Angiogenesis, Vascular Endothelial Growth Factor and Platelet-Derived Growth Factor-BB Expression, Iron Deposition, and Oxidation-Specific Epitopes in Stented Human Coronary Arteries

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Abstract—Pathogenesis of in-stent restenosis remains poorly understood because information from human histopathologic studies is scarce. We used an improved saw-grinding and cutting method on methacrylate-embedded samples containing metal stents, which allows in situ hybridization and immunohistochemical analysis of in-stent restenosis. Twenty-one samples were collected 3 hours to 3 years after stenting from 6 patients aged 36 to 81 years. Except in very early samples collected within hours after the stent deployment, neovascularization was present in all segments studied. At advanced stages, extensive neovascularization was located mainly at the luminal side of the stent struts and was only rarely accompanied by inflammatory cells. The neovessels colocalized with vascular endothelial growth factor (VEGF)-A mRNA and protein expression as well as with iron deposits and oxidation-specific epitopes, which imply the presence of chronic oxidative stress. VEGF-A expression was detected in the same areas containing macrophages, endothelial cells, and, to a lesser extent, smooth muscle cells, which also showed platelet-derived growth factor-BB expression. We conclude that in-stent restenosis features neovascularization, VEGF-A and platelet-derived growth factor-BB expression, and iron deposition, which is most probably derived from microhemorrhages. These mechanisms may play an important role in the development of neointimal thickening and could provide useful targets for the prevention and treatment of in-stent restenosis. (Arterioscler Thromb Vasc Biol. 2001;21:1720-1726.)

Key Words: in-stent-restenosis ■ immunochemistry ■ in situ hybridization ■ pathology ■ methylmethacrylate embedding

Percutaneous transluminal coronary angioplasty (PTCA) is the treatment of choice in short single coronary lesions. Acute complications, dissections, and early recoil can be treated or avoided with intracoronary stents, which also reduce the incidence of late restenosis. Nevertheless, angiographic restenosis is seen in 20% to 30% of ideal lesions treated with stents.1,2 The angiographic restenosis rate with more complex lesions is even higher.3 Treatment of in-stent restenosis by balloon dilation, rotablation, and coronary atherectomy leads to unsatisfactory results in at least 50% of the cases.4 Thus, prevention of in-stent restenosis is an important goal.

See cover

In-stent restenosis is thought to be a reparative process resembling wound healing, involving a cascade of traumatic, thrombotic, granulating, and proliferative phases as well as late remodeling, with final accumulation of smooth muscle cells and matrix components.5,6 Glycoprotein IIb/IIIa antagonists aiming at blocking early platelet deposition/thrombus formation and the proliferation and migration of smooth muscle cells reduced but failed to fully inhibit in-stent restenosis.7,8 The amount of medial damage and stent oversizing have been shown to be correlated with the degree of in-stent restenosis, suggesting that the extent of vessel trauma plays a crucial role in in-stent restenosis.9,10

One of the major problems in understanding in-stent restenosis is the lack of histopathologic and gene expression information from human stented vessels. Analysis of samples containing both soft tissues and metal stents is technically demanding.11 Stent strut removal and embedding with ordinary paraffin or snap-freezing result in loss of constituents and artifacts from tissue rupture. In situ implants can be embedded in resin polymers, such as methylmethacrylate (MMA), and processed by hard-tissue cutting.5 We used a saw-grinding method, which improves the quality of the
tissue sections and permits the study of in-stent restenosis by use of immunohistochemistry and in situ hybridization.

In the present study, we examined characteristics of human in-stent restenosis at different time points after stenting. It was found that vessel wall thickening is accompanied by extensive neovascularization, vascular endothelial growth factor (VEGF)-A and platelet-derived growth factor (PDGF)-BB expression, iron deposits, and epitopes characteristic of oxidative stress, which may play important roles in the pathogenesis of in-stent restenosis.

**Methods**

Stented coronary artery segments were obtained during autopsy at the Institute for Pathology of the University Hospital of Hamburg at a mean of 10±4 hours after death. All protocols were approved by the ethics committee of the University Hospital of Hamburg. A total of 21 stented segments (16 from stented coronary arteries and 5 from stented restenotic venous coronary bypass grafts) from 6 patients were studied. In addition, 3 samples from a nonstented coronary artery were analyzed (Table). Intact vessel segments were removed en bloc and immersion-fixed in 4% neutral buffered formalin for 24 hours. Fixed samples were dehydrated in a graded series of ethanol and infiltrated with a 1:1 solution of MMA and xylene and, finally, polymerized blocks were initially ground to bring the tissue components closer to the cutting surface. After the opposite side of the block was glued to a Plexiglas slide with Technovit 4000 (Kulzer & Co) in vacuum, a 1200-grit grinding in a micro parallel grinding system (EXAKT, Apparatebau) was used, followed by 4000-grit sandpaper grinding, which smoothed the final lower surface of the samples. After it was cleaned with benzene, this surface was glued to a second glass slide in vacuum. Subsequently,
a 100-μm-thick section attached to the second slide was sawed by using a diamond blade saw and 50- to 100-g pressure. The final thickness of ~10 μm was achieved by grinding and final polishing steps with 1200-, 2400-, and 4000-grit sandpaper according to methods described. It should be noted that because of the saw-grinding method, adjacent serial sections were 200 to 300 μm apart. Sections from each block were used for toluidine blue staining without deplastination. Additional sections were deplastinized and used for immunocytochemistry and in situ hybridization analysis.

Serial sections (5 μm) of the same MMA blocks were cut on a Leica 2500 SM sliding microtome with hard tissue blades (Leica). After immersion in a drop of 80% ethanol, sections were stretched to a fold-free state on Superfrost glass slides (Menzel-Gläser), covered with a polyethylene sheet and several layers of filter paper, and tightly pressed on the glass slides, followed by overnight drying at 42°C under pressure. Deplastination was carried out in 2-methoxyethyl acetate for 45 to 90 minutes. Rehydration of the sections was performed in graded ethanol solutions and 1 mmol/L PBS. Toluidine blue, hematoxylin and eosin, and Masson's trichrome stainings and Prussian blue reaction for iron/hemosiderin detection were performed according to standard histopathologic methods.

**Immunocytochemistry**

Sections were heated for 10 minutes at 90°C in 0.1 mol/L citrate buffer for antigen retrieval. Immunohistochemical stainings were performed with a Vectastain Elite kit (Vector Laboratories). Endogenous peroxidase was blocked by incubation for 20 minutes with 0.3% H2O2 in methanol, followed by 30 minutes of incubation with Zymed CAS blocking solution (Zymed Laboratories). Sections were then incubated for 1 hour with primary antibody and rinsed, and secondary antibody was added for 30 minutes. Avidin-biotin complex was added for 30 minutes, and signals were detected by using 3,3-diaminobenzidine tetrahydrochloride (Zymed Laboratories), SG (Vector Laboratories), or 3-amino-9-ethylcarbazole (Zymed Laboratories). Smooth muscle cells were detected with monoclonal antibody (mAb) HHF-35 against α-actin (dilution 1:50, Enzo Diagnostics), macrophages with mAb recognizing CD68 (1:50, Dako), endothelial cells with mAbs recognizing CD31 (1:20, Dako) and CD34 (1:10, Dako), T cells with mAbs recognizing CD3 (1:100, Novocastra), VEGF-A with mAb sc-7269 (1:100, Santa Cruz Biotechnology), PDGF-BB with goat antisem (1:200, R&D Systems), and oxidized epitopes with anti–hydroxynonenal (HNE)-LDL (HNE-7) antisem (1:1000). Biotinylated secondary antibodies were purchased from Vector Laboratories and used at a dilution of 1:200; FITC-labeled anti-mouse secondary antibody was from Dako (1:100). Controls for immunostaining included sections incubated with class- and species-matched irrelevant antibodies and incubations in which the primary antibody was omitted.

**In Situ Hybridization**

VEGF-A mRNA was localized by in situ hybridization with riboprobes as described. Human VEGF-A cDNA was subcloned in pBluescript SK (Stratagene) by using standard techniques, and full-length antisense and sense riboprobes were synthesized by using T3 and T7 polymerases with [33P]UTP (NEN Life Science Products). Deplastinized and rehydrated sections were pretreated with proteinase K (37°C, 30 minutes), dehydrated, and dried. After hybridization, the sections were washed with 4× standard saline citrate (SSC) at 37°C. Unspecifically bound probes were digested with RNase A treatment at 37°C for 30 minutes, followed by washes in decreasing SSC concentration at 37°C, with the final wash at 52°C in 0.1× SSC for 15 minutes. Slides were then coated with Kodak NTB-2 autoradiographic emulsion (Eastman-Kodak), exposed for 2 to 6 weeks, and counterstained with hematoxylin-eosin or Prussian blue. For nonradioactive probes, signal detection was carried out by using an...
Early Phase With Thrombus Deposition

Under microscopic examination, all stented segments from coronary arteries and venous bypass grafts were affected by fragmentation of elastic lamellae, necrosis of the media, and tears of fibrous caps and vessel walls (Figures 2a through 2c). Freshly placed stents showed thrombotic material adjacent to almost every stent strut, which was still seen 2 weeks after the stenting (Figures 2c, 3a, and 3b). Within 2 weeks, the struts were covered by highly cellular neointimal tissue (Figures 2a through 2c, 3a, and 3b). At regions of relatively normal vessel wall, high neointimal cellularity showing mainly smooth muscle cells (HHF35). The presence of oxidation-specific epitopes was detected around the stent struts (Figures 2h, 2k, 2l, and 3e). VEGF-A protein was detected in serial sections. Multinucleated giant cells were also detected around some stent struts (Figures 2h, 2k, 2l, and 3e). The tissue consisted of a diffuse macrophage, smooth muscle cell, and endothelial cell mixture resembling granulation tissue (Figure 2e through 2g). The same regions showed iron deposition, which was also seen at nearly every stent strut (Figure 2d, 2k, and 2l). VEGF-A mRNA and protein were detected around the stent struts (Figures 2h, 2k, 2l, and 3e). Macrophages were detected at every stent strut (Figures 2f, 3c, and 3d). The presence of oxidation-specific epitopes was detected in early restenotic tissue (Figure 3f and 3g). Complete thrombotic occlusion and early thrombus organization due to disruption of the neointimal intima occurred in 2 of the 5 venous bypass graft segments investigated.

Late Phase With In-Stent Restenosis

Two to 3 years after stenting, extensive in-stent restenosis was found in all investigated segments. The neointimal thickness was maximally 10 times that of the media (Figure 4a). The luminal part of the neointima was composed of dense connective tissue, containing tightly layered smooth muscle cells (Figure 4d and 4k). PDGF-BB colocalized to smooth muscle cells beneath this tightly layered area, to the endothelium, and to cells of the deeper parts of restenotic tissue (Figure 4f) at the same locations where macrophages were detected in serial sections. Multinucleated giant cells resembling foreign body reaction were detected around some stent struts (Figure 4f). Multinucleated giant cells were also positive for PDGF-BB immunostaining (data not shown). The

Results

We found that improved tissue processing techniques (ie, saw-ground preparations) preserved original morphology much better than cutting in a hard tissue microtome, in which section scratch artifacts, folds, and distortions were seen in every section, whereas in saw-ground sections, these were absent, and only 22% of the stent struts were lost. Patient data and general microscopic characteristics of the analyzed sections are listed in the Table. As a control, we obtained 3 samples from a patient who died 2 days after PTCA without stent placement (Table, Figure 1). These segments showed a fibroatheroma with thromboemorrhagic complications (Figure 1a). Abundant iron deposition was detected within the lesion extending to the fibrous cap, shoulder region, atheromatous core, and the adjacent media (Figure 1a and 1b). In the shoulder region, neovascularization was present in the same areas as VEGF-A mRNA expression (Figure 1b through 1g).
deep neointima near the stent struts was composed of loose connective tissue containing intense neovascularization at the luminal side of the struts, as shown by CD31 and CD34 immunostaining (Figure 4c and 4k). At all cross-sectional levels investigated, most of the struts were accompanied by multiple capillaries (Figure 4c and 4k). There was abundant iron deposition (Figure 4b) in the proximity of the stent struts accompanied by capillaries. Within the neovessel areas, cells expressed VEGF-A mRNA, as detected by in situ hybridization (Figure 4h and 4i). Oxidation-specific epitopes were detected in iron-storing cells in neovessel areas (Figure 4j). The mixed hypercellularity of early restenotic lesions was changed in the late stages toward a dense accumulation of smooth muscle cells in the upper luminal part of the lesions (Figure 4d and 4k), whereas scattered macrophages were present near the stent struts (Figure 4e and 4k). Late in-stent restenosis also contained fibrous tissue, especially in areas between the struts where cellularity was low. In these regions, no neovessel formation was observed.

Discussion

The present study focused on histological phenomena and gene expression in in-stent restenosis. We used an improved saw-grinding method for the analysis of stented coronary artery samples. This technology allowed excellent preservation of tissue morphology and the use of immunohistochemistry and in situ hybridization on methacrylate-embedded sections. We found that neovascularization was a prominent feature in in-stent restenosis. Iron deposits, VEGF-A and PDGF-BB expression, and oxidation-specific epitopes were also present in the same areas.

The first event in restenosis is believed to be thrombus formation, followed by growth factor release from platelets. We found thrombus deposition on stent struts in every section of early lesions. However, glycoprotein IIb/IIIa receptor antagonists, which inhibit platelet deposition and the proliferative stimulus of platelets on smooth muscle cells and their migration by cross-reaction with vitronectin receptors, fail to fully inhibit clinically relevant restenosis formation. Thus, other contributing factors are also involved in the formation of in-stent restenosis.

Immunohistochemically detectable proliferative cellular nuclear antigen has proven the proliferative nature of in-stent restenosis. We found that PDGF-BB was involved in the proliferative response by immunohistochemistry. Macrophages were common, with the formation of multinucleated giant cells typical of a foreign body reaction covering nearly every stent strut investigated. The foreign body reaction probably contributes to the pathogenesis of restenosis, inasmuch as we detected PDGF-BB protein expression in the multinucleated giant cells. Neovessels have also been described as part of the neointimal formation by others, who have suggested that granulation tissue reaction is involved in the formation of in-stent restenosis, consistent with our observations in early lesions. In general, foreign body reaction should lead to granuloma and/or scar tissue formation encapsulating the foreign body. This was not the case; instead, in late stages, we detected loose highly capillarized tissue with less inflammatory cell infiltrates. Expression of VEGF mRNA and protein near stent struts could provide a mechanism for the formation of neovessels. In the samples obtained 2 years after stenting, histology around the stent struts was still characterized by multiple capillaries with very wide lumina and loose connective tissue without scar tissue formation, which would be the physiological consequence of granulating trauma healing.

Rigid metal stents may possibly cause continuous mechanical trauma in the vessel wall, followed by microhemorrhages around the stent struts. This is supported by the abundant iron deposits found in the stented vessels. In atherosclerosis, iron deposition is an established histological feature generated by repeated hemorrhagic episodes that are due to ulcerations and ruptures of the fibrous cap. Iron is a potent pro-oxidant, and oxidation-specific epitopes were detected within and
around iron-containing cells. Iron may induce oxidation of lipoproteins, which might amplify proinflammatory signals in stented vessels.24,25 Consistent with these findings, the antioxidative drug probucol has been reported to reduce the rate of restenosis after PTCA, and iron chelation has inhibitory effects on smooth muscle cell proliferation.26,27 Interestingly, angiogenesis inhibitors reduce atherosclerosis in atherosclerosis-prone mice.28

The process leading to in-stent restenosis is a multifactorial cascade involving thrombosis, neovascularization, and VEGF-A and PDGF-BB expression, as well as sustained tissue damage caused by rigid metal struts. We show in the present study that neovascularization, VEGF-A expression, iron deposition, and oxidized epitopes colocalize in proximity to the stent struts. It is likely that these factors play an important role in the development of in-stent restenosis and could provide useful targets for prevention and treatment of in-stent restenosis.

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