Angiopeptin Inhibits Oncogene Induction in Rabbit Aorta After Balloon Denudation

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Background Angiopeptin, a synthetic cyclic octapeptide analogue of somatostatin, reduces neointimal hyperplasia after balloon denudation of rabbit aorta if administered before injury. The aim of this study was to analyze the effect of angiopeptin pretreatment on the level of expression of the c-fos and c-jun protooncogenes, early markers of smooth muscle cell proliferation, after balloon denudation of rabbit aorta.

Methods and Results For histological analysis of the effect of angiopeptin on neointimal thickening after aortic balloon denudation, rabbits were randomized into three groups: group 1 (controls), twice-daily injections of saline begun 24 hours before balloon denudation (n=9); group 2, twice-daily injections of angiopeptin 10 µg/kg begun 24 hours before balloon denudation (n=9); and group 3, twice-daily injections of angiopeptin 10 µg/kg begun 1 hour after balloon denudation (n=7). The degree of neointimal thickening 28 days after balloon denudation was significantly less in group 2 than in group 1 (neointimal area: group 1, 0.59±0.11 mm²; group 2, 0.22±0.05 mm²; P<.05. Neointima/media: group 1, 0.85±0.17; group 2, 0.23±0.05; P<.05). When angiopeptin was started 1 hour after denudation (group 3), however, the neointimal area (0.52±0.09 mm²) and the neointima/media ratio (0.76±0.10) were not statistically different from the control group. For analysis of protooncogene induction, rabbits received twice-daily subcutaneous injections of saline (n=7), angiopeptin 10 µg/kg (n=8), or angiopeptin 100 µg/kg (n=4) begun 24 hours before balloon denudation. The animals were killed 30 minutes after balloon denudation, and total aortic RNA was hybridized with fos and jun probes. Expression of c-fos and c-jun was detected 30 minutes after injury; angiopeptin pretreatment at 20 µg·kg⁻¹·d⁻¹ induced a 41% reduction in c-fos expression and a 42% reduction in c-jun expression compared with control animals. The inhibitory effect at the higher dose of angiopeptin was similar.

Conclusions Our results show that the inhibitory effect of angiopeptin on neointimal thickening is related to events that occur very early after injury and suggest that the inhibition of smooth muscle cell activation may be responsible, at least in part, for this effect. (Circulation. 1994;89:2327-2331.)

Key Words • restenosis • arteries • oncogenes

The major disadvantage of the use of percutaneous transluminal coronary angioplasty in the treatment of patients with atherosclerotic coronary disease is the frequent occurrence of restenosis after an initially successful procedure. Improvements in technology or pharmacological interventions have had no significant impact on the rate of restenosis.

Studies in animals and histological observations in humans have demonstrated that restenosis is characterized by neointimal hyperplasia caused by smooth muscle cell proliferation and by the synthesis of extracellular matrix.

Angiopeptin, a synthetic cyclic octapeptide analogue of somatostatin, is a drug that may potentially be useful in the prevention of restenosis in humans. Although animal models have important differences from the human situation, angiopeptin is unique in that it has been shown to reduce neointimal hyperplasia in several different animal models of angioplasty. The mechanism of this effect is unknown but is thought to be related to a local inhibition of growth factors responsible for smooth muscle cell activation. In a rabbit model of balloon angioplasty, angiopeptin significantly reduced intimal hyperplasia if administered before the injury; however, if its administration was delayed for 8 hours after injury, no such beneficial effect was seen. This lack of effect after delayed administration suggests that the antiproliferative effect of angiopeptin is due to an action on events that occur very early after balloon injury.

We recently showed that expression of the c-fos and c-jun protooncogenes, whose induction is one of the earliest events associated with growth factor stimulation, can be demonstrated in a rabbit model of balloon denudation within hours of the procedure. Similar findings have been reported in a rat model. To test the hypothesis that the antiproliferative effects of angiopeptin in vivo are related to an inhibitory effect on smooth muscle cell activation, we examined the effects of pretreatment with angiopeptin on the level of expression of the c-fos and c-jun protooncogenes in a rabbit model of balloon denudation.

Methods

Balloon Denudation

Male New Zealand White rabbits (2 to 2.5 kg) were used in all experiments. The animals were anesthetized with ethyl
carbamate (1 g/kg IV). After exposure of the right femoral artery, a 3F Fogarty balloon catheter was passed retrogradely to a distance of 25 cm and inflated until contact was made with the endothelium. The aortas were denuded by gentle advancement and withdrawal of the catheter three times. The femoral artery was subsequently ligated and the wound closed. All the studies described conform to the position of the American Heart Association on research animal use.

**Study Groups**

**Analysis of Protooncogene Induction After Injury**

Eight rabbits were killed at various times (30 minutes, 1 hour, 2 hours, and 5 hours) after balloon denudation. Another group of eight animals who underwent a sham operation served as controls.

**Effects of Angiopeptin on Protooncogene Induction**

To investigate the effects of angiopeptin, 15 rabbits were randomized to receive twice-daily subcutaneous injections of either 0.5 mL saline (n=7) or angiopeptin 10 μg/kg (n=8). A third group of animals received twice-daily injections of angiopeptin 100 μg/kg (n=4). Angiopeptin was provided by Ipsen Biotech. Aortic balloon denudation was performed on the second day of treatment, 2 hours after the third injection of angiopeptin or of saline. The animals were killed 30 minutes after balloon denudation.

**Effects of Angiopeptin on Neointimal Thickening 28 Days After Injury**

To analyze the in vivo inhibitory effect of angiopeptin on neointimal thickening after balloon denudation, 25 rabbits were randomized into three groups to receive the following treatment regimens: group 1, twice-daily subcutaneous injections of 0.5 mL saline begun 24 hours before balloon denudation (n=9); group 2, twice-daily injections of angiopeptin 10 μg/kg begun 24 hours before balloon denudation (n=9); and group 3, twice-daily injections of angiopeptin 10 μg/kg begun 1 hour after balloon denudation (n=7). The animals were killed 28 days after balloon denudation for histological analysis.

**RNA Extraction and Northern Blot Analysis**

Immediately after the animals were killed, the abdominal aorta was removed, stripped of periaortic fat and connective tissue, and frozen in liquid nitrogen until assayed. RNA extraction was performed by the technique described by Chomczynski and Sacchi. Northern blot analysis (using 25 to 30 μg total RNA per lane) was performed as previously described. The RNA content was verified by use of ethidium bromide. Blots were hybridized with a fos probe (1.06-kb Pst I fragment, corresponding to a v-fos) or a jun probe (2.1-kb EcoRI and Pvu II fragment, corresponding to a v-jun). In addition, blots were hybridized with the constitutive probe specific for 18S ribosomal RNA to monitor the RNA content of the different lanes. The probes were labeled with [32P]dCTP by use of the multiprime DNA labeling reaction kit (Amersham Corp). Hybridization was carried out for 12 to 16 hours at 42°C as previously described. The membranes were washed for two 10-minute periods in 200 mL of 2× standard sodium citrate (SSC)/0.1% SDS at room temperature and then for two 15-minute periods in 0.1× SSC/0.1% SDS at 50°C. Autoradiography was performed with Dupont Cronex 4 x-ray films at −70°C for 1 to 3 days.

The relative abundance of specific mRNA transcripts was quantified by radioanalytic scanning with a spectrophotometric scanner (Schimadzu Corp). The results were expressed as a fraction of the levels of 18S ribosomal RNA in relative densiometric units.

**Histological Analysis**

The vessels were perfusion-fixed with 4% paraformaldehyde (in a saline phosphate buffer solution) at a pressure of 110 mm Hg as previously described. After further immersion in 4% paraformaldehyde for 24 hours, the vessels were embedded in paraffin. Aortic cross sections were cut and stained with hematoxylin-eosin-saffron-astra blue or with Mallory's phosphotungstic hematoxylin. Morphometric analysis of the histological cross sections was performed with digital microscopic planimetry (Morphometry-System, Biocom). We analyzed three histological cross sections per aorta (2 cm below the junction of the aorta with the lower renal artery, 2 cm above the aortoiliac bifurcation, and midway between these two points). The mean areas of the neointima and of the media (in square millimeters) were calculated. The neointima/media ratio was defined as the neointimal area divided by the area of the media. All these measurements were performed by a pathologist who was unaware of the design of the protocol.

**Statistical Analysis**

Results are expressed as mean±SEM. The Mann-Whitney rank-sum test was used for between-group comparisons of mean levels of protooncogene expression, expressed in relative densiometric units. Differences in intimal thickening were assessed by ANOVA. A value of P<.05 was considered to indicate statistical significance.

**Results**

**Oncogene Induction**

Representative examples of the results of RNA hybridization with the c-fos and c-jun probes 30 minutes, 1 hour, 2 hours, and 5 hours after balloon denudation are shown in Fig 1. In sham-operated animals, c-jun was expressed at very low levels, and expression of c-fos was not detected. After balloon denudation, expression of the two protooncogenes was evident at 30 minutes, remained at a high level at 1 and 2 hours, but was undetectable at 5 hours.

**Effects of Angiopeptin on Protooncogene Induction**

Representative examples of the results of RNA hybridization with the two probes 30 minutes after balloon denudation are shown in Fig 2. A reduction in mRNA level was found for c-fos and for c-jun with 20 μg·kg⁻¹·d⁻¹ and with 200 μg·kg⁻¹·d⁻¹ of angiopeptin.
This was confirmed by semiquantitative analysis: angiopeptin pretreatment at the lower dose used (20 \( \mu \)g \( \cdot \) kg\(^{-1} \cdot d^{-1} \)) was associated with a 41% reduction in c-fos expression and a 42% reduction in c-jun expression compared with the control animals (\( P<.05 \)). The inhibitory effects of the higher dose (200 \( \mu \)g \( \cdot \) kg\(^{-1} \cdot d^{-1} \)) of angiopeptin were similar (38% reduction in c-fos expression, 44% reduction in c-jun expression).

Histological Studies

Pretreatment with angiopeptin 20 \( \mu \)g \( \cdot \) kg\(^{-1} \cdot d^{-1} \) was associated with a highly significant reduction in neointimal thickening and in neointima/media ratio compared with the saline group (Table). By contrast, when the angiopeptin treatment was begun 1 hour after balloon denudation, no reduction was observed.

Discussion

Angiographic studies show that restenosis occurs in 30% to 40% of patients who undergo coronary angioplasty in the months that follow the procedure.\(^1\) Recent studies in animal models of balloon denudation demonstrate that cellular events ultimately associated with intimal proliferation are detectable within hours of the procedure.\(^11\)\(^,\)\(^12\) Our results show that in this rabbit model of balloon denudation, a pretreatment with angiopeptin was associated with marked inhibition of c-fos and c-jun induction 30 minutes after injury and with a highly significant reduction in neointimal thickening 28 days after injury. By contrast, if the angiopeptin treatment was begun 1 hour after injury, no effect on neointimal thickening was observed. These findings suggest that the inhibitory effect of angiopeptin in this model may be related, at least in part, to an inhibitory effect on early (G1) events of the cell cycle.

We and others have previously demonstrated that balloon denudation of rabbit aorta, as performed in the present study, causes complete deendothelialization and that smooth muscle proliferation, migration, and intimal thickening subsequently occur.\(^9\)\(^,\)\(^10\)\(^,\)\(^18\) The activation of nuclear oncogenes occurs at a very early stage in this process.\(^11\)\(^,\)\(^12\)\(^,\)\(^18\) It has recently been demonstrated that the distribution of c-fos and c-jun products after balloon injury of rat aorta was concentrated in smooth muscle cell nuclei.\(^19\) The induction of nuclear oncogenes is one of the earliest transcriptional events associated with growth factor stimulation, and the increased expression of these genes is a transient response to mitogenic stimulation persisting for at most a few hours after exposure to growth factors.\(^20\)\(^,\)\(^21\) The protooncogenes c-fos and c-jun are two prominent members of the nuclear oncogene family. The corresponding oncoproteins function together as a dimeric complex that binds to a specific DNA consensus sequence (AP-1) of target genes to stimulate their transcription and are involved in the G1 phase of the cell cycle.\(^22\) Experiments in cell culture models have shown that the use of antibodies or of antisense RNA against nuclear oncogenes prevents cell entry into the S phase and the subsequent synthesis of DNA.\(^23\)\(^,\)\(^24\) It is therefore plausible that this expression of nuclear oncogenes in the hours after angioplasty is associated with the early G1 events preceding DNA synthesis in smooth muscle cells.

Previous studies\(^8\)\(^,\)\(^10\)\(^,\)\(^25\)\(^,\)\(^26\) have shown that angiopeptin specifically inhibits the growth of vascular smooth muscle cells after arterial injury. Using the same experimental model of balloon denudation of rabbit aorta, Conte et al\(^8\) demonstrated a significant reduction in neointimal thickening 3 weeks after injury in animals treated with 10 \( \mu \)g/kg of angiopeptin twice daily compared with control animals. Asotra et al\(^20\) showed that 100 \( \mu \)g/kg of angiopeptin twice daily markedly inhibits \([3H]\)thymidine incorporation into balloon-denuded rabbit aorta, thus demonstrating that the effect of angiopeptin on neointimal formation is due to inhibition of vascular smooth muscle cell proliferation. Moreover, in the same model, Howell et al\(^10\) demonstrated that when the angiopeptin (20 \( \mu \)g \( \cdot \) kg\(^{-1} \cdot d^{-1} \)) was administered just before angioplasty, it inhibited neointimal thickening; however, when administration of angiopeptin was delayed for 8 hours after balloon injury, there was no reduction in intimal thickening. Our results confirm and extend these findings by showing that angiopeptin inhibits neointimal thickening if begun before the injury but has no effect if the first dose is given 1 hour after balloon denudation. These findings suggest that the inhibitory effect of angiopeptin is related to cellular events that occur at the time of injury or in the first hour after injury.

The exact role of c-fos and/or c-jun in the growth response of cells to different agonists is still debated. It has been reported that the growth of embryonic stem cells was not affected by a specific inhibition of c-fos by homologous recombination\(^27\) and that a null mutation in the c-fos protooncogene had no effect on the replication of most
cells in prenatal and later mouse development. Conversely, experiments on fibroblasts using anti-fos antibodies or c-fos antisense RNA inhibited cell proliferation and the ability to reenter the cell cycle from quiescence. One explanation for these apparent discrepancies is that introduction of the fos-deficient state early in development may allow the cells to compensate by upregulation of genes that have a similar function. The large size of the AP-1 factor family makes it plausible that one or more genes such as fos B, fra-1, fra-2, jun B, or jun D may be upregulated to permit cell growth to proceed normally. Conversely, acute inhibition by use of antibodies or of antisense RNA may not allow the cell to compensate. Recently, the use of antisense oligonucleotides against c-myc, another member of the nuclear oncogene family, was shown to inhibit restenosis in vivo in a rat model of balloon denudation, suggesting that the inhibition of a single gene, if important, may significantly affect the vascular response to injury. Taken together, these findings are consistent with the hypothesis that the early reduction in c-fos and c-jun expression induced by angiopeptin pretreatment may contribute to the reduction in neointimal thickening observed 28 days later; however, the inhibition of other immediate early genes might also explain the efficacy of angiopeptin pretreatment. The specific aim of this study was not to demonstrate that the induction of c-fos and c-jun was necessary for restenosis but rather to use these genes as early markers of smooth muscle cell activation; our results show that angiopeptin pretreatment significantly affects the immediate response of the smooth muscle cell to injury. The lack of effect on neointimal thickening of angiopeptin treatment begun 1 hour after balloon denudation may be because at this point, the cascade of events leading to smooth muscle cell proliferation has already begun. The effect of angiopeptin may be related to a local inhibition of growth factors responsible for smooth muscle cell proliferation. It has been suggested that protein tyrosine phosphatase activation may be involved in the antiproliferative signal induced by somatostatin analogues. Numerous growth factors, including platelet-derived growth factor, epidermal growth factor, and insulin growth factor-1, stimulate tyrosine kinase activity after binding to their specific receptors. These growth factors have been implicated in the pathogenesis of the response to injury after balloon denudation and are candidates for protooncogene induction. An attractive hypothesis for the findings of this study would be that angiopeptin is capable of inhibiting the stimulatory effect of growth factors on the phosphorylation of their specific receptors, thus inhibiting subsequent cell growth.

Although animal models of myointimal proliferation, such as balloon injury of normal arterial segments, do not reproduce the clinical situation, they provide valuable insights into potential mechanisms of cell growth regulation. The potential of candidate drugs for the prevention of restenosis in animal models is generally assessed by the determination of their effect on neointimal proliferation. The recognition that events ultimately associated with neointimal proliferation are detectable in the hours after balloon injury provides another potentially useful method to assess possible antiproliferative activity. To the best of our knowledge, our findings are the first demonstration of an inhibitory drug effect on the early steps of the vascular wall response to injury. Interestingly, a relatively low dose of 20 μg · kg⁻¹ · d⁻¹ was sufficient to obtain a significant inhibition with no apparent additional benefit for higher doses; this is of great potential clinical relevance, since there are usually marked discrepancies between the doses of drugs used in animal models of restenosis and the doses used in clinical trials in humans. Clinically relevant doses of angiopeptin are similar to those we used in our experimental study. Finally, the demonstrated inhibitory effect of angiopeptin as early as 30 minutes after injury emphasizes the need for pretreatment to maximize a potential beneficial effect on restenosis.

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