Virchows Archiv (1994) 425:41–47

ORIGINAL ARTICLE

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The relationship between pre-existing subendothelial smooth muscle cell accumulations and foam cell lesions in cholesterol-fed rabbits

Received: 11 February 1994 / Accepted: 13 May 1994

Abstract We investigated whether pre-existing subendothelial smooth muscle cell (SMC) accumulations in cholesterol-fed rabbits are transformed into foam cell plaques. Twenty-four rabbits received a standard diet supplemented with 2% cholesterol for 4 or 8 weeks. Six rabbits received a supplement of 0.3% cholesterol for 35 weeks. The aorta and other systemic and pulmonary vessels were studied by immunohistochemistry for smooth muscle cells SMC (α-SMC actin), macrophages (RAM11), cell replication (proliferating cell nuclear antigen) and endothelial cells (von Willebrand factor; vWF). Initially the foam cell plaques were composed exclusively of foam cells of macrophage origin (MFC). In more advanced lesions SMC and collagen fibres were also present, leading to a fibrous transformation of the plaque. Cell replication was mainly located in the MFC. The endothelial cells covering the plaques showed an increased immunoreactivity for vWF which was also deposited in the interstitium between the FC. Pre-existing subendothelial SMC did not transform into FC. The newly formed FC plaques remained clearly separated from the pre-existing subendothelial SMC. The development of the plaques can be attributed not only to monocyte recruitment but also to macrophage multiplication.

Key words Cholesterol · Foam cells · Atherosclerosis
Smooth muscle cells · Endothelium
von Willebrand factor

Introduction

Spontaneous intima formation of the eccentric type (intimal cushions) is present at the orifices of branches in different arteries of the rabbit. These intimal cushions consist of longitudinal orientated smooth muscle cells (LSMC) and are associated with bundles of LSMC in the media [13]. At sites distant from branches and bifurcations rabbit arteries demonstrate solitary and small clusters of subendothelial LSMC. Since the rabbit is generally used for the induction of atheroma-like lesions by hypercholesterolaemia, the aim of the present study was to investigate the relationship between the pre-existing LSMC accumulations and newly formed atheromatous plaques in cholesterol-fed rabbits. In particular, we studied whether plaques are formed by incorporation of these LSMC.

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Material and methods

Twenty-four New Zealand white male rabbits, weighing between 2.8 and 3.5 kg received a standard diet supplemented with 2% cholesterol. Twelve animals were sacrificed after 4 weeks, the other 12 after 8 weeks. Four additional untreated animals served as controls. Because animals on a diet with such a high cholesterol content show a high mortality rate after 26 weeks [2] a separate group of six animals were fed a diet with 0.3% cholesterol for 35 weeks. Three additional untreated animals served as controls. Before the animals were sacrificed by an intravenous overdose of sodium pentobarbital (Nembutal), blood was taken for determination of cholesterol by an enzyme assay [16]. For histological examination vessels were dissected and fixed in situ for 30–60 min in methacarn fixative (60% methanol, 30% 1-1-1-trichloroethane, 10% acetic acid) or in 4% neutral formalin. After removal fixation was continued for 24 h for methacarn fixed tissue and for 3–5 weeks for formalin fixed tissue until frozen sections for fat stains were made. The left common carotid artery of four animals of the 8 week group were perfused in situ with glutaraldehyde 1.2% in a cacodylate buffer for electron microscopy (EM).

The following vascular segments collected at the same sites in each animal were examined (Fig. 1): coronary arteries and adjacent myocardium of the cranial part of the ventricles, both carotid arteries at three levels, ascending aorta and adjacent trunk of the pulmonary artery, aortic arch between the orifices of the carotid arteries and the left subclavian artery, descending thoracic aorta just distal to the left subclavian artery and just above the diaphragm, coeliac axis, superior mesenteric artery, left renal artery, abdominal aorta and its bifurcation, iliac and femoral arteries at two levels.

Standard stains on 5 µm thick sections of paraffin embedded tissues were the Sirius haematoxylin stain, Verhoef's elastica stain

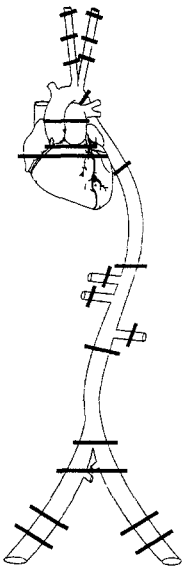

Fig. 1 Scheme indicating the topography of the vascular segments used in this study

and alcian blue stain at pH 3.2. Immunohistochemistry was done on polylysine coated slides by a direct immunoperoxidase technique with diamino-benzidine as chromogen. The following antibodies were used: monoclonal anti-—SMC actin (Sigma, A-2547) diluted 1:2000, monoclonal anti-swine vimentin (Dako, M725) diluted 1:120, monoclonal anti-proliferating cell nuclear antigen (PCNA; Dako, PC 10) diluted 1:300, polyclonal anti-von Willebrand factor (vWF; Binding Site, Birmingham, UK) diluted 1:250 and the rabbit monospecific monocyte-macrophage monoclonal antibody RAM 11 (Dako) diluted 1:1000. Oil red-O was used for the demonstration of neutral fat on frozen formalin fixed tissues.

For EM fragments were postfixed in 1% osmic acid and embedded in Epon. Ultrathin sections were stained with 2% uranyl acetate and examined in a JEOL 1200 EX EM at 80 kV.

The widely used descriptive term of foam cell (FC) refers to a large polygonal cell with a diameter between 20–40 μm and repleted with fat globules or containing many optically empty spaces after processing through organic solvents. Whether small cells containing a few fat globules are considered as FC is not specified in the literature. We decided to call FC those cells of any size whose cytoplasm was entirely filled with fat globules. Only SMC derived FC contain α-SMC actin: we call those cells AFC and the other FC which are monocyte derived, RAM 11 positive and α-SMC actin negative, MFC. Cells containing discrete fat globules are called "fat containing cells". Cyclic growth activity was assessed by counting the nuclei immunoreactive for PCNA. In order to localize the positive nuclei in MFC, AFC or SMC a double immunostaining for PCNA and α-SMC actin was applied. In one animal of the 35 week group ten different localizations were studied and per plaque six successive non overlapping sections 30 μm apart were used. The areas were measured with a digitizing tablet and a morphometry program by Osteometrics. The six areas of each plaque were added. The nuclei immunoreactive for PCNA were expressed as a percentage of the total number of nuclei in the area. Nuclei displaying immunoreactivity for PCNA will be referred to as PCNA positive nuclei.

Results

Serum cholesterol levels in controls at 4 weeks showed a mean of 30.1 [standard deviation (SD 9.4)] mg/dl, at 8 weeks 31.4 (SD 20.6) mg/dl, in cholesterol fed animals at 4 weeks 905.3 (SD 255.0) mg/dl and at 8 weeks 1041.0 (SD 226.2) mg/dl. The values in the 35 week group were in the controls 16.0 (SD 3.5) mg/dl and in the experimental animals 468.5 (SD 158.3) mg/dl. In the high cholesterol group of 4 and 8 weeks lesions were found in all the vascular segments examined, although more frequent and more severe in those animals fed for 8 weeks. However the extent and the severity of the involvement was very variable between animals and in each animal. In the low cholesterol group of 35 weeks lesions were more widespread, less patchy and hence more diffuse with respect to the intimal area involved and above all they were more fibrotic. But even in these animals small lesions mainly composed of macrophages were present. Therefore the lesions will be described according to their severity and their temporal relationship with the duration of the experiment will only be mentioned if relevant.

The minimal abnormality noted was the presence of discrete small globules in and underneath endothelial cells (EC) without any topographical predilection. A further stage was characterized by the presence of these fat particles in the SMC adjacent to the internal elastic lamina (IEL). LSMC, either diffusely spread or concentrated in cushions contained identical fat globules: the fat was present in the cells closest to the lumen, whatever their direction. Interstitial fat accumulation occurred around SMC and was bound by the IEL and if severe by deeper elastic laminae.

The appearance of MFC between the EC and the IEL was a first indication for the development of plaques. The MFC showed a preferential localization on the slopes of bifurcations and upon protruding LSMC in subendothelial cushions and in medial LSMC columns at branchings but were found in many straight non branch-

Fig. 2 Example of a pre-existing intima cushion (*IC*) covered by a fibrous plaque (*FP*) with superficial foam cells. Orifice of a branch of a coeliac artery, 8 weeks 2% cholesterol. Internal elastic lamina (*IEL*). Sirius haematoxylin (*bar* is 100 μm)

Fig. 3 Example of a pre-existing diffuse subendothelial smooth muscle cell (SMC) accumulation limited by a duplicated IEL *(arrows)*. It is covered by a cellular plaque stained for the rabbit macrophage marker RAM 11 demostrating that the majority of the foam cells are monocyte derived. Upper thoracic aorta, 35 weeks 0.3% cholesterol. Immunohistochemical staining for RAM 11 (*bar* is 100 μm)

Fig. 4 Example of a pre-existing medial column of longitudinally orientated SMC covered by a fibrous plaque (*FP*). Aorta bifurcation, 4 weeks 2% cholesterol. Sirius haematoxylin (*bar* is 100 μm)

Fig. 5 Pulmonary vein demonstrating foam cell plaques *(arrows)* at the junction of a small and a large branch. The dark cells in the plaques are SMC. Animal of the 4 weeks, 2% cholesterol group. Immunohistochemical staining for α-SMC actin (*bar* is 50 μm)

Fig. 6 Small cellular plaque composed of a few foam cells showing strong positivity for von Willebrand factor (vWF) in the endothelium and in between foam cells. Lower abdominal aorta, 8 weeks 2% cholesterol. Immunohistochemical staining for vWf (*bar* is 50 μm)

Fig. 7 Large fibro-cellular plaque showing increased positivity for vWF especially at the slopes where the foam cells are concentrated. Lower abdominal aorta, 8 weeks 2% cholesterol. Immunohistochemical staining for vWf (*bar* is 50 μm)

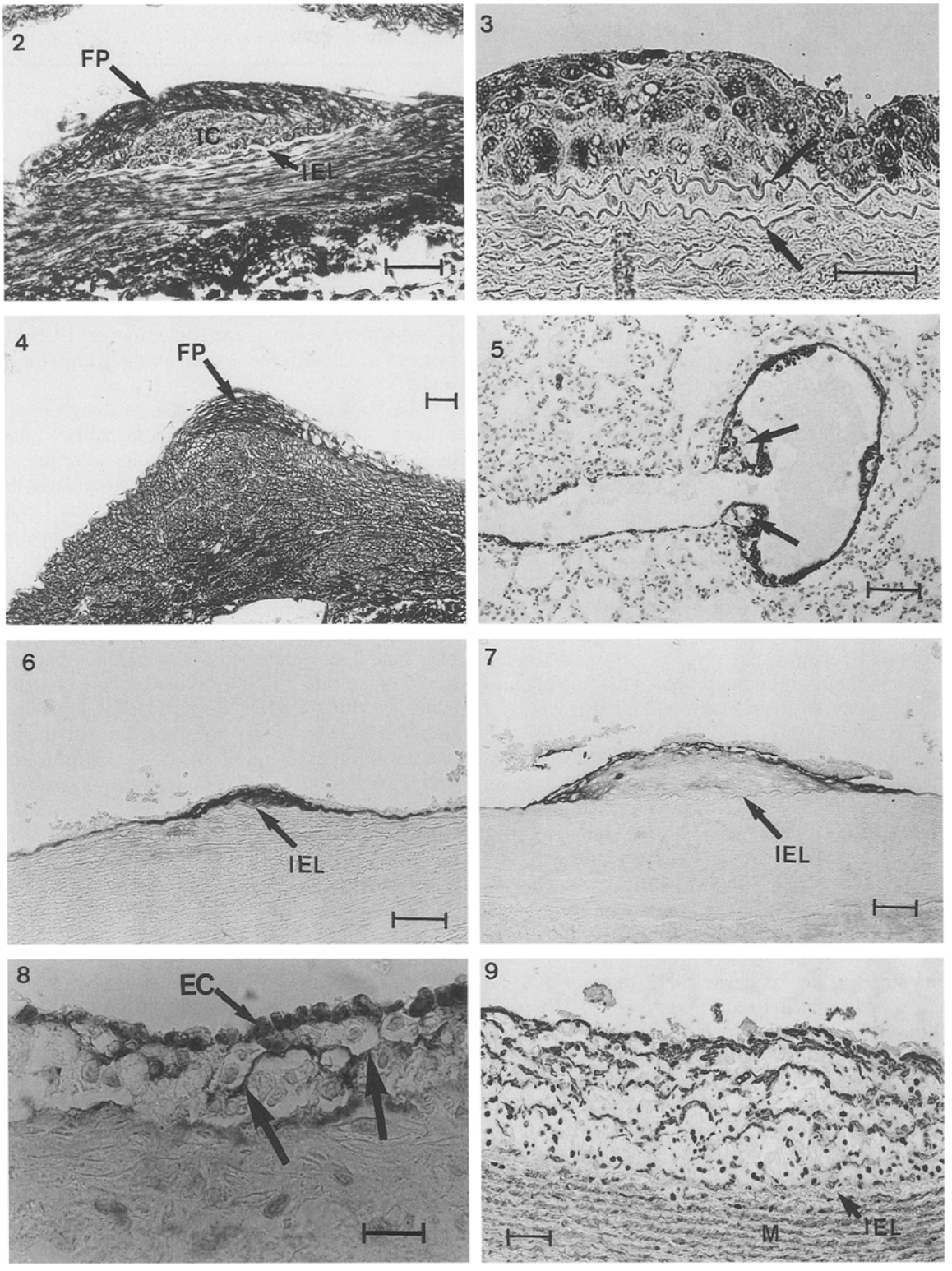

Fig. 8 High power photomicrograph of the endothelial cell (EC) layer, covering a large fibro-cellular plaque, stained for vWf. The EC's demonstrate an enhanced dense granulofibrillar immunoreactivity for vWF. Subendothelial accumulation of vWF is present spreading in between macrophage derived foam cells (MFC; *arrows*). (*Bar* is 20 μm)

Fig. 9 Plaque demonstrating the successive layers of foam cells and SMC (dark cells). The proliferating cell nuclear antigen (PCNA) positive nuclei *(black dots)* are mainly located in the optically empty foam cells. Media (*M*). Carotid artery, 8 weeks 2% cholesterol. Combined immunohistochemical staining for α-SMC actin and PCNA (*bar* is 50 μm)

Table 1 Quantitative data of proliferating cell nuclear antigen (PCNA) nuclei. The lower numbers of the subdiaphragmatic vessels are the expression of the decreased number of foam cells and the fibrotic transformation. (*L* Length of plaque base in circular direction, *H* maximal height of plaque, *A* total area measured per plaque, *N* number of nuclei, *P* percentage of PCNA positive nuclei)

	L (μm)	H (μm)	A ($\times 10^3$ μm^2)	N (per 10^5 μm^2)	P (%)
Carotid	600	230	133	401	74
Coronary	520	480	L51	344	51
Aorta thoracic	3000	330	990	245	27.7
Aorta abdominal (superior)	600	350	209	436	15
Aorta abdominal (inferior)	1500	370	284	161	11.6
Coeliac	1300	705	917	313	8.4
Iliac	650	850	554	359	4
Left renal	450	560	254	380	4
Pulmonary artery	650	540	350	492	19.4
Pulmonary vein	700	390	275	536	18.2

ing segments as well. In the branches of the pulmonary artery they were found on the spurs of the bifurcations. FC were rarely single, but even then they were always covered by EC. FC covered, as a single cell layer, segments occupying up to half of the inner circumference of the vessel. Gradually they presented as segmental accumulations forming five or six layers mainly composed of MFC and forming the macroscopically recognizable foam cell plaques. These could always been clearly recognized whether they were lying on top of a cushion (Fig. 2), a diffuse accumulation (Fig. 3) or medial columns (Fig. 4). FC plaques were also present in intrapulmonary veins around the junctions, between the mergers of smaller and larger branches (Fig. 5). Since LSMC are never present in rabbit pulmonary veins [13], the plaques develop independently, as in many arterial segments not containing LSMC.

Staining for vWF demonstrated a conspicuous increase of immunoreactive material in the EC covering the plaques compared to the adjacent EC (Figs. 6, 7). The positive granules were more numerous and coarser. Subendothelial accumulation of vWF was present and spreading in between MFC was evident (Fig. 8). This increased accumulation was already found in zones with only a few MFC where no SMC or collagen fibres were found. In the 35 week group vWF immunoreactivity was clearly enhanced in and underneath EC even when the plaque was transformed into a fibrous nodule. The positive reaction correlated with the bulging character of the lesions, the non involved segments showing the normal pattern present in the controls.

Collagen fibres appeared in lesions formed by one or two rows of MFC, both underneath and in between the MFC. If the plaque was multilayered the collagen content increased accordingly and each layer was separated from the next by a horizontal (circumferential) sheet of collagen fibres resulting in the formation of a three to four tiered structure. Staining for α-SMC actin disclosed the presence of a dense network of SMC which colocalized exactly with the collagen fibres (Fig. 9). They formed a particularly dense layer just underneath the EC. Many of the SMC contained discrete fat globules but only few were transformed to small or large AFC. Often the media underneath plaques was thinned.

PCNA positive nuclei were found in a very variable number in many plaques. An example is given in Table 1, which represents the quantitative PCNA data of plaques in ten different localizations within the same animal.

The large majority of PCNA positive nuclei were localized in MFC (Fig. 9). The less MFC a plaque contained the less PCNA positive nuclei were present. Within each plaque the MFC were very irregularly distributed but more frequent at the borders or in the "roof". In the endothelium and in the media, PCNA positive nuclei were infrequent.

Progressive replacement of MFC by SMC and collagen fibres was frequent. It was more pronounced in larger plaques but was found in small plaques only composed of five to ten cells. The plaques became finally transformed into a fibromuscular nodule containing remnants of MFC. Often their surface was covered by one row of MFC surrounded by some thin collagen fibres repeating the process of fibrogenesis. Fibrotic transformation was pronounced in all localizations under the diaphragm. The low percentages of PCNA positive nuclei in the foam cell plaques under the diaphragm is due to the low number of MFC and the concomitant fibro-muscular transformation. The bulk of large plaques above the diaphragm, (thoracic aorta and in the pulmonary artery) in the 4 and 8 week groups, was formed by MFC. Fibromuscular plaques which developed on top of LSMC cushions or medial columns could always be well delineated: no incorporation of the pre-existing SMC structures into organized plaques were found.

EM of plaques in the carotids at 4 and 8 weeks showed an uninterrupted layer of EC. They were higher than normal and contained more organelles. Underneath the EC, MFC and SMC were found. The MFC were crowded with large non membrane bound vacuoles, presenting ramified lamellipodia, containing lysosomes and non descript cellular debris, all characteristics of monocyte derived macrophages (Figs. 10, 11). The SMC contained large often dilated profiles of rough endoplasmic reticulum, medium sized optically empty vacuoles not bound by a membrane, few filaments, few pinocytotic vesicles and a fragmented basal lamina, all stigmata of cells with the synthetic phenotype and fat accumulation. The interstitium contained normal collagen fibres amongst irregular moderately electron dense granular material.

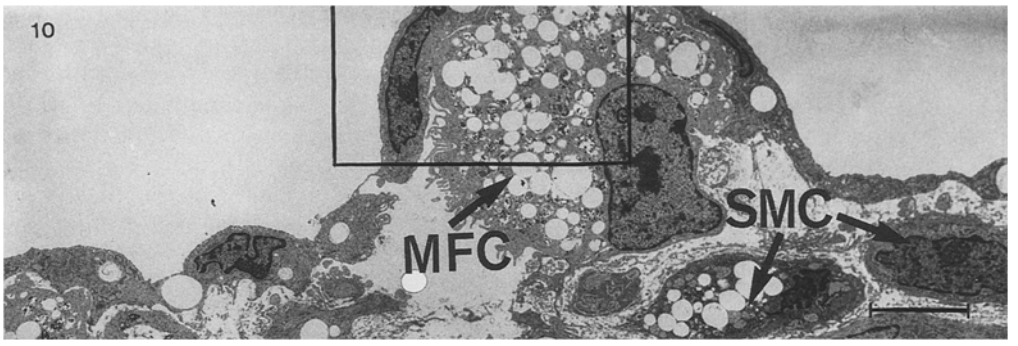

Fig. 10 Transmission electron micrograph of a foam cell plaque in the carotid artery at 4 weeks 2% cholesterol diet. MFC and SMC are present beneath the endothelial cell layer. (*Bar* is 5 μm)

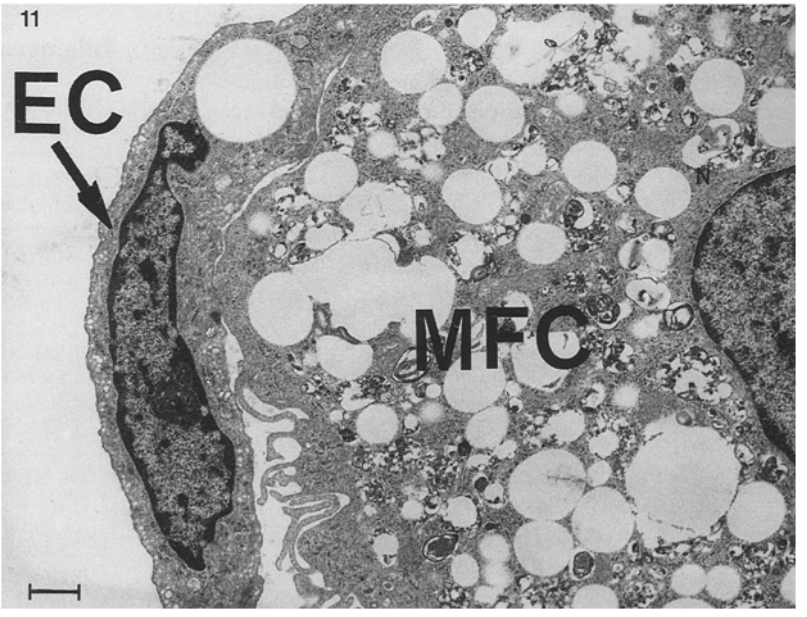

Fig. 11 High power photomicrograph of the boxed area of Figure 10. The cytoplasm of a MFC is crowded with large non membrane bound vacuoles; lysosomes and non descript cellular debris. The cell presents ramified lamellipodia. (*Bar* is 1 μm)

Discussion

This study demonstrates that pre-existing subendothelial LSMC, either diffusely spread or in cushions, are not transformed into FC plaques. This finding is consistent with the results obtained by Stary [22] in human coronary arteries. Fat accumulation in the arterial wall correlates with the distance from the lumen, shows in the initial stages no topographical predilection and is not associated with the LSMC. In our previous study [13] we presented evidence that LSMC structures are an expression of increased circumferential stress implicating a haemodynamic mechanism. FC plaques are frequently associated with anatomical structures like curves or branchings but they occur also frequently in straight non branching arteries of which the common carotid is a good example. It must be stressed that each plaque in each animal has its proper structure and hence comparison between lesions in different animals subjected to different diets during different periods is not indicated. The development of plaques is asynchronous.

The distribution pattern and the cellular composition of the FC lesions have been described with varying emphases [1, 4, 7, 11, 17, 19, 23]. Our study disclosed specific features of the dynamics of plaque development. At the stage of a few FC covered by an uninterrupted layer of EC, SMC and collagen fibres are absent and many MFC contain PCNA immunoreactive nuclei. Thus the lesion not only expands by recruiting monocytes but also by considerable multiplication of FC. Our results confirm to a certain extent those obtained by incorporation of tritiated thymidine [18]. We could not, however, demonstrate a high replication activity in SMC as was reported by these authors, whereas our data for MFC are within their range. As a control of the reliability of PCNA immunoreactivity for demonstration of replicating cells, we compared it with the results obtained after bromodeoxyuridine administration in a previous study [12]. When so many MFC are replicating the question arises about the stimulus for cell replication. Macrophage colony stimulating factor has been demonstrated in endothelial cells and SMC in atheromatous plaques of both human and rabbit arteries [5, 20]. Since monocyte infiltration precedes the SMC infiltration [10], it is possible that the stimulus for MFC replication comes from the EC.

The enhanced vWF content of the EC, covering FC accumulations, and the presence of the factor between

MFC precedes the appearance of SMC in the plaques. Because in the EC adjacent to the plaques the vWF content is not increased it is obvious that the increase is associated with the presence of FC and may constitute a reactive phenomenon or be a manifestation of a change in EC function. Several studies have reported an increased number of Weibel-Palade bodies, which are known to store vWF, in EC on top of atheromatous plaques [3, 8, 24, 25]. The possibility that the vWF may act as an attractant for SMC migration must be considered. Since collagen fibres only appear when SMC are present it is likely that SMC and not the FC are responsible for collagen production, and that a stimulation of the SMC through cytokines of EC or MFC may play a role [21]. However FC are not obligatory to induce vWF production by EC and collagen formation by SMC. In our studies on neo-intimal formation in cuffed carotid arteries in rabbits we have shown the same pattern of vWF reactivity and the formation of collagen fibres in the absence of FC [12, 14].

Plaques grow and MFC with PCNA positive nuclei are most frequent on the slopes and the margins. During plaque development SMC progressively increase in number but show less PCNA positive nuclei than the MFC, so that the possibility of a continuing recruitment from the media must be considered [6].

The fibrous transformation is more pronounced in the abdominal vessels resulting in extensive rather flat lesions comparable to the diffuse fibrous intimal thickening in humans. A preponderance of fibrosis in the abdominal aorta has also been reported in rats [15] and in primates [9]. Some of these superficial MFC contain PCNA positive nuclei, which indicates that even on a fibrous soil which is very different from a normal vessel wall the FC replication continues. In the 35 weeks group the fibrosis was more pronounced and involved the supradiaphragmatic vessels as well. The fibromuscular transformation of the FC plaques has been considered to be a proliferative lesion [9]. However the growth of the plaques is dependent on the multiplication of FC and they stop their expansion once the SMC and their secreted collagen replace the MFC. The process more closely resembles scar formation.

An important practical question is to what extent the hypercholesterolaemic rabbit can be used as a reliable model for human atheromatosis. The lesions, mainly composed of large FC accumulations in the high, short duration, cholesterol fed group are clearly different from human lesions. But the extensive fibrous lesions present in the low, long duration cholesterol fed group mimic human fibrous lesions very well and may be used as a model. A study of rabbits receiving a low cholesterol supplement for a 5 year period gave similar results [26].

We conclude that the pre-existing LSMC either spread diffusely or present in intimal cushions do not transform into FC plaques in cholesterol fed rabbits. The newly formed atheromatous plaques remain clearly separated from the underlying pre-existing LSMC. Moreover FC plaques develop also frequently in vessels without LSMC. We advance the hypothesis that EC react to the presence of underlying MFC by producing or releasing more vWF which diffuses already in the initial stages of FC accumulation and may be a manifestation of a change in EC function. Once SMC are present, the FC plaques acquire collagen fibres, leading to the transformation of the FC plaques into a fibrous scar. The development of the plaques can be attributed not only to monocyte recruitment but also to macrophage multiplication. SMC multiplication is low and the possibility of a persisting migration from the media must be considered.

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