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Antithrombotic Activity of a 2-kDa Heparin Fragment in an Experimental Model of Carotid Artery Thrombosis in Rats

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Abstract

The antithrombotic activity of a 2-kDa heparin fragment was studied in a rat model of common carotid artery thrombosis that causes a completely occlusive thrombus with cessation of the blood flow within 10–15 min.

The compound reduced thrombus formation in a dose-dependent manner, starting from an intravenous dose of 5 mg kg^{-1}. A dose of 20 mg kg^{-1} completely prevented thrombus formation and apparently induced the almost complete lysis of the already formed occlusive thrombus. At none of the doses used did the compound cause increased bleeding or the formation of haematomas.

The present results indicate that low molecular weight heparins, which have an established, highly beneficial effect in venous thromboembolism, are also highly effective in an animal model of arterial thrombosis.

Heparin is a heterogeneous mixture of sulphated polysaccharides (glycosaminoglycans) consisting of chains of alternating residues of D-glucosamine and either glucuronic or iduronic acid, ranging in molecular weight from 5 to 30 kDa with an average molecular weight of 15 kDa (Johnson & Mulloy 1979; Lindahl et al 1986). The anticoagulant activity of heparin is accounted for by a unique pentasaccharide with a high-affinity binding sequence to antithrombin III (AT III) (Rosenberg & Lam 1979; Lindahl et al 1979; Choay et al 1983). Only about one-third of the heparin molecules contain the unique pentasaccharide sequence, whose distribution along the heparin chain appears to be random (Lindahl et al 1979). The major anticoagulant effect of heparin is accomplished through its interaction with AT III. This interaction produces a conformational change in AT III and markedly accelerates the ability of AT III to inactivate the coagulation enzymes thrombin, factor Xa and factor IXa (Rosenberg 1987). Heparin potentiates the inactivation of thrombin by serving as a template to which both AT III and thrombin bind to form a ternary complex (Lindahl et al 1979; Rosenberg 1987).

Heparin has been for more than 50 years the predominant drug for the treatment and prevention of venous thrombosis and thromboembolism. However, an obvious side effect of heparin administration is haemorrhage (Levine et al 1989).

Heparin molecules with fewer than 18 saccharides (molecular weight < 5·4 kDa, low molecular weight heparins, LMWH) are unable to bind thrombin and AT III simultaneously and, therefore, are unable to accelerate the inactivation of thrombin by AT III, but retain their ability to catalyse the inhibition of factor Xa (Andersson et al 1976; Johnson et al 1976; Jordan et al 1980; Lane et al 1984; Ofosu & Barrowcliffe 1990; Green et al 1994). Thus, the risk of haemorrhage is greatly reduced, while the therapeutic potential of these compounds is increased by their more favourable bioavailability after subcutaneous administration, and longer half-life, so that, at present, LMWH may be considered as the drugs of choice for the treatment of venous thrombosis (Green et al 1994; Weitz 1994).

On the other hand, few data are available concerning the activity of these compounds in arterial thrombosis.

We present here the results obtained in an experimental model of arterial thrombosis with a 2-kDa heparin fragment, prepared by free-radical depolymerization of the heparin molecule (Bergonzini et al 1992; Volpi et al 1992).

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Materials and Methods

Animals

Male and female rats of a Wistar strain (Morini, S. Polo d'Enza, Reggio nell'Emilia, Italy), 260–320 g, were housed, five per cage (44 × 35 × 20 cm) (males and females separately), in air-conditioned colony-rooms (temperature: 22 ● 1°C; humidity: 60%) with free access to standard food in pellets and tap water, on a natural light-dark cycle. They were acclimatized to our standard housing conditions for at least 1 week before experimental use. The experiments were conducted in compliance with the standards and suggestions established by the CIOMS (Conseil des Organizations Internationales des Sciences Médicales).

Experimental model of arterial thrombosis

Rats, anaesthetized with ethylurethane (1·25 g kg^{-1}, i.p.), were fixed in the supine position. A polyethylene catheter

was inserted into a femoral vein, for drug administration. A segment of a common carotid artery, 15–20 mm long, was then exposed and dissected free of surrounding tissue to allow the placing of a miniaturized 20-MHz pulsed Doppler flow probe (0·75 mm i.d.). The flow probe was connected to a flowmeter (Crystal Biotech, Holliston, MA, USA). Pulsatile and mean Doppler shifts (kHz) were recorded simultaneously on a polygraph (Battaglia-Rangoni, Bologna, Italy). Velocities were calculated from the measured Doppler shifts using the Doppler equation in the conventional manner (Hartley & Cole 1974), and estimates of volume flow were obtained by multiplying the mean velocity by the estimated internal cross-sectional area. After 10-min stabilization, the baseline blood flow was determined.

Thrombus formation was induced by simultaneous mechanical constriction and electric current application as suggested by Hladovec (1974), with modifications by ourselves (Guarini 1996). In brief, upstream of the flow probe, a 5-mm wide polyacrylate holder, forming a hook and fixed by a pinion with possibility of up and down movement, was inserted under the exposed carotid artery. The holder incorporated two stainless-steel electrodes (diam. 1 mm) exposed on the inner surface at the base of the hook so as to make electrical contact with the artery, connected to a constant-current generator. A current of 2 mA was delivered for 5 min. Concurrently, and for the same time-lapse, the carotid artery was clamped with a small haemostatic clamp placed downstream from the holder (between the holder and the flow probe).

The degree of thrombotic occlusion was expressed by the decrease in carotid blood flow, continuously recorded, and at the end of the experiment the carotid artery segment was removed, opened lengthwise, and examined under a stereomicroscope.

The thrombi were then removed and weighed wet and dry (after 24 h at 37°C). In some cases the arterial segment was cut and prepared for histological examination (haematoxylin-eosin and May Grünwald-Giemsa staining).

Drug and treatment

The 2-kDa heparin fragment (Oligo-H), obtained by depolymerization of heparin induced by the free radical HO· in the presence of cupric acetate at 60–70°C (Volpi et al 1992), was supplied by Opocrin (Corlo, Modena, Italy). Thirty minutes before producing the arterial thrombus, the animals were randomly assigned to intravenous treatment with Oligo-H, 5, 10 or 20 mg kg^{-1} or saline, 1 mL kg^{-1}. In another set of experiments, the animals were randomly treated with either Oligo-H (20 mg kg^{-1}), or saline (1 mL kg^{-1}), 15 min after thrombus production, in order to better detect a possible thrombolytic effect. Eight to ten rats per dose were used.

Statistical analysis

All data were analysed for statistical significance by analysis of variance followed by Student-Newman-Keuls test. When appropriate, a Student's *t*-test was also used. Values are mean ± s.e.m.

Results

As shown in Fig. 1, our model of arterial thrombogenesis in the rat, employing a combination of electrically-induced vascular damage and stasis, is highly effective and reproducible. For all saline-treated animals a completely occlusive thrombus was consistently produced. A progressive decrement in blood flow was observed starting a few minutes after removing the clamp and cutting off the electric current, and an almost complete cessation of blood flow was recorded starting 10 min after the thrombogenic lesion (Fig. 2).

Postmortem examination of the carotid lumen (45 min after thrombogenic lesion) showed the presence of a completely occlusive thrombus (wet weight: 1,150 ± 130 μg; dry weight: 405 ± 45 μg; n = 10), firmly anchored to the damaged arterial inner wall. The histological examination showed the prevailing presence of fibrin and platelets, with small piles of red blood cells trapped into the fibrin network. The administration of the 2-kDa heparin fragment (Oligo-H) 30 min before the lesion of the carotid wall reduced thrombus formation in a dose-dependent manner. There was only a reduction in blood flow (Figs 1 and 2), but in no case was a complete cessation observed: with the lowest dose (5 mg kg^{-1}) the maximum reduction was about 80%, while with the highest dose (20 mg kg^{-1}) it was about 50%. Moreover, in these animals a progressive restoration of blood flow was observed starting 10–20 min after thrombus production; in rats treated with a dose of 20 mg kg^{-1} the blood flow returned to the baseline level about 30 min after the thrombogenic lesion, and no thrombi were detected as postmortem examination of the carotid lumen.

An apparent thrombolytic activity of the compound was observed in rats with a practically complete occlusion of the carotid lumen (15 min after clamp and electric current removal) (Fig. 3). Under these experimental conditions the injection of the 2-kDa heparin fragment, at a dose of 20 mg kg^{-1}, almost completely restored the blood flow (about 85% of the basal value, 90 min after treatment).

Neither increased bleeding nor haematomas were observed in our conditions after any dose of the 2-kDa heparin fragment.

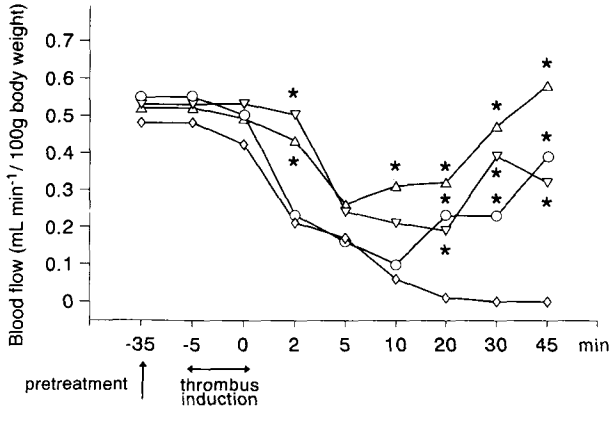

FIG. 1. Time-course of mean blood flow in a common carotid artery after thrombus induction in rats pretreated intravenously with saline 1 mL kg^{-1} (◇) or Oligo-H 5 (○), 10 (▽), and 20 (△) mg kg^{-1}. Mean values for 8–10 rats per group; s.e.m. (not represented for sake of clarity) was < 10% of the mean values in all cases. *$P < 0·01$ compared with corresponding value for saline-treated rats (Student-Newman-Keuls test).

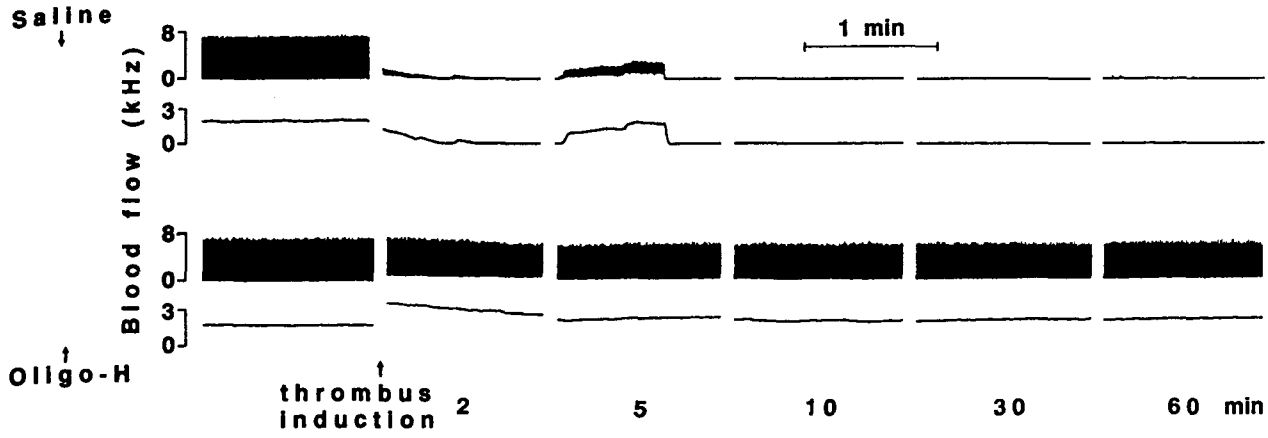

FIG. 2. Representative recordings showing the effect of pretreatment with saline (1 mL kg^{-1} i.v.) or Oligo-H (20 mg kg^{-1} i.v.), on pulsatile and mean carotid blood flow after thrombus induction, in rats. Pretreatments were performed 30 min before thrombus induction.

Discussion

Our model of arterial thrombogenesis combines electrically-induced vascular damage (Hladovec 1974) and complete mechanical constriction (Guarini 1996). In this way we have consistently obtained the formation of a completely occlusive and persistent thrombus. In control rats the blood flow is practically undetectable after about 10 min, and remains so for the whole observation period (2 h). In such an experimental model of occlusive thrombosis of the common carotid artery, the intravenous administration of a 2-kDa heparin fragment (Oligo-H) not only prevents thrombus formation but apparently also produces lysis of the thrombus. This latter effect may be due to the Oligo-H-induced prevention of fibrin accretion; since thrombus growth occurs when fibrin accretion is faster than fibrinolysis, the inhibition of fibrin accretion in the presence of an unaltered fibrinolysis can lead to a thrombus reduction.

The maximum effective dose used in this study (20 mg kg^{-1}) is well below the toxic level (Bergonzini et al 1992) and did not produce bleeding or haematomas.

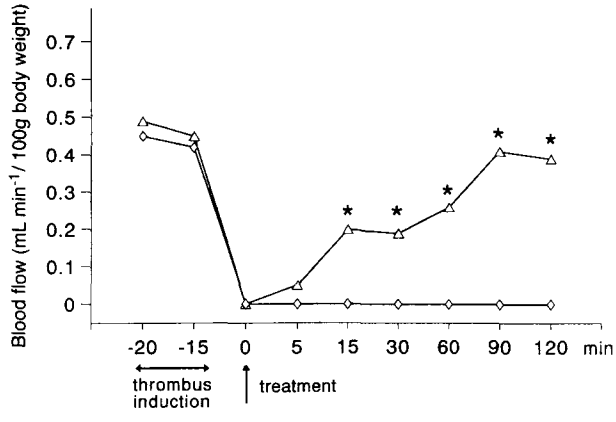

FIG. 3. Time-course of mean blood flow in a common carotid artery after thrombus induction in rats treated intravenously 15 min later with saline 1 mL kg^{-1} (◇) or Oligo-H 20 mg kg^{-1} (△). Mean values for 8–10 rats per group; s.e.m., not represented for sake of clarity, was < 10% of the mean values in all cases. *$P < 0.01$ compared with corresponding value for saline-treated rats (Student's t-test).

Thus, our present data show that the LMWH fragment Oligo-H is highly effective in preventing and curing arterial thrombosis, in the rat.

LMWH are an important, relatively new, class of antithrombotic agents (Weitz 1994; Green et al 1994). They differ from unfractionated heparin in having relatively more anti-Xa activity, a reduced haemorrhagic to antithrombotic ratio, greater bioavailability, longer half-life, and more predictable anticoagulant response.

The incidence of heparin-induced thrombocytopenia also appears to be lower with LMWH than with heparin. So far, available clinical trials (Green et al 1994) indicate that LMWH are drugs of choice for the treatment of venous thromboembolism; they are also safe and effective for the treatment of acute deep-vein thrombosis, and for the prophylaxis of thromboembolism after major orthopaedic surgery of the lower limb as well as in general and pelvic surgery; they seem to be more effective than unfractionated heparin in the prophylaxis of thromboembolism in patients with strokes or spinal cord injury. Another potential indication for LMWH is clotting prevention in cardiopulmonary bypass, haemodialysis, and arterial angioplasty.

Our present animal data, if confirmed, may also suggest a beneficial therapeutic activity of these drugs in arterial thrombosis.

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