Rabbit Ear Model of Injury-Induced Arterial Smooth Muscle Cell Proliferation
Kinetics, Reproducibility, and Implications

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Recently, considerable interest has focused on the vascular smooth muscle cell (SMC) response to injury, particularly as it relates to restenosis after angioplasty. In an effort to find an optimal experimental model of arterial SMC proliferation after injury, we examined the effects of external injury to the central artery of the rabbit ear and assessed the reproducibility, morphological changes, and time course of cellular proliferation after such an injury. With rabbits under general anesthesia, direct pressure was applied at two sites along the central artery of the ears of 19 New Zealand White rabbits. Rabbits were maintained on a diet of 2.4% fat and 0.001% cholesterol throughout the experiment. In seven rabbits examined after 21 days, marked SMC proliferation with neointimal formation was observed at all 28 sites (100%). Mean neointimal area, expressed as a percentage of the area of the tunica media, was 82±40% (range, 21–203%). Compared with the uninvolved artery displaced 2 mm from the injury site, mechanical crush caused a 38% increase in total vessel area (p<0.001), a 40% decrease in luminal area (p<0.002), and no change in the area of the media. Serial histological studies were performed 1–42 days after injury, using light and electron microscopy and bromodeoxyuridine immunohistochemistry. Beginning at day 3, activated medial SMCs were noted to migrate through defects in the internal elastic membrane, with a gradual increase in neointimal area between days 5 and 12. Peak DNA synthesis was identified in the media 5 days after injury, with proliferative activity shifting almost exclusively to the neointima thereafter. We conclude that mechanical crush injury is a potent stimulus for SMC proliferation. The method is simply employed, multiple lesions can be created in a single animal with high yield, and therapeutic end points can be easily quantified. The lesions so produced are superficial and easily accessible; therefore, agents with the potential to prevent SMC proliferation can be targeted locally by subcutaneous injection or topical application. (Circulation Research 1991;69:748–756)

Establishment of a simple and reproducible animal model of vascular smooth muscle cell (SMC) proliferation in response to injury is essential for understanding the natural history and pathophysiology of restenosis after coronary angioplasty, accelerated arteriosclerosis in cardiac allograft recipients, and neointimal proliferation in saphenous vein grafts. Development of such a model would make possible in vivo screening of therapies with the potential to prevent SMC proliferation, a fundamental component of these syndromes. Currently available models use vessel overdistension, endothelial denudation (in the presence or absence of an atherogenic diet), chronic electrical stimulation, or deployment of oversized metal coils to initiate SMC proliferation.1–6 These methods produce neointimal hyperplasia to varying degrees, but all suffer from a relatively high degree of complexity and invasiveness.

External crush is a simple and direct method of arterial injury that has been shown to initiate SMC proliferation and neointimal hyperplasia in the central artery of the rabbit ear.7,8 We examined the reproducibility of this technique and investigated the natural history of the morphological and proliferative changes that follow such an injury.

Materials and Methods

Nineteen male New Zealand White rabbits were used in this study. The experimental protocol was

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approved by the Animal Care and Use Committee of the National Heart, Lung, and Blood Institute. It was performed in accordance with the Guide for the Care and Use of Laboratory Animals (Department of Health and Human Services publication No. [NIH] 86-23, revised 1985).

Creation of the Lesions

General anesthesia was induced in rabbits with ketamine HCl (44 mg/kg i.m.) and xylazine (4 mg/kg i.m.). Bilateral auricular nerve block was established with 1% lidocaine administered subcutaneously at the base of each ear. Polyethylene disks (5 mm diameter x 2 mm thick) were placed on each side of the ear over the central artery. Kelly clamps (Biomedical Research Instruments, Inc., Rockville, Md.) oriented perpendicular to the artery were used to crush the arterial segments interposed by the disks. Pressure was maintained for 30 minutes, after which the sites were marked with indelible ink.

Study Protocol

Four crush lesions were performed simultaneously in each animal, two on each ear. After recovery from anesthesia, the rabbits were maintained on NIH-09 ration formula (2.4% fat, 0.001% cholesterol) for the duration of the experiment. Forty-eight lesions were examined serially, 1, 2, 3, 5, 7, 10, 12, and 42 days after injury. Twenty-eight additional lesions were examined after an interval of 3 weeks.

Incorporation of 5-Bromo-2'-deoxyuridin

Four hours before death, each rabbit received 5-bromo-2'-deoxyuridine (United States Biochemical Corp., Cleveland, Ohio) (20 mg s.c.) to determine the extent of SMCs undergoing DNA synthesis. Bromodeoxyuridine (BrdU) is a thymidine analogue that is incorporated into the DNA of S-phase proliferating cells.

Tissue Preparation

Ears were fixed by immersion in McDowell-Trump fixative. Each site, approximately 1 cm in length, was cut into 10 cross-sectional segments and embedded together in paraffin. Five-micrometer sections were removed from the top of the block and at two additional points displaced 100 and 200 mm from the first section. Samples were stained with hematoxylin and eosin, Movat, and Masson’s trichrome, and additional sections were reserved for anti-BrdU immunohistochemistry. By removing sections from three locations along each block, each lesion was effectively sampled at 30 sites. Samples from six additional rabbits were embedded in epoxy resin, sectioned at 700 Å, and stained according to the electron microscopic method of Kajikawa et al.10

Identification of Proliferating Cells Using Anti-Bromodeoxyuridine Immunohistochemistry

Immunohistochemistry was performed as described by McDowell et al.11 Sections were deparaf-
finized and hydrated in graded ethanol. Endogenous peroxidases were quenched with 1% H2O2 in absolute methanol. After one rinse in distilled water and three rinses in phosphate buffered saline (pH 7.4), the sections were incubated with 0.4 mg/ml pepsin (Sigma Chemical Co., St. Louis, Mo.) in 0.1 M HCl for 30 minutes at 37°C. Sections were washed in 0.5% Tween 20 (Sigma) in phosphate buffered saline and hydrolyzed in 4 M HCl for 20 minutes at 25°C. They were neutralized in 0.1 M borax buffer (pH 8.5) and incubated with mouse anti-BrdU monoclonal antibody diluted 1:40 (Becton Dickinson Immunocytochemistry Systems, Mountain View, Calif.) for 16–20 hours at 25°C. Sections were washed three times with phosphate buffered saline and incubated with biotinylated horse anti-mouse IgG (Vector Laboratories, Inc., Burlingame, Calif.) for 30 minutes at a dilution of 1:200. Localization of the secondary antibody was achieved using the avidin biotin method. Sections were incubated with the ABC complex (Vectastain ABC kit, Vector Laboratories) 60 minutes at 25°C and subsequently with 0.01% diaminobenzidine-HCl with 0.02% H2O2 in 0.05 M Tris buffer for 7 minutes. Sections were counterstained with hematoxylin, dehydrated in graded ethanol, displaced in xylenes, and mounted with Entellan (EM Science, Gibbstown, N.J.).

Morphometric and Statistical Analysis

Digital planimetry of tissue sections was accomplished using a Joyce Loebel Magiscan System, Tyne & Wear, UK. Planimetry was performed at the site of the lesion and at an adjacent normal arterial segment displaced 2 mm from the location of the injury. Selection of the normal site was random; approximately one half were proximal and one half distal to the injury site. The lumen, the area circumscribed by the internal elastic lamina (“original lumen”), and the area circumscribed by the external border of the media (total vessel area) were measured directly. The area of the media and neointima were deduced by subtraction: medial area=total vessel area-area bounded by internal elastic lamina; neointimal area=area bounded by internal elastic lamina-area of lumen. The neointimal area was indexed to the size of the vessel by expressing the neointimal area as a percentage of the area of the tunica media. The neointimal area also was expressed as a percentage of the total arterial area. In all cases, the single section demonstrating the greatest extent of neointimal proliferation was selected for planimetry. Proliferating SMCs were quantified by expressing the total number of cells with labeled nuclei divided by the area in which they were identified.

All data are reported as mean±SD. The statistical significance of differences between the sites of vascular injury and normal adjacent arterial segments was evaluated using two-tailed Student’s t tests for paired observations.
Results

All rabbits underwent the above procedures uneventfully. No mortality and no apparent morbidity were evident in any animal.

Light Microscopy

Three weeks after crush injury, significant neointimal formation was identified in 100% (28 of 28) of the lesions analyzed (Figure 1). Figure 2 shows the luminal, neointimal, and medial areas for the lesions, as well as these parameters from adjacent normal vessels 3 weeks after vascular injury. The mean neointimal area comprised 82±40% of the area of the media. Compared with the normal artery displaced 2 mm from the injury site, the injured vessel was significantly larger in total cross-sectional area (p<0.001), with a smaller luminal area (p<0.002). The area of the media at the site of vascular injury was preserved, unchanged from that of the normal artery.

Importance of Serial Sectioning

In the preparation of samples for histological analysis, each arterial injury site was divided into 10 cross-sectional segments and embedded in a single block. Thus, observation of the topmost section of the block provided sampling at 10 sites more or less evenly distributed throughout the lesion. Analysis of sections from two additional block locations, displaced 100 and 200 μm from the first, provided sampling at a total of 20 and 30 sites, respectively. To assess the practical limits to which serial sectioning should be carried out, we compared the apparent maximal stenosis determined after analysis of one section (10 sample sites) to that ascertained after analysis of the second and third sections (20 or 30 total sample sites, respectively). Analysis of a second section increased the apparent degree of neointimal proliferation by 7%, relative to analysis of a single section. Analysis of a third section increased the perceived degree of proliferation by an additional 5%, relative to analysis of two sections.

Serial Studies and Quantification of Smooth Muscle Cell Proliferation

In the tunica media, cellular proliferation was first identified 2–3 days after vascular injury, with proliferative activity peaking on day 5 and declining rapidly thereafter (Figure 3). Events in the neointima can be summarized as follows: The neointima first appeared as zero to two SMC layers on the third day after injury, but evidence of proliferation was absent at this stage. On the fifth and seventh days, two to six and three to eight SMC layers were identified, respectively. Peak proliferative activity occurred on day 7, with labeling throughout the neointima. By the 10th and 12th days, the neointima consisted of eight...
Electron Microscopy

Three to 5 days after crush injury, endothelial cell loss or damage was apparent. Cells were rounded in shape, with mild to moderate dilation of the endoplasmic reticulum and swelling of mitochondria. Disappearance of basement membrane was apparent in some sections. Seven to 12 days after injury, endothelial cell damage was still evident; however, basement membrane was clearly apparent. Mild to moderate SMC injury was evident in all specimens examined, with dilation of the endoplasmic reticulum and mitochondrial swelling. SMC basement membrane appeared well developed. The internal elastic lamina was disrupted at numerous sites, and the orientation of the SMCs suggested that migration from the media to the subintimal space was occurring (Figures 5 and 6). Three to 5 days after injury, scant neutrophils and eosinophils could be identified in the arterial wall. Between days 7 and 12, few lymphocytes and macrophages predominated in the adventitia and the perivascular area. Fibrosis was not a feature of these lesions.

### Table 1

<table>
<thead>
<tr>
<th></th>
<th>Normal Site</th>
<th>Injury Site</th>
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<tbody>
<tr>
<td>Lumen</td>
<td>0.060±0.026</td>
<td>0.036±0.024 *</td>
</tr>
<tr>
<td>Neointima</td>
<td>0</td>
<td>0.085±0.041</td>
</tr>
<tr>
<td>Media</td>
<td>0.123±0.041</td>
<td>0.128±0.061</td>
</tr>
<tr>
<td>Total Vessel</td>
<td>0.184±0.063</td>
<td>0.250±0.092 **</td>
</tr>
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**Figure 2.** Schematic illustration of the differences in vessel geometry and composition between the site of injury (right) and a normal arterial segment displaced 2 mm from the lesion (left). Areas depicted in bar graphs and bull's-eye plots represent the mean±SD from all lesions analyzed 3 weeks after injury. The bar graph expresses area in square millimeters. The bull's-eye plot depicts relative areas. With the development of neointimal proliferation, the total vessel cross-sectional area increased (p<0.001) and luminal area decreased (p<0.002). The medial area at the site of vascular injury was unchanged from that of the normal artery. *p<0.002; **p<0.001.

### Discussion

The proliferative response of vascular SMC to injury presents a formidable obstacle to the long-term success of percutaneous transluminal coronary angioplasty (PTCA). This process causes restenosis in 25–49% of patients within 6 months of angioplasty. Recent studies also have shown similar rates of restenosis after directional atherectomy, laser angioplasty, and endovascular stent deployment. Efforts to elucidate the pathophysiological mechanisms underlying the vascular SMC response to injury and to develop effective treatment strategies have been hampered by the lack of an appropriate experimental model.

### Other Models of Smooth Muscle Cell Proliferation

Several experimental models have been used to emulate the pathophysiology of restenosis after angioplasty, and all share in common some variety of vascular injury. This would seem appropriate, because endothelial injury with some degree of medial damage is believed to be the initiating event leading to restenosis after transcatheter endovascular interventions. The ideal restenosis model would involve the performance of angioplasty on an underlying fibromuscular plaque, histologically similar to that found in human coronary atherosclerosis.
The majority of investigators who have attempted to produce lesions suitable for angioplasty have coupled arterial injury with an atherogenic diet, primarily in rabbits. Unfortunately, the resulting intimal lesions have been characterized by an abundance of foam cells and therefore are distinctly different from those in humans. Such models therefore have major limitations as analogues of human disease.

Recently, Hanke et al. initiated plaque formation in the rabbit carotid artery by applying constant-current DC impulses for 28 days and subsequently subjecting the lesions to balloon angioplasty. Of 18 arteries examined 21 or more days after balloon dilatation, three demonstrated more than a 50% compromise of luminal area. This preparation is attractive because it is analogous to PTCA, superimposing balloon dilatation on a preexisting fibromus-
FIGURE 5. Electron micrograph of an arterial lesion 3 days after crush injury. The arterial lumen (L) is at the top. Endothelial cells (EC) show a mild degree of damage with cytoplasmic swelling. Disruption of the internal elastic membrane (IEM) is apparent, through which a smooth muscle cell (SMC) is crossing. Magnification, ×6,350.
The Rabbit Ear Model

As originally described by Silver et al., the technique of forceps-induced vascular injury was used to study the acute interaction of platelets with damaged arterial wall. In an extension of these studies, Ingerman-Wojenski and Silver used repetitive forceps-induced injury to mimic the initial events in arteriosclerosis.

Using a single external crush injury to the central artery of the rabbit ear, we were successful in producing vascular SMC proliferation with significant luminal compromise. SMC proliferation occurred at 100% of injury sites, and morbidity was essentially zero. The simplicity of this model is underscored by the fact that four or more lesions can be created in each of several rabbits in a time span of only one hour. The ear is accessible to subcutaneous or topical therapy, and treatment can be directed locally, such that one ear can be treated with an experimental agent and the other with placebo. Thus, the statistical power of the model can be increased by using each animal as its own control. For all of these reasons, it is feasible to screen an intervention for possible therapeutic effects in a relatively short period of time, obtaining meaningful statistical results from a limited number of animals.

Analysis of End Points

For each lesion, we expressed the neointimal area as a percentage of the area of the media. The medial area at the injury site was virtually identical to that of the normal artery displaced 2 mm from the lesion, providing a constant denominator against which the area of the neointima could be indexed. Other investigators have used perfusion fixation and based quantification of neointimal area on percent stenosis using the formula 100 × neointimal area ÷ (luminal area + neointimal area). Calculation of percent stenosis therefore is critically dependent on vessel circularity, which is in turn dependent on fixation conditions and subject to compression artifact. We attempted to circumvent these variables by expressing the subintimal area as a percentage of the medial area.

Cellular Proliferation

The time course of cellular proliferation after arterial injury was defined by measuring layers of SMCs in the neointima over time and changes in proliferative activity using BrdU immunostaining (Figure 4). Smooth muscle cells first were identified in the neointima on day 3 and continued to accumulate throughout the 3 weeks of observation. We
found that a peak in proliferative activity occurred in the tunica media on day 5, with a subsequent peak in the neointima on day 7. The simultaneous occurrence of SMC proliferation and migration through fenestrations in the internal elastic lamina suggests that cellular migration and proliferation collectively lead to neointimal formation. The neointimal area/medial area was virtually equivalent at 3 and 6 weeks, suggesting that regression of SMC proliferation does not occur spontaneously in this interval. Furthermore, the lack of cellular proliferation at 6 weeks suggests that, to be successful, therapies to prevent neointimal formation must be directed early.

The time course of SMC proliferation after vascular injury has been studied previously by Clowes and coworkers\(^{19,20}\) using tritiated thymidine and by Hanke et al\(^{6}\) using bromodeoxyuridine. With the use of a single balloon injury in the rat carotid artery, the results of Clowes and Clowes\(^{19}\) were identical to ours: maximal SMC proliferation was found in the media and intima at 4 and 7 days, respectively. Hanke et al\(^{6}\) used a staged model in the rabbit carotid artery, where electrical stimulation was used to cause intimal plaque formation, and subsequent balloon dilatation was used to mimic the injury of PTCA. A peak in proliferative activity occurred in the intima 3 days after balloon dilatation; the peak in medial SMC proliferation occurred later and was far less pronounced. Their results suggest that balloon injury at the site of a preexisting plaque initiates SMC proliferation predominantly in the neointima and therefore differs from primary arterial injury.

It could be argued that injury of a normal artery induced by its external compression has little relevance to the pathophysiology of vascular SMC proliferation after transcather endovascular inventions, during which vascular damage is initiated from within the lumen. Pathological studies and experimental investigations suggest that whatever the mode of injury—mechanical, thermal, or immunological—the end result is the same: SMC proliferation and luminal obstruction.\(^{21}\) Although the method used to initiate neointimal proliferation in this model was somewhat unorthodox, external crush injury effectively induced SMC proliferation with a high degree of reproducibility. The vascular lesions so produced were indistinguishable from those produced after experimental endovascular injury. Because SMC proliferation is the key element of restenosis, we therefore believe that the rabbit ear model can be used to elucidate further the cellular and molecular mechanisms involved in the proliferative response of SMCs to vascular injury, and to screen potential therapeutic strategies designed to inhibit injury-induced SMC proliferation.

Conclusion

The rabbit ear experimental model of vascular SMC proliferation affords an excellent opportunity to study the pathophysiology of vascular wall syndromes caused by SMC proliferation and to test potential therapeutic modalities to inhibit this process. The method is simply employed, multiple sites of vascular injury can be created in a single animal, and a large number of lesions can be produced quickly and efficiently. The lesions so produced are superficial and easily accessible; therefore, agents with the potential to prevent neointimal hyperplasia can be targeted locally by subcutaneous injection or topical application. We believe that the technical simplicity of this model and the accessibility to local treatment that it provides are key factors that will prove helpful in the investigational efforts to elucidate the cellular and molecular mechanisms responsible for SMC proliferation and to test therapeutic strategies designed to inhibit this process.

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References


KEY WORDS: smooth muscle cells • accelerated arteriosclerosis • neointima • restenosis • animal model
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