Heparin Regulates Smooth Muscle S Phase Entry in the Injured Rat Carotid Artery

Mark W. Majesky, Stephen M. Schwartz, Monika M. Clowes, and Alexander W. Clowes

Smooth muscle cell (SMC) proliferation in injured arteries is inhibited by heparin, but the mechanism of inhibition is unknown. In particular, it is not clear whether heparin prevents exit of quiescent SMC from the resting state, inhibits progression through the prereplicative (G₁) sequence, or acts during DNA synthesis itself. In this study, induction of ornithine decarboxylase (ODC) activity was used as a marker of SMC entry into the cell cycle in an attempt to localize the site of heparin action during the initial hours after rat carotid injury. Rapid and transient induction of ODC activity was observed that reached a maximum (twenty-three-fold) 6 hours after wounding. Heparin failed to prevent ODC induction but greatly reduced frequencies of [³H]thymidine-labelled SMC nuclei 33 hours after injury. Moreover, heparin infusion could be delayed for up to 18 hours after the injury event with no significant loss of antiproliferative effect. Further delays resulted in marked loss of growth inhibition. The results of these studies show that SMC rapidly and synchronously leave the resting state after injury and suggest that heparin acts late in the prereplicative (G₁) sequence or early in S phase to inhibit SMC proliferation in damaged arteries. (Circulation Research 1987;61:296–300)

Wound repair in injured arteries leads to accumulation of smooth muscle cells (SMC) in the intima and may contribute to the formation and localization of atherosclerotic plaques. 1–3 However, simple endothelial denudation, even with platelet adherence and release of granule contents, does not stimulate smooth muscle proliferation or intimal migration. 4–6 Thus, a more extensive injury, perhaps involving release of a growth factor endogenous to the vessel wall or release from a local growth inhibitor, is required.

This concept is supported by analysis of the kinetics of SMC proliferation after extensive injury to the rat carotid artery. Initial increases in [³H]thymidine-labelled SMC nuclei are found 24–48 hours after wounding, 7 and entry of SMC into the growth fraction is essentially complete 72 hours after carotid injury. 8 Further proliferation of SMC occurs by repeated division of this initial cohort. 4 This suggests some form of commitment event occurring early after the injury.

Replication of SMC can be nearly totally ablated if heparin is given at the time of carotid injury. 9,10 The possibility that this effect of heparin mimics some endogenous heparin-like molecule is supported by the extraction of a proteoglycan with growth inhibitory activity from intact aorta 11 and by observations that cultured SMC and endothelial cells can synthesize a heparin-like glycosaminoglycan with the ability to inhibit SMC proliferation in vitro. 12,13 These results suggest that a heparin-like molecule present in the extracellular matrix of the artery wall serves to maintain SMC in a quiescent state. If true, one might expect exogenous heparin to prevent any of the series of events that lead to DNA synthesis in injured arteries. The studies reported here were designed to explore this possibility by examining the effects of heparin on an early biochemical marker of SMC growth initiation after carotid injury. Ornithine decarboxylase (ODC) activity in injured arteries was used as an indicator of SMC entry into the prereplicative (G₁) phase. 14,15 ODC activity is rate limiting for the biosynthesis of putrescine, a precursor for the polyamines spermidine and spermine, and has been shown to be necessary for stimulation of DNA synthesis in another model of tissue response to acute injury, regenerating rat liver after partial hepatectomy. 16 Since the enzyme has an extremely short half-life, changes in rates of synthesis and breakdown quickly appear as fluctuations in enzyme activity. 17 The results obtained suggest that SMC rapidly and synchronously leaves the resting state after carotid injury. Heparin does not prevent early changes in ODC activity in injured arteries and appears to act late in the sequence leading to DNA synthesis.

Materials and Methods

Arterial Injury Model

Male Sprague-Dawley rats (500 g, 5 months old) (Tyler Laboratories, Bellevue, Wash.) were anesthetized with Innovar (0.1 ml i.m.) (Pittman-Moore, Inc., Washington Crossing, N.J.) and pentobarbital (5 mg i.p.) (Veterinary Laboratories, Lenexa, Kan.), and acute injury to the left common carotid artery was made with an inflated balloon catheter as previously described. 7 Either heparin (0.3 µg/g body w/hr) (Sigma Chemical Co., St. Louis, Mo., type II)
dissolved in lactated Ringer's solution or lactated Ringer's solution only was infused for the indicated times through a catheter inserted into the left jugular vein and connected to a Harvard constant infusion pump. Heparin delivery was verified by demonstration of appropriately prolonged clotting times after killing. When heparin infusion was started at varying times after injury, catheters were placed at the time of injury, and rats were infused with lactated Ringer's solution for the interval prior to the start of heparin infusion.

Preparation of Arterial Soluble Fractions

Rats were killed by exsanguination at the indicated times after injury, and both carotids were rapidly excised, rinsed with ice-cold lactated Ringer's solution, and cleaned of periadventitial fatty and connective tissue. Endothelium of the right carotid was removed by gently scraping the luminal surface with a scalpel blade. Right and left carotid arteries were separately pooled (4 rats per group) and homogenized at 0–4°C in 1 ml of buffer A (in mM: Tris HCl 50, pH 7.2; dithiothreitol 5; EDTA 1; and pyridoxal-5'-phosphate 0.5). The homogenates were centrifuged at 30,000g for 30 minutes at 0–4°C. The supernatant (soluble fraction) was collected and stored at −70°C until use. Carotid ODC activities were stable for at least 2 weeks under these storage conditions.

ODC Assay

L-Ornithine decarboxylase (EC 4.1.1.17) (ODC) activity in carotid artery soluble fractions was determined by measuring the CO₂ produced from L-[1-¹⁴C]ornithine HC1 (55 mCi/mmol) (Amersham, Arlington Heights, Ill.) essentially as described. A final reaction volume of 1.0 ml contained 0.1 mM L-ornithine, 0.25 μCi L-[¹^-¹⁴C]ornithine, and 0.2–0.4 mg of carotid artery soluble protein in buffer A. Incubations were carried out in miniglass scintillation vials equipped with rubber stopper/center well assemblies (Kontes Co., Vinland, N.J.) for 2 hours at 37°C. Evolution of CO₂ from ornithine was linear for the 2-hour period, was accompanied by stoichiometric formation (1:1) of putrescine, and was inhibited >95% by DL-α-difluoromethylornithine (25 μM, 60 minutes) (Merrell-Dow Research Institute, Cincinnati, Ohio). Protein concentrations were determined by the dye-binding assay of Bradford using bovine gamma globulins (Bio-Rad Laboratories, Richmond, Calif.) as standard.

 Autoradiography

Rats were given a single intramuscular injection of [¹^-H]thymidine (0.5 μCi/g body wt, 6.7 Ci/mmol) (New England Nuclear, Boston, Mass.) 1 hour prior to death. Both carotid arteries were retrieved, and 5-mm segments were cut from the central region of the vessel, fixed in 1.5% glutaraldehyde in 0.1 M phosphate-buffered saline (pH 7.4), and embedded in methacrylate. Cross sections of 1 μm were cut. Slides were dipped in Kodak NTB-2 emulsion and were exposed for 2 weeks at 4°C. Slides were developed using Kodak D19 developer and were stained with toluidine blue. Thymidine index values (percent) were determined by counting at least 1,000 cells in the medial segment per rat.

Results

Carotid ODC Activity

Rapid and transient increases in ODC activity were found in the damaged left carotid artery during the initial hours after injury (Figure 1). Significant increases in enzyme activity were evident 3 hours after wounding. This was followed by a sharp rise and then a rapid fall in decarboxylase activity that returned to near basal levels by 9 hours after injury. Increases in ODC activity reached maximum (twenty-three-fold) 6 hours after wounding. Following these initial large changes, enzyme activity remained at a slightly elevated level (about fourfold) for the remainder of the 48-hour period studied. No detectable changes in ODC activity were observed in the uninjured right carotid artery at any of the times examined. These changes in decarboxylase activity suggest that SMC responds rapidly and synchronously to signals for stimulation of cell growth produced by the injury event.

SMC Thymidine Index

The entry of SMC into S phase was examined in rats injected with [¹^-H]thymidine at various times after injury. The frequency of labelled SMC nuclei was very low (about 0.06%) during the first 18 hours (Figure 1). A significant increase in SMC thymidine index (0.4–0.6%) was first detected 24 hours after injury. This was followed by a steady increase during the next
9 hours that reached a maximum (about 25%) at 33 hours and then declined over the next 12–15 hours. Thymidine indexes of about 6% were obtained 48 hours after injury. The uninjured right carotid displayed very low frequencies of labelled SMC nuclei (0.05–0.1%) throughout the 48-hour period. These data suggest that SMC in injured carotids begins to enter S phase 24–27 hours after injury and synthesize DNA with considerable synchrony over the next 12–18 hours.

**Effects of Heparin on SMC Growth Initiation**

To examine the effects of heparin on SMC growth initiation, peak increases in ODC activity and SMC thymidine index were measured with and without heparin treatment. Heparin, in doses that inhibit intimal thickening, was found to have no effect on ODC induction 6 hours after carotid injury (Table 1). ODC activities in uninjured arteries were not significantly altered by heparin treatment. In contrast, heparin markedly inhibited SMC thymidine index values 33 hours after injury (Figure 2). Low thymidine index values in heparin-treated rats reflects inhibition of SMC entry into S phase, not simply a shift in the time point of maximal thymidine labelling. No evidence of a shift in peak labelling time was found in rats killed at 27 hours (3.6 ± 3.1%), 33 hours (2.7 ± 4.0%), and 39 hours (3.6 ± 1.4%) after carotid injury (n = 4 for each group). Moreover, continuous [3H]thymidine infusion for up to 7 days after injury shows reduced entry of SMC into the growth fraction in heparin-treated rats with no indication of delayed entry into S phase. In addition, total DNA content in injured arteries from heparin-treated rats 2 weeks after injury is significantly reduced, which is consistent with inhibition of SMC proliferation. These results suggest that either 1) heparin does not inhibit the transition of quiescent SMCs into early stages of the growth response to injury or 2) ODC induction proceeds independently of the critical events for DNA synthesis inhibited by heparin.

**Effects of Delay of Heparin Treatment**

To help distinguish between these possibilities, the start of heparin infusion was delayed for varying periods of time, and the effect of delay on SMC proliferation was examined by thymidine autoradiography 33 hours after wounding. The start of heparin infusion could be delayed for up to 18 hours after injury with no significant decrease in antiproliferative effect (Table 2). However, a delay of 27 hours before heparin infusion was begun resulted in SMC thymidine index values that were not significantly different from values obtained in rats infused only with lactated Ringer’s solution. These data, together with results shown in Figure 1, suggest that heparin can be delayed until late G1 or S phase and still produce significant inhibition of DNA synthesis.

**Discussion**

In the studies reported here, heparin failed to inhibit ODC induction at early times after carotid injury. This was not due to inadequate delivery of heparin because clotting times were prolonged and numbers of thymidine-labelled SMC nuclei were reduced using identical heparin infusion regimens. These results suggested that heparin does not prevent initiation of SMC growth after injury but rather acts at later stages in progression to DNA synthesis. This interpretation is supported by the finding that heparin can be delayed for up to 18 hours after injury with little or no loss of antiproliferative activity. The possibility that additional heparin-sensitive events, which do not affect the regulatory pathway for ODC induction, occur at very early times after injury cannot be ruled out. Similar results have recently been reported for heparin inhibition of SMC proliferation in vitro following addition of fresh serum-containing medium to growth-arrested cultures of rat aortic SMC. These findings point to events closely related to progression of SMC through

![Figure 2](image-url)

**FIGURE 2.** Effect of heparin on carotid ODC activity and SMC thymidine index after injury. Heparin (0.3 μg/g body wt/hr) was infused intravenously beginning at time of injury. Panel A: Carotid ODC activities were determined at 6 hours. Panel B: SMC thymidine indexes determined at 33 hours in control (vertical lines) and heparin-treated (stippled) rats as described in “Materials and Methods” section. Values shown represent mean ± SEM of at least duplicate determinations from 4 rats per treatment group. *p<0.01, significantly different from normal saline-treated controls.

**Table 1. Effect of Heparin on Carotid ODC Activity After Injury**

<table>
<thead>
<tr>
<th>Artery</th>
<th>ODC Activity (pmol CO2/mg protein/30 min)</th>
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<tbody>
<tr>
<td>Right carotid</td>
<td>38 ± 20</td>
</tr>
<tr>
<td>Left carotid</td>
<td>770 ± 72</td>
</tr>
<tr>
<td>Left–right</td>
<td>23 ± 8</td>
</tr>
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</table>

Ten minutes before left carotid injury, rats (4 per group) were given either an intravenous injection of heparin (10 μg/g body wt) dissolved in lactated Ringer’s solution or lactated Ringer’s solution only. At the time of injury, a cannula was inserted into the left jugular vein, and heparin was infused at a rate of 0.3 μg/g body wt/hr for 6 hours. Control rats were infused with an equal volume of lactated Ringer’s solution for 6 hours. Rats were then killed, soluble fractions were prepared from a pool of 4 right or left carotid arteries per experiment, and ODC activities were determined as described in the “Materials and Methods” section. Values shown are mean ± SD of 4 separate experiments. Left–right ratios refer to fold increases in ODC activities calculated by dividing the indicated specific activities of left carotids by those of right carotids for each treatment group.
Table 2. Effect of Delay of Heparin Infusion on SMC Thymidine Index

<table>
<thead>
<tr>
<th>Time heparin infusion started after injury</th>
<th>SMC thymidine index (%) ± SEM</th>
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<tr>
<td>0 Hours</td>
<td>2.1 ± 1.9</td>
</tr>
<tr>
<td>8 Hours</td>
<td>3.1 ± 3.1</td>
</tr>
<tr>
<td>18 Hours</td>
<td>4.9 ± 2.2</td>
</tr>
<tr>
<td>27 Hours</td>
<td>10.6 ± 3.5</td>
</tr>
<tr>
<td>Saline infusion</td>
<td>15.0 ± 7.3</td>
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At the indicated times after arterial injury, heparin infusion was begun (0.5 μg/g body wt i.v.). Heparin infusion was continued until 33 hours after injury. In this time, rats were given a single injection of [3H]thymidine (0.5 μCi/g body wt i.m.) and killed 1 hour later. Autoradiography on cross sections of injured and uninjured arteries was carried out as described in “Materials and Methods” section. Values shown represent mean thymidine index (%) ± SEM in traumatized arteries. Saline infusion, rats that were given infusion of an equal volume of lactated Ringer’s solution for the 33-hour period after injury.

the prereplicative (G₁) period as potential targets for inhibition of SMC proliferation by heparin.

We have previously shown, using a continuous [3H]thymidine delivery method, that entry of SMC into the growth fraction in injured arteries is largely complete by 3 days after injury.1 In those studies, the estimated growth fraction was between 20 and 40%. The results reported here using single, 1-hour pulses of [3H]thymidine show a similar fraction of SMC nuclei labelled as early as 33 hours after injury. These data suggest that an initial group of SMC rapidly and synchronously enters the cell cycle after injury and that this cohort serves as progenitors for a majority of new SMC in the restored media and thickened intima. This contrasts with the suggestion that SMC must undergo extensive changes in its state of differentiation prior to responding to exogenous mitogens.2 A requirement for “modulation” was suggested based on the observation that cells in culture lose their myofibrils and acquire large amounts of endoplasmic reticulum before being able to replicate. That process can be separated from replication and takes several days.22 In contrast, the cohort of cells forming the replicative response in vivo appears to enter S phase synchronously within 33 hours after the injury. This implies that modulation is either not required or much more rapid in vivo or that a subset of cells is already present with the ability to respond to mitogenic stimuli.

The rapid, transient changes in ODC activity observed in the initial hours after carotid injury are similar to the rapid changes in decarboxylase activity following partial hepatectomy in vivo14 and after addition of serum or defined growth factors to quiescent cells of a wide variety in culture.15 Induction of ODC activity after mitogen stimulation occurs coordinately with changes in expression of a number of genes as cells progress through G₁ in preparation for DNA synthesis and cell division. We have interpreted the large induction of ODC activity 6 hours after carotid injury as indicative of the exit of SMC from the quiescent state characteristic of the adult artery wall. We suggest that the failure of heparin to prevent induction of ODC activity argues against one possible mechanism for the antiproliferative effect, i.e., that heparin maintains SMC in the quiescent (G₀) state after arterial injury. Because heparin can apparently be delayed for up to 18 hours after carotid injury without significant loss of growth inhibitory effect, we have suggested that heparin acts late in the prereplicative (G₁) sequence or early in S phase to inhibit SMC proliferation. However, the SMC prereplicative period after rat carotid injury has not been sufficiently characterized to assign specific cell cycle periods to the events we have described here.

The relation of these findings to the postulated role of endogenous heparin-like molecules in local control of arterial SMC growth in vivo18,20,23 is not clear. Our finding that the time point(s) at which heparin acts to inhibit DNA synthesis in injured arteries localizes to the late prereplicative (G₁) period is surprising. It seems likely that many of the events associated with the transition from quiescence (G₀) to growth (G₁), based on cell culture studies, would have already occurred.24 If we try to reconcile these results with the hypothesis that heparin mimics the action of an endogenous growth inhibitor in the vessel wall, then we are led to conclude that the critical step or steps involved in control of SMC DNA synthesis in vivo are late in the prereplicative (G₁) period. This is in contrast to the usual view, based on studies in vitro, that growth is controlled by critical processes occurring many hours prior to initiation of DNA synthesis. Yet, evidence for control of SMC replication at the level of S phase entry has previously been reported. Clemmons showed that factors present in plasma, particularly insulin-like growth factor 1/somatomedin-C, are necessary for initiation of DNA synthesis in porcine aortic SMC previously exposed to platelet-derived growth factor.25 Further studies of the SMC responses at early times after arterial injury may provide additional clues as to the nature and timing of heparin-sensitive events involved in the control of SMC proliferation in damaged arteries.

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References


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