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Chapter 6

Hematopoietic stem cell derived factor VIII

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In preparation
ABSTRACT

The liver is the main site of factor VIII production in mammals but previous studies have shown that multiple other tissues like kidney, spleen, and lymph nodes also express factor VIII. In the present study we determined whether hematopoietic cells are capable of factor VIII production as well.

Factor VIII deficient mice underwent a bone marrow transplantation receiving bone marrow from wildtype littermates. Six weeks later blood and tissues were collected for analysis of factor VIII activity in plasma and the presence of factor VIII mRNA in blood cells and/or organs.

After bone marrow transplantation, 9 out of 11 of the factor VIII deficient mice showed low, but detectable plasma factor VIII levels (mean +/- SEM: 4.2% +/- 1.0% of normal). Furthermore, factor VIII mRNA was detected in both bone marrow and peripheral blood cells, whereas no factor VIII mRNA was present in the liver. Factor VIII mRNA was also detected in the heart, spleen and lung of transplanted factor VIII deficient mice suggesting transdifferentiation and/or fusion of hematopoietic stem cells.

In summary, transplantation of wildtype bone marrow into hemophilia A mice partly restores factor VIII plasma levels. The source of plasma factor VIII remains difficult to define, however. After transplantation the presence of factor VIII mRNA was not limited to hematopoietic cells, most likely due to stem cell plasticity.

INTRODUCTION

Factor VIII is a plasma protein that plays an essential role in the hemostatic system. It acts as the cofactor of factor IX in the activation of factor X, thereby promoting fibrin formation (1). The clinical relevance of factor VIII is evident from the fact that a deficiency of factor VIII results in the bleeding disorder hemophilia A.

To date there still is controversy regarding which tissues are capable of factor VIII production. Without doubt, the liver is the principal site of factor VIII synthesis (2,3). This is based on studies, which demonstrated the correction of factor VIII levels in hemophilic dogs and humans upon liver transplantation (4-6). Furthermore, the presence of factor VIII mRNA and antigen was observed in sinusoidal endothelial cell and hepatocytes (3,6-9). In addition, extra-hepatic tissues, i.e. spleen, kidneys, and lymph nodes, have been shown to be capable of factor VIII mRNA synthesis (2,10-13). Although factor VIII mRNA in blood cells is regularly used to screen for genetic abnormalities in hemophilia A patients (14-17), it is doubtful whether blood cells are capable of producing factor VIII. As already suggested in the early nineties, factor VIII mRNA found in the circulation might be an example of ectopic or "illegitimate" mRNA (15,17). In the sixties and seventies it was demonstrated that (sub-cellular fractions of) granulocytes contain factor VIII protein (18-20). However, convincing evidence that functional factor VIII is synthesized by hematopoietic cells and secreted into the circulation is lacking.
In the present work we address the question whether hematopoietic cells are capable of factor VIII production by performing bone marrow transplantsations into hemophiliic mice. Indeed, bone marrow transplantation increased circulating factor VIII levels in hemophiliic mice, but the cellular source remains to be defined.

**METHODS**

*Mouse strains*

The generation of factor VIII deficient mice (exon 16 disrupted) has been described in detail by Bi and co-workers (21,22). The factor VIII deficient mice we used are direct descendents from an F1-cross, and thus genetically 50% C57Bl/6 and 50% 129Sv. Recipient mice were hemizygous offspring of heterozygous factor VIII knockout mice. Donor mice were wildtype siblings of the recipients. Genotyping was performed as described before (21). The mice were bred and maintained at the animal care facility at the Academic Medical Center. All mice were housed according to institutional guidelines, with free access to food and water. Animal procedures were carried out in compliance with the Institutional Standards for Humane Care and Use of Laboratory Animals.

*Bone marrow cell preparation*

Bone marrow cells were harvested from 10-12 weeks old wildtype males. Cells were isolated by flushing tibia and femurs with phosphate buffered saline (PBS, NPBI, Emmercompascuum, The Netherlands) containing 10% fetal calf serum (FCS, BioWitthaker, Heidelberg, Germany), 100 U/ml penicillin (BioWitthaker), and 100 μg/ml streptomycin (BioWitthaker), and single cells were prepared by pulling the tissue clumps three times through a 25-gauge needle. Next, the cells were centrifuged at 250 x g for 10 minutes, aspirated, washed, and resuspended in PBS.

*Bone marrow transplantation*

Eight to twelve week-old, male factor VIII-deficient mice received a total body irradiation of two times 4.0 Gy with three hours between the two doses (sublethal dose), using an X-ray source at a dose rate of 0.88 Gy/min, followed by i.v. injection of 10^7 bone marrow cells. To protect the irradiated recipients from infections, the mice were supplied with autoclaved, acidified (pH 2.5) drinking water containing 2% neomycin (Sigma Chemical Co, St.Louis, MO, USA) from one week before until six weeks after transplantation, and they were housed in sterile filter top cages in a laminar flow chamber.

*Sample preparation*

Six weeks after transplantation, the mice were sacrificed, blood was drawn via a heart puncture and collected into tubes containing 0.32% sodium citrate, and various tissues
Hematopoietic stem cell derived factor VIII were surgically removed and immediately frozen in liquid nitrogen. Blood was centrifuged twice at 1,000 x g for 10 min. The plasma layer was carefully aliquoted and stored at -80°C until subsequent analysis. The remaining pellet was resuspended in 200 μl PBS. DNA was isolated from these cells using the QIAamp DNA blood mini kit (Qiagen, Hilden, Germany).

Total RNA was isolated from snap frozen tissue using guanidine isothiocyanate (Trizol®, Gibco)/ chloroform extraction followed by precipitation with 2-propanol. After washing with 80% ethanol, the isolated RNA was dissolved in RNase free water and stored at -80°C until usage. cDNA was made by reverse transcription from total RNA using random hexamer primers (Life Technologies) and Superscript II RNase H reverse transcriptase (Life Technologies).

Measurement of factor VIII activity

Factor VIII activity was measured in citrated blood using a chromogenic factor VIII assay (Dade Behring) on a Behring Coagulation System analyzer (BCS, Dade Behring). Murine wildtype plasma diluted in murine factor VIII deficient plasma was used as a calibration curve.

Analysis of factor VIII mRNA synthesis

Factor VIII mRNA levels were measured by a quantitative real-time RT-PCR using LightCycler technology (Roche Molecular Biochemicals) with SYBR Green II detection. Primers for factor VIII were chosen in exon 16/17 based on the murine factor VIII mRNA sequence (Genbank accession number NM-007977) and the mouse genome database. The amplification products were analyzed on a 2% agarose gel. The primer pair (forward 5'-GCTTATTTCCTCTGATGTTGATCTTG-3' and reverse 5'-CATCAAGATAGTGAAAAGCAGAGC-3') produced a single band of 110 bp, which, because exon 16 is disrupted in factor VIII deficient mice, was absent in the liver of these mice. Relative amounts of mRNA were semi-quantified by comparing the sample curve with the positive control curve (wildtype liver). The cycle number in which the signal becomes above the predefined threshold is denoted CN. By using the following equation: \[ \% \text{mRNA} = 2^{(\text{CN}_{\text{positive control}} - \text{CN}_{\text{sample}})} \times 100, \] the percentage of tissue specific factor VIII mRNA as compared to the positive control was calculated. The obtained percentages were then converted into relative amounts as compared to the bone marrow mRNA level by defining the average bone marrow signal as 100%. We opted to represent mRNA levels relative to bone marrow mRNA levels because that shows the difference in transplantation efficiency between the different mice.

RESULTS

In two separate experiments, we transplanted 11 factor VIII deficient mice with wildtype bone marrow and all transplanted mice survived the six week-recovery period. As is shown in figure 1, nine of the transplanted factor VIII deficient mice showed detectable factor VIII activity levels (ranging from 1.7 to 10.4%; mean ± SEM: 4.2 % ± 1.0 %), whereas two of
the 11 transplanted factor VIII deficient mice showed no detectable factor VIII activity 6 weeks after transplantation. Therefore, bone marrow transplantation increased factor VIII levels in 82% of the transplanted mice.

![Figure 1. Effect of bone marrow transplantation on plasma concentration of factor VIII.](image)

To determine whether hematopoietic cells produce the observed plasma factor VIII activity levels, we analyzed blood cells, bone marrow and liver of five transplanted factor VIII deficient mice for the presence or absence of factor VIII mRNA. Figure 2A shows the predicted PCR product using liver mRNA of wildtype mice. No product was seen using liver mRNA from factor VIII deficient mice. Factor VIII mRNA was detected in bone marrow and blood cells, but not in liver. These results suggest that hematopoietic cells are indeed the source of factor VIII upon bone marrow transplantation (fig.2B).

Recent work has suggested that adult bone marrow-derived hematopoietic stem cells might transdifferentiate into other cell types than the anticipated bone marrow and blood cells (23-29). To verify whether transplanted stem cells were migrated and/or differentiated into other tissues and also expressed factor VIII mRNA, we determined factor VIII mRNA in brain, heart, kidney, lung and spleen. In addition to bone marrow and blood, low but detectable levels of factor VIII mRNA were observed in heart, lung and spleen of all mice (fig.2C). Maximal factor VIII mRNA levels were present in bone marrow, followed by spleen, lung and blood cells. In only one mouse factor VIII mRNA was detected in kidney and in another mouse in muscle. Liver and brain were negative for factor VIII mRNA in all five mice.

**DISCUSSION**

To date there is still controversy about the tissues capable of producing factor VIII. Without discussion, the liver is the principal site of factor VIII synthesis, demonstrated by
transplantation studies in both hemophilic animals and patients with hemophilia A (4-6,9). But in addition, extra-hepatic tissues, i.e. spleen, kidneys, and lymph nodes, express factor VIII mRNA as well (2,3,10-13). Factor VIII mRNA is also present in circulating blood cells. However, factor VIII mRNA found in the circulation is often thought to be an example of ectopic RNA (15,17). It is unclear whether blood cells are able to produce functional factor VIII. Previous studies demonstrated factor VIII-like activity in leukocytes (lymphocytes or granulocytes) suggesting that these cells are capable of factor VIII synthesis (18-20). In the present work, we show that bone marrow transplantation of hemophilic mice results in low levels of circulating factor VIII, as factor VIII activity in hemophilic mice increased up to 4.2% ± 1.0% upon wildtype bone marrow transplantation. In contrast, hematopoietic grafting in hemophilic dogs showed no evidence of factor VIII synthesis (30). We have no explanation for these apparently disparate observations. It is possible that small changes of factor VIII levels in the canine study have escaped detection.

Figure 2. The presence of factor VIII mRNA in factor VIII deficient mice 6 weeks after bone marrow transplantation using wildtype donor cells. A. Factor VIII deficient mice show no factor VIII mRNA. Lane 1, 2 and 5 show PCR products from livers of factor VIII deficient mice, lane 3, 4 and 6 show PCR products from livers of wildtype mice and lane 7 shows the negative control. B. Representative RT-PCR of blood (striped black), bone marrow (black) and liver (dotted black) of a factor VIII deficient mouse that underwent bone marrow transplantation. The positive control (wildtype liver) is depicted as dashed gray and the negative control as gray. C. Graphical representation of average relative amounts of factor VIII mRNA present in tissue of factor VIII deficient mice 6 weeks after bone marrow transplantation. Results are depicted relative to the amount of factor VIII mRNA present in bone marrow. Shown are mean ± SEM of 5 mice.

Based on our observation that bone marrow transplantation results in elevated factor VIII activity in hemophilic mice, we tried to identify the cellular source of plasma factor VIII in our transplanted mice. Initial experiments showed factor VIII mRNA in both bone marrow and blood cells but not in liver, suggesting that hematopoietic cells do
produce factor VIII. Whether these factor VIII producing hematopoietic cells are leukocytes as suggested already in the 1960's (18-20) will be subject of future experiments. Megakaryocytes and platelets, however, are probably not the cells producing factor VIII after transplantation (31).

Our conclusion that hematopoietic cells produce factor VIII resulting in elevated plasma levels after transplantation is only partly the answer. Low levels of factor VIII mRNA were also observed in heart, lung and spleen. The most logical explanation would be that factor VIII mRNA in heart, lung and spleen is due to the presence of blood cells in these tissues. However, the observations that not all tissues analyzed showed detectable factor VIII mRNA levels, e.g. liver and that the amount of factor VIII mRNA detected in lung and spleen was higher than the amount detected in blood cells argue against this explanation.

Alternatively, transdifferentiation of hematopoietic stem cells into factor VIII producing cells could be responsible for factor VIII synthesis upon bone marrow transplantation. During the last years there has been a debate about whether adult bone marrow-derived stem cells are capable of differentiation into other cells than bone marrow and blood cells (23-29). It has often been described that bone marrow transplantation results in the presence of donor cells in liver, skin and several other tissues (32-35). This phenomenon has been extensively explored for its potential therapeutic use in renewing damaged tissue as for instance during ischemic heart disease (36). However, it remains unclear whether the presence of donor cells in other tissues than bone marrow or blood is the result of transdifferentiation of hematopoietic cells into for instance cardiomyocytes or of fusion of the stem cells with cells of the recipient (23,25). Evidently, if bone marrow-derived stem cells are capable of differentiation into or fusion with for instance cardiomyocytes or spleen cells, our observed results do not indicate that hematopoietic cells produce factor VIII, but that bone marrow-derived cells can produce factor VIII. Based on our current experiments it is not possible to distinguish between factor VIII production by hematopoietic cells and other stem cell derived cells.

We do not yet have data about the phenotype of the mice after bone marrow transplantation. In patients factor VIII levels below 1% are characteristic for severe hemophilia, whereas factor VIII levels above 5% are characteristic for a mild form of hemophilia with less clinical signs (37). The observed plasma factor VIII activity levels of on average about 4% might thus be clinically relevant. Whether conventional techniques, such as tail bleeding time will demonstrate phenotypic improvement after transplantation remains to be demonstrated. However, Sarkar et al. (38) described the partial correction of the hemophilia A phenotype in 65% of hemophilic mice having approximately 7 % factor VIII activity after administration of an adeno-associated virus vector containing murine factor VIII.

In conclusion, we demonstrated that transplantation of wildtype bone marrow into hemophilia A mice partly restores factor VIII plasma levels. However, the source of plasma factor VIII remains to be identified.
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