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Dynamics of bone marrow changes in patients with chronic idiopathic myelofibrosis following allogeneic stem cell transplantation

J. Thiele[1], H.M. Kvasnicka[1], H. Dietrich[1], G. Stein[1], M. Hann[1], A. Kaminski[1], N. Rathjen[1], K.A. Metz[2], D.W. Beelen[3], M. Ditschkowski[1], A. Zander[4] and N. Kroeger[4]

Institutes of Pathology, Universities of [1]Cologne, Cologne and [2]Essen, Essen and Departments of Bone Marrow Transplantation, [3]University of Essen, Essen and [4]University Hospital Hamburg, Germany

Summary. Scant knowledge exists about the dynamics of fibro-osteosclerotic bone marrow (BM) lesions and regeneration of hematopoiesis following allogeneic peripheral stem cell transplantation (SCT) in chronic idiopathic myelofibrosis. Therefore, an immunohistochemical and morphometric study was performed on BM biopsies in 20 patients before and at standardized intervals (days 30 through 384) following SCT. In responding patients, a total regression of the pretransplant increased fibrosis was completed in the posttransplant period after about six months, while the extent of osteosclerosis did not change significantly during observation time. The quantity of $CD61^+$ megakaryocytes including precursors was strikingly variable after SCT and, by using planimetric methods, atypical microforms exhibiting a dysplastic aspect could be demonstrated. These anomalies may be responsible for posttransplant thrombocytopenia. $CD34^+$ progenitor cells were increased before transplantation, however, their number declined rapidly to normal values in responding patients. Nucleated erythroid precursors revealed a decreased amount before and after SCT accounting for anemia. Large clusters of this cell lineage indicated an initial hematopoietic reconstitution comparable with the expansion of the neutrophil granulopoiesis. Proliferative activity and apoptosis showed an increase until one year after SCT that implied a still regenerating hematopoiesis in keeping with an enhanced cell turnover.

Key words: Chronic idiopathic myelofibrosis, Stem cell transplantation, Fibro-osteosclerosis, Erythropoiesis, $CD34^+$ progenitors, Megakaryocytes, Proliferation, Apoptosis

Offprint requests to: Juergen Thiele, M.D., Institute of Pathology, University of Cologne, Joseph-Stelzmannstr. 9, D-50924 Cologne, Germany. e-mail: j.thiele@uni-koeln.de

Introduction

Allogeneic peripheral hematopoietic stem cell transplantation (SCT) has been increasingly applied in recent years as a curative treatment option for chronic idiopathic myelofibrosis (CIMF) or myelofibrosis following polycythemia vera and essential thrombocythemia (Guardiola et al., 1999; Daly et al., 2003; Deeg et al., 2003; Ditschkowski et al., 2004; Cervantes, 2005; Rondelli et al., 2005). However, there is little information available concerning the dynamics of bone marrow (BM) changes during the posttransplant period. In particular following SCT, controversy and discussion arises over alterations as regards the quality and quantity of the fibro-osteosclerotic BM lesions characterizing this disorder (Dickstein and Vardiman, 1993; Georgii et al., 1998; Barosi, 1999; Thiele et al., 2001a). Because megakaryocytes have been shown to be the source of abnormal cytokines involved in the generation of the fibrous myeloid matrix (Le Bousse-Kerdiles and Martyre, 1999; Schmitt et al., 2002), it seems reasonable to investigate this cell lineage in the context of presumptive stromal changes that may occur after transplantation. Additionally, other constituents of hematopoiesis like $CD34^+$ progenitor cells, nucleated erythroid precursors as well as proliferative activity and programmed cell death (apoptosis) may play a crucial role in this concert of complex functional cell to cell interactions composing the microenvironment of the BM.

For this purpose, we performed an immunohistochemical and morphometric study on BM biopsy samples derived at standardized endpoints in a cohort of 20 patients with CIMF following SCT to elucidate transplant-related changes.

Materials and methods

Patients

A total of 20 patients (17 men, 3 women) with CIMF

and a median age of 50 years (range 13 to 64 years) were enrolled in this prospective study between January 1999 and July 2003. The treatment protocol was approved by the local ethics committee and all patients had given written informed consent. Only five of these patients received no pretransplant cytoreductive therapy while the others had a history of various therapeutic regimens including hydroxyurea, busulfan as well as radiation and splenectomy or a combination of these. Following a reduced-intensity conditioning regimen (Kroeger et al., 2005), all patients received peripheral blood stem cells with a median number of transplanted $CD34^+$ progenitors of $8x10^6$ per kg body weight (range 0.9-15.6) derived either from HLA-identical siblings or matched unrelated donors. Relevant clinical data at the standardized endpoints are given in Table 1. For further clinical details, especially response criteria and outcome, we are referring to a recently published report on this series of patients (Kroeger et al., 2005). As a control group specimens of 25 patients were entered without evidence for any hematological disorder or osteopathy.

Bone marrow biopsies

Representative BM trephine biopsies were performed at standardized intervals (Table 1) from the posterior iliac crest, fixed in formalin, decalcified and embedded in paraffin wax. Staining techniques involved Giemsa, PAS (periodic acid Schiff reagent), tatrate-resistant acid phosphatase, naphthol-AS-D-chloroacetate esterase, Perls' reaction for iron and the silver impregnation method following Gomori's technique. Immunohistochemistry with monoclonal antibodies was applied for a proper identification of $CD34^+$ progenitors (Soligo et al., 1991), $CD61^+$ megakaryocytes (Gatter et al., 1988) and nucleated erythroid precursor cells (monoclonal antibody Ret40f). To determine proliferative capacity a monoclonal antibody (MIB1) against Ki 67 was applied (Budke et al., 1994). Monoclonal antibodies and other reagents were purchased from Dako-Diagnostica GmbH, Hamburg, Gemany. Apoptosis was visualized by the specific monoclonal antibody (Frankfurt, 2004) Apostatin (Bender MedSystems, Vienna, Austria). Details of staining procedures (APAAP-method) were reported previously (Cordell et al., 1984; Thiele et al., 1999).

Morphometric analysis was carried out by two planimeters (MOP-A-MO1-Kontron and VIDAS-Zeiss-Kontron) with a standard program set on large (18.4x3.9 mm^2) trephine biopsies with an artefact-free BM mean area of the pre- and posttransplant specimens ranging between 12±7 mm^2 and 14±4 mm^2. First of all, we evaluated the frequencies of immunostained $CD61^+$ megakaryocytes including precursors (promegakaryoblasts, megakaryoblasts), and their planimetric variables (size, form factor). To enhance the discriminating impact of individual measurements concerning planimetry, i.e. the evaluation of megakaryopoiesis, all values were pooled (total of 3,408 megakaryocytes) and descriptive statistics were calculated for the corresponding endpoints, accordingly. In addition, quantities of $CD34^+$ progenitors and nucleated erythroid precursors as well as the number of proliferating ($Ki67^+$) and apoptotic cells were determined at x500 magnification by regarding the total biopsy and those areas occupied by hematopoiesis. Measurements per cellularity were carried out to avoid an undue influence of therapy-related lesions (interstitial edema, expansion of the adipose tissue, reduction in cellularity) during the posttransplant period on the quantification of variables. Moreover, density of reticulin-collagen (argyrophilic) fibers was determined by counting the number of intersections with the lines of an ocular grid at x500 magnification and density was expressed as number of intersections (i) per square millimeter of hematopoietic tissue (Thiele et al., 2000a). Finally, the extent of osteosclerotic bone changes and number of acid phosphatase expressing osteoclasts was measured. The latter included the multi- and uninucleated osteoclasts as well as anuclear cytoplasmic fragments lying along the endosteal border which were evaluated by techniques described in detail in a previous communication (Thiele et al., 1989).

Results

In comparison with normal BM specimens before SCT, a significant increase in fiber density was recognizable in all pretransplant samples, revealing CIMF often associated with endophytic bone formation - osteosclerosis (Fig. 1a). However, following myeloablative treatment and transplantation (Fig. 1b,c), a relatively rapid reversal to normal values (i.e. upper limit of the control group) within the third to fifth posttransplant month was detectable (Table 2). A significant (>10 i $x10^5$) regression of fiber density was shown to occur between the 1st and the 2nd biopsy endpoint in 40% and between the 1st to 3rd examination in more than 80% of patients. Regarding osteosclerosis, no significant decrease in the trabecular bone area (endophytic bone formation) was detectable even after one year of observation (Fig. 1b). On the other hand, in

Table 1. Hematological findings before and at standardized intervals following allogeneic stem cell transplantation (SCT) in chronic idiopathic myelofibrosis. Note: most patients received a variety of pretransplant cytoreductive therapies and all multiple transfusions following SCT.

	ENDPOINTS			
	Before SCT	Posttranslationa period (days)		
	1	2	3	4
Intervals (median)	-14	30	157	384
Erythrocytes ($x10^{12}$/l)	3.6±1.1	3.8±1.0	3.2±1.0	3.7±1.2
Hemoglobin (g/dl)	10.7±2.7	10.8±1.3	10.3±1.0	12.6±4.0
Hematocrit (%)	32.4±8.3	31.6±4.3	30.4±5.9	39.5±11.6
Thrombocytes ($x10^9$/l)	379±365	202±225	121±113	247±125
Leukocytes ($x10^9$/l)	16.8±11.3	10.5±8.0	8.2±4.3	6.9±3.2

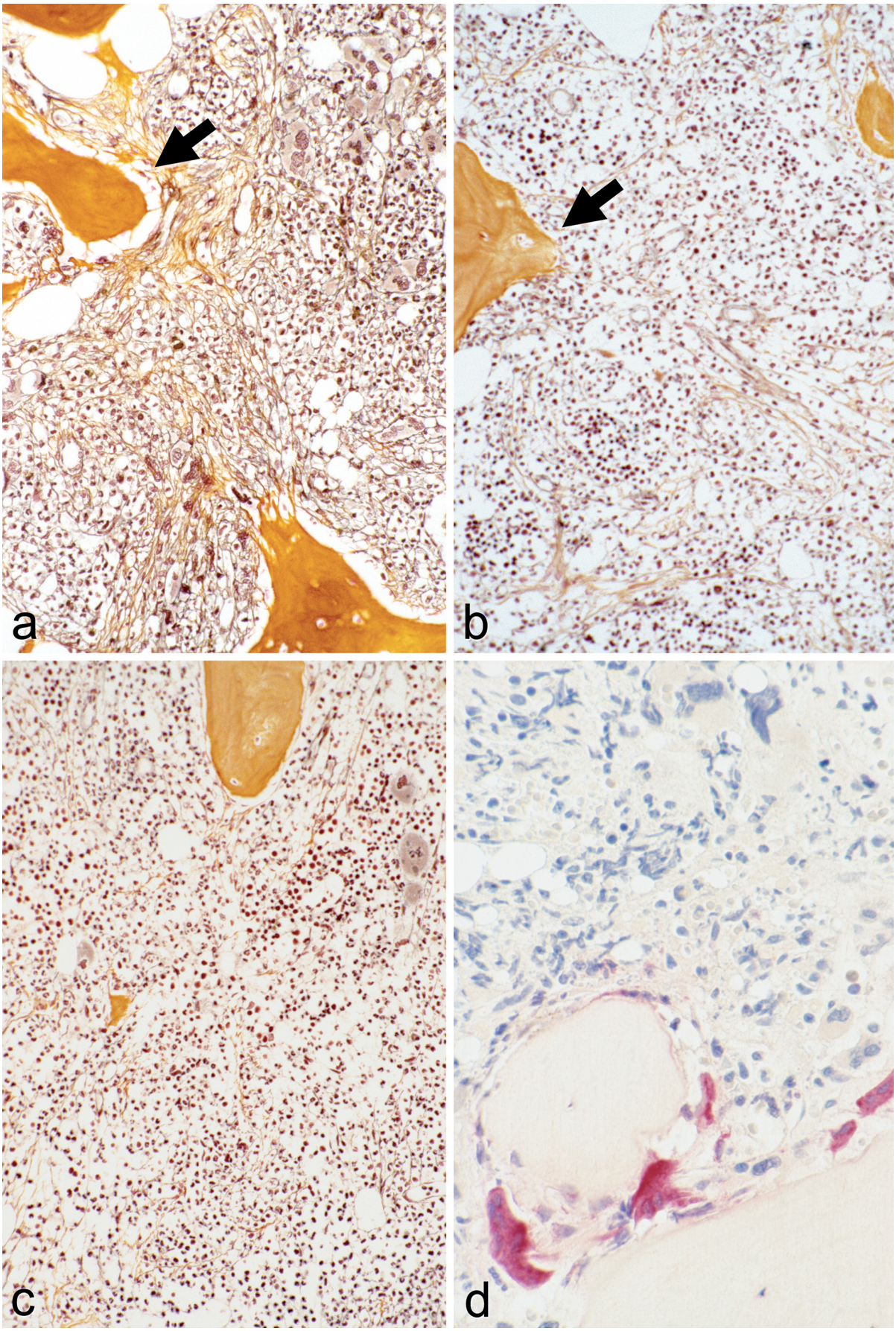

Fig. 1. Fibro-osteosclerotic bone marrow lesions in CIMF before and after allogeneic peripheral stem cell trans-plantation (SCT). Before SCT, a marked dense collagen fibrosis is recognizable with endophytic (osteo-sclerotic) bone lesions (arrow) **(a)**. About 3 months after SCT (same patient), a significant regression of fibrosis is apparent, but osteosclerotic lesions are still detectable (arrow) **(b)**. After 6 months (same patient), a normal density of single reticulin fibers is present **(c)**. Osteosclerotic bone formation with increase in osteoclasts after SCT **(d)**. a-c, Gomori's silver impregnation; d, tartrate-resistant acid phosphatase reaction. a-c, x 180; d, x 380

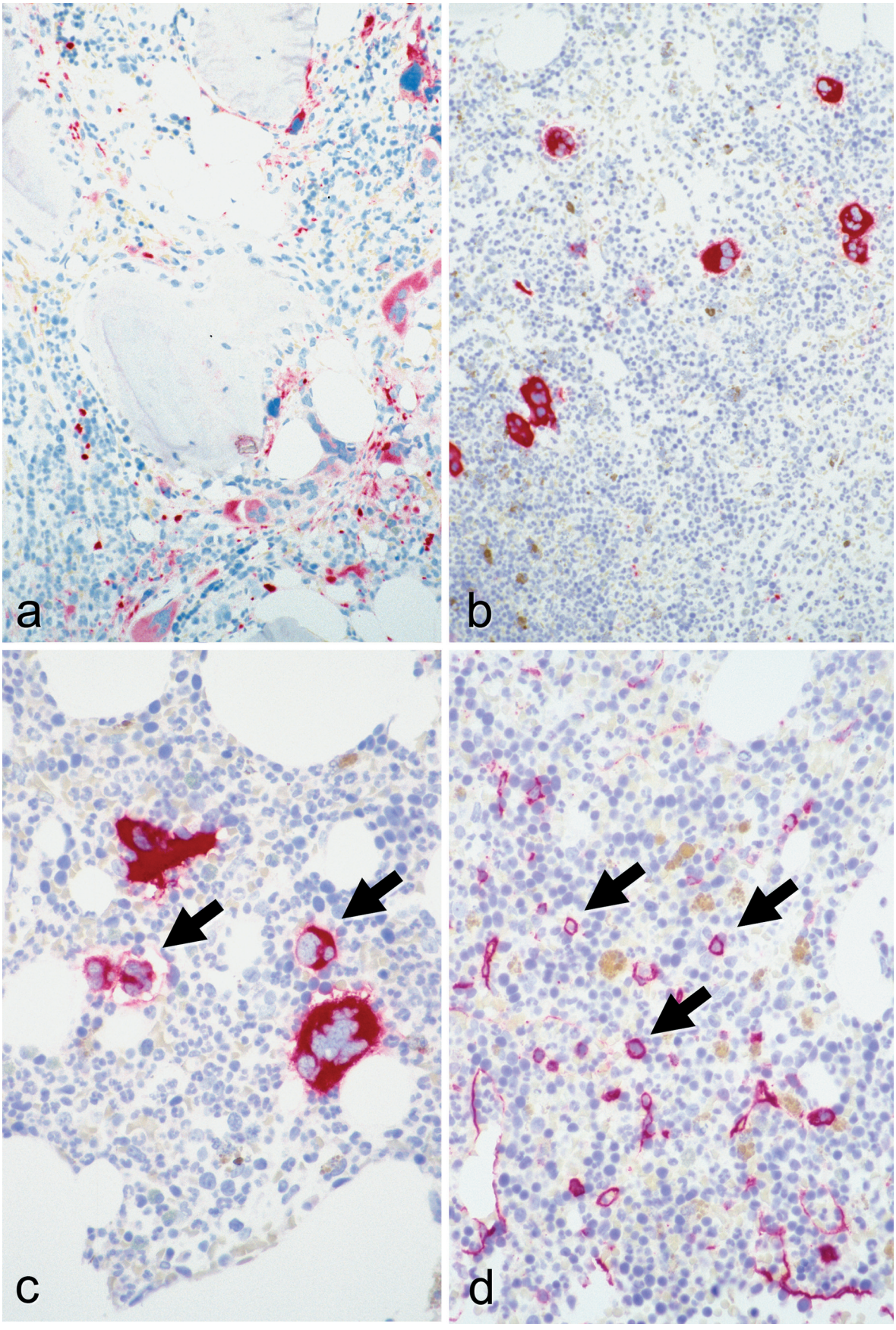

Fig. 2. Megakaryocytes and CD34$^+$ progenitor cells in CIMF before and after allogeneic peripheral stem cell transplantation (SCT). Before SCT, clusters of CD61$^+$ megakaryocytes may be observed showing an abnormal dislocation along the endosteal borders of the osteosclerotic bone and anomalies of differentiation after cytoreductive therapy **(a)**. Following hematopoietic reconstitution after SCT number of megakaryocytes maintained to be increased exhibiting also striking differences in size and maturation **(b)**. Megakaryocytes reveal not only a loose clustering, but also a mild to moderate aberration of normal development, occasionally resulting in a dysplastic appearance of microforms (arrows) **(c)**. In comparison to the normal bone marrow, CD34$^+$ progenitor cells (arrows) are not significantly increased in responding patients after SCT **(d)**. a-c, CD61 immunostaining; d, CD34 immunostaining. a, b, x 180; c, d, x 380

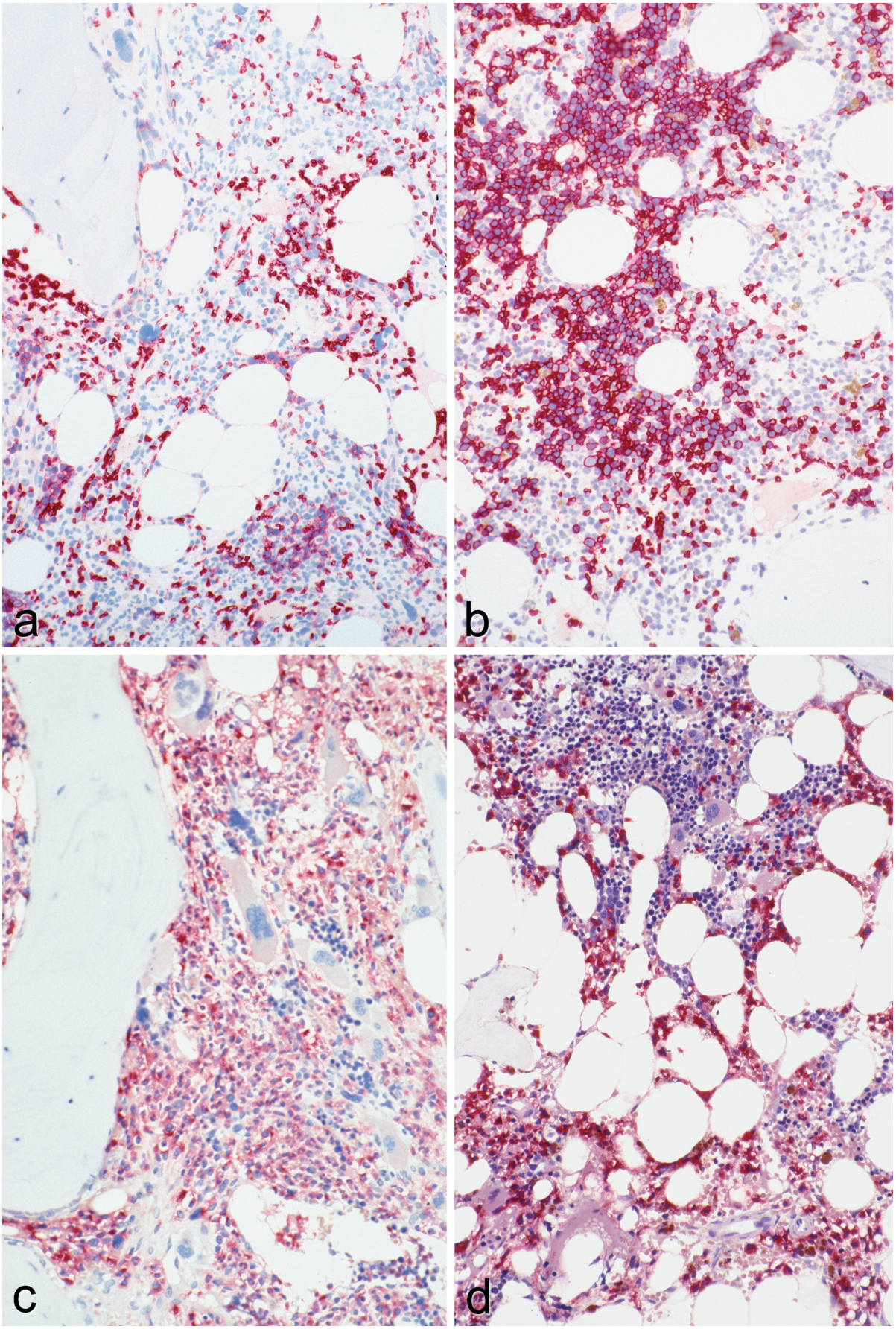

Fig. 3. Erythro- and granulopoiesis in CIMF before and after allogeneic peripheral stem cell trans-plantation (SCT). Before SCT, there are only small clusters of erythroid precursor cells observable with random distribution in the fibro-osteosclerotic marrow **(a)**. After SCT, dense confluent clusters of regenerating erythropoiesis is the first indication for successful hematopoietic reconstitution **(b)**. In CIMF before SCT hematopoiesis is characterized by a granulocytic and mega-karyocytic myelo-proliferation **(c)**. After SCT, retrieval of neutrophil granulopoiesis is not so conspicuously expressed in the early posttransplant period (see above) in comparison to the red cell lineage **(d)**. a, b, immuno-staining with Ret40f; c, d, AS-D-chloroacetate esterase reaction. a-d, x 180

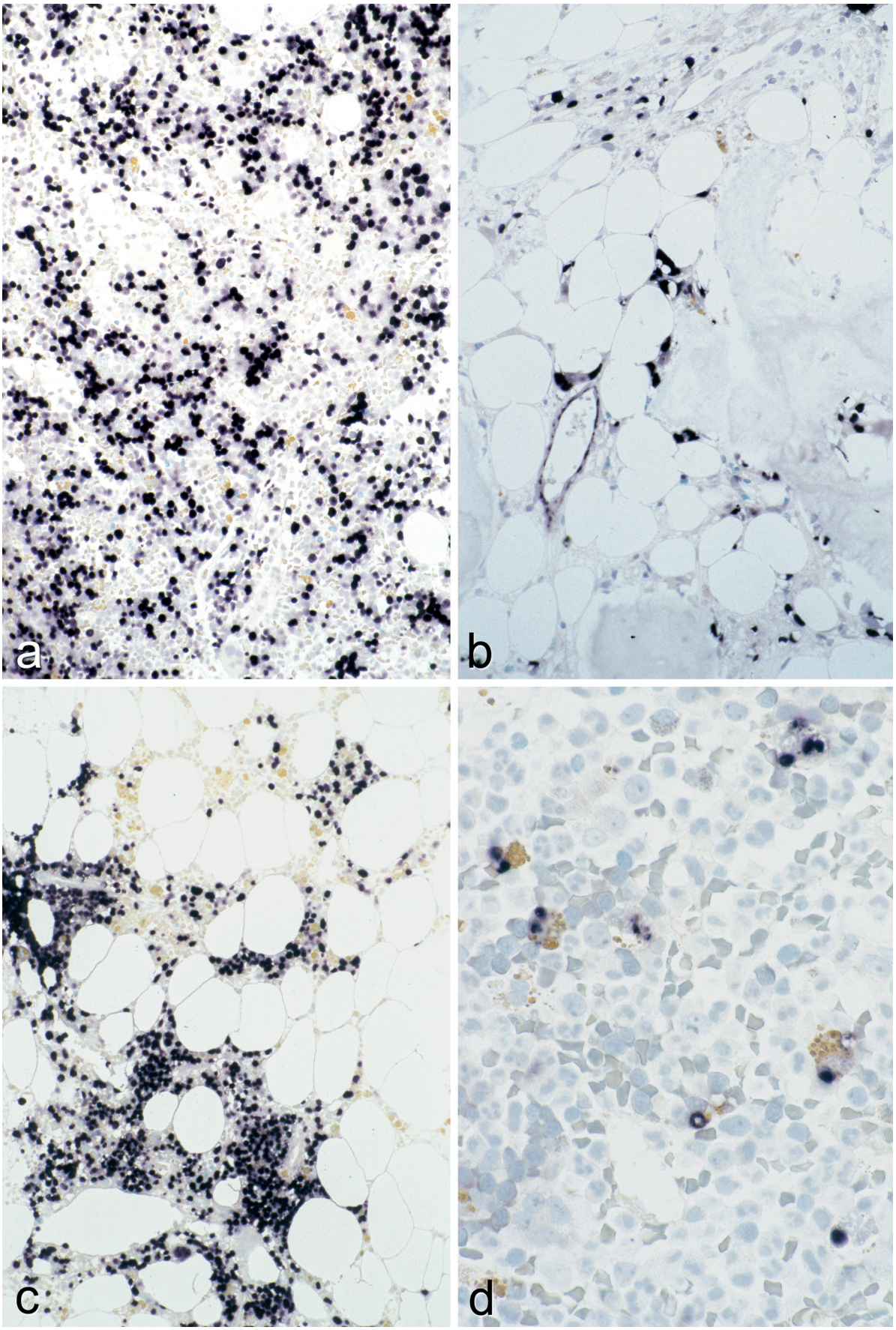

Fig. 4. Proliferation and apoptosis in CIMF before and after allogeneic peripheral stem cell transplantation (SCT). Before SCT, a significantly increased capacity for proliferation is recognizable in CIMF with cytoreductive pretreatment **(a)**. Marked delay in engraftment is associated with decrease in proliferating (marked) cells **(b)**. Successful engraftment is characterized by a dense cluster-like labeling of proliferating cells, most probably erythropoiesis (see also Fig. 3b) **(c)**. Apoptotic cell engulfed by macrophages including hematocrit deposits are frequently encountered after SCT **(d)**. a-c, immunostaining with Ki67; d, immunostaining with apostatin. a-c, x 180; d, x 870

comparison to control specimens (Table 2), the number of osteoclasts per bone area remained increased after SCT (Fig. 1d). Quantity of $CD61^+$ megakaryocytes including immature forms (promegakaryoblasts, megakaryoblasts) was significantly enhanced during cytoreductive therapy in the pretransplant trephines (Fig. 2a). With reference to total hematopoiesis (cellularity) during the posttranplant period, this cell population showed a striking variety in number (Table 2). Frequently small megakaryocytes were observable, displaying an obvious disturbance of maturation and occasionally, a loose clustering was indicated (Fig. 2b). In addition to a descriptive calculation of absolute values and in comparison with normal BM parameters (Table 3), a more detailed planimetric analysis of this cell lineage with the use of certain cut-off points was performed (Table 4). This elaborate evaluation disclosed an increased number of abnormal, immature microforms of megakaryocytes exhibiting a more rounded shape of the nucleus, and a deviation of the nuclear/cytoplasmic ratio implying a relative increase in nuclear size (Table 4). Taken together, these features account for an atypical appearance of this cell lineage, especially in the early posttransplant period consistent with a borderline to mild degree of dysplasia (Fig. 2c). In comparison with the normal BM that showed a relative frequency of micromegakaryocytes (size <150 μm^5) of only 12%, in CIMF a significantly higher incidence could be found before and after SCT (Table 4). Contrasting the increase of $CD34^+$ progenitors in the pretransplant period, their amount was not strikingly enhanced (Fig. 2d) in patients with normal posttransplant hematopoietic regeneration (Table 2). Nucleated erythroid precursor cells revealed a markedly decreased amount before (Fig. 3a) and especially after SCT (Table 2). However, in a number of patients early reconstitution of hematopoiesis was characterized by a pronounced clustering of these cells forming extensive aggregates or erythrons (Fig. 3b).

Table 2. Endpoints of bone marrow biopsy examinations before and at standardized intervals following allogeneic stem cell transplantation (SCT) in chronic idopathic myelofibrosis. Frequency of parameters was determined by morphometric analysis per mm^2 hematopoietic area or cancellous bone, respectively.

	ENDPOINTS				
	Before SCT	Posttransplant period (days)			Controls
	1	2	3	4	
Intervals (median)	-14	30	157	384	-
Ratio biopsy/bone area (%)	5.5±2.5	5.7±2.2	6.7±4.3	6.4±2.0	6.4±2.1
Osteoclasts	10.8±11.8	16.4±16.1	23.2±22.0	12.4±9.4	3.3±2.8
Fibers (x10^5)	44±22	32±15	25±9	21±15	16±5
$CD61^+$ megakaryocytes	108±73	64±30	81±78	80±86	52±12
Erythroid precursors (x10^2)	5±3	6±4	6±4	8±4	27±8
$CD34^+$ progenitors	29±64	9±5	13±14	7±4	9±3
Proliferating ($Ki\ 67^+$) cells (x10^2)	6±3	5±4	6±3	5±3	2±0.5
Apoptotic cells	7±5	10±6	8±3	12±6	4±1

Table 3. Features of $CD61^+$ megakaryopoiesis before and at standardized intervals following allogeneic stem cell transplantation (SCT) in chronic idopathic myelofibrosis as determined by morphometry.

	ENDPOINTS				
	Before SCT	Posttransplant period (days)			Controls
	1	2	3	4	
Intervals (median, days)	-14	30	157	384	-
No. of evaluated megakaryocytes	1,245	830	863	684	870
Cell size (μm^2)	311±234	328±211	185±182	293±202	266±123
Nuclear size (μm^2)	85±67	81±63	47±42	67±56	77±56
Form factor (score x 10^{-2})*:					
Cytoplasm	77±14	76±14	77±13	77±13	78±14
Nucleus	62±21	58±22	65±23	60±23	59±23
Ratio of nuclear/cytoplasmic area (x10^{-2})	27±13	26±13	32±38	26±25	30±12

*: circular perimeter 100 x10^{-2}

Similarly, engraftment was accompanied by an extended focal proliferation of neutrophil granulopoiesis (Fig. 3c, d), which was usually not so conspicuously expressed in the early posttransplant period (Fig. 3d). In line with a chronic myeloproliferative disorder and previous applications of different therapeutic regimens, proliferation was enhanced (Fig. 4a). Even after SCT overall proliferating activity persisted (Table 2), depending on the success of transplantation (Fig. 4b,c). On the other hand, apoptosis (Fig. 4d) remained significantly increased.

Discussion

The presence of BM fibrosis has previously been assumed to be a relative contradiction to transplantation, because of concerns about insufficient marrow space or an inadequate function of the microenvironment to allow an undisturbed engraftment (Rajantie et al., 1986; Soll et al., 1995). In patients with chronic myeloid leukemia (CML), the degree of pretransplant fibrosclerotic BM changes was associated with an increased delay or failure of engraftment (Thiele et al., 2000a). Similarly, in CIMF, advanced fibro-osteosclerosis was considered to be related to a worse outcome (Guardiola et al., 1999; Deeg et al., 2003). On the other hand, the striking advances concerning SCT in patients with full-blown CIMF and related disorders recently achieved by several groups (Daly et al., 2003; Deeg et al., 2003; Rondelli et al., 2005) offer long-term relapse-free survival (Fruchtman, 2003; McCarty, 2004). In this context, one has to keep in mind that BM fibrosis does not present a stable process. This phenomenon is regulated by complex, only partially understood interactions between cytokine release, myeloid stroma and a variety of cells (megakaryocytes, macrophages, fibroblasts, endothelial cells). These have been found to exert a close relationship between increase in collagen (Reilly, 1994; Martyre, 1995; Le Bousse-Kerdiles and Martyre, 1999, 2001; Chagraoui et al., 2002; Schmitt et al., 2002) and formation of endophytic bone or osteosclerosis (Yan et al., 1996; Takai et al., 1998; Chagraoui et al., 2003). It is reasonable to assume that removal of the abnormal, clonally transformed cell population with replacement by normal precursors from healthy donors should eliminate the cytokine-mediated stimulus, and consequently regression of myelofibrosis has to be expected. In CML following full BM transplantations, reversal of myelofibrosis was reported to occur in the posttransplant period (McGlave et al., 1982; Oblon et al., 1983; Thiele et al., 2000a). On the other hand, in CIMF until now, no systematic investigation, in particular involving morphometry focused explicitly on this phenomenon. In only four patients, a serial analysis of the volume density of fibers in the BM was performed at standardized intervals post transplantation demonstrating the progressive resolution of marrow fibrosis (Rondelli et al., 2005). According to descriptions that mostly included single patients (Dokal et al., 1989; Creemers et al., 1992; Byrne et al., 2000; Cervantes et al., 2000; Devine et al., 2002; Hessling et al., 2002; Greyz et al., 2004) or small groups with repeatedly performed posttransplant examinations after BM (Singhal et al., 1995; Anderson et al., 1997) or peripheral SCT, the majority of cases revealed a striking regression of fibers within one year (Anderson et al., 1997; Guardiola et al., 1999; Daly et al., 2003). In the largest series, comprising 49 patients, semiquantitative evaluation showed no relevant fibrosis in 30 patients as early as 12 months after SCT and in 19 patients a persistent considerable degree of myelofibrosis even two years after transplantation (Deeg et al., 2003). Altogether, these data fit well with our results and that of others (Guardiola et al., 1999; Daly et al., 2003; Deeg et al., 2003; Rondelli et al., 2005) implicating that myelofibrosis is reversed in responding patients predominantly between the 6th to

Table 4. Relative frequency (%) of CD61^{+} megakaryocyte features before and at standardized intervals following allogeneic stem cell transplantation (SCT) in chronic idiopathic myelofibrosis. Significant anomalies of cytology - dysplastic aspects (cut-off points were chosen, according to mean values of parameters - see Table 3) are demonstrable. In comparison with the different checkpoints, abnormalities were most conspicuously expressed in the early posttransplant period after about 4 to 6 months (endpoint 3).

		ENDPOINTS			
		Before SCT	Posttransplantat period (dasy)		
		1	2	3	4
Intervals (median, days)		-14	30	157	384
Frequency (%):					
Normal megakaryocytes (>250 μm^2)		50	58	23	52
Micromegakaryocytes and precursors (<150 μm^2)		29	16	62	26
Form factor (score x10^{-2})*:					
Megakaryocytes	<78 ***	46	48	48	49
Nucleus	<59 **	57	50	66	53
Ratio of nuclear/cytoplasmic area (x10^{-2})	<30 **	37	36	47	35

*: circular perimeter 100 x10^{-2}. Level of significance: **, $p < 0.001$; ***, $p > 0.05$

12th posttransplant months. Regarding osteosclerosis, only scant knowledge exists about changes associated with transplantation in CML (McGlave et al., 1982). Our data on the extent of pretransplant endophytic bone formation in CIMF and the number of osteoclasts are in keeping with a previous report (Thiele et al., 1989). Although in a very few patients following SCT a partial resolution of osteosclerosis or even a complete disappearance was reported (Cervantes et al., 2000; Tanner et al., 2003), a significant regression of the newly-formed (woven) bone was not recognizable in our cohort even after one year of observation. However, this feature is in need of further long-term investigation.

Although pathogenesis of myelofibrosis is functionally linked with megakaryopoiesis (Martyre et al., 1994; Reilly, 1994; Le Bousse-Kerdiles and Martyre, 1999, 2001; Schmitt et al., 2002), no significant correlation between the regression of fiber density and quantity of megakaryocytes was evident in our series. On the other hand, appearance of this cell lineage was not completely in keeping with a normal state. Maturation defects and conspicuous varieties in size and differentiation were observed, in particular by applying CD61 immunostaining (Gatter et al., 1988) in combination with an elaborate planimetric evaluation of cytological variables (Table 4). Our findings resemble reports on dysplastic changes of megakaryocytes occurring in the early posttransplant period in CML patients following full BM transplantation (Van den Berg et al., 1990; Rousselet et al., 1996; Hurwitz, 1997; Thiele et al., 2000a, 2001b). This feature may be responsible for the decline in function (platelet shedding) and therefore results in corresponding defects, i.e. thrombocytopenia in many patients independently of the number of megakaryocytes. Focal groupings of (left-shifted) nucleated erythroid precursor cells are usually indicative for an initial hematopoietic regeneration following transplantation (Van den Berg et al., 1990; Rousselet et al., 1996; Thiele et al., 2000b, 2001b). However, in CIMF the reconstitution of this cell lineage is relatively slow and even after one year amount of erythropoiesis does not reach the normal level (Table 2), which is clinically consistent with mild to marked anemia in most patients (Table 1). In the context of transplantation, it has been speculated that various subpopulations of $CD34^+$ progenitors serve different functions in reconstituting normal hematopoiesis (Thiele et al., 2002). It appears reasonable to assume that the more committed progenitor cell population may be important for an early engraftment and hematopoietic recovery, while the pluripotent stem cell with its potency for self-renewal may be responsible for a long-term establishment of hematopoiesis after transplantation (Messner, 1998). In our series of patients, the increased amount and wide ranges of $CD34^+$ cells before SCT are probably due to different disease stages and the varieties of pretransplant cytoreductive therapies applied. Contrasting this finding, in the posttransplant period quantity of progenitors is in line with the normal BM and therefore reflects a steady state of cell turnover in this compartment except for one patient that apparently developed an acceleration at day 157. Little information exists concerning proliferation and apoptosis in the posttransplant period in correspondence with the regenerating hematopoiesis. In comparison with the pretransplant values an increase in these parameters is still recognizable after one year and possible accounts for a general enhancement of cell turnover, i.e. a still persistent stimulation of hematopoietic regeneration. On the other hand, a failing reconstitution of hematopoiesis is associated with a significant decrease in proliferative capacity.

In conclusion, allogeneic SCT in CIMF causes an almost total regression of myelofibrosis after about 6 months, that is not associated with a significant reversal of osteosclerosis. However, SCT fails to retrieve completely the normal amount of erythropoiesis and generates minimal to mild dysplastic effects on megakaryocytes, at least in the early posttransplant period. Proliferative activity and programmed cell death are still enhanced in the late posttransplant period and probably associated with an increased cell turnover, while $CD34^+$ progenitors usually display a normal quantity in responding patients.

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