Summary. Scant knowledge exists concerning lineage-restricted mixed chimerism (mCh) after allogeneic peripheral blood stem cell transplantation (PSCT) in patients with chronic idiopathic myelofibrosis (CIMF). Following a sex-mismatched PSCT, a combined immunopheno- and genotyping by fluorescence in-situ hybridization (FISH) was performed on sequential bone marrow (BM) biopsies at standardized intervals. Results were compared with PCR analysis of corresponding peripheral blood samples in five patients. According to FISH, pretransplant specimens revealed a gender congruence of more than 99%, while in the first three months the total BM exhibited a persistent fraction of host cells (30% to 40%) with a tendency to decline after about one year. It is noteworthy that the majority of endothelial cells maintained a recipient origin, whereas CD34+ progenitors and especially CD61+ megakaryocytes exhibited only very few host-derived cells. In keeping with the prevalence of donor cells in the hematopoietic compartment, PCR analysis of peripheral blood cells displayed a non-significant degree of mCh.

In conclusion, according to FISH and PCR analysis, successful PSCT in CIMF results in an almost complete chimeric (donor-derived) state of the hematopoietic cell population. The non-transplantable stromal compartment includes the vascular endothelium with a predominance of recipient cells. The minimal mCh of this population implies probably a donor-derived origin (endothelial progenitor cells).

Key words: Mixed chimerism, CD34+ progenitors, Megakaryocytes, Endothelial cells, Peripheral stem cell transplantation, Chronic idiopathic myelofibrosis, Bone marrow biopsies

Introduction

In chronic idiopathic myelofibrosis (CIMF) relatively little information exists regarding mixed chimerism (mCh) following allogeneic bone marrow (BM) or stem cell transplantation (Deeg et al., 2003; Rondelli et al., 2005). A characteristic feature of histopathology in this disorder is the fibrous matrix of the BM that usually includes prominent vessels (Thiele et al., 2001). On the other hand, as has been repeatedly demonstrated, fibroblasts are no part of the leukemogenic (clonally transformed) process underlying the myeloproliferative disorders (Greenberg et al., 1978; Golde et al., 1980; O'Brien et al., 1988). However, controversy continues about the donor or host (recipient) origin of stroma constituents following myeloablative therapy and subsequent transplantation (Simmons et al., 1987; Athanasou et al., 1990; Agematsu and Nakahori, 1991; Santucci et al., 1992). It has been argued that engraftment of BM stroma cell precursors does not occur and that host stromal cells survive the various conditioning regimens applied before transplantation procedures (Athanasou et al., 1990). In this context, angiogenesis in CIMF is of special interest not only for its relationship with the progression of myelofibrosis (Reilly et al., 1985; Thiele et al., 1992; Kvasnicka and Thiele, 2004), but for its crucial role concerning restitution and maintenance of hematopoiesis (Davis et al., 1995; Mohle et al., 1999). A wealth of data has been accumulated concerning the functional properties of vascular structures, in particular the endothelial cells that serve as gatekeepers by controlling the trafficking and homing of progenitors (Simmons et al., 1992; Mohle et al., 1999). For this reason, a study was performed on gender-related mCh involving patients with CIMF in the early and late posttransplant period. Analysis included peripheral blood cells determined by the polymerase chain reaction (PCR) technique in comparison with the lineage...
restricted chimeric state of CD34+ endothelial and progenitor cells as well as megakaryocytes by using the fluorescence in-situ hybridization (FISH) method.

**Material and methods**

**Patients**

A total of five patients (four men, one woman; age 45 years) with CIMF and a sex-mismatched transplantation constellation were enrolled into this study. All patients had a history of various therapeutic regimens including hydroxyurea, busulfan as well as radiation or a combination of these. Following dose-reduced myeloablative therapy that has been described in detail in previous studies (Kröger et al., 2005; van Besien and Deeg, 2005), patients received a peripheral blood stem cell transplantation (PSCT) with a median number of transplanted CD34+ progenitors of 8x106 per kg body weight (range 0.9-15.6) derived either from HLA-identical siblings or matched unrelated donors. Further information of this cohort which was derived from a larger prospective and clinically controlled trial have already been reported (Kröger et al., 2005). Explicit approval of this study was obtained from the Institutional Review Board on Medical Ethics at Essen University Hospital.

**Bone marrow biopsies**

Representative BM trephine biopsies (mean size 17.5±1.8 mm²) were performed at standardized intervals from the posterior iliac crest. The fixation of samples was carried out in a low-concentrated phosphate-buffered formalin solution for 12-48 hours. Further processing included decalcification for 3-4 days in 10% buffered ethylene-diamine tetra-acetic acid (EDTA), pH 7.2, paraffin wax embedding, and employment of several staining techniques, involving Giemsa, PAS (periodic acid Schiff reagent), naphthol-AS-D-chloroacetate esterase, Perls’ reaction for iron and a silver impregnation method (Gomori’s technique).

**Simultaneous immunostaining and dual color FISH**

For a simultaneous immunophenotypic and genotypic evaluation, 4 µm paraffin-embedded sections were dewaxed through xylene, air-dried, microwave-heated in an appropriate buffer (100mM Tris/50mM EDTA, pH 7.0) for 4 min, cooled down and rinsed twice in Tris (HCl/0.05% Tween 20 buffer, pH 7.6) at 4°C. Subsequently, to detect progenitor cells and endothelial cells immunostaining with CD34+ (Soligo et al., 1991) was performed and to identify megakaryopoiesis CD61+ (Gatter et al., 1988) monoclonal antibodies were applied by using the APAAP method (Cordell et al., 1984). 5-Bromo-4-chloro-3-indolyl-phosphatase (BCIP) and nitroblue tetrazolium (NBT; Sigma, Deisenhofen, Germany) were used as chromogenic substances. Tissue sections were dehydrated through graded ethanol solutions (70%, 90% and 100%), air-dried and permeabilized by microwave treatment, followed by 0.25 mg/ml proteinase K digestion (Sigma) for 3 min at 37°C before hybridization (Bull and Harnden, 1999). After this procedure, the slides were quickly transferred to 70% ethanol at 4°C, dehydrated in 100% ethanol and then air-dried. A 10-µl aliquot of a directly conjugated satellite probe mix for chromosomes x and y (CEP X Spectrum Orange/CEP Y Spectrum Green DNA Probe Kit or CEP X Spectrum Green/CEP Y Spectrum Orange DNA Probe Kit, respectively; Vysis, Bergisch Gladbach, Germany) was placed on a coverslip, picked up onto the slide and incubated at 73°C for 5 min on a hot plate. Because green fluorescence is more conspicuous than a red signal, following a pilot study (Table 1), correspondingly mismatched probes for the x- and y-chromosomes (green versus red signals) were also applied. After denaturation, the slides were quickly brought to 37°C, sealed with rubber cement, and then placed in a humidified box for 16 h at 42°C. Coverslips were removed, and slides were placed in wash buffer (0.4xSSC) at 73°C for 2 min, immersed in 2xSSC/0.01% NP 40 at room temperature for 1 min and then air-dried, before mounting in 0.125Fg DAPI/ml Antifade (DAPI II; Vysis). The slides were evaluated with an Aristoplan® microscope (Leitz, Wetzlar, Germany) equipped with an optimized triple bandpass filter and imaged with a digital camera (Photometrics SenSys; Tucson, Ariz., USA) and appropriate software (IPLab Spectrum P, Vienna, Va., USA). Accordingly, positive (red: x-chromosome and green: y-chromosome signals) signals were differentiated following DNA hybridization (sex typing) at the various endpoint intervals of pre- and posttransplant examinations (sequential biopsies) by counting explicitly only those cells containing two marked signals.

**PCR analysis**

Real-time quantitative PCR for the detection of the y-chromosome was performed on peripheral blood cells by following methods that were formerly described (Fehse et al., 2001). According to this technique, sensitivity was very high to identify male cells present in very low proportions (i.e. 1 male in 10⁶ female cells) (Fehse et al., 2001).

**Results**

Immunophenotyping of CD34+ endothelial cells, progenitors and CD61+ megakaryocytes revealed a distinctive staining pattern which was a basic mean for further FISH analysis. Thus, dual color FISH with a simultaneous demonstration of proper signals visualizing the x- and y-chromosomes was easily accomplished (Fig. 1a-h). A pilot study in a female patient with CIMF that received an allogeneic PSCT from a male donor showed a strikingly variable extent of mCh in the
different cell populations and also during the posttransplant period (Table 1). A total congruence with the female gender of this patient was shown in the 120 cells evaluated in the pretransplant specimen. On the other hand, in the early posttransplant period (up to about three months), about one third of all evaluated BM cells still displayed a host-derived sextyping that significantly declined in quantity after one year. A very different constellation was found in the vascular endothelium, where less than 10% of cells exhibited a donor origin. In contrast to the stroma cell compartment and the total cell count hematopoiesis, i.e. CD34+ progenitors and CD61+ megakaryocytes that include also precursors, showed an only minor fraction (less than 10%) of residual host-derived cells.

On the other hand, PCR analysis of peripheral blood cells exhibited a percentage of recipient cells ranging between 0% and 1% at the corresponding checkpoints of the early and late posttransplant period, corresponding with an almost complete chimeric state.

Following this preliminary investigation which clearly demonstrated a strikingly expressed discrepancy of mCh, we analyzed a significantly larger number of cells in four male patients with CIMF and a gender-mismatched graft constellation after PSCT (Table 2). Here, first of all, the sensitivity of our FISH method according to gender congruence was established to exceed 99%. The total cell count, including hematopoietic as well as stroma cells, revealed a persistent host origin ranging between 30% to 40% with a tendency to decrease after about one year (Table 2). Again, the vast majority of endothelial cells maintained a recipient type with only very few samples apparently generated from the donor (Fig. 1a,b). This incidence differed significantly when studying CD34+ progenitor cells (Fig. 1c,d) and megakaryopoiesis including precursors, i.e. CD61+ immature micromegakaryocytes (Fig. 1e,f) as well as mature megakaryocytes (Fig. 1g,h). In the hematopoietic cell compartment the situation was reversed, with less than 10% of host-derived cells in the

Table 1. Female patient with a sex-mismatched (donor male) graft constellation following allogeneic peripheral blood stem cell transplantation (PSCT) for chronic idiopathic myelofibrosis - pilot study with relative incidence (%) of sextyping based on the evaluation of at least 100 (up to 600) bone marrow (BM) cells in each category.

<table>
<thead>
<tr>
<th>Endpoint (day)</th>
<th>All BM cells</th>
<th>CD34+ progenitor cells</th>
<th>CD61+ megakaryocytes</th>
<th>Endothelial cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>xy xx</td>
<td>xy xx</td>
<td>xy xx</td>
<td>xy xx</td>
</tr>
<tr>
<td>Before PSCT</td>
<td>190</td>
<td>0 100</td>
<td>0 100</td>
<td>0 100</td>
</tr>
<tr>
<td>After PSCT</td>
<td>20</td>
<td>74 26</td>
<td>100 0</td>
<td>100 0</td>
</tr>
<tr>
<td></td>
<td>81</td>
<td>69 31</td>
<td>92 8</td>
<td>100 0</td>
</tr>
<tr>
<td></td>
<td>290</td>
<td>63 17</td>
<td>91 9</td>
<td>100 0</td>
</tr>
<tr>
<td></td>
<td>376</td>
<td>88 12</td>
<td>94 6</td>
<td>97 3</td>
</tr>
</tbody>
</table>

Table 2. Relative incidence (%) of mixed chimerism and evaluated number (No.) of cells at each checkpoint in the bone marrow (BM) following sex-mismatched allogeneic peripheral stem cell transplantation (PSCT) in four men with chronic idiopathic myelofibrosis (gender-graft constellation: host male - donor female).

<table>
<thead>
<tr>
<th>Endpoint (ranges, days)</th>
<th>All BM cells</th>
<th>CD34+ progenitor cells</th>
<th>CD61+ megakaryocytes</th>
<th>Endothelial cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. xx xy</td>
<td>No. xx xy</td>
<td>No. xx xy</td>
<td>No. xx xy</td>
</tr>
<tr>
<td>Before PSCT</td>
<td>6-40</td>
<td>601 3 997</td>
<td>92 0 100</td>
<td>174 0 100</td>
</tr>
<tr>
<td>After PSCT</td>
<td>20-50</td>
<td>363 60 40</td>
<td>61 95 5</td>
<td>118 100 0</td>
</tr>
<tr>
<td></td>
<td>80-200</td>
<td>380 64 36</td>
<td>77 95 5</td>
<td>94 98 2</td>
</tr>
<tr>
<td></td>
<td>300-400</td>
<td>507 67 33</td>
<td>58 90 10</td>
<td>82 99 1</td>
</tr>
</tbody>
</table>

Fig. 1. Sex-genotyping of endothelial cells and hematopoiesis in the bone marrow after PSCT in patients with CIMF. Immunophenotyping of the different cells is shown in the left panel and FISH analysis (right panel) reveals either red signals (x-chromosomes) or green signals (y-chromosomes) indicating mCh. a. CD34+ Endothelial lining of a vascular lumen (L) is surrounded by hematopoietic cells in a female host. A donor origin (b) is displayed in two endothelial cells (arrows) and a maintained recipient genotype in a hematopoietic cell (arrow head). c. CD34+ progenitor cell in a female host (arrow) revealing a still persistent recipient genotype (arrow) after transplantation. d. Immature CD61+ megakaryocyte precursor in a male patient (e) demonstrates (arrow) a host origin (f), in contrast to a large hyperlobulated mature megakaryocyte (g) showing (arrow) a female donor genotype (h). a-d, x 670; e-h, x 1,500
primitive precursor cell population and only single recipient cells in the more differentiated megakaryopoietic lineage (Table 2). Relating this very low frequency to the corresponding data of the evaluable total cell count, it is reasonable to assume that the majority of maintained host cells in the posttransplant period belong to the stromal compartment.

In keeping with the prevalence of complete donor chimerisms in the hematopoietic cell population (progenitors, megakaryocytes) PCR analysis performed exactly at the same checkpoints revealed an incidence of mCh ranging between 0% to 6%.

**Discussion**

Following a gender-mismatched transplantation, concurrent characterization of genotype and cell lineage provides a suitable means for the identification of chimeric states and thus, the host/donor origin of a certain cell population (Thiede et al., 2004). Although a number of techniques are currently available to document definitely donor cell engraftment and residual host cells, PCR and FISH are the most popular methods. It is well known that PCR analysis yields results readily and in a short time, and is certainly the more sensitive technique, because it allows the detection of one abnormal cell in 10⁶ cells (Fehse et al., 2001; Alizadeh et al., 2002; Thiede et al., 2004). However, a significant disadvantage is that in CIMF related to BM fibrosis (dry tap) this method is usually applied on peripheral blood cells and that no characterization of lineage-restricted mCh is possible. This shortcoming precludes, among others, the labeling of endothelial cells and the recognition of their chimeric state indicating their CD34+ progenitor cell origin (Gehling et al., 2000). In contrast, FISH allows the identification of mCh in single immunophenotyped cells (Kvasnicka et al., 2003). On the other hand, this technique may lead to false-positive and false-negative results ranging between 0.2% and up to 4.0% based on results gained from smears or diluted cell preparations (Koegler et al., 1995; Rondon et al., 1997). Moreover, the results of FISH analysis (Rondon et al., 1997; Smith et al., 1999; Tamura et al., 2000) have to be discussed very critically (Kvasnicka et al., 2003), especially in cases where the investigators restrict their evaluations only on the identification of the Y-chromosome (Mackinnon et al., 1994). We would like to pinpoint that our applied dual-colored sextyping analysis failed to reveal any relevant discrepancies (false positive signals 0.3%) between gender and positive labeling in the pretransplant specimens (Tables 1, 2). Finally, it has to be emphasized that the methodology of transplantation exerts a significant impact on the extent of mCh (Elmaagacli et al., 2001), and therefore only results derived from series with corresponding therapeutic strategies, especially including conditioning regimens, are comparable.

The labeling of peripheral blood cells and genotyping by FISH has been repeatedly performed to monitor mCh in CML patients (Smith et al., 1999; Tamura et al., 2000) and posttransplantation donor-recipient constellations were assessed by means of DNA microsatellite analysis (Thiede et al., 1999) in CIMF (Deeg et al., 2003; Rondelli et al., 2005). In CML the presence of a small amount of host-derived (clonally transformed) CD34+ progenitors that significantly exceeded the more differentiated cell lineages like the megakaryopoiesis, stimulated a discussion about so-called tumor dormancy even in patients after successful transplantations (Uhr et al., 1997; Holyoake et al., 1999). Some data indicate that in CML patients with a complete cytogenetic remission, a minority of bcr/abl+ cells were maintained (Chomel et al., 2000; Kitzis et al., 2001). It is tempting to speculate that these clonally transformed progenitor cells may possibly be the source of a later relapse and presumably may also differentiate into endothelial cells. This complex, until now ill-defined, pathomechanism determining the oncogenic potential of hematopoietic progenitors is probably governed by a variety of mediators, such as specific immune response (Oka et al., 1998). When compared with the relevant data on the CD34+ progenitor cell compartment (Table 2) results gained from this study were consistent with a significantly lower incidence of persistent host-derived megakaryocytes that nearly reached the methodological limit (White and Sweeney, 1993; Koegler et al., 1995; Rondon et al., 1997). This striking quantitative difference points towards a minor degree or almost complete mCh in the more differentiated hematopoietic cell lineages, while a very small amount of the primitive cell population still harbors a clonally transformed (leukemogenic) fraction as a possible source of relapse.

By using long-term cell culture techniques and genotyping with x- and y-specific probes, a host (recipient) origin of BM fibroblasts has been reported after allogeneic BM transplantation (Simmons et al., 1987; Athanasou et al., 1990; Agematsu and Nakahori, 1991; Santucci et al., 1992). These findings supported the argument of a non-transplantability of stroma cells even in patients with a maintained hematopoietic reconstitution (Agematsu and Nakahori, 1991; Santucci et al., 1992). In CIMF with a prevalent fibrous matrix, the discrepancy between the extent of mCh concerning the quantity of analyzed cells derived from the peripheral blood (PCR analysis) versus all BM cells that definitely included a significant proportion of stroma cells (FISH analysis), are generally in keeping with this finding. It is reasonable to assume that a leukemic relapse will initiate in the BM and therefore the very first stages of transformation are hardly detectable by performing PCR on peripheral blood cells, at least in CIMF.

Regarding the stroma compartment, the vascular structures have to be discriminated. With the exception of the sinusoids, these are usually composed of at least two components of different origin, i.e. the endothelium and the cells of the larger vessel walls (i.e. myofibroblasts, adventitial cells of the capillaries and
arterioles). Following BM transplantation in CML, only a fraction of endothelial cells was demonstrated to be host-derived (bcr/abl+) and maintained, even after more than one year (Kvasnicka et al., 2003). Regarding this point, a conflict of opinion exists, because another group applying also the FISH method identified this cell population as being totally of host origin (Athanasou et al., 1999). In contrast, the results of several authors on the CD34+ progenitor origin of endothelial cells provided persuasive evidence that this peculiar cell compartment is not only the source of hematopoiesis, but plays a pivotal role in angiogenesis (Asahara et al., 1997; Choi et al., 1998; Gehling et al., 2000; Gunsilius et al., 2000). For this reason, as shown in this study, a minor degree of mCh has to be expected following transplantation procedures. All these results are in keeping with the finding of a certain, although small, quantity of donor-derived endothelial cells in the BM after transplantation (Gehling et al., 2000; Gunsilius et al., 2000; Kvasnicka et al., 2003) contrasting with the host origin of the myofibroblasts of the larger vessel walls (Kvasnicka et al., 2003). Moreover, convincing data have been recently accumulated supporting this concept that even in adult life so-called hemangioblasts or endothelial precursor cells of the peripheral blood are present, and contribute to the formation of new blood vessels (Shi et al., 1998; Hristov and Weber, 2004; Iwami et al., 2004; Schatteman, 2004; Murasawa and Asahara, 2005). Therefore, it is reasonable to assume that the minor mCh of the endothalial layer may probably derive from transplanted donor endothelial progenitor cells.

In conclusion, contrasting PCR on peripheral blood cells, FISH analysis of the BM reveals a striking quantity of mCh in the stroma compartment that survives conditioning. In this context, only a small amount of donor-derived endothelial cells is recognizable, while the chimeric state of the engrafted hematopoietic cell population is almost complete (less than 10%).

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References


polycythaemia vera: an immunomorphometric study on trephine biopsies of the bone marrow. Br. J. Haematol. 80, 227-234.

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Dualism of chimerism