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CHAPTER 6

LMW HEPARIN-COATED ERYTHROCYTES ATTENUATE THROMBUS GROWTH AND THROMBIN GENERATION IN A RABBIT JUGULAR VEIN THROMBOSIS MODEL

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Summary

Low molecular heparins (LWM heparin) are known as potent anticoagulants used in the treatment of thrombotic disease. However, their short half-life requires daily injections. Hence, prolonging the half-life of LMW heparin might be of benefit for the treatment of thrombotic disease. Therefore, LMW heparin was linked to rabbit erythrocytes ('heparinocytes') according to a recent report, and their maintenance of \textit{ex vivo} and \textit{in vivo} antithrombotic properties in rabbits was demonstrated. Methods: New Zealand White rabbits received either autologous heparinocytes, LMW heparin, or 2 ml saline. Whole blood thrombin generation was assessed at different timepoints after heparinocyte administration and thrombus growth two hours after heparinocyte administration in a jugular vein thrombosis model. Results: Reduction of thrombus growth after administration of heparinocytes or LMW heparin, respectively, was significantly effective with 36\% (high dose heparinocytes), 22\% (low dose), and 22\% (LMW heparin) compared to saline. Thrombin generation was significantly lowered 2, 4 and 6 hours following heparinocyte administration, anti-Xa-activity levels were 0.5 and 0.3 IU $\alpha$Xa after 2 and 4 hours, with still lowered levels of thrombin generation after 24 hours and 0.1–0.2 IU $\alpha$Xa. Radioactive labeling of HC and nuclear scanning showed presence of HC in the circulation for at least 72 hours with decreasing concentrations and increasing retention of HC in the liver and spleen 2 and 6 hours after administration. Conclusion: Autologous HC maintain their antithrombotic properties in a rabbit in vivo model for at least 6 hours following administration, but are retained by the endoreticular system of the liver and spleen.
Introduction

Heparin or its derivative LMW heparin is a potent anticoagulant frequently used in the prevention and treatment of venous and arterial thrombotic disease. Treatment with heparin includes daily one or two subcutaneous injections of LMW heparin or continuous intravenous infusion of unfractionated heparin (UH) because of its short half-life. Patients, however, need heparin usually for prolonged periods of time. Hence, prolonging the half life of this agent might be of benefit and may facilitate heparin treatment.

In a recent study, LMW heparin was successfully bound to human homologous erythrocytes, without losing its antithrombotic properties in vitro for several days. The LMW heparin-coated erythrocytes, so-called ‘heparinocytes’, were prepared firstly by modification of LMWH through the introduction of a sulphydryl group (LMWH-SH), which then covalently binds to the surface exposed amino groups of the erythrocyte membrane by means of a crosslinker.

It was the aim of the present study to evaluate, whether 1) heparinocytes preserve their antithrombotic effect ex vivo and in vivo and maintain a prolonged half-life in vivo, compared to conventional LMW heparin. We demonstrate that LMW heparin-coated erythrocytes display a strong and sustained antithrombotic effect after autologous administration in a well standardized in vivo thrombosis model.

Methods

Preparation of rabbit heparinocytes

LMW heparin-coated erythrocytes (heparinocytes) were prepared according to the protocol of Müller et al from New Zealand White rabbit erythrocytes. Thereby, 25 ml of whole blood were drawn from an ear artery into a citrated tube (3.2% Tris-Na-dihydrate citrate, 1:10) and centrifuged with 800 g for 10 minutes at 4°C. Plasma was separated and erythrocytes were resuspended in a wash buffer (WB) consisting of 140 mM NaCl, 5 mM KCl, 10 mM Glucose, 10 mM NaPi, pH 7.4, and centrifuged for 5 minutes. Supernatant was then removed and erythrocytes resuspended in WB. LMW heparin was modified by the addition of a thiol group (LMW heparin-SH), thus allowing chemical binding to the amino-group of the erythrocyte surface, and was then stored as a dried-frozen powder. Coupling of the erythrocyte to LMW heparin-SH was achieved by dissolution of dried-frozen LMW heparin-SH in WB in a 10% concentration, resulting in a 500-fold excess of the number of maximally possible binding NH₂ groups on the erythrocyte membrane (see below). EMCS, a heterobifunctional crosslinker between LMW heparin-SH and the amino-groups of the erythrocyte surface, was dissolved in a 10% solution with DMSO and added to the LMW heparin-SH solution. The washed
erythrocytes were then incubated with the LMW heparin-SH/EMCS-solution during 60 minutes at room temperature for the linking process. This was followed by 4 to 5 times washing with WB, and assaying supernatants each time for thrombin clotting time (TCT). Washing was stopped, when TCT was within the normal range (18-20 sec; 6 IU thrombin, 60" incubation time). Heparinocytes were then resuspended to a total volume of 15 ml with saline and administered autologously to the rabbits through an ear vein. Blood samples were collected before and after administration of HC at different timepoints, depending on the parameters that were tested.

Study design and dose administration
For the assessment of bilateral jugular vein thrombus growth 22 New Zealand white rabbits were randomized to either receive heparinocytes (56.5 nmol LMW heparin-SH), LMW heparin (Nadroparin calcium 1500 IU αXa and 500 IU αXa), or saline. The maximal amount of amino binding sites accessible for succinimidyl on the human erythrocyte membrane is approximately 10 nmol/ml erythrocytes. For an estimation of the amount of amino binding sites in the rabbit erythrocyte, some morphological interspecies differences have to be taken into account. Rabbit erythrocytes are smaller than human red blood cells, expressed as the mean corpuscle volume (MCV), which was 59.6 (57.5-61.5) fl/EC in our rabbits compared to 88 (80-96.1) fl/erythrocyte in humans, while red blood cell counts are equally high with $5.5 \times 10^{12}/l$ versus $5.2 \times 10^{12}/l$, respectively. Due to 32.2% lower MCV-values in rabbits, we can claim a proportionally smaller total erythrocyte surface and number of amino binding sites, which is approximately 7 nmol/ml erythrocytes. The rabbits treated in vivo for the evaluation of thrombus growth inhibition received a total of either 8.3 ml of heparinocytes, which means a maximally possible amount of 56.5 nmol LMW heparin-SH molecules bound to the erythrocyte membranes or of 5.5 ml, i.e. 37.3 nmol/l, respectively.

Assessment of thrombus growth; jugular vein thrombosis model
Animal experiments were approved by the Institution Review Board for Animal Experiments of the Academic Medical Center and performed according to the guidelines of the American Physiological Society and the Dutch Law for Animal Experiments. New Zealand white rabbits (± 2.5 kg) were anesthetized with 0.5 ml xylazine 2% (Rompun, Bayer AG, Leverkusen, Germany) and 9 mg ketamine/atropin (Aescoket plus, Boxtel, The Netherlands) intramuscularly. A repeat injection of xylazine was administered after 30 minutes, followed by repetitive intravenous injections of thiopentalsodium (Nesdonal) to maintain anesthesia. After a median ventral neck incision the left carotid artery and both jugular veins were bluntly isolated. A catheter (Baby Feeding Tube, 1.6 mm Ø) was inserted into the carotid artery and kept open with saline as an intravascular access line. A second infusion line was installed at one of the ear
marginal veins. Both jugular veins were cleared along a distance of about 2 cm, including the ligation of all side branches. The veins were clamped both proximally and distally, and the isolated vein segment was injected with nonradiolabeled thrombi formed by 150 µl of autologous citrated blood, aspirated into a 1-mL syringe containing 25 µl of human thrombin (Human Thrombin T7009, Sigma Chemical Company, St. Louis, USA, 150 U/mL) and 45 µl CaCl₂ (0.25 M), injected into the isolated venous segments. After 30 minutes of aging, the vessel clamps were removed, blood flow restored and 100 µl $^{125}$I -radiolabeled human fibrinogen (Amersham, 2 µCi/L) was injected systemically, followed immediately by the administration of heparinocytes or other study compounds, respectively. At timepoint 2 hours following drug intervention, the jugular veins were ligated proximally and distally to the clot and the vessel segment containing the whole thrombus was removed. Accretion of $^{125}$I -radiolabeled human fibrinogen onto the preformed non-radioactive thrombi was measured by a γ-counter and thrombus growth was expressed as a percentage of the initial thrombus volume.

*Whole blood thrombin generation time*

For the assessment of whole blood thrombin generation time (TGT) 1.5 mL of citrated whole blood was recalcified with 20 µl of CaCl₂ (1 M) while shortly vortexing the sample. The blood was aliquoted into 200 µl portions in 500 µl plastic tubes, which were then placed in a shaker at 37 °C. At timepoints before, 15, 30, 45, 60, 75, and 90 minutes after recalcification samples were spun for 2 minutes at 9000 rpm, and 20 µl of plasma were added to 180 µl of EDTA-buffer and placed on ice to stop coagulation. 150 µl of this solution containing the plasma were filled into microtiter plates and thrombin generation was assayed by the use of the chromogenic substrate S-2238 (Chromogenix, Instrumentation Laboratory Nodia BV, Amsterdam, the Netherlands) in a kinetic measurement. Thrombin generation was expressed in activity mOD and standardized by a dose response curve of a known thrombin concentration.

*Thrombin clotting time and Factor anti-Xa activity*

Thrombin clotting time (TCT) was assayed in plasma, washed HC, and erythrocytes performed on a Coagulometer KC-10 (Amelung, Lehbrincksweg, Germany). For merely plasma TCT measurements 100 µl plasma and 100 µl wash buffer were incubated for 60 seconds at 37°C. Coagulation was started by the addition of 100 µl of a bovine thrombin solution (6 IU/mL, Behring, AG, Marburg, Germany). To assess TCT of heparinocytes, the test was modified by using 100 µl of plasma and various amounts of washed heparinocytes, e.g. 5 µl, 10 µl, 50 µl, and 95 µl, 90 µl, 50 µl, respectively, resuspended in wash buffer. Coagulation was started as described for the evaluation of plasma TCT. Blood samples collected after heparinocyte injection at different timepoints were separated from plasma by centrifugation, and the erythrocyte portion, containing heparinocytes, was washed as described above; 100 µl of these cells were
added to 100 μl of plasma for the test. Anti-Xa-activity (αXa) levels were measured amidolytically according to previously described method (12). Thereby, 100 μl of heparinocytes were first incubated for 60 seconds with 100 μl of plasma and 100 μl of FXa-solution followed by the chromogenic substrate. The measurements were obtained from the supernatant after centrifugation.

**Red blood cell labeling with 111Indium-Oxinate**

Freshly prepared heparinocytes were washed as described and labeled with 111Indium (111In) before administration. Thereby, 1 mL of washed heparinocytes were centrifuged with 200 x g for 5 minutes and the supernatant was discarded. 37MBq 111Indium Oxinate was added to the sample and incubated at room temperature for 15 minutes. Centrifugation was repeated and supernatant discarded. The labeled heparinocytes or erythrocytes samples were then resuspended in 1 ml physiological saline and ready for injection. At timepoints 5, 30, 60, 120, 240, 360 minutes and 24, 48 and 72 hours after intravenous autologous injection of the labeled heparinocytes to the animals, citrated blood samples were collected and radioactivity measured on a gamma counter expressing radioactivity in cpm. Distribution measurement of labeled heparinocytes or erythrocytes (control) was performed in a whole body scan using an Arc camera (ADAC) with a low energy high resolution (LEHR) collimator. Scantime was 5 minutes for each anterior and posterior section at matrix 128 x 128.

**Methodology and statistical analysis**

An investigator who was not aware of the treatment assignment performed counting of the thrombi radioactivity. Statistical analysis was performed by the student's t-test. A p-value of 0.05 was considered statistically significant. All values are presented as mean ± SD.

**Results**

**Thrombus growth**

Thrombus growth *in vivo* was evaluated in 20 heparinocyte- (10 high, 10 low dose), 12 LMW heparin- (6 high dose, 6 low dose), and 12 control jugular vein thrombi. The reduction of thrombus growth in the heparinocyte group was 36 % for the high dose, 22 % for the low-dose, and 22 % for the LMW heparin group as compared with the control group (figure 1). Two hours following drug administration mean thrombus growth in the high-dose heparinocyte group was 63.7 % ± 7.7, in the lower-dose heparinocyte group 77.8 % ± 4.1, in the 1500 IU and 500 IU anti-Xa-activity of the LMW heparin group 78.0 % ± 3.6 and 78.0 % ± 0.3, respectively, and in the control group 99.94 ± 7.0 (Fig. 1). All intervention groups presented significantly different
results compared to the control group, and the high-dose heparinocyte group was significantly more efficient than both the LMW heparin group and the lower dose HC group. In this model, heparinocytes at a dose of 56.5 nmol/l LMW heparin-SH was even more effective than high doses of intravenous LMW heparin.

Figure 1. The antithrombotic effect of heparinocytes (HC) on thrombus growth in the jugular vein of New Zealand White rabbits was higher than that of free low molecular weight heparin (Fraxiparine). Two hours following thrombus induction and drug administration HC attenuate thrombus growth with a 36.3% effect at higher dose and 22.1% at the lower dose, whereas LMWH (Fraxiparine) has a comparable effect of 22% at doses of 1500 or 500 IU anti-Xa-activity.

Whole Blood Thrombin Generation
For kinetic evaluations of whole blood thrombin generation, blood samples were collected before, 5, 30, and 60 minutes, 2, 4, 6, 24, 48, 72, 96, 120, and 168 hours following drug administration. Figure 2 shows the mean values and standard errors of thrombin generation in mOD activity, 60 minutes after recalcification, as measured for the different follow-up timepoints. Results demonstrate, that thrombin generation after high dose heparinocyte injection is reduced by 80% during the first 1 to 6 hours, with still a 40% reduction effect at later timepoints up to six days, while the effect of LMW heparin has mostly diminished by 6 hours.
**Figure 2** Whole blood thrombin generation was strongly attenuated after administration of HC with a lasting effect of several days.

*Thrombin Clotting Time and Anti-Xa-Activity*

Rabbit heparinocytes reached unclottable TCT in vitro with as little as 5 µl added to 95 µl of plasma, and a dose-dependent response was observed, as shown in figure 3. TCT in plasma after heparinocyte injection was not prolonged. In vivo, 5 minutes after heparinocyte administration, TCT was prolonged for more than 500 seconds, whereas at 30 minutes the effect had decreased to 61.2 seconds (100% longer than controls) returning to normal values within one to two hours. However, no clear dose dependency could be recognized in the in vivo measurements.

Means of anti-Xa-activity were between 0.3 and 0.5 IU five to 60 minutes following heparinocyte injection, decreasing to 0.1 – 0.2 after 4 and 24 hours (figure 4). These results show the presence of anticoagulant activity of heparinocytes in the circulation system for at least 24 hours, and are in consistency with the results of the thrombin generation test.
Figure 3  Heparinocytes prolong in vitro thrombin clotting time more than tenfold at low doses

![Graph showing the prolongation of in vitro thrombin clotting time by heparinocytes.](image)

Figure 4  Anti-Factor Xa-activity following HC-administration was still measurable at 24 hours

![Graph showing anti-Factor Xa-activity over time after HC-administration.](image)
Red blood cell labeling with $^{111}$Indium-Oxinate

Two rabbits were injected with $^{111}$Indium-Oxinate labeled heparinocytes and one rabbit with labeled erythrocytes. Blood samples collected at 5, 30, 60, 120, 240, 360 minutes, and 24, 48, and 72 hours showed presence of radioactivity in blood cells up to 72 hours. Nuclear scanning revealed radioactive label to be present in the heart, liver and spleen 2 hours and 6 hours after heparinocyte injection, whereas the control rabbit had only a labeled blood pool in the heart and almost no liver/spleen staining (Figure 5).

**Figure 5** Two hours after intravenous administration of radiolabelled heparinocytes the liver and spleen are clearly stained (A), while no radiolabelled staining of the liver and spleen can be detected in the control rabbit (B).
Discussion

Low molecular weight heparins have a relatively short half-life, requiring daily injections for the treatment of venous or arterial thromboembolic disease. The aim of this study was to achieve a prolongation of LMW heparin half-life by coating autologous erythrocytes with LMW heparin. In vitro studies of human heparinocytes have shown maintenance of the antithrombotic activity of LMW heparin conjugated to erythrocytes, in terms of a dose-dependent prolongation of clotting time and whole blood thrombin generation.

In the present study, heparinocytes were prepared from rabbit erythrocytes and first tested in vitro, followed by intravenous administration for kinetic and antithrombotic evaluations, and for the assessment of their thrombus inhibiting efficacy in a model of jugular vein thrombosis. The in vitro studies showed that rabbit heparinocytes display a strong dose-dependent antithrombotic effect in TCT, that is comparable to the effect seen with human heparinocytes. After venous administration to the rabbits, the prolongation of TCT persisted for at least 24 hours. Similarly, whole blood thrombin generation was reduced to 20 to 40%. Later on, the antithrombotic effect is diminishing with still measurable values for up to 7 days. Thrombus growth was significantly inhibited two hours after heparinocyte administration with an even stronger effect than the one achieved by LMW heparin. It is of particular interest that the same amount of immobilized heparin on red cells appeared more effective than heparin in solution. One possible interpretation is, that soluble heparin binds more rapidly to cells and molecules of the vessel wall.

However, the presented data show, that the strongest antithrombotic effect, as expressed in whole blood thrombin generation, was observed within two and six hours following intravenous administration and decreased thereafter to a much more attenuated effect. This observation was additionally supported by the anti-Xa-activity measurements displaying only low levels after 24 hours and non-measurable levels lateron. The main reason for this relatively fast attenuation of the antithrombotic effect is explained by the observation obtained in the nuclear scan of radioactively labelled heparinocytes. Thereby, it seems that most of the heparinocytes are retained in the liver and spleen, probably due to the binding of LMW heparin to specific receptors of the hepatic endothelial cells. However, despite the retention of heparinocytes in the liver and spleen, radioactivity measurements of indium-labeled heparinocytes gave evidence for the presence of circulating heparinocytes for at least three to five days. Fractionated heparin is taken up by subpopulations of liver cells. It is therefore possible, that heparinocytes, once bound to the hepatic endothelial cells, are fragmented and endocytosed by the local macrophage system and that parts of the heparinocyte membrane containing the LMWH-SH portion are being taken up by the parenchymal and non-parenchymal hepatic cells.

We did not try to attenuate heparinocyte binding to endothelial liver cells by first injecting a bolus dose of free LMW heparin to saturate the receptors before administration of heparinocytes, because changes in TCT, thrombin generation and thrombus growth would have
been difficult to interpret. But this step might lead to half-life prolongation and could be evaluated in a quantitative radioactive assessment of heparinocyte half-life. It was, however, not the aim of this study, to evaluate the precise half-life of heparinocytes, but to show qualitatively, whether heparinocytes can survive in a mammalian circulation system and maintain their antithrombotic properties. Based on our data, this question can be confirmed. The described method could be a promising new strategy, especially in the prophylactic treatment of thrombosis. Orthopedic patients undergoing elective hip surgery might be an ideal target group, who would receive autologous heparinocytes perioperatively, replacing repetitive administrations of LMW heparin. Heparinocytes could easily be compared to other new LMW heparin derivatives like PEG-heparin that were designed with the intention to achieve prolonged half-lives. Although, the method at its present state has its disadvantages and affords expertise know-how, the preparation of heparinocytes could be performed in larger amounts and standardized for clinical use. Finally, the principle of binding a drug to autologous erythrocytes using the described method by Müller et al could be an interesting approach for specific organ cell targeting, as for example in oncological chemotherapy.

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