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

Jan Hinrich Bräsen, Antti Kivelä, Kerstin Röser, Tuomas T. Rissanen, Mari Niemi, Friedrich C. Luft, Karl Donath, Seppo Ylä-Herttuala

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 ▪ immunohistochemistry ▪ 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

Received June 20, 2001; revision accepted July 5, 2001.
From the A.I. Virtanen Institute (J.H.B., A.K., T.T.R., M.N., S.Y.-H.) and the Department of Medicine (A.K., S.Y.-H.), University of Kuopio, Kuopio, Finland; Franz Volhard Clinic (J.H.B., F.C.L.), Department of Medicine Charitè, Humboldt University, Berlin, Germany; and the Department for Pathology (K.R., K.D.), University of Hamburg, Hamburg, Germany.
Reprint requests to Seppo Ylä-Herttuala, MD, PhD, A.I. Virtanen Institute, University of Kuopio, PO Box 1627, FIN-70211 Kuopio, Finland. E-mail seppo.ylaherttuala@uku.fi
© 2001 American Heart Association, Inc.
Arterioscler Thromb Vasc Biol. is available at http://www.atvbaha.org

1720
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 at MMA and xylene (4°C, 12 hours each). Infiltrated specimens were placed into embedding molds, and polymerization was performed at 1:1 solution of MMA and xylene and, finally, with 0.5% (4°C, 12 hours each). Infiltrated specimens were placed into embedding molds, and polymerization was performed at −15°C.13 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 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 deplastinated 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-Glaser), 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 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/γ-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 antiserum (1:200, R&D Systems), and oxidized epitopes with anti–hydroxynonenal (HNE)-LDL (HNE-7) antiserum (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 [33 P]UTP (NEN Life Science Products). Deplastinated 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

Figure 1. Histological features of serial sections of nonstented left anterior descending coronary artery 2 days after PTCA from a 63-year-old man. Orange color is contrast material; arrows indicate colocalization of the stains described. a, Overview exhibiting a cross section of the whole circumference with abundant iron deposition (blue). A thrombus adhering to the fibrous cap is present. The latter shows layers of iron deposition and enhanced eosinophilia due to fibrinoid necrosis. Frame indicates location shown in panels b through g (Berlin blue reaction, original magnification ×1.25). b, Higher magnification of the marked area. c, Immunostaining for macrophages (CD68), which can be observed in the shoulder region and necrotic fibrous cap, where smooth muscle cells are not present. d, Immunostaining for smooth muscle cells (HHF35). e, Immunostaining for endothelial cells (CD31) in the marked area. f, In situ hybridization for VEGF-A mRNA with an antisense riboprobe (dark blue spots). g, Negative sense control (the same area as shown in panel f). Original magnifications were as follows: ×1.25 (a), ×10 (b through d), and ×20 (e through g). Stains are as follows: a and b, Berlin blue reaction; c through e, hematoxylin counterstain; and f and g, nonradioactive in situ hybridization.
Under microscopic examination, all stented segments from patients who died 2 days after PTCA without stent placement (Table, Figure 1) showed a fibroatheroma with thrombohemorrhagic 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).

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 and 3c). 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, 3c, and 3d). At regions of relatively normal vessel wall, high neointimal cellularity showing mainly smooth muscle cells (HHF35). The neointima 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, 2l, and 3e). These 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, 2l, and 3e). The region of inflammation was composed of dense connective tissue, containing tightly layered smooth muscle cells (Figure 4d and 4k). PDGF-BB localized 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 4l). Multinucleated giant cells were also positive for PDGF-BB immunostaining (data not shown). The

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 localized 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 4l). Multinucleated giant cells were also positive for PDGF-BB immunostaining (data not shown).
deeper 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.

Acknowledgments

This study was supported by the Deutsche Forschung Gemeinschaft (grant Br 1616/2-1), the Finnish Academy, Sigrid Juselius Foundation, and EVO grant 5130 from Kuopio University Hospital. We thank Dr Joe Witztum for kindly providing HNE-7 antibody and Marja Poikolainen for preparing the manuscript.

References


van Breusekom HM, van der Giessen WJ, van Suylen R, Bos E, Bosman FT, Serruys PW. Histology after stenting of human saphenous vein bypass grafts: observations from surgically excised grafts 3 to 320 days after stent implantation. \(1993;21:45–54.\)


Angiogenesis, Vascular Endothelial Growth Factor and Platelet-Derived Growth Factor-BB Expression, Iron Deposition, and Oxidation-Specific Epitopes in Stented Human Coronary Arteries
Jan Hinrich Bräsen, Antti Kivelä, Kerstin Röser, Tuomas T. Rissanen, Mari Niemi, Friedrich C. Luft, Karl Donath and Seppo Ylä-Herttuala

doi: 10.1161/hq1101.098230

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/21/11/1720

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Arteriosclerosis, Thrombosis, and Vascular Biology can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online at:
http://atvb.ahajournals.org/subscriptions/