Preparation and characterization of 'heparinocytes': erythrocytes with covalently bound low molecular weight heparin

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Abstract In an attempt to create the possibility of stable, long acting, intravascular anticoagulation, low molecular weight heparin was modified by introducing a sulfhydryl group into the molecule (LMWH-SH). Human erythrocytes were covalently grafted with LMWH-SH by the use of a heterobifunctional coupling reagent which reacts with the SH group of LMWH-SH and surface exposed amino groups of erythrocytes now called 'heparinocytes' (HC). HC were morphologically indistinguishable from untreated erythrocytes and displayed identical osmotic resistance. The functionality of HC was analyzed by classical coagulation tests in which they dose dependently inhibited clot formation. HC were also functional in recalcified whole blood inhibiting thrombin formation as assessed by the cleavage of the chromogenic substrate S-2238. The system appears applicable as a potential autologous, long-term anticoagulant treatment or prophylaxis.

Key words: Heparin; Erythrocyte; Coagulation; Antithrombin III; Thrombin

1. Introduction

Heparin and its low molecular weight derivatives (LMWH) are widely used to treat and prevent thromboembolic disorders [1]. Despite its generic name, LMWH represents a class of heterogeneous molecules generated by chemical or enzymatic depolymerization of unfractionated heparin with a typical molecular weight distribution, covering the range of approximately 1800–12 000 with an average of 5000 [2]. The molecular weight and the anticoagulatory properties of the various preparations differ conspicuously not only from unfractionated heparin but also between themselves. The advantages of LMWH include a better bioavailability, a longer half-life in plasma, a superior reproducibility of the weight-adjusted anticoagulation response, a more selective way of action and a better side-effect profile [3–6].

It would be useful to have a way to produce anticoagulation with a stable effect over several days without the need for daily injection(s) and with a strictly intravascular mode of action. Eruthrocytes (EC) with an active substance immobilized on their surface might be good candidates for this purpose. EC have been used earlier for such purposes: covalently bound fibrin monomers were used for the detection of fibrinogen–fibrin intermediates [7,8]. More recently, Coller et al. [9] produced so-called thromboerythrocytes by covalently attaching arginine-glycine-aspartic acid (RGD) peptides of different lengths onto the surface of EC, more precisely to the amino groups of glycophorin A. Such thromboerythrocytes were functional in aggregation tests in vitro and in the animal model [10].

The present study describes a new modification of LMWH which displays a thiol group suitable for chemical coupling. This property was applied to covalently bind the modified LMWH onto the surface of EC with the ultimate aim of generating a model for anticoagulation exclusively confined to the circulating blood and with the perspective of a long-term anticoagulant effect.

2. Materials and methods

2.1. Materials

Coagulometer according to Schnitger-Gross was from Amelung, Lemgo, Germany; dialysis membranes, cut-off 10 000–20 000, Servapor, Serva, Heidelberg, Germany; diode array spectrophotometer, Hewlett Packard HP 8452A, Waldbronn, Germany; flow cytometer, EPICS Profile II, Coulter Corp., Hialeah, FL, USA; microtiter plate reader, SLT-Labinstruments EAR 340 AT, Grödig, Austria; thermomixer 5437, centrifuge 5415c, Eppendorf, Hamburg, Germany; APTT reagent, actin FS, Baxter, Miami, FL, USA; cystamine, dithionitrobenzoate, 2-ethoxy-1-ethoxycarbonyl-1,2-dihydroquinoline, LMWH, Fluka, Buchs, Switzerland; fluorescein-conjugated anti-rabbit antibodies, Sigma, St. Louis, MO, USA; rabbit anti-human AT-III antiserum, Behring, Marburg, Germany; S-2238, human fibrinogen, Fragmin, Heparin Coatest, Chromogenix, Mölndal, Sweden; thrombin, Diagnotek AG, Liestal, Switzerland; trinitrobenzoic acid, monothioglycerol, Serva, Heidelberg, Germany. All other reagents were obtained in the highest purity grade available commercially.

2.2. Synthesis of a thiol derivative of LMWH

About 41 μmol of LMWH (calculations made on the basis of an average molecular weight of 5000) were dissolved in 4 ml 20 mM morpholinoethanesulfonic acid (MES), pH 5, and 1.6 ml dimethylsulfoxide (DMSO). Cystamine (245 μmol) was dissolved in 0.4 ml 20 mM MES, pH 5, and added to the LMWH solution. The reaction was started by the addition of 250 μmol EEDQ dissolved in 0.4 ml DMSO under vigorous stirring. The pH was corrected to 5 by the addition of 1 M HCl and the mixture was left at room temperature without stirring overnight. The solution was then dialyzed against 1 l 0.1 M NaP_i, pH 9, for 1 day with changes of buffer after 4 and 10 h. The

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Abbreviations: APTT, activated partial thromboplastin time; AT-III, antithrombin III; DMSO, dimethylsulfoxide; EC, erythrocytes; EMCS, succinimidyl 6-maleimidylhexanoate; FITC, fluorescein isothiocyanate; HC, heparinocytes; MES, morpholinoethanesulfonic acid; TT, thrombin time

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reduction of cystamine was achieved by the addition of 55 mM monothioglycerol (final concentration). To improve reduction, a few grains of sodium dithionite were also added to the solution which was left for 2 h at room temperature. The solution was dialyzed against 1 l 50 mM NaP_i, pH 4.5, for 4 h, 1 l 25 mM NaP_i, pH 4.5, for 4 h, and finally against 1 l of double distilled water overnight. After an additional change of dialysis water for 4 h the solution was lyophilized. The content of substance was measured by weight and by a colorimetric method with Azur A [11]; SH group content was determined by reaction with dithionitrobenzoate [12]. The molar ratio between LMWH and SH groups was 0.9–1.5. The powder was kept under argon at room temperature until use. Under these conditions the substance was stable for over a year.

2.3. Preparation of heparinocytes (HC)

Citrated blood (25 ml, 1:10, 110 mM sodium citrate) was taken from healthy volunteers taking no medication by venipuncture of an antecubital vein in the supine position, in polypropylene syringes (1×5 ml, 2×10 ml). A standardized pressure of 50 mm Hg was installed using a sphygmomanometer for less than 1 min. One 10 ml syringe was kept at 4°C until use for recalcification experiments (see Section 2.7); the second 10 ml syringe was used as a source of plasma (centrifugation: 2000×*g* for 20 min); the 5 ml syringe was used as a source of washed EC. Washed EC were prepared according to Coller et al. [9] and resuspended in 140 mM NaCl, 5 mM KCl, 10 mM glucose, 10 mM sodium phosphate, pH 7.4 (buffer A), to the original whole blood volume. Based on work of Coller et al. [9] we estimated the maximal amount of amino groups accessible to the succinimidyl ester chemistry on the EC surface to be about 10 nmol/ml EC at a hematocrit of 40%. Typically, an excess of 200 times of LMWH-SH on a molar basis over the maximally accessible number of amino group content of EC was chosen, whereas the molar ratio between the crosslinker succinimidyl 6-maleimidylhexanoate (EMCS) and LMWH-SH was 1.

2.4. Osmotic resistance

Control EC and HC were added to decreasing concentrations of NaCl ranging from 8.5 to 0 g/l in double distilled water. After 20 min at room temperature the solutions were gently mixed and centrifuged at 1700×*g* for 10 min. The optical density of the supernatants was read at 540–546 nm to determine the concentration of free hemoglobin. The measurement was repeated after 24 h during which time the cells were kept at 4°C.

2.5. Flow cytometry

HC (10 μl) were diluted 1:10 with buffer A and incubated with 50 μl autologous plasma for 10 min at 37°C. In this step antothrombin III (AT-III) present in the plasma was allowed to bind to the LMWH molecules covalently bound to the EC surface. After centrifugation (8000×*g* for 2 min) the HC pellet was resuspended with 100 μl of buffer A (this step was repeated twice) and incubated with 10 μl of anti-human AT-III rabbit antibodies for 1 h at 37°C. HC were washed as before and incubated with anti-rabbit fluorescein isothiocyanate (FITC)-labeled goat antibodies for 1 h at 37°C. For flow cytometry samples were washed as described before, resuspended to 100 μl and further diluted at 1:100 with buffer A. Control experiments were performed (i) by omitting the primary antibody, (ii) by omitting the secondary antibody, (iii) using a non-specific rabbit IgG as primary antibody, and (iv) using untreated EC instead of HC.

2.6. Coagulometric assays

Thrombin time (TT) and activated partial thromboplastin time (APTT) were measured with a coagulometer according to Schnitger-Gross. For the TT, 100 μl plasma were incubated with 100 μl of buffer B (50 mM Tris–HCl, 100 mM NaCl, pH 7.4 at room temperature) and varying amounts of EC or HC for 3 min at 37°C. Coagulation was started by the addition of 100 μl of bovine thrombin solution (10 U/ml). For the APTT, 50 μl of plasma was incubated with 50 μl APTT reagent, 100 μl of buffer B and varying amounts of EC or HC for 2 min at 37°C. Coagulation was started by the addition of 100 μl 20 mM $CaCl_2$.

2.7. Thrombin generation in whole blood after recalcification

Citrated whole blood (1.5 ml) was recalcified by the addition of 20 μl 1 M $CaCl_2$ (= time zero) under vigorous vortexing for 2–3 s, and distributed as 200 μl aliquots in five Eppendorf tubes. The tubes were incubated at 37°C in an Eppendorf thermomixer under continuous agitation at 600 rpm. At the times indicated, the samples were centrifuged for 2 min at 8000×*g*. 10 μl plasma was added to 275 μl of 100 mM NaCl, 50 mM Tris, pH 8.5, and 15 μl 2 mM chromogenic substrate S-2238 in a microtiter plate. Absorbance was read at 405 nm at 30 s intervals for 3–4 min in a microtiter plate reader. Samples were measured in duplicate and rates were calculated from kinetic data.

3. Results

3.1. LMWH modification

The LMWH (Fluka, art. no. 51550) had an anti-factor Xa activity of 166–210 U/mg as assessed by Chromogenix Coatest spectrophotometric determination ($n = 3$). The measurements

Table 1
Coagulation tests: inhibitory effect of heparinocytes

Test	(s)					
Thrombin time						
Control, no addition	21.4	20.7	18.9	18.6	20.0	15.6
Third wash solution	19.2	19.0	19.1	18.6	19.1	18.6
Control EC, 100 μl	19.0	18.8	17.4	16.6	21.1	20.7
HC 10 μl	196.6	215.0	45.8	71.4	105.4	43.6
HC 20 μl	>1000	>1000	>1000	>1000	>1000	
HC 30 μl	>1000	>1000	>1000	>1000	>1000	
Activated partial thromboplastin time						
Control, no addition	31.0	30.7				
HC 60 μl	301.0	317.1				
HC 80 μl	767	486				
HC 100 μl	>1000	>1000				
Activated clotting time (RACT cuvettes)						
Control, citrated whole blood	135	131	129			
10% HC	151	143	171			
25% HC	182	174	198			
50% HC	212	217				
100% HC	252	252				

Tests were performed as described in Section 2. In order to assess that HC during their preparation were well washed and free of unbound LMWH, the supernatants were routinely tested by thrombin time for its presence. The values obtained from the third supernatant of the washing procedure showed that HC were free of unbound LMWH. Addition of unmodified EC to the test solution did not significantly modify the control times. Incremental additions of HC to the different test solutions showed a prolongation of the clotting times. In the presence of sufficient amounts of HC the test solutions were also unclottable. The results were representative of two to six different preparations of HC.

were calibrated using LMWH Fragmin (Kabi) as standard which has an anti-factor Xa activity of 156 U/mg. The introduction of cystamine molecules in LMWH was catalyzed by the carboxyl group-activating reagent EEDQ (Fig. 1). A molecule of LMWH is composed of four to six pentasaccharide units each of which have two carboxyl groups. The intact presence of these groups in at least one pentasaccharide unit is essential to preserve anti-factor Xa activity. The modification used in this work and the subsequent reduction of the disulfide bridge of cystamine resulted in the loss of $\leq 50\%$ of the original anti-factor Xa activity. The molar ratio between SH groups and LMWH varied from 0.9 to 1.5 ($n = 6$). This indicates that only a part of the essential carboxyl groups were modified and therefore the modified LMWH still possessed valuable activity.

3.2. Clotting tests

Each preparation of HC and the last two wash supernatants were routinely tested for the prolongation of TT as described in Section 2. Control EC (i.e. EC which were incubated and washed as HC without addition of crosslinker but in the presence of LMWH-SH) and HC were resuspended with wash buffer A to their original concentration in whole blood (hematocrit 40%). As shown in Table 1, control EC gave TTs very similar to control times obtained with plasma alone whereas HC showed a dose dependent prolongation of the TT. On average 20–30 μl of HC in a total volume of 400 μl was sufficient to give unclottable TTs. Even smaller amounts of HC also clearly prolonged the APTT and the activated clotting time.

3.3. Thrombin generation in recalcified whole blood

Increasing amounts of HC were added to autologous citrated whole blood and after recalcification, the thrombin

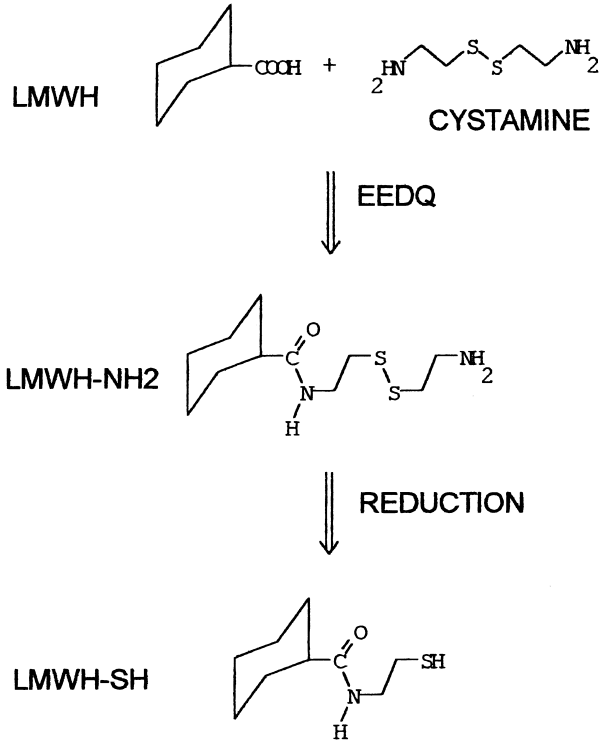

Fig. 1. Scheme of the LMWH modification.

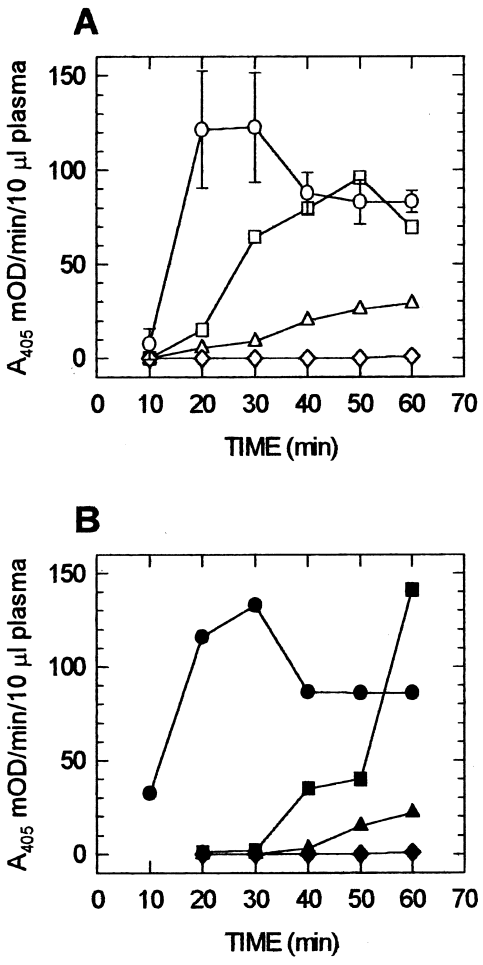

Fig. 2. Kinetics of thrombin generation in recalcified whole blood. A: Open symbols represent the control experiment without any addition (○, $n = 8$), in the presence of free LMWH Fragmin 0.1, 0.3, 0.6 U/ml blood (□, △, ◇, $n = 2$ for all other measurements). B: Closed symbols represent a control experiment performed in the presence of control EC (●), 5, 10, 20% HC (hematocrit 40%) mixed with whole blood before recalcification (■, ▲, ◆).

generation in plasma was measured using the chromogenic substrate S-2238 (Fig. 2). When 20% of the red cells in whole blood were HC, it was sufficient to block thrombin generation completely over 60 min after recalcification.

To further extrapolate the anticoagulatory potency of 20% HC, experiments were performed in the presence of increasing amounts of free LMWH (Fragmin; 156 anti-Xa U/mg) added to the citrated whole blood before recalcification. An equivalent blockade of thrombin generation (as produced by 20% HC) was achieved by 0.6 anti-Xa U/ml whole blood. This means that fully modified blood at a hematocrit of 40% would reach a functional potency as high as 3 anti-Xa U/ml. Prophylactic anticoagulation reaches peak levels of ~ 0.2–0.4 anti Xa U/ml 4–6 h after the injection and may reach up to 0.75 U/ml during therapy. Thus, heparinocytes may achieve the levels required with a reasonable amount of blood modified.

3.4. Binding of AT-III to heparinocytes

The anticoagulatory mechanism of LMWH consists of increasing the inhibitory constant of AT-III for its targets, mainly factor Xa. Therefore HC must bind AT-III in order

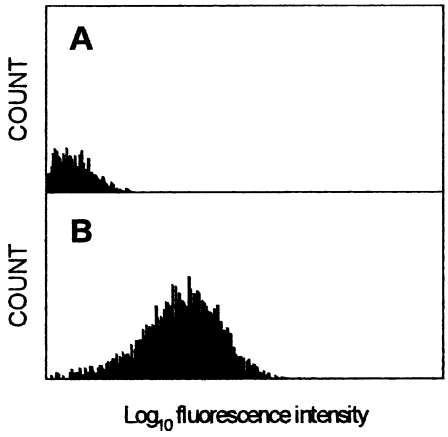

Fig. 3. Flow cytometric analysis of heparinocytes. HC were incubated with plasma (during this step AT-III bound to the LMWH molecules on the surface of HC), rabbit anti-human AT-III antibodies, and FITC-labeled goat anti-rabbit antibodies. A: control EC; B: HC.

to exert their anticoagulatory effect. This step was analyzed by detecting the fluorescence of secondary antibodies directed against anti-AT-III antibodies bound to HC (Fig. 3). Omitting the incubation step with plasma or the anti-AT-III antibodies resulted in negative controls as also seen for control EC. In addition clotting tests (TT) using AT-III-depleted plasma incubated with HC have shown that the coagulation times were equal to the control times (not shown). Therefore we conclude that (1) LMWH was indeed covalently bound to the surface of EC and (2) the immobilized LMWH was still functional; thus, the concept of 'heparinocytes' with the potential of pure intravascular anticoagulation at a stable level of heparin action was realized.

4. Discussion

The modification strategy of LMWH presented in this study differs substantially from the modifications published until now [11,13–16]. Under controlled conditions it was possible to modify carboxyl groups of LMWH leaving the major amount of original anti-factor Xa activity (≥50%). The covalently coupled cystamine (H_2N–CH_2–CH_2–S–S–CH_2–CH_2–NH_2) in a molar ratio of about 1 has the intrinsic advantage of carrying two different chemical groups (–NH_2 and –SH) that are easily coupled to other targets, i.e. in our study to the surface of EC.

We have successfully attached LMWH to red cells by the use of the heterobifunctional cross-linker EMCS. This strategy has been used previously with small peptides by one of us (J.H.B.) in Coller et al. [9], who showed that the main reactive amino groups modified were located on glycophorin A. A similar modification site can therefore be assumed in our experiments. We confirmed that such modified EC were unaffected by the procedure as shown by light microscopy, degree of hemolysis and osmotic resistance, and this is also in agreement with the results of Coller et al. [9]. HC were functionally active in all coagulation tests used. In order to be closer to the in vivo situation and not to exclude potential interactions of red blood cells with other blood cells, we used a method of measuring the thrombin generation in whole blood after recalcification. The comparison of the effect of free LMWH with a known activity allowed us to quantify the anticoagulatory potency of HC although we cannot exclude a different specific activity of the covalently attached LMWH, since the surface of EC displays a large number of carbohydrates. Nevertheless, it was shown that HC exert anticoagulatory properties very similar to free LMWH with the difference that LMWH bound to a 'solid phase' now has the potential half-life of the red cells.

This in vitro model appears attractive because of its *clinical potential*: (a) HC with the potential half-life of red cells would allow a stable, prophylactic heparinization of up to 2–4 months, i.e. during the entire red cell life span; the gradual disappearance seems to be an additional, interesting option. Ideally, a patient could donate 20–30 ml of blood, his red cells would be washed, modified with LMWH-SH and reinfused within a few hours. Such a patient would remain anticoagulated over the period of about 2 months as desired. This new approach could make today's 1–2 injections for anticoagulation unnecessary and could offer stable levels of anticoagulation over longer periods of time. (b) HC will exert their anticoagulatory activity strictly intravascularly and there should be less interaction with endothelial cells with the advantage that the dosage could be calculated more precisely. Ideally, primary hemostasis due to small vascular injuries could proceed unaffected because HC have no immediate access to these sites as easily as free LMWH molecules, particularly in a laminar flow. On the other hand they would inhibit intravascular initiation and progression of clotting. This could imply the potential of anticoagulation without an increased excess bleeding risk.

The issues that we will address in a second step include the survival of HC in vivo (an animal model with 'thromboerythrocytes' showed encouraging results [10]); and just recently, preliminary data of a rabbit jugular model of thrombosis indicated that the heparinocytes appeared to be very effective in vivo as well [17].

A realistic scenario seems to be the following clinical application: a patient comes in for orthopedic surgery and therefore receives a single dose of modified autologous erythrocytes and then remains prophylactically anticoagulated for at least 4 weeks, without the need for daily injection of warfarin treatment. The immunogenicity, the potential proteolytic cleavage of the new ligand in vivo, and, most importantly, the therapeutic effectiveness and its reversal e.g. by protamine will be additional, important questions. Preliminary in vitro data suggest partial reversibility by protamine (data not shown).

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