Mouse Model of Arterial Injury

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In the present study, we established an injury model of the mouse carotid artery. Complete removal of the endothelium was achieved with a flexible wire. A platelet monolayer covered the denuded surface, and damage to underlying medial smooth muscle cells (SMCs) was detected. Injection of [³H]thymidine was used to determine the replication index for medial SMCs, which was found to be 1.6% at 2 days after denudation and 9.8% at 5 days. SMCs were observed in the intima by day 8 (replication index, 66%), and by 2 weeks the intimal lesion had a similar cell content as the media. In most animals, repair of the endothelial lining was complete 3 weeks after injury. The present model will allow us to use transgenic animals to address questions relevant to vascular biology and atherosclerosis. (Circ Res. 1993;73:792-796.)

KEY WORDS • mouse • carotid artery • denudation • smooth muscle • endothelium • proliferation • intima

M any questions in vascular biology require studies at the level of endogenous molecules. To gain insight into the mechanisms underlying intimal lesion growth, various strategies of testing the function of these molecules have been used in rat models of arterial injury.¹² These approaches include the administration of factors directly to animals⁴⁻⁶ and the elimination of endogenous factors using antibodies⁷⁻⁸ or antisense oligonucleotides.⁹⁻¹⁰ An alternative approach is to use transgenic animals in which particular genes are overexpressed or have been deleted; thus, concerns regarding adequate delivery of these specific factors or their inhibitors are eliminated. Mice are the species of choice for transgenic or gene knockout experiments; therefore, we decided to develop a model of vascular injury in the mouse that would allow us to take advantage of transgenic animals. In the present study, we report on a mouse model of endothelial denudation in which the response of smooth muscle cells (SMCs) and endothelium in the common carotid artery was characterized.

Materials and Methods

Animals

Female Swiss Webster mice (Simonsen Laboratories, Inc, Gilroy, Calif) weighing 25 to 35 g were used in these experiments. The animals were anesthetized by intraperitoneal injection with a solution composed of ketamine (80 mg/kg body wt, Ketaset, Aveco Co, Inc, Fort Dodge, Iowa) and xylazine (5 mg/kg, AnaSed, Lloyd Laboratories, Shenandoah, Iowa) diluted in an equal volume of 0.9% sodium chloride solution.

Denudation of the Carotid Artery

Surgery was carried out using a dissecting microscope (Stemi SR, Zeiss, Germany). The left carotid artery was exposed via a midline incision on the ventral side of the neck. The bifurcation of the carotid artery was located, and two ligatures (surgical silk, size 6-0, Deknatel, Queens Village, NY) were placed around the external carotid artery, which was then tied off with the distal ligature. An incision hole was made between the two ligatures to introduce the denudation device. The curved flexible wire (0.35-mm diameter) was introduced into the common carotid artery (Fig 1) via the incision, and under rotation the wire was passed along the vessel three times. The wire was then removed, and the external carotid artery was tied off proximal to the incision hole with the proximal ligature. The skin incision was closed using two suture clips (7.5×1.75 mm, Michel, Aesculap, San Francisco, Calif).

SMC Proliferation and Endothelial Outgrowth

A minimum of six animals was studied in each experimental group. Replication of SMCs was determined by labeling SMCs in S phase with three doses of 50 μCi [³H]thymidine [6.7 mCi/(mmol/L), Du Pont, NEN, Boston, Mass] given 17, 19, and 1 hour before death. Ten minutes before death by an overdose of pentobarbital (210 mg/kg IP), the animals received an injection of Evans blue (50 μL of a 5% solution in saline) to mark the denuded area of the carotid artery. The dye was injected into the tail vein using a 30-gauge needle and avoiding injection of any air. Perfusion fixation under physiological pressure with phosphate-buffered paraformaldehyde (4%, 0.1 mol/L, pH 7.3) was carried out via a 24-gauge intravenous catheter placed into the left ventricle. The carotid arteries were excised, and the endothelial regrowth occurring from the aortic arch and the carotid bifurcation was measured. For determination of SMC replication, the denuded segment of the vessel (blue) was embedded in paraffin, and four sections (each ≥200 μm apart) were cut. For autoradiography, the slides were coated with emulsion (NTB-2, Kodak, Rochester, NY) and developed after 2 weeks. The replication was measured for medial SMCs of unmanipulated as well as
injured vessels and, when present, also for intimal SMCs. The replication index was determined by counting the total number of SMCs and the number of radiolabeled SMCs on four sections per animal [(radiolabeled nuclei/total nuclei)×100]. The loss of SMCs from the media due to the denuding injury was assessed by counting the number of medial SMCs at various times in arteries after denudation and in uninjured carotid arteries. As an indirect measure for intimal lesion size, the total number of intimal cells is presented. In addition, for the 2-week time point, the intimal lesion of the denuded segment was also quantified by measuring the intimal area as previously described.3 In the initial denuding experiments, a small number of animals showed total thrombotic occlusion of the vessel. These animals were not included in the studies. With increased experience in the denuding technique, the thrombotic occurrences are now a rare finding.

**Scanning Electron Microscopy**

To verify that complete endothelial denudation of the carotid artery was achieved, six animals were killed 30 minutes after the denuding procedure. Perfusion fixation was carried out as described above using phosphate-buffered 0.25% glutaraldehyde/2% paraformaldehyde as fixative. The vessels were cut open longitudinally, pinned flat onto Teflon sheets, and dehydrated through a series of ethanol. The tissue was critical point–dried and mounted on scanning electron microscopy stubs with colloidal silver paste. After sputter-coating with gold/palladium, the specimens covering the entire length of the carotid artery were examined in a Joel 35C microscope at 15 kV.

**Immunocytochemistry**

Immunocytochemical staining for SMC a-actin was carried out on paraffin sections of carotid arteries using a mouse monoclonal antibody (HHF-35, kindly provided by Dr Gown, University of Washington, Seattle) at a 1:1000 dilution.9 Subsequent incubations with biotinylated horse anti-mouse immunoglobulin G (1:2000 dilution) and ABC-Elite kit (both from Vector Laboratories, Inc, Burlingame, Calif) were performed as previously described.10 In control vessels, the primary antibody was replaced with matching concentrations of nonimmune mouse immunoglobulin G. A biotinylated rat monoclonal antibody recognizing mouse leukocytes (common leukocyte antigen CD45, GIBCO BRL, Gaithersburg, Md) was used on cryostat sections at a 1:500 dilution11 followed by application of the ABC-Elite kit (Vector Laboratories).

**Statistical Analysis**

Fisher's least significant difference test for multiple comparisons (significance level, 95%) was used to compare means of endothelial regrowth, medial and intimal SMC replication index, and means of medial SMC numbers. The t test (unpaired, two tailed) was used to compare means of intimal SMC numbers of 8-day and 14-day time points.

**Results**

The initial characterization of the injury model of the mouse carotid artery focused on whether the denuding procedure removed all the endothelial cells from these arteries. Scanning electron microscopy was used to

![Graph showing endothelial regrowth in the mouse carotid artery measured at the indicated times after denudation. Total endothelial regrowth from the aortic arch and the carotid bifurcation is expressed as a percentage of the length of the entire length of the vessel. In most cases, complete reendothelialization occurred within 3 weeks. Endothelial regrowth was significantly different between all time points (*). Data are mean±SEM.](image-url)
Fig 4. Photomicrographs of mouse carotid arteries after denuding injury. a, Normal unmanipulated carotid artery is shown with no smooth muscle cells in the intima. b, Carotid artery is shown 2 days after denudation. Note the loss of some smooth muscle cells in the media below the internal elastic lamina (arrow). c, A substantial intimal thickening is observed in the carotid artery 2 weeks after injury (arrow marks the internal elastic lamina). d, Only faint immunoreactivity is shown for smooth muscle cell α-actin, and only the outer layer of the media and parts of the inner layer of the media show strong staining for smooth muscle cell α-actin (arrowheads). Arrow marks internal elastic lamina. e, Immunostaining for the common leukocyte antigen revealed only few positive cells (arrowheads) in the 2-week intimal lesion (arrow marks the internal elastic lamina). Nuclei were stained with hematoxylin only in a through c. Original magnification ×400.

Examine the surface of the vessel 30 minutes after the denudation, and three passes of the wire removed all of the endothelium from these arteries (Fig 2). If the wire was passed only once down the carotid artery, occasional endothelial cells, which provide foci for reendothelialization, were still present on the surface. In subsequent experiments, denudation was always carried out with three passes of the wire. As shown in Fig 2, the denuded surface is covered with a monolayer of platelets adhering to the exposed subendothelial matrix.

After the denuding procedure was established, four groups of animals were subjected to denudation of the
carotid artery. The animals were injected with Evans blue to differentiate between denuded (blue) and reendothelialized (white) surfaces before death at 5, 8, 14, and 21 days after denudation. Reendothelialization of the vessel from the aortic arch and the carotid bifurcation was measured on the excised vessels. Complete reendothelialization (approximately 9 mm) of the vessel usually occurred within 3 weeks (Fig 3).

As shown in Fig 4, the uninjured mouse carotid artery consists of two layers of medial SMCs (Fig 4a) with no SMCs found in the intima. Loss of SMCs from the layer closest to the lumen was apparent on some sections after denudation (Fig 4b). Statistical analysis, however, revealed there was no significant decrease in medial cell number between any group (Fig 5). Within 8 days after denudation, SMCs were present in the intima, and by 2 weeks the number of intimal SMCs had increased significantly to an average of 133 nuclear profiles per cross section (Fig 5). The average intimal area in the denuded segment of the vessel at 2 weeks was 0.012±0.002 mm² (mean±SEM) (Fig 4c). An antibody against SMC α-actin (HHP-35) was used to determine the nature of the cells present in the vessel wall 2 weeks after injury. Cells in the outer layer of the media stained positive while cells in parts of the inner media and intima showed only faint immunoreactivity for α-actin (Fig 4d). To further characterize the cellular composition of the intimal lesion, immunostaining was carried out with an antibody against the common leukocyte antigen (CD 45), which recognized all species of leukocytes including macrophages. Staining with this antibody revealed very few immunoreactive cells in the intima (Fig 4e).

Replication of SMCs in the media was determined by autoradiography after giving three doses of [3H]thymidine before killing of the animals (Fig 6). The replication index for medial SMCs 2 days after denudation was 1.6%. Maximal replication of medial SMCs at nearly 10% was seen at 5 days after injury, which was significantly higher than replication in uninjured and 2-day vessels. Replication in the media had decreased significantly at 8 days after denudation, and at the same time SMCs began to appear in the intima. At 8 days after injury, these intimal cells showed high replication rates (66%), and intimal SMC replication was still elevated 2 weeks after injury (Fig 6).
Discussion

The interest in defining the roles of endogenous factors in systems in vivo has led many investigators to develop genetic models in which genes are either over-expressed, deleted, or mutated. This approach has been most successful in the mouse, since murine genetics are well characterized and embryonic stem cell lines are readily available. The possibility of using genetic models to study factors thought to be important in vascular biology prompted us to develop an arterial injury model in the mouse. The use of a transgenic mouse model has considerable advantages over other animal systems in that it overcomes the necessity of administering factors or their inhibitors, which can be problematic and often difficult to quantify. Other advantages are that, compared with the rat model, smaller quantities of reagents, such as antibodies, are needed when their effects are studied in the mouse. It would also be possible to prolong any monoclonal antibody treatment, since it is probable that long-term infusions of mouse antibodies would be better tolerated in experimental mice. Further, since the adventitia and the tunica media are considerably thinner in the mouse carotid artery, delivery of agents from the adventitial side will be more likely to penetrate the vessel wall, which may be important when endothelial cells, intimal SMCs, or even medial SMCs are targeted.

With this model of mouse arterial injury, we have shown that complete endothelial denudation of the mouse carotid artery can be achieved, leading to the formation of an intimal lesion. The procedure presented here caused little damage to the tunica media, which may explain the relatively low replication of medial SMCs 2 days after denudation. This form of injury to the mouse carotid artery appears to be similar to the filament loop denudation that we reported in rat arteries in which medial trauma was minimized. The increase in medial SMC replication was followed by migration of SMCs into the intima at 8 days after injury. Those SMCs that migrated into the intima replicated at a high rate, and the 15% replication rate seen at 2 weeks suggested that the lesion continued to increase in size. Indeed, in one animal in which complete reendothelialization had not occurred within 3 weeks, the numbers of SMCs in the intima were as high as 750 per cross section (data not shown). When staining for α-actin was used as an SMC marker, only faint immunoreactivity was found in the intima and inner layer of the media, ie, in those parts of the vessel wall where, presumably, replication of SMCs has recently occurred or is still ongoing. A similar reduction in expression of α-actin by proliferating (synthetic phenotype) SMCs in injured arteries has also been reported for rat and rabbit, and the identity of these cells is problematic. Despite the lack of staining with the SMC α-actin antibody, we believe that these cells are SMCs, as only a few cells in the intima were stained with the antibody directed against the common leukocyte antigen.

One difference from the rat balloon injury model is the fact that complete reendothelialization of the mouse carotid artery usually occurs within 3 weeks. In contrast, complete reendothelialization never occurs in the balloon-injured rat carotid artery. The reason for this is probably related to the shorter length of the vessel, which measures approximately 9 mm in the mouse versus 25 mm in the rat. In the rat, the balloon catheter denudation usually also removes endothelium from the aortic arch around the branch point of the common carotid artery. The denudation device used in our experiments, however, cannot remove endothelium in this location, and rapid regrowth from the arch may thus explain why complete reendothelialization occurs earlier in the mouse carotid artery. From our studies in the rat, we know that SMC replication stops when reendothelialization has occurred. A similar finding was observed in the reendothelialized portion of the mouse carotid artery (Fig 6). This fact is important because it points out that SMC replication, in the absence of endothelial cells, can be studied for only a limited time.

In summary, the model presented here demonstrates for the first time that complete endothelial denudation and intimal lesion formation can be induced in the mouse carotid artery in a predictable manner. With the number of transgenic, knockout, or mutant mouse strains available today, it will be possible to use this model to address the role of specific molecules in vascular biology and atherosclerosis.

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References

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