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

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

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 metacarn 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 left ventricle, ascending aorta and adjacent trunk of the pulmonary artery, aortic arch, ascending thoracic and descending thoracic aorta, descending thoracic aorta and left subclavian arteries, 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, Verhoeff's elastica stain...
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 &mu;m 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 &alpha;—SMC actin: we call those cells AFC and the other FC which are monocyte derived, RAM 11 positive and &alpha;—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 &alpha;—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 &mu;m 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 90.5 [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-