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Research Article

Anti-Inflammatory Effects of Heparin: Do they Influence Murine Acute GvHD?

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Abstract

Background

Graft-versus-host disease (GvHD) is still one of the most challenging complications after allogeneic hematopoietic stem cell transplantation. Steroid-resistant or steroid-refractory patients with acute GvHD have limited therapeutic options and there is a strong demand in clinics for new therapeutic approaches. Due to the published anti-inflammatory effects of heparin, we hypothesized that either unfractionated (UFH) or low-molecular-weight heparin (LMWH) could be a novel treatment option for patients with acute GvHD.

Methods

We performed dose finding studies with both UFH and LMWH via intravenous (iv) and subcutaneous (sc) routes of application in a mouse model of acute GvHD. Survival rates, clinical GvHD score, $CD4^+$ and $CD8^+$ T cell levels were determined.

Results

Iv administration of UFH caused slightly but no statistically improved survival rates or clinical scores of the GvHD animals. Neither sc administration of UFH, nor sc or iv administration of LMWH was linked to improved survival or clinical GvHD score. $CD4^+$ and $CD8^+$ T cell levels were not influenced by UFH or LMWH treatment.

Conclusion

UFH seems to be more effective than LMWH. However, the therapeutic effect of UFH on acute GvHD is limited and heparin alone is probably not sufficient for the therapy of acute GvHD.

Keywords:

GvHD; Heparins; Immune-Suppression; Bone Marrow Transplantation

Introduction

Many patients with leukemia require allogeneic hematopoietic stem cell transplantation (HSCT), which is in most cases the only possibility to cure the haematological malignancy. However, allogeneic HSCT is frequently accompanied by severe side-effects such as graft-versus-host disease (GvHD) [1]. GvHD is still one of the most challenging complications after HSCT. The pathophysiology is based on allo-reactive $CD4^+$ and $CD8^+$ donor T cells, causing severe inflammatory reactions against host tissues such as skin, liver and gut [2]. The first-line therapy of GvHD is based mainly on immune-suppressive corticosteroids [3]. However, corticoid-treatment of patients after HSCT is linked to several side-effects. Additionally, some patients are steroid-resistant or get steroid refractory during the corticoid treatment [4]. Hence, there is a considerable demand for new GvHD treatment options.

Heparin is one of the most used anticoagulants worldwide. In addition to its anti-coagulatory effect, anti-inflammatory effects of heparin were published already in 1959 [5, 6]. One of the underlying effects, the inhibition of leukocyte adhesion to the blood vessel endothelium, was described in 1962 [7]. Later Nelson et al. found that heparin is a potent inhibitor of endothelial P-selectin and leukocytic L-selectin binding [8]. These findings are very interesting in the case of GvHD, since in a double P-selectin knock-out mouse model reduced GvHD mortality and an improved clinical course were observed [9]. Furthermore several effects of heparin were described on the inhibition of pro-inflammatory cytokines and Th-1, Th-2 and Th-17 expression levels in general [10, 11]. These effects were described for both unfractionated (UFH) and low-molecular-weight heparin (LMWH). Clinical studies revealed beneficial anti-inflammatory effects of heparin in corticoid-resistant patients with asthma or ulcerative-colitis [12-14]. In addition, several studies describe a beneficial impact of heparin treatment in sepsis [15, 16].

Despite these positive findings, a lack of larger randomized trials and investigations on the incidence of adverse bleeding complications caused by heparin treatment still exists [15, 17].

According to the described anti-inflammatory potential of heparin, we wondered whether heparin could exert similar beneficial effects on GvHD which is featured by severe inflammatory reactions within different tissues. In the literature we found a single study describing heparin treatment in a GvHD mouse model, only [18]. In this study no dose finding experiments and no investigations with LMWH were performed. Especially the differentiation of UFH and LMWH seems to be important due to their differences in bioavailability, pharmacokinetics, and bleeding risk [19].

We hypothesized that heparin could have positive effects on survival and severity of acute GvHD. Furthermore we investigated whether UFH or LMWH is more favorable in case of GvHD treatment and which application route is most promising. To answer these questions we used an established mouse model of acute GvHD and performed a dose finding study with both LMWH and UFH administered via the intravenous (iv) or subcutaneous (sc) route of application.

Methods

Heparin

We used heparin sodium (Merckle GmbH, Blaubeuren, Germany) as unfractionated heparin and enoxaparin (Clexane, Sanofi-Aventis, Frankfurt am Main, Germany) as low-molecular weight heparin. Heparin was diluted in sodium chloride (NaCl) to a final concentration of 20-200 international units (IU) per kg body weight and 100 µl of the solution were injected intravenously (iv) into the tail vein or subcutaneously (sc) into the nuchal fold or flexure of the mice. As corresponding controls we performed iv or sc injections with NaCl only.

Mice

All mice were male and 10 weeks of age at the time point of bone marrow transplantation (BMT). C57BL/6J donor mice (B6) were purchased from Janvier (St Berthevin, France) and BALB/c recipient mice from Charles River Laboratories (Sulzfeld, Germany). Mice were housed in individual-ventilated cages. All animal experiments were conducted according to ethical regulations after permission from the local authorities of Lower Saxony/Germany.

Bone Marrow Transplantation

We used the B6→BALB/c mouse model of BMT for the induction of severe acute GvHD. BALB/c recipient mice were conditioned 24h prior to BMT with a total body irradiation dose of 9.5 gray. Bone marrow cells were isolated from femur and tibia and T cells were depleted from the bone marrow cell suspension by using CD90.2 MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany). Afterwards a defined number of T cells were added to the cell suspension. These T cells were purified from spleen and lymph nodes with the Pan T-cell isolation Kit (Miltenyi Biotec). For BMT 10^7 B6 bone marrow and $2x10^6$ B6 T cells were injected iv into the tail vein of each recipient BALB/c mouse. To avoid bacterial infections, neomycin was added to the drinking water.

GvHD Scoring

GvHD clinical score was set to 8 points maximum. For each criteria which were based on posture, activity, skin/fur condition and diarrhea 0, 1 or 2 points were counted according to disease severity. For ethical reasons animals were sacrificed when a score of 6 or higher was reached. The GvHD score and the weight of the animals were assessed daily.

Flow cytometry

Cellular stainings were performed at the end of each experiment at day 45 or when animals reached a score of 6 before. Cells were prepared from spleen and lymph-nodes of the animals. Anti-mouse CD3ε (Pacific Blue, clone 145-2C11), anti-mouse CD8 (APC, clone 53-6.7) and anti-mouse CD4 (FITC, clone RM4-5) were purchased from eBioscience (San Diego, CA, USA). Cell suspensions were incubated with corresponding antibodies for 10 min at room temperature and washed with phosphate buffered saline (Lonza, Verviers, Belgium). Analysis was performed by using a FACS Canto II flow cytometer (BD Biosciences, San Jose, CA, USA).

Statistical Analysis

Log-rank test was used for statistical p-value calculation of *in vivo* survival rates. P-values of clinical score and cellular *in vitro* data were calculated with the two tailed students t-test. Graphs show mean values + SD.

Results

Marginal Therapeutic Effects of Intravenous UFH Treatment

Corresponding to our hypothesis that the anti-inflammatory effects of heparin could exert beneficial effects on GvHD, we performed heparin treatment after murine BMT. The iv application was based on injection of either 100 IU/kg UFH or 20 IU/kg LMWH once a week each. The survival rate and the clinical score of the LMWH group did not differ from the control group during the observation period of 45 days (Figures 1a and 1b). The group treated with 100IU/kg UFH iv showed a tendency for a better survival rate compared to the control group (Figure 1a). The improvement in the survival rate did not reach statistical significance according to the log-rank test (p=0.28) but the percentage of living animals from day 10 to day 45 after BMT was constantly about 12% higher or more with a final survival rate at day 45 of 29% in the UFH group and 17% in the control group respectively (Figure 1a). The corresponding clinical GvHD score of the UFH iv treatment group was lower compared to the control group (p=0.04 at day 14, Figure 1b). The percentages of the $CD4^+$ and $CD8^+$ T cells were not different in the treatment and control groups, respectively (Figures 1c and 1d).

Tendency for Adverse Effects of Subcutaneous Applied LMWH on GvHD *in vivo*

Due to the widespread sc application of LMWH in clinics we went on with sc injections of Clexane (enoxaparin). We did not observe positive therapeutic effects of the LMWH (Figures 2a and 2b). The survival rates of both 20 IU/kg and 200 IU/kg LMWH treatment groups were even decreased from day 8 to day 38 in contrast to the NaCl control group (Figure 2a). Looking at the total 45 day period the survival rates were not statistically different according to the log-rank-test (Figure 2a). The clinical GvHD scores of the 20 IU/kg LMWH and the control group were not different whereas the score of the high dose 200 IU/kg LMWH group was even worse compared to the control group (p=0.04 at day 15; Figure 2b). The T cell analysis revealed a tendency for increased $CD4^+$ and $CD8^+$ T cell percentages in the lymph nodes of the LMWH groups with a statistical significant up-regulation of $CD8^+$ T cells in the lymph nodes of the 20 IU/kg LMWH group compared to the control group (p=0.03; Figures 2c and 2d). T cell levels in spleen showed no significant differences between the groups (Figures 2c and 2d).

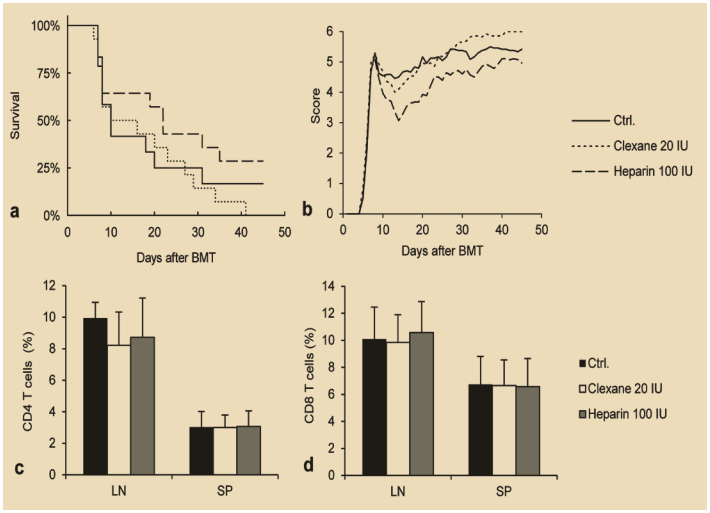

Figure 1. Therapeutic impact of intravenous LMWH (Clexane) and UFH (Heparin) on acute murine GvHD.

Survival rates of mice after B6→BALB/c bone marrow transplantation (a). GvHD severity score of acute murine GvHD (b). Cellular analysis of $CD4^+$ and $CD8^+$ T cell percentages of lymph nodes (LN) and spleen (SP) of GvHD mice (c and d; n=12-14; * $p<0.05$).

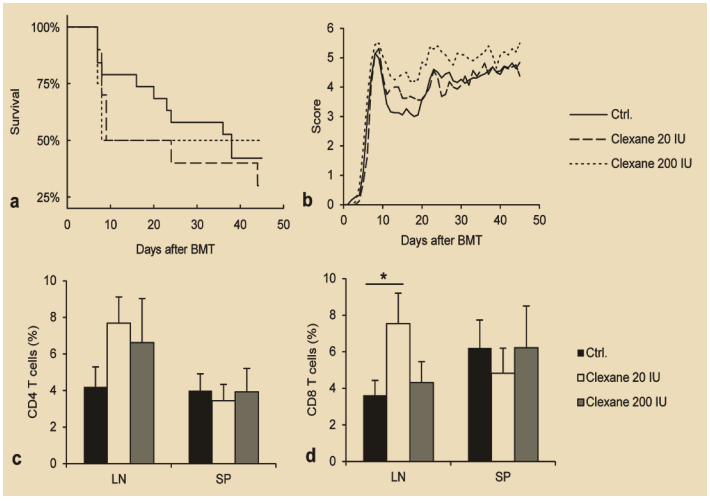

Figure 2. Effect of subcutaneous LMWH (Clexane) on acute murine GvHD.

Survival of mice after allogeneic B6→BALB/c bone marrow transplantation (BMT) (a). Clinical GvHD severity score of mice after BMT (b). Percentages of $CD4^+$ and $CD8^+$ T cells of the lymph nodes (LN) or spleen (SP) of GvHD mice (c and d; n=8-19; * $p<0.05$).

No Improvement of GvHD Characteristics after Subcutaneous UFH Treatment

After using LMWH we changed to UFH sc administration with dosages of 50, 100 and 200 IU/kg body weight. Neither in the overall survival rate nor in the clinical GvHD score we found significant differences between the UFH treatment and the control groups (Figures 3a and 3b). The survival rate of the control group appeared to be more in the upper range of the survival curves and the corresponding clinical score had a tendency for slightly lower disease scores in the control group compared with the UFH treatment groups (Figures 3a and 3b). The cellular analysis of $CD4^+$ and $CD8^+$ cells revealed no changes in the T cell levels of the UFH treatment groups and the control group (Figures 3c and 3d).

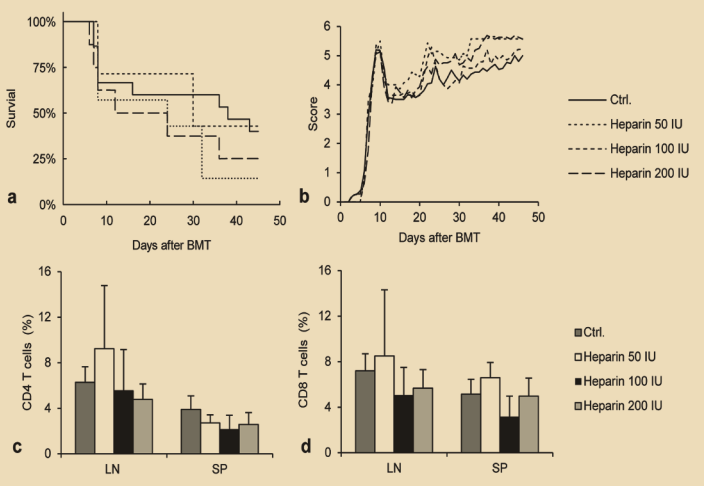

Figure 3. Effect of subcutaneous UFH (Heparin) on acute murine GvHD.

Kaplan-Meier Plot after bone marrow transplantation (BMT) in the B6→BALB/c mouse model of acute GvHD (a). GvHD score after BMT (b). $CD4^+$ and $CD8^+$ T cell percentages in lymph nodes (LN) or spleen (SP) of GvHD mice (c and d; n=7-15).

Discussion

Both LMWH and UFH have been reported to down-regulate inflammatory parameters such as pro-inflammatory cytokines [20-22]. However our results on the efficacy of LMWH and UFH were only modest. Since some of the gut associated GvHD symptoms such as inflammation of the gut mucosa associated with potential bleedings are similar compared to ulcerative colitis (UC) it is interesting to compare our results with studies about heparin treatment in UC. Interestingly the results of studies on heparin treatment in UC are quite different. Whereas some studies found positive effects of heparin both in UC animal models and clinical studies [13, 14, 23], other clinical investigations showed no benefit of LMWH or UFH treatment on UC [24, 25]. However, the application route has a major impact on the therapeutic outcome shown by beneficial LMWH application via colon-release tablet on UC patients compared to subcutaneously given LMWH without beneficial effects [24].

In general we did not observe significant therapeutic effects on the survival time in the GvHD mouse model. However, UFH treatment in the GvHD mouse model is dependent on the dosage and route of application. Although iv application was performed only once a week and only within the first four weeks of the experimental period, the survival showed a small tendency for better survival compared with the control group. According to the algorithm of the log-rank test, it is likely that further experiments with higher experimental numbers could result in significant p-values. The tendency for a better survival points to the importance of iv application of UFH which is linked to better bioavailability. The effect was achieved without maintaining a constant pharmacological level of UFH. Therefore the anti-inflammatory effect of UFH seems to be more persistent than its anti-coagulatory effect which decreases already a few hours after application. When applying LMWH via iv route, we did not observe positive effects on survival or clinical score but there was also no indication for adverse effects.

In general LMWH is applied subcutaneously. The bioavailability is higher and the half-life is longer than of UFH. Furthermore LMWH shows fewer side effects such as heparin-induced thrombocytopenia [26, 27]. Additionally and perhaps most important, Constantino et al. observed a tendency for less bleeding risks in patients treated with LMWH compared to UFH [28]. Based on these characteristics LMWH could maybe more beneficial for treatment of GvHD than treatment with UFH. Interestingly our results showed a rather contrary tendency. Subcutaneous application of either low dose (20 IU/kg) or high dose (200 IU/kg) LMWH showed no beneficial effects on survival or clinical score of GvHD in mice. Although not significant, the survival rate of the LMWH treated mice seemed to be lower compared with the control animals. The clinical GvHD score of the high LMWH dose group (200 IU/kg) was significantly higher at day 15 after BMT. Both LMWH and UFH treatment are always linked to the risk of bleeding especially at the site of inflammatory lesions in case of GvHD. Although we did not observe enhanced bleeding in our GvHD mice, this side effect could account for the slightly worse outcome in the LMWH treatment group since the acute GvHD mouse model is often associated with bloody diarrhea up to 10 days after BMT.

Since we did not observe beneficial effects of sc LMWH we also had a look on sc UFH treatment. In contrast to LMWH, application of UFH is preferred via iv route due to better bioavailability of the larger molecules. However, we also performed sc

UFH injections since the number of iv injections is limited in the mouse model and sc injections can be performed more frequently. The sc application of UFH is probably linked to a lower bioavailability of each heparin dose but the therapy could be performed every second day during the whole experimental period of 45 days. In brief, we did not observe any beneficial effects on the survival rate or clinical score of the GvHD mice after sc UFH treatment with different dosages from 50 IU/kg up to 200 IU/kg. A similar experimental approach was performed by Naparstek et al. [18]. They treated GvHD mice with UFH sc daily for 30 days after allogeneic BMT. In this experiment the survival of the UFH and the control group did not differ from day 0 to day 22 with a tendency for slightly improved survival after 30 days in the UFH group.

Conclusion

We did not observe statistically significant therapeutic effects after UFH or LMWH treatment on acute murine GvHD. The iv application seems to be more favorable compared with the sc application and UFH is maybe more effective than LMWH. Although the therapeutic potential of heparin for GvHD treatment seems to be limited, further *in vivo* studies with higher animal numbers could perhaps uncover the complete anti-inflammatory effect of heparin on GvHD.

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