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Low Molecular Weight Dextran Sulfate: A Strong Candidate Drug to Block IBMIR in Clinical Islet Transplantation

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The instant blood-mediated inflammatory reaction (IBMIR) is triggered in clinical islet transplantation when human pancreatic islets come in contact with blood and may explain the initial tissue loss associated with this procedure. Low molecular weight dextran sulfate (LMW-DS; MM 5000), today available for clinical use, inhibits both complement and coagulation activation. In a tubing loop model, LMW-DS at concentrations ranging from 0.01 to 1 g/L showed a dose-dependent inhibition of IBMIR with an inhibition of coagulation and complement activation and less consumption of platelets and other blood cells. In blood or plasma APTT was demonstrated to be an excellent method for monitoring the LMW-DS concentration both *in vitro* and *in vivo* in a nonhuman primate model. The toxicity was assessed using a glucose challenge test and the pharmacokinetics was tested in the nonhuman primate model. Here, we present a tentative protocol for using LMW-DS in clinical islet transplantation.

Key words: Dextran sulfate, IBMIR

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Introduction

With islet cell transplantation insulin-independence in Type 1 diabetes was recently shown to be possible if more than one donor was used (1). Reports demonstrate that only a small fraction of the transplanted islets successfully engraft, which is confirmed by experimental islet transplantation where 50–70% of the transplanted islets are lost in the immediate post-transplantation period (2,3). We have in a series of reports demonstrated that a thrombotic/inflammatory reaction is elicited when islets come in direct contact with ABO-compatible blood: characterized by a rapid binding, and activation of platelets to the islet surface, infiltration of the islets by leukocytes, particularly granulocytes and activation of the coagulation and complement systems. We call this an instant blood-mediated inflammatory reaction (IBMIR) (4).

The presence of IBMIR was confirmed *in vivo* in an allogeneic pig model of intra-portal islet transplantation (4). The occurrence of IBMIR *in vivo* was further established in patients who underwent clinical islet transplantation with 26 grafts (5). Fifteen to 60 min after the islet infusion, thrombin-antithrombin (TAT) and FVIIa-AT complex levels peaked followed by a rise in D-dimer 24 h after the transplantation in caval vein samples reflecting an ongoing clotting process. Slightly delayed to the increase of TAT the C-peptide started to rise, indicating that the islet cells were damaged. We could also demonstrate that tissue factor (TF) expressed by the islet cells was the trigger of IBMIR, and blockade of TF activity abrogated IBMIR in our experimental models (6). A link between a strong IBMIR i.e. high TAT levels and a poor C-peptide response 1 week after transplantation was established (5). The detrimental effects of IBMIR provide one of explanations for the relatively low success rate of clinical islet transplantation and an explanation for the need of islets from several donors in order to obtain normoglycemia in the transplantation series performed by the Edmonton group and others (1,3,7).

We believe that inhibition of IBMIR would improve the outcome of clinical islet transplantation. Specific inhibition of TF in blood requires the use of recombinant proteins, e.g. FVIIai and TFPI (8), that are not readily available for clinical use. An alternative candidate is low molecular weight dextran sulfate (LMW-DS, MM 5000). The LMW-DS which has previously been shown to be a potent inhibitor of the activation of both the coagulation and the complement systems (9,10) and unlike high molecular weight DS does not activate the fibrinolytic system (11). The substance has also been shown to have direct effects on cell interactions, such as inhibition of E-selectin-mediated adhesion of neutrophils

to endothelial cells (12). We have recently demonstrated the efficacy of LMW-DS in preventing xenogeneic IBMIR *in vitro* and *in vivo* (11). The improved morphological findings of intraportally transplanted islets correlated with a substantial prolongation of the survival of the transplanted adult porcine islets. Apart from IBMIR, LMW-DS also has other positive effects, it potentiates the mitogenic effect of hepatocyte growth factor on hepatocytes that might favor engraftment of the islets (13,14) and protect intra-hepatic islet grafts *in vivo* (15).

In this study we show that LMW-DS inhibits the allogeneic IBMIR *in vitro* and propose an *in vivo* protocol for the administration of LMW-DS in which the concentration of LMW-DS is monitored using APTT.

Materials and Methods

Islet isolation

Pancreases were obtained from cadaver donors after consent was obtained either from the organ donor registry or from relatives. The organs were obtained from two female and three male normoglycemic donors (aged 41–65 years, one with blood group O and four with blood group A). The islets were isolated using a modification of previously described semiautomated digestion-filtration methods (16–18). This isolation procedure using Liberase (Roche Diagnostica, Indianapolis, USA) was followed by further purification on a continuous density gradient in a refrigerated COBE 2991 centrifuge (COBE Blood Component Technology, Lakewood, CO, USA). The islets were maintained in suspension culture at 37°C (5% CO_2) for 1–5 days. The culture medium (CMRL 1066; ICN Biomedicals, Costa Mesa, CA, USA) was changed every other day. The volume and purity of the islets were determined by microscopic sizing on a grid after staining with diphenylthiocarbazone. The purity of the islet preparations ranged from 50% to 90%. Viability was assessed in terms of insulin secretion in response to a glucose challenge in a dynamic perfusion system (in 1.67 mM, 16.7 mM and then 1.67 mM glucose).

Tubing loops as a model

A modification of the model previously described was used (4,19,20). This device consisted of loops made of PVC (diameter, 6.3 mm; length, 380 mm) with an inner surface furnished with immobilized heparin (Corline, Uppsala, Sweden). A rocking device, placed in a 37°C incubator, was used to generate blood flow inside the loops. Six 60-min islet experiments were performed, with islets isolated from five donors.

The LMW-DS (MM 5000, Sigma Chemical CO., St Louis, MO, USA), dissolved in saline, was tested at 0, 0.01, 0.1, and 1 g/L, HMW-DS (Pharmacia, Uppsala, Sweden) and LMW Dextran (Fluka Chemical, Buchs, Switzerland) was only tested at 1 g/L. As control for each experiment one loop containing CMRL-1066 without islets was included. In two experiments one loop with 1 g/L LMW-DS and one with 1 g/L HMW-DS were included without islets. Fresh ABO-compatible human blood from the same donor (7 mL) was added to each loop. The loops were placed on the rocking device for a 5-min pre-incubation with LMW-DS or saline. Thereafter, the loops were opened, and 100 μL of CMRL-1066, with or without 5 μL of islets (~5000 IEQs), was added to the loops and followed by 60-min incubation on the rocking device at 37°C. Blood glucose levels were measured with a glucometer (Glucometer Elite; Bayer Diagnostics, Leverkusen, Germany) before the perfusion. One milliliter samples were taken from each loop after 5, 15, 30 and 60 min and collected tubes containing 6.1 mM K_3-EDTA (final concentration). Samples taken at 0 min were also included, in these samples the blood was immediately transferred to the EDTA-containing tubes and not added to the tubing loops. The blood was used directly for hematologic analysis (platelets, lymphocytes, monocytes and granulocytes) or centrifuged at 4°C at 3290*g* for 15 min. The plasma was collected and stored at –70°C until analyzed for markers of coagulation and complement activation (TAT, complement factor C3a [C3a], and factor XIa-antithrombin complexes [FXIa-AT]) using EIA.

Enzyme immunoassays (EIA)

(A) Thrombin-Antithrombin (TAT)
Plasma levels of TAT were quantified using commercially available EIA kits (TAT, Behringswerke, Marburg, Germany). The values are given as μg/L.

(B) Factor XIa-antithrombin (FXIa-AT)
Complexes between FXIa and AT were measured in plasma according to the method of Sanchez et al. (21). Values are expressed as μmol/L.

(C) C3a
C3a generation was measured in plasma according to the method of Nilsson Ekdahl et al. (22). Values are expressed in ng/mL.

Activated prothrombin time in plasma and whole blood

Fresh human blood was collected in nonheparin coated polypropylene tube containing 10% citrate, using an open system. To obtain platelet-poor plasma blood was centrifuged at room temperature at 4500*g* for 5 min. Activated prothrombin time (APTT) was analyzed in blood or plasma using the Cephotest kit (Nycomed, Stockholm, Sweden) in the presence of clinically used dose of heparin (LEO, 5000 IU/mL, Lowen, Sweden) or 0–0.05 g/L LMW-DS. Whole blood (200 μL) or 100 μL plasma was incubated 5 min with heparin or LMW-DS in 37°C water bath, 100 μL Cephotest was added and after 6 min pre-warmed (37°C) $CaCl_2$ was added to a final concentration of 20 mM. The clotting time of the samples was measured in seconds.

PFA100

Platelet function was assessed using a PFA-100® (Dade Behring, Düdingen, Switzerland). The analyses were carried out according to the manufacturer's instructions on whole blood containing varying concentrations of LMW-DS and heparin.

APTT measurement in vivo study

All procedures using animals were approved by the Swedish council on medical ethics. Six cynomolgus monkeys weighing 2.5–7.0 kg were fasted overnight. Before each experiment, animals were sedated with 6 mg/kg Zoletil® 100 (Virbac S.A., Carros, France) intramuscularly and general anesthesia was maintained with inhalation of 1–3% enflurane. During the experiment, electrocardiogram, blood pressure and pulse were continuously monitored. Intravenous infusion of LMW-DS was performed via an indwelling catheter placed in the jugular vein (n = 3) or via a catheter in the portal vein (n = 3). All blood samples were drawn from a femoral vein catheter and plasma APTT was assessed in the same way as in the *in vitro* study. The first monkey received a bolus dose of LMW-DS (3 mg/kg) i.v. and in the two following experiments, a bolus dose of 4.5 mg/kg LMW-DS was given followed by a continuous i.v. infusion of LMW-DS (0.4–1.1 mg/kg/h). In order to mimic the proposed LMW-DS protocol, the following three animals were given a bolus dose of 1.5 mg/kg + 3 mg/kg given as an infusion over 20 min. This was followed by 0.75–1.5 mg/kg/h up to 120 min. The LMW-DS were given the intra-portal route.

Statistical analysis

Because of individual variation in blood cell counts and plasma parameters, the changes were either calculated as a percentage of the values obtained in the pre-sample (for blood cells) or in the islet loop with no LMW-DS.

All results are expressed as mean ± SEM. Mean values were compared using Friedman ANOVA (Analyse-It, version 1.44, Software Ltd, UK). The significance was determined at $\alpha = 0.05$.

Results

Dose-dependent inhibition of the IBMIR elicited by LMW-DS in the tubing loop model

In tubing loops containing human islets, a 60-min perfusion without LMW-DS resulted in a marked consumption of platelets and granulocytes, combined with a pronounced activation of the coagulation system (Figure 1). In tubing loops without LMW-DS, macroscopic clotting was seen and was accompanied by a significant rise in TAT (Figure 1A), FXIa-AT (not shown) and C3a (Figure 1B) levels. In addition, in the absence of LMW-DS, nearly all the platelets were consumed after 60 min (Figure 1C). Granulocytes were also partially consumed after 60 min, while lymphocytes were essentially unaffected (not shown).

The LMW-DS diminished cell consumption and cascade system activation in a dose-dependent manner (Figure 1). A beneficial effect on most parameters was observed at LMW-DS concentrations as low as 10 mg/L. The cell counts were fully restored at 100 mg/L. The coagulation parameters FXIa-AT and TAT decreased to nearly the same level as the medium control at 100 mg/L LMW-DS. The complement parameter C3a was generated also in the loops without islets (probably due to activated platelets) but was suppressed to almost background levels in the presence of 100 mg/L LMW-DS.

Functional evaluation of islets cultured in LMW-DS

Culturing human islets in LMW-DS at concentrations of 0.5 and 1 g/L for 4 and 16 h produced no toxic effects on islet function reflected as unaffected insulin release in response to a glucose challenge (Figure 2).

APTT and PFA100 assessments in the presence of LMW-DS or heparin in vitro

APTT in human plasma or in whole blood and the PFA100 time in whole blood were assessed after the addition of various concentrations of LMW-DS and heparin. The APTT obtained with heparin at concentrations routinely used in clinical islet transplantation (i.e. $\leq$1 IU/mL) reached an APTT of almost 400 s, which corresponded to a concentration of about 30 mg/L of LMW-DS (Figure 3A). A close relationship between APTT and the concentration of LMW-DS was established. The APTT was almost identical in plasma and whole blood in the presence of LMW-DS (Figure 3B) and heparin (Figure 3C).

The *in vitro* bleeding time assessed by PFA100 was evaluated in blood from five individuals. The *in vitro* bleeding time was normal and unaffected by increasing concentration of LMW-DS (Figure 3B) and heparin (Figure 3C) up to 20 mg/L and 0.5 IU/mL, respectively, except for one

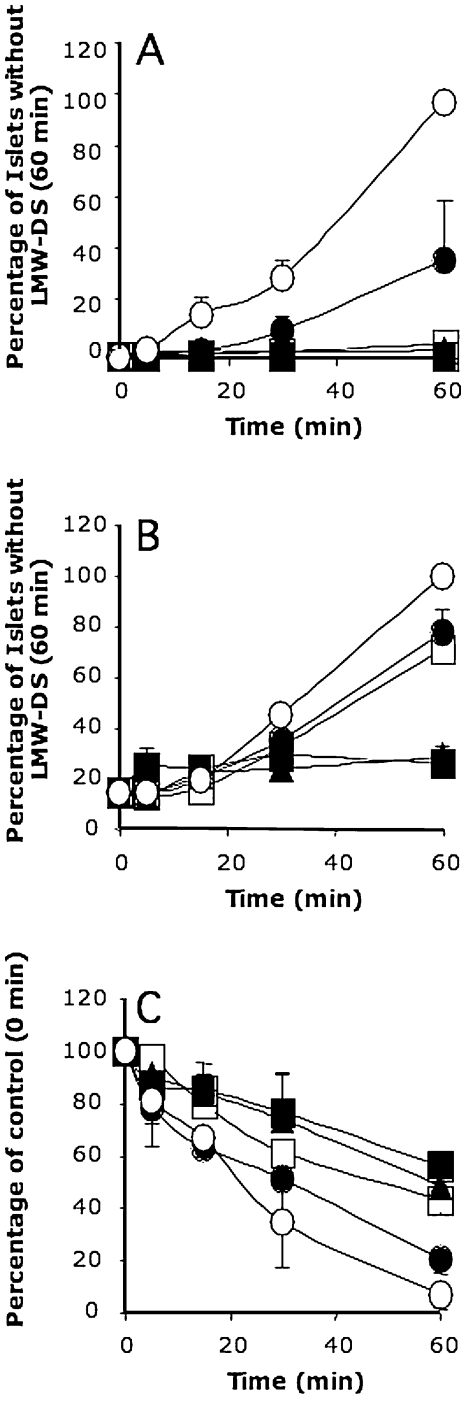

Figure 1: The effect of LMW-DS on IBMIR in the tubing loop assay. Human islets incubated in the tubing loop model with ABO-compatible blood containing LMW-DS at various concentrations: 0 g/L (open circles), 0.01 g/L (closed circles), 0.1 g/L (closed triangles) and 1 g/L (closed squares); control loops without islets and LMW-DS (open squares). Panel A, TAT; and Panel B, C3a; were expressed as percentage of the values for the loops of the positive control containing islets but no LMW-DS at 60 min. Panel C, the platelet count; was expressed as percentage of the pre-sample at 0 min.

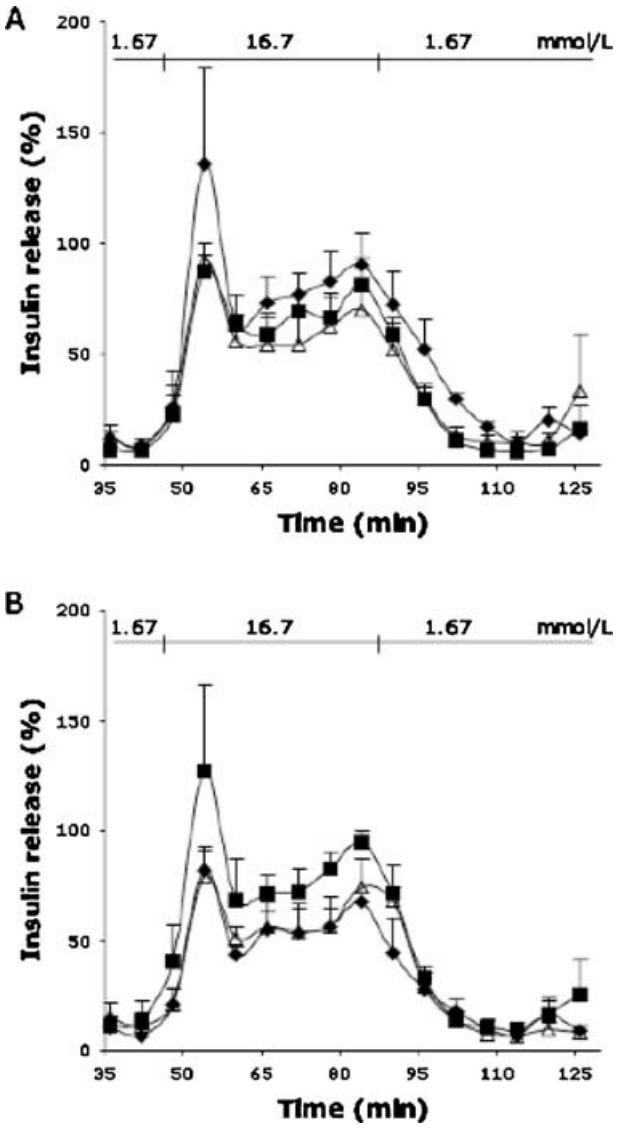

Figure 2: Insulin release to a glucose challenge. Insulin release from islets that have been cultured in the presence of 0 g/L (close diamond), 0.5 g/L (closed square) and 1 g/L (open triangle) of LMW-DS for 4 (Panel A) and 16 h (Panel B). The islets were stimulated with 1.67, 16.7 and 1.67 mmol/L of glucose. The insulin release was expressed as percentage of the peak value of each stimulation (n = 4).

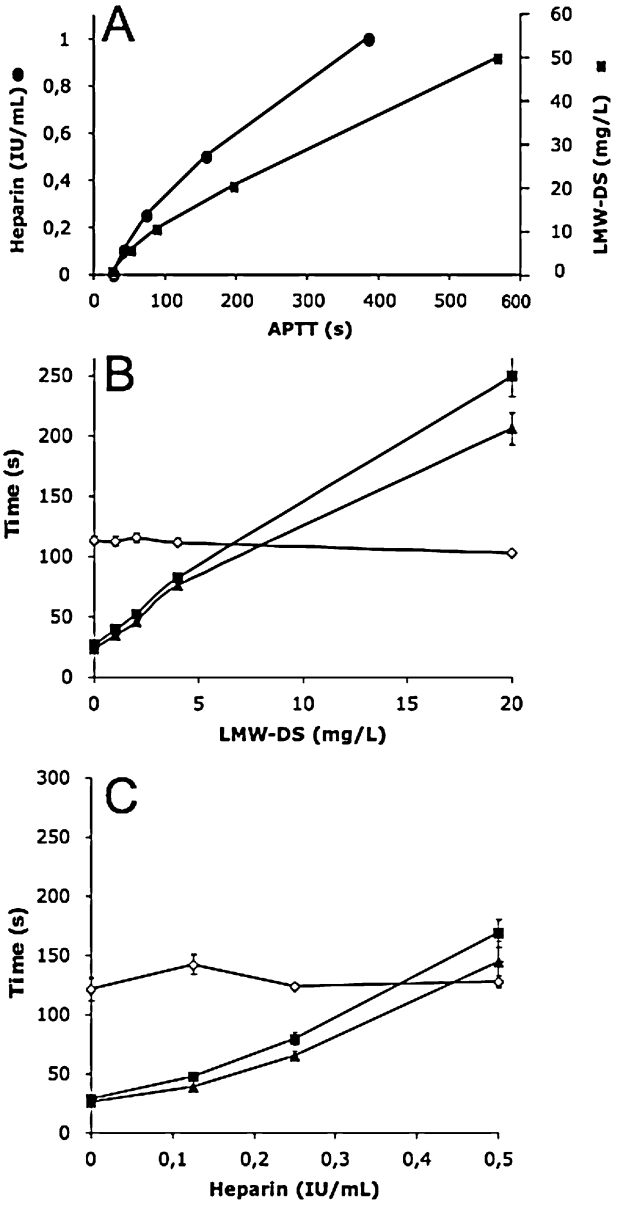

Figure 3: APTT and PFA100 assessments in plasma or whole blood treated with LMW-DS. Citrated blood was drawn from five normal subjects. Citrated plasma and whole blood was treated with LMW-DS. The APTT and PFA100 were assessed as described in materials and methods. Panel A shows APTT in plasma treated with increasing concentrations of heparin (closed circles) or LMW-DS (closed squares). Panels B (LMW-DS) and C (heparin) show PFA100 (open diamonds) in blood and APTT in blood (closed squares) and plasma (closed triangles). The PFA100 curve was constructed from four individuals, since one subject had taken ibuprofen before the experiment.

individual who had taken ibuprofen before the experiment (excluded). The PFA100 values for this individual increased to approximately 250 s but was unaffected by the increasing concentrations of LMW-DS.

Pharmacokinetics of LMW-DS in cynomolgus monkeys

In an initial experiment a bolus dose of 3 mg LMW-DS/kg body weight was administered i.v. The APTT reached >900 s but dropped back rapidly to the normal levels (Figure 4). The initial half-life was estimated to be 5–10 min. In two additional experiments a bolus dose (4.5 mg/kg) combined with a continuous infusion was evaluated. In both experiments the continuous infusion of dose ranged from 0.3 to

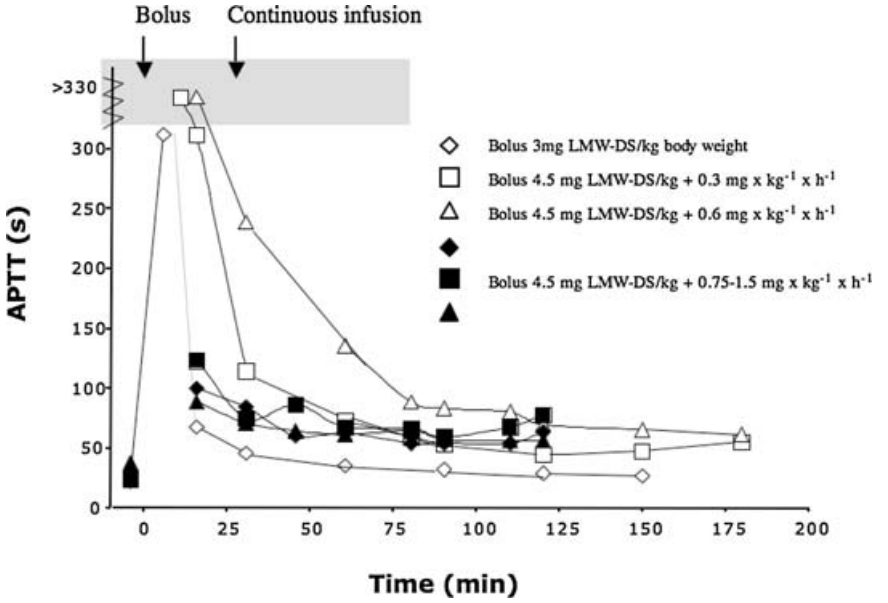

Figure 4: Pharmacokinetics of LMW-DS in cynomolgus monkeys monitored by APTT. Six different cynomolgus monkeys were treated with LMW-DS. In the first monkey, a bolus was administered intravenously and in the following five both a bolus dose and a continuous infusions were given according to the legend in the figure. The concentration was monitored by assessing the APTT in plasma.

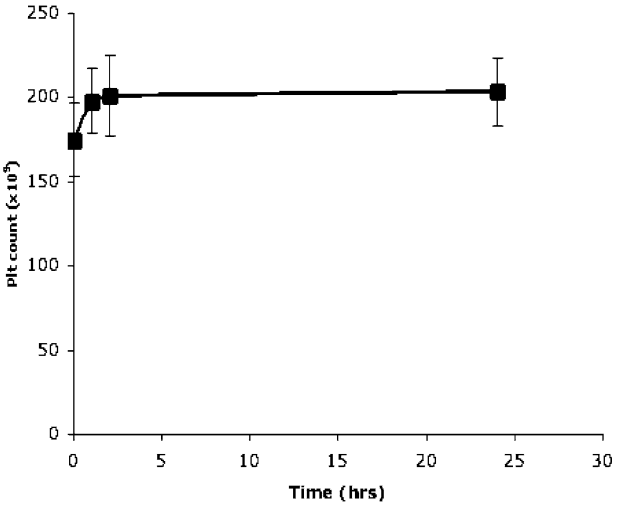

Figure 5: Platelet counts in LMW-DS treated cynomolgus monkeys. The platelet counts were assessed in cynomolgus monkeys treated with continuous LMW-DS infusion, targeting an APTT of 60–90 s. No significant change in platelet count was observed (n = 4).

0.6 mg/kg/h and the APTT was kept constant at 50–60 s in the first experiment and at 70–80 s in the second (Figure 4). From these data and an assumed distribution volume of 6.4 L (23), the half-life of the drug was estimated to be ≈15–20 min. In the following three monkeys the bolus was divided into two portions which were given intraportally; the first dose (1.5 mg/kg) was given as a bolus dose followed by an infusion (3 mg/kg) given over a 20 min time period. The dose of the continuous infusion ranged from 0.75 to 1.5 mg/kg/h. The platelet count (Figure 5), and the liver enzymes (ALT and AST) and creatinine levels (not shown) were all kept within normal ranges throughout the experiments. No bleedings or other adverse reactions were observed.

Discussion

In the present study we demonstrate that LMW-DS is a potent inhibitor of, not only xenogeneic (24) but also of allogeneic IBMIR. LMW-DS inhibited macroscopic clotting, abrogated platelet consumption and completely blocked the generation of C3a, FXIa-AT and TAT when human islets came in contact with ABO-compatible blood.

The exact mechanism by which LMW-DS inhibits IBMIR is unclear. Unlike heparin, the state-of-the-art anti-coagulant today, LMW-DS does not contain any specific anti-thrombin binding sites. Despite this LMW-DS is far more effective in inhibiting the IBMIR than heparin, which needs to be combined with the complement inhibitor sCR1 in order to obtain any significant effect (4). The LMW-DS, at concentrations between 2 and 20 g/L, has been shown in two other studies to be effective in preventing hyperacute rejection (HAR) of vascularized discordant xenografts (10,25). In allogeneic IBMIR, complement activation also occurs, but secondarily to coagulation (4,26). This finding explains why complement activation is reduced parallel with the reduction in TAT at much lower concentrations (0.01–1 mg/L) of LMW-DS compared to those used in the HAR studies. Considering that IBMIR is triggered by TF, it is unlikely that the previously identified agonistic effect on C1-INH is able to mediate an effect on IBMIR.

The half-life of DS (MM 8000) has been reported to be up to 1.6 h (30 min initially) in humans, but our cynomolgus monkey experiments point to a shorter half-life (≈15–20 min). The difference may be explained by the

difference in molecular mass of the LMW-DS. Hartmann and coworkers treated rats with single doses of i.v. DS (DS 8000) (27). Only a small fraction of the i.v. injected DS was eliminated by the kidneys, suggesting that the rest was eliminated by other means, most likely by the liver endothelial cells (28). The DS eliminated by the kidneys had a molecular mass of 3000–4000 Da, indicating that the cutoff for elimination by the kidney is <4000 Da. The average molecular mass (5000 Da) of the preparation of LMW-DS used in the present study is close to this cutoff, which implies that LMW-DS may be eliminated by the kidneys to a greater extent.

Our data show that LMW-DS up to the highest concentration tested (1 g/L) did not exert any toxic effects on the islets reflected in normal glucose-induced insulin secretion. We have shown similar data for porcine islets (11). At present, immobilized dextran sulfate is used to treat patients with hyperlipidemia by plasmapheresis (29). In patients treated with ACE-inhibitors, anaphylactoid reactions with hypotension have been reported due to activation of the contact system. Unlike immobilized DS, soluble LMW-DS does not activate the contact system within the dose interval that inhibits IBMIR and is therefore not anticipated to give this side effect (11).

The major concern using LMW-DS is the potential risk of bleeding and it is therefore appropriate to compare LMW-DS with heparin which is routinely used in clinical islet transplantation. As in the case of heparin, a direct correlation between the blood concentration of LMW-DS and APTT in normal individuals was demonstrated by Lorentsen et al. (23). This is confirmed in the present study and is also shown to be applicable on citrated blood which facilitates bed-side monitoring of LMW-DS. We assume that the APTT should not be prolonged more than 4-fold in clinical islet transplantation, in order to avoid bleeding complications, but a transient APTT similar to that obtained with currently applied dosages of heparin can be accepted. In our *in vitro* studies we have seen that blood concentrations between 10 and 50 mg/L corresponds to an APTT between 100 s and 400 s which give a substantial reduction of the IBMIR. This equals the APTT obtained with 1 IU/mL of heparin, which is theoretically the maximum blood concentration of heparin that is obtained with the present protocols for clinical islet transplantation (30).

The LMW-DS has been used in clinical trials both as an anticoagulant combined with kallikrein in stroke patients and as an anti-viral agent for treatment of HIV (31–33). These studies have given us a hint about the side effect of the drug. Severe thrombocytopenia has been observed after several days of treatment both during prolonged peroral administration (weeks) (34) and during infusion (7 days) (31) of LMW-DS (MM 8000). For the use in clinical islet transplantation LMW-DS will according to the proposed clinical protocol below be used for only 6 h and side effects of that kind are therefore not expected. This is supported by the constant platelet count in the cynomolgus monkeys during the LMW-DS infusions and by no influence of LMW-DS (or heparin) on the *in vitro* bleeding time (PFA100). The latter finding supports that platelet function (35,36) is not severely affected by LMW-DS (or heparin) at doses used during clinical islet transplantation.

Inhibition of IBMIR is probably most critical for the first hours (3–6 h) after infusion of the islets into the portal vein. A tentative model of how the LMW-DS should be administered during the initial stages of clinical islet transplantation would be to infuse the islets in the presence of a bolus dose of LMW-DS followed by a continuous infusion of LMW-DS intraportally. The infusion of islets in the presence of LMW-DS would lower the risk of clotting further since the portal concentration can be kept high. Our experiments in cynomolgus monkeys support such a treatment regimen: a bolus dose of 3–4.5 mg/kg body weight (LMW-DS; MM 5000) gave a transient peak of APTT more than 330 s. In the final three monkeys the bolus dose was given over a longer time period to mimic the proposed administration protocol below, where the LMW-DS is given together with the islets. This procedure blunted the initial peak substantially. The cynomolgus experiments also demonstrated that a continuous infusion, added after that the APTT elicited by the initial bolus dose had reached the targeted APTT, maintained the APTT at a constant level.

Table 1: Contribution of the priming dose and the continuous infusion of LMW-DS to the portal blood concentration

	Dose	Distribution	Concentration (container) (g/L)	Time (min)	mg/min	mL/min	Contribution to the portal LMW-DS concentration
Bolus[1]	4.5 mg/kg (=315 mg)	105 mg i.v.	20	1	105	5.3	18 μg/mL[2]
		140 mg infusion bag	0.7	30	4.7	6.7	15 μg/mL[3]
		70 mg wash bag	0.7	15	4.7	6.7	15 μg/mL[3]
Continuous infusion	0.75 mg/kg/h (=53 mg/h)		0.75	314	0.9	1.2	2.6 μg/mL[3]

[1]The calculation is based on a body weight of 70 kg and that one-third is given directly into the portal vein and the other two-thirds are distributed to the same concentration in the islet infusion bag and the wash bag which carries the wash buffer to rinse the infusion bag.
[2]This is the concentration in the whole body distribution volume of 6.4 L (23).
[3]Estimated portal flow of 600 mL/min (36) and a hematocrit of 40%.

The present study was aimed to give safe guidelines for the dosing of LMW-DS during clinical islet transplantation and based on our experimental findings we propose an initial protocol that consist of (1) one-third of a bolus of 3–6 mg LMW-DS/kg given before transplantation and two-thirds together with the islet intraportally. (2) Continuous infusion of 0.3–1.2 mg LMW-DS mg/kg/h started immediately after the islet infusion is stopped and maintained for 6 h, carefully adjusted to target an APTT of 50–100 s which according to our *in vitro* studies corresponds approximately to 5–10 mg/L of LMW-DS. In Table 1, the contribution to the intra-portal LMW-DS concentrations provided by the bolus dose and the continuous infusion are calculated. This shows that the intra-portal concentration of LMW-DS is well within therapeutic concentration during islet infusion. The effect on hemostasis with this regimen will be very similar to that of heparin treatment and therefore not anticipated to increase the risk of bleeding more than heparin. If bleeding nevertheless would occur during the LMW-DS infusion, the short half-life of the drug implies that any hemostatic effects of LMW-DS can be abrogated by stopping or reducing the infusion rate.

Based on the experimental data presented, LMW-DS will be used in an attempt to down-regulate IBMIR and enhance islet engraftment in clinical islet transplantation.

Acknowledgments

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