American Journal of Pathology, Vol. 129, No. 3, December 1987

RAPID COMMUNICATION

Endothelial Regeneration

IX. Arterial Injury Followed by Rapid Endothelial Repair Induces Smooth-Muscle-Cell Proliferation but Not Intimal Thickening

TOYOHIRO TADA, MD,
and MICHAEL A. REIDY, PhD

From the Department of Pathology, University of Washington, Seattle, Washington

Rat thoracic aortas were denuded of endothelial cells with a fine nylon filament, which removed a row of endothelium approximately 100 μ in width and caused platelets to adhere to the exposed subendothelium. A further group of rats was subjected to a sham operation where only the abdominal aorta was injured, and another group of rats was used as controls. Each animal was continuously labeled with ^{3}H-thymidine (6.7 Ci/mM, 10 μCi/hr) for 7 days, at which time they were killed and the aortas perfusion-fixed with aldehydes. Sections of each aorta were processed for autoradiography. The thoracic aortas from animals subjected to the nylon filament injury showed no intimal thickening, but there was a significant increase in the thymidine index of the medial smooth muscle cells (SMCs) as compared with aortas from sham operation or control animals (1.02% ± 0.44% versus 0.19% ± 0.13% versus 0.15% ± 0.12%). This experiment showed that continuous administration of ^{3}H-thymidine permitted the detection of a small but significant increase in replication rates that could not be detected by the standard bolus administration of ^{3}H-thymidine, and that selected loss of endothelium with minimal trauma to the vessel wall caused SMC proliferation without intimal thickening. These findings suggest that platelets may indeed provide SMC proliferation, but that migration of these cells into the intima may be controlled by different factor(s). (Am J Pathol 1987, 129:429–433)

THERE HAS BEEN much debate over whether endothelial denudation with ensuing platelet interaction with the vessel wall initiates proliferation of vascular smooth muscle cells (SMCs) *in vivo*.[1,2] In recent years work from this laboratory has shown that limited loss of endothelium does not lead to intimal thickening despite the presence of platelets.[3,4] This finding was in contrast to that obtained with other models of injury where vessels were totally denuded, such as with a balloon catheter, and a profound SMC proliferation was obtained.[5–7] Why two models of denudation in the same species and even in the same vessel gave such differing results was unclear, but injury to the medial cells and/or slow regrowth of endothelial cells have been discussed in this context.[5–7]

In this study we reexamined the cellular response after a limited denuding injury and focused our attention on the proliferation of the medial SMCs. This is because in other *in vivo* models we have observed that medial proliferation can occur, but without any detectable endothelial loss and without migration of cells into the intima.[8] Furthermore, we have greatly increased our ability to detect replicating cells *in vivo* by continuous infusion of ^{3}H-thymidine with an in-

Supported in part by National Institutes of Health Grants HL-30203 and HL-03174. Dr. Reidy is an Established Investigator of the American Heart Association and is supported in part from funds contributed by the Washington Affiliate.

Accepted for publication October 6, 1987.

Address reprint requests to Michael A. Reidy, PhD, Department of Pathology, SJ-60, University of Washington, Seattle, WA 98195.

dwelling osmotic pump.[9] In this experiment, we found that a transient loss of endothelium, 2–3 days, led to an increase in SMC proliferation, but without any migration of cells into the intima and, hence, no thickening of the intima. These results suggest that endothelial loss can induce SMC proliferation, but that other factors may be necessary for cell migration. Alternatively, rapid repair of wounds by endothelium may play a role in inhibiting the process of SMC movement into the intima.

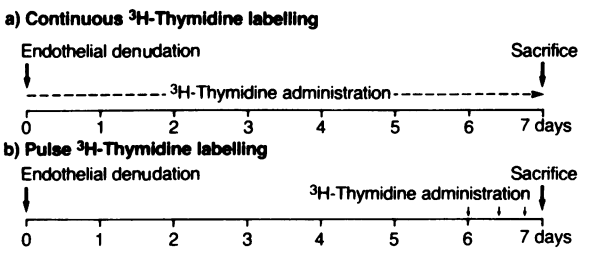

Figure 1—Schematic diagram illustrating the protocols for ^{3}H-thymidine administration. **A**—The implanted osmotic pump continuously infuses ^{3}H-thymidine for the entire experimental period of 7 days. **B**—^{3}H-thymidine is administered during the last 24 hours of the experiment by three bolus injections.

Materials and Methods

Animals

Male Sprague–Dawley rats (Tyler Laboratories Inc., Bellevue, Wash), approximately 20 weeks old, were used in this study.

Induction of Injury of Aortas

The thoracic aortas of 8 rats were subjected to a defined injury by means of a nylon filament (diameter 0.39 mm) inserted into a polyethylene tubing (PE50, Clay Adams, Parsippany, NJ). The tip of the nylon filament was bent such that it would come into contact with the inner surface of the aorta. The catheter, with the nylon filament withdrawn into the PE50, was inserted into the aorta via the right femoral artery; and at the level of the diaphragm, the filament was extended out of the polyethylene tubing to the level of the aortic arch. The catheter was then slowly withdrawn, and the incision closed. At the same time, in 5 of the rats an osmotic minipump was implanted into the peritoneal cavity (model 2001, Alzet Co., Palo Alto, Calif). These osmotic pumps contained 0.2 ml of ^{3}H-thymidine (6.7 Ci/mmol, 10 mCi/ml, New England Nuclear, Boston, Mass) and pumped at a rate of 10 uCi/hr for 7 days. This protocol is outlined in Figure 1. A further 5 rats received no injury, but identical osmotic pumps filled with ^{3}H-thymidine were implanted as above. Another 5 animals were subjected to a sham operation in which the polyethylene tubing alone was inserted into the abdominal aorta to the level of the diaphragm and then withdrawn. As above, in these animals osmotic pumps containing ^{3}H-thymidine were implanted into the intraperitoneal cavity. After 7 days, all animals were perfused with 2% glutaraldehyde, 1% paraformaldehyde in 0.15 M phosphate buffer for 5 minutes, as previously described.[10] A further 3 rats were injure with the nylon filament but received no ^{3}H-thymidine and were perfusion-fixed as above 30 minutes after injury and stained with 20 ml silver nitrate (0.25%) for approximately 15 seconds. The aorta was then perfusion-fixed for a further 2 minutes and excised.

Light Microscopy and Autoradiography

The aortas of all rats were carefully excised and immersion-fixed for a further 24 hours. These aortas, except the 3 rats stained with silver nitrate, were prepared for histology and autoradiography. Three separate segments of each thoracic aorta were removed and embedded in paraffin. Four 5-μ cross-sections were cut from each specimen with a distance of 100 μ between sections. The sections were dipped in Kodak NTB-2 emulsion and exposed for 2 weeks. The slides were then developed, stained with hematoxylin, and mounted under coverslips; and the number of replicating cells was counted. A cell was counted if three distinct silver grains were observed over each nucleus.

Scanning Electron Microscopy (SEM)

Pieces of thoracic aorta were cleared of periadventitial fat, opened longitudinally, and pinned flat onto Teflon sheets. The tissue was dehydrated through a series of ethanol and dried in a critical-point dryer as previously described.[3] Subsequently, the specimens were mounted on an SEM stub with colloidal silver paste, sputter-coated with gold/palladium, and examined in a JEOL 35C microscope, at a voltage of 15 kv.

Results

The nylon filament removed a row of endothelial cells, approximately 100 μ wide, along the length of the thoracic aorta, and the exposed subendothelium was covered by adhering platelets (Figure 2). In a previous study,[4] we showed that an injury of this size completely reheals with endothelium after approximately 90 hours. For the purposes of this experiment,

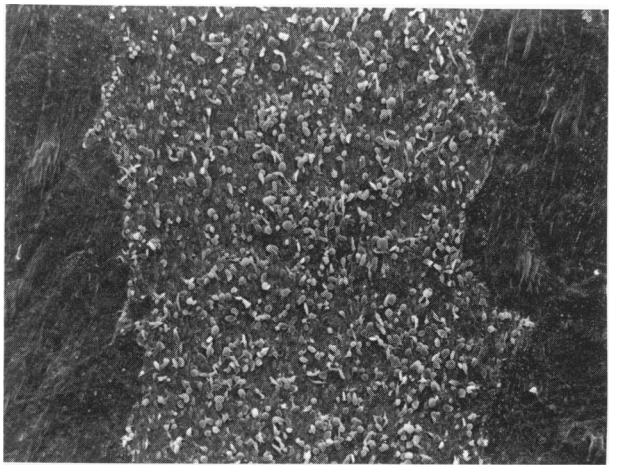

Figure 2—Scanning electron micrograph of thoracic aorta 30 minutes after injury with the nylon filament. A row of endothelium was removed and the exposed subendothelium covered by platelets. (×830)

it was not deemed necessary to repeat this work; however, Figure 3A shows the rehealed surface after 7 days. The regenerated endothelial cells that repopulated the wound were densely packed and aligned with flow. In all respects, these injured vessels were identical to those subjected to a similar injury previously described by us.[4] The thoracic aorta of sham and control animals showed no morphologic changes.

Histologic sections cut through the site of injury, which was easily recognized by the endothelial cell density and the ^{3}H-thymidine labeling, showed no intimal thickening after 7 days (Figure 3B). For comparison, balloon catheter injured vessels could show extensive intimal thickening at this time.[5] Autoradiography, however, showed that in those animals subjected to the nylon filament injury, there was a very significant increase in medial SMC replication (Table 1 and Figure 3B) that was not observed in either the aortas of the sham operation animals or the control animals. We did not observe a close association between the location of replicating SMCs and the sites of denudation. Most of the replicating SMCs were not closely associated with those sites showing endothelial cell replication (Table 2 and Figure 3B). As previously published.[3,4] transmission electron microscopy revealed no morphologic evidence of medial injury at early or late time periods.

Discussion

The results of this study showed that endothelial cell loss followed by platelet adherence resulted in a significant increase in the proliferation of SMCs in the media of rat aortas. These data are of importance

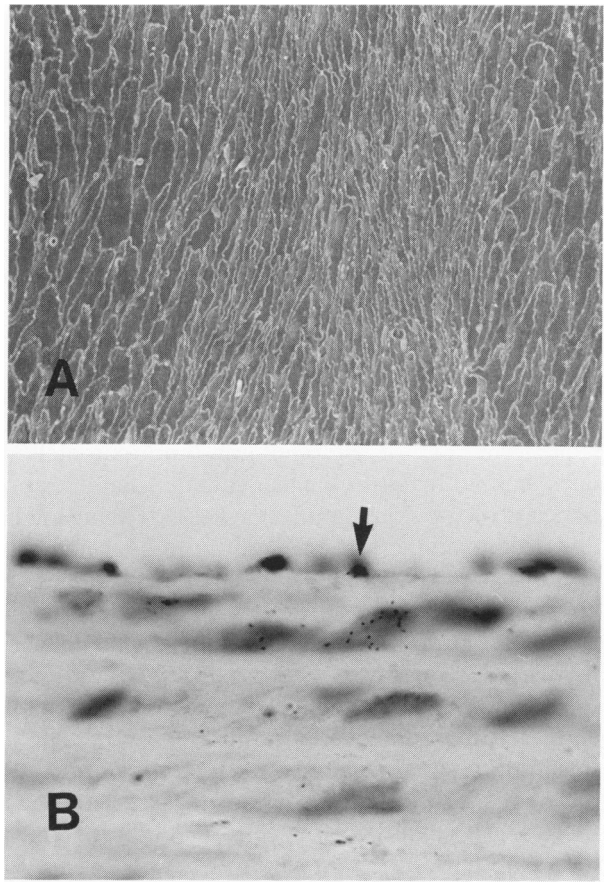

Figure 3A—Thoracic aorta of animal 7 days after injury with the nylon filament. The vessel was stained with silver nitrate (0.25%) to outline the cell borders. The site of injury can be easily recognized by the densely packed endothelial cells. Normal-sized endothelial cells are present on either side of the injury. **B**—Section of thoracic aorta at the site of denudation 7 days after injury with the nylon filament. The vessel showed no thickened intima, but several labeled SMCs (silver grains over nuclei) are clearly visible in the media. There is one labeled endothelial cell, and the cell with two grains (*arrow*) was not counted. (×900)

because they offer one possible explanation why in the past different techniques for denuding a vessel of endothelium have given such opposing results.[3-5,7] For example, balloon catheter denudation of an artery causes very profound medial proliferation followed by intimal thickening[2,5-7,11]; yet narrow catheter denudation, which also causes endothelial loss and platelet adhesion, was found to initiate no detect-

Table 1—SMC Replication in Rat Aortas with Continuous Thymidine Labeling for 7 Days

	Denuding injury	Sham operation	Control
Thymidine index*	1.02 ± 0.44†	0.19 ± 0.13	0.15 ± 0.12

*The cumulative thymidine index is the number of replicating cells/total number of cells counted ×100. (See text for details.)

†Mean ± standard deviation (SD) ($n = 5$ for each group); $P < 0.005$.

Table 2—Location of Replicating SMCs With Sites of Endothelial Replication

	Number of replicating smooth muscle cells (%)*
Adjacent to endothelial replication†	34.5 ± 26.7%
Distant to endothelial replication	65.5 ± 26.7%

*Data expressed as a percentage of total labeled SMCs ± SD.
†Includes those SMCs that were directly covered by replicating endothelial cells or lay within one cell length of a replicating endothelial cell.

able smooth-muscle-cell replication in the underlying media. In this experiment we have now shown that short-term loss of endothelium (approximately 3 days) caused by a mechanical catheter that does not appear to do obvious damage to the underlying media does lead to an increase in smooth-muscle-cell proliferation. A key difference with these experiments as compared with those previously published by us[3,4] is the administration of ^{3}H-thymidine. In this experiment, ^{3}H-thymidine was administered continuously throughout the entire 7 days; whereas in the latter study ^{3}H-thymidine was administered in three bolus injections over the last 24 hours of the experiment. In our hands, continuous labeling with ^{3}H-thymidine is more sensitive in detecting cells undergoing replication than conventional techniques.[9] One reason for this is that ^{3}H-thymidine is always present, and therefore multiple rounds of division will be detected. It is interesting to speculate what this value really means, because multiple doublings of a few cells or single replication of many cells would give the same cumulative thymidine index. Without detailed information on the concentration of ^{3}H-thymidine in each cell, it is not possible to suggest which pathway occurred. For the purposes of this study, this was not relevant, because our aim was to detect whether cells underwent any replication at all. It should be noted that the cumulative thymidine index obtained by this procedure should not be compared with data obtained by means of single or multiple injections of ^{3}H-thymidine over a 24-hour period.

The data presented here could be used to support the reaction to injury hypothesis[2]; however, surprisingly, the majority of SMC replication took place at sites not directly adjacent to sites of endothelial cell denudation. This lack of any clear association of replicating SMCs with replicating endothelial cells might reflect that in some sites endothelial repair took place without any replication or that we may not have recognized all replicating endothelial cells. We consider this latter possibility quite plausible because in cross-sections of these arteries only a small profile of each endothelial cell nucleus was visible as compared with the large nuclear profile of the underlying SMCs (Figure 3), and if we assume a uniform nuclear distribution of ^{3}H-thymidine, then the fractional area of each nucleus displayed in each cross-section could profoundly influence the number of silver grains and thus our ability to determine cell replication. This is especially true where the amount of ^{3}H-thymidine incorporated might be minimal. Finally, there is the fact that a wave of cell replication spreads along the vessel, and therefore cells at distant sites might be induced to proliferate because of a signal that originated at some distance away.[16]

Of great interest to us was the finding that SMC replication was not followed by migration of cells into the intima. These data are in striking contrast to the results obtained with balloon catheter injury, where SMCs were observed in the intima by Day 4, and a very substantial intimal thickening was observed by Day 7 (Table 3).[5] At the present time there are no firm data to explain these differences, but one possibility relates to the speed of endothelial regrowth. After injury with the nylon filament, endothelial regrowth occurred within approximately 3 days[4]; but after balloon catheter denudation, endothelial regrowth was slow.[5,11,12] *In vitro,* endothelial cells have been shown to inhibit the growth of SMCs, possibly by the action of a secreted heparinlike molecule.[13–15] Thus, the presence of endothelial cells over the proliferating SMCs may be an important controlling factor in intima lesion development. Some support for this concept can be found in the study of Haudenschild and Schwartz,[16] where it was observed that after balloon denudation, regions of the rat thoracic aorta closest to the intercostal arteries, which were rapidly repopulated with endothelium, showed little or no intimal thickening. Areas away from the intercostal branches, which were slow to be repopulated, showed extensive intimal thickening.

Another possible explanation of why the nylon fila-

Table 3—Summary of Rat Vessel Wall Response After Denuding Injury

	Small nylon filament	Balloon catheter†
Extent of endothelial cell loss	10–15 cells wide	Total denudation
Injury to vessel media	Not detected	Acute loss of DNA
Time for complete reendothelialization	72–96 hours*	>1 year
SMC proliferation	Medial only	Intimal + medial
Intimal thickening after denudation	No	Yes

*See Reidy and Schwartz.[3]
†See Clowes et al.[5]

ment does not provoke intimal thickening could be that this injury does not damage the underlying medial cells.[4] In contrast, the balloon catheter causes detectable injury with cell death and possible release of intracellular contents (Table 3).[5] Thus, it is possible to speculate that mitogenic factors such as fibroblast growth factor (FGF) are released from injured cells directly into the media where they might stimulate cell migration as well as cell proliferation.[17-19] Severe stretching of the vessel caused by the balloon catheter, possibly in connection with release of proteolytic enzymes from dead cells, may also facilitate the destruction of the matrix and thereby allow medial cells to gain access to the intima. We consider this unlikely, because recent work by one of us (M.A.R.) has shown that *in vivo* stretching of an artery without widespread endothelial denudation stimulates SMC proliferation but no intimal thickening.[20]

In summary, loss of endothelium using a nylon filament caused a significant increase in the proliferation of the underlying medial SMCs. This was only detected when ^{3}H-thymidine was administered continuously for the duration of the experiment. This cellular proliferation was not accompanied by intimal thickening. One explanation for these findings is that platelet interaction with the vessel stimulates cell proliferation, but that rapid regrowth of the endothelium prevents the migration of SMCs to the intima.

References

1. Schwartz SM, Campbell GR, Campbell JH: Replication of smooth muscle cells in vascular disease. Circ Res 1986, 58:427–444
2. Ross R: The pathogenesis of atherosclerosis. An update. N Engl J Med 1986, 314:488–500
3. Reidy MA, Schwartz SM: Endothelial regeneration: III. Time course of intimal changes after small defined injury to rat aortic endothelium. Lab Invest 1981, 44:301–308
4. Reidy MA, Silver M: Endothelial regeneration: VII. Lack of intimal proliferation after defined injury to rat aorta. Am J Pathol 1985, 118:173–177
5. Clowes AW, Reidy MA, Clowes MM: Kinetics of cellular proliferation after arterial injury: I. Smooth muscle growth in the absence of endothelium. Lab Invest 1983, 49:327–333
6. Schwartz SM, Stemerman MB, Benditt EP: The aortic intima: II. Repair of the aortic lining after mechanical denudation. Am J Pathol 1975, 81:15–42
7. Spaet TH, Stemerman MB, Veith FJ, Lejnieks I: Intimal injury and regrowth in the rabbit aorta: Medial smooth muscle cells as a source of neointima. Circ Res 1975, 36:58–70
8. Reidy MA: *In vivo* proliferation of vascular smooth muscle cells in vessels with intact endothelial cover (Abstr). Fed Proc 1986, 45:683
9. Clowes AW, Schwartz SM: Significance of quiescent smooth muscle migration in the injured rat carotid artery. Circ Res 1985, 56:139–145
10. Schwartz SM, Benditt EP: Studies on aortic intima: I. Structure and permeability of rat thoracic aortic intima. Am J Pathol 1972, 66:241–264
11. Clowes AW, Reidy MA, Clowes MM: Mechanisms of stenosis after arterial injury. Lab Invest 1983, 49:208–215
12. Reidy MA, Clowes AW, Schwartz SM: Endothelial regeneration: V. Inhibition of endothelial regrowth in arteries of rat and rabbit. Lab Invest 1983, 49:569–575
13. Castellot JJ Jr, Addonizio ML, Rosenberg R, Karnovsky MJ: Cultured endothelial cells produce a heparinlike inhibitor of smooth muscle cell growth. J Cell Biol 1981, 90:372–379
14. Castellot JJ Jr, Favreau LV, Karnovsky MJ, Rosenberg RD: Inhibition of vascular smooth muscle cell growth by endothelial cell-derived heparin: Possible role of a platelet endoglycosidase. J Biol Chem 1982, 257:11256–11260
15. Majack RA, Clowes AW: Inhibition of vascular smooth muscle cell migration by heparin-like glycosaminoglycans. J Cell Physiol 1984, 118:253–256
16. Haudenschild CC, Schwartz SM: Endothelial regeneration: II. Restitution of endothelial continuity. Lab Invest 1979, 41:407–418
17. Abraham JA, Mergia A, Whang JL, Tumolo A, Friedman J, Hjerrild KA, Gospodarowicz D, Fiddes JC: Nucleotide sequence of a bovine clone encoding the angiogenic protein, basic fibroblast growth factor. Science 1986, 233:545–548
18. Schweigerer L, Neufeld G, Friedman J, Abraham JA, Fiddes JC, Gospodarowicz D: Capillary endothelial cells express basic fibroblast growth factor, a mitogen that promotes their own growth. Nature 1987, 325:257–259
19. Gospodarowicz D, Neufeld G, Schweigerer L: Molecular and biological characterization of fibroblast growth factor, an angiogenic factor which also controls the proliferation and differentiation of mesoderm and neuroectoderm derived cells. Cell Diff 1986, 19:1–17
20. Clowes AW, Clowes MM, Reidy MA: Role of acute distension in the induction of smooth muscle proliferation after endothelial denudation (Abstr). Fed Proc 1987, 46:720

Acknowledgments

The authors would like to thank to Stella Chao for her excellent technical assistance.