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Low molecular weight heparins prevent thrombin-induced thromboembolism in mice despite low antithrombin activity. Evidence that the inhibition of feed-back activation of thrombin generation confers safety advantages over direct thrombin inhibition

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Background and Objectives. Thrombin-induced thromboembolism in mice is a model in which the feed-back clotting activation produced by the injected enzyme greatly contributes to fibrin accumulation in lungs and to mortality. Using this model we have previously shown that activated human protein C (aPC), by interrupting endogenous clotting activation at a high level (factors Va and VIIIa), prevents mortality inducing only a minor hemostatic impairment. With the same model we have now compared the antithrombotic and prohemorrhagic effects of two low molecular weight heparins (LMWHs), reviparin and tinzaparin, which are expected to inhibit preferentially the positive feed-back triggered by thrombin (anti Xa activity), with those of unfractionated heparin (UFH) and PEG-hirudin, which inhibit mainly or exclusively thrombin activity (anti IIa activity).

Design and Methods. Pulmonary thromboembolism was induced in mice by i.v. injection of bovine thrombin (1,000U/kg). Drugs (from 0.12 to 1.2 mg/kg) were given as bolus injection 2 min prior to thrombin challenge and mortality was assessed within 15 min. The bleeding time was assessed by a tail tip transection model. Activated partial thromboplastin time (aPTT), thrombin clotting time (TcT), fibrinogen assay and anti Xa activity determination were performed in citrated plasma from saline- or drug-treated animals.

Results. All drugs protected mice from thrombin-induced mortality in a dose-dependent way. At comparable antithrombotic dosages, the anti IIa activity generated in plasma (assessed by TcT) was highest with UFH, intermediate with tinzaparin and very low with reviparin. Accordingly, the fibrinogen drop, which is caused mainly by the injected thrombin, was prevented by the heparins to an extent that was fairly well related to their anti IIa activity. aPTT and bleeding time, used as measures of hemorrhagic risk, were markedly more prolonged by UFH than by reviparin. Tinzaparin, instead, had an intermediate effect. Interestingly, PEG-hirudin, at equipotent antithrombotic dosages, caused a prolongation of bleeding time comparable to that observed with UFH.

Interpretations and Conclusions. Our data show that, in our model, drugs acting at a high level of the blood clotting cascade, like LMWHs with a high anti Xa/anti IIa ratio, display a better antithrombotic/prohemorrhagic profile than drugs acting prevalently on thrombin.

Key words: heparin, low molecular weight heparin, antithrombotic activity, animal model

Thrombin-induced thromboembolism in mice is a model of acute and massive intravascular fibrin deposition, mainly within the pulmonary arteries, that leads to death of the animals within a few minutes.[1,2] Using this model we have previously shown that thrombin injection causes feed-back blood clotting activation via the contact system and that the additional thrombin generated in this way greatly contributes to fibrin accumulation within lung vessels and to mortality.[3] In line with this finding, administration of activated human protein C (aPC) prevented thrombin induced mortality by inhibiting factor Va and factor VIIIa generated by the feed-back activation of the coagulation cascade induced by injected thrombin.[3] Interestingly, the protection by aPC against mortality was accompanied by an only limited hemorrhagic tendency, as shown by minor activated partial thromboplastin time (aPTT) and bleeding time prolongations. In the same context unfractionated heparin (UFH), which efficiently inhibits thrombin by potentiating the activity of antithrombin III,[3] also prevented mortality but exposed the animals to a higher risk of hemorrhage as indicated by the very marked prolongation of aPTT and of bleeding time. These observations suggest that the pharmacologic inhibition of the feed-back mechanisms leading to the generation of new thrombin may offer safety advantages over drugs acting directly against thrombin, at least in a system in which blood clotting activation takes place mainly in solution and not on a

damaged surface.

Among the currently available anticoagulant drugs, low molecular weight heparins (LMWHs) act at a higher level of the clotting cascade, thus inhibiting thrombin formation more than thrombin activity. These anticoagulants are obtained from UFH by enzymatic or hydrolytic cleavage[4] and have an average molecular weight of around 5,000 Daltons.[5] LMWHs maintain a specific sequence with high affinity for ATIII but, unlike UFH, exert a prevailing inhibitory effect on factor Xa and consequently show an anti Xa/anti IIa ratio higher than that of UFH.[6] This peculiarity has been claimed to confer them an advantage over UFH in terms of lower hemorrhagic risk.[7] It should be noted, however, that the various preparations of LMWHs have markedly different physico-chemical characteristics and such heterogeneity is reflected in different anti Xa/anti IIa ratios and thus, likely, in different antithrombotic/prohemorrhagic patterns.[8] To test the hypothesis that the inhibition of endogenous thrombin generation may be safer than direct inhibiton of thrombin activity in mice we evaluated the antithrombotic and prohemorrhagic effects of two LMWHs, with different anti Xa/anti IIa ratios (reviparin and tinzaparin), and compared them to those of UFH and recombinant hirudin, a direct thrombin inhibitor that inactivates the enzyme by blocking its substrate binding site in a 1:1 stoichiometric complex.

Design and Methods

In vivo thrombosis model in the mouse

Pulmonary thromboembolism in mice was induced by a method described previously.[1,3] Briefly, male CD-1 mice (Charles Rivers, Calco, Como, Italy), weighing 20-25 g, were used. Mice were caged and fed a regular diet for at least one week before use. The drugs to be tested (unfractionated heparin, reviparin, tinzaparin and PEG-hirudin) or solvent were administered intravenously (i.v.) in a fixed volume of 100 µL, 2 min before the thrombotic challenge which was induced by the rapid i.v. injection into a tail vein of bovine thrombin.[1,3] The dose of thrombin used was selected from a concentration/response curve as the minimal dose giving a reproducible over 90% mortality in the control group (data not shown). In each experimental session at least five animals per treatment group were tested; control groups were run at the beginning and at the end of every experimental session. Mice were accustomed to handling by the investigators and the injections were carried out by skilled investigators with minimal disturbance to the animals. The total duration of each experiment was 15 min. The animals which did not die within this time were sacrificed by exposure to ether vapors and were recorded as survivors. No anesthesia was used during the experiment because of the short duration and because anesthesia interferes with thromboembolism in this model.[1] This study was approved by the Committee on Ethics of Animal Experiments of the University of Perugia and by the Italian Ministry of Public Health. The evaluation of the effect of drug treatments on the intravenous challenge with thrombin was carried out as previously described:[1,9] the cumulative end point to be overcome was death of the animal or prolonged paralysis of the hind limbs (for more than 15 min). The data are presented as number of animals dead/number of animals tested or as% of total. Protection against thrombin was expressed as $(1-T_{DRUG}/T_{SAL})x\ 100$, where T_{DRUG} is the mortality rate in treated mice, and T_{SAL} is the mortality rate in controls.

Assays

Blood was collected from ether-anesthetized mice by cardiac puncture and anticoagulated with 4% trisodium citrate (1/10 vol). Anticoagulated blood was immediately centrifuged for 5 min at 12,000 x g in an Eppendorf microfuge and the supernatant platelet-poor plasma (PPP) was separated and transferred onto melting ice until tested (generally within 1 h) or frozen at –80° C. Activated partial thromboplastin time (aPTT) and thrombin clotting time (TcT) were measured by standard assays, in an automatic coagulometer (ACL 300R, Instrumentation Laboratory, Milan)[3] using reagents from Instrumentation Laboratory. The TcT was measured using bovine thrombin at a final concentration of 15 U/mL. Plasma fibrinogen was measured by the Clauss method in a Coagulab MJ coagulometer (Ortho Diagnostics, Milan, Italy) using bovine thrombin. Anti Xa activity was measured according to the Teien and Lie method[10] using a chromogenic substrate (S-2222) (Ortho).

Bleeding time

Bleeding time was assessed by a tail transection method, as previously described.[3,11] Briefly, mice pretreated with the tested drugs or their vehicle were positioned in a special immobilization cage that keeps the tail steady and immersed in saline thermostated at 37°C. After 2 min the tip of the tail was transected with a razor blade, at approximately 2 mm from its end. The tail was immediately reimmersed in warm saline and the bleeding time recorded. The end point was the arrest of bleeding lasting for more than 30 s.

Chemicals

Bovine thrombin (T-4265) was purchased from Sigma Chemicals (St. Louis, USA); unfractionated heparin was from Novo Nordisk (Bagsvaerd, Denmark); reviparin (Clivarin, from Schwarz Pharma, Milan, Italy); PEG-hirudin from Knoll AG (Ludwigshafen, Germany) and tinzaparin (Innohep) from Leo Pharmaceutical Products (Copenhagen, Denmark).

Statistics

Data are expressed as mean ± SEM. A Chi-squared test with Bonferroni's correction was used to analyze mortality data and one-way ANOVA followed by Student Newman Keuls' test for multiple comparison was used for all other studies. For all data, a p value < 0.05 was considered as statistically significant.

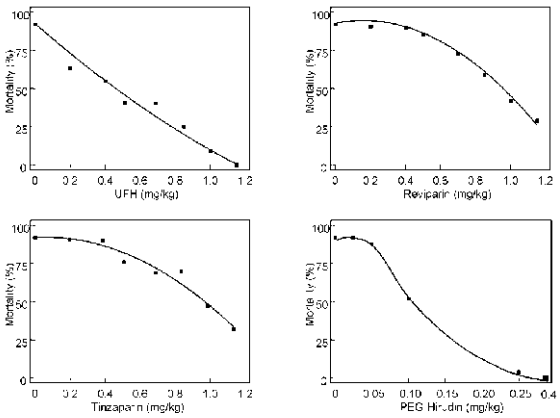

Figure 1. UFH, LMWHs and PEG-hirudin dose-dependently prevent thrombin-induced death in mice. Drugs were given as i.v. bolus 2 min before thrombin injection (1,000 U/kg) and the mortality rate was evaluated as reported in the *Design and Methods* section. ED_{50} were calculated as the dose reducing mortality by half that in controls (0 mg/kg). Ten to 50 animals were tested in each group.

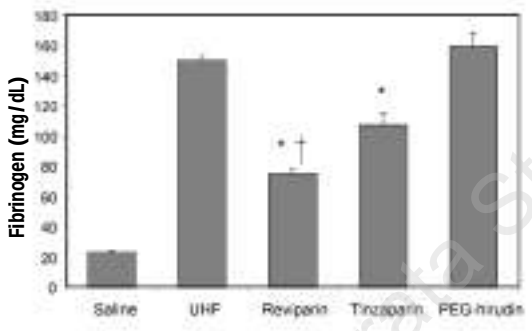

Figure 2. Fibrinogen levels in mice injected with thrombin (1,000 U/kg) after pretreatment with UFH (0.5 mg/kg), reviparin (1 mg/kg) tinzaparin (1 mg/kg), and PEG-hirudin (0.1 mg/kg). Data are the mean ± SEM (six animals for each group). The basal level of fibrinogen in mice was 422±12.5 s (n=6). Results in all groups are significantly different from those in saline-treated animals. *$p < 0.05$ vs UFH and PEG-hirudin; †$p < 0.05$ vs tinzaparin.

Results

In vivo pulmonary thrombembolism in mice

The dose of bovine thrombin used was established, from a dose-response curve (data not shown), at 1,000 U/kg and caused mortality in 92% of mice (296/323). Unfractionated heparin (UFH) (from 0.2 to 1.2 mg/kg) dose-dependently protected mice from death induced by thrombin. The first dose that significantly protected animals was 0.4 mg/kg (p<0.05 vs control), while the dose protecting 50% of animals from death (ED_{50}), was ~0.5 mg/kg (Figure 1).

The two low molecular weight heparins, reviparin and tinzaparin, were both dose-dependently able to prevent the mortality induced by i.v. injection of bovine thrombin although, on a weight basis, less effectively than UFH. The first dose that significantly protected against mortality was 0.7 mg/kg (p<0.05 vs control) while the ED_{50} was ~1 mg/kg for both LMWHs. Under the same conditions, PEG-hirudin also dose-dependently protected the animals from death, although much more effectively on a gravimetric basis, the ED_{50} being ~0.12 mg/kg (Figure 1).

Fibrinogen consumption

Thrombin injection reduced the circulating fibrinogen level by more than 90% (from 422±12.5 to 23.6±0.3). We previously showed that this drop is rapid and largely due to a direct effect of the injected thrombin on fibrinogen.[3] We, therefore, evaluated in this study the capability of heparins and hirudin to prevent fibrinogen consumption as an *in vivo* measure of thrombin inhibition. In these experiments mice were treated with the ED_{50} of each drug and plasma fibrinogen was determined on samples taken 2 min after thrombin challenge. Both heparin (0.5 mg/kg) and hirudin (0.1 mg/kg) effectively attenuated fibrinogen consumption by preserving about 35-40% of the circulating protein (Figure 2). LMWHs, on the contrary, had a clearly weaker effect, residual fibrinogen amounting to 25% and 19% of normal with tinzaparin and reviparin, respectively. The difference in fibrinogen levels between tinzaparin- and reviparin-treated animals was statistically significant (p< 0.05).

Effects on coagulation parameters

The anticoagulant effects of UFH, LMWHs and PEG-hirudin were studied in mice not challenged with thrombin. Clotting assays (aPTT and TcT) as well as assay of anti-Xa activity were carried out on samples collected 2 min after drug injection. UFH, reviparin and tinzaparin all induced a dose-dependent prolongation of aPTT. However, at doses corresponding to ED_{50} against thrombin-induced mortality, UFH (0.5 mg/kg) and tinzaparin (1 mg/kg) prolonged the aPTT by more than 10 fold while reviparin (1 mg/kg) only caused a prolongation of about 4-fold (Table 1). At these dosages, plasma anti-Xa activity was 0.6±0.02 for UFH, 1.07±0.02 for tinzaparin, and 1.05±0.06 for reviparin. Under the same experimental conditions, PEG-hirudin, at a dose protecting 90% of animals from mortality (0.25 mg/kg), prolonged the aPTT to only 52±2 sec (Table 1).

The effect of UFH on thrombin clotting time (TcT) was completely different from that of reviparin. While UFH induced a dose-dependent prolongation of TcT that became unmeasurable at the dose of 1 mg/kg, reviparin, at the tested doses (from 0.25 to 1 mg/kg), had a very limited effect on TcT (Table 2). On the other hand, tinzaparin induced a prolongation of TcT to an extent intermediate between UFH and reviparin. PEG-hirudin prolonged the TcT dose-dependently (Table 2).

Table 1. Effect of UFH, reviparin, tinzaparin and PEG-hirudin on aPTT.

	aPTT ratio (n)			
	Dose mg/kg			
	0.25	*0.5*	*0.7*	*1*
UFH	7.2 ± 0.7 (28)	10.1 ± 0.8 (19)	> 12 (7)	>12 (5)
Reviparin	1.5 ± 0.04 (8)	2.1 ± 0.14 (13)	2.9 ± 0.23 (12)	4.0 ± 0.19 (7)
Tinzaparin	2.4 ± 0.14 (11)	4.5 ± 0.3 (13)	7.2 ± 0.4 (10)	> 12 (13)
PEG-hirudin	2.6 ± 0.10 (5)	3.2 ± 0.26 (4)	5.5±0.6 (3)	11.3±1.2 (3)

Anticoagulant activity of UFH, LMWHs and PEG-hirudin was assessed ex vivo by aPTT prolongation. The test was performed on samples collected 2 min after the iv injection of the different drugs. Control animals had an aPTT of 19.9±1.3 sec (n=48).

Table 2. Effect of UFH, reviparin, tinzaparin and PEG-hirudin on TcT.

	TcT ratio			
	Dose mg/kg			
	0.25	*0.4*	*0.7*	*1*
UFH	3.4±0.8	6.3±2.3	9.8±0.5	>25
Reviparin	1.1±0.04	1.3±0.3	1.7±0.2	1.8±0.3
Tinzaparin	1.3±0.1	1.6±0.1	3.1±0.9	7.1±1.3
PEG-hirudin	4.2±0.3	6.1±0.4	10.2±0.7	16±0.6

The effect of UFH, LMWHs and PEG-hirudin was assessed ex vivo by measuring the TcT. The test was performed on samples collected 2 min after the iv injection of the different drugs. Control animals had a TcT of 6.56±0.24 s. Data are the mean ± SEM of 6 experiments.

Bleeding time

The tail transection bleeding time was measured in animals receiving the ED_{50} of each drug, two minutes after treatment. In line with the aPTT results, the bleeding time was significantly more prolonged in mice treated with UFH and tinzaparin (7.5- and 5.9-fold increase, respectively) than in mice receiving reviparin (3.6-fold increase). PEG-hirudin (0.1 mg/kg), despite the weak effect on aPTT, prolonged the bleeding time by 6.7-fold (Figure 3).

Discussion

Thrombin-induced thromboembolism in mice is a model in which the massive deposition of fibrin and the persistence of fibrin emboli within the pulmonary vasculature do not appear to be the mere consequence of fibrinogen-to-fibrin conversion induced by the injected thrombin but are rather the result of the combined effects of exogenous and endogenous thrombin, the latter generated by the feed-back activation of blood clotting activation, partly via a FXI-mediated mechanism.[3] In the present study we comparatively tested the antithrombotic activity of two LMWHs possessing different anti Xa/anti IIa activity ratios (reviparin=5.3; tinzaparin=2),[12-15] with that of UFH, which has an anti Xa/anti IIa activity ratio of 1, and with that of hirudin,[16] a direct thrombin inhibitor. These experiments had two aims: a) to provide additional evidence on the importance of the feed-back clotting activation induced by thrombin *in vivo*, and b) to see whether inhibition of the clotting cascade at a higher level confers safety advantages over drugs acting preferentially (UFH) or exclusively (PEG-hirudin) against thrombin.

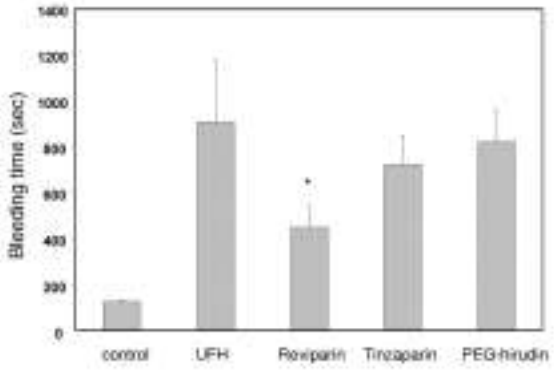

Figure 3. Tail transection bleeding time of animals pretreated with UFH (0.5 mg/kg), reviparin (1 mg/kg), tinzaparin (1 mg/kg), and PEG-hirudin (0.1 mg/kg). Data are the mean ± SEM (6 animals per group). Results in all groups are significantly different from those in saline-treated animals. *$p < 0.05$ vs UFH, tinzaparin and PEG-hirudin.

All drugs tested inhibited thrombin-induced mortality in a dose-dependent way. The two LMWHs showed a fairly comparable dose-response profile with an ED_{50} of ~ 1 mg/kg. On a gravimetric basis UFH was more effective than LMWHs, the ED_{50} being ~0.5 mg/kg. Determination of anti Xa and anti IIa activity in plasma of mice receiving an ED_{50} of each drug indicated that UFH preferentially inhibited the injected thrombin (high anti IIa activity) whereas reviparin acted mainly at the level of factor Xa. Tinzaparin behaved in an intermediate way: indeed at comparable anti Xa activity, the TcT was prolonged by tinzaparin but not by reviparin.

The different mechanisms of clotting inhibition by the various heparins is also inferred from their ability to prevent fibrinogen consumption upon thrombin challenge. In our model most of the circulating fibrinogen is converted to fibrin by the injected thrombin[3] and the observation that UFH is very efficient in preventing fibrinogen drop indicates that it inhibits exogenous thrombin effectively. Similarly, hirudin, a direct thrombin inhibitor, strongly prevented fibrinogen drop. In contrast, reviparin only moderately prevented fibrinogen drop and tinzaparin again behaved in an intermediate way, indicating

that also *in vivo* the latter exerts a more pronounced anti IIa activity than reviparin.

Taken together these results support the hypothesis that the thrombin-dependent feed-back mechanism leading to the generation of additional thrombin plays an important role in the pathogenesis of pulmonary embolism in mice in our model. Most likely, the newly formed thrombin serves as a fibrin stabilizer via the enhancement of TAFI (thrombin actifiable fibrinolysis inhibitor) and factor XIII activation.[3] Accordingly, drugs with a high antiXa/anti IIa ratio, similarly to activated PC,[3] will prevent the formation of lysis-resistant fibrin by inhibiting the positive feed-back activation of blood clotting, thereby allowing the endogenous fibrinolytic system to work more efficiently.

Another relevant finding of our work is that, in our model in which blood clotting activation takes place mainly in solution and not on a damaged surface, the inhibition of the clotting cascade at a higher level is safer than inhibition of thrombin activity. At comparable antithrombotic dosages (ED_{50}) the prolongation of aPTT and, more importantly, the prolongation of the tail transection bleeding time were fairly well related to the anti IIa activity of the drugs tested: they were, indeed, highest with UFH and progressively less marked with tinzaparin and reviparin. Moreover, the observation that a specific thrombin inhibitor like hirudin also caused a more pronounced prolongation of bleeding than reviparin, further supports the hypothesis that inhibition of thrombin is by itself a condition associated with a higher risk of hemorrhage.

In conclusion, our data suggest that a pharmacologic antithrombotic approach that works at a high level of the blood clotting cascade, on the positive feed-back mechanisms involved in the propagation of thrombin generation, may display a better efficacy/safety profile than drugs acting at the level of thrombin. Although extrapolating results obtained in the present model to clinical use is too far-reaching, clinical trials employing low molecular weight heparins for the treatment of acute venous thromboembolism suggest a trend towards them being safer than UFH.[17,18]

Contributions and Acknowledgments

PG designed the study. SM and MN carried out all the experiments. SM, PG and MC prepared the manuscript. PG, MC and GGN were involved in critically revising the content of the manuscript and gave the final approval for its submission. The order in which the Authors appear depends on the importance of their contributions. PG was the most important contributor to the study design and critical revision and final approval of the manuscript: his name, therefore, appears last.

Disclosures

Conflict of interest: none.

Redundant publications: no substantial overlapping with previous papers.

Manuscript processing

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Potential implications for clinical practice

A pharmacologic antithrombotic approach that works at a high level of the blood clotting cascade, on the positive feed-back mechanisms involved in the propagation of thrombin generation, may have a better efficacy/safety profile than drugs acting at the level of thrombin.

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