Inhibition of Integrin Function by a Cyclic RGD-Containing Peptide Prevents Neointima Formation

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**Background** RGD-containing peptides are able to prevent binding of ligands to certain integrins such as αIIbβ3 (glycoprotein IIb/IIIa) and αβ1, and as such are inhibitors for platelet aggregation and smooth muscle cell migration, both of which are involved in neointima formation.

**Methods and Results** Hamster carotid arteries were damaged, and neointima formation was determined at different time points. G4120, a cyclic RGD-containing peptide, was administered continuously intravenously by an implanted osmotic pump. Neointima formation was inhibited dose dependently. The inhibition was strongest when treatment was started before the vascular injury and continued for the full observation period. Treatment started after the damage and maintained until neointima assessment or started before and stopped earlier was less effective.

**Conclusions** Inhibition of integrin function by an RGD-containing peptide results in reduction of the development of a neointima. This effect is due both to an early event, which could be due to inhibition of secretion of PDGF by the platelets with blocked αIIbβ3, and to a late event, possibly by interference with smooth muscle cell αβ1.

**Key Words** • platelets • muscle, smooth • cells • stenosis • carotid arteries

The long-lasting success of percutaneous transluminal coronary angioplasty, a common treatment for many patients with coronary artery disease, is hampered by acute reclosure due to thrombosis and by late restenosis due to smooth muscle cell (SMC) migration and proliferation. At present, successful experimental approaches to prevent neointima formation have been described using platelet-derived growth factor (PDGF) antagonists, angiotensin-converting enzyme (ACE) inhibitors, and antisenes c-myc oligonucleotides. Some evidence has been provided that platelets are also involved in the process of restenosis: Thrombocytopenic animals exhibited significantly less intimal thickening after balloon angioplasty than normal controls, the frequency and severity of platelet-dependent flow variations were closely related to the severity of neointimal proliferation after experimental coronary stenosis and endothelial injury, and this proliferation could be retarded by treating the animals with platelet inhibitors such as a dual thromboxane A2 synthase/receptor blocker and a serotonin S2 receptor antagonist. These findings to some extent can be explained by the platelets providing both competence (eg, PDGFs) and progression factors (eg, epidermal growth factor, β-transforming growth factor) leading to SMC migration and proliferation. However, clinical trials using various classic antiplatelet drugs presently available did not show a beneficial effect.

More potent antiplatelet agents such as inhibitors of the platelet fibrinogen receptor glycoprotein IIb/IIIa or αIIbβ3 have become available and are highly effective both in vitro and in vivo in animal models and in humans in preventing platelet aggregation. One such compound, G4120 (N-mercaptoacetyl-D-Tyr-Arg-Gly-Asp-sulfoxide) has been studied extensively as an antithrombotic agent but is not specific for the αIIbβ3-integrin. Indeed, several integrins, including the related vitronectin receptor αβ3, present, for example, on platelets and SMCs, recognize the Arg-Gly-Asp (RGD) sequence present in many adhesive molecules, and short synthetic RGD-containing peptides inhibit the binding of ligands to both integrins. SMC adhesion to vitronectin, mediated by αβ3, can be blocked by RGD-containing peptides, and smooth muscle cell αβ3 has been implicated in the migration of the cells over fibronectin, laminin, and collagen I and IV.

**Methods** Hamsters (Gold, pfd, KU Leuven) with a body weight of 100 to 130 g were anesthetized with sodium pentobarbital. The distal right common carotid artery and the region of the bifurcation were exposed. A 2FG Portex Green catheter with a roughened tip was inserted through the external carotid artery and advanced into the thoracic aorta, where it was left in position for 30 seconds, rotated three times completely, after which it was slowly withdrawn. The external carotid artery was ligated, and the wound was closed. G4120 (Genentech Inc) was administered by a continuous intravenous infusion using an implanted osmotic pump (2ML1, Alzet). The infusion of 1 to 10 μg/h G4120 was either started 1 hour before or 30 minutes after denudation and was continued for 3, 7 (infusion rate, 10 μL/h), or 14 days (infusion rate, 5 μL/h), at which time the animals were anesthetized, blood was collected, and the common carotid artery was removed, immersion fixed, divided into several cross sections, and stained with hematoxylin and eosin (Fig 1). Finally, the light microscopical picture was analyzed using a computer-assisted image analyzer, and the ratio between neointimal area and the total area
within the internal elastic lamina was determined on three cross sections each.

Blood was collected by aortic puncture on sodium citrate (0.011 mol/L, final concentration) and centrifuged for 10 minutes at 150g to obtain platelet-rich plasma. Platelet aggregation was induced by 1 μmol/L ADP (Sigma Chemical Co) and followed in a dual-channel aggregometer (Elvi, Pabisch) at 37°C with 1000 rpm stirring speed. Aggregation is expressed as percentage of the maximum light transmission obtained in the absence of G4120.

In vitro effects of G4120 on the growth of hamster SMCs were studied essentially as described by Yamamoto et al21: DDTMF2 hamster SMCs (ATCC-1701) were grown to confluence in 75-cm² culture flasks (Costar 3375) in Dulbecco's modified Eagle’s medium (Gibco BRL) (5% fetal calf serum, 100 μg/mL streptomycin, 100 U/mL penicillin, 4 mmol/L glutamin) at 37°C under humidified 5% CO₂–95% air. Cells were then treated with 0.25% trypsin in phosphate-buffered saline (pH 7.4), washed, and counted, and 8x10⁴ isolated cells were cultured as above in the absence or presence of increasing concentrations of G4120 for 8 days in 16-mm Falcon multiple-well dishes (3047) that were coated with fibronectin. Human fibronectin (ICN Biomedicals) was dissolved at 20 μg/mL in saline and adsorbed onto the dishes (4 μg/cm²) at 25°C for 60 minutes, after which they were rinsed twice with medium containing 0.2% BSA before use. Cell culture medium containing G4120 was renewed every 2 or 3 days. On day 8, cells were detached with trypsin and counted.

Data are given as mean±SEM; statistically significant differences (P<.05) were determined using ANOVA followed by the Student-Newman-Keuls test.

**Results**

In a first series of experiments, continuous intravenously administration of G4120 to hamsters with denuded carotid arteries was started 1 hour before the damage and was continued for up to 7 days. Effective delivery of G4120 by implanted osmotic pumps was maintained throughout the experimental period because ex vivo ADP-induced platelet aggregation was still dose-dependently inhibited at the end of the observation period (Fig 2b). G4120 treatment reduced the neointima formation in hamsters (Fig 1) dose dependently (Fig 2a), with an estimated IC₅₀ of 90 μg/kg per hour. Unlike what was observed under control conditions (Fig 1a) or after inhibition of neointimal formation with a PDGF antagonist or an ACE inhibitor (unpublished observations), G4120 treatment did not result in a densely packed neointima but rather in what appears to be an open network composed of SMC-containing structures (Fig 1b).

Next, the effect of a 3-day treatment with a 7-day treatment on neointima formation after 7 days was compared (Fig 2c): The shorter treatment period was no
longer effective, indicating that integrin inhibition was still needed after the initial period in which platelet activation and thrombus formation is believed to occur. Similar observations were made when vessels were examined 14 days after vessel denudation after a 14- or 7-day treatment period: Inhibition of neointima formation only was significant with sustained treatment (Fig 2c). The mazelike structure was consistently observed in all 14 treated animals and in none of the controls.

In addition, in the same set of experiments, the need for pretreatment of the animals with G4120 was investigated by delaying the start of administration until 30 minutes after injury. This treatment was significantly less effective to prevent neointima formation at 7 days than the one started before the injury. However, when treatment was continued until the 14th day, both treatment schedules were equally effective.

At the doses used, no bleeding complications apart from some minor bleedings at the sites of surgery were observed.

G4120 not only inhibits both ex vivo (see above) and in vitro (IC_{50} = 0.12 μg/mL) aggregation of hamster platelets but also reduces the growth of the hamster SMC line DDT1MF2 on fibronectin during an 8-day observation period in vitro dose dependently (Fig 3, IC_{50} = 70 μg/mL).

**Discussion**

In this study, we report on dose- and time-dependent inhibition of neointima formation in damaged hamster carotid arteries by a cyclic RGD-containing peptide, G4120. G4120 is a nonspecific inhibitor of integrins such as α_{IIb}β_{3} and α_{IIb}β_{1} or glycoprotein IIb/IIa is the platelet receptor for, among others, fibrinogen, and as such is responsible for platelet aggregation and thrombus formation.

Activation of platelets is accompanied by secretion of vasoactive substances such as vasoconstrictive agents (thromboxane A2, serotonin) as well as growth factors, with PDGF one of the more prominent ones in the formation of neointima. That platelets and platelet-derived products do play a role in the latter process was already demonstrated. Also, recently published data on a clinical trial using a chimeric monoclonal antibody Fab fragment, c7E3, which strongly inhibits platelet GPIIb/IIIa receptors but also cross-reacts with α_{IIb}β_{3}, indicated that with a bolus injection followed by a 12-hour infusion of c7E3, a significant reduction of the need for repeat target vessel revascularization was obtained. However, no angiographic measurements to demonstrate a reduced narrowing in the treatment groups were performed.

On the other hand, α_{IIb}β_{3}, present on numerous cells including platelets, has been shown to be involved in migration of melanoma cells and in the growth and the PDGF-stimulated migration of SMCs. In the latter study, an antibody against α_{IIb}β_{3} or an α_{IIb}β_{3}-specific RGD-containing peptide both were able to inhibit in vitro SMC migration, whereas continuous local administration of the peptide to rabbits with carotid artery balloon angioplasty also resulted in a significant reduction in neointimal lesion formation.

We could confirm and extend these findings: G4120 dose-dependently inhibited neointima formation when administration was started 1 hour before the damage and maintained for the complete observation period. When treatment was started only 30 minutes after the vessel wall damage and continued for up to 7 days, the inhibitory effect was less pronounced. Preliminary data had indicated that thrombus formation in the hamster carotid artery is fast, reaching a maximum near 10 minutes after the damage and gradually decreasing thereafter. G4120 effectively prevented this acute effect when administered beforehand and prevented ex vivo platelet aggregation. These results are in line with the ones obtained after a short treatment with c7E3: Inhibition of early massive platelet activation has a beneficial effect on clinical events, which, in view of our findings, can be explained at least in part by the prevention of renarrowing.

On the other hand, earlier studies had revealed that suppression of SMC migration and/or proliferation for only 2 to 3 days after arterial injury was sufficient to reduce late cell proliferation. However, when G4120 administration was started before the vessel wall damage but interrupted before the end of the observation period, the effect was largely lost, implying that the triggers necessary for smooth muscle cell migration/proliferation were still present. Since no evidence exists that platelets still would play a major role at this stage, and in view of the data obtained with an α_{IIb}β_{3}-blocker, the late effect of G4120 most probably may be ascribed to inhibition of SMC α_{IIb}β_{3}. And, although we cannot completely exclude the possibility of non-integrin-related actions of G4120 on the development of intimal thickening in this model, we could confirm the action of G4120 on the growth of SMCs under conditions similar to those in which GRGDSP but not a control peptide GRGESP was active: Hamster SMC growth was reduced dose dependently by G4120. Whether or not the more elevated concentrations (IC_{50} = 70 μg/mL) needed to observe this effect as compared with the inhibition of platelet aggregation (IC_{50} = 0.12 μg/mL) is an indication that G4120 would be more specific for GPIIb/IIIa or may be due to the high proliferating capacity of the leiomyosarcoma-derived DDT1MF2 cell line is at present unclear. On the other hand, the peculiar mazelike structure of the neointima as observed in hamsters...
after G4120 treatment also could be due to a defective adhesion of SMCs. However, further studies are needed to fully understand this phenomenon.

Conclusions

Our findings indicate that a nonspecific RGD peptide, by acting both at the platelet and the SMC level, may have a dual beneficial effect in the prevention of neointimal lesion formation, which opens a new incentive for the development of compounds with this combined action.

Acknowledgments

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