Predominant Hematopoietic Origin of Cell-free DNA in Plasma and Serum after Sex-mismatched Bone Marrow Transplantation

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Background: Despite current interest in the biology and diagnostic applications of cell-free DNA in plasma and serum, the cellular origin of this DNA is poorly understood. We used a sex-mismatched bone marrow transplantation model to study the relative contribution of hematopoietic and nonhematopoietic cells to circulating DNA.

Methods: We studied 22 sex-mismatched bone marrow transplantation patients. Paired buffy coat and plasma samples were obtained from all 22 patients. Matching serum samples were also obtained from seven of them. Plasma DNA, serum DNA, and buffy coat were quantified by real-time PCR of the SRY and β-globin gene DNA. To investigate the effects of blood drawing and other preanalytical variables on plasma DNA concentrations, blood samples were also collected from 14 individuals who had not received transplants. The effects of blood sampling by syringe and needle, centrifugation, and time delay in blood processing were studied.

Results: The median percentage of Y-chromosome DNA in the plasma in female patients receiving bone marrow from male donors (59.5%) differed significantly (P <0.001) from that in the male patients receiving bone marrow from female donors (6.9%). This indicated that plasma DNA in the bone marrow transplantation recipients was predominantly of donor origin. Compared with paired plasma samples, serum samples had a median 14-fold higher DNA concentration, with the additional DNA being of donor origin. Control experiments indicated that none of the three tested preanalytical variables contributed to a significant change in cell-free DNA concentration.

Conclusions: After bone marrow transplantation, the DNA in plasma and serum is predominantly hematopoietic in origin. Apart from the biological implications of this observation, this finding suggests that plasma and serum can be used as alternative materials for the study of postbone marrow transplantation chimerism.

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Recently, much interest has been focused on the biology and diagnostic applications of cell-free DNA in plasma and serum (1). For example, tumor-derived DNA has been found in the plasma and serum of cancer patients (1). In pregnancy, fetal DNA has been found in maternal plasma and serum (2). Following liver and kidney transplantation, donor-derived DNA has been found in the plasma of recipients (3). Although these studies indicate that tumor-, fetus-, and graft-derived DNA can be found in plasma, they provide no information regarding the tissue of origin of other DNA species in the plasma. In particular, the tissue origin of plasma DNA found in apparently healthy individuals remains unclear.

Because blood cells are the cell types that are in the closest proximity to plasma, we reasoned that it would be logical to ask whether any plasma DNA is of hematopoietic origin. In this study, we developed a sex-mismatched bone marrow transplantation model to answer this question. In this model, the hematopoietic system of the transplant recipient was predominantly of donor origin, whereas the nonhematopoietic tissues were recipient in origin. Because either the donor or recipient was male, we used molecular methods based on detection of Y-chromosome DNA to investigate the proportion of male DNA in the cell-free (plasma and serum) and cellular compartments.
Materials and Methods

Sample Collection
We recruited 22 sex-mismatched bone marrow transplantation patients at the Department of Pediatrics of the Prince of Wales Hospital for the study. Informed consent was obtained from all individuals. Of these 22 patients, 14 were males who had received bone marrow from female donors, whereas the remaining 8 were females who had received bone marrow from male donors. All study participants had previous evidence of hematopoietic engraftment by fluorescence in situ hybridization analysis using X- and Y-chromosomal probes. None of the transplant recipients had evidence of graft rejection or graft-vs-host disease. Peripheral blood samples were collected into EDTA tubes from all participants and processed as described below for the harvesting of plasma and buffy coat samples. For seven transplant recipients, blood samples were also collected into a paired plain tube for the preparation of serum samples. Five of these transplant recipients were males who had received bone marrow from female donors, and the remaining two were females who had received bone marrow from male donors. Blood samples were drawn from a peripheral vein with a 23-gauge needle, with immediate transfer into the collection tubes. Control blood samples were also obtained from 14 healthy volunteers.

Processing of Blood Samples from Bone Marrow Transplantation Patients
Blood samples were processed within 6 h of collection. Between blood collection and processing, the samples were left at room temperature. Blood samples were then centrifuged at 3000g for 10 min. Serum samples (only from the seven cases mentioned above) and buffy coat samples were then collected. Plasma samples were further centrifuged in a microcentrifuge (16 000g; Eppendorf Centrifuge 5415D) for 10 min to remove the remaining blood cells (4). DNA extraction was then performed immediately.

DNA Extraction
DNA was extracted using the QIAamp Blood Kit (Qiagen) according to the “blood and body fluid protocol” as recommended by the manufacturer (5). For DNA extraction, we used 400 or 800 μL of the plasma and serum samples, or 400 μL of the buffy coat sample for each column. The DNA was then stored at –20 °C until further processing.

Real-Time Quantitative PCR

The theoretical and practical aspects of real-time quantitative PCR have been described in detail elsewhere (6–8). Real-time quantitative PCR analysis was performed using a PE Applied Biosystems 7700 Sequence Detector.

Plasma and serum DNA was subjected to real-time quantitative PCR assays for the SRY and β-globin genes as described previously (6). For each amplification, we used 5 μL of extracted plasma or serum DNA or 200 ng of buffy coat DNA. Duplicate amplification was performed for each sample, and the mean result was used for further analysis. In addition, a calibration curve was prepared and included with each amplification.

The expression of quantitative results was as described previously (6). The DNA concentration was expressed as genome-equivalents/mL by use of a conversion factor of 6.6 pg of DNA per cell (6). One genome-equivalent was defined as the amount of a particular target sequence contained in a single diploid human cell.

Numerical Analysis of Results

Because the SRY gene is found in all nucleated cells of males only, whereas the β-globin gene is present in all nucleated cells of both males and females (6), we calculated the percentage of male DNA in a particular plasma or buffy coat sample, denoted as Y%, using the following equation:

\[
Y\% = \frac{SRY}{\beta\text{-globin}} \times 100\%
\]

where SRY and β-globin denote the quantities of the SRY and β-globin sequences measured by the respective real-time PCR assays.

Assay Imprecision

To determine the imprecision of the Y% values, buffy coat DNA from a healthy male and a healthy female was mixed in different proportions to obtain DNA mixtures with different SRY concentrations. Three DNA mixtures were prepared, with 50%, 10%, and 1% Y-chromosome DNA, respectively. The DNA mixtures were then subjected to 20-replicate SRY and β-globin PCRs, and the percentage of male DNA was calculated for each replicate. A total of 200 ng of DNA was used for each PCR reaction.

Effects of Blood Sampling and Processing

To exclude the possibility that the release of cell-free DNA was an in vitro phenomenon, we performed three control experiments. We proposed that blood collection using a syringe and needle and blood-processing procedures after venesection might cause the liberation of DNA from blood cells, leading to an apparent change in the cell-free DNA concentration. Blood-processing steps that were studied included the time delay in processing of the blood samples and centrifugation. Plasma samples were obtained from healthy volunteers for investigation of these three factors.

Collection using a syringe and needle. To examine whether blood collection using a syringe and needle might lead to the in vitro release of DNA, we performed the following experiment. After blood was collected from the control individuals, an aliquot was immediately subjected to
repeated aspiration by a syringe with a 23-gauge needle. The needle size was identical to that used for blood collection in the study involving bone marrow transplantation patients. The two aliquots were then immediately centrifuged at 3000g for 10 min and microcentrifuged at full speed (16 000g; Eppendorf Centrifuge 5415D) for 10 min to obtain the plasma. Plasma DNA was extracted from the aliquots and analyzed using real-time PCR for the \( \beta \)-globin gene.

**Time delay.** To determine whether a delay in processing of blood samples could contribute to the release of DNA from blood cells into the plasma, we performed a second experiment. After collection from healthy volunteers, the blood samples were immediately divided into three aliquots. The three aliquots were processed immediately, after a 3-h delay, or after a 6-h delay. The aliquots not processed immediately were left at room temperature. To obtain the plasma, the blood from samples were centrifuged at 3000g for 10 min and then microcentrifuged (Eppendorf Centrifuge 5415D) at full speed (16 000g) for 10 min to obtain the plasma. Plasma DNA was extracted from the aliquots and analyzed using real-time PCR for the \( \beta \)-globin gene.

**Centrifugation.** For the third experiment, we studied whether centrifugation of blood samples might cause the release of DNA from the blood cells. Immediately after venesection, blood samples from healthy volunteers were divided into three aliquots. The first aliquot was centrifuged at 3000g for 10 min and then microcentrifuged (Eppendorf Centrifuge 5415D) at full speed (16 000g) for 10 min to obtain the plasma. For the second aliquot, after the blood sample was centrifuged at 3000g for 10 min, it was remixed by gentle shaking of the tube. The blood was then recentrifuged at 3000g for 10 min before it was microcentrifuged (Eppendorf Centrifuge 5415D) at full speed (16 000g) for 10 min to obtain the plasma. The third aliquot was processed similar to the second aliquot, except that the pellet was resuspended by gentle pipetting after the first microcentrifugation and then the sample was subjected to a second microcentrifugation step. Plasma DNA was extracted from the aliquots and analyzed using real-time PCR for the \( \beta \)-globin gene.

**Statistical analysis**

Statistical tests were carried out using SigmaStat 2.0 software.

**Results**

**Assay imprecision**

The analytical intraassay CVs for the percentage of male DNA (Y%) in the DNA mixtures with different SRY concentrations were determined. The CVs for the DNA mixtures containing 50%, 10%, and 1% Y-chromosome DNA were 5.0%, 10%, and 17%, respectively.

**Genetic origin of plasma DNA in bone marrow transplantation recipients**

The results for the 22 sex-mismatched bone marrow transplantation cases are shown in Table 1. The percent-

<table>
<thead>
<tr>
<th>Patient</th>
<th>Gender of recipient</th>
<th>Gender of donor</th>
<th>Diagnosis</th>
<th>Month(s) after transplantation</th>
<th>Y cell % of buffy coat</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMT 1</td>
<td>M</td>
<td>F</td>
<td>AML</td>
<td>2</td>
<td>0.2</td>
</tr>
<tr>
<td>BMT 2</td>
<td>M</td>
<td>F</td>
<td>Wiskott–Aldrich syndrome</td>
<td>4</td>
<td>0.2</td>
</tr>
<tr>
<td>BMT 3</td>
<td>M</td>
<td>F</td>
<td>( \beta )-Thalassemia major</td>
<td>100</td>
<td>0.0</td>
</tr>
<tr>
<td>BMT 4</td>
<td>F</td>
<td>M</td>
<td>Aplastic anemia</td>
<td>14</td>
<td>73.8</td>
</tr>
<tr>
<td>BMT 5</td>
<td>F</td>
<td>M</td>
<td>AML</td>
<td>12</td>
<td>68.1</td>
</tr>
<tr>
<td>BMT 6</td>
<td>F</td>
<td>M</td>
<td>CML</td>
<td>37</td>
<td>100.0</td>
</tr>
<tr>
<td>BMT 7</td>
<td>F</td>
<td>M</td>
<td>ALL</td>
<td>10</td>
<td>68.4</td>
</tr>
<tr>
<td>BMT 8</td>
<td>M</td>
<td>F</td>
<td>ALL</td>
<td>24</td>
<td>0.0</td>
</tr>
<tr>
<td>BMT 9</td>
<td>M</td>
<td>F</td>
<td>( \beta )-Thalassemia major</td>
<td>45</td>
<td>12.2</td>
</tr>
<tr>
<td>BMT 10</td>
<td>F</td>
<td>M</td>
<td>Fanconi anemia</td>
<td>10</td>
<td>63.4</td>
</tr>
<tr>
<td>BMT 11</td>
<td>M</td>
<td>F</td>
<td>ALL</td>
<td>2</td>
<td>0.2</td>
</tr>
<tr>
<td>BMT 12</td>
<td>M</td>
<td>F</td>
<td>( \beta )-Thalassemia major</td>
<td>99</td>
<td>1.5</td>
</tr>
<tr>
<td>BMT 13</td>
<td>M</td>
<td>F</td>
<td>( \beta )-Thalassemia major</td>
<td>54</td>
<td>0.3</td>
</tr>
<tr>
<td>BMT 14</td>
<td>F</td>
<td>M</td>
<td>AML</td>
<td>28</td>
<td>81.2</td>
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<tr>
<td>BMT 15</td>
<td>M</td>
<td>F</td>
<td>( \beta )-Thalassemia major</td>
<td>103</td>
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</tr>
<tr>
<td>BMT 16</td>
<td>M</td>
<td>F</td>
<td>MDS</td>
<td>28</td>
<td>0.0</td>
</tr>
<tr>
<td>BMT 17</td>
<td>F</td>
<td>M</td>
<td>AML</td>
<td>53</td>
<td>56.9</td>
</tr>
<tr>
<td>BMT 18</td>
<td>F</td>
<td>M</td>
<td>( \beta )-Thalassemia major</td>
<td>42</td>
<td>18.1</td>
</tr>
<tr>
<td>BMT 19</td>
<td>M</td>
<td>F</td>
<td>( \beta )-Thalassemia major</td>
<td>72</td>
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<tr>
<td>BMT 20</td>
<td>M</td>
<td>F</td>
<td>Chronic granulomatous disease</td>
<td>116</td>
<td>0.1</td>
</tr>
<tr>
<td>BMT 21</td>
<td>M</td>
<td>F</td>
<td>ALL</td>
<td>13</td>
<td>0.0</td>
</tr>
<tr>
<td>BMT 22</td>
<td>M</td>
<td>F</td>
<td>ALL</td>
<td>1</td>
<td>0.0</td>
</tr>
</tbody>
</table>

*AML, acute myelogenous leukemia; CML, chronic myelogenous leukemia; ALL, acute lymphoblastic leukemia; MDS, myelodysplastic syndrome.*
age of Y-chromosome DNA (Y cell%) in the Buffy coat, as illustrated in Table 1, was determined by real-time PCR and verified by fluorescence in situ hybridization. There was a significant correlation between the concentration of the SRY gene as determined by real-time PCR and the Y cell% as determined by fluorescence in situ hybridization (Pearson product moment correlation, $r = 0.878$; $P = 7.74 \times 10^{-8}$). Analysis of the percentage of Y-chromosome DNA in plasma indicated that this variable was different between the group of female patients receiving bone marrow from male donors and the group of male patients receiving bone marrow from female donors (Fig. 1). The median percentages of Y-chromosome DNA in the plasma in the former and the latter groups were 59.5% and 6.9%, respectively (Fig. 1). The difference between these two groups was highly significant (Mann–Whitney rank-sum test, $P < 0.001$). In the former group, the SRY genes detected originated from the hematopoietic cells of the male donors. Hence, the percentage of Y-chromosome DNA in the plasma in this group of patients indicated the percentage of plasma cell-free DNA originating from the hematopoietic system. On the other hand, in the latter group, both the nonhematopoietic cells and the remaining hematopoietic cells of the male recipients could account for the SRY genes detected. However, in these male patients, the hematopoietic system was predominantly, and in many cases close to 100%, converted into female after transplantation (Table 1). Therefore, in this group of patients, the free SRY DNA mainly originated from the nonhematopoietic cells of the male recipients; hence, the percentage of Y-chromosome DNA in the plasma in these cases served as an indicator of the percentage of free plasma DNA originating from nonhematopoietic cells. We therefore concluded that there was a significant difference between the percentage of free plasma DNA arising from the hematopoietic system and that arising from the nonhematopoietic system, with the predominant source of free plasma DNA being hematopoietic cells.

**Comparison of Plasma DNA Concentrations in Transplant Recipients and Controls**

To determine whether bone marrow transplantation was associated with a quantitative aberration in the concentrations of circulating DNA, we compared the total plasma DNA concentrations of the bone marrow transplantation patients and controls. The median DNA concentrations of the controls and the patients studied were 1257.6 and 1171.4 genome-equivalents/mL, respectively (Fig. 2). No significant difference was observed in these values (Mann–Whitney rank-sum test, $P = 0.833$).

**Concentration and Origin of Serum DNA**

We also compared the total DNA concentrations in the paired plasma and serum samples (Fig. 3, top panel). Compared with paired plasma samples, serum samples had a median 14-fold higher DNA concentration, with the median DNA concentrations in plasma and serum being 1195.1 and 16 344.8 genome-equivalents/mL, respectively. The difference was statistically significant (Mann–Whitney rank-sum test, $P < 0.001$). To determine the origin of these increased amounts of DNA in the serum, we compared the SRY DNA concentrations in paired plasma and serum samples. In those male patients receiving bone marrow from female donors (patients BMT 16 and BMT 19–22), the hematopoietic systems were converted, nearly 100%, to female (Table 1). Hence, the SRY genes detected in these serum samples predominantly originated from the nonhematopoietic cells of the male recipients. In these cases, the SRY DNA concentrations in plasma and serum showed no obvious difference (Fig. 3, bottom panel). For the cases involving female recipients and male donors (patients BMT 17 and 18), the SRY genes detected in the serum samples were derived from the hematopoietic cells of the male donors. In these cases, the SRY DNA concentrations in serum were obviously much higher than those in the matched plasma sample (Fig. 3, bottom panel). We, therefore, concluded that the higher

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**Fig. 1.** Box plot showing the percentage of Y-chromosome DNA (Y%) in the plasma of bone marrow transplantation patients.

The subject categories are shown on the x axis. The percentage of Y-chromosome DNA in the plasma (Y% of plasma) is plotted on the y axis. The upper and lower limits of the boxes and the lines inside the boxes indicate the 75th and 25th percentiles and the median, respectively. The upper and lower horizontal bars denote the 90th and 10th percentiles, respectively. ● indicate outliers.

**Fig. 2.** Box plot of plasma DNA concentrations in controls and bone marrow transplantation patients.

The subject categories are shown on the x axis. Plasma DNA concentrations (genome-equivalents/mL) as determined by real-time quantitative PCR for the β-globin gene are plotted on the y axis. The upper and lower limits of the boxes and the lines inside the boxes indicate the 75th and 25th percentiles and the median, respectively. The upper and lower horizontal bars denote the 90th and 10th percentiles, respectively. ● indicate outliers.
total DNA concentrations in serum compared with plasma was attributable to the release of DNA by hematopoietic cells during the clotting process.

PREANALYTICAL VARIABLES

Syringe and needle. We postulated that blood drawing using a syringe and needle might cause the ex vivo release of DNA from the blood cells. We therefore performed a control experiment to investigate this preanalytical variable. The DNA concentrations in the plasma samples obtained from blood aspirated into the syringe different numbers of times did not demonstrate any statistically significant difference (Wilcoxon signed-rank test, \( P = 0.844 \); Fig. 4).

Time delay. A control experiment was carried out to examine the possibility that a delay in processing blood samples might lead to the release of DNA in vitro before analysis. Quantitative results for the \( \beta \)-globin gene in the plasma samples obtained from blood processed at different time points are shown in Fig. 5. No significant difference was found between the DNA concentrations in the plasma samples obtained at 0 and 3 h after collection (Wilcoxon signed-rank test, \( P = 0.383 \)). The same result was observed when we compared the DNA concentrations of the plasma samples obtained at 0 and 6 h after collection (Wilcoxon signed-rank test, \( P = 0.250 \)).

Centrifugation. Another control experiment was performed to investigate whether centrifugation during the processing of blood samples might cause the release of DNA from blood cells. Fig. 6 shows the quantitative
results for the \( \beta \)-globin gene in the plasma samples obtained by centrifugation of blood different numbers of times. We compared the DNA concentration in the plasma samples obtained by centrifugation once and that obtained by centrifugation twice. No significant difference was observed (Wilcoxon signed-rank test, \( P = 0.563 \)). There also was no significant change in the DNA concentration when the plasma samples were centrifuged twice and microcentrifuged twice (Wilcoxon signed-rank test, \( P = 0.563 \)).

**Discussion**

In this study, using a sex-mismatched bone marrow transplantation model, we have shown that most of the plasma DNA is of hematopoietic origin in these transplant recipients. Apart from answering a fundamental question in the plasma DNA field, this work also has several biological and technical implications.

We have painstakingly taken precautions to address the possibility that the generation of cell-free DNA may be an in vitro phenomenon attributable to manipulations during the blood-collection and sample-processing steps. We studied three possible factors that might contribute to our observations: blood collection using a syringe and needle, a delay in processing of blood samples, and centrifugation during blood sample processing. With regard to the first factor, we considered the possibility that blood collection using a syringe and needle might damage blood cells, thus causing their DNA to be released into the plasma. Regarding the second factor, time delay, neutrophils in EDTA blood could undergo apoptosis when stored at room temperature (9). This might lead to the release of DNA in vitro before analysis and an overestimation of the proportion of free circulating DNA originating from hematopoietic cells.

Another factor that had attracted our interest was the effect of centrifugation during blood sample processing. During centrifugation, cells might be damaged and DNA released. This might falsely increase the proportion of plasma DNA originating from hematopoietic cells. We therefore performed control experiments to investigate these factors. There was no significant difference in the DNA concentrations in samples either with or without these treatments. These results suggested that the three factors proposed did not measurably contribute to the preanalytical release of DNA in vitro. In other words, the release of cell-free DNA was probably an in vivo phenomenon. Although it is impossible for us to investigate all potential preanalytical variables that could lead to the release of DNA from blood cells, we believe that we have studied the three most likely candidate variables. Furthermore, this sex-mismatched bone marrow transplantation model can provide a platform to test other potential preanalytical variables in the future.

An important question that has arisen from our results is whether these data can be extrapolated to the situation in healthy individuals who have not had bone marrow transplantation. In this regard, we compared the concentrations of plasma DNA in patients after bone marrow transplantation and in healthy individuals and did not find a significant difference in circulating DNA concentrations between these two groups (Fig. 2). Thus, we believe that our results have general implications to circulating DNA in healthy individuals. However, whether the results of this study can be generalized to all cases of free circulating DNA requires further investigation. For example, whether DNA derived from hematopoietic cells contributes a significant proportion of plasma DNA in individuals with cancer (1) or during pregnancy (6) remains to be elucidated. In this regard, it would be informative to analyze sex-mismatched bone marrow transplantation recipients whose transplant is complicated with a secondary malignancy (10) as well as those who become pregnant after transplantation (11).

It has been known for quite some time that serum contains a higher DNA concentration than plasma (6). Previous publications have hypothesized that the release of DNA during blood clotting was a potential mechanism for this observation (6). However, others have interpreted these results differently. For example, Honda et al. (12) have attributed these results to a higher efficiency of DNA extraction from serum. Using sex-mismatched bone marrow transplantation model, we have shown conclusively that the additional DNA in serum, compared with a paired plasma sample, was of hematopoietic origin. Lee et al. (13) have recently attempted to prove the same point by adding male cells to female blood. However, in comparison with this highly artificial system, we believe that our approach is a much more “physiologic” one for elucidating this important point.

To date, studies on bone marrow transplantation chimerism have relied on the investigation of whole blood samples (14–17). Our study suggests that plasma could act as an alternative sample type for such investigations.
Hence, similar to whole blood samples (16), plasma may also be used clinically for the evaluation of relapse after bone marrow transplantation. It would also be very interesting to obtain serial data on the variation in plasma DNA concentrations during the bone marrow transplantation procedure. It would be particularly informative to document the temporal variation in the ratio of donor to recipient DNA, from the marrow ablation stage to the eventual complete hematopoietic reconstitution.

In conclusion, we have described a bone marrow transplantation model for investigation of the main source of free circulating DNA. We showed that most of the free DNA in plasma and serum originated from hematopoietic cells. However, the process by which DNA is released from hematopoietic cells remains to be elucidated. One possibility is the apoptosis of hematopoietic cells. Another possibility is active release by certain cell populations, such as lymphocytes (18). Future work can also be performed to investigate the proportion of free circulating DNA accounted for by the different lineages of hematopoietic cells. In addition, apart from the bone marrow transplantation model described, it would be interesting to look at the proportions of free circulating DNA originating from different organs, using different sex-mismatched organ transplantation models (3).

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References