed by a non-occlusive, biologically inert, flexible silastic collar in contact with the surface of the artery for 1 mm at each end and the space between the collar and the artery was filled with sterile saline (0.9% w/v). For controls, the collar was placed around the contralateral arteries and was immediately removed. The carotids were collected at 1, 7, 14 and 56 days for transmission electron microscope (TEM) studies

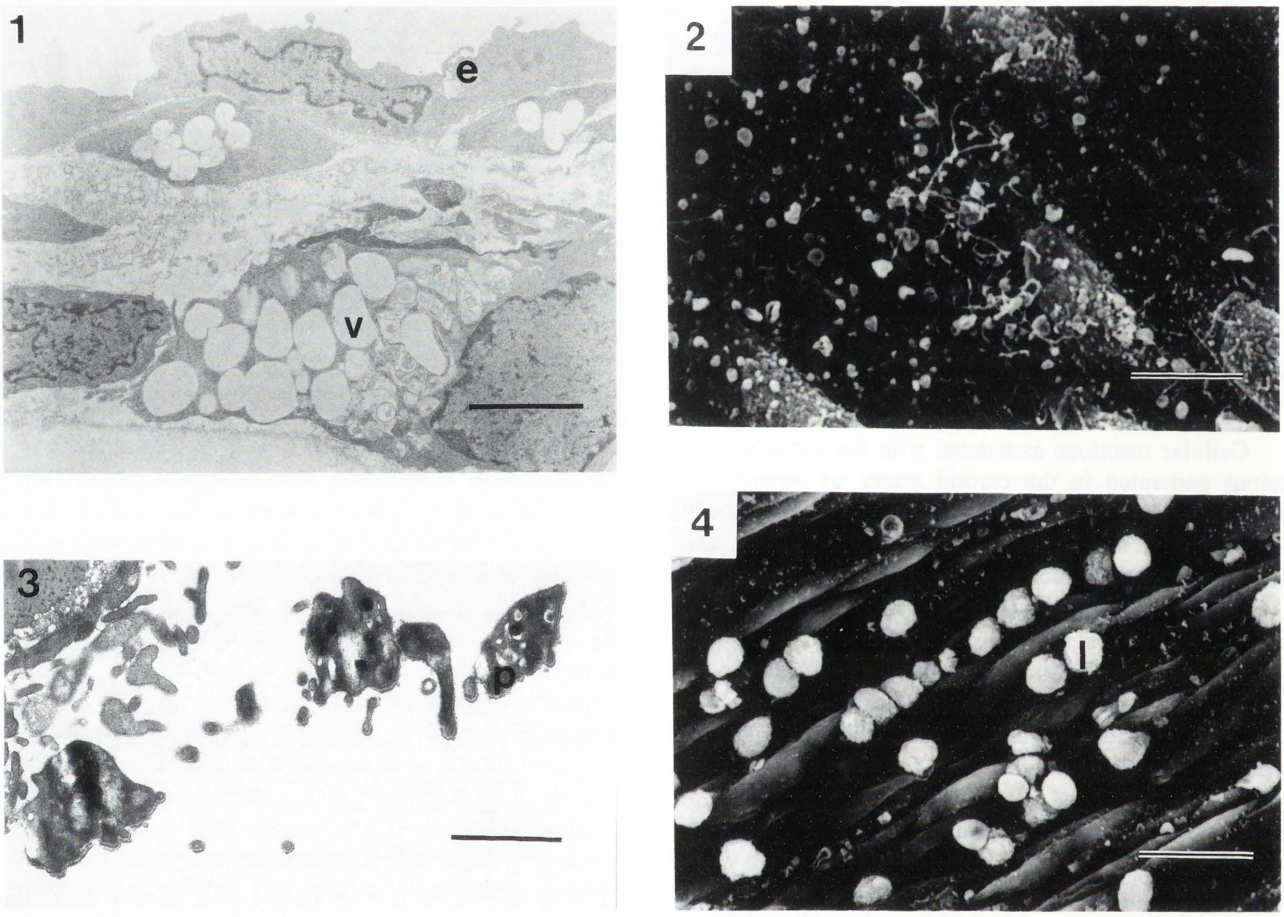

Figure 1. Throughout lesion formation endothelial cells (e) remain as a continuous layer one cell thick, the tips of each endothelial cell interdigitating with those of adjacent cells. Morphological differences could not be observed between endothelial cells overlaying lesions and those from control arteries. This lesion shows early foam cell development with small vesicles (v) prominent in many cells (bar = 2.5 μm).

Figure 2. Scanning electron microscopy of the sham operated arteries at 4 hours revealed a thin scattering of platelets adhering to the endothelium (bar = 1 μm).

Figure 3. TEM preparation of areas shown in Figure 2 confirm the identification of platelets (p) observed by SEM (bar = 1 μm).

Figure 4. By 8 hours after collar placement SEM revealed leukocytes (l) adhering in rows along the endothelial cell boundaries (bar = 1 μm).

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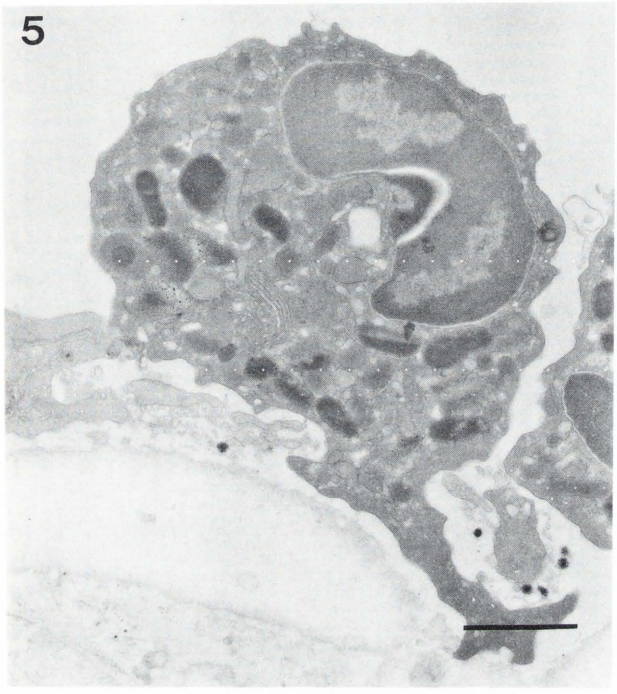
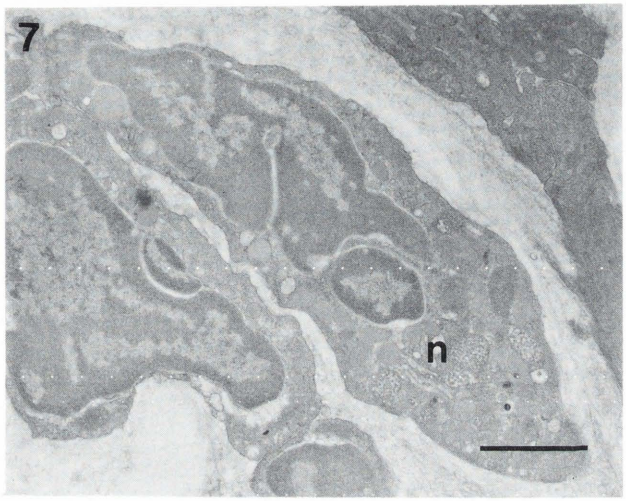
and at 30 minutes, 4 hours, and 8 hours for scanning electron microscope (SEM) observation.

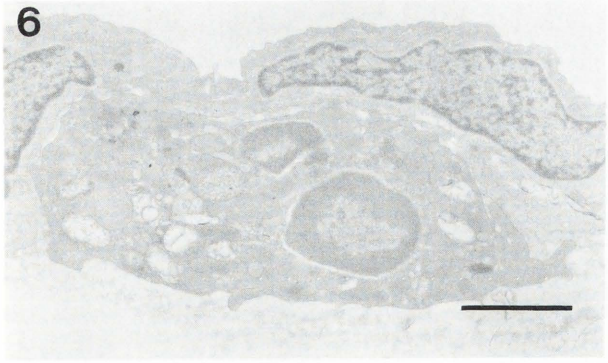
Microscopy

At each selected time after the initiation of the experiment 6 rabbits from each dietary group were anaesthetised (pentobarbital, 30 mg kg^{-1}, i.v.) and both carotid arteries were cannulated. The arteries were perfused *in situ* (37 °C, 100 mm Hg, 2 minutes) with Krebs-Ringer and with Karnovsky's modified fixative (4% glutaraldehyde, 3% formaldehyde) for 30 minutes. The fixed arteries were removed and stored in this fixative at 4 °C until required for further processing. The mid region from the collared areas were processed from each artery.

For TEM studies each sample was embedded in epoxy resin. The tissues were rinsed in 0.1 M phosphate buffer and fixed sequentially with 1% buffered osmium tetroxide and 2% aqueous uranyl acetate, dehydrated in a graded series of ethanol, followed by propylene oxide and finally embedded in EMix resin (Bio-Rad Microscience Division, Watford, UK). Semi-thin survey sections of transverse aspects of the arteries were cut







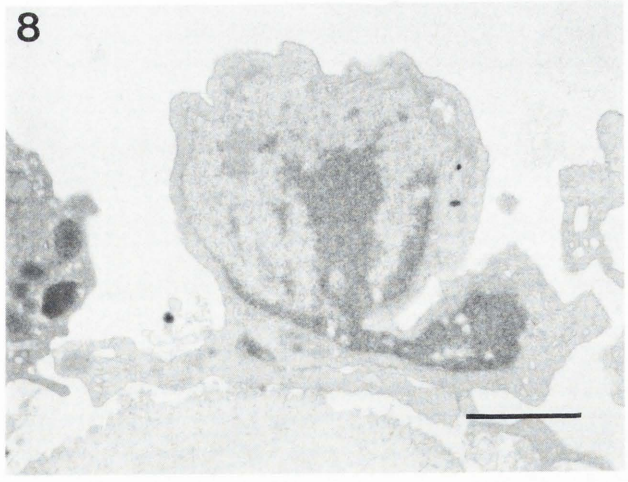


Figure 5. A neutrophil in close contact with the endothelial cell. The neutrophil is flattening on the endothelial cell and appears to be inserting cytoplasmic extrusions beneath the endothelial cell and into discontinuities in the internal elastic lamina (bar = 1.5 μm).

Figure 6. Neutrophils were often observed flattened between the endothelial cell and the internal elastic lamina. The neutrophil and endothelial cells appear to be in close association with each other (bar = 1.5 μm).

Figure 7. TEM confirmed that neutrophils (n) had migrated in clusters several cell layers into the medial layer. The neutrophils were not in close contact with the smooth muscle cells (bar = 2 μm).

Figure 8. Monocytes, recognised by their characteristic nucleus, few cytoplasmic granules and Golgi apparatus were observed spreading over the internal elastic lamina as early as 1 day after placement of the collar (bar = 1.5 μm).

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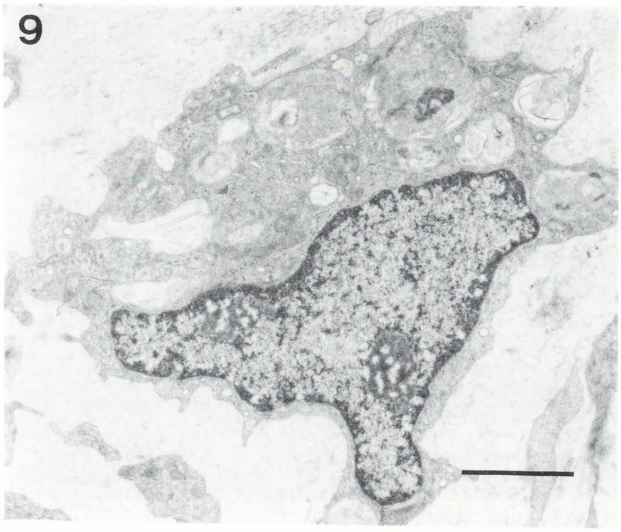
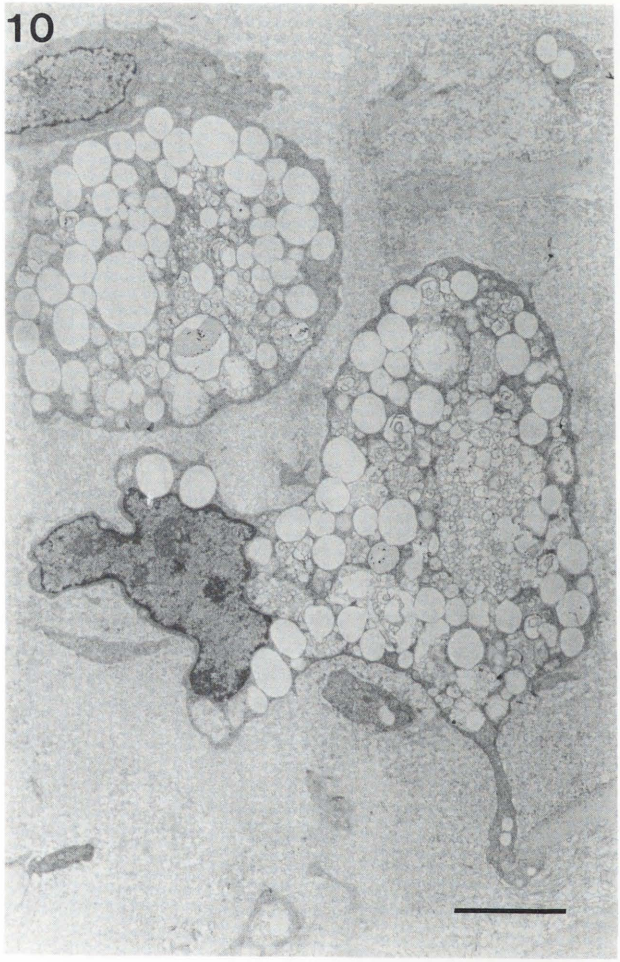
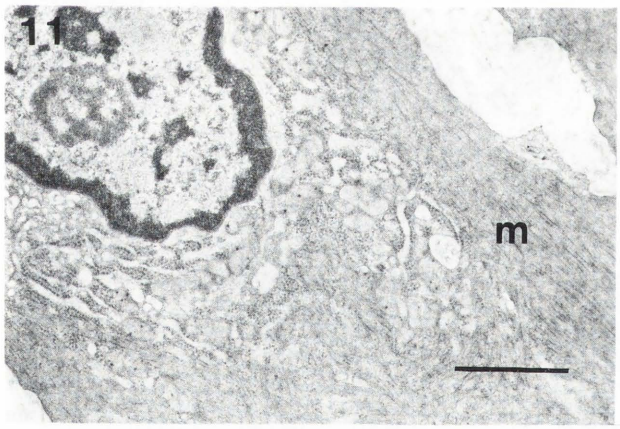
with an LKB Historange (Cambridge Instruments, Bar Hill, Cambridge) between 0.5 and 3 μm and stained with methylene blue-azure II/basic fuchsin. All the samples from each group were studied by semi-thin sections. The most appropriate area from each animal was identified and ultra-thin sections were prepared and stained with uranium and lead salts before examination with a Philips 300 TEM.







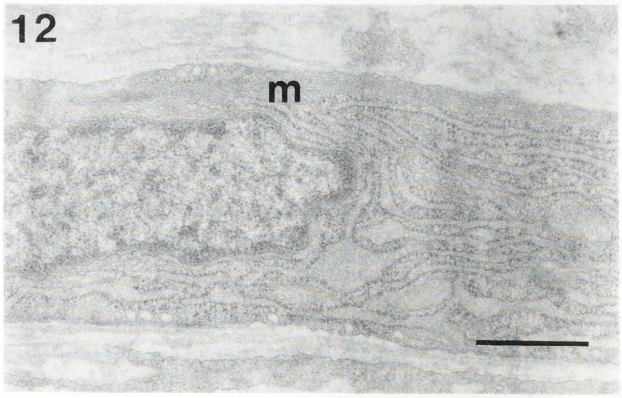


Figure 9. Distinct macrophages were not found although macrophage-like cells with pseudopodia and some morphological characteristics resembling macrophages were occasionally observed in those animals maintained on a high cholesterol diet (bar = 2 μm).

Figure 10. Large foam cells were present in the lesions of cholesterol-fed animals by 14 days. The foam cell cytoplasm contained a mass of vacuoles and an eccentric nucleus. Processes of the foam cells were often in intimate contact with the internal elastic lamina (bar = 3.5 μm).

Figure 11. A smooth muscle cell with morphological features of a contractile cell. The majority of the cytoplasm contains abundant, parallel myofilaments (m). A myofilament-free area in the centre of the cell contains the nucleus and cell organelles (bar = 1 μm).

Figure 12. A synthetic smooth muscle cell. The myofilaments (m) characteristic of contractile cells are considerably reduced and the cytoplasm contains organelles associated with synthesis. These cells were present in both the medial and the intimal layers (bar = 0.5 μm).

For SEM the carotid arteries were processed whole. The aldehyde-fixed tissues were post-fixed with 1% osmium tetroxide in 0.1 M sodium phosphate buffer for 1 hour, treated with a saturated solution of thiocarbohydrazide and after a rinse in water, again treated with 1% osmium tetroxide for 1 hour. They were dehydrated in a graded series of methanol, through acetone, and after critical point drying were mounted on SEM stubs. The

luminal surface was exposed by length-wise dissection and coated with carbon and gold. The preparations were examined with a Philips 500 SEM.

Results

Endothelial cells formed a single continuous layer which remained essentially intact throughout the experiments although in areas of intense cellular adhesion and migration of adherent blood cells they were displaced by the invading cells. Despite careful examination no difference in organelle distribution or cell ultrastructure could be found between endothelial cells from control experiments and those cells surmounting a lesion (Figure 1).

Platelets had accumulated in patches, not thrombi, by 4 hours on the endothelium of both collared and sham operated arteries (Figure 2). Part of the tissue that had been examined with the SEM, was embedded in resin and examination of ultra-thin sections confirmed the presence of platelets (Figure 3). The number of platelets decreased rapidly after 8 hours.

Leukocytes had attached to the endothelial cell layer by 4 hours in both the experimental and sham operated carotids (Figure 4) in rows along the edges of the endothelial cells and were commonly observed with microvilli inserted between the endothelial cells. It was not possible to differentiate neutrophils from monocytes by SEM but this differentiation could be made by TEM. The neutrophil possesses a highly-lobed nucleus with densely staining heterochromatin, two populations of granules and rod shaped mitochondria. In contrast, monocytes have a kidney-shaped nucleus, less lobed than the neutrophil, lacey heterochromatin, lightly staining round mitochondria in abundance, and a single population of azurophilic granules (Figures 5-8).

Neutrophils accumulated until 7 days after the placement of the collar on the arteries of animals fed the normal diet, but by the 7-days sample, neutrophils were not detected in the cholesterol-fed animals. They were in close contact with the luminal surface of the endothelial cells and thin processes from the neutrophils were invading the endothelial cell monolayer (Figure 5) and into discontinuities in the internal elastic lamina. Neutrophils beneath the endothelial cells were flattened onto the internal elastic lamina and were spreading beneath the endothelial cells (Figure 6). Regions of the neutrophil membrane were in very close apposition to the endothelial cell plasma membrane.

The neutrophils were found 7-10 cell layers deep in the medial layer (Figure 7). They were not closely associated with the smooth muscle cells. Some of the granules of the neutrophils were reticulated although this may be an artefact of fixation. There was no disruption of the smooth muscle cells.

Monocytes were relatively few and were found in the collared areas of animals maintained on both normal and high cholesterol diets (Figure 8). At 1 day they were detected on the luminal surface of endothelial cells and on the internal elastic lamina.

After extensive searching macrophage-like cells were found, in cholesterol-fed animals only, 56 days after placement of the collar, both in the intimal layer and in the medial layer (Figure 9). Macrophage-like cells were identified by their shape. Unlike smooth muscle cells and endothelial cells which were elongate the macrophage-like cells appeared to be spreading, with their pseudopodia insinuating between adjacent cells.

By 7 days after the collar placement a lesion, consisting of approximately four cell layers, was present in the collared region in both cholesterol-fed and normal diet animals. Electron microscopy showed the cells within the lesion to be elongate, with distinct nuclei. Some possessed considerable endoplasmic reticulum while others also contained abundant Golgi and vesicles.

Foam cells were present only in the cholesterol-fed animals. They were observed by 7 days after the placement of the collar. Study of semi-thin sections showed an abundance of foam cells within the intimal layer and also, to a lesser extent, along the luminal edges of the medial layer. There were no foam cells within lesions of animals fed the normal laboratory diet nor within the contralateral arteries, even at 56 days, in animals given the cholesterol diet and with the collar in position on the opposite artery.

There was a graded foam cell development culminating in the presence of large foam cells 14 days after the placement of the collar. Initially the cells contained small vesicles, presumably accumulated cholesterol (Figure 1). These cells were often present just beneath the endothelial cells. The vacuoles increased in size with concomitant increase in size of the foam cell. Large foam cells were present at 14 days (Figure 10). The foam cell cytoplasm contained a mass of vacuoles and an eccentric nucleus. Foam cells were often in intimate contact with the internal elastic lamina and in places the foam cell membrane appeared to spread over the internal elastic lamina.

Some foam cells may have been degrading, since the extracellular matrix of the intimal thickening consisted of a mass of small vesicles, very similar to those enclosed within the foam cells. There was in addition, abundant proteinaceous material indicating that some cells in the intimal region were capable of producing high amounts of matrix material. In contrast, however, the medial layer contained mainly collagen, small amounts of which, leaked into the intimal layer whenever the internal elastic lamina was discontinuous.

Smooth muscle cells in the medial layer of control arteries were elongate, circumferentially positioned and the majority of the cytoplasm contained abundant, parallel myofilaments indicating their contractile phenotype (Figure 11). As the lesion developed, a proportion of the smooth muscle cells changed into another distinct phenotype, the synthetic cells (Figure 12). The contractile myofilaments were considerably reduced in synthetic cells. These cells were still elongate, but, instead of the abundance of fine myofilaments so characteristic of the contractile cells, the cytoplasm of the synthetic cells consisted almost entirely of organelles associated with synthesis. Myofilaments, when present, were restricted to a thin parallel band at the periphery of the cell.

The numbers of synthetic and contractile cells in the medial and intimal layers was counted, any intermediate state being assigned to one or other of the categories. Between 38 and 205 smooth muscle cells from each animal in each group at 1, 14 and 56 days were assessed. As expected from such a small group, the results are variable but nonetheless general trends can be discerned. The medial layer of contralateral arteries contained almost exclusively (95-100%) contractile cells throughout the experiment. At 1 day there were 6% synthetic cells in the high cholesterol fed animals in the medial layer but this rose to 54% at 14 days and 55% at 56 days. In the medial layer of animals fed normal diet the number of synthetic cells were higher at 1 day (43%) although lower at 14 days (44%) and 56 days (35%). The majority of the cells in the intimal lesion were synthetic in both the high cholesterol fed animals (99% at 14 days, 76% at 56 days) and the normal fed animals (97% at 14 days, 83% at 56 days).

Examination of relevant semi-thin and ultra-thin sections revealed that despite lesion formation in the intimal layers the number of cell layers in the medial layer remained constant.

Discussion

The observations made in this study show that a localised lesion can be induced in the carotid artery of the rabbit. The development of foam cells and a change in the phenotype of smooth muscle cells in the medial and intimal layers are associated with lesion formation. We have found evidence of an early involvement of platelets and neutrophils with few monocytes and macrophage-like cells.

We have not detected gross endothelial damage or stripping of cells. This of course does not imply that there is no functional alteration of the cells within the collared regions since changes in endothelial cell glycocalyx and permeability have been shown, by ruthenium red staining, to be transient (Viele and Betz 1985, Gerrity *et al.*, 1979).

The lesion developed in the collared region of both cholesterol-fed animals and normocholesterolaemic animals, but foam cells appeared only in those animals fed the high cholesterol diet, implying that two factors are involved in lesion development. The first, a stimulus generated by the presence of the collar initiates lesion formation and the second, in association with a raised cholesterol level stimulates foam cell formation.

It is conceivable that the early presence of platelets and neutrophils in the collared and sham operated arteries may be a short term effect of our surgical procedures since the appearance of these cells is a feature of damaged vessel walls (Haudenschild and Studer 1971). It is also possible that the appearance of the platelets and the neutrophils may be associated with the early development of the lesion and may be indicative of changes, associated specifically with the endothelial cells, which are significant in attracting platelets and neutrophils. Early transient monocyte and neutrophil attachment to intact endothelial cells was observed by Kling *et al.* (1987) in their transmural electrical stimulation carotid model of experimental atherogenesis. They feel that the presence of these cells reinforces the view that the initial phases of atherosclerosis may represent a special form of an inflammatory response. Our model, showing the early presence of platelets, monocytes and neutrophils, may indeed support this view.

Alternatively, the presence of neutrophils in our model may represent a distant effect of hypoxia produced in the adventitial and medial layers through occlusion of the vasa vasorum by the collar, as hypothesised by Martin *et al.* (1990). In addition, the attachment of leukocytes to the isolated rat superior mesenteric artery (Grossman and Zambetis, 1989) and the presence of neutrophils in and around the site of myocardial infarction (Mullane *et al.*, 1984), have both been shown to relate to local ischaemia and would support this hypothesis. It is, of course, a tantalising question as to whether neutrophils actively stimulate the processes involved in intimal thickening.

Ross *et al.* (1984) have documented smooth muscle cell, macrophage and foam cell appearance in atherosclerotic lesions. Macrophages are distinguished by numerous lamellipodia at their surface, they are not surrounded by a basal lamina, and usually contain dense bodies representing primary and secondary lysosomes. In addition, the chromatin distribution in the periphery of the nucleus of the macrophage is less coarse than in the smooth muscle cell. Furthermore, the rough endoplasmic reticulum and the Golgi complex of the macrophage are poorly developed and macrophages possess fewer polysomes than smooth muscle cells. Our electron microscope studies did not reveal distinct macro-

phages according to the above criteria but we did find some macrophage-like cells. Electron microscopic identification of macrophages may be difficult in atherosclerotic lesions and an immunocytochemical study would be necessary to resolve this anomaly as well as the origin of the foam cells.

Smooth muscle cells in the medial layer of the contralateral (control) arteries were almost exclusively contractile. During lesion development however, approximately half the smooth muscle cells in the medial layer became synthetic while the greater proportion of cells forming the lesion in the intimal layer were synthetic. This occurred in the arteries of both normal and cholesterol-fed animals. We did not wish to carry out a fully quantitative assessment of synthetic cell appearance but our findings, however, strongly suggest that the presence of a collar around the arteries induces high levels of synthetic cells. Phenotypic variation associated with lesion development has been reported previously by Ross *et al.* (1984) who describe the two cell types as classical and modified. Glukhova *et al.* (1988) consider these two distinct phenotypes, correlated with changes in cytoskeleton and contractile apparatus, to be at opposing extremities on a continuous spectrum of intermediate states of differentiation available to the smooth muscle cell. The modified cells, with an extensive complement of dilated rough endoplasmic reticulum and Golgi complex, are similar to the synthetic cells described in our study. The change from contractile to synthetic phenotype has previously been noted in cultured smooth muscle cells (Schwartz *et al.*, 1986). Dilley *et al.* (1987) suggest that vascular smooth muscle cells change into the synthetic phenotype which produce intimal thickening. They further suggest that after changing to the synthetic phenotype, migration and proliferation are enhanced, for contractile cells do not migrate. In addition, most smooth muscle cells observed in mitosis are synthetic. This supports our findings that the greater proportion of the cells forming the lesion are synthetic, an observation also recorded by Kling *et al.* (1987). It is interesting to question whether the stimulus for smooth muscle cell migration also initiates the change from contractile to synthetic or whether a stimulus causes the change and in consequence the synthetic smooth muscle cells migrate.

In conclusion, we appreciate that the intimal thickening observed in our model system may be a response to one or many forms of arterial injury and the early appearance of platelets and neutrophils may indicate a possible trauma, exacerbated by hypercholesteraemia. Studies such as these, however, will each contribute to our knowledge of the underlying mechanisms of lesion formation in these models and which may be extrapolated to further our understanding of development of lesions in man.

Acknowledgements

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Discussion with Reviewers

Reviewer I. When was cholesterol feeding started in relation to the surgical procedures and what levels of hypercholesterolemia were reached in the course of the experiments?

Authors: There was no pre-feeding. As soon as the collar was in position the animals were allowed access to the high cholesterol diet. Plasma cholesterol reached 30 mM in animals maintained on the high cholesterol diet compared with less than 2 mM on the normal laboratory diet.

Reviewer I: Please provide additional details related to the collar placement.

Authors: Surgical exposure of the carotid artery removed some of the adventitial layer. The cylindrical collar, 1.5 cm in length and slit longitudinally, was positioned over the artery. The collar was held open while the chamber was filled with sterile saline. The slit was sealed with elastomer taking care not to allow fluid elastomer to contact the artery.

W.G. Jerome: Do you think collaring the artery could be damaging the smooth muscle cells leading to cellular proliferation?

Authors: There was some smooth muscle degradation and this could be a factor.