Mithramycin Inhibits Myointimal Proliferation After Balloon Injury of the Rat Carotid Artery In Vivo

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**Background** Smooth muscle proliferation and extracellular matrix formation in the subintimal region of blood vessels that have been subjected to intimal injury are responsible for restenosis following balloon angioplasty of the coronary arteries and for accelerated atherosclerosis in a variety of other pathophysiological states. The immediate early-response gene c-myc is overexpressed in proliferating vascular smooth muscle cells in vitro, and c-myc antisense oligomers have been shown to reduce c-myc expression and to inhibit proliferation of vascular smooth muscle cells in culture. Mithramycin is a commercially available G-C-specific DNA binding drug that selectively inhibits transcription of genes, such as c-myc, that have G-C-rich promoter sequences. This study tested the hypothesis that mithramycin inhibits transcription of the c-myc proto-oncogene and prevents myointimal proliferation after balloon injury of the rat carotid artery in vivo.

**Methods and Results** Ten-week-old male Sprague-Dawley rats received mithramycin (150 μg/kg/IP) or distilled H2O 1 hour before and 1 hour after balloon injury of the right common carotid artery. After 2 weeks, the rats were killed by overdose of pentobarbital, and the injured right and uninjured control left arteries were pressure-fixed and subjected to morphological analysis for evaluation of the degree of myointimal thickening. Separate groups of rats were killed at 2 and 6 hours after vascular injury, and total RNA from injured and control vessels of mithramycin- and vehicle-treated rats was subjected to Northern analysis for assessment of steady-state c-myc mRNA levels. The areas of neointima and the ratios of neointimal to medial area were significantly less in mithramycin-treated than in control rats (0.6±0.1 versus 1.2±0.2 mm², P<.01 and 95±16% versus 190±14%, P<.01). Lumen size was significantly greater in mithramycin-treated than in control rats (1.5±0.1 versus 0.8±0.1 mm², P<.01). Steady-state c-myc mRNA levels were increased 10-fold and 2-fold (compared with undamaged carotid arteries) at 2 and 6 hours after balloon injury, respectively; mithramycin treatment reduced c-myc mRNA levels at 2 and 6 hours by 66% and 53%, respectively.

**Conclusions** These results support the hypothesis that systemic administration of mithramycin immediately (1 hour before and after intervention) effectively inhibits transcription of the c-myc proto-oncogene and prevents myointimal proliferation after balloon injury of the rat carotid artery in vivo. Because mithramycin has been shown to be well tolerated by humans and to effectively inhibit transcription of c-myc in proliferating human cells, this agent may be useful in the prevention of coronary restenosis. (Circulation. 1994;90:2468-2473.)

**Key Words** • vascular endothelial injury • restenosis • mithramycin • c-myc oncogene

Endothelial injury resulting from balloon angioplasty or following vascular surgery and cardiac transplantation leads to smooth muscle cell proliferation and migration into the subintimal region, accompanied by local extracellular matrix formation in muscular arteries.1 This process is responsible for the high (30% to 50%) incidence of restenosis following balloon angioplasty of the coronary arteries and for the accelerated atherosclerosis observed in coronary artery bypass grafts.2 Balloon injury of the common carotid artery of the rat has been developed as an experimental model of localized and highly controllable vascular damage in which smooth muscle cell proliferative responses can be studied in vivo.3,4 In this model, balloon inflation denudes endothelium and induces a highly reproducible intimal migration/proliferation of smooth muscle cells over the entire length of the affected vessel.5

The mechanisms responsible for smooth muscle cell proliferation following endothelial injury remain incompletely understood. Injury to the arterial wall triggers the synthesis and release of various mitogens, which in turn promote migration/proliferation of smooth muscle cells through receptor specific interactions.6-8 Attempts to inhibit vascular smooth muscle cell migration/proliferation with prolonged infusions of specific antibodies against individual mitogens have given mixed results.9,10 Several large-scale clinical trials testing pharmacological agents, such as thromboxane antagonists, heparin fragments, corticosteroids, and angiotensin-converting enzyme inhibitors, have failed to reduce the rate of restenosis.11-15

Recent advances in cellular and molecular biology have provided insight into the molecular mechanisms of smooth muscle cell proliferation, which is caused by the transduction of mitogenic signals from growth factors to the nucleus.16-19 An immediate early response proto-oncogene, c-myc, plays an important role in the regulation of cellular proliferation and differentiation.20 Var-
ious mitogens, such as platelet-derived growth factor, basic fibroblast growth factor, transforming growth factor-β, and insulin-like growth factor-1, can induce c-myc transcription after balloon denudation. Transient increases in c-myc mRNA after mitogenic stimulation have been demonstrated as the cells enter the G1 phase and appear to be necessary for the G1-to-S-phase transition. It has also been demonstrated that the c-myc and c-fos proto-oncogenes are activated shortly after serum stimulation of smooth muscle cells in culture or balloon injury of rat and rabbit aorta. The c-myc antisense oligomers have been shown to reduce c-myc expression and to inhibit growth of HL-60 promyelocytic cells and human smooth muscle cells in vitro, indicating that the c-myc proto-oncogene plays an important role in regulating the proliferation in a number of cell types, including vascular smooth muscle cells.

Mithramycin is a G-C-specific DNA binding drug that selectively inhibits transcription of genes, such as the human c-myc gene, that have G-C-rich promoter sequences. Mithramycin prevents the binding of Sp1 and other proteins to the c-myc P1 and P2 promoters. Previous studies have demonstrated that mithramycin prevents serum-induced proliferation of rat aortic smooth muscle cells and the concomitant increase in expression of c-myc proto-oncogene in vitro.

The current study tested the hypotheses that (1) mithramycin can prevent myointimal proliferation after balloon injury of rat carotid artery in vivo, (2) myointimal proliferation after vascular injury is associated with enhanced expression of the c-myc proto-oncogene, and (3) mithramycin inhibits the expression of c-myc mRNA in the damaged carotid artery. Our results suggest that c-myc participates in the proliferative response of the vascular wall to arterial injury and that overexpression of c-myc in the damaged artery can be effectively inhibited by mithramycin, and therefore that inhibition of c-myc with mithramycin may be therapeutically useful in preventing the proliferative lesion that occurs after coronary angioplasty.

Methods

Animal Model

Ten-week-old male Sprague-Dawley rats weighing 300 to 350 g were obtained from Charles River Breeding Laboratories. Mithramycin (Sigma Chemical Co) in a dose of 150 μg/kg was dissolved in distilled H2O (120 μg/mL) and administered by intraperitoneal injection 1 hour before and 1 hour after balloon injury of the right carotid artery. Control animals received injections of an equal volume of distilled H2O. Animals were assigned at random to the mithramycin treatment or to distilled-H2O control groups. Rats were anesthetized with sodium pentobarbital (50 mg/kg IP), and the right carotid artery of each animal was isolated by a middle cervical incision, suspended on ties, and stripped of adventitia. The distal right common carotid artery and region of the bifurcation were exposed. A 2F Fogarty balloon catheter (Baxter V. Mueller, Irvine, Calif) was introduced through the external carotid artery and advanced into the thoracic aorta. The balloon was inflated with saline to distend the common carotid artery and then was pulled back to the external carotid artery. After six repetitions of this procedure, the endothelium was removed completely, and some injury to medial smooth muscle layers throughout the common carotid artery resulted. After removal of the catheter, the external carotid artery was ligated and the wound closed. The left carotid artery was not damaged and served as the control.

Animals were given standard rat chow and tap water ad libitum for 2 weeks, at which time they were killed with an overdose of sodium pentobarbital (75 mg/kg) and perfused with 10% formalin at a pressure of 120 mm Hg. The vascular system was rinsed with 10 mL PBS before infusion of fixative solution. Both carotid arteries were isolated from adherent tissue and fixed in 10% formalin for morphometric analysis. All protocols were approved by the Institutional Animal Care and Use Committee at the University of Alabama at Birmingham and are consistent with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication No. 85-23, revised 1985).

Morphometric Analysis

For histological analysis, vessels were embedded in paraffin and the middle fifth (0.2 cm) of the damaged right carotid artery was serially sectioned (30 μm). The left carotid artery was not damaged and served as a control. Morphometric analysis of each arterial segment was performed with a computer-based Bioquant II morphometric system. Tissue was stained with Verhoef's elastic tissue staining, which demonstrated several layers of elastic laminae (Fig 1). At least five sections of each vessel were examined, and the measurements were averaged for statistical analysis. All morphometric analyses were carried out by a single examiner, who was blinded with respect to the experimental group to which each sample belonged. The cross-sectional surface areas of the vessel with the external elastic lamella (the total area), within the internal elastic lamina (the intimal area), and within the lumen (the lumen area) were measured. The degree of myointimal proliferation of the injured carotid artery was expressed as the absolute area of neointima and the ratio of the neointimal area to the medial area.

RNA Isolation and Northern Blot Analysis

In a separate experiment, 10-week-old male Sprague-Dawley rats received mithramycin (150 μg/kg IP) or distilled H2O 1 hour before and 1 hour after balloon catheterization. At 2 and 6 hours after vascular injury, rats were killed by an overdose of sodium pentobarbital (75 mg/kg). Fresh tissue was collected, rapidly frozen in liquid N2, and stored at −70°C. Total RNA was extracted from undamaged left carotid arteries and balloon-injured right carotid arteries by the method of Chomczynski and Sacchi. Then 30 μg of total RNA (pooled from six animals) was denatured at 65°C for 5 minutes in 50% formamide and 6% formaldehyde (in 22.5 MOPS with 1.2 mmol/L EDTA), size fractionated by electrophoresis through 1.5% agarose-3% formaldehyde gels (in 20 mmol/L MOPS, 5 mmol/L sodium acetate, 1 mmol/L EDTA, pH 7.0), and blotted onto Nytran membrane (0.45 μm, Schleicher & Schuell Inc) in 20 × SSC (1 × SSC=0.15 mol/L NaCl, 15 mmol/L sodium citrate, pH 7.0) according to a modification of the technique of Thomas. Northern blots were UV cross-linked (Bio-Rad), prehybridized in QuickHyb hybridization buffer (Stratagene) for 15 minutes, and hybridized in the same buffer with a 32P-labeled mouse c-myc cDNA probe at 68°C for 1 hour. After hybridization, blots were washed twice in 500 mL 2 × SSC, 0.1% SDS at room temperature for 15 minutes and twice in 0.1 × SSC, 0.1% SDS at 60°C for 20 minutes. Blots were partially dried and exposed to x-ray film (Kodak X-OMAT film, Sigma). To quantitate total RNA loaded on each lane, blots were stripped by washing in 5 mmol/L Tris-HCl (pH 8.0), 0.2 mmol/L Na2EDTA, 0.05% sodium pyrophosphate, 0.002% polyvinyl-pyrrolidone, 0.002% BSA, and 0.002% Ficoll at 70°C for 3 hours and rehybridized with a 32P-labeled 18S rRNA-specific oligonucleotide probe (5'-ACGGTATCTGATCGTCTTCGAACC-3') under the conditions specified above. The 18S rRNA levels were used as internal controls to normalize the densitometric data to account for variations in RNA loading. The density of autoradiographic signals was measured with an optical densitometer.
**Statistical Analysis**

Results were expressed as mean±SEM. Data were analyzed with the CRUNCH statistical package on an IBM PC/AT computer. Statistical comparisons of neointimal area, medial area, total area, lumen area, ratio of neointimal area to medial area, and ratio of c-myc mRNA to 18S rRNA between mithramycin-treated and control groups were performed with Student’s unpaired t test. Differences were reported as significant at a value of P<.05.

**Results**

Two weeks after balloon injury of rat carotid artery, the body weight of mithramycin-treated rats was not different from that of control rats (389±9 g, n=6 versus 378±22 g, n=7, respectively), indicating that 150 μg/kg of mithramycin administered 1 hour before and 1 hour after carotid arterial injury was not toxic to the rats. In the undamaged left carotid artery of both mithramycin-treated and control groups, the intima was only a single cell layer thick; the internal elastic lamina was intact, and the external elastic lamina was in contact with the adventitia (Fig 1, top). Two weeks after balloon injury of the right carotid artery, significant proliferation of the neointima consisting of circumferentially uniform, multiple layers of smooth muscle cells was observed in the damaged vessel (Fig 1, middle). Furthermore, the internal elastic lamina was disrupted. The extensive neointimal proliferation was associated with a reduced residual lumen area (Fig 1, middle). In mithramycin-treated rats, the degree of neointimal proliferation of the damaged carotid artery was much less extensive than in vehicle-treated control rats (Fig 1, bottom).

Morphometric analysis showed significant suppression (=50%) of myointimal formation in mithramycin-treated compared with vehicle-treated control rats (0.6±0.1 versus 1.2±0.1 mm², respectively, P<.01) (Fig 2, top). The ratios of neointimal to medial area were markedly less in mithramycin-treated rats than in vehicle-treated control rats (95±16% versus 190±14%, respectively, P<.01) (Fig 2, middle). The lumen sizes were significantly decreased in the vehicle-treated control rats compared with the mithramycin-treated rats (0.8±0.1 versus 1.5±0.1 mm², P<.01) (Fig 2, bottom). Compared with vehicle-treated control rats, there was no significant change in the total area (2.70±0.17 [n=6] vs. 2.76±0.10 [n=7] mm², control versus mithramycin-treated group, respectively) or medial area (0.65±0.03 [n=6] versus 0.63±0.01 [n=7] mm², control versus mithramycin-treated group, respectively) of the carotid artery in mithramycin-treated rats.

Northern analysis showed that expression of c-myc mRNA in undamaged carotid artery was low and barely detectable but that steady-state c-myc mRNA levels increased significantly following vascular injury. The c-myc signal was maximal at 2 hours and persisted 6 hours following balloon injury. Compared with the undamaged carotid artery, the magnitude of the increase of c-myc mRNA, measured by densitometric scanning, was 10-fold and 6-fold at 2 and 6 hours after balloon injury, respectively. Mithramycin significantly inhibited expression of c-myc gene transcription levels by 66% and 53% at 2 and 6 hours after balloon injury, respectively, compared with damaged control vessels from vehicle-treated rats (Fig 3).
Proliferation and Myointimal Proliferation

By guest on May 2, 2017

Fig 2. Effects of mithramycin on neointima formation 14 days after balloon injury of the right common carotid artery of male Sprague-Dawley rats. Control rats received distilled H2O vehicle. Cross-sectional areas of neointima (top), neointimal area to medial area ratios (middle), and cross-sectional lumen (bottom) are presented as mean±SEM. **P<.01 compared with control groups.

Discussion

This study demonstrated that (1) mithramycin, a G-C-specific DNA binding drug, significantly inhibits myointimal proliferation following balloon injury of the rat carotid artery in vivo, (2) expression of the c-myc proto-oncogene is increased acutely (10-fold increase at 2 hours) following balloon injury, and (3) mithramycin significantly inhibits c-myc expression in damaged vessels when administered systemically immediately before and after injury of the carotid artery in doses that are not toxic to rats.

An immediate early-response gene, c-myc is expressed in nearly all cell types and appears to play an important role in regulating cellular proliferation and differentiation. The c-myc gene product forms a sequence-specific DNA binding heterodimer with its counterpart protein, Max. This complex appears to be involved in the induction of replication competence (S phase) during the cell cycle. Cell culture experiments suggest that c-myc expression is a necessary component of smooth muscle cell proliferation and migration. The levels of c-myc mRNA and protein are highest before DNA synthesis (S phase) and remain increased throughout the cell cycle in the presence of growth factors. Studies using other cell systems have shown that downregulation of c-myc expression is invariably associated with the inhibition of cell growth and induction of differentiation.

Antisense inhibition of c-myc expression in cultured vascular smooth muscle cells is associated with inhibition of cell proliferation and migration, as well as decreased expression of c-myc protein as detected by Western immunoblotting and immunocytochemical staining. Serum-stimulated induction of c-myc expression is blunted by treatment with antisense oligonucleotides. Shi and coworkers reported that antisense oligomers directed toward the translation initiation region of the human c-myc gene have a significant antiproliferative effect on human vascular smooth muscle cells in culture, whereas the sense and mismatched oligomers do not have a growth-inhibitory effect. Simons and Rosenberg showed that the c-myb proto-oncogene, which is homologous to the transforming gene product of the avian myeloblastosis virus, is over-expressed in proliferating vascular smooth muscle cells in vitro. Simons et al showed that suppression of the increase in c-myb mRNA by local application of antisense oligonucleotide inhibits smooth muscle proliferation both in vitro and in a carotid artery injury model in vivo. Thus, inhibition of both c-myc and c-myb prevents proliferation of smooth muscle cells, confirming their essential roles in vascular smooth muscle cell growth stimulation.
This study has demonstrated that c-myc expression is increased acutely as much as 10-fold in damaged carotid artery compared with the undamaged control vessels. The levels of c-myc mRNA were considerably increased 2 hours following balloon injury of rat carotid artery and remained high 6 hours after injury. This observation is consistent with previous reports of increased c-myc expression within 1 hour in serum-stimulated or growth factor-stimulated vascular smooth muscle cells in culture and on balloon injury of rat and rabbit aorta. The time course of induced overexpression of c-myc in damaged carotid artery is quite similar to the temporal pattern of c-myc expression following partial hepatectomy in rats.

Mithramycin is a DNA binding compound that selectively inhibits expression of genes with G-C–rich promoters, including c-myc. Although it is commonly used to treat hypercalcemia in clinical settings, it has also been shown to induce terminal differentiation of myeloid leukemia cells, which is accompanied by selective inhibition of c-myc expression. Mithramycin prevents the binding of regulatory proteins to both the P1 and P2 promoters of the c-myc gene, inhibiting in vitro transcriptional activity of both promoters. It also prevents binding of the Sp1 regulatory protein to consensus binding sites in the promoters of the dihydrofolate reductase gene and the SV40 early promoter, resulting in complete transcriptional inhibition.

Previous studies have demonstrated that mithramycin prevents the serum-induced proliferation of rat aortic smooth muscle cells in vitro. Studies of the effect of mithramycin on the transcriptional activity of the c-myc gene assayed by dot hybridization and Northern analysis show that mithramycin inhibits the serum-induced increase in the c-myc transcription after 4 hours of exposure to serum, indicating that mithramycin may act by preventing stimulation of c-myc transcription. Our data provide the first definitive evidence that G-C–specific DNA binding drugs can prevent the proliferative response of vascular smooth muscle cells to balloon injury.

Pharmacological inhibition of proto-oncogene expression has several advantages over the antisense approach. Mithramycin has been used clinically for more than 20 years. Although cases of significant hepatic toxicity have been reported, they have been rare. Mithramycin is relatively stable, with transcriptional effects that may last as long as 12 to 24 hours. In addition, transcriptional inhibition involves the interaction of the ligand (drug) with a fixed number of target sequences, which do not increase in response to treatment. In contrast, antisense treatment may stimulate the transcriptional activity of the target gene, resulting in many more target sequences. The selective inhibition of c-myc transcription by G-C–specific DNA binding drugs may allow the development of more specific “antigene therapies,” such as triplex-forming transcriptional inhibitors. Furthermore, because mithramycin is known to be an effective inhibitor of human c-myc gene transcription in vivo in doses that are safe and well tolerated, it offers promise for the prevention of coronary artery restenosis in human subjects.

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