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Influence of Hypercholesterolemia on the Morphology of In-Stent Stenosis in an Experimental Animal Model

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Abstract: Post-mortem studies show that in-stent stenosis is characterized by a prolonged proliferation and inflammatory reactions around the stent struts. The aim of this study was to evaluate the influence of hypercholesterolemia on the specific characteristics of in-stent stenosis in an experimental animal model. Palmaz-Schatz stents were placed in the iliac arteries of 20 white New Zealand rabbits. One half of the animals were fed an 0.5% hypercholesterolemic diet, the other half were normocholesterolemic. After 28 days the animals were killed and the stented segments were excised. Immunohistochemistry with antibodies against bromodeoxyuridine, alpha-actin, RAM 11-macrophages and Von Willebrand factor was performed. Morphometric analysis was done on cuts with the stent left in place. Intimal and medial smooth-muscle-cell proliferation was significantly increased in hypercholesterolemic animals, mainly around the stent struts. Inflammatory infiltrates and clusters of macrophages were markedly increased as well, however, they were found predominantly at the edge of the stent, whereas proliferation was highest in the center of the stented segments. These effects contributed synergistically to a marked increase in neointimal stenosis (39% in hypercholesterolemic animals versus 26% in controls).

Key Words: Lipids, atherosclerosis, stent, restenosis, animal model

INTRODUCTION

Atherosclerosis is the disease with the highest mortality and morbidity in the western world. In the US., coronary artery disease, the main manifestation of atherosclerosis, causes two thirds of all deaths already, and the need for revascularization procedures is still increasing. Although several clinical trials show that stent placement reduces restenosis compared to balloon angioplasty, in-stent restenosis, which occurs in 20-50% of cases in daily clinical practice, is a persistent clinical and huge economic problem[1-4]. Several years ago we already demonstrated in rabbits that in-stent restenosis differs from restenosis after conventional PTCA because of an elevated and prolonged proliferation of smooth muscle cells[5], but it has also been shown that restenosis in stents is characterized by a marked inflammatory reaction with infiltrates surrounding the stent wires, composed of lymphocytes, macrophages and foam cells, histiocytes and eosinophils[6,7]. Examinations of human vessels 6 months after stent implantation revealed considerable amounts of extracellular lipids and cholesterol crystals[7]. The pathophysiological link of the vascular LDL and oxidized LDL content with the occurrence of macrophages and foam cells is very important in the development of atherosclerotic lesions[8]. There is increasing evidence and also first clinical data that statins have beneficial effects on the development of in-stent-stenosis[9,10], however, these effects might be, at least in part, explained by other effects of statins beyond lipid lowering, like direct antiproliferative effects[11-13]. Since there is only limited data from clinical and post-mortem studies how much elevated lipid levels contribute to these special characteristics of in-stent stenosis, the aim of this study was to evaluate the effects of hypercholesterolemia on the specific morphology and characteristics of in-stent stenosis in an experimental animal model.

MATERIALS AND METHODS

Animal study: White New Zealand rabbits (2.6-3.3 kg) were obtained by Thomae, Biberach, Germany and kept in our institution for 2 weeks before inclusion in the study. The rabbits were housed individually with 12 h light periods. Animal weights and behavior were monitored daily during the whole study, and the ongoing study was closely supervised by the veterinarian of our institution.

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The study protocol was reviewed by the ethical committee on animal research of our institution and was found to conform to the guidelines on animal care. Twenty animals were used for this study, which were divided randomly into two groups, normocholesterolemic or hypercholesterolemic animals. In order to induce hypercholesterolemia, the animals were fed an 0.5% cholesterol diet during the whole study, starting on the day of intervention. The animals were anaesthetized with ketamine (35 mg kg^{-1}), xylazine (5mg kg^{-1}) and atropine (0.5 mg) IM. Then the femoral artery was exposed, and a 4F sheath was introduced after arteriotomy. Palmaz-Schatz Stents (Johnson and Johnson, Warren, New Jersey, USA) were premounted on a 2.0 mm standard balloon angioplasty catheter (Schneider, Buelach, Switzerland). Before stent implantation, the animals were given 800 IU heparin and 60 mg aspirin intravenously via the sheath. Stents were advanced into the right common iliac artery and expanded at 8 atm for 2x2 min. The resulting stent to artery ratio was 1.3:1. After removal of the sheath, the femoral artery was ligated and the wound was closed. All animals received antibiotic therapy for the following 2 days. The rabbits were looked after for 4 weeks, during this period they were given 60 mg aspirin IM every 3rd day for prevention of stent thrombosis. Four weeks after intervention the animals were given a lethal dose of sodium pentobarbital, and the iliac arteries were excised. During the whole study no animal died on account of anaesthesia, surgical or postsurgical complications.

Histological preparation: Bromodeoxyuridine (BrdU), a thymidine analogue that is incorporated in cells undergoing DNA synthesis, was given to each animal before the excision of the vessels. As described earlier in detail[14], 100 mg kg^{-1} b.w. BrdU and 75 mg deoxycytidine (d-Cyt) (Sigma, Deisenhofen, Germany) were given as a subcutaneous neck depot 18 h before the vessels were excised. 18 and 12 h before the excision of the vessels, additional 30 mg BrdU/kg b.w. and 25 mg d-Cyt/kg b.w. were injected intramuscularly. Before excision, the arteries were perfused in situ with 500 mL of 0.1 M cacodylate-buffered 2% paraformaldehyde solution at a pressure of 80 mm Hg via a catheter inserted into the aorta. After overnight immersion-fixation two thirds of each stented segment were cut off. After step-by-step dehydration the specimens were embedded in methylmethacrylate (Merck, Darmstadt, Germany). Each vessel segment was serially sectioned in 10-15 slices (60-70 µm). Each of the slices per segment was then stained with toluidine blue (cf. also Fig. 1). For quantitative analysis, at least 3 serial cuts at plaque maximum were used and averaged, as well as 3 cuts at the edge of the stented segment.

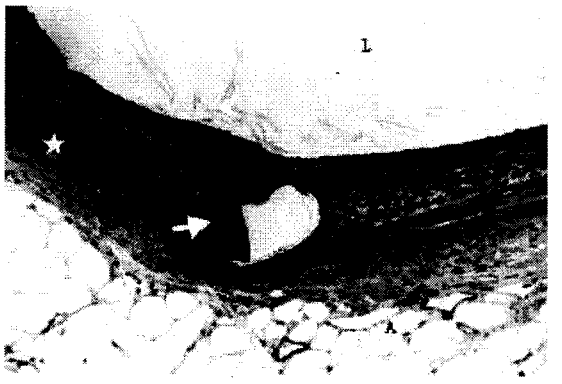

Fig. 1: Close-up of a segment of a normochlesterolemic animal with stent left in place. Toluidine blue–staining of a methylmethacrylate-embedded section. Magnification: x 125. L = Lumen, I = neointima, Δ = internal elastic layer, M = media, A = adventitia, Ø = stent strut

The remaining third of each vessel segment was prepared for immunohistochemistry. After the wires had been removed from the stented region, the specimens were embedded in paraffin. These segments were serially cut in 10 sections (4 µm) at intervals of 50 µm at both ends of the segment. The resulting slices were then used for 5 different staining procedures. Each staining was performed in cross sections of each vessel segment. Histological sections were stained with hematoxylin-eosin and elastica-van Gieson's stain for histomorphometry and histological orientation. In order to identify replicating cells, immunohistological staining was performed with a monoclonal antibody against BrdU (Bio Cell Consulting, Grellingen, Switzerland). Smooth muscle cells were identified with immunohistological alpha-actin staining (Renner, Dannstadt, Germany) and macrophages were detected with RAM 11 antibody[15,16]. As chromagen, either 3-amino-9-ethylcarbazole or 3,3'-diaminobenzidine were used for all the immunohistological stainings. Controls were done by performing the identical staining procedure but omitting the first antibody. The number of proliferating cells were determined by counting the number of BrdU-positive cells, and relating it to $1mm^2$. Toluidine blue-stained sections were quantitatively analyzed by computerized morphometry (microscope from Olympus, Hamburg, Germany, planimetry unit from Summagraphics Inc., Seymour, USA, software from Bilaney Consulting, Duesseldorf, Germany). The perimeters of the luminal border, internal elastic lamina and external elastic lamina as well as neointimal thickness at each wire site were manually traced. Consecutively the area of intima, media, residual lumen and degree of

stenosis (% stenosis = intimal area x 100 / intimal + luminal area) was determined. Furthermore, a semiquantitative inflammation score was assessed[17]: 0: no inflammatory cells around the stent struts, 1: light localized infiltration, 2: dense but localized infiltration, 3: dense infiltration around the whole stent strut.

Statistical evaluation: Mean, median and standard deviation were calculated per animal from the single results, the data are presented as median. The results of the morphometric analysis were quantitatively analyzed with the Wilcoxon test. P-values < 0.05 were considered to identify relevant differences. All analyses were performed with the statistical software GraphPad Prism 2.01 (GraphPad Software, San Diego, CA).

RESULTS AND DISCUSSION

Morphological results: In hypercholesterolemic animals macrophages and foam cells were found in nearly every single cut that was studied, in contrast to animals with normal lipid levels, where virtually no foam cells and very few macrophages were found (cf. Fig. 2). It was impossible to count the number of macrophages because of cluster formation, however, using a semiquantitative scaling score (0 = no macrophages, 1 = macrophage present in up to 25% of the circumference, 2 = 50%, 3 = 75% and 4 = 100% of the circumference) it became obvious that significantly more macrophages were found at the border between stent and normal vessel segment, mainly around the stent struts (cf. also Fig. 3). BrdU-positive cells were also concentrated around the stent struts, but the number of proliferating cells differed markedly between the center portion of the stented segment and the edges of the stent. Figure 4 shows a close-up of a hypercholesterolemic animal (center portion) where many proliferating cells were found, mainly around the stent struts. Both intimal and medial smooth-muscle-cell proliferation was significantly increased in hypercholesterolemic rabbits (H) compared to animals with normal (N) cholesterol levels (mean of intimal proliferation in N: 28 cells/mm^2, in H: 87 cells/mm^2, p=0.008, mean of medial proliferation in N: 39 cells/mm^2, in H: 90 cells/mm^2, p=0.046). The exact data of the proliferation rates are given in Fig. 5. In normocholesterolemic animals both intimal and medial proliferation was significantly higher (p=0.027) in the central parts of the stented region when compared to the edges of the same segment, whereas in hypercholesterolemic animals only medial proliferation was significantly higher in the central parts. Cellular infiltrates around the stent struts as, marker for an inflammatory reaction were reduced slightly in normocholesterolemic animals when compared to hypercholesterolemic animals (mean inflammatory score in N: 1.0, in H: 1.3, p=n.s).

Morphometric Analysis

Four weeks after intervention the residual lumen was smaller in hypercholesterolemic animals than in animals with normal lipid levels (median N: 5.8 mm^2, H: 4.5 mm^2). Together with an increased intimal area (median N: 1.8 mm^2, H: 2.6 mm^2) this led to a marked increase in the

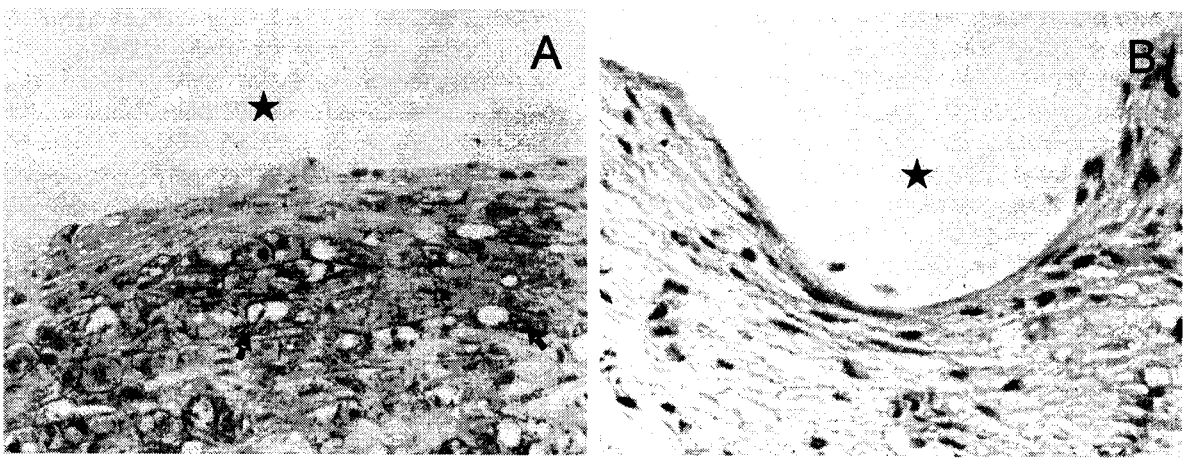

Fig. 2: Area around the stent strut (removed). A:) close-up of a hypercholesterolemic animal. RAM-11-staining. Red-stained cells (Ø) show macrophages that are difficult to count because of cluster formation and foam cells. B: normocholesterolemic anmial with compressed, hypocellular tissue around the (removed) stent strut, but no foam cells and macrophages. Magnification: x 250. * = stent strut (removed)

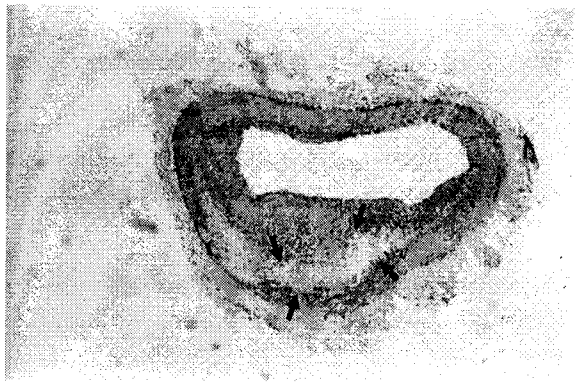

Fig. 3: Hypercholesterolemic animal. Close-up of a section that was taken from the vessel wall immediately behind the edge of the stent. Accumulation of foam cells (Ø) with empty cytoplasm, because the lipid content has been washed out during preparation within the exxentric plaque formation. Alpha-actin staining. Magnification: x 50

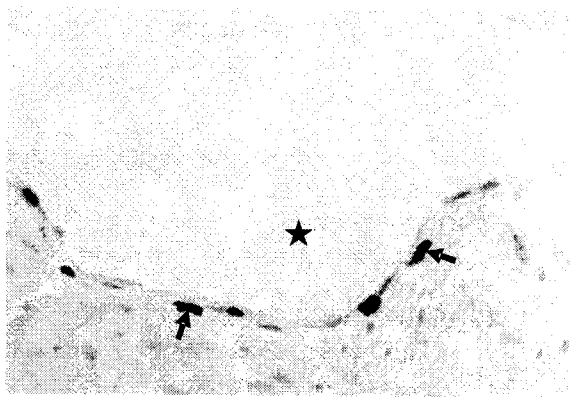

Fig. 4: Close-up of a section of a hypercholesterolemic animal. Numerous red-stained, proliferating cells can be seen aorund the (removed) stent strut. BrdU-staining. Magnification: x 250. L = Lumen, I = neointima, Δ = internal elastic layer, M = media, A = adventitia, * = stent strut (removed), Ø = BrdU-positive cells

extent of neointimal stenosis (median N: 26%, H: 39%). However, these results are not statistically different. The morphometric data are graphically presented in Fig. 6.

The problem of transferring animal data to the human situation is well known. It is obvious that the

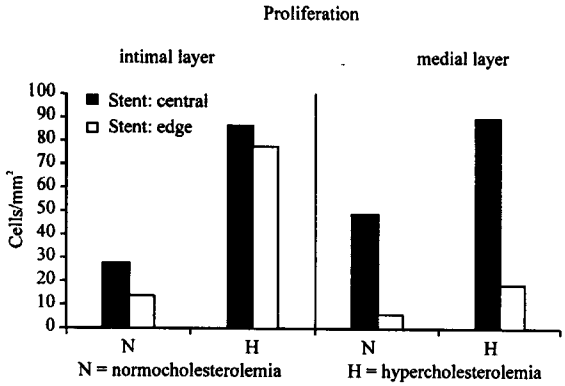

Fig. 5: Proliferation rates in intimal and medial layer. Data are expressed as median

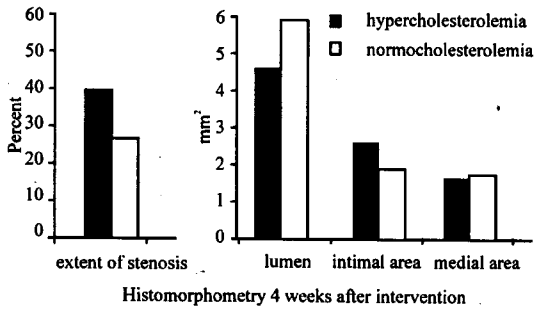

Fig. 6: Morphometric analysis 4 weeks after intervention. White bars stand for normocholesterolemic and black bars for hypercholesterolemic animals. Data are expressed as median

development of plaque formation as well as its composition differs strongly in a herbivore species. However, when looking at the specific influence of elevated lipid levels, this artificial situation as an exaggerated prototype model may also be of some advantage. Therefore we chose rabbits, which rapidly react to atherogenous stimuli and develop extreme hypercholesterolemia of about 650 mg dL^{-1} when being fed a low 0.5% cholesterol diet[14], as species for this animal study. It is known that an additional vascular trauma (e.g. induced by stent implantation) will lead to more cellular atherosclerotic plaques, but the resulting lesions are still rich in foam cells and differ strongly from advanced human plaques with calcifications, central necrosis, ulcerations and thrombus formation[18,19].

As expected, hyperlipidemia led to an increased intimal and medial smooth muscle cell proliferation in this study as well as to an increased neointima formation, as shown in numerous studies before. Therefore the question was raised if a lipid-lowering therapy could

reverse this development and lead to plaque regression. However, it was argued that the positive effects of statins can be observed within 4-6 months, whereas morphological alterations of the coronaries were observed after years[20,21]. The AFCAPS/TexCAPS study indicates that even in primary prevention healthy persons without hypercholesterolemia could benefit from a statin therapy[22,23] and a recent study showed beneficial effects of statin therapy after successful coronary stent implantation in unstable angina pectoris, which were most prominent during the first 4 weeks after the ischemic disease[24]. So there is evidence that the beneficial effects of a statin therapy cannot be explained with the lipid-lowering properties of these substances alone. A very likely explanation is that statins lead to a stabilization of the plaque contributing to the rapid transformation of unstable coronary artery disease into a stable condition[24]. It is known that a high cholesterol content reduces the mechanic stability of the plaque and triggers inflammatory reactions that further destabilize the plaque[25] and even worse, especially in-stent stenosis is characterized by a marked inflammatory reaction with infiltrates surrounding the stent wires, composed of lymphocytes, macrophages and foam cells, histiocytes and eosinophils[6,7]. In this study virtually no macrophages and foam cells were detected in animals with normal lipid levels, which may also be indicative of a strong role of lipoproteins in the above mentioned processes. Interestingly, macrophages and foam cells were predominantly found at the border between stent and normal vessel, although smooth muscle cell proliferation was highest in the central portions of the stent. There is reason to assume that the abrupt gap in vessel wall diameter between overstretched stent and normal vessel wall triggers this accumulation of inflammatory cells. At least, the data of this study may indicate that the advantage of a big initial lumen after stent deployment could be associated with the risk of creating a potentially unstable region at the border between stent and vessel. Therefore clinical studies in patients with different cholesterol levels that underwent coronary stenting are desirable.

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