Extracellular Matrix Changes in Stented Human Coronary Arteries

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Background—Restenosis after stenting occurs secondary to the accumulation of smooth muscle cells (SMCs) and extracellular matrix (ECM), with the ECM accounting for >50% of the neointimal volume. The composition of the in-stent ECM has not been well characterized in humans.

Methods and Results—Postmortem human coronary arteries (n=45) containing stents underwent histological assessment of neointimal proteoglycans, hyaluronan, collagen (types I and III), SMCs, and CD44 (a cell surface receptor for hyaluronan). The mean duration of stent implantation was 18.7 months; stents in place ≥3 to <9 months (n=17) were assigned to group 1, stents ≥9 to <18 months old (n=19) to group 2, and stents ≥18 months old (n=9) to group 3. In groups 1 and 2, neointimal versican and hyaluronan staining was strongly positive, colocalized with α-actin–positive SMCs, and was greater in intensity compared with group 3. Conversely, decorin staining was greatest in group 3. The neointima of both group 1 and 2 stents was rich in type III collagen, with reduced staining in group 3. Type I collagen staining was weakest in group 1 stents, with progressively stronger staining in groups 2 and 3. SMC density and stent stenosis were significantly reduced in group 3 stents compared with groups 1 and 2. CD44 staining colocalized with macrophages and was associated with increased neointimal thickness.

Conclusions—The ECM within human coronary stents resembles a wound that is not fully healed until 18 months after deployment, followed by neointimal retraction. ECM contraction may be a target for therapies aimed at stent restenosis prevention. (Circulation. 2004;110:940-947.)

Key Words: atherosclerosis ■ pathology ■ restenosis ■ stents
microscopy and paraffin embedding. (The remainder of the stent was submitted for methylmethacrylate embedding.) Sections were cut at 5 μm and stained with hematoxylin and eosin, Movat pentachrome, and picrosirius red.

Immunohistochemistry
Paraffin-embedded sections were evaluated for neointimal macrophages, SMCs, and vascular channels with CD68, smooth muscle α-actin, and factor VIII immunostains, respectively. Neointimal ECM molecules (versican, hyaluronan, inter-α-trypsin, biglycan, and decorin) were stained with methods outlined previously. Inter-α-trypsin inhibitor was detected with a polyclonal antibody (Dako) at a dilution of 1:100. CD44 was detected with a monoclonal antibody (Binding Site Ltd) diluted 1:80.

Histological Assessment
Immunohistochemical staining of the neointima was semiquantitatively graded on a 0 to 3+ scale corresponding to no staining (grade 0), variably detectable (weak) staining (grade 1), moderate staining (grade 2), and strong staining (grade 3). Picrosirius red–stained sections were viewed under polarized light to assess the presence of type I (yellow) and III (green) neointimal collagen. For morphometric measurements, sections were digitized, and computer-guided measurements were performed (IPLab Spectrum Software, Scanalytics Inc) to measure stent area, lumen area, and neointimal thickness at each stent strut (with mean neointimal thickness calculated). Four ×40 high-power fields from the mid portion of the neointima were selected, and nuclei were counted to calculate cell density. The total area of neointimal SMCs was measured from α-actin–stained sections (BioQuant, R&M Biometrics). The presence or absence of CD44 immunostaining was assessed at each stent strut.

Statistical Analysis
Numerical data are presented as mean±SD. Continuous variables were compared by use of ANOVA. A value of P≤0.05 was considered significant.
Results

Patient and Stent Characteristics

Forty-five stented coronary arteries from 36 patients (22 men, 14 women; mean age, 61±14 years) with stents in place 18.7±18.5 months (range, 3 to 60 months) were analyzed. There were 17 group 1 stented arteries (≥3 to <9 months’ implant duration), 19 group 2 arteries (≥9 to <18 months), and 9 group 3 arteries (≥18 months). The indications for stenting were unstable or post–myocardial infarction angina (n=14), stable angina (n=9), acute myocardial infarction (n=1), and unknown (n=12). The following stents were present: MULTI-LINK, n=29; AVE, n=7; Palmaz-Schatz, n=4; Gianturco-Roubin, n=1; and Gianturco-Roubin 2, n=1. Arteries stented were left anterior descending (n=21), right coronary (n=15), left circumflex (n=4), ramus intermedius (n=2), left obtuse marginal (n=2), and left diagonal (n=1) arteries. Patient mortality was due to sudden cardiac death (n=15), postcoronary revascularization (n=9), acute myocardial infarction (n=1), and noncoronary death (n=11).

ECM Staining

Strong neointimal staining for versican was observed in group 1 and 2 stents; the mean versican staining grade was significantly greater than in group 3 (Figure 1). In group 2, versican staining was relatively stronger in the mid and superficial neointima compared with the deep neointima, with a similar distribution (but fainter) of staining in most group 3 stents (Figure 2). Hyaluronan deposition was similar in intensity for both group 1 and 2 stents and was significantly greater than in group 3 (Figure 1). The distribution of hyaluronan staining within the neointima (superficial, mid, and deep regions) paralleled versican staining (Figure 2). Versican and hyaluronan staining colocalized with α–actin–positive SMCs (Figure 2). Immunostaining for inter–α–trypsin inhibitor, a serum protease inhibitor, colocalized with hyaluronan (Figure 3). Staining for biglycan was relatively stronger in groups 2 and 3 (P for trend<0.06, group 3 versus 1; Figure 1) and was equally distributed throughout the neointima (Figure 4). Decorin staining was absent to weak in most group 1 and 2 stents (graded as 0 or 1+ in 93% and 94%, respectively), with significantly increased staining in group 3 (Figures 1 and 4). When present, decorin staining was also observed within the walls of microvessels in the deep neointima.

The neointima of both groups 1 and 2 was rich in type III collagen (91% and 95%, respectively, having 2 to 3+ staining) associated with a significantly higher staining grade compared with group 3 stents (Figure 5). Neointimal type I collagen was sparse in group 1 stents (67% with 1+ staining), with significantly stronger staining in group 2 (81% with 2+ staining) and group 3 (89% with 3+ staining; Figure 5) stents.

Neointimal SMC Density, Stent Stenosis, and CD44 Staining

SMCs in the versican- and hyaluronan-rich regions (groups 1 and 2) of the mid portion of the neointima were stellate in shape and randomly arranged within the ECM. SMC content was similar in group 1 and 2 stents; 57±25% of the neointima was α–actin positive (cell density, 2979±586 cells/mm²) in group 1 stents versus 66±22% α–actin positive (cell density, 3111±510 cells/mm²) in group 2 stents (Figure 6). Neointimal cell density within group 3 stents (2109±835 cells/mm²) was significantly reduced compared with groups 1 and 2, with only 12±13% of the neointima being α–actin positive (P<0.001 versus groups 1 and 2). Maximal stent area stenosis was 76.8±15.7% (diameter stenosis, 54.6±16.6%) for group 1 stents, 69.5±16.4% (diameter stenosis, 47.6±18.0%) for group 2 stents, and 48.0±17.7% (diameter stenosis, 28.8±12.0%) for group 3 stents (P<0.005 group 3 versus groups 1 and 2; Figure 6). Immunostaining for CD44 colocalized with KP-1–positive macrophages deep in the neointima (Figure 7). Positive CD44 staining was present in 48% and 42% of group 1 and 2 stent struts, respectively, compared with 12% of group 3 struts (P<0.0001). Neointimal thickness was greater in overlying struts associated with CD44–positive cells (0.68±0.31 mm) compared with CD44–negative struts (0.45±0.29 mm; P<0.0001).
Discussion

Experimental animal and human autopsy studies have demonstrated that local arterial responses to stenting follow a response-to-injury sequence of events.\textsuperscript{4,5,14–16} Initially, successful stenting always produces arterial injury (intimal plaque disruption typically accompanied by medial injury). In humans, coronary stents elicit platelet/fibrin deposition around struts and an initial acute inflammatory cell response within 0 to 3 days. By 2 to 4 weeks, acute inflammation subsides and is replaced by chronic inflammatory cells; proliferating SMCs are seen in the early neointima associated with organizing thrombus and a thin provisional ECM. Beyond 30 days, fibrin and chronic inflammation may persist, and SMCs and ECM further enrich the expanding neointima.\textsuperscript{17}

The present study extends these observations to a more chronic phase of neointimal evolution. The neointima in stents in place up to 18 months (groups 1 and 2) remains hypercellular (confluent SMCs) and rich in type III collagen, versican, and hyaluronan with relatively little type I collagen and decorin. In contrast, stents ≥18 months old (group 3) demonstrated weaker staining for versican, hyaluronan, and type III collagen and stronger staining for type I collagen and decorin. Neointimal cell density, area occupied by SMCs, and in-stent stenosis were smaller in group 3 stents compared with group 1 and 2 stents. These data demonstrate that neointimal lesions at 18 months after stenting resemble wounds that are not fully healed and suggest that neointimal retraction occurs subsequently.

A previous study from our laboratory demonstrated positive associations among arterial injury, inflammation, and neointimal growth.\textsuperscript{18} Specifically, the neointimal area occupied by macrophages was 3-fold higher in restenotic versus nonrestenotic cases. In the present report, CD44 colocalized with neointimal macrophages deep in the hyaluronan-rich neointima near stent struts and was associated with increased neointimal thickening. Inter-α-trypsin inhibitor, which cross-links hyaluronan into stable structures and facilitates macrophage adhesion,\textsuperscript{19} colocalized with hyaluronan staining. CD44 is associated with inflammatory cell recruitment and vascular cell activation and is a cell surface receptor for hyaluronan.\textsuperscript{21–23} Expression of CD44 on activated macrophages that bind to hyaluronan promotes tyrosine kinase activity and mediates macrophage growth factor and chemokine synthesis, leading to continued SMC synthesis of proteoglycans.\textsuperscript{23}

Wound Healing

In wound healing, best characterized by dermal injury responses, a thrombotic and acute inflammatory reaction is followed by a granulation tissue phase (macrophages infiltration, myofibroblast in-growth, and angiogenesis).\textsuperscript{24} The early ECM consists of fibrin, fibronectin, versican, and hyaluronan.\textsuperscript{25,26} Hyaluronan provides the matrix into which mesenchymal cells migrate, promotes cell proliferation, and supplies feedback regulation of growth factor synthesis.\textsuperscript{27–30} Versican binds to hyaluronan and affords viscoelasticity to healing tissues.\textsuperscript{3} Over time, there is progressive hyaluronan degradation, reabsorption of a portion of type III collagen, and synthesis of type I collagen, decorin, and biglycan.\textsuperscript{6,31} These later changes, with cross-linking of type I collagen, are associated with wound contraction.\textsuperscript{24,32} In uncomplicated dermal wounds, healing is usually complete by 2 weeks; in contrast, the present study demonstrates that complete healing after coronary stenting requires at least 18 months, and the presence of a foreign body (stent) and underlying atherosclerosis may further delay the process.
Experimental Animal Studies of ECM Development

Balloon-injured rat carotid arteries demonstrate hyaluronan staining around proliferating medial SMCs at 3 days, with peak staining of the developing neointima (colocalizing with peak SMC proliferation) at 7 days. There was a progressive decline in neointimal hyaluronan up to 8 weeks after injury. In the rabbit double-iliac injury model, Strauss et al observed that SMC proliferation peaked at 1 week, but there was a progressive increase in lesion size until 12 weeks because of glycosaminoglycan and collagen synthesis. In the atherosclerotic primate iliac artery angioplasty model, intimal thrombus is replaced by proliferating actin-positive cells from day 7 to day 14 after injury. Hyaluronan was observed in the neointima at 7 days after injury and persisted for at least 20 days. Staining for versican was similar to that of hyaluronan at 28 days but diminished by 120 days. Neointimal decorin accumulation is a late event after arterial injury and is not upregulated up to 8 weeks after balloon denudation. From 2 to 6 months, stented porcine coronary arteries show a reduction in neointimal hyaluronan associated with reduced neointimal type III and increased type I collagen.

Human Studies of ECM Development

Analysis of coronary and peripheral atherectomy specimens from restenosis lesions, obtained 5 days to 17 months after angioplasty, showed strong hyaluronan staining around stellate SMCs. Hyaluronan staining was strongest where collagen staining was weakest and vice versa. In a
follow-up study of peripheral artery restenosis, atherectomies (obtained 13 days to 36 months after angioplasty) demonstrated strong versican and biglycan staining associated with SMCs with little type I collagen.35 Furthermore, patchy biglycan staining interwoven with type I and III collagen with negative decorin staining was observed in atherectomies from coronary restenotic lesions (1 to 19 months after angioplasty).36 Studies of coronary in-stent restenosis atherectomy specimens suggest that over time, the myxomatous composition of the neointima (proteoglycans and hyaluronan) decreases with an increase in collagen content.37 It should be noted that atherectomy studies are limited because of sampling (blind biopsies of restenosis tissue and native plaque) and an inability to orient samples for optimal sectioning. The present postmortem study provides greater insights into the neointimal remodeling process via study of complete arterial cross sections.

Neointimal Regression
Several preclinical and clinical studies have documented neointimal regression after long-term follow-up after catheter-based interventions. Rat carotid artery in-stent neointimal size decreases between days 28 and 60, which is associated with reduced versican and hyaluronan content and increased type I collagen.9 There is a gradual thinning of the in-stent neointima in canine coronary arteries by 12 months after stenting, accompanied by reduced cellularity and increased fibrosis.38 In a study of stented porcine coronary arteries, Kim et al34 reported a 33% reduction in stent area stenosis at 6 compared with 2 months.

In humans, coronary arteries with Palmaz-Schatz stents showed a small but significant increase in angiographic minimal lumen diameter at 3 years versus 6 months.39 Kuroda et al40 performed 6- and 12-month follow-up imaging studies in patients with coronary artery stents. Angiography demonstrated a significant 10% increase in minimal lumen diameter corresponding to a 22% reduction in diameter stenosis at 12 months. Over the same time interval, intravascular ultrasound showed a 13% increase in lumen volume and a 28% reduction in neointimal volume.40 Thinning of the in-stent neointima (increased transparency) 3 years after stenting has been visualized by coronary angioscopy.41 The present study provides pathological insights into these observations of neointimal regression: beyond 18 months, the stent neointima becomes less cellular (potentially via SMC apoptosis) and richer in type I collagen.

Clinical Implications
Most published clinical trials of coronary stenting evaluate target vessel success or failure and major adverse clinical events at ≤12 months’ follow-up. Taken together, the results of the present study, previous investigations of the neointimal ECM, and reports of late neointimal regression suggest that at least until 18 months, the neointima remains incompletely healed and retains the potential for shrinkage and further morphological changes.42 Late neointimal ECM contraction (negative remodeling) in which water-trapping proteoglycans (hyaluronan and versican) are replaced by decorin and type 1 collagen should reduce in-stent stenosis and is the likely mechanism for neointimal regression. These data should further discourage the “oculostenotic reflex” as a justification for repeated intervention in patients with angiographic in-stent restenosis in
the absence of symptoms or objective evidence of ischemia. Furthermore, these data may be relevant to the emerging field of drug-eluting stents, in which few long-term data have been presented and publications of larger trials report ≤12 months of follow-up.43,44 It can be postulated that the advantage of a drug-eluting device over a bare metal stent at 9 months may be reduced by regression of the neointima in the bare metal stent (as a result of neointimal regression) at a later time point (perhaps 2 years) when the neointima may be increasing within a drug-eluting stent.

Furthermore, the in-stent ECM should be considered a potential target for new approaches for in-stent restenosis prevention or treatment. Currently, promising antirestenosis therapies use stents that elute antiproliferative drugs (sirolimus and paclitaxel). The goal of a novel ECM modification treatment would be to accelerate the wound healing process via reductions in neointimal SMC synthesis of water-entrapping ECM proteins (versican and hyaluronan), accompanied by a relative increase in decorin and type I collagen. For example, rat balloon-injured carotid arteries that overexpress bovine decorin via gene transfer demonstrate reduced neointimal size and reduced versican staining with increased type I collagen.33

Study Limitations
As an autopsy study, the results presented may not be representative of persons who receive stents and survive without stent-associated morbidity. However, ECM remodeling is likely to be a universal finding in human coronary stents.

Conclusions
The neointima retains the morphology of a nonhealed wound consisting of SMCs associated with water-entrapping matrix macromolecules (hyaluronan and versican) up to 18 months after stent deployment. These features suggest the potential for late neointimal regression beyond 18 months after stenting and offer an alternative target to antiproliferative and antiinflammatory therapies for restenosis prevention.

References
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