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Review

Dynamics of lineage-restricted mixed chimerism following sex-mismatched allogeneic bone marrow transplantation

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Summary. Scant knowledge is available about the dynamics of lineage-specific mixed chimerism (Ch) following bone marrow transplantation (BMT). This review is focused on findings derived from bone marrow (BM) biopsies in patients with chronic myeloid leukemia (CML) including a sex-mismatched host/donor constellation. Appropriate techniques involved immunophenotyping by monoclonal antibodies to identify the various cell lineages, dual color fluorescence in situ hybridization (FISH) with x- and y-chromosome-specific DNA-probes and a proper detection system for a simultaneous labeling of the bcr/abl locus. A significant degree of Ch with more than 20% host CD34+ progenitors was found in the early and late (up to 200 days after BMT) posttransplant period. However, only 10% of these cells harbored the bcr/abl translocation gene. This result fits well with corresponding molecularbiological findings of so-called minimal residual disease. Conversion of Ch evolved during leukemic relapse with 90% host progenitors of which 50% revealed the bcr/abl locus. A Ch of nucleated erythroid percursors (5%) and CD68+ macrophages (8%) was expressed to a significantly lower degree. The slightly increased frequency found in CD61+ megakaryocytes (16%) was probably due to the polyploid state of these cells. Similar to the CD34+ progenitor cells abrupt changes from donor to host type was associated with an insidious transformation into recurrent leukemia. The CD34+ endothelial cells showed a minor degree of Ch, because donor-derived elements ranged from 18% to 25%. Leukemic relapse was characterized by an almost complete conversion of the endothelial cells to a host type. These findings point towards a CD34+ progenitor cell origin of the (leukemic) endothelial cell layer and suggests that their dysfunction may contribute to an expansion of the neoplastic clone.

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Introduction

Efficacy of myelo-ablative chemo- and radiotherapy preceding allogeneic stem cell and bone marrow transplantation (BMT) may be determined by the stage of chimerism (Ch). The persistence of only donor cells (full Ch) is consistent with a more effective conditioning regimen than when a mixture of both host (recipient) and donor cells (mixed Ch) is detectable. The relationship between mixed Ch and leukemic relapse continues to be a matter of controversy (Elmaagacli et al., 2001; Kitzis et al., 2001). Although this condition is not necessarily associated with a poor outcome, several groups have suggested that mixed Ch is often associated with an increased risk to develop leukemia (Offit et al., 1990; Sawyers et al., 1990; Mackinnon et al., 1994; Radich et al., 1995; Roman et al., 1998; Serrano et al., 2000; Tamura et al., 2000). In this context with the exception of T- and B-lymphocytes (Przepiorka et al., 1990; Mackinnon et al., 1994; Rondon et al., 1997; Zetterquist et al., 2000) and rather ill-defined myelomonocytic cells (Roux et al., 1992; Koegler et al., 1995; Serrano et al., 2000) in most studies only peripheral blood cells were evaluated. On the whole the issue of lineage-restricted Ch of bone marrow (BM) cells has been rarely and until now not very systematically addressed in the pertinent literature or was limited to the time of leukemic relapse and a very few patients (Baurman et al., 1998).

A variety of techniques are available to determine the state of Ch of the recipient patient after BMT, including conventional cytogenetics, red blood cell antigen typing, molecularbiological methods (restriction fragment length polymorphism, real-time quantitative polymerase chain reaction - PCR) and genotyping in

patients with a sex-mismatched donor/host constellation (Fishleder et al., 1992; Roux et al., 1992; Martinelli et al., 1993; Wilborn et al., 1993; Radich et al., 1995; Hessel et al., 1996; Serrano et al., 2000; Elmaagacli et al., 2001; Alizadeh et al., 2002). In these patients fluorescence in-situ hybridization (FISH) technique involving y- and x-specific probes are frequently applied to document Ch and thus the host or donor origin (Durnam et al., 1989; Przepiorka et al., 1990; Dewald et al., 1993; Wessman et al., 1993; Nagler et al., 1994; Koegler et al., 1995; Palka et al., 1996; Rondon et al., 1997; Smith et al., 1999; Tamura et al., 2000). In combination with appropriate immunophenotyping FISH analysis may be used to investigate and monitor lineage-restricted mixed Ch in blood and BM cells during the posttransplant period and especially in patients with suspected or evolving leukemic relapse. Scant knowledge about the involvement of properly defined cell populations and their presumptive differences regarding the extent and dynamics of mixed Ch certainly warrants a review on recently obtained data on this fascinating phenomenon.

Methodology

When trying to compare the results communicated in the relevant literature certain aspects of applied techniques have to be taken into account. First of all quality of the chromosome probes should be discussed more critically and related to suitable control specimens. According to corresponding data approximately 0.1 to 3% false positive or negative labelings were found when using either y- or x-specific probes alone (Przepiorka et al., 1990; Wessman et al., 1993; Koegler et al., 1995). To improve this shortcoming dual color fluorescence technique applying both gene probes simultaneously and in combination with immunostaining for detection of distinctive cell lineages should be entered into the study design (Johnson et al., 2000; Wickenhauser et al., 2002). Contrasting the situation in smears, in histological slides the adverse influence of a certain section level on the recognition of positive stainings should be neutralized by regarding only cells that show two characteristic signals. Consequently, opposed to the significantly larger number of specific BM cells that may be easily identified by morphometry, evaluations have to focus on the usually smaller fraction of cells revealing two corresponding labelings (Fig. 1a). When following these stringent procedures more than 99.5% of 1,383 counted hematopoietic cells composing normal BM and CML specimens were reported to reveal an appropriate sex-typing (Wickenhauser et al., 2002). After BMT in several patients with CML persistence of residual host hematopoiesis including lineage-restricted cell compartments was repeatedly noticed. This phenomenon occurred not only in the B- and T-lymphocyte population (Przepiorka et al., 1990; Mackinnon et al., 1994; Koegler et al., 1995; Rondon et al., 1997; Serrano et al., 2000; Alizadeh et al., 2002), but also in myelomonocytic cells at day +7 (16%), day +18 (7 %) and even beyond day +28 (0.5-1%) following BMT (Koegler et al., 1995). Comparison of these data with other results on $CD34^+$ progenitors demonstrates a relatively higher degree of mixed Ch in our patients, in particular in the late posttransplant period (Table 1). On the other hand, even during complete remission a relatively stable and significant number of host cells could be detected until two years after successful BMT, thus indicating that this feature is normally developed in the mononuclear or myeloid cell series (Roux et al., 1992). Although quantity of BM mononuclear cells of the host type decreases steadily, in general agreement with our calculations comprising very different hematopoietic cell lineages (Tables 1-4) more than 1% were still identifiable as late as day +252 after BMT (Przepiorka et al., 1990). Therefore the rather frequently occurring incidence of cell lineage specific mixed Ch can be regarded as a hallmark to determine the effectiveness of various conditioning regimens (myelo-ablative treatment modalities) to eradicate the clonally (leukemic) transformed cells and (as an unwanted side effect) also the residual normal hematopoiesis.

Moreover, it should be explicitly ruled out that other potential causes of mixed Ch which might be associated with blood transfusion and especially pregnancy (bidirectional fetomaternal cell trafficking) or both may impair the assessment of this phenomenon. Because fetal $CD34^+$ and $CD38^+$ stem cells persist in the peripheral

Table 1. Mixed chimerism of $CD34^+$ progenitor cells in the bone marrow derived from 10 patients with CML after sex-mismatched bone marrow transplantation (BMT) and successful engraftment. CML control specimens before BMT revealed an appropriate genotyping of the 60 $CD34^+$ progenitor cells evaluated.

DAYS AFTER BMT	No. OF PATIENTS	No. OF PROGENITORS	PROGENITOR CELLS	
			Host-type	Donor-type
≥ 10 - 40	6	74	16 (22%)	58 (78%)
> 60 - 200	4	114	30 (26%)	84 (74%)
Total	10	241	54 (22%)	187 (78%)

blood in healthy women for many years following delivery of a male child (Bianchi et al., 1996; Johnson et al., 2001; Srivatsa et al., 2001) or artificial termination of pregnancy with a male fetus (Bianchi et al., 2001), one should always enter male recipient patients into relevant investigations. Finally, it was speculated that the often reported positive PCR findings in the posttransplant period (minimal residual disease) could be due to bcr/abl transformed cells that are not clonogenic, but may be more resistant to myelo-ablative treatment than others and thus linger as a quiescent cell population in the BM after transplantation (Kohler et al., 1990; Sawyers et al., 1990; Delage et al., 1991; Roth et al., 1992; Cross et al., 1993; Miyamura et al., 1993; Miyamura et al., 1994; Radich et al., 1995; Chomel et al., 2000; Serrano et al., 2000; Alizadeh et al., 2002). For this reason, interpretation of results should not only include immunopheno- and sex-typing, but also the visualization of the relevant bcr/abl translocation gene during the posttransplant period (successful engraftment versus leukemic relapse). Accordingly, the bcr/abl gene fusion was analysed performing FISH with a special extrasignal translocation probe (Sinclair et al., 1997). This probe produces a red and a green signal for the nontranslocated genes, a yellow mixed signal for the translocated locus and an additional smaller red signal for the truncated abl residue in cells possessing the translocation. Therefore, a random overlap of red and green markers can be excluded.

Successful engraftment

CD34⁺ progenitor cells

The CD34$^+$ progenitor cells play a pivotal role during BMT including homing, self-renewal, proliferation and differentiation into various lineages. However, until now there is very little information available about a putative mixed Ch and bcr/abl expression of this most important BM cell population

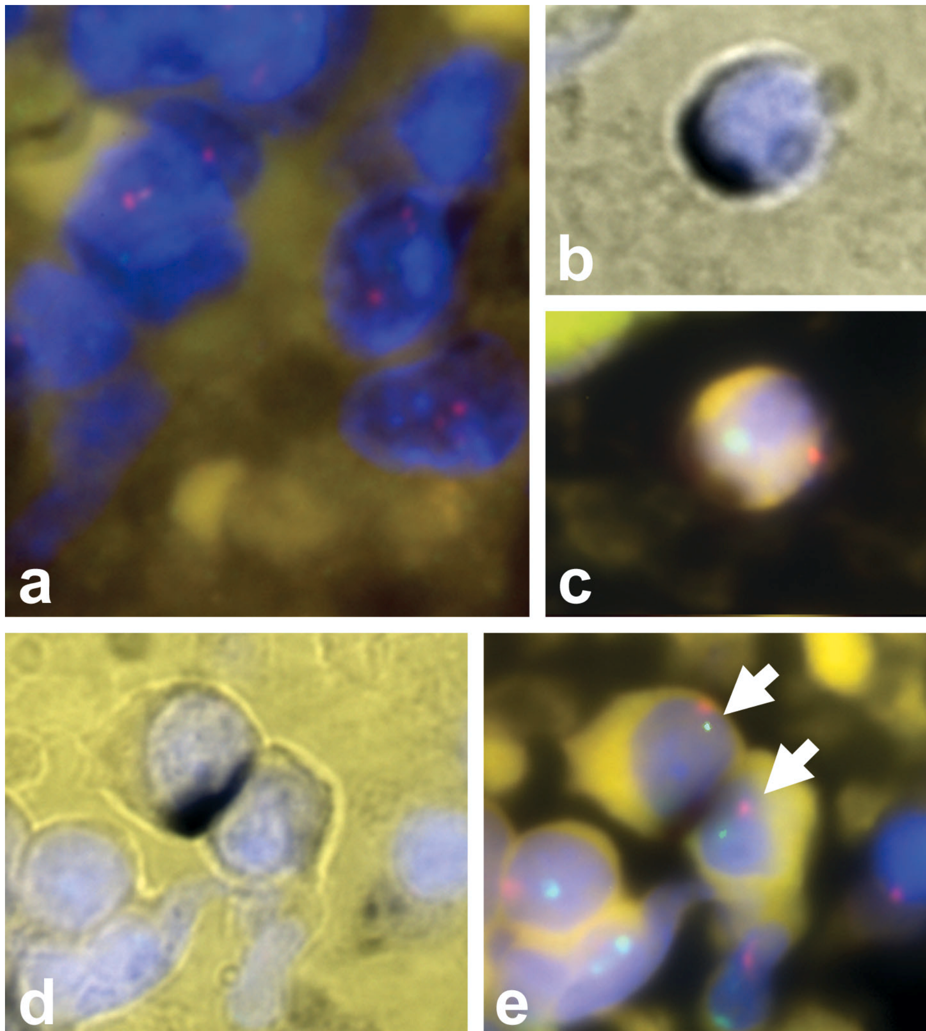

Fig. 1. CD34$^+$ progenitor cells (red signal: x-chromosome; green signal: y-chromosome) **a.** Survey of successfully engrafted bone marrow showing proper female signals of various hematopoietic cells in a male host. **b and c.** Residual (male) host progenitor cell after BMT with a female donor. **d and e.** Retrieval of two male adjacent host progenitor cells (arrows) together with other hematopoietic cells in a female graft situation during leukemic relapse.

(Koegler et al., 1995). This lack of knowledge is especially obvious in the early stages of hematopoietic reconstitution when blood and BM are still very hypocellular and limited methods for the identification of such subpopulations exist (Thiele et al., 2002a). Cell culture studies are in keeping with the assumption that CD34$^+$ cells of the blood and BM are not only precursors of sustained hematopoiesis, but can be stimulated to produce endothelial cells (Asahara et al., 1997; Choi et al., 1998; Shi et al., 1998). Following myelo-ablative therapy significant changes in the functional capacity of BM stroma cells composing the microenvironment are encountered that affect long-term engraftment and maintenance of hematopoiesis (Van den Berg et al., 1992; Neben et al., 1993; Novitzky and Mohamed, 1995; O'Flaherty et al., 1995; Van Hennik et al., 2000; Banfi et al., 2001). Therefore, these precursors are also held to be responsible for the undisturbed reconstitution of the vascular compartment of the BM (Gehling et al., 2000; Gunsilius et al., 2000). Regarding the homing phenomenon various ill-defined properties adhering to the fibrous BM stroma and vessel walls including a release of adhesion molecules by CD34$^+$ precursors were described to be involved in this very complex pathomechanism (Liesveld et al., 1991; Dercksen et al., 1995; Verfaillie et al., 1997; Obinata et al., 1998; Gordon et al., 2000). According to experimental studies in CML therapeutic regimens like interferon are known to exert an enhancing effect on the close contact between the neoplastic progenitors and the fibrous BM matrix and thus maintain the regulating influence of the microenvironment (Dowding et al., 1993; Santucci et al., 1993; Bhatia et al., 1995).

So far, concerning the exact quantity of a host/donor type origin of CD34$^+$ precursors no reliable data are available. Therefore one may speculate about the extent of mixed Ch of this peculiar cell population during the posttransplant period with respect to successful engraftment and in particular impending or manifest leukemic relapse (Thiele et al., 2002b). First of all, CD34 immunohistochemistry shows a distinctive staining of the progenitor cells which are easy to discriminate from BM endothelial cells (Thiele et al., 2001). Following BMT sex-typing exhibits a strikingly expressed mixed Ch of this peculiar cell population (Fig. 1b,c) with an approximate incidence of 22 % in the early posttransplant period (Table 1). In comparison with the signals indicating x- and y-chromosomes, bcr/abl gene expression visualized by orange and green closely adjacent signals or completely fused yellow dots was detectable in only 10 % of the CD34$^+$ cell compartment following successful engraftment (Thiele et al., 2002b). For this reason, caution should be applied about the issue that all retrieved host cells in the course of mixed Ch should be considered as neoplastic in nature or to determine the extent of minimal residual disease according to the quantity of the recipient cell population. In this regard only a very small fraction of progenitors (and other corresponding marrow constituents - see later) are responsible for this most important clinical phenomenon, while the majority of CD34$^+$ host cells probably represent regenerating hematopoiesis (Thiele et al., 2002b). Finally, concerning the probability of mixed Ch eventually generated in female recipient patients following pregnancy with a male fetus before BMT (Bianchi et al., 1996, 2001), one has to realize that the quantity of donor progenitor cells (and also the other hematopoietic cells under study) is not different when considering male and female patients separately (Table 1).

Erythroid precursors

The nucleated immature cells of erythropoiesis may be easily detected and differentiated from the precursors of the neutrophil granulocytic lineage by applying immunohistochemistry (Fig. 2a,c,e) involving a monoclonal antibody against glycophorin C (Gatter et al., 1988). Following myelo-ablative therapy and BMT together with the pronounced decrease in cellularity (Rousselet et al., 1996), the amount of erythroid precursors was also significantly reduced, but revealed the sex of the donor in the majority of cells (Fig. 1a,b). Furthermore, a striking correlation between the quantity

Table 2. Mixed chimerism of nucleated erythroid precursor cells in the bone marrow derived from 12 patients with CML after sex-mismatched bone marrow transplantation (BMT) and successful engraftment. CML control specimens before BMT revealed no donor type (sex-mismatched) cells in 782 evaluated erythroid precursors.

DAYS AFTER BMT	No. OF PATIENTS	No. OF ERYTHROID PRECURSORS	ERYTHROID PRECURSOR CELLS	
			Host-type	Donor-type
≥ 10 - 40	5	744	19 (3%)	725 (97%)
> 40 - 60	3	250	9 (4%)	241 (96%)
> 60 - 200	4	579	53 (9%)	526 (91%)
Total	12	1,573	81 (5%)	1,492 (95%)

of this cell population and the resident CD68+ macrophages was calculable (Thiele et al., 2000b). This feature of a mutual numerical relationship is in keeping with important functional aspects regarding these cell lineages (Wang et al., 1992; Hanspal, 1997). For this reason, it is not astonishing that both series revealed a mixed Ch that was also maintained and even increased in the late posttransplant period (Table 2). However, overall incidence of this phenomenon (Fig. 2c,d) was strikingly lower than in the CD34+ progenitor cell compartment (about 5% versus 24%) and probably also less expressed in comparison to the megakaryocyte compartment when regarding their polyploid state (see later). Amongst other explanations these features point to the possibility of an inhibition for further differentiation and proliferation of host-derived or residual recipient CD34+ progenitor cells, in particular concerning the erythroid and to a lesser extent also the megakaryocytic (Table 3) and monocytic macrophage (Table 4) series. Finally, the slight increase of lineage-restricted myeloid cells like erythroid precursors and megakaryocytes in the late posttransplant period (Tables 2 and 3) warrants explanation. These striking, although insignificantly expressed features point to an insidiously occurring recovery of corresponding committed recipient cells after severe damage of the bone marrow by myelo-ablative therapy (Neben et al., 1993; Novitzky and Mohamed, 1995; Henon et al., 1998). On the other hand, overall incidence of these cells does by no means reach the level of impending leukemic relapse, but might be detectable as minimal residual disease with evidence of bcr/abl transcriptions when performing RT-PCR (Van Leeuwen et al., 1991; Miyamura et al., 1994; Pichert et al., 1994; Radich et al., 1995; Schulze et al., 1995; Chomel et al., 2000; Serrano et al., 2000). Applying the latter method it is well-known that single cells among 106 total cells are identifiable (Lawler et al., 1991; Stuppia et al., 1993).

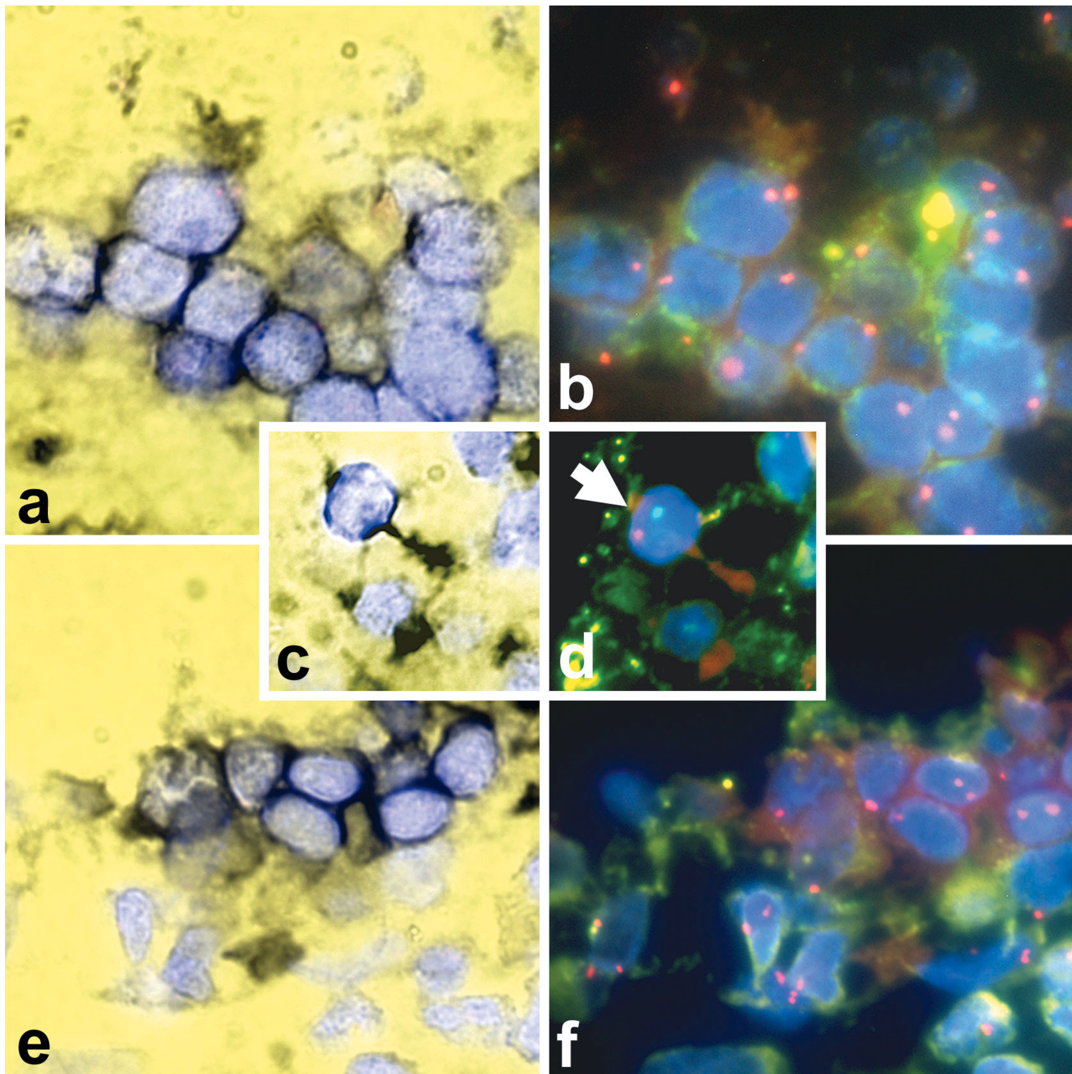

Fig. 2. Erythroid precursor cells (red signal: x-chromosome; green signal: y-chromosome). **a and b.** Cluster of female donor erythroblasts in a male host patient with successful engraftment. **c and d.** Single residual male host erythroblasts in a female graft situation (arrow). **e and f.** Leukemic relapse with groups of retrieved recipient (female) erythroblasts in a male graft.

Megakaryopoiesis

The CD61$^+$ megakaryocytes (Gatter et al., 1988) encompass all stages of maturation ranging from promegakaryoblasts to mature granulated and platelet-shedding cells (Thiele et al., 1990). In the early posttransplant period the atypical small megakaryocytes characteristic for CML (Georgii et al., 1990; Thiele and Fischer, 1991; Bartl et al., 1993) disappear and a patchy regeneration of a disarranged hematopoiesis occurs including dysplastic megakaryocytes (Van den Berg et al., 1990; Rousselet et al., 1996; Hurwitz, 1997; Thiele et al., 2000a). On the other hand, normalization of megakaryocyte size and cytological appearance was considered as presenting a hallmark of successful engraftment (Thiele et al., 2000a). This effect may be also observed after interferon therapy in patients with a major hematological response (Thiele et al., 1998). Regarding mixed Ch of this lineage after BMT (Fig. 3a,b), the quantity of the residual host-derived micromegakaryocytes indicating CML (Georgii et al., 1990; Thiele and Fischer, 1991; Bartl et al., 1993) was slightly higher (Table 3) than in the macrophage series (Table 4), but less expressed than in the CD34$^+$ progenitor cell compartment. On the other hand, by contrasting routine staining methods, CD61 immunohistochemistry also significantly facilitates the detection of the smaller elements of this cell lineage (Fig. 1a) thus increasing the probability of identifying not only larger numbers, but also precursors (Thiele and Fischer, 1991). In this context, some caveats have to be taken into account regarding evaluation and quantity of positive signals in this cell population. Because megakaryocytes are (hyper)polyploid cells (Levine et al., 1982; Williams and Levine, 1982) one may expect several sets of sex-chromosomes and thus the probability for more hits in a certain histological section does arise including several signals (Fig. 3c,d). Consequently, in comparison with the other (diploid) cells of hematopoiesis, overall incidence for obtaining two signals is significantly increased at a given section level and therefore frequency may be unduly overrated. Quantity of sex-chromosome labeling in (polyploid) megakaryocytes is presumably enhanced to a two- to three-fold degree which should be realized when calculating the extent of mixed CH in this cell lineage.

CD68$^+$ resident macrophages

The resident mature CD68$^+$ mononuclear -

Table 3. Mixed chimerism of CD61$^+$ megakaryocytes also including precursors in the bone marrow of 12 patients with CML after sex-mismatched bone marrow transplantation (BMT) and successful engraftment. CML control specimens before BMT revealed no donor type (sex-mismatched) cells amongst the 488 counted CD61$^+$ megakaryocytes.

DAYS AFTER BMT	No. OF PATIENTS	No. OF MEGAKARYOCYTES	CD61$^+$ MEGAKARYOCYTES	
			Host-type	Donor-type
≥10 - 60	5	210	26 (12%)	184 (88%)
>60 - 300	4	194	31 (16%)	163 (84%)
>300 - 600	3	59	18 (31%)	41 (69%)
Total	12	436	73 (17%)	363 (83%)

Table 4. Mixed chimerism of CD68$^+$ resident bone marrow macrophages derived from 13 patients with CML after sex-mismatched bone marrow transplantation (BMT) and successful engraftment. CML control specimens before BMT revealed an appropriate genotype of 356 CD68$^+$ macrophages evaluated.

DAYS AFTER BMT	No. OF PATIENTS	No. OF MACROPHAGES	MACROPHAGES	
			Host-type	Donor-type
≥ 10 - 40	4	178	21 (12%)	157 (88%)
>40 - 60	5	122	9 (7%)	113 (93%)
>60 - 200	4	100	4 (4%)	96 (96%)
Total	13	400	34 (9%)	366 (81%)

macrophage cell compartment of the BM has long been recognized as a potentially important population in CML patients, in particular regarding the expansion of the leukemic cell clone which may be mediated by malignant stromal macrophages (Bhatia et al., 1995). According to their monocyte-derived progeny and therefore clonal transformation (Golde et al., 1977; Thiele et al., 1998) BM macrophages in CML are further characterized by a striking variety of differentiation including the peculiar phenomenon of acquired lipidosis (Hayhoe et al., 1979). Amongst others this feature generates the Pseudo-Gaucher cells and the sea-blue (blue-pigmented) histiocytes (Kattlove et al., 1969; Hayhoe et al., 1979; Kelsey and Geary, 1988; Buesche et al., 1997; Thiele et al., 1999). Although resident (mature) macrophages constitute an important part of the BM microenvironment and are presumed to establish an intimate spatial contact and relevant functional

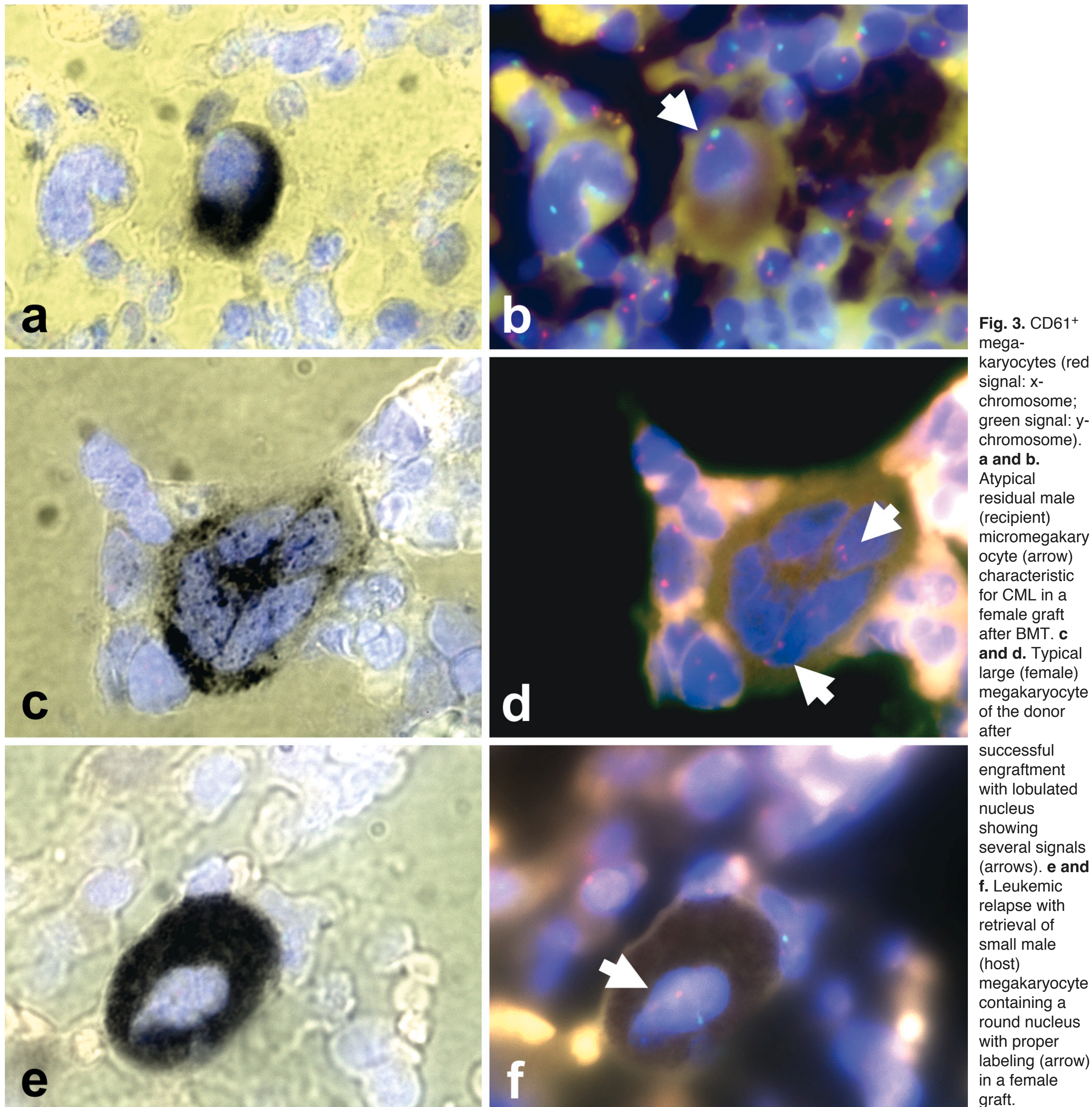

Fig. 3. $CD61^{+}$ megakaryocytes (red signal: x-chromosome; green signal: y-chromosome). **a and b.** Atypical residual male (recipient) micromegakaryocyte (arrow) characteristic for CML in a female graft after BMT. **c and d.** Typical large (female) megakaryocyte of the donor after successful engraftment with lobulated nucleus showing several signals (arrows). **e and f.** Leukemic relapse with retrieval of small male (host) megakaryocyte containing a round nucleus with proper labeling (arrow) in a female graft.

relationship with other cells, in particular erythropoiesis (Rich, 1986; Wang et al., 1992; Wilson and Tavassoli, 1994; Hanspal, 1997; Obinata et al., 1998), their role during hematopoietic reconstitution after BMT has been rarely investigated (Anastasi et al., 1998; Thiele et al., 2000c). Recently, concern has been expressed that a posttransplant recurrence of host-derived Pseudo-Gaucher cells indicates an unfavorable outcome because they may harbor the bcr/abl translocation (Anastasi et al., 1998). Following proper immunostaining and phenotyping of CD68$^+$ resident BM macrophages, distinctive signals were shown that facilitated the identification of this peculiar cell population (Anastasi et al., 1998; Thiele et al., 1998; Wickenhauser et al., 2002). In sex-mismatched allogeneic BMT a mixed Ch of CD68$^+$ macrophages together with the other hematopoietic cells was clearly evident (Fig. 4a,b) including Pseudo-Gaucher cells (Fig. 4c,d). Persistence of host cells in the engrafted BM of 13 male and female patients in the early posttransplant period amounted to between 8% and 10% (Wickenhauser et al., 2002). However, this extent of host-derived macrophages was time-dependent, because a gradual decrease was recognizable, with an average of 12% less than two months and a decline to 4% more than 3 months (median 5 months) after BMT, also including the fraction of Pseudo-Gaucher cells (Table 4). When comparing the incidence of mixed Ch in the myeloid cell compartment (Tables 2-4) CD34$^+$ progenitors are clearly outstanding according to their quantity of residual host cells at the various checkpoints of evaluation. For this reason, it is tempting to speculate about differences regarding the peculiar lineage-restricted pathways of development that in the context of hematopoietic reconstitution following BMT may include inhibition or stimulation of a specific cell series.

Endothelial cells

Regarding undisturbed engraftment of host-derived hematopoiesis the rapid recovery of an intact microenvironment is an essential feature (Van den Berg et al., 1990; Novitzky and Mohamed, 1995; O'Flaherty et al., 1995; Domenech et al., 1998; Van Hennik et al., 2000). Within this very complex functional network the microvascular endothelial cells of the BM sinusoidal vessels act not only as gatekeepers controlling the trafficking and homing of hematopoietic progenitors, but also provide multiple cellular interactions including release of cytokines that are responsible for the preservation of steady-state hematopoiesis and immune recovery (Abboud, 1995; Rafii et al., 1997). According to in vitro studies angiogenesis which is a most important factor for restitution and maintenance of hematopoiesis (Simmons et al., 1992; Davis et al., 1995; Rafii et al., 1995; Shalaby et al., 1995) implies firstly in situ differentiation of endothelial cells from hemangioblasts (Asahara et al., 1997; Gehling et al., 2000) and furthermore their proper organisation into a primary vascular plexus. In a second step formation of new blood vessels is performed by sprouting from pre-existing BM sinusoids and capillaries (Risau, 1997). In the context of BMT the first process seems to play a crucial role in the very early posttransplant period following marrow-ablative regimens. In vitro experiments are in keeping with the assumption that

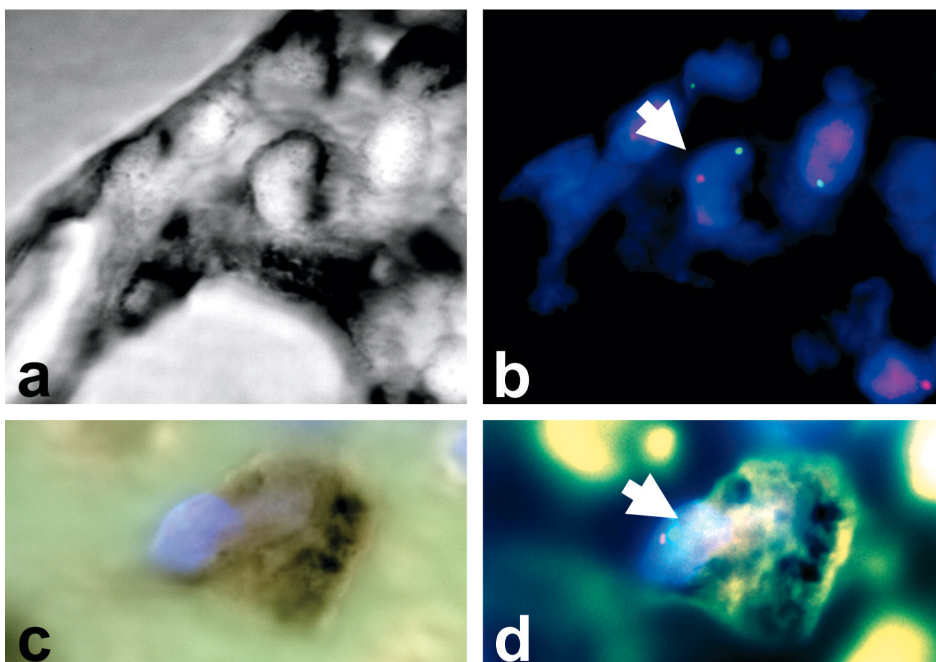

Fig. 4. CD68$^+$ macrophages (red signal: x-chromosome; green signal: y-chromosome). **a and b.** Residual male (host) macrophage with proper labeling (arrow) in a female donor situation. **c and d.** Pseudo-Gaucher cell with extensive area of cytoplasm revealing a male recipient origin (arrow) after BMT with a female graft.

$CD34^+$ progenitor cells from the peripheral blood and BM are not only precursors of sustained hematopoiesis, but can be stimulated to produce endothelial cells (Asahara et al., 1997; Choi et al., 1998; Shi et al., 1998). Therefore, several lines of evidence suggest that even in adult life hemangioblasts or more mature endothelial cell progenitors are maintained and contribute to the formation of new blood vessels (Gehling et al., 2000). Recently, it has been shown that in CML patients a bipotent marrow-derived hemangioblastic progenitor cell exists that is capable of producing both blood cells and endothelial cells (Gunsilius et al., 2000). By using current BMT procedures several studies support the argument of a non-transplantability of the fibroblast and myofibroblast population resulting in a host-derived origin of stroma cells of the vessel wall, even in patients showing a complete long-lasting hematopoietic reconstitution (Agematsu and Nakahori, 1991; Santucci et al., 1992). However, some caution is required when analyzing the chimeric state in vascular structures. Endothelial cells as the only, but functionally very important cellular constituents of the BM sinusoids have to be clearly separated from the other components of the larger vessels (capillaries, arterioles) that additionally encompass various layers of myofibroblasts and adventitial cells (Kvasnicka et al., 2003). Without applying appropriate immunohistochemical labeling in small vascular structures or in corresponding cell culture settings, it may become difficult to distinguish unequivocally between these various cellular compartments (Fig. 5a,c,f).

After BMT the $CD34^+$ endothelial cell population revealed a peculiarly expressed mixed Ch (Fig. 5b) with an overall incidence of about 20% donor-type origin (Kvasnicka et al., 2003) accounting for a predominance of surviving host-derived cells (Fig. 5c,d). However, when regarding the length of the posttransplant period a tendency for an increase in the donor-type endothelial cells up to 25% was evident after the third month (Table 5). Following myelo-ablative therapy survival of a considerable number of endothelial cells and also myofibroblasts of the vessel walls has been described, which implies a persistence of host-derived vascular structures of the BM stroma. However, in only a very small proportion (about 2%) of bcr/abl$^+$ endothelial cells (Fig. 5f,g) and thus minimal residual disease was detectable (Kvasnicka et al., 2003), which is significantly less than the situation encountered in the $CD34^+$ progenitor cell population (Thiele et al., 2002b) and the other myeloid cell populations (erythroid precursors and megakaryocytes).

In this context it should not be overlooked that one group reported a host origin of endothelial cells by using FISH technique on histological slides (Athanasou et al., 1990). This adverse result contradicting the hypothesis of a hemangioblastic precursor cell (Asahara et al., 1997;

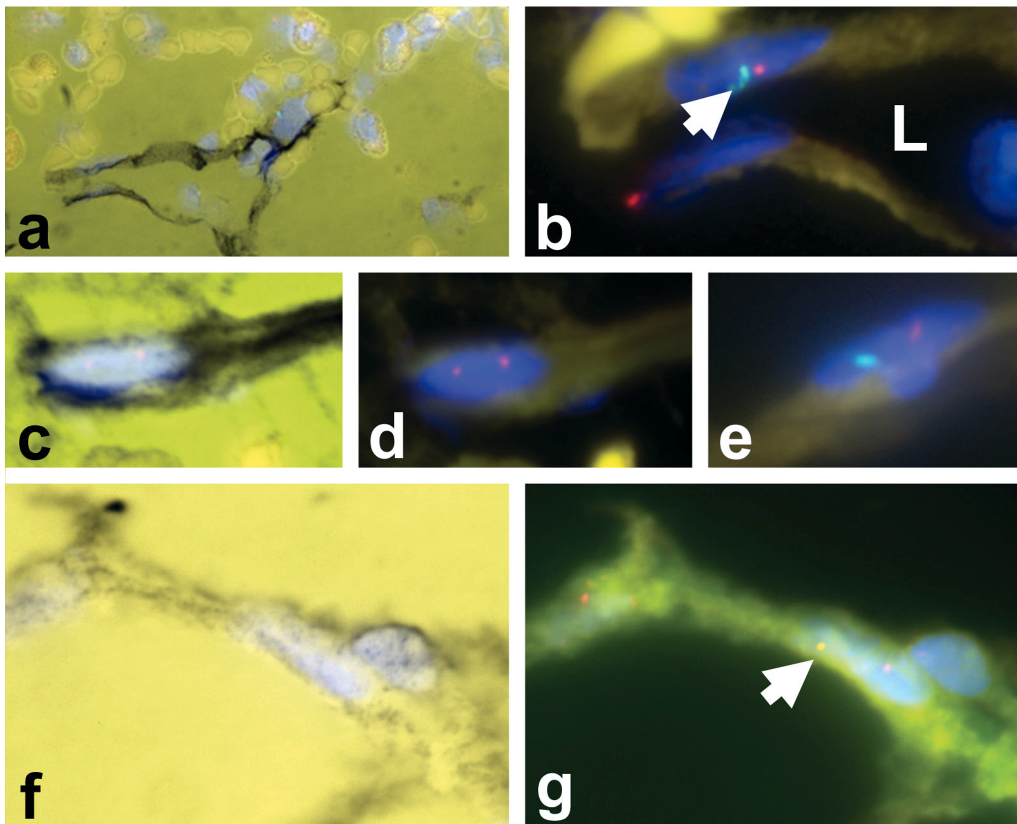

Fig. 5. $CD34^+$ endothelial cells (red signal: x-chromosome; green signal: y-chromosome). **a.** Survey of small sinusoidal vessels marked by immunohistochemistry. **b.** Endothelial cells lining a sinusoidal lumen (L) and exhibiting a male donor type origin (arrow) in a female host after BMT. **c and d.** Remaining female host endothelial cell in a male graft constellation. e. Retrieved male recipient endothelial cell during leukemic relapse with a female graft. **f and g.** Endothelial cell with yellow fusion signal (arrow) indicating a bcr/abl gene locus in a residual (male) host cell after BMT (female donor).

Gehling et al., 2000; Gunsilius et al., 2000) may be caused by several factors acting alone or conversely. First of all, only a single y-specific probe was used for FISH analysis opposed to our method (dual color FISH with x- and y-specific probes). Furthermore, no specific immunohistochemical labeling of the endothelial layer and no quantitative evaluation of the various cell populations has been performed. In comparison to our cohort of patients of whom several encountered a leukemic relapse with almost complete retrieval of host-derived endothelial cells, this significant point was not investigated in the latter study (Athanasou et al., 1990). After using a factor VIII-related antigen antibody morphometric analysis was in keeping with a significantly increased density of vascular structures in CML associated with a high level of vascular endothelial growth factor (VEGF) expression (Aguayo et al., 2000). Accordingly, in this condition the BM stroma compartment is significantly altered, because its composition includes a mixture of clonally transformed and non-leukemic cells as well (Bhatia et al., 1995). Cytokine-secreting cells within this complex microenvironmental setting provide a suitable surrounding for homing, self-renewal, proliferation and differentiation of hematopoietic stem cells (Verfaillie et al., 1997) that may be significantly influenced by interferon therapy (Dowding et al., 1993; Bhatia and Verfaillie, 1998). Prominent members of this compartment like resident macrophages (Anastasi et al., 1998; Thiele et al., 1998), osteoclasts (Thiele et al., 1998) and endothelial cells (Gunsilius et al., 2000) have been found to harbor the bcr/abl translocation, thus contrasting the non-clonally transformed fibroblasts (Greenberg et al., 1978; Golde et al., 1980; O'Brien et al., 1988). It is noteworthy that only a very small fraction of host-derived endothelial cells also carried the bcr/abl translocation gene (Fig. 5f,g). Using peripheral blood cells similar discrepancies between persistent host cells and their bcr/abl expression were recorded after BMT in CML patients (Nagler et al., 1994; Palka et al., 1996). This finding clearly implies that one should be reluctant to apply sex-mismatched probes alone to determine residual tumor cells that lack specific markers.

Leukemic relapse

Monitoring lineage-restricted Ch during the posttransplant period seems to be very important. Patients with impending relapse show a consistent evolution pattern featured by increased mixed Ch in myeloid cells (Roux et al., 1992; Miyamura et al., 1993, 1994; Baurmann et al., 1998; Serrano et al., 2000). However, it should be emphasized that degree of mixed Ch may be dependent on the various methods applied for allogeneic BMT procedures (Elmaagacli et al., 2001). The finding of significantly increased host hematopoietic cells after BMT characterizes an insidious conversion from donor to host origin of the reconstituting BM and therefore is usually indicative of leukemic relapse or graft rejection. Until now little data are available about quantitative aspects of this phenomenon and especially dynamic features in the different cell populations. In one male patient with acute lymphoid leukemia (ALL) and a female graft constellation simultaneously performed genotypic and immunophenotypic analysis of the mononuclear cell population of the BM during clinical relapse revealed a y-chromosome in about 90 % (Koegler et al., 1995). However, controversy and discussion continues to persist regarding the involvement of the various cell lineages in the retrieval of clonally transformed (leukemic) host cells.

1. CD34$^+$ progenitor cells

Generally, the evaluation of both mixed Ch and the presence of the bcr/abl gene in hematopoietic precursor cells after BMT could be helpful in the understanding of the biology of leukemic relapse. It is reasonable to assume that the small fraction of host-derived, clonally transformed (bcr/abl-positive) CD34$^+$ progenitors are the possible source of minimal residual disease and, depending on a certain threshold number, implicate leukemic relapse (Thiele et al., 2002b). Moreover, regarding this fatal complication it is noteworthy that a high proportion (about 90%) of CD34$^+$ host cells were retrieved during leukemic transformation (Baurmann et al., 1998). This significant amount contrasts to only about a 50% incidence of the CD68$^+$ resident (mature)

Table 5. Mixed chimerism of CD34$^+$ endothelial cells mostly derived from sinusoidal vessels of the bone marrow in 9 patients after sex-mismatched bone marrow transplantation (BMT) and successful engraftment. CML control specimens before BMT revealed no donor type (sex-mismatched) cells in 38 evaluated endothelial cells.

DAYS AFTER BMT	No. OF PATIENTS	No. OF ENDOTHELIAL CELLS	ENDOTHELIAL CELLS	
			Host-type	Donor-type
≥ 10 - 60	4	94	77 (82%)	17 (18%)
>60 - 200	5	52	39 (75%)	13 (25%)
Total	9	146	116 (79%)	30 (21%)

macrophage population of the BM during leukemic relapse (Wickenhauser et al., 2002). One may discuss that these differences are probably associated with the peculiar cell kinetics of both compartments. Turnover rate is assumed to be relatively high in the progenitor cell population, whereas mature BM macrophages have been found to be quiescent cells representing endstages (Titius et al., 1994). Therefore, it is most likely that leukemic expansion of the host-derived mature macrophages occurs at a significantly lower rate. According to our results lineage-restricted mixed Ch is a relatively frequent finding and involves about one fourth of the CD34+ progenitor cell population in the early posttransplant period. The frequency reported in this study is apparently higher than that indicated by a previous investigation (Koegler et al., 1995) in which it

Table 6. Conversion of mixed chimerism during leukemic relapse in the CD34+ progenitor cell population after sex-mismatched bone marrow transplantation (BMT) for CML.

DAYS AFTER BMT	No. OF PATIENTS	No. OF PROGENITOR CELLS	PROGENITOR CELLS	
			Host-type	Donor-type
≥ 190 - 600	4	154	131 (85%)	23 (15%)
>600 - 1,172	4	149	146 (98%)	3 (2%)
Total	8	303	277 (91%)	26 (9%)

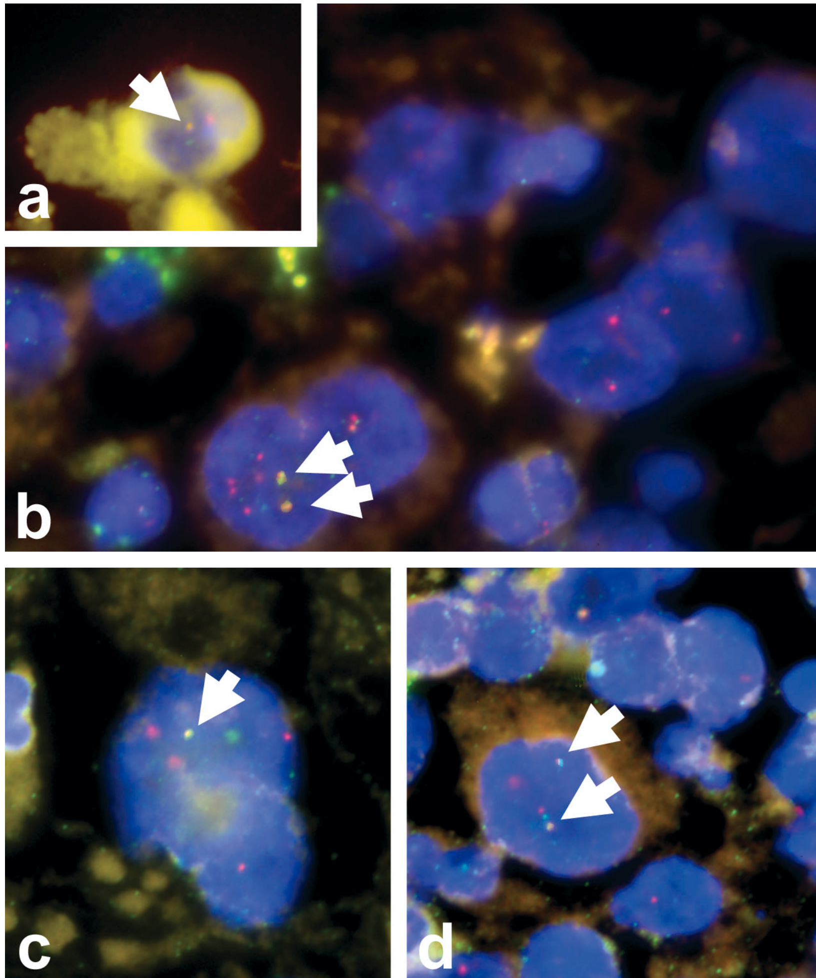

Fig. 6. Labeling of the bcr/abl gene locus (arrows) during leukemic relapse indicated by overt conversion of mixed Ch (red signal: bcr gene; green signal: abl gene). **a.** CD34+ progenitor cell. **b-d.** Megakaryocytes of different sizes corresponding with (hyper)ploidy show one to several signals (either adjacent red/green or yellow fusions spots) in addition also to the bcr and abl labeling of the surrounding hematopoietic (leukemic) cells.

is not clear whether peripheral blood or BM specimens were evaluated. In comparison with the large number of recipient CD34+ cells, only a small fraction of leukemic (bcr/abl+) primitive precursors survive myelo-ablative treatment. The latter are assumed to present the source for recurrent leukemia indicated by a conspicuous conversion of mixed Ch. In patients with a manifest leukemic relapse according to clinical and cytogenetic definition, this feature was evident in the 303 evaluated progenitors (Fig. 1d,e) by displaying up to 90% cells of recipient origin (Table 6). This population also showed the bcr/abl translocation (Fig. 6a), but only in approximately 50% of the precursor host cells (Thiele et al., 2002b). Therefore, it is reasonable to assume that quantity of mixed Ch alone, even in the situation of impending conversion, does not neccessarily imply a neoplastic nature.

In the context of mixed Ch of the CD34+ progenitor cell compartment, certain experimental models suggested the presence of a small amount of bcr/abl+ host-derived cells after successful BMT and thus were the basis for the tentative explanations of tumor "dormancy" (Uhr et al., 1997; Holyoake et al., 1999). Following BMT even in patients with complete cytogenetic remission, a significant minority of cells were shown to be of host origin (Kitzis et al., 2001). These results were interpreted as implicating a persistence of a quiescent and transcriptionally silent population of primitive leukemic cells after BMT, independently of treatment modalities (Holyoake et al., 1999; Kitzis et al., 2001). Interestingly there are in vitro findings suggesting a dormancy of progenitor cells with bcr/abl transcriptions after curative regimens like BMT and interferon treatment (Pichert et al., 1994; Talpaz et al., 1994; Wandl et al., 1994; Reiter et al., 1998; Serrano et al., 2000) or even following application of the tyrosine kinase inhibitor STI571 (Graham et al., 2002). It is tempting to speculate that a small amount of primitive CD34+ progenitors survive in the recipient patients after myelo-ablative therapy by being inactive and not proliferating. However, when stimulated ex vivo in a corresponding colony assay by appropriate growth factors, cells do proliferate and produce detectable amounts of bcr/abl mRNA (Schulze et al., 1995).

Erythroid precursors and megakaryocytes

In keeping with the CD34+ progenitor cells a similar conversion of mixed Ch is also found with evolving leukemic relapse in the erythroid (Fig. 2e,f) and megakaryocyte cell series (Fig. 3e,f). Dynamic features of recurrent leukemia are readily apparent in repeatedly performed bone marrow examinations before and following BMT. Especially in the late posttransplant period after approximately two years incidence of host type, nucleated precursors of the red lineage changed from 5% to 91% (Table 7) which supports previous data (Baurmann et al., 1998). A similar result was obtained in the megakaryocyte population which revealed an abrupt conversion of mixed Ch from 7% donor to 93% host cells in patients with clinical and cytogenetic evidence for leukemic relapse (Table 8). In line with relevant alterations of the CD34+ progenitor cell population (see above) similar features emerging in the more

Table 7. Conversion of mixed chimerism during leukemic relapse in the erythroid precursor cell compartment after sex-mismatched bone marrow transplantation (BMT) for CML.

DAYS AFTER BMT	No. OF PATIENTS	No. OF ERYTHROID PRECURSORS	ERYTHROID PRECURSORS	
			Host-type	Donor-type
≥ 180 - 560	1	109	109 (100%)	0 (0%)
>560 - 1,938	5	455	402 (88%)	53 (12%)
Total	6	564	511 (91%)	53 (9%)

Table 8. Conversion of mixed chimerism during leukemic relapse in the CD61+ megakaryocyte population after sex-mismatched bone marrow transplantation (BMT) for CML.

DAYS AFTER BMT	No. OF PATIENTS	No. OF CD61+ MEGAKARYOCYTES	CD61+ MEGAKARYOCYTES	
			Host-type	Donor-type
≥ 190 - 560	3	235	220 (94%)	15 (6%)
>560 - 1,938	2	107	99 (93%)	8 (7%)
Total	5	342	319 (93%)	23 (7%)

differentiated lineage-restricted cell compartments of hematopoiesis could be expected. This was especially evident concerning the detection of the bcr/abl gene locus which, depending on the polyploid state of megakaryocytes, was relatively easy to identify at a given section level of the bone marrow specimens (Fig. 6b-d).

CD68⁺ resident macrophages

In comparison with the other cell lineages, in particular the $CD34^+$ progenitors, leukemic relapse of the mature (resident) bone marrow macrophages is characterized by a lesser degree of host cell retrieval. Postgraft leukemia by clinical and cytogenetic standards (Appelbaum et al., 1995; Clift and Anasetti, 1997; Hansen et al., 1998) is associated with approximately one third to one half of this cell population showing a host origin (Table 9). This relatively small proportion of neoplastic macrophages may be due to certain peculiarities of cell kinetics in this compartment. According to relevant studies, $CD68^+$ macrophages are quiescent cells representing an endstage (Titius et al., 1994) and therefore substitution and expansion of this population is prolonged, opposed to the $CD34^+$ progenitor cell pool and also the erythroid and megakaryocyte precursors.

Endothelial cells

In patients with insidious evolution into manifest leukemia a conversion of mixed Ch with retrieval of host-derived endothelial cells ranging up to almost 100% was observable (Table 10). These changes from donor to host type origin of the endothelial cell compartment (Fig. 5e) were accompanied by an increased incidence (about 10%) of cells that carried the bcr/abl fusion gene (Kvasnicka et al., 2003). These features are consistent with a relatively small quantity of a leukemic endothelial cell population. However, it may not be ruled out that dysfunction of the neoplastic endothelial cells in chronic and relapsing CML following BMT may exert a stimulating impact on the expansion of the clonally transformed cell population. This assumption seems to be reasonable, because BM microvasculature (endothelial cells, basement membrane, adventitial cell layer) significantly monitors adhesion, regulation, trafficking, proliferation and differentiation of lineage-committed and pluripotent stem cells (Rafii et al., 1994, 1995; Abboud, 1995; Davis et al., 1995; Turner and Sweetenham, 1996).

In conclusion, mixed Ch is a striking phenomenon of reconstituting hematopoiesis after BMT and involves all cell lineages derived from the $CD34^+$ progenitor cell pool, as was shown by dual color FISH technique combined with immunophenotyping of the various cell compartments. In this context, most remarkable was also the involvement of the endothelial cell layer. These features are in keeping with a significant plasticity of human adult stem cells, even if mixed Ch is rare, implicating that the environment is able to reprogram the fate of a cell, as has been recently shown for hepatocytes

Table 9. Conversion of mixed chimerism during leukemic relapse in the $CD68^+$ resident bone marrow macrophage population after sex-mismatched bone marrow transplantation (BMT) for CML.

DAYS AFTER BMT	No. OF PATIENTS	No. OF MACROPHAGES	MACROPHAGES	
			Host-type	Donor-type
≥ 70 - 550	3	68	19 (28%)	49 (72%)
>550 - 1,938	3	90	47 (52%)	43 (48%)
Total	6	158	66 (42%)	82 (58%)

Table 10. Conversion of mixed chimerism during leukemic relapse in the $CD34^+$ endothelial cells (mostly derived from sinusoidal vessels) of the bone marrow after sex-mismatched bone marrow transplantation (BMT) for CML.

DAYS AFTER BMT	No. OF PATIENTS	No. OF ENDOTHELIAL CELLS	ENDOTHELIAL CELLS	
			Host-type	Donor-type
≥ 190 - 600	3	30	30 (100%)	0 (0%)
>600 - 1,172	3	42	38 (90%)	4 (10%)
Total	6	72	68 (94%)	4 (6 %)

and epitheloid cells of the skin (Koerbling et al., 2002). On the other hand, in the BM, evolving leukemic relapse was always accompanied by a significant conversion of mixed Ch to the host (recipient) cell type. A simultaneous visualization of the bcr/abl translocation gene was in keeping with the finding that only a certain fraction of the maintained host cells indicated a neoplastic nature or minimal residual disease, even in the case of recurrent leukemia.

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