Neointima Formation After Vascular Stent Implantation
Spatial and Chronological Distribution of Smooth Muscle Cell Proliferation and Phenotypic Modulation
Hong-zhi Bai, Junichi Masuda, Yoshiki Sawa, Susumu Nakano, Ryota Shirakura, Yasuhisa Shimazaki, Jun Ogata, Hikaru Matsuda

Abstract Intravascular stents have proved useful as angioplasty devices, but intimal hyperplasia after stent implantation remains an unsolved problem. In the present study, we analyzed the spatial and chronological distribution of proliferation and phenotypes of smooth muscle cells (SMCs) in rabbit aortas during the process of neointima formation after stent implantation (Gianturco’s Z type) by immunohistochemistry for proliferating cell nuclear antigen (PCNA) and myosin heavy chain isoforms (SM1, SM2, and SMemb). Stent implantation induced regional injury in the arterial wall. Medial SMCs then began to proliferate adjacent to the injured SMCs, maximally on day 4 (PCNA index in the media: 3.9±3.4% [mean±SD]), and were modulated to the embryonic phenotype (SMemb-positive and SM2-negative). They migrated into the intima and proliferated most frequently on day 7 (PCNA index in the intima: 20.3±5.5%) and subsequently led to fibrocellular neointima formation at 2 weeks and later. At 1 month after implantation and later, SMC proliferation was rare, and the phenotype of intimal SMCs was gradually returning to the adult type (SMemb-negative and SM2-positive). Thus, this stent implantation model demonstrates that the regional effect on arterial wall by stenting leads to neointima formation through transient and regional proliferation and migration of SMCs and their phenotypic modulations. (Arterioscler Thromb. 1994;14:1846-1853.)

Key Words • stent • restenosis • smooth muscle • myosin heavy chain isoform • proliferating cell nuclear antigen

Intravascular stents have been extensively investigated as tools of angioplasty and proved useful in elastic recoil, restenosis, and dissection after balloon angioplasty.1 They have also been applied to various sites of stenotic lesions, including coronary arteries.1-4 Several clinical trials have demonstrated that intracoronary stenting may be more effective than percutaneous transluminal coronary angioplasty (PTCA) in maintaining long-term vessel patency and may reduce the incidence of restenosis after angioplasty.5-9 Such reduction in the restenosis rate is considered to be due to a larger acute gain obtained by stent implantation, although late loss of diameter is greater after stenting than that after PTCA.5-9 Thus, restenosis after stenting still remains an unsolved problem.

Pathological specimens of restenotic lesions after stenting show intimal hyperplasia, composed of abundant smooth muscle cells (SMCs) mixed with various degrees of connective tissue matrix.2,10-12 Therefore, many attempts have been made to reduce the intimal hyperplasia—systemic or local drug administration,13-15 stent coating with heparin16 or genetically engineered cells,17 and modification of stent material such as tantalum18 and biodegradable polymer19—but no clinical success has been achieved. Extensive investigation on the processes of neointima formation after stenting is thought to be necessary to make such attempts successful.

In the present study, therefore, we histologically examined the process of neointima formation in rabbit aortas after stent implantation and analyzed the spatial and chronological distribution of the cellular components as well as SMC proliferation and phenotypic modulation with immunohistochemistry.

Methods

Animals
Twenty-six adult male New Zealand White rabbits weighing 3.4 to 4.0 kg (Kbl-NZW, Nagano, Japan) fed on a standard diet (ORC4, Oriental Yeast Corp) were used. Procedures were in accordance with the Guide for Care and Use of Laboratory Animals issued by the US Institute of Laboratory Resources.

Stent Description
The stent used was a 12-mm-long self-expandable device with an expanded diameter of 7.0 mm made of 0.2-mm stainless steel wire (SUS304) with five bends in a zigzag pattern, known as Gianturco’s Z type stent (Fig 1).1,20 Two stents were implanted in the descending thoracic aorta of each rabbit.

Stent Implantation
General anesthesia was induced with xylazine (5 mg/kg IM) and ketamine (5 mg/kg IM) followed by additional adminis-
Adjoining aortic tissue, both proximal and distal to the stented site, was also examined in the same manner. Paraffin sections were stained with hematoxylin and eosin, Elastica van Gieson, and by immunohistochemistry as described below. The other half was frozen in OCT compound and the specificities in immunostaining were identical to those of the previously described polyclonal antibodies generated in rats or mice.21-23

Immunohistochemistry on Paraffin-Embedded Sections

Electron micrographs were taken at 3,000× magnification. The middle of the three paraffin sections from each aorta with stent were used for quantification because the wires of the stent aligned at an equal distance. Intimal and medial areas were measured by a computerized image-analysis system (Image Command 5098, Olympus) on the sections with Elastica van Gieson staining, and the area of the stent wires was excluded from the measurement. Intimal thickening was expressed as intimal index by intimal/medial area. The number of PCNA-positive nuclei was counted in the intima and media separately by the same observer. The number of PCNA-positive and methyl green-positive nuclei was also expressed as intimal index by intimal/medial area. The number of PCNA-positive nuclei was expressed as the PCNA index, defined as the ratio of the number of PCNA-positive nuclei to that of all the nuclei, in the intima and media.

Quantification of Intimal Thickening and Cell Proliferation

The middle of the three paraffin sections from each aorta with stent was used for quantification because the wires of the stent aligned at an equal distance. Intimal and medial areas were measured by a computerized image-analysis system (Image Command 5098, Olympus) on the sections with Elastica van Gieson staining, and the area of the stent wires was excluded from the measurement. Intimal thickening was expressed as intimal index by intimal/medial area. The number of PCNA-positive and methyl green-positive nuclei was also counted in the intima and media separately by the same image-analysis system at ×400 magnification. The frequency of cell proliferation was expressed as the PCNA index, defined as the ratio of the number of PCNA-positive nuclei to that of all the nuclei, in the intima and media.
Results

Early Death or Complications

None of the animals died or experienced serious complications throughout the experimental period, and all were killed on schedule. The aortic diameter was 6.3±0.2 mm (mean±SD) on angiography before stenting, and no significant change was observed after stent placement.

Histology of Control Animals

Gross examination of the arterial segments from the two nonoperated control animals demonstrated no macroscopic abnormalities. Microscopic observation of paraffin sections showed the presence of no SMCs in the intima except for a few layers in cushions at branching sites. Very few PCNA-positive SMCs were observed, and the surface endothelial cells were rarely stained with anti-PCNA antibody. Neither intimal nor medial cells were stained with RAM11. In frozen sections, all medial SMCs were positive for both SM1 and SM2 and negative for SMemb, whereas endothelial cells were positive for SMemb.

Chronological Changes in Intimal Thickening After Stent Implantation

Fig 2A shows chronological changes in intimal thickening expressed as intimal index. Light microscopic examination showed that stent implantation produced thrombus formation around the wire on day 2 (Fig 3A) that was subsequently organized and replaced by newly formed fibrocellular tissue until day 28 (Fig 3B). The intimal area was defined to include both thrombus and fibrocellular tissue because the boundaries between them were poorly delineated. The intimal index gradually increased with time, and the increase was the most remarkable between days 10 and 14.

Chronological Changes in Histological and Immunohistochemical Findings

Two to 4 Days After Stent Implantation

The stent wire compressed the aortic wall, and the underlying medial SMCs appeared to be injured, as indicated by loss of nuclei and loss or diminishment of α-actin (Fig 4, A and B). Medial elastic fibers were oppressed and gathered, although apparent disruption of internal elastic lamina was rarely observed (Fig 3A). On the luminal surface, the stent wire was surrounded by a fresh white thrombus composed of fibrin and platelets, the surface of which was partly covered by regenerated endothelium. Macrophages, indicated by positive staining with RAM11, were rarely observed in the thrombus or media. Immunostaining for PCNA showed that SMC proliferation began to appear in the media adjacent to the injured area on day 2 and that these proliferating SMCs increased in number on day 4. PCNA-positive cells tended to accumulate in the shoulder portion adjacent to the injured area rather than beneath the stent as shown in Fig 4C.

By MHC isofrom staining on frozen sections, anti-SM2 antibodies showed positive immunoreactivity in the media, except in the injured area around the wire (Fig 5B). In contrast, cells around the injured area were stained positively with the anti-SMemb antibody (Fig 5A), and this distribution was consistent with that of PCNA. Endothelial cells were stained positively with SMemb as observed in the control animals.

In the sham-operated animal killed on day 4, no medial or endothelial injury was observed in paraffin sections, and PCNA-positive SMCs or endothelial cells were rarely observed. SMemb-positive SMCs were not observed in frozen sections.

Seven to 10 Days After Stent Implantation

The thrombus adhered to the stent was gradually replaced by fibrocellular tissue from day 7 (Fig 4D) and disappeared with the exception of the area near the wire by day 10. This fibrocellular response in the neointima was also observed in some animals on day 4. Extracellular matrix surrounding intimal SMCs contained discontinuous elastic fibers as shown by Elastica van Gieson staining, and the endothelial cells appeared to cover most of the surface of the neointima. By immunohistochemical staining with RAM11, a few macrophages...
were recognized in the thrombus near the wire. Most of the spindle-shaped cells observed in the fibrocellular tissue were labeled with anti-α-actin antibody (Fig 4E), but some were not positive for either α-actin or RAM11. Immunostaining for PCNA showed that proliferating SMCs continued to be present in the media but were reduced in number compared with those on day 4. Instead, many PCNA-positive cells appeared in the fibrocellular tissue in the neointima (Fig 4F), most of which were identified as SMCs by double immunostaining for α-actin and PCNA.

In frozen sections, the cells in the fibrocellular tissue in the neointima stained positively for SM1 and SMemb (Fig 5C) and were negative for SM2 (Fig 5D). In the media, SMemb-positive cells remained around the injured area as observed on day 2 or 4.

Two Weeks After Stent Implantation

Intimal thickening was markedly increased compared with that on days 7 and 10 and almost replaced by newly formed fibrocellular tissue (Fig 4G), where most of the intimal cells were stained for α-actin (Fig 4H). Macrophages were present in the organizing thrombus near the wires, but no foreign-body reaction such as appearance of multinucleated giant cells was detected. PCNA-positive cells became scarce, not only in the media but also in the neointima compared with those at earlier periods (Fig 4I).

In frozen sections, medial SMCs around the stent and intimal SMCs remained positive for SMemb (Fig 5E) and negative for SM2 (Fig 5F) as on days 7 and 10.

In the sham-operated animal killed on day 14, no neointima formation was observed, and PCNA-positive cells were rarely observed in the media.

One to 2 Months After Stent Implantation

The neointima was mostly composed of fibrocellular tissue and showed little increase in thickness. The extracellular matrix surrounding intimal SMCs became rich and contained densely and continuously arranged elastic fibers (Fig 3B). RAM11-positive macrophages remained around the base of the wire but were reduced in number compared with those on day 14. The number of PCNA-positive cells was markedly reduced at this stage and returned to the basal level observed in the control animals.

The phenotypes of intimal SMCs appeared to have reverted to the adult type according to immunohistochemistry for the different MHC isoforms. After 1 month, SM2-positive cells were observed at the foot of the neointima, where immunostaining for SMemb was lost instead. After 2 months, almost all of the neointimal and medial SMCs, with the exception of those in a limited area around the stent, reverted to being negative for SMemb (Fig 5G) and positive for SM2 (Fig 5H).

Quantification of Proliferating Cells

Fig 2B shows the chronological changes in PCNA index, which represents the frequency of PCNA-positive cells. The appearance of PCNA-positive cells in the media preceded that in the neointima. The PCNA index was maximal on day 4 in the media (3.9%) and gradually decreased thereafter. In the neointima, the PCNA index reached a maximal level on day 7 (20.3%) and decreased to the baseline value by 28 days.

Discussion

Several animal studies have histologically investigated the process of neointima formation after stenting. In canine coronary arteries, Schatz et al11 reported that neointima reached maximal thickness by 8 weeks after stenting and consisted mostly of SMCs and extracellular matrix. In an atherogenic swine model, the coronary arteries showed maximal intimal thickening at 4 weeks after stenting,27 and Santoian and King12 stated that stent compression of the arterial wall may create a continuous stimulus for smooth muscle proliferation. Although SMC proliferation has been assumed to occur during the process of neointima formation after stenting, most studies have not applied techniques to detect proliferating cells in vivo directly. Strauss et al11 examined seven human restenotic lesions with PCNA immunostaining, but specimens taken 47 to 609 days after stenting contained no PCNA-positive cells. Therefore, no information has been available concerning when and where cell proliferation is induced and how it contributes to intimal hyperplasia formation after stenting.

In the present study, we applied PCNA immunostaining to identify proliferating cells, but this staining needs careful consideration. PCNA is an auxiliary protein of DNA polymerase-δ, and its immunostaining labels not only cells in the S phase of the cell cycle but also those in the late G1 and early G2 phases, thus staining a larger population than [3H]thymidine or bromodeoxyuridine labeling techniques.28 Nevertheless, recent comparative studies showed that PCNA staining favorably compares
with $[\text{H}]$thymidine and bromodeoxyuridine labeling and becomes a reliable marker for cell proliferation after arterial balloon injury.\textsuperscript{28-29} Furthermore, we fixed the tissues no longer than 20 hours to avoid loss of staining caused by prolonged fixation and immuno-stained the specimens together with the same positive control specimens at each time to obtain constant results. Therefore, we believed that the PCNA staining in the present study represented the kinetics and distribution of cell proliferation after stent implantation.

In the present study, we demonstrated that neointima formation after stenting is preceded by SMC proliferation in the media and intima. From the spatial correlation of SMC proliferation and arterial injury caused by stenting, it is feasible to speculate that arterial injury caused by stent compression induces SMC proliferation and contributes to the formation of intimal hyperplasia after stenting. Our results also suggest that stent implantation does not cause infinite or prolonged SMC proliferation, with implantation of the proper stent size to a nonatherogenic artery. The chronological sequences of SMC proliferation and intimal thickening in our stent model proved to be generally in agreement with those observed in the rat carotid injury model using a balloon catheter.\textsuperscript{30-31} However, Duprat et al\textsuperscript{20} reported that excessive eccentric intimal thickening was observed when the stent-artery ratio was greater than 1.2, and thus SMC proliferation might be prolonged in cases of stent size mismatch or overdilatation by ballooning.

Arterial SMCs show phenotypic modulation not only in vitro but also in vivo such as neointima formation after arterial injury.\textsuperscript{32-34} Similar phenotypic modulation was demonstrated in primary atherosclerotic lesions and in restenotic lesions after PTCA in humans.\textsuperscript{35,36} Recently, novel MHC isoforms, SM1, SM2, and SMemb, were cloned by Nagai and colleagues\textsuperscript{21-23} and determined to be developmentally regulated. Furthermore, immunohistochemical staining for these isoforms has been shown to be a good molecular marker for phenotypic modulation of arterial SMCs not only in rabbits but also in humans.\textsuperscript{21-24,36} SMemb is a nonmuscle MHC isoform, known to be identical to nonmuscle MHC-B.\textsuperscript{23,37} and is expressed in SMCs at embryonic and neonatal stages and when they are dedifferentiated toward the embryonic phenotype.\textsuperscript{23,36,38} Therefore, SMemb is a useful molecular marker for the embryonictype SMCs. We took advantage of the unique features of these MHC isoforms for analysis of the spatial and chronological correlation of proliferation and phenotypic modulation of SMCs in stent-implanted aortas.
Fig 5. Photomicrographs show immunohistochemical stainings for myosin heavy chain isoforms, SMemb (A, C, E, G) and SM2 (B, D, F, H), in frozen sections of thoracic aortas. Aortic tissues were dissected from animals killed on days 4 (A and B), 7 (C and D), 14 (E and F), and 56 (G and H) after stent implantation. Note inverse expression of SMemb and SM2 at each time point. (Original magnification, ×180.)
In the present study, proliferating SMCs showed the embryonic phenotype as indicated by loss of SM2 and expression of SMemb, and such embryonic-type SMCs continued to be present in the neointima even after cessation of cell proliferation. On day 28 after stenting, the neointimal SMCs began to return to the adult phenotype, as indicated by the reappearance of SM2 and loss of SMemb, and this process was almost completed by 56 days. This phenomena indicates that phenotypic modulations of SMCs are reversible during the process of neointima formation.

Proliferation and migration of SMCs together with production of extracellular matrix are considered to play critical roles not only in neointima formation after stenting but also in atherogenesis and restenosis after PTCA.39-42 According to analysis performed in the balloon-injury model in rat carotid arteries, platelet-derived growth factor released from platelets and SMCs themselves and basic fibroblast growth factors released from injured SMCs play significant roles in inducing migration and proliferation of SMCs.33-46 Macrophages have also been observed in the neointima in the balloon-injury model.31 Macrophages were immunostained in our stent implantation model but were localized only in the thrombi remaining around the base of the wire on day 14 when SMC proliferation had almost ceased. Therefore, we did not consider the cytokines and growth factors secreted from macrophages to play a major role in our model. Double immunostaining for PCNA and macrophages and for PCNA and α-actin showed that proliferating cells were exclusively SMCs in our model. In the hypercholesterolemic state, macrophages are known to accumulate and proliferate during atherogenesis,55,41,42 so we are currently investigating the effect of stent implantation on the cellular responses in aortas of cholesterol-fed rabbits to determine the role of macrophages in the formation of restenotic lesions after stenting.

Neointima formed after stenting or PTCA contains significant amounts of extracellular matrix produced and secreted from intimal SMCs and endothelial cells. Such matrix may be involved in the control of proliferation and phenotypic modulation of vascular SMCs.43-47 Recent studies have proposed that some proteoglycans in the extracellular matrix may modulate growth factor activities through their involvement in the binding mechanisms of the growth factors to their receptors.48 Therefore, extracellular matrix and its components are possibly involved in the control of SMC proliferation and phenotypic modulation. This stent implantation model may become useful to demonstrate the pathological correlation of the components of extracellular matrix with cell growth activities and phenotypic modulation during the process of neointima formation.

In the present study, we attempted to perform immunohistochemical analysis of the spatial and chronological distribution of cellular components and of SMC proliferation and phenotypic modulation. We used Gianturco's Z type stents because other types of coronary stents, such as those of Palmaz-Schatz, Gianturco-Roubin, and Wiktor, are difficult to process for immunohistochemistry because of their complex structure. We considered that any type of stent may induce similar responses in the arterial wall because all inevitably produce compression injury when expanded.

The cellular responses when the stents are implanted in rabbit aorta may not represent those when implanted in human atherosclerotic arteries, and porcine coronary arteries are more suitable for the restenosis model. Nevertheless, we chose rabbit aorta because immunohistochemistry for cell type-specific markers, PCNA and MHC isoforms, is readily applicable. Furthermore, the rabbit model enabled us to examine a sufficient number of animals to observe the chronology of the cellular responses, and we are currently investigating responses when the stents are implanted in atherosclerotic aortas of rabbits. These data should be valuable for the establishment of local delivery systems and administration protocols of prophylactic agents in future investigations.

In conclusion, the present study demonstrated that the compression injury by stent implantation led to neointima formation through regional proliferation and migration of SMCs and their phenotypic modulation to the embryonic type. SMC proliferation and progression of intimal thickening were transient within 1 month, and the intimal SMCs returned to the adult phenotype 1 to 2 months after implantation. This stent implantation model is useful as a vascular injury model for the investigation of atherogenesis and restenosis after angioplasty, because it clearly demonstrates the spatial correlation of the regional injury with the cellular responses that subsequently lead to neointima formation.

Acknowledgments

This study was supported in part by research grants from the Ministry of Education (No. 06557707) and from the Kanae Research Foundation. We thank Dr Ryozo Nagai for the generous gifts of antibodies for MHC isoforms and helpful comments on the manuscript, and Gunpei Kikuchi for manufacturing the stents used in this experiment.

References


Neointima formation after vascular stent implantation. Spatial and chronological distribution of smooth muscle cell proliferation and phenotypic modulation.
H Bai, J Masuda, Y Sawa, S Nakano, R Shirakura, Y Shimazaki, J Ogata and H Matsuda