Prevention and Therapy of Experimental Venous Thrombosis in Rabbits by Desmin 370

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Summary

Desmin 370 (D370), a low molecular weight dermatan sulfate, has been shown to reduce the size of preformed thrombi in rats, via a mechanism largely independent of its anticoagulant activity. In the present study we investigated the therapeutic efficacy of D370 in rabbits with experimental jugular vein thrombosis. Experiments performed to evaluate the antithrombotic dosages in rabbits indicated that D370 prevented the formation of venous thrombi (Wessler model) in a dose-dependent manner with complete inhibition at 20 mg/kg. When injected to rabbits bearing a 30 min aged thrombus, D370 caused a time- and dose-dependent reduction in thrombus weight. Thrombi harvested 2 h after injection of 50 mg/kg of D370 were 71% smaller than thrombi from saline-treated rabbits and 50% smaller than pretreatment thrombi, suggesting a double effect of the drug: inhibition of thrombus accretion and reduction of the existing thrombus. Interestingly, pretreatment with the fibrinolytic inhibitor EACA (1 g/kg), significantly attenuated the therapeutic efficacy of D370, suggesting a possible involvement of the fibrinolytic system. Heparin (50 and 200 U/kg) was less active as therapeutic agent, the maximal decrease in thrombus weight, as compared to untreated rabbits, amounting to 38%. Heparin, moreover, caused a more pronounced prolongation of APTT than comparable antithrombotic dosages of D370. Our present data extend previous results on the therapeutic efficacy of D370 and underscore its potential as an alternative antithrombotic drug.

Introduction

Dermatan sulfate (DS) is an endogenous glycosaminoglycan which inhibits blood coagulation via the enhancement of thrombin inactivation by heparin cofactor II (1, 2). In experimental models DS prevents thrombus formation and inhibits thrombus growth (3-5). Desmin 370 (D370) is a low molecular weight dermatan sulfate (6) that exerts an antithrombotic activity similar to the parent preparation but has better pharmacokinetic properties (7, 8). We have previously shown that, in rats, D370 markedly reduced the weight of preformed venous thrombi (9, 10), and enhanced the lysis of experimental pulmonary emboli (11). Both effects were largely independent from inhibition of thrombus accretion and appeared to be mediated by a fibrinolysis-dependent mechanism. Since the evidence supporting the therapeutic efficacy of D370 is, so far, limited to the rat, we wished to study the thrombolysis promoting capacity of this low molecular weight DS in another animal species, frequently used to investigate thrombolytic agents. We report here the results obtained in rabbit jugular vein thrombosis model which confirm that D370 not only prevents thrombus formation but also reduces the weight of pre-existing thrombi. These antithrombotic actions are achieved with minor changes of blood clotting, thus lending further support to the concept that this glycosaminoglycan might be an alternative drug for the treatment of thrombosis.

Materials and Methods

Reagents. The preparation of D370 and its physicochemical properties are described elsewhere (6, 9). Heparin (Liquemin) and hirudin were purchased from Roche, Basel and Boehringer Mannheim, Milan, respectively. EACA (e-aminocaproic acid) was from Sigma Chimica, Milan, and Hypnorm from Duphar, Amsterdam. Experimental models. Male New Zealand rabbits (Morini, Bologna) weighing 2-2.5 kg were used. They were anesthetized by intramuscular injection of Hypnorm (0.4 ml/kg). When necessary, additional Hypnorm (0.1-0.2 ml) was given to maintain anesthesia. Two different experimental models were used to study the ability of D370 to prevent and to treat venous thrombosis, respectively. The first one was a slight modification of the jugular vein thrombosis model described by Wessler and coworkers (12). Briefly, the external jugular veins were exposed through a medial incision in the neck. Each vein was cleared and isolated with two loose sutures, placed two cm apart. One min later, 0.8 ml of brain thromboplastin was injected via a cannulated femoral vein. After an additional 30 s, both jugular vein segments were occluded by distal and proximal sutures. The thrombi formed in the occluded segments were harvested after 30 min, rinsed in saline and dried in an oven for 18 h. Thrombus weight was then evaluated using an analytical balance. For each animal, the mean weight of the two thrombi was recorded. In these experiments, the drugs were given i.v. via a femoral vein, 10 min before induction of thrombosis. To evaluate the therapeutic effect of D370, a modification of the thrombosis model described by Collen et al. (13) was used. After anesthesia, an external jugular vein was exposed, as detailed above, over a distance of 4 cm up to the main bifurcation with the facial vein. Small side branches were ligated and the facial vein was cannulated with a Portex “pink gauge” cannula (Portex, Hythe, UK). A woollen thread was inserted in the lumen of the jugular vein, over a distance of 4 cm, using an ordinary needle. The vein was clamped both proximally and distally to isolate a segment of 2 cm in length, which was then emptied of all blood by suction via the catheter. To produce an occlusive thrombus, rabbit blood, freshly collected from a femoral vein, was rapidly mixed with thrombin (2 U/ml) and thromboplastin (0.01 ml per ml of blood), aspirated in a graduated microsyringe and then injected in the jugular vein segment through the facial vein cannula until the vessel was fully distended. Attention was paid to record the volume of injected blood. In all instances the clot formed rapidly and was allowed to age for 30 min before both vessel clamps were removed. Drugs were injected as a bolus, immediately after removing the clamps, via the contralateral marginal ear vein. At a predetermined interval (ranging from 0 to 2 h) the thrombus was surgically removed, rinsed in saline and weighed after drying as detailed above. To reduce the intra-animals variability, the results were normalized to the volume of blood injected to produce the intravenous clot. Therefore, data are expressed as mg/ml of injected blood.
Blood collection and assays. Blood samples (2 ml) were collected via a cannulated femoral vein, anticoagulated with 4% trisodium citrate (9 vol blood + 1 vol citrate) and kept on melting ice until processed. Plasma was prepared by centrifugation (20 min at 1000 × g) and immediately tested or stored at –20 °C for less than two months.

Activated partial thromboplastin time (APTT) was performed on fresh plasma samples by standard laboratory technique using optimized thrombofax (Ortho Diagnostic Systems, Milan) as reagent. Results are expressed as ratio between the APTT after treatment and the APTT before treatment. Plasminogen activator (PA) activity and plasminogen activator inhibitor (PAI) activity in plasma were measured spectrophotometrically using commercially available kits (Menarini Diagnostici, Florence), according to the manufacturer’s instructions. PA activity in the euglobulin fraction, prepared from fresh plasma, was assayed by the fibrin plate method (14) with slight modifications (15). Fibrin autography was carried out as previously reported (16).

Statistical analysis. Results are expressed as mean ± SEM. Difference among groups was evaluated by one way analysis of variance followed by Duncan’s multiple comparison test (Stat 100 software). p < 0.05 was considered as statistically significant.

Results

Prevention of thrombus formation. Bolus injection of D370, given 10 min before the thrombotic challenge, caused a dose-dependent inhibition of thrombus formation (Fig. 1). At the lowest dose tested (2.5 mg/kg) the mean weight of thrombi was reduced by 80% while at 20 mg/kg no visible thrombi were observed. In these experimental conditions a complete inhibition of thrombus formation was also observed with heparin and hirudin at doses of 50 U/kg and 0.4 mg/kg, respectively.

Therapy of venous thrombosis. In these experiments the drugs were injected in animals bearing a 30 min aged thrombus. Figure 2 illustrates the weight of thrombus harvested 2 h after treatment and shows that both D370 and heparin significantly reduced the mean size of preformed thrombi as compared to that in saline-treated rabbits. At doses producing a comparable inhibition of thrombus formation, D370 (20 mg/kg) and heparin (50 U/kg) caused 49% and 38% reduction in weight, respectively. At higher dosages we observed an increase in the therapeutic efficacy of D370, with a 71% reduction in thrombus weight at the dose of 50 mg/kg. In contrast, 200 U/kg heparin produced the same effect as 50 U/kg heparin (Fig. 2). In this model hirudin was poorly effective, an 18% reduction in thrombus weight being observed at a dose about 3 times higher than that producing complete inhibition of thrombus formation.

To determine the possible mechanism of action of D370 we evaluated the changes in weight of preformed thrombi at different intervals after treatment. The mean weight of thrombi harvested immediately after the 30 min aging interval (time zero of Fig. 3) was 51 mg. In saline-treated animals, mean thrombus weight increased markedly over time, reaching 171% of the initial value after 120 min. On the contrary, following D370 injection (50 mg/kg), the mean thrombus weight decreased progressively over time and had reached the low value of 25.6 mg (corresponding to 50% of the initial weight) at the end of the experiment.
To see whether endogenous fibrinolysis contributes to thrombus weight reduction we tested the effect of the fibrinolysis inhibitor EACA. Administration of 1 g/kg of EACA, immediately before treatment, resulted in a significant attenuation of the therapeutic effect of D370 (Fig. 4) but did not influence to a significant extent the thrombus weight of control animals.

Anticoagulant effect. Fig. 5 shows the profiles of APTT changes after injection of two different doses of D370 and heparin, selected on the basis of their antithrombotic activity. At doses totally preventing thrombus formation, D370 (20 mg/kg) and heparin (50 U/kg) induced a peak increase in APTT ratio of 1.6 and 2.3, respectively. The highest dose of D370 (50 mg/kg) prolonged the APTT only by 2.3-fold.

Plasma fibrinolytic activity. Chromogenic assay on plasma samples revealed that neither PA nor PAI activity was affected by the injection of 20 or 50 mg/kg of D370 (not shown). On the contrary, PA activity in the euglobulin fraction of plasma increased rapidly after D370 (20 mg/kg) injection, reaching a peak value of 400% after 10 min, and then declining progressively towards the preinjection value within 60 min (Fig. 6). The time course of PA activity changes in D370-treated rabbits was very similar to that of APTT (Fig. 5), suggesting a possible direct effect of D370 on PA measurements. To test this possibility we added the drug (100 μg/ml) to rabbit plasma before euglobulin preparation or directly to the euglobulin fraction. As shown in Fig. 6, a significant increase in fibrinolytic activity, comparable to that observed in the ex vivo experiments, was observed only if D370 was added to plasma. Fibrin autography of the euglobulin fraction derived from normal rabbit plasma or from plasma supplemented with D370 did not reveal any difference in the pattern and intensity of the lysis bands (not shown).

Discussion

Our data show that D370 dose-dependently inhibited the formation of jugular vein thrombi in rabbits. The antithrombotic dosages were similar to those previously reported in rats (9) and ranged between 2.5 mg/kg and 20 mg/kg, the latter being the dose totally preventing thrombosis. In the same model, also heparin and hirudin prevented thrombus formation completely at the doses of 50 U/kg and 0.4 mg/kg, respectively.

The main goal of our study was to evaluate the ability of D370 to reduce the size of preformed thrombi. In these experiments the drug was administered to rabbits bearing a 30 min aged thrombus. Thrombi harvested 2 h after D370 injection were significantly smaller than control thrombi. The degree of thrombus weight reduction was dose-dependent and ranged from 38% to 71%, with 10 and 50 mg/kg of D370, respectively. Heparin was also effective in reducing the size of preformed thrombi but with a lower efficacy, the maximum decrease in weight amounting to 38%. This effect was obtained with a dose of 50 U/kg and could not be enhanced any further by a 4-fold higher dose. It should be noticed that during the 2 h experimental period there was a spontaneous and significant growth of the jugular thrombi. This implies...
that inhibition of thrombus accretion by the anticoagulants will suffice to produce a pronounced “therapeutic” effect. In heparin-treated rabbits, the fact that the weight of thrombi after treatment was very similar to that recorded at time zero suggests that the main mechanism of action of heparin is inhibition of thrombus growth. This might also explain why the increase in heparin dosage up to 4-fold is not associated with an enhancement of the therapeutic efficacy as complete inhibition of thrombus accretion is already achieved with 50 U/kg. D370 behaved differently in that the reduction in weight after treatment was markedly higher than expected by simple inhibition of thrombus accretion. Indeed, 2 h after the injection of 50 mg/kg of D370, thrombi were smaller than the pretreatment ones by 50 %, thus implying an additional mechanism. This contention is supported by the finding that EACA attenuated the therapeutic effect of D370. It should be noticed that, under these conditions, thrombi collected after D370 treatment had a mean weight comparable to that of pretreatment thrombi, suggesting that inhibition of thrombus growth still occurred in EACA-treated animals. This is in agreement with previous observations showing that the antifibrinolytic agent does not affect the anticoagulant activity of D370 (9). Moreover, the observation that EACA did not enhance thrombus weight in control rabbits makes unlikely a “generalised” detrimental effect of the drug on thrombus accretion and suggests that the endogenous fibrinolytic system does not participate to a significant extent in the turnover of thrombi under normal conditions. It is suggested, therefore, that EACA nullifies the stimulation of the fibrinolytic process induced by D370 injection. This interpretation, however, remains speculative since the mechanism by which D370 promotes fibrinolysis is still unknown. As in previous studies (9, 11), we were unable to show an enhancement of plasma fibrinolytic activity by chromogenic assays. On the other hand, the increase in PA activity in the euglobulin fraction seems to be an in vitro artefact, likely related to the influence of D370 on the precipitate formed after acidification of plasma.

A surprising finding in our study is that hirudin was weakly active in the therapy of venous thrombosis notwithstanding it was shown to be an efficient inhibitor of thrombus growth (9, 17, 18). A possible explanation might be the rather high content of exogenous thromboplastin within the thrombus that makes a direct inhibitor of thrombin, like hirudin, less active than anticoagulants inhibiting the clotting cascade at multiple levels, such as heparin and D370. The latter, indeed, besides its HCII-dependent activity, has been shown to possess anti-factor X activity (19), because of the presence of trace amounts of low molecular weight heparin, and to induce the release of TFPI (20) as well as the release of additional anti-factor X activity (19, 21). It should be concluded that the combination of these different anticoagulants may contribute to enhance the therapeutic efficacy of D370 as suggested by previous studies showing that the coadministration of heparin and dermatan sulfate (devoid of heparin contamination) resulted in a more prolonged antithrombotic effect as compared to the single drugs (22).

In conclusion our data show that D370 is efficacious both in the prevention and therapy of rabbit jugular vein thrombosis. As compared to heparin, D370 was superior in that, at similar antithrombotic dosages, it produced lesser anticoagulation as assessed by APTT prolongation. Moreover, when given to rabbits with preformed thrombi it showed a higher efficacy than heparin, likely because, besides preventing thrombus accretion, it also promoted the reduction of the pre-existing thrombus. Whatever the mechanism of this additional effect, our present data further underscores the potential use of this antithrombotic agent in clinical setting.

References

Received January 27, 1998 Accepted after revision April 17, 1998