Mechanisms of Neointima Formation and Remodeling in the Porcine Coronary Artery

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Background—To characterize the cells responsible for neointima formation after porcine coronary artery wall injury, we studied the expression of smooth muscle cell (SMC) differentiation markers in 2 models: (1) self-expanding stent implantation resulting in no or little interruption of internal elastic lamina and (2) percutaneous transluminal coronary angioplasty (PTCA) resulting in complete medial rupture and exposure of adventitia to blood components.

Methods and Results—The expression of α-smooth muscle (SM) actin, SM myosin heavy chain isoforms 1 and 2, desmin, and smoothelin was investigated by means of immunohistochemistry and Western blots in tissues of the arterial wall collected at different time points and in cell populations cultured from these tissues. The expression of smoothelin, a marker of late SMC differentiation, was used to discriminate between SMCs and myofibroblasts. Both stent- and PTCA-induced neointimal tissues and their cultured cell populations expressed all 4 markers. The adventitial tissue underlying PTCA-induced lesions temporarily expressed α-SM actin, desmin, and SM myosin heavy chain isoforms, but not smoothelin. When placed in culture, adventitial cells expressed only α-SM actin.

Conclusions—Our results suggest that SMCs are the main components of coronary artery neointima after both self-expanding stent implantation and PTCA. The adventitial reaction observed after PTCA evolves with a chronology independent of that of neointima formation and probably corresponds to a myofibroblastic reaction. (Circulation. 2001; 103:882-888.)

Key Words: angioplasty ■ stents ■ muscle, smooth ■ myofibroblasts ■ smoothelin

Percutaneous transluminal coronary angioplasty (PTCA) is essential in the treatment of coronary disease.1 Restenosis, occurring in 30% to 40% of patients, represents a major limitation of this procedure. The advent of coronary stenting has resulted in a significant (30% to 50%) decrease of restenosis rate.2 However, the new entity of “in-stent restenosis” resistant to any available therapy has emerged.3

According to a contemporary paradigm, several phenomena are involved in restenosis development after PTCA.4 One is proliferation and migration of medial smooth muscle cells (SMCs) into the intima.5 It was recently suggested that adventitial myofibroblasts are capable of traversing the ruptured media and thus participate in the formation of neointimal thickening.6,7 Another is unfavorable constrictive remodeling of the artery, possibly related to activation and proliferation of adventitial fibroblasts.6,7 It is noteworthy that stent implantation eliminates the constrictive remodeling of the artery but does not interfere with neointima formation.4

It is conceivable that implantation of a self-expanding stent (Wallstent) into a porcine coronary artery leads to a chronic injury of the wall, resulting in the migration of medial SMCs into the intima.8 Conversely, a balloon overstretch injury, resulting in complete rupture of the media and exposure of the adventitia to blood components, could lead (in addition to SMC stimulation) to activation and proliferation of adventitial fibroblasts, followed by their inward migration and participation in neointima formation.6,7 To acquire more knowledge on the origin of cells responsible for neointima formation in both models, we studied chronologically the expression of well-established SMC differentiation markers, ie, α-smooth muscle (SM) actin,9 SM myosin heavy chain (MHC) isoforms 1 and 2,9 desmin,9 and smoothelin,10 in the arterial wall layers after stent implantation or PTCA. We also studied the same differentiation markers in cell populations cultured from these tissues under normal conditions and after both injuries. The expression of smoothelin, a marker of late SMC differentiation, was used to discriminate between SMCs and myofibroblasts.11

We demonstrate that (1) a self-expanding stent induces a neointima composed of cells expressing all SMC markers...
tested, without producing a myofibroblastic reaction in the adventitia; (2) PTCA-induced neointima essentially contains cells with SMC features; and (3) the myofibroblastic reaction taking place in the adventitia after PTCA evolves independently of neointima formation.

Methods

Animals and Standard Procedure Protocol
Domestic crossbred pigs (Sus scrofa) weighing ~40 kg were premedicated with azaperone (60 mg/10 kg; Janssen Pharmaceutica), midazolam (10 mg; Roche Pharma), and 0.25 mg atropine IM. The pigs were sedated by continuous injection of fentanyl (0.2 mg/h IV). After endotracheal intubation, the animals were ventilated with halothane (1.5 vol%; Zeneca), nitrous oxide, and oxygen to maintain anesthesia throughout the experiment. The left common carotid artery was surgically exposed, and after insertion of the introducer (8F, Inuit, PS; Bard), acetylsalicylic acid (1 g; Synthelabo) and heparin (Liquemine 100 IU/kg; Roche Pharma) were administered intra-arterially. The coronary ostia were cannulated, and arteries were then injured either by direct self-expanding stent implantation (Wallstent, Schneider) without subsequent balloon inflation or by an oversized PTCA balloon inflation (Bonnie, Schneider) with a balloon-to-artery diameter ratio of 1.2 to 1.3. The balloon was inflated 3 times for 30 seconds. Noninstrumented coronary arteries and coronary arteries of pigs from a nearby slaughterhouse served as controls. After closure of cervicotomy and local antibiotic treatment, the animals received 10^6 IU of penicillin G (Hoechst-Pharma) IM. The pigs were euthanized under general anesthesia by whole-heart exsanguination, and coronary arteries were dissected from the surrounding myocardium. Histology and immunohistochemistry were performed on specimens collected at 4 (stent and PTCA, n=1), 7 (stent, n=3; PTCA, n=4), 15 (stent and PTCA, n=3), and 30 (stent, n=3; PTCA, n=4) days. Tissues for digestion and protein extract preparation were harvested from normal media (n=5), 30-day-old stent- and PTCA-induced neointimas (stent, n=3; PTCA, n=1 for protein extraction and n=3 for digestion), and adventitial tissue either normal (n=3) or underlying 7-day-old PTCA-induced injury (n=3). As controls for adventitial fibroblasts, we placed in culture subcutaneous fibroblasts from the skin of the neck. All animal procedures were performed according to federal veterinary guidelines and approved by the Ethical Committee of the Geneva Medical Faculty.

Histology and Immunohistochemistry
Normal coronary arteries and segments containing a stent- or a PTCA-induced lesion were fixed with 4% neutral buffered formalin and embedded in paraffin or snap-frozen in precooled liquid isopentane and embedded in OCT resin (Miles Scientific). Paraffin-embedded sections were stained with hematoxylin and eosin and Miller, Landrum, or Alcian blue stains. For indirect immunofluorescence, cryostat sections were stained with mouse monoclonal IgG2a specific for a-SM actin,9 mouse monoclonal IgG1 specific for desmin (Clone D33; Dako), rabbit polyclonal IgG specific for SM MHC (Biomedical Technologies Inc; this antibody does not recognize nonmuscle isoform B1), or mouse monoclonal IgG1 specific for smoothelin10 as previously described.9 Appropriate second antibodies coupled to fluorescein and rhodamine were applied as previously described.9 Slides were observed with a Zeiss Axioshot photomicroscope (Carl Zeiss) equipped with a Plan-NeoFluar ×63/1.4 objective as previously described.9 A 3D image corresponding to the projection of all optical sections in 1 plane was reconstructed with the Imaris program (Bitplane) running on an Octane Silicon Graphics Workstation. Images were processed with Adobe Photoshop 5.0 (Adobe System). Cell Culture
The intimal thickening present between the struts and the lumen was separated from the stent and the underlying media with 2 ophthalmologic tweezers. Lesion sites induced by PTCA were localized with the help of binoculars once the vessel was opened longitudinally, and the intimal thickening was collected with a sharp ophthalmologic curette. Normal adventitia was stripped free of medial tissue. All dissected tissues were validated by histology. Normal media, intimal thickenings, and adventitia were digested, and isolated cells were cultured in DMEM (Gibco BRL, Life Technologies) containing 10% FCS (HyClone) as previously described.9

Protein Extraction, Electrophoresis, and Western Blotting
Dissected tissues were immediately frozen in liquid nitrogen and crushed. Tissues and cell cultures were processed for SDS-PAGE, with protein load adjusted according to the method of Bradford, and immunoblotted as previously described.9 In separate experiments, filters were incubated with either anti-a-SM actin, anti-SM MHC, anti-desmin, or anti-smoothelin antibodies.9,10,12 Signals were digitized by means of an Arcus II scanner (Agfa) and analyzed with Image Quant software (Image Quant Analysis; Molecular Dynamics). Values were normalized to the pixel values measured in normal media.

Results

Histology of Neointima Formation After Stenting or PTCA
Representative pictures of coronary artery segments with stent- or PTCA-induced lesions, stained for elastin and collagen at 7, 15, and 30 days, are shown in Figure 1. The
internal elastic lamina was only slightly compressed in stented arteries (Figure 1, a, c, and e), whereas both internal elastic lamina and media were interrupted in all arteries after balloon angioplasty (Figure 1, b, d, and f). In both models, deposition of fibrin and accumulation of inflammatory cells were seen at 4 days (data not shown); from 7 days on, spindle-shaped cells started to accumulate within the neointima (Figure 1, a and b) and gradually increased in quantity up to 30 days. Stent implantation induced an inflammatory reaction characterized by the presence of macrophages around the metallic struts. Fifteen days after PTCA, spindle-shaped cells infiltrated the space left by media interruption and extended below the external elastic lamina (Figure 1d, arrow). Thirty days after stent implantation or PTCA, the neointima was always thicker than the adjacent media (Figure 1, e and f).

Remarkably, no significant changes were observed in the adventitia of stented arteries (Figure 2a). In contrast, the adventitia underlying PTCA-induced lesions showed clear inflammatory and fibrotic changes with a progressive accumulation of collagen, whereas the neointima was strongly positive for Alcian blue staining, as previously described. At 30 days, the adventitia of PTCA-treated arteries was always thicker than that of noninjured or stented arteries (Figure 2b).

Expression of SMC Differentiation Markers After Stent or PTCA
Stented coronary arteries were double-stained for either α-SM actin and smoothelin or SM MHC and desmin at 7, 15, and 30 days (Figure 3; Table). At 7 days, 16% of the cells of the thin neointima expressed α-SM actin and SM MHC (Figure 3, a and g, respectively; Table). The same pattern of staining was observed for smoothelin and desmin; however, only 44% and 58% of SMCs positive for α-SM actin and SM MHC were also positive for smoothelin and desmin, respectively (Figure 3, d and j; Table). At 15 days, 93% of the neointimal cells were positive for α-SM actin and SM MHC (Figure 3, b and h; Table), of which 99% and 76% were positive for smoothelin and desmin, respectively (Figure 3, e and k; Table). At 30 days, 95% of neointimal cells were positive for α-SM actin, SM MHC, and smoothelin (Figure 3, c, i, and f, respectively; Table), but only 49% were positive for desmin (Figure 3l; Table); areas negative for SMC markers were limited around the struts of the stent. Practically all SMCs in the underlying media expressed all 4 differentiation markers regardless of the time of study; only areas around the stent struts, containing inflammatory cells,
The adventitial fibroblasts in stented arteries remained negative for all differentiation markers at 4 (data not shown), 7, 15, and 30 days. Taken together, these data suggest that a self-expanding stent induces a neointima composed of SMCs derived from the underlying media.

In 7-day-old PTCA-induced lesions, 26% of the cells filling in the gap induced by media interruption expressed \( \alpha \)-SM actin and SM MHC (Figure 4a and g; Table); of these, 44% were positive for smoothelin and 94% positive for desmin (Figure 4d and j, Table). Smoothelin was more frequently observed in neointimal cells adjacent to the interrupted media (Figure 4d). After 15 days, 94% of the cells filling the gap were expressing \( \alpha \)-SM actin and SM MHC (Figure 4b and h; Table), of which 95% and 70% were positive for smoothelin and desmin, respectively (Figure 4, e and k; Table). After 30 days, the pattern of staining was similar to that of 15 days (Figure 4, c, f, i, and l). \( \alpha \)-SM actin and SM MHC were present in 98% of neointimal cells (Figure 4, c and i; Table); of these, 96% and 55% coexpressed smoothelin and desmin, respectively (Figure 4, f and l; Table). The intensity of smoothelin staining was variable; in \( \approx \)23% of intimal cells, smoothelin staining, although appreciable, was weaker than that of the remaining intimal cells. This could be related to a variable degree of differentiation of intimal SMCs.

Seven days after PTCA, the adventitia contained cells positive for \( \alpha \)-SM actin (Figure 4a) and desmin (Figure 4j); and to a lesser extent SM MHC (Figure 4g). At 15 days, the adventitial cells expressing \( \alpha \)-SM actin (Figure 4b) were still numerous, whereas only a few expressed SM MHC and desmin (Figure 4, h and k, respectively). At 30 days, only a few adventitial fibroblasts expressed \( \alpha \)-SM actin, and all were negative for SM MHC and desmin (Figure 4, c, i, and l, respectively). No smoothelin-positive cells were detected in the adventitia at any time point (Figure 4, d, e, and f). These data are compatible with a myofibroblastic reaction of the adventitia after PTCA.

For a more detailed analysis of \( \alpha \)-SM actin and smoothelin expression in PTCA-induced neointima, CLSM was used. In 7-day-old PTCA-induced neointima, all spindle-shaped cells expressed \( \alpha \)-SM actin. Among them, some coexpressed smoothelin importantly (Figure 5, double arrow, yellow color), and others showed either weak (single arrow, yellow and green) or no (arrowhead, only green) smoothelin expression.

Immunoblots of total protein extracts obtained from the normal media, intimal thickening 30 days after stent implantation or PTCA, normal adventitia, and adventitia underlying 7-day-old PTCA confirmed the results obtained by immunofluorescence staining (Figure 6). \( \alpha \)-SM actin, SM MHC isoforms 1 and 2, desmin, and smoothelin were clearly expressed in both stent-induced (Figure 6, lane g) and

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<th>Days After Injury</th>
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Percentages of Neointimal SMCs Expressing Differentiation Markers at Different Times After Injury

![Figure 4.](http://example.com/figure4.png) Expression of differentiation markers in coronary arteries after PTCA. Immunofluorescence staining showing expression of \( \alpha \)-SM actin (a through c) and smoothelin (d through f) or SM MHC (g through l) and desmin (j through l) at 7 (a, d, g, j), 15 (b, e, h, k) and 30 (c, f, i, l) days. Cells in neointima express all differentiation markers, including smoothelin, whereas adventitial fibroblasts express differentiation markers to some extent, with exception of smoothelin. Note that at 30 days, adventitial staining has practically disappeared. This evolution is similar to that of a reactive granulation tissue. Dotted lines highlight limit of media/neointima. L indicates lumen. Bar=200 \( \mu \)m.
PTCA-induced (Figure 6, lane h) neointima. In stent-induced neointima, the content of $\alpha$-SM actin was decreased to 70%, that of SM MHC 1 to 60% and of SM MHC 2 to 30%, that of desmin to 8%, and that of smoothelin to 25% compared with normal media (Figure 6, lanes g and f). In PTCA-induced neointima, the content of $\alpha$-SM actin was decreased to 40%, that of SM MHC 1 to 40% and of SM MHC 2 to 35%, that of desmin to 6%, and that of smoothelin to 20% compared with normal media (Figure 6, lanes h and f). The normal adventitia was negative for all 4 differentiation markers (Figure 6, lane i), whereas in adventitia underlying 7-day-old PTCA-induced lesions, the content of $\alpha$-SM actin was 3% and that of desmin 8% compared with normal media (Figure 6, lanes j and f), and no SM MHC (despite some cells positive by means of immunofluorescence, see Figure 4g) or smoothelin was observed (Figure 6, lane j).

Cells were isolated by enzymatic digestion from media and adventitia of normal coronary arteries and from stent- or PTCA-induced neointima at 30 days. At passage 5, immunobots showed that cells from the stent-induced neointima expressed all 4 markers, including smoothelin (Figure 7, lane f). The content of $\alpha$-SM actin was decreased to 40%, that of SM MHC 1 to 70% and of SM MHC 2 to 75%, that of desmin to 15%, and that of smoothelin to 40% compared with normal media (Figure 7, lanes f and e). Cells derived from PTCA-induced neointima also expressed all 4 markers (Figure 7, lane g). The content of $\alpha$-SM actin was decreased to 50%, that of SM MHC 1 to 30% and SM MHC 2 to 2%, that of desmin to 15%, and that of smoothelin to 35% compared with normal media (Figure 7, lanes g and e). Cultured adventitial fibroblasts (h) express only $\alpha$-SM actin.

Discussion

To efficiently prevent and/or cure restenosis, it is of paramount importance to identify cells responsible for both
neointima formation and constrictive remodeling. For this purpose, we studied 2 distinct types of injury chronologically in the pig (currently a well-accepted animal model in this respect\(^\text{14}\)): (1) self-expanding stent implantation, inducing a chronic injury without rupture of the internal elastic lamina and of the media and (2) PTCA-induced lesions, which result in complete medial interruption and expose the adventitia to the circulating blood, thus facilitating fibroblast activation in the adventitia.\(^\text{6,7}\) Our results suggest that SMCs are the main component of the neointima after both self-expanding stent and balloon overstretch injuries. They are in agreement with current views concerning the pathogenesis of stent-induced lesions,\(^\text{9}\) whereas the pathogenesis of PTCA-induced lesions has up to now remained controversial.\(^\text{6,7,15–17}\)

**SMC Marker Expression in Tissues of Porcine Coronary Arterial Wall**

We have used well-established markers of SMC differentiation in general and of vascular SMC differentiation in particular, such as \(\alpha\)-SM actin, SM MHC, desmin, and smoothelin. These markers have been extensively characterized during the embryonic and fetal development of smooth muscle.\(^\text{18}\) The notions that \(\alpha\)-SM actin is the earliest marker to appear during SMC development\(^\text{18}\) and that smoothelin is a late differentiation marker of SMCs are currently well accepted.\(^\text{10,11}\) These markers, with few exceptions for \(\alpha\)-SM actin,\(^\text{19}\) are not expressed in fibroblasts of normal tissues but can be expressed in myofibroblasts present in granulation tissue or fibrocontractive diseases,\(^\text{19}\) with the exception of smoothelin, which represents a selective SMC marker.\(^\text{11}\)

As previously described in other models,\(^\text{20}\) cells accumulating in the neointima after PTCA first dedifferentiated, then gradually acquired a positive staining for SMC differentiation markers, including smoothelin, with practically the same time course as neointimal cells after stent implantation. At no time after stent implantation did adventitial fibroblasts express 1 of the 4 markers. In contrast, after PTCA, adventitial fibroblasts modulated into myofibroblasts, as indicated by the positive staining for \(\alpha\)-SM actin, SM MHC, and desmin and the negative staining for smoothelin. It is noteworthy that the chronology of adventitial myofibroblastic modulation was clearly different from that of SMC differentiation within the neointima; moreover, this adventitial reaction was reversible, as seen during granulation tissue formation or many fibrotic reactions.\(^\text{21}\)

In an elegant study in which adventitial fibroblasts were labeled with bromodeoxyuridine, Shi et al\(^\text{15}\) showed that during the remodeling of an autologous vein graft, a proportion of neointimal cells was derived from perivascular fibroblasts. It has been shown that an important degree of apoptosis takes place in vein graft SMCs\(^\text{22}\); thus, the recruitment of perivascular fibroblasts could be specific for this procedure. Further studies using smoothelin antibodies may be useful to clarify this point.

When proliferation and SMC marker expression were investigated in the rabbit carotid artery injury model, a myofibroblastic reaction appeared to take place in the adventitia, and neointima formation consisted of medial SMCs with only a modest adventitia-to-media cell migration.\(^\text{16}\) When fibroblasts transduced with retroviral particles encoding \(\beta\)-galactosidase were implanted in the adventitia of the rat carotid artery, some of these cells could be found in neointima formation after balloon injury.\(^\text{17}\) The relative proportions of SMCs and fibroblasts participating in neointima formation in that model remain to be established.

**SMC Marker Expression in Cultured Cells**

We and others recently reported that cultured porcine coronary artery SMCs maintain a high degree of differentiation in vitro.\(^\text{8,23}\) contrary to what has been reported for rodent SMCs.\(^\text{18,24}\) In the present study, SMC marker expression was very similar in the populations of neointimal SMCs cultured after either stent or PTCA. Both populations maintained the expression of all markers, including smoothelin, at passage 5, indicating a SMC rather than a fibroblastic origin. In contrast, cultured adventitial fibroblasts showed a SMC marker expression profile clearly different from that of neointimal cells and similar to that of subcutaneous fibroblasts; in particular, they did not show smoothelin expression. Moreover, it has been reported that porcine coronary SMCs behave differently in vitro from adventitial fibroblasts and from SMCs of elastic arteries.\(^\text{23}\) All these data support, albeit indirectly, the SMC origin of intimal lesions.

**Study Limitations**

We cannot exclude the possibility that in our model, a small proportion of fibroblasts participate actively in neointima formation. It is conceivable, although far from proven, that once myofibroblasts have migrated within the media, they are submitted to the influence of local microenvironmental factors facilitating full SMC differentiation, including smoothelin expression. As discussed above, this possibility is not reflected by the results of experiments in culture, where adventitial fibroblasts never expressed smoothelin, whereas neointimal cells derived from both stent lesions and balloon overstretch lesions expressed all SMC markers, including smoothelin. Coculture experiments of SMCs and adventitial fibroblasts may provide interesting information on the possibility that SMCs modulate the phenotypic profile of fibroblasts and vice versa. Another limitation of our study is that injuries were performed on nonatherosclerotic arteries.

**Conclusions**

Our results support the view that SMCs are the main contributor to neointima formation after stent implantation and balloon overstretch injury and thus appear to represent an appropriate target for preventive and therapeutic interventions based on the modulation of their mitotic, apoptotic, and motile activities. In contrast, adventitial fibroblast activation, and in particular the fibrotic and retractile activities of these cells,\(^\text{19}\) may be involved in negative remodeling after PTCA and hence should be considered in preventive and therapeutic strategies of restenosis after this intervention.

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