Pituitary Factors in Blood Plasma Are Necessary for Smooth Muscle Cell Proliferation in Response to Injury In Vivo

Jurgen Fingerle, Andreas Faulmuller, Gottfried Muller, Daniel F. Bowen-Pope, Monika M. Clowes, Michael A. Reidy, and Alexander W. Clowes

Intimal thickening in response to vascular injury is inhibited in animals previously subjected to hypophysectomy. We have investigated the nature and cell kinetics of this effect in a balloon catheter model of injury to the rat carotid artery. The ability of injury to stimulate \(^{3}H\)thymidine labeling 48 hours after injury was almost completely eliminated in hypophysectomized (hypox) compared with control animals (0.1% versus 32.1%). Total DNA content of the developing neointima 14 days after injury was only 30% of the values found in ballooned carotid arteries of normal rats. If hypox rats were treated with recombinant human growth hormone, the proliferative response was not restored. There are two possible general explanations for the reduction of proliferative response in hypox animals: 1) that smooth muscle cells in the hypox animals have lost the ability to respond to the stimulus of injury or 2) that the ability of the smooth muscle cells to respond has not been reduced by prior hypophysectomy, but that the response itself requires the presence of pituitary-dependent factors. Transplantation experiments were performed in vivo to distinguish between these possibilities. Carotid arteries in inbred Lewis rats were excised 1 hour after balloon injury to give platelets the opportunity to adhere. These vessels were then transplanted from hypox into control animals and vice versa. At 48 hours, proliferation of smooth muscle cells in “control-to-hypox” transplants was 0.3% compared with 14.3% in “control-to-control” transplants, whereas vessels from hypox rats increased their indices to 4.8% if transplanted into control animals. This indicates that the ability of the smooth muscle cells to proliferate depends on their hormonal environment after injury rather than on the past history or state of the smooth muscle cells. This conclusion is supported by in vitro experiments that show no difference in the growth properties of primary cultures of carotid smooth muscle cells from control and hypox animals but that demonstrate less growth-promoting activity in plasma-derived serum from hypox animals. We conclude that one or more hormonal factors in the plasma, dependent on the pituitary, are required for smooth muscle proliferation in response to injury and that hypophysectomy does not produce a stable change in the properties of the smooth muscle cells themselves. (Arteriosclerosis and Thrombosis 1992;12:1488-1495)

KEY WORDS • smooth muscle cells • rats • carotid artery • injury • balloon catheter • smooth muscle cell proliferation • pituitary • plasma

The response of medial smooth muscle cells to arterial damage is probably regulated by the combined action of a number of growth factors. Some of these factors originate within the vessel wall, and some are present in the blood. The early wave of proliferation in smooth muscle cells in carotid arteries of rats after balloon catheter injury is largely driven by basic fibroblast growth factor (bFGF) present in the vessel wall. Subsequent migration from the media to the intima is controlled by products released from platelets. Platelet-derived growth factor (PDGF), which was originally characterized for its growth-promoting properties for cultured cells, has recently been shown to stimulate smooth muscle cell migration in the injured rat carotid artery, but compared with bFGF, it has a weak direct effect on smooth muscle cell proliferation. Interferon gamma released from intimal T lymphocytes inhibits intimal smooth muscle cell proliferation. In addition to these potential autocrine or paracrine mechanisms of smooth muscle cell stimulation (growth and migration), hormonal factors circulating in the blood plasma, such as insulin and insulin-like growth factor 1 (IGF-1), are important for cellular growth and have been shown to act as progression factors for proliferating smooth muscle cells in tissue culture. For example, IGF-1 in combination with PDGF is as effective as serum in eliciting a mitogenic response. A similar situation appears to exist after vascular injury in vivo, where factors that are locally...
present (e.g., bFGF and PDGF) might act in concert with plasma factors to induce proliferation.9,10

In hypophysectomized (surgically hypox) rats, injury-induced neointimal formation is inhibited.11 Bettmann et al12 added pharmacological doses of glucocorticoids, thyroxin, and growth hormone (GH) but were unable to restore the normal neointimal thickening response in these animals. This could be because the maintenance of a normal responsive smooth muscle cell phenotype requires the continuous presence of pituitary-dependent factors. The vessel wall in a hypox rat might then become intrinsically unresponsive to the growth stimulus of injury. Alternatively, pituitary-dependent factors might not be necessary to maintain the smooth muscle cell phenotype but might be necessary cofactors in a proliferative response to injury.

To distinguish between these possibilities, we transplanted acutely denuded rats (including adherent platelets) from hypox rats into normal rats and vice versa and determined the number of proliferating cells in the media in the early proliferative response to injury. We also evaluated the effects of hypophysectomy on the growth capacity of medial smooth muscle cells and the growth-promoting activity of blood components. All of these approaches suggest that hypophysectomy reduces the levels of growth-promoting factors, which must be present after injury to support a normal proliferative response.

Methods

Male Sprague-Dawley rats weighing about 300 g (Tyler Laboratories, Bellevue, Wash.) were used for the experiments. For the cross-transplantation studies described below, inbred Lewis rats weighing about 250 g (Majlegard, Copenhagen, Denmark) were used. Hypophysectomy was performed by the company supplying the rats. Rats were kept for 3 weeks, and hypox animals received special water (35 mM NaCl, 1.1 mM KCl, 20 mM CaCl2, 180 mM MgCl2, and 140 mM sucrose). Body weight was measured every second day.

Hypophysectomy was considered successful if the rats did not gain weight and remained active with no signs of apparent sickness. Only animals fulfilling these criteria were used for the study (about 85% of all animals supplied). GH-treated animals received two subcutaneous injections per day (at 12-hour intervals) of 0.125 unit/kg body wt (equals 250 milliunits per kg per day) of human recombinant GH (a gift from Genentech, South San Francisco, Calif.). Twice-daily injections were used to mimic the pulsatile secretory pattern of GH. Treatment was started 2 days before ballooning. Balloon catheter injury was performed within 1 hour after the morning dose.

Balloon Catheter Denudation

Balloon catheter injury of the left common carotid artery was performed as described earlier.15 Rats were anesthetized with Innovar-Vet, Pitman-Moore, Ill. (0.03 ml/kg body wt, a dose equivalent to fentanyl 12 mg/kg), the artery was exposed, and a 2F embolectomy catheter (Edward Labs, Santa Ana, Calif.) was introduced through the external branch, pushed forward to the aortic arch, and withdrawn under inflation with saline three times. The external carotid artery was permanently ligated and the wound closed.

Cross Transplantation of Rat Carotid Arteries

Cross transplantation of 7-mm segments of common carotid arteries between normal and hypox Lewis rats was performed without anticoagulation by using interrupted sutures (end-to-end anastomosis with seven 10/0 nylon monofilament sutures; Braun, Melsungen, FRG) with the help of an operating microscope (Zeiss, Oberkochen, FRG). Before transplantation, the vessel in the donor animal was denuded of endothelium as described above and left there for 1 hour to allow the platelets to adhere. The rats were killed with an intravenous dose of 0.2 ml ketamine hydrochloride (100 mg/ml). The abdominal aorta was cannulated, the vessels were flushed free of blood by perfusion with N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES)–buffered tissue-culture medium (Dulbecco's modified Eagle's medium [DMEM], from Gibco), and the carotid arteries were harvested. Under these conditions, the patency rate of transplanted carotid arteries was 80%.

Morphometry

Intimal and medial areas of carotid arteries were determined by morphometric analysis of paraffin-embedded cross sections after perfusion-fixation in vivo at 100 mm Hg with 4% paraformaldehyde.15 Eosin/hematoxylin-stained cross sections were projected onto a digitizing board (Summascetch II, Summagraphics, Bilany, Düsseldorf, FRG) with a ×10 objective and a camera lucida (Olympus). To calculate areas a computer-aided morphometry system (Bioquant IV, Bilany, Düsseldorf, FRG) was used.

DNA Content of Carotid Arteries

DNA content of carotid arteries was determined from 5-mm pieces cut from unfixed vessels, which were homogenized in Dounce homogenizers (Braun).1617 DNA was stained with Hoechst stain.18 Fluorescence was determined in a minifluorometer (Hoefer Scientific Instruments, Heidelberg, FRG) with herring sperm DNA (Boehringer Mannheim, Mannheim, FRG) as a standard.

S-Phase Labeling

In most experiments, proliferating smooth muscle cells were detected by autoradiography on histological sections of carotid artery from animals receiving titrated thymidine (0.5 mCi/kg i.m. at 17, 9, and 1 hour before sacrifice; New England Nuclear, Inc.).10 Cell proliferation in the set of animals used for cross-transplantation studies was analyzed by the alternative technique of bromodeoxyuridine (BrdU) labeling (17 and 1 hour before they were euthanized, the animals were injected with 30 mg/kg body wt BrdU and 25 mg/kg body wt deoxycytidine). BrdU was detected by means of a monoclonal anti-BrdU antibody (Boehringer Mannheim) by the avidin-biotin method (Vecastain ABC-Kit, Vector Laboratories, Burlingame, Calif.). The proliferative rates of tissues were determined on cross sections as percentages of labeled nuclei.

Plasma, Serum, and Platelet Preparation

Serum from platelet-poor plasma of rats was obtained from puncture of the abdominal aorta after overdosing...
the animals with 0.2 ml ketamine hydrochloride i.v. Blood was collected into acid-citrate-dextrose solution (ACD, 9:1 vol/vol; 85 mM sodium citrate, 111 mM glucose, and 71 mM citric acid, pH 6.8, 4°C). Individual blood samples were immediately spun down in a Sorvall centrifuge (15,000g). Coagulation was initiated with addition of 50 μl of 1 M CaCl2 per milliliter of plasma for 3 hours at 37°C. The samples were centrifuged and stored at −70°C until use.19,20 Platelet lysates were made from platelets isolated from platelet-rich plasma (ACD-blood spun at 400g for 10 minutes) after a double wash in ACD buffer containing 1% bovine serum albumin (BSA; Sigma Chemical Co., St. Louis, Mo.) and centrifugation for 15 minutes at 1,000g. Platelets were counted with a hemocytometer under phase-contrast illumination and a ×40 objective. The concentration was then adjusted to about 1010/μl. Lysates were made by three freeze/thaw cycles in liquid nitrogen and sonication (Braun, Melsungen, three times, 30 seconds each) and stored at −70°C until use.

Smooth Muscle Cell Culture

Smooth muscle cells were isolated by enzymatic digestion of carotid media with collagenase/elastase. Vessels were preincubated in enzyme solution (collagenase CLASS II, Worthington, 167.5 units/ml; elastase, Boehringer Mannheim, 15 units/ml; soybean trypsin inhibitor, Serva, 0.03 IE/ml; and 2% BSA in Waymouth’s complete medium with 10 mM CaCl2) for 30 minutes, and the adventitia was carefully removed by using watchmaker’s forceps (Dumont No. 7). Medial tissue was minced with small scissors and digested further for 1 hour at 37°C or until single cells were released from the tissue. Cells were plated on six-well plates (Falcon, Becton Dickinson, Heidelberg, FRG) at a density of approximately 2,000/cm2 and kept at 37°C in an incubator with 21% O2 and 5% CO2 in Waymouth’s complete medium supplemented with 10% fetal bovine serum (GIBCO), penicillin (100 units/ml) and streptomycin (0.1 mg/ml).21 Cell counts were determined in an electronic cell counter (Coulter counter, Coulter Electronics, Harpenden, Herts, England).

Mitogenesis Assay

Mitogenic activity of rat whole-blood serum, plasma-derived serum, or platelet lysates was tested on Swiss 3T3 cells.20,22 Cells were maintained in DMEM with 5% fetal calf serum in 24-well plates (Costar) until reaching confluence. Before addition of the test medium, cells were rendered quiescent in low serum (0.5% for 48 hours). Test plasma was added for the following 22 hours, after which time the cells were exposed to [3H]thymidine (2 μCi/ml) for 1 hour. Cells were then treated with 5% ice-cold trichloroacetic acid, and after three washes any insoluble material on the plates was dissolved in 0.8 ml of 250 mM NaOH and counted in a liquid scintillation counter (Beckmann LS 1801) with Aqualos (New England Nuclear).

Determination of PDGF Levels

PDGF levels of whole-blood serum and platelet lysates were determined by a receptor competition assay as described earlier.21,22 PDGF-free serum was obtained from calf plasma after carboxymethyl Sepharose treatment (CMS I).20 Confluent monolayers of diploid hu-

<table>
<thead>
<tr>
<th>Thymidine indices</th>
<th>Left carotid artery (ballooned)</th>
<th>Right carotid artery (uninjured)</th>
</tr>
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<tbody>
<tr>
<td>Hypophysectomized rats</td>
<td>0.1±0.2%*</td>
<td>0.1±0.1%</td>
</tr>
<tr>
<td>Normal rats</td>
<td>32.1±16.3%</td>
<td>0.1±0.1%</td>
</tr>
</tbody>
</table>

Twenty-one days after removal of the pituitary gland, the endothelium was denuded from left carotid arteries and tritiated thymidine was injected between 24 and 48 hours later. S-phase nuclei were counted from autoradiographs of cross sections, and the indices were expressed as (labeled nuclei/total nuclei)×100 for every cross section examined (four per animal). Induction of proliferation in hypophysectomized rats was significantly reduced (hypophysectomized versus normal, p<0.005, t test, n=4).

Results

Proliferation of Smooth Muscle Cells in Injured Carotid Arteries of Hypophysectomized Rats

In normal animals thymidine labeling of medial smooth muscle cells is maximal at 24–48 hours after carotid artery injury.9,24 This early proliferative response of medial smooth muscle cells was completely eliminated by prior hypophysectomy (Table 1). After 14 days, the neointima that developed in the hypox animals was greatly reduced (37% of control DNA content and 28% of control area; Table 2). Hypophysectomy was much less inhibitory for proliferation of smooth muscle cells within the neointima (Table 3) than it was for the initial stimulation of cells within the media. Thymidine labeling indices were reduced only about 50% from 12.4% to 6.1%.

| Table 1. Proliferation of Medial Smooth Muscle Cells 48 Hours After Balloon Catheter Injury |
|---------------------------------|-----------------------------------|
| Neointima after 14 days (left carotid arteries) | |
| DNA content | Intimal area |
| Hypophysectomized rats | 2.2±0.2 μg* | 0.031±0.009 mm²* |
| Normal rats | 5.9±0.3 μg† | 0.112±0.014 mm²† |

Fourteen days after balloon catheter injury of the left carotid artery, the DNA content and the area of the neointima were determined. DNA content was obtained from 3-mm segments of artery by assuming that DNAarea=DNAnuc=DNAmuscle (two segments per carotid artery were analyzed, n=4). Neointimal areas were obtained from morphometric analysis of histological cross sections (four for each carotid artery, n=4). Neointimal formation was significantly reduced in hypophysectomized rats (*versus‡, p<0.01, t test).

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Effect of GH in Hypophysectomized Rats

GH has been reported to be a direct mitogen for vascular smooth muscle cells in culture. In addition, GH regulates IGF-I synthesis in the liver, and a decrease in GH and IGF-I in the plasma after hypophysectomy might account for the decrease in smooth muscle cell proliferation. To test this hypothesis, rats were given twice-daily injections of human recombinant GH at doses that have been described to be sufficient to restore GH and IGF-I levels in tissues and plasma. GH treatment caused the animals to gain weight but did not affect the thickening response (Table 4). Medial smooth muscle cell proliferation 48 hours after carotid artery balloon catheter injury as measured by thymidine incorporation was also not restored to levels obtained from normal rats (control rats, 17±7.7%; saline-injected hypox rats, 3.7±2.3%; and GH-supplemented hypox rats, 3.4±1.8%; data are presented as mean±SD, n=5 for each group).

Cross Transplantation of Injured Carotid Arteries

The hypothesis that factors present in blood plasma might be important for smooth muscle cell proliferation was tested in vivo by cross transplanting balloon-injured carotid arteries from inbred hypox into normal rats and vice versa. We waited 1 hour after injury to allow platelet adhesion to develop fully and then transplanted the carotid arteries and measured [3H]thymidine incorporation 24–48 hours later. When injured vessels were transplanted from hypox to normal animals the proliferative response was restored (4.8% versus 14.3%). In the converse experiment, transplantation of an injured vessel from a normal to a hypox animal, the proliferative response was almost entirely eliminated (0.3%; Table 5). These findings demonstrate that the recipient animal and factors in its blood play an important role in the first wave of smooth muscle cell proliferation.

Effect of Hypophysectomy on PDGF Levels

To determine whether the removal of the pituitary gland would change the PDGF content of blood platelets, washed platelets as well as whole-blood serum were analyzed. No statistically significant difference was found in the PDGF content of platelets from normal or hypox rats (Table 6). Because of the low PDGF content of rat platelets and possible degranulation artifacts produced during platelet preparation, the results for PDGF levels from washed platelets were somewhat variable. Samples of whole-blood serum gave more reproducible estimates of PDGF content and also revealed no difference in free PDGF levels between normal and hypox rats (Table 6).

Effect of Hypophysectomy on Mitogenic Activity of Blood Components in Culture

To detect differences in the overall growth factor content of blood components, we used a mouse 3T3 cell culture mitogenesis assay. Platelet lysates from hypoxic animals below a concentration of 5% contained about 30% mitogenic activity as compared with platelet lysates from normal animals. Above 7%, however, no statistical difference was detectable anymore between the two lysates (Figure 1). A similar decrease of about 30% over the whole range of concentrations tested was detected in plasma-derived serum and whole-blood serum from hypox animals (Figure 2).

Effect of Hypophysectomy on Growth of Smooth Muscle Cells in Culture

This experiment tested the possibility that the removal of the pituitary gland affected the growth capac-

### Table 3. Thymidine Indices in Intimal Smooth Muscle Cells 14 Days After Balloon Catheter Injury

<table>
<thead>
<tr>
<th></th>
<th>Thymidine indices in left carotid artery 14 days after denudation</th>
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</thead>
<tbody>
<tr>
<td>Hypophysectomized rats</td>
<td>6.1±4.9%</td>
</tr>
<tr>
<td>Normal rats</td>
<td>12.4±5.6%</td>
</tr>
</tbody>
</table>

Between days 13 and 14 after endothelial denudation, tritiated thymidine was injected and S-phase nuclei were detected as described in Table 1. Proliferative activity was reduced in hypophysectomized animals (n=4, not significant, p>0.05).

### Table 4. Effect of Human Recombinant Growth Hormone on Neointimal Thickening in Hypophysectomized Rats 14 Days After Balloon Catheter Injury of the Left Carotid Artery

<table>
<thead>
<tr>
<th></th>
<th>DNA content (μg/5 mm)</th>
<th>Intimal area (mm²)</th>
<th>Change in body weight (g/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hypophysectomized rats</td>
<td>2.3±0.5*</td>
<td>0.030±0.0124</td>
<td>0.5±0.94#</td>
</tr>
<tr>
<td>Normal rats</td>
<td>5.6±0.4f</td>
<td>0.109±0.0086</td>
<td>1.44±1.06**</td>
</tr>
<tr>
<td>Hypophysectomized+</td>
<td>3.2±0.5*</td>
<td>0.039±0.0164</td>
<td>2.94±0.44**</td>
</tr>
</tbody>
</table>

GH, growth hormone. Fourteen days after balloon catheter injury the development of the neointima was determined by means of DNA content and morphometric analysis of intimal areas (see Table 3, *versus, fversus§, and #versus** are significantly different, p<0.01, t test, n=5).

### Table 5. Proliferative Indices in Transplanted Carotid Arteries

<table>
<thead>
<tr>
<th></th>
<th>Recipient rats</th>
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<tbody>
<tr>
<td></td>
<td>Normal</td>
</tr>
<tr>
<td>Donor rats</td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>14.3±0.5%</td>
</tr>
<tr>
<td>Hypophysectomized</td>
<td>4.8±1.3%</td>
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</table>

Carotid arteries were deendothelialized by means of a balloon catheter, removed from the donor animal 1 hour later, and used for transplantation immediately. From each transplanted carotid artery, four cross sections were analyzed (n=6 animals). Proliferative indices of medial smooth muscle cells 48 hours after transplantation are given in percent as shown in Table 1. All indices are significantly different from each other (p<0.05, t test). ND, not determined.

### Table 6. Platelet PDGF Content in Hypophysectomized Rats

<table>
<thead>
<tr>
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<th>Washed platelets (ng PDGF/109 platelets)</th>
<th>Whole-blood serum (ng PDGF/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hypophysectomized rats</td>
<td>0.71±0.71</td>
<td>1.93±0.45</td>
</tr>
<tr>
<td>Normal rats</td>
<td>1.34±0.89</td>
<td>2.20±0.28</td>
</tr>
</tbody>
</table>

The platelet-derived growth factor (PDGF) content of washed platelets (10^9/ml) was determined in freeze/thaw lysates from three independent preparations of platelet-rich plasma (pooled from six to 12 animals). PDGF serum levels were derived from individual blood samples from 13 animals per group. The differences are not significant.
FIGURE 1. Line plot of lysate concentration versus [3H]thymidine incorporation. 3T3 cells were stimulated with platelet lysate from either normal (control) or hypophysectomized (hypox) rats to incorporate [3H]thymidine (n=6). The concentration of the lysate was based on the number of platelets from which it was made. A concentration of 100% refers to a lysate made from 10^6 platelets/ul, which is the number of platelets present in normal rat blood. At concentrations below 7%, hypox platelet lysate was significantly less mitogenic (p<0.05, t test).

FIGURE 2. Line plot of serum concentration versus [3H]thymidine incorporation. Mouse 3T3 cells were stimulated to incorporate [3H]thymidine by using whole-blood serum and plasma-derived serum from normal (control) and hypophysectomized (hypox) rats (n=3). The incorporation was threefold smaller when stimulated with serum from hypophysectomized rats.

FIGURE 3. Line plots of time in culture versus cell number or [3H]thymidine uptake. Primary cultures of enzymatically isolated carotid medial smooth muscle cells from hypophysectomized (hypox) and normal (control) animals were stimulated to grow with 10% bovine serum. The increase in cell number (upper panel) and [3H]thymidine incorporation (lower panel) was monitored as a function of time. Cells were plated in 24-well plates (21×10^3 cells/well), and three wells per time point were analyzed in each experiment by either electronic cell counting (upper panel) or scintillation counting (lower panel).

Discussion

In this study the importance of plasma factors for vascular smooth muscle cell proliferation was studied in hypox rats. From our data we conclude that hormonal factors dependent on the pituitary gland are involved in medial smooth muscle cell growth after arterial injury. Autocrine and paracrine mechanisms in the vessel wall are believed to govern proliferation of smooth muscle cells. Two growth factors, PDGF2^-4,28,29 and bFGF1,20 have already been implicated in the proliferative response of the rat carotid artery to balloon catheter injury. PDGF was shown to be important for the response to injury but has been suggested to be a minor mitogen for early medial smooth muscle cell proliferation.2,4 In addition, experiments with platelet-depleted rats indicate that platelets, a main source for PDGF, were less important for medial proliferation.2 bFGF present within the vessel wall appears to be a key mitogen for the first wave of medial smooth muscle cell proliferation after injury.90 This growth factor lacks a signal sequence and is probably not actively released,
except when cell membranes are disrupted during tissue injury. It is not detectable in normal blood plasma. These observations have been interpreted to mean that the amount of bFGF available to cell-surface FGF receptors is determined by the extent of medial injury or whether hormonal factors are contributing as well. Previous studies have shown that injury-induced neointimal thickening is reduced in hypox rats.\textsuperscript{11,12} Using pulse labeling with $^3$Hthymidine, we could demonstrate that the initial proliferative response of medial smooth muscle cells was inhibited in hypox rats. This suggests that some hormonal mechanisms dependent on the pituitary gland, in addition to autocrine/paracrine mechanisms, might be essential.

To test this hypothesis we performed transplantation experiments in vivo, during which we injured a carotid artery by means of a balloon catheter. These vessels were harvested 1 hour after injury and cross-transplanted between normal and hypox individuals of an inbred Lewis rat strain. Our results indicate that the proliferative rates of medial smooth muscle cells are mainly dependent on the animal into which the vessel is transplanted rather than the origin of the segments of carotid arteries. After transplantation of balloon catheter-injured carotid arteries from normal rats into hypox rats, the proliferation of medial smooth muscle cells decreased to zero. The result of the converse experiment, in which we transplanted segments of injured carotid arteries from hypox rats into normal rats, showed a proliferative rate of 4.8%. Therefore, we postulate that in addition to the known bFGF-driven paracrine mechanism, hormonal factors are important.

Removal of the pituitary gland, however, is a very severe intervention and could cause changes in autocrine/paracrine mechanisms. This might include changes in growth factors like bFGF, PDGF, and IGF-1 or possibly changes in smooth muscle cell status leading to decreased responsiveness of these cells.

Addressing the question of a changed smooth muscle cell status, we found no differences in growth capacity when stimulated with calf serum between primary cultures of vascular smooth muscle cells obtained from hypox rats and cells obtained from control rats, thus providing no evidence for altered smooth muscle cell responsiveness after hypophysectomy.

As mentioned above, PDGF seems to be of minor importance as a medial smooth muscle cell mitogen. Nevertheless, we compared overall platelet mitogens and platelet PDGF content in normal versus hypox rats and did not find differences, suggesting that the removal of the pituitary gland did not alter these paracrine parameters.

Adenocorticotropin has been reported to regulate fetal adrenal gland bFGF expression.\textsuperscript{37} Therefore, it is possible that hypophysectomy results in a decreased amount of bFGF in the carotid artery wall and that this alteration in the bFGF content of the carotid artery causes the lower proliferative response after injury in hypox rats. When we transplanted balloon catheter-injured rat carotid arteries from hypox into normal animals, we found a high proliferative activity in hypox tissue. Since bFGF is the main mitogen for medial smooth muscle proliferation in normal rats and since it is not present in blood plasma, this growth factor most probably was present in hypox tissue.

This experiment does not demonstrate whether bFGF is partly reduced in hypox vessel wall tissue. Reduced bFGF content in hypox tissue could explain why, after transplantation of carotid arteries from hypoxic animals into control rats, proliferative activity was not restored completely.

If bFGF or any other autocrine/paracrine factor would have been sufficient for the early proliferative response, we would have expected to observe a normal thymidine labeling response after 48 hours in injured vessels transplanted (with adhering platelets) from normal into hypox rats. In fact, however, we observed that the proliferative index in normal injured vessels was reduced to almost zero after transplantation into hypoxic rats. This suggests that paracrine mitogens need the support of hormonal factors in plasma to drive medial smooth muscle cells through the S phase.

An alternative possibility could be that after hypophysectomy an inhibitor is produced. In our experience, hypox plasma was mitogenic for cultured smooth muscle cells, thus providing no evidence for the presence of an inhibitor. Although the existence of such a molecule cannot be excluded, it is more likely that a mitogenic hormonal factor(s) is missing.

Of the multitude of potentially involved molecules, we can discuss only a few candidates. Observations on the effect of hormonal reconstitution in balloon-injured rats were previously made by Bettmann et al.\textsuperscript{12} Focusing on neointimal thickening, they found that a combination of thyroxin, corticosterone, deoxycorticosterone acetate, and GH could not restore the inhibited reaction in hypoxic animals. In vitro it appears that glucocorticoids inhibit rather than stimulate vascular growth,\textsuperscript{38-40} although their role in vivo is unclear; a suppressive activity might have overcome a potential positive effect of the other three hormones tested.

We therefore addressed the question of whether GH alone could restore medial smooth muscle cell proliferation in injured carotid arteries in hypox rats. In our experiments, GH was ineffective. Our animals gained weight during treatment, and therefore it seems unlikely that inadequate application of GH was responsible for the lack of an effect, thus confirming earlier results.\textsuperscript{14} Similar treatment with GH was shown to restore IGF-I levels in heart tissue,\textsuperscript{13} in the aorta, and in plasma\textsuperscript{20} and was equivalent to daily injections of 1 mg/kg IGF-I.\textsuperscript{14} GH was also demonstrated to be directly involved in the differentiation of preadipose embryonic mouse fibroblasts.\textsuperscript{41} During this differentiation process, GH was shown to induce a mitogen-resistant state in these cells. This effect of GH, however, was only observed if it was applied as a single factor in a chemically defined medium. After addition of insulin, adipocyte differentiation could be completed and cells were susceptible to mitogens.\textsuperscript{32} It could be argued that in our reconstituted hypox animals, GH may have had similar effects on vascular smooth muscle cells. However, GH was cer-
Failure to restore smooth muscle cell growth was due to an antimitotic effect of GH.

IGF-I has to be considered as an important molecule, since it has been shown to act as a progression factor for smooth muscle cell proliferation. It has also been shown that expression of IGF-I in arterial tissue after vascular injury is substantially enhanced. A protocol similar to ours for reconstitution of GH in hypox rats resulted in an almost complete restoration of plasma levels of IGF-I. Nevertheless, GH treatment did cause the animals to gain weight and induced IGF-I mRNA expression in the liver (data not shown). Autocrine/paracrine IGF-I in injured walls of carotid arteries from control rats should have been transferred into hypox rats, thus possibly leading to enhanced proliferative activity. As described above, proliferative activity was decreased to zero. Furthermore, it was shown by Khorsandi et al. that after vascular injury, IGF-I mRNA is increased in normal as well as in hypox rat arteries. Yet despite the presence of IGF-I mRNA in the hypox rat vessel wall, we found virtually no cell proliferation. Together these data suggest that IGF-I is present in the arteries of hypox animals treated with GH but is insufficient to stimulate proliferation.

In summary, our results indicate that circulating factors from the pituitary gland (or dependent on pituitary function) are important mitogens or comitogens for the initial proliferative response of the carotid artery to balloon catheter injury. These factors remain unidentified but might be independent of GH. Identification of these important factors could shed light on the nature of growth regulation in the vessel wall.

Acknowledgments

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