Intimal Lesion Formation in Rat Carotid Arteries after Endothelial Denudation in Absence of Medial Injury

Jürgen Fingerle, Y.P. Tina Au, Alexander W. Clowes, and Michael A. Reidy

Injury of an artery by passage of a balloon catheter causes both endothelial denudation and medial damage and produces a marked smooth muscle cell (SMC) proliferative response. In this study, the endothelium from rat carotid arteries was removed by use of a rotating loop of 5/0 monofilament suture (gentle denudation technique), which did not cause any detectable damage to the underlying medial cells but did cause platelet adherence. Expression of platelet-derived growth factor (PDGF) A-chain and PDGF receptor mRNA was comparable to that seen in ballooned carotids, but the medial SMC proliferative response to gentle denudation was markedly reduced when compared to that observed after balloon denudation (1.4% vs. 13.6%). Intimal lesions were only observed in those zones that remained denuded for more than 7 days. These results demonstrate that a denuding injury with no medial trauma is sufficient to induce intimal lesions and that the significantly higher proliferation seen in ballooned vessels might reflect a response of the medial cells to trauma that occurred during denudation.


The balloon catheter-injured artery has become the standard model for studying smooth muscle cell (SMC) proliferation in vivo, and yet there is still little information as to what factors are involved in this process. One early hypothesis was that smooth muscle replication, which occurs soon after denudation, is initiated by platelet factors, but work by several groups has shown that platelet adherence to exposed subendothelium causes neither SMC replication nor intimal lesion formation. Furthermore, in the total absence of platelets, balloon-injured arteries show a dramatic increase in SMC proliferation similar to that found in denuded arteries from control animals. These data strongly suggest that the SMC proliferation induced by mechanical injury is modulated by other, undefined factors.

A recent study by Majesky et al. has shown that within hours after balloon injury, rat carotid arteries increase their expression of mRNA for platelet-derived growth factor (PDGF) A-chain and for the PDGF receptor. Therefore, injury to the arterial wall might induce an autocrine pathway in which SMC stimulate their own proliferation by synthesis of PDGF. Yet another explanation for the rapid increase in SMC proliferation might be that the process of balloon denudation disrupts the arterial wall and so releases growth factors into the extracellular milieu. In this regard, there is a body of evidence suggesting that basic fibroblast growth factor (FGF) is indeed localized in the extracellular matrix of arteries, and both endothelial cells and SMC are known to synthesize FGF. Injury to the vessel wall could therefore release this stored FGF and stimulate smooth muscle growth.

In this study, we compared SMC proliferation in arteries subjected to endothelial denudation in a manner known to cause widespread cell death (balloon catheter) with the proliferation in arteries denuded by a new technique (gentle denudation), which causes no detectable trauma to the SMC. Both these procedures caused equal loss of endothelium, platelet adherence to exposed subendothelium, and similar expression of PDGF and its receptor, and yet the early medial proliferative responses were markedly different (13.6% vs. 1.4%). These data demonstrate that loss of endothelium for several days is followed by SMC proliferation, but that the extent of cell replication seems to correlate with the extent of trauma inflicted on the vessel wall.

Methods

Experimental Design

A total of 149 Sprague-Dawley rats weighing 400 g (Tyler Labs, Bellevue, WA) were used in this study. The animals were anesthetized with Innovar (0.1 ml/kg body weight, Pitman-Moore, Washington Crossing, NJ), and their left external carotids were exposed in a way that minimized trauma to the common carotid and removed the endothelium as described below. For comparison studies, rat carotid arteries were subjected to balloon catheter injury with

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Gentle Denudation of Carotid Artery

The endothelium was denuded from a rat carotid by use of the device shown in Figure 1. A loop of 5/0 nylon monofilament suture (Dermalon, Davis and Geck, Wayne, NJ) was held open by inserting its ends into a polyethylene tubing (PE 10, Clay Adams, Parsippany, NY), and the loop was introduced into the left external carotid artery via a trocar made of polyethylene tubing (PE 60). The device was pushed through the trocar into the common carotid to the aortic arch. Since the loop measured 3 cm, it was long enough to reach the arch while the supporting PE 10 tubing remained inside the trocar not touching the common carotid. The device was steadily pulled back along the carotid with constant rotation. After retrieving the trocar, the external carotid was tied off, and the incision was closed. We evaluated the extent of endothelial denudation in 10 animals with only one single passage of the loop and found three carotids with residual patches of endothelial cells. In all subsequent experiments, three passages of the loop were passed through the carotid artery.

DNA Assay

The DNA content was determined on unfixed duplicate segments (5 mm in length) of cleaned common carotid, as described earlier. 8

Measurement of Smooth Muscle Cell Proliferation

The proliferative response was assessed in the arteries of animals fixed by perfusion with 1% paraformaldehyde and 2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) at different intervals after gentle denudation (1, 2, 4, 7, and 14 days and 4 and 12 weeks; a minimum of four animals per time point). All animals received 0.5 ml of 0.5% Evans blue intravenously at 0.5 hours before sacrifice.

Two protocols for labeling were used: 1) To permit determination of cumulative proliferation rate over the first 14 days, osmotic minipumps (2022, minipumps volume 250 μl, Alza, Palo Alto, CA) were used. The pumps were filled with tritiated thymidine (6.7 mCi/mM; 10 mCi/ml, New England Nuclear). Two pumps per animal were implanted intraperitoneally at the time of gentle denudation. The arteries were fixed by perfusion 14 days after injury. 2) To determine the cumulative proliferation rate at 17 hours, 9 hours, and 1 hour before sacrifice. Autoradiography was performed on paraform-embedded sections dipped in Kodak NTB2 emulsion and developed after 14 days at 4°C. The thymidine index (percent) was measured and expressed as labeled nuclei/total nuclei x 100.

Endothelial Morphology

Silver staining of endothelial surfaces was performed by interruption of the perfusion fixation after the first minute and injection of 20 ml of 1% silver nitrate in water followed by continuation of fixation for another 5 minutes. The excised vessels were pinned out on Teflon sheets and were examined under a light microscope.

Area Measurements

Intimal and medial cross-sectional areas were obtained from hematoxylin/eosin-stained paraffin sections. Cross-sectional images were traced on a digitizing pad (GTCo, Rockville, MD) by means of a camera lucida (Leitz, Wetzlar, FRG), and calculations were made with a software package (Image Measure, Microscience, Seattle, WA).

Northern Hybridization

The carotids were subjected either to gentle denudation with the new loop device or to balloon catheter injury. The total RNA was extracted from the carotid arteries of rats killed at 6 hours, or at 4, 7, or 14 days after injury (a minimum of six carotids per time point). The vessels were quickly removed, stripped of the adventitia, rinsed in phosphate-buffered saline (pH 7.4), and then were snap-frozen in liquid nitrogen. The frozen tissue was ground to a fine powder with a mortar and pestle while being constantly cooled with liquid nitrogen and was then homogenized in 5 M guanidine isothiocyanate buffer with a polytron (Kinematica, Lucerne, Switzerland). The total RNA was selectively precipitated by the addition of lithium chloride according to the procedure of Cathala et al. 16

The RNA concentration was determined by spectrophotometry. Northern blot analysis was performed as described earlier. 17 The cDNA probes for PDGF A-chain, 18 PDGF receptor (beta-subunit), 11 and actin 19 were labeled by nick translation. 20 The 32P-labeled cDNA was added to the hybridization solution at 2 × 10^6 cpm/ml. After hybridization at 42°C for 20 to 24 hours, blots were washed in 0.3 M NaCl/0.03 M Na-citrate/0.1% sodium dodecyl sulfate (SDS) (pH 7.0) at room temperature for 30 minutes, followed by washing in 0.015 M NaCl/0.0015 M Na-citrate/0.1% SDS (pH 7.0) at 85°C for 10 minutes. The signals were detected by autoradiography.
Table 1. Effect of Different Methods of Endothelial Denudation on DNA Content of Carotid Arteries

<table>
<thead>
<tr>
<th>Carotid treatment</th>
<th>Uninjured</th>
<th>Denuded</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gentle denudation</td>
<td>2.65±0.25</td>
<td>2.37±0.24</td>
</tr>
<tr>
<td>Balloon denudation</td>
<td>2.60±0.3</td>
<td>1.95±0.5</td>
</tr>
</tbody>
</table>

The values are the means±SD of the DNA content (μg/5 mm length of carotid).

Two segments per animal were evaluated (four animals in each treatment).

*p<0.005 vs. left carotid.

Results

The effectiveness of the new catheter in removing endothelial cells from rat carotid arteries was assessed by examining the full length of 10 arteries at 1 hour after denudation. No endothelial cells were seen by scanning electron microscopy, and the luminal surface was covered with platelets. As previously noted, platelet thrombi were frequently observed immediately after denudation of these vessels, but at later times these denuded surfaces were identical to balloon catheter-denuded arteries. In all subsequent experiments, one or two animals from each group were randomly selected, were killed immediately after denudation, and were examined by scanning electron microscopy. These vessels showed that total and complete loss of endothelium was always achieved with this new catheter. An important difference with this new denuding technique, as compared to the balloon catheter, was that endothelial cells continued to grow on the denuded surface of these arteries until re-endothelialization was complete. Our understanding of this occurrence is incomplete, but pertinent to this issue is our observation that these regenerating endothelial cells always stain strongly for basic FGF.

To assess the extent of injury inflicted on medial SMC by the denuding procedure, the DNA content of known lengths of five carotids was measured (Table 1). No significant loss of DNA was detected when compared to normal untraumatized vessels, and the slight reduction in DNA in these denuded arteries could be accounted for by the removed endothelial cells (2.2×10^3 endothelial cells/mm²). These results, however, do not eliminate the possibility that medial SMC were subjected to some trauma that could not be detected.

Response of Smooth Muscle Cells

In a previous report on studies of the balloon denudation model, we documented changes in the expression of medial SMC of genes thought to be associated with the progression of cells through the cell cycle. Of particular interest was the mRNA for PDGF A-chain which increased sixfold within 6 hours of denudation. In the present study, gentle denudation also increased the mRNA for PDGF A-chain by 6 hours and, in a similar manner to balloonened arteries, the transcript declined by day 4 and remained constant at 7 days (Figure 2). The PDGF-receptor mRNA decreased by 6 hours, but expression increased again by day 4 to control levels. Therefore, the mRNA of the transcript remained constant or was elevated. An important feature of this experiment is that the changes in mRNA seen after gentle denudation were identical to those observed in a balloon catheter-denuded artery, where there is a significantly greater increase in proliferation (see below).

SMC proliferation in these denuded carotids was determined by autoradiography at various times after injury. At 2, 4, and 7 days after gentle denudation, there was a significant increase in the thymidine index of the media, although the replication did not exceed 2% in a 24-hour time period (Table 2). By 2 weeks and at subsequent later times, the medial cell proliferations dropped to levels observed in control tissue. Intimal SMC were detected in these denuded carotids by day 7 and had a high proliferative rate (48.1%). This intimal proliferation remained significantly elevated at 2 weeks after denudation, but by 12 weeks had declined to approximately background levels (Table 2). For comparison, after balloon catheter injury, we previously showed that the medial SMC proliferation was approximately 40% by 4 days after injury. The intimal proliferative response in both models was similar.

The size of the intimal lesion produced by gentle denudation was measured at various times (Figure 3). After gentle denudation, no SMC were observed in the intimas at day 4, but cells were present by day 7. By 2 weeks, the intimas had grown substantially and remained at the same size for the remaining experimental period. DNA measurements of these injured vessels
The regrowth of endothelium over these sites, therefore, of the artery by balloon catheter, and yet the reason appears to have suppressed SMC migration. Areas with or those without new endothelium (Table 4). We observed no difference in the replication rate of medial SMC at 14 days after denudation in either the lesion after gentle denudation was about 50% smaller than that observed after balloon catheter injury (Figure 3). A comparison of these lesions with those formed after balloon catheter injury showed that at 1, 4, and 12 weeks, gentle denudation induced a significantly smaller lesion. At 12 weeks after injury, the size of the lesion after gentle denudation was about 50% smaller than that observed after balloon catheter injury (Figure 3).

After gentle denudation, those areas that were rapidly repopulated by endothelial cells (in less than 7 days) showed no intimal thickening (data not shown). Regrowth of endothelium could influence both cell proliferation and cell migration, but using continuous thymidine labeling, we observed no difference in the replication rate of medial SMC at 14 days after denudation in either the areas with or those without new endothelium (Table 4). The regrowth of endothelium over these sites, therefore, appears to have suppressed SMC migration.

Discussion
SMC proliferation in vivo can be initiated by denudation of the artery by balloon catheter, and yet the reason for this dramatic response is poorly understood. Early work focused on the role of platelets, but it soon became apparent that release of their mitogens on exposed subendothelium could not readily explain the increase in smooth muscle replication observed in denuded arteries. Indeed, in a recent study, we found that the total absence of platelets made no difference to the first wave (24 to 48 hours) of SMC proliferation induced by the use of a balloon catheter. Furthermore, in this study a relatively small increase in cell proliferation was observed after denudation, and yet large platelet thrombi were present on the exposed subendothelium. This latter data should be contrasted with the marked increase in SMC proliferation seen after balloon catheter denudation, where only a monolayer of platelets was observed. Other hypotheses that try to explain the dramatic increase in SMC replication have included 1) release of endogenous mitogens from injured smooth muscle and endothelial cells and 2) the de novo synthesis of mitogens by the arterial wall cells. This latter suggestion was of interest because SMC in vitro have been shown to synthesize PDGF, and a recent study by Majesky et al. showed that soon after balloon catheter injury, marked changes in mRNA levels for the PDGF A-chain and for the PDGF receptor were induced. Moreover, the rapidly proliferating intimal cells continued to express high levels of mRNA PDGF A-chain. One interpretation of these results is that a PDGF-dependent endogenous growth regulatory pathway exists in cells from injured arteries. In this study, the two denuding injuries stimulated SMC at very different rates; after gentle injury, the thymidine index never exceeded 2.0%, but after balloon catheter injury, the index was 13.6%. Of particular interest to the above discussion is the finding that mRNA expression for PDGF A-chain and the receptor were identical for both sets of arteries. Thus, despite significantly different proliferation rates, these two sets of denuded arteries showed similar
changes in their mRNA for at least one important mitogen, which suggests that either endogenous PDGF synthesis does not play a key role in cell replication or that translation of this peptide is differentially regulated after these two injuries.

The data presented here would suggest that the degree of injury (assessed by DNA content) to the vessel wall is related to the extent of SMC proliferation. On first consideration, it is difficult to see how cell death might be a mitogenic stimulus, but one explanation is that dead cells and their matrices may release one or more mitogenic factors. Of particular interest is the suggestion that FGF can be released from injured cells. Both endothelial cells and SMC can synthesize FGF, and there is evidence that the subendothelial matrix is a rich source of basic FGF. Using Western blot analysis, we have found basic FGF in rat arteries. It is also important to remember that SMC respond to FGF, and that exogenous basic FGF has been shown to cause a significant increase in the replication rate of SMC in rat arteries. Our data show that balloon catheter denudation causes widespread cell death and causes a highly significant increase in the proliferation rates of medial smooth muscle cells, whereas after gentle denudation, there is no detectable trauma to the arterial wall, and the proliferation response is significantly smaller (13.6% vs. 1.4%). Thus, the release of intracellular factors after injury might offer an explanation for the induction of SMC proliferation after arterial injury. Studies are currently underway to provide evidence for such a pathway.

One reason for initiating this study was to answer the question of whether endothelial cell loss (in the absence of medial trauma) is important for arterial SMC proliferation. In the past, major differences were found in experiments where arteries were subjected to balloon catheter injury as compared to other experiments in which denudation without medial trauma caused no intimal thickening. The results of this study highlight the fact that the duration of endothelial denudation is an important factor in controlling the development of intimal lesions. This was clearly shown in the experiment in which arteries subjected to gentle denudation and rapidly repopulated with new endothelium (in less than 7 days) developed no intimal thickening despite a significant increase in smooth muscle proliferation. Interestingly, the adjoining areas that were not yet re-endothelialized by day 7 and that had an identical labeling index did go on to develop intimal lesions. Thus, it would appear that loss of endothelial cells does initiate intimal lesion formation, provided the period of endothelial loss is sustained for several days. These data also emphasize that SMC replication and cell movement are different processes and that the endothelium strongly influences cell movement. In retrospect, it is therefore not surprising that small denuding injuries followed by rapid re-endothelialization never formed intimal lesions.

In summary, this article has shown that the loss of endothelium without detectable trauma to the media and without rapid rehealing of endothelium initiated a small, but significant, increase in SMC proliferation and the formation of an intimal lesion. The magnitude of this response both in terms of SMC replication and lesion formation was significantly less than after endothelial denudation with a balloon catheter. Both groups of denuded vessels showed similar changes in mRNA expression for the PDGF A-chain and for the PDGF receptor. These data, summarized in Table 5, suggest that injury to the vessel with a possible release of intracellular mitogens may be an important factor controlling smooth muscle proliferation seen so reproducibly after the mechanical denudation of arteries.

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References


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