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Long-lasting decrease of marrow and circulating long-term culture initiating cells after allogeneic bone marrow transplant

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Summary:

We investigated bone marrow (BM) and circulating (PB) hematopoietic progenitor cells in 37 normal donors and in 25 patients 1 to 8 years after successful allogeneic bone marrow transplant. At the time of testing, transplanted patients had normal blood counts and bone marrow cellularity. By flow cytometry, BM CD34$^+$ cells were found to be three- to four-fold decreased in transplanted patients compared to normal donors, while the number of PB CD34$^+$ cells was the same as in normal donors. Using a methylcellulose colony assay, primary BM colony-forming cells (CFU-GM) were decreased 2.1-fold, whereas PB CFU-GM were only marginally decreased. In a long-term culture initiating cell (LTC-IC) assay, an eight-fold decrease of early progenitor cells was observed in the marrow of transplanted patients compared to normal donors, and a five-fold decrease was documented in peripheral blood. We found that the BM LTC-IC cell number correlated with concurrently determined BM CD34$^+$ cells and committed progenitor cell number (measured as CFU-GM) and with PB LTC-IC number, but not with PB CFU-GM and CD34$^+$ cells. We conclude that marrow and circulating early stem cell compartments, as measured by the LTC-IC assay, are greatly and permanently depressed following bone marrow transplant. The correlation between BM and PB LTC-IC indicates that the enumeration of circulating LTC-IC can be used as a measure of the stem cell compartment in the bone marrow after transplant. It seems that the deficiency of the most immature progenitor cells persists forever after successful bone marrow transplant; this means that a complete hematopoietic reconstitution can be sustained by a reduced stem cell pool.

Keywords: CD34$^+$; LTC-IC; BMT

Long-term survivors after allogeneic bone marrow transplantation (BMT) have normal marrow cellularity and normal peripheral blood cell counts. Due to intrinsic experimental problems with measurement of the human stem cell compartment, the majority of studies on stem and progenitor cells after BMT deal only with more mature committed colony forming-units (CFU-GM).[1–3] These studies suggest that the recovery of committed progenitors such as CFU-GM may be protracted and take longer than normalization of peripheral blood counts and marrow cellularity; however, they do not allow conclusions with regard to the extent of damage and the degree of reconstitution of the true stem cell compartment. In addition, neither CD34$^+$ cells nor CFU-GM numbers have been reported as suitable parameters to predict engraftment after unmanipulated allogeneic transplant.[4–7] In aplastic anemia, which is characterized by greatly depleted progenitor and stem cell compartments, we and others have observed only a partial restoration of the number of LTC-IC and committed progenitor cells, despite full recovery of PB counts after immunosuppressive therapy;[8] a numerical defect in these cells persists even years after the initial successful treatment.[9,10] The causes for the inability to regenerate a normal stem cell pool are not clear on the cellular level. A sustained quantitative defect in the progenitor cell compartment despite normal graft function after BMT may suggest either intrinsic inability of a limited stem cell pool to expand (only less than 1/200 of the donor hematopoietic system is allografted), or a stromal defect reducing the 'seeding efficiency' and thus impairing the expansion.[11–13] Indeed, a tremendous expansion of the allografted stem cells would be needed to restore a normal LTC-IC frequency. With the exception of a few recent studies,[14,15] attempts at *in vitro* expansion of stem cells have been unsuccessful.[16,17] Recently, it has been reported that telomere shortening occurs during *in vitro* and *in vivo* expansion of hematopoietic stem cells, suggesting that the ability to proliferate may be limited.[18] After transplant, a significant reduction of telomere length has been documented, and this phenomenon may impair stem cell long-term replicative capacity.[19–21] Impaired function of stromal cells supporting maintenance and proliferation of hematopoietic progenitor and stem cells may also be responsible for their long-lasting quantitative defect: chemotherapy and radiotherapy have both been reported to induce a long-lasting impairment in marrow stroma function.[22–24]

Unlike murine models, where hematopoietic stem cells can be measured using *in vivo* competitive repopulation assays, only a few methods exist for the measurement of human stem cells. Assessment of the repopulating capacity of early hematopoietic progenitors can be indirectly perfor-

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med utilizing pre-CFU or LTC-IC assays.[6,25] At present, LTC-IC are believed to be the best *in vitro* surrogate of the stem cells which allow long-term engraftment after transplant.[26,27] Recently, deficient reconstitution of BM LTC-IC after allogeneic BMT has been reported.[9,10] Although LTC-IC can also be measured in the peripheral blood, to our knowledge no study of long-term reconstitution of circulating early hematopoietic progenitors has been reported so far. As compared to the determination in BM, a PB-based LTC-IC assay has several obvious advantages, which are related to inaccuracy due to the variable dilution of marrow samples with blood, difficult assessment of marrow cellularity and limitation in frequent BM sampling. To investigate the stem compartment after transplant and to assess the value of the measurement of LTC-IC in peripheral blood, we have checked the number of stem cells and committed progenitors in transplanted patients in comparison with normal subjects, by measuring LTC-IC, CFU-GM and $CD34^+$ cells concurrently in BM and PB.

Materials and methods

Specimen collection

Bone marrow (BM) and peripheral blood (PB) samples were obtained after informed consent from 37 bone marrow donors and 25 patients who had been successfully allotransplanted with unmanipulated marrow from an HLA-identical sibling. Allotransplanted patients included one patient with severe aplastic anemia (SAA), 10 with acute myeloid leukemia (AML) and 14 with chronic myeloid leukemia (CML) (Table 1). There were 13 females and 12 males, with a median age of 31 years (range 15–51). Patients were infused with a median number of 2.72×10^8/kg mononuclear cells (range 0.9–7.12) and 2.90×10^4/kg (range 1.3–4.6) granulocyte–macrophage colony-forming cells (CFU-GM). All patients were conditioned with the BUCY2 regimen, except the patient with SAA who received only cyclophosphamide (200 mg/kg); no patient received prophylactic growth factors after BMT. The median interval between BMT and the present analysis was 4 years (range 1–8). At the time of testing, all transplanted patients had normal blood counts and bone marrow cellularity (median value: 70% with a range 50–100%) with a normal distribution of all hematopoietic lineages (Table 2).

Cytogenetics

In gender mismatched transplants, the engraftment was documented by cytogenetic analysis according to the standard GTG-banding technique.

Flow cytometry analysis

Phycoerythrin (PE)-conjugated monoclonal antibody (moAb) to CD34 (clone HPCA-1; Becton Dickinson, Mountain View, CA, USA) was used to identify $CD34^+$ cells. BM or PB samples were diluted to a white blood cell (WBC) count of about $2 \times 10^4/\mu$l and stained with the moAb for 20 min at 4°C. After staining, samples were subjected to red blood cell lysis with ammonium chloride buffer (Ortho, Raritan, NJ, USA) and washed with phosphate-buffered saline. Flow cytometry was performed using

Table 1 Characteristics of bone marrow transplanted patients

UPN	*Age*	*Gender patient/donor*	*Disease*	*Mononuclear cells infused $\times 10^8$/kg*	*CFU-GM infused $\times 10^4$/kg*	*aGVHD grade*	*cGVHD grade*	*CMV infection*
4	31	F/F	SAA	3.70	4.58	2	3	no
5	17	M/M	AML	3.20	4.25	1	0	no
6	32	F/M	CML	3.70	6.12	0	0	no
7	15	F/F	AML	4.60	7.10	1	0	no
8	23	F/F	CML	2.71	2.91	2	1	yes
9	17	F/M	AML	3.10	3.97	2	0	no
10	25	F/F	CML	3.40	4.62	0	1	yes
12	37	F/F	CML	1.90	2.49	0	1	no
13	21	M/F	CML	2.70	3.76	2	0	no
15	20	M/F	CML	3.30	NA	1	3	no
16	36	M/M	CML	2.70	NA	0	3	no
19	51	F/M	AML	3.80	NA	1	0	no
22	35	M/F	AML	1.90	NA	0	3	no
24	20	F/F	AML	2.80	NA	1	0	no
33	45	F/F	AML	2.80	NA	0	1	yes
45	19	M/M	CML	3.60	1.65	0	0	yes
46	35	M/F	CML	1.30	0.90	1	0	yes
49	38	F/M	CML	2.60	2.70	0	3	no
61	19	F/M	CML	1.40	1.40	0	1	yes
68	17	M/F	CML	1.74	2.90	0	1	no
75	45	M/F	AML	2.10	1.60	3	1	yes
78	33	M/F	CML	2.00	1.83	2	3	yes
82	16	F/F	AML	2.60	2.80	0	1	no
91	50	M/F	CML	1.60	NA	2	3	no
95	38	M/M	AML	2.40	3.10	1	3	yes

M = male; F = female; SAA = severe aplastic anemia; AML = acute myeloid leukemia; CML = chronic myeloid leukemia; NA = not available.

Table 2 Characteristics of bone marrow transplanted patients at the time of study

UPN	*Time from transplant (years)*	*WBC $\times 10^9$/l*	*Plt $\times 10^9$/l*	*BM cellularity (%)*	*BM CD34$^+$*	*PB CD34$^+$*	*BM CFU-GM*	*PB CFU-GM*	*BM LTC-IC*	*PB LTC-IC*
					$\times 10^5$ mononuclear cells					
4	8	7.9	220	70	400	50	10.8	4.25	0.140	0.010
5	7	5.0	175	80	1400	111	51.0	21.25	1.020	0.370
6	7	6.9	274	70	200	69	6.1	10.25	0.010	0.080
7	7	5.4	145	90	500	137	17.5	14.00	0.250	0.040
8	6	4.1	124	70	594	100	ND	8.75	ND	0.190
9	5	6.9	201	80	400	54	40.2	7.25	0.160	0.090
10	5	6.2	185	100	700	172	31.0	10.00	ND	0.035
12	5	4.6	158	90	400	153	18.0	6.25	0.400	0.020
13	4	5.1	110	70	900	149	27.6	15.50	0.510	0.020
15	4	3.7	128	70	600	85	7.7	10.00	ND	0.020
16	4	7.4	357	80	400	97	24.6	11.00	0.150	0.060
19	4	6.0	214	100	1300	52	48.4	8.50	0.810	ND
22	4	5.0	117	100	277	158	14.0	8.50	0.015	ND
24	3	6.6	235	90	800	102	44.2	11.50	0.500	0.095
33	3	3.6	197	100	700	167	23.5	16.25	0.350	0.010
45	3	6.1	163	90	1100	74	25.2	4.25	0.520	0.140
46	2	7.5	418	70	200	98	10.8	7.75	0.100	0.040
49	2	3.8	124	70	ND	52	ND	5.00	ND	0.040
61	2	4.7	228	90	600	80	30.6	5.50	0.260	0.045
68	2	9.5	162	70	100	119	3.0	11.25	0.040	0.120
75	2	7.6	143	50	ND	145	2.7	9.50	0.100	0.035
78	2	5.1	159	100	300	55	8.5	3.50	0.120	0.030
82	1	3.8	212	50	ND	109	ND	5.75	ND	0.060
91	1	6.6	201	80	ND	ND	ND	ND	0.172	ND
95	1	5.8	148	100	ND	ND	ND	ND	0.081	ND

a flow cytometer (Cytoron Absolute, Ortho Diagnostic Systems) equipped with the ARS software (Ortho Diagnostic Systems). During acquisition, a threshold was set on forward light scatter to exclude cell debris from analysis. A rectangular analysis region on sideward light scatter was drawn to include all MNCs for determinations of CD34-expressing cells, and live gate acquisition was performed on cellular events that fell into that region. Ten thousand events were acquired in the live gate, with a minimum of 60 000 events in the entire population.

Cell separation

BM was aspirated from the posterior iliac crest into syringes containing Iscove's modified Dulbecco's medium (IMDM) supplemented 1:10 with heparin (O'Neil and Feldman, St Louis, MO, USA). Mononuclear cells (MNC) from BM and PB were isolated by density gradient centrifugation using lymphocyte separation medium (Organon, Durham, NC, USA). After washing with Hanks' balanced salt solution (HBSS), cells were resuspended in IMDM supplemented with 5% fetal calf serum (FCS). HBSS, IMDM and FCS were purchased from Life Technologies, Gaithersburg, MD, USA.

Hematopoietic colony assay

Isolated BM and PB cells were plated in methylcellulose (Stem Cell Technologies, Vancouver, Canada) at a concentration of 2×10^5 cells/ml of medium (35 mm dishes; 1 ml of medium/dish). The growth factor cocktail consisted of 10 ng/ml IL-3, 50 ng/ml G-CSF, 50 ng/ml GM-CSF, 20 ng/ml stem cell factor (SCF) and 2 U/ml erythropoietin (EPO) (Amgen, Thousand Oaks, CA, USA). All cultures were performed in duplicate. For estimation of circulating progenitors per milliliter, the following equation was used: LTC-IC/ml = LTC-IC/10^5 MNC $\times$ WBC/μl $\times$ %MNC/10^4.

LTC-IC assay

PB and BM MNC were plated on irradiated (15 Gy of 250 kV rays) allogeneic stromal cells (3×10^4/cm^2) at cell densities varying from 1×10^6 to 5×10^6 per well. To ensure consistency in all experiments, stromal cells from only four normal donors were used, and these layers showed comparable feeder function as measured by the number of LTC-IC grown from BM and PB of controls. At least three and often more cell concentrations were applied on pre-established irradiated stromal feeder layers. Culture medium consisted of long-term stem cell medium (LTC; Stem Cell Technologies) supplemented with 1×10^{-6} mol/l hydrocortisone sodium hemisuccinate (Sigma, St Louis, MO, USA). These cultures were maintained at 37°C for 3 days and then switched at 33°C for 5 weeks with weekly withdrawal of half of the medium, which was replaced by fresh LTC medium. At the end of the 5 weeks, all nonadherent cells were removed and combined with cells harvested from the adherent fraction by trypsinization; these cells were then washed three times and assayed for their CFU-GM content in the methylcellulose colony assay as described above. The method we used to calculate LTC-IC number is described at the end of the next section.

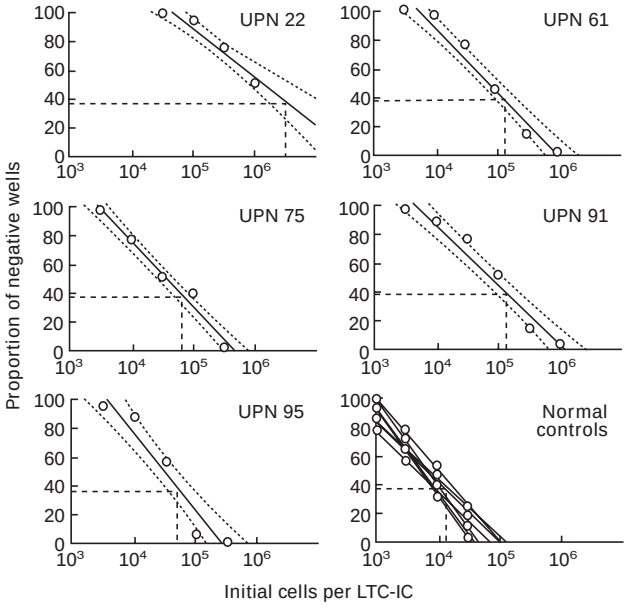

Figure 1 Limiting dilution analysis. Bone marrow cells from five representative transplanted patients and from five normal controls were serially diluted and plated on irradiated allogeneic bone marrow stroma. The proportion of wells containing secondary colonies after 5 weeks of long-term culture was assessed and the absolute frequency of LTC-IC was determined at the cell dilution with ⩽37% negative wells according to the Poisson equation.

Limiting dilution analysis

BM and PB MNC were cultured in 96-well flat bottom plates containing pre-irradiated bone marrow allogeneic stroma. At each dilution, 24 wells were seeded. After 5 weeks, wells were overlaid with semisolid medium containing the cocktail of growth factors described above. The frequency of LTC-IC was calculated by determining the cell dilution that resulted in ⩽37% negative wells according to the Poisson equation ($m = -1/f \ln P_0$, where m is the frequency of clonogenic cells, f is the number of cells seeded into each well, ln is the natural log, and P_0 is the proportion of empty wells). The clonogenic capacity of a single LTC-IC was measured as the mean number of colonies per positive wells at dilution where the proportion of empty wells was >0.75, and the probability of each well containing the progeny of a single cell was >85%, equivalent to single hit kinetics (one LTC-IC/well), according to the gamma distribution.[28] The clonogeneic capacity of a single LTC-IC was also calculated by dividing the number of colonies derived from bulk cultures by the frequency of LTC-IC (Figure 1). By limiting dilution analysis we determined that from normal donors a median of 3.5 colonies (range 1–24) were generated by a single LTC-IC (n = 15, data not shown). The clonogenic potential of LTC-IC *in vitro* from 10 transplanted patients was lower than normal donors, but not statistically different ($P > 0.5$). Since in the LTC-IC assay the relation between cell input and the output of secondary colonies after a 5 week culture was linear (Figure 2), for cultures in which the limiting dilution analysis was not possible because of a limited cell number, the number of clonogenic cells was converted to the absolute number of LTC-IC by dividing the number of secondary CFU-GM in bulk culture by the calculated *in vitro* clonogenicity.

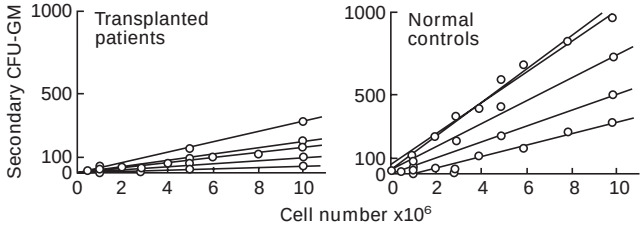

Figure 2 Relationship between day 1 cell input in long-term culture and number of secondary colonies measured after 5 weeks of culture. Five representative transplanted patients and five normal controls.

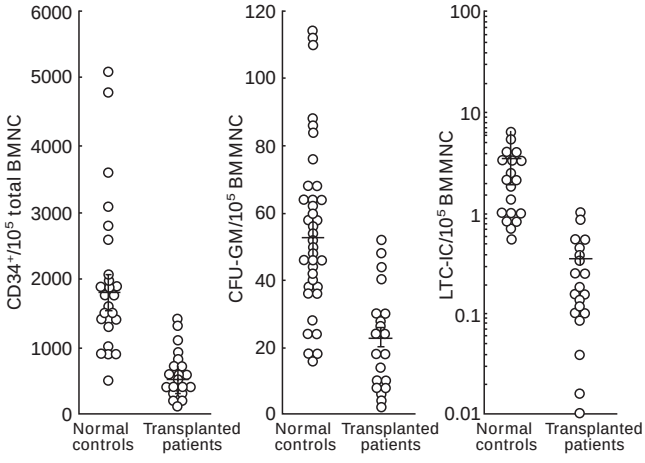

Figure 3 Hematopoietic progenitors in bone marrow of transplanted patients and normal controls. Total bone marrow nucleated cells (BMNC) were used for flow cytometric evaluation of CD34⁺ cells; bone marrow mononuclear cells (BMMNC) were used for colony-forming cells (CFU-GM) and for long-term culture initiating cell (LTC-IC) measurement. Each dot represents a subject studied.

Statistical analysis

All results were expressed as mean ± standard error of the mean (s.e.m.). Differences between groups were evaluated using the Student's *t*-test. Correlation between variables was assessed using Pearson's linear regression. The gamma distribution was calculated using the GAMMADIST function in Microsoft Excel version 5.0 (Wokingham, UK).

Results

Marrow CD34⁺ cells, CFU-GM and LTC-IC in transplanted patients

BM and PB total nucleated cells (TNC) were used for flow cytometry evaluation of CD34⁺ cells, while mononuclear cells were used for the measurement of primary and secondary CFU-GM. All transplanted patients had normal PB leukocyte counts and BM cellularity at the time of the analysis (Table 2), and showed complete engraftment at the cytogenetic level when this test was informative. CD34⁺ cells from BM were decreased three- to four-fold in trans-

Table 3 Stem cells and hematopoietic progenitors in bone marrow of transplanted patients

	CD34⁺ $\times 10^5$ *total nucleated cells*	*CFU-GM* $\times 10^5$ *mononuclear cells*	*LTC-IC* $\times 10^5$ *mononuclear cells*
Normal subjects	1976 ± 229 *n* = 25	55.02 ± 4.13 *n* = 37	2.465 ± 0.370 *n* = 20
Transplanted patients	594 ± 85 *n* = 20	22.27 ± 3.35 *n* = 20	0.285 ± 0.060 *n* = 20
P[a]	<0.0001	<0.0001	<0.0001

[a]Student's *t*-test.

Table 4 Approximate percentage of progenitor cells in BM and PB of normal subjects and transplanted patients

	Normal subjects	*Transplanted patients*
Bone marrow		
CD34⁺[a]	2%	0.6%
CFU-GM[b]	0.05%	0.02%
LTC-IC[b]	0.002%	0.0003%
Peripheral blood		
CD34⁺[a]	0.1%	0.1%
CFU-GM[b]	0.015%	0.010%
LTC-IC[b]	0.0004%	0.00007%

[a]% of total nucleated cells.
[b]% of mononuclear cells.

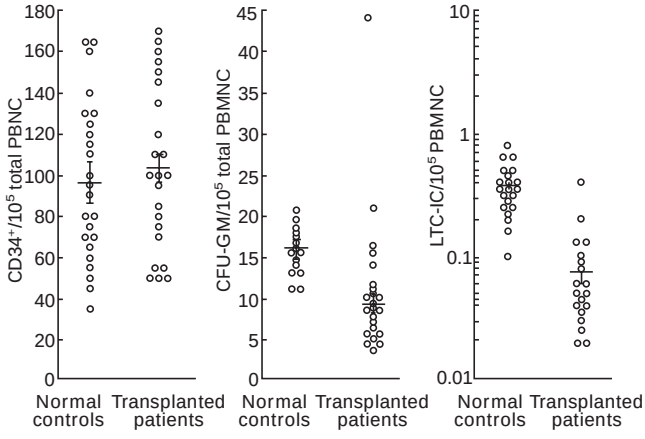

Figure 4 Circulating hematopoietic progenitors of transplanted patients and normal controls. Total peripheral blood nucleated cells (PBNC) were used for flow cytometric evaluation of CD34⁺ cells; peripheral blood mononuclear cells (PBMNC) were used for colony-forming cells (CFU-GM) and for long-term culture initiating cell (LTC-IC) measurement. Each dot represents a subject studied.

planted patients compared to normal donors (594 ± 85/10^5 total nucleated cells *vs* 1976 ± 229; $P < 0.0001$). Similarly, primary CFU-GM from BM were decreased 2.1-fold (22.3 ± 3/10^5 MNC plated *vs* 55 ± 4; $P < 0.0001$). The depletion of the LTC-IC compartment appeared to be more pronounced: we observed an eight-fold decrease of early progenitor cells, measured as LTC-IC, in the BM of transplanted patients compared to normal donors (0.28 ± 0.06/10^5 MNC plated *vs* 2.46 ± 0.37: $P < 0.0001$) (Tables 3 and 4, Figure 3). Marrow LTC-IC cell number in transplanted patients was not influenced by the number of CFU-GM infused: using as a cut-off the median value (2.93×10^4/kg CFU-GM), there was no difference in LTC-IC frequency between the groups of patients who had received fewer or more CFU-GM ($P = 0.74$). Any grade of acute or chronic graft-versus-host disease as well as cytomegalovirus infection did not affect LTC-IC frequency after BMT ($P = 0.38$, $P = 0.06$ and $P = 0.43$, respectively). In addition, we found that LTC-IC cell number in BM showed a good correlation with concurrently determined BM CD34⁺ cells and CFU-GM ($P < 0.0001$): CFU-GM *vs* CD34⁺ cells ($r = 0.786$), LTC-IC *vs* CD34⁺ ($r = 0.954$), and LTC-IC *vs* CFU-GM ($r = 0.810$).

Circulating CD34⁺, CFU-GM and LTC-IC in transplanted patients

Quantitative assessment of LTC-IC in the BM may be affected by several factors such as a variable level of contamination with blood or heterogeneous distribution of cellularity within the aspirated marrow area. We therefore measured CD34⁺, CFU-GM and LTC-IC cell numbers in PB from transplanted patients and normal subjects. The measured numbers were adjusted for white blood cell count and the proportion of blood mononuclear cells as described in the section Hematopoietic Colony Assay in Materials and methods. We also evaluated the absolute frequency of CD34⁺, CFU-GM and LTC-IC per milliliter of blood; the two measurements had a good correlation ($P < 0.0001$). At variance with the findings in BM, the number of circulating CD34⁺ cells was similar in transplanted patients and normal donors (103.8 ± 8.4 *vs* 97.6 ± 7.7) and primary CFU-GM were only slightly decreased (9.3 ± 0.9 *vs* 15.6 ± 0.9). By contrast, a five-fold decrease of LTC-IC was documented in PB (0.074 ± 0.018 *vs* 0.374 ± 0.043) (Table 5, Figure 4). We found that PB LTC-IC cell number correlated with concurrently determined BM LTC-IC number ($P = 0.003$), but not with BM CFU-GM and CD34⁺ cells.

Effect of time elapsed from transplant on LTC-IC number in blood and bone marrow

BM and PB CD34⁺ and CFU-GM cell numbers showed a progressive improvement in the first 2–5 years after transplant. Although the median number was reduced as com-

Table 5 Stem cells and hematopoietic progenitors in peripheral blood of transplanted patients

	CD34$^+$		*CFU-GM*		*LTC-IC*	
	$\times 10^5$ *TNC*	$\times$ *ml*	$\times 10^5$ *MNC*	$\times$ *ml*	$\times 10^5$ *MNC*	$\times$ *ml*
Normal subjects	97.6 ± 7.7	5656 ± 474	15.6 ± 0.9	334 ± 38.4	0.37 ± 0.04	8.11 ± 1.24
	n = 25		*n* = 20		*n* = 21	
Transplanted patients	103.8 ± 8.4	6191 ± 603	9.3 ± 0.9	246 ± 28.7	0.074 ± 0.02	1.94 ± 0.49
	n = 23		*n* = 23		*n* = 21	
P[a]	0.59	0.49	<0.001	0.07	<0.001	<0.001

[a]Student's *t*-test.

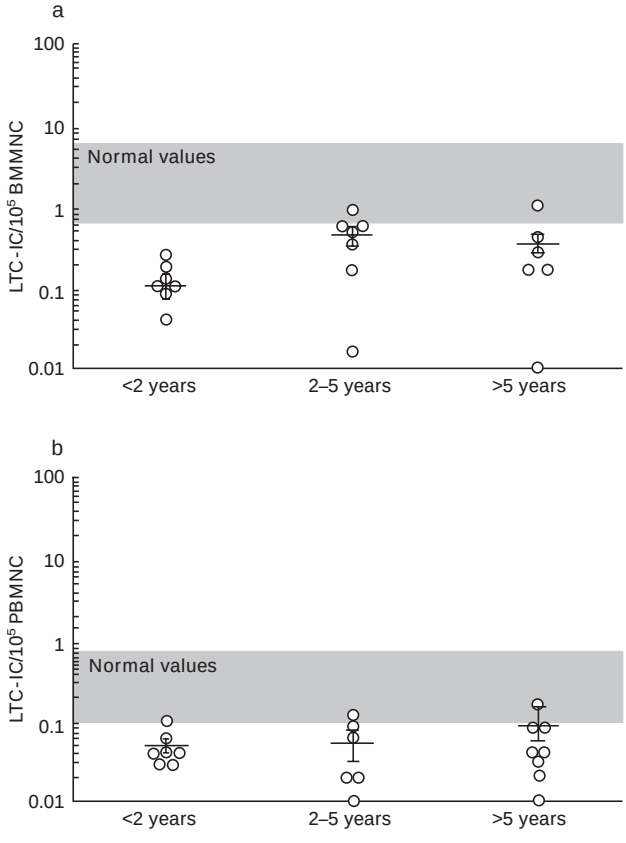

Figure 5 Number of BM (**a**) and PB (**b**) LTC-IC according to time from transplant. Grey area: number of LTC-IC in 20 normal donors.

pared to normal subjects, there was some overlap with the normal range that was not detected when LTC-IC were evaluated (data not shown). The BM LTC-IC compartment was markedly depleted during the first 2 years from transplant. Between 2 and 5 years from transplant, the mean marrow LTC-IC cell number tended to rise (0.124 ± 0.026 *vs* 0.371 ± 0.083 before and after 2 years, respectively; $P = 0.018$), but the majority of patients showed LTC-IC numbers markedly below those seen in normal controls. There was no further improvement in LTC-IC number up to 8 years after transplantation (Figure 5). Unlike those in BM, circulating LTC-IC did not improve with time after transplant: LTC-IC were only slightly higher, but not statistically different, 5 years after transplant (1.34 ± 0.38 *vs* 2.25 ± 0.71 before and after 2 years, respectively; $P = 0.40$).

In vitro and in vivo proliferative potential of LTC-IC from transplanted patients and normal subjects

Stem cell clonogenic capacity *in vivo* was indirectly evaluated by dividing the number of LTC-IC by the number of CFU-GM. Using this proportion, we documented that in transplanted patients ($n = 17$) 14.43 ± 1.89 marrow LTC-IC were necessary to obtain 10^3 CFU-GM, in comparison to 40.75 ± 20 in healthy subjects ($n = 14$; $P = 0.014$), suggesting that *in vivo* LTC-IC in transplanted patients have higher clonogenic capacity than controls (LTC-IC/CFU-GM 1/70 *vs* 1/25 in transplanted patients and healthy subjects, respectively). Similar results were observed in peripheral blood: 8.39 ± 1.68 LTC-IC/10^3 CFU-GM ($n = 21$) *vs* 27.09 ± 3.29 ($n = 18$) in transplanted patients and in healthy subjects, respectively ($P < 0.001$). To determine the proportion of CFU-GM in relation to the stem cell compartment *in vitro*, we also measured the clonogenic capacity of individual LTC-IC. In addition to the direct determination by limiting dilution analysis, where decreasing numbers of cells are plated to define the concentration achieving single hit kinetics by the Poisson distribution, an indirect technique was also applied. In this method the clonogenic capacity of LTC-IC was obtained by dividing the number of colonies derived from bulk cultures by the frequency of LTC-IC. Using both methods, the median number of CFU-GM per LTC-IC was about 2.5 ($n = 10$) and not significantly different from normal controls ($n = 15$; $P = 0.3$) (Table 6). We also calculated the frequency of marrow LTC-IC in relation to the CD34$^+$ cell compartment: the number of LTC-IC/10^5 MNC was divided by CD34$^+$/10^5 MNC. Marrow LTC-IC within the compartment of CD34$^+$ did not show significant differences between transplanted patients and normal controls: 0.198 ± 0.020 LTC-IC/10^3 CD34$^+$ *vs* 0.275 ± 0.077 ($P = 0.19$).

Discussion

The analysis of stem and progenitor cell compartments after allogeneic bone marrow transplant may give clues on the potential and kinetics of stem cell expansion which starts from the finite number of cells used for a bone marrow

Table 6 *In vitro* secondary CFU-GM production from single LTC-IC by limiting dilution analysis

UPN	*Number of positive wells*[a]	*P for each well containing a single clonogenic cell*[a]	*Number of secondary CFU-GM per positive well*	*Measured clonogenicity*[b]	*Calculated clonogenicity*[c]
12	6	0.91	1,1,2,3,5,7	2.9	3.1
19	6	0.89	1,2,4,5,5,8	3.8	3.3
22	3	0.90	1,2,5	2.43	1.95
33	8	0.95	1,2,2,3,3,3,4,5	2.77	2.68
45	7	0.93	1,3,4,5,6,8,9	4.8	4.3
46	4	0.90	1,1,1,3	1.3	1.5
61	5	0.89	1,1,1,1,7	1.99	2.69
75	5	0.89	1,1,1,1,2	1.08	1.82
91	9	0.91	1,1,1,1,2,2,3,3,4	1.84	1.945
95	4	0.95	1,3,4,5	3.08	2.12
Median				2.6	2.4
Range				1.08–4.8	1.5–4.3
Normal controls ($n = 15$)					
Median				3.5	3.3
Range				1.2–8.1	1.8–6.7

[a]Positive wells at dilution where the proportion of empty wells was >0.75 and P for each well containing the progeny of a single cell was >0.85, according to gamma distribution.
[b]Clonogenicity was measured as mean of secondary CFU-GM per positive wells, adjusted according to the P for each well containing a single clonogenic cell.
[c]Clonogenicity was calculated dividing secondary CFU-GM derived from bulk cultures by the frequency of LTC-IC calculated using the Poisson equation.

graft. Our study shows that despite full hematologic recovery, the number of the most immature hematopoietic cells remains permanently below that observed in normal donors. The results also suggest that a greatly and permanently depleted stem cell compartment is compatible with sufficient production of mature blood cells to cause full hematological recovery. These findings are in agreement with previous results showing deficient post-transplant recovery of BM committed progenitors, measured as $CD34^+$ cells or as CFU-GM, and of stem cells, measured as LTC-IC.[1–3] Markedly decreased numbers of BM and PB LTC-IC in transplanted patients suggests that either the self-renewal ability of hematopoietic stem cells is in some way impaired or that a biologically determined limit of expansion of stem cells does exist *in vivo*. In addition, the lack of correlation between the number of repopulating cells transplanted and the number of progenitors measured after recovery suggests that the repopulation is attributable to a small number of early progenitors. Recently, it has been hypothesized that hematopoietic stem cells have only a limited replicative potential (<100 divisions) and that 55 divisions can yield 4×10^{16} cells over a lifetime.[29] Impairment of the self-renewal capacity under conditions of a depleted stem cell pool may explain the inability to restore the stem cell levels while ensuring complete hematological recovery. This possibility is consistent with our finding *in vivo* of lower LTC-IC/CFU-GM ratios after transplant, and implies that primitive cells tend to differentiate into committed progenitor cells rather than to self-renew. Telomere shortening after transplant also supports our conclusions: comparison of telomere length concomitantly measured in peripheral blood granulocytes from the donor and from the recipient years after successful transplant revealed that telomere length was significantly reduced in the recipients.[19–21] It was also documented that the extent of telomere shortening after transplant was inversely correlated with the number of stem cells infused, suggesting a reduction of their proliferative drive.[19] A possible upregulation of telomerase activity in response to cytokine-induced proliferation may only reduce, but not prevent, telomere shortening of hematopoietic cells after *ex vivo* expansion.[18,22] All these observations may suggest that in transplanted patients the self-renewal ability of hematopoietic stem cells *in vivo* during bone marrow repopulation may be shifted towards differentiation or maturation. Recently, in a murine model it has been reported that LTC-IC expansive capacity is inversely correlated with the number of infused LTC-IC, suggesting that the repopulative stress does not reduce the long-term repopulating activity of stem cells; nevertheless, such an increased repopulating capacity is not sufficient to restore a normal stem cell compartment.[30] We found that in transplanted patients the clonogenic capacity of LTC-IC *in vitro* is within the normal range; thus, there is an apparent discrepancy between *in vivo* and *in vitro* studies. The discrepancy could be due to a peculiar steady-state condition of the transplanted stem cells. In the experimental *in vitro* system, the proliferation kinetics of transplanted stem cells seems identical to that of stem cells derived from normal donors, while *in vivo* the type and concentration of endogenously produced growth factors as well as a T cell-mediated host tolerance to the graft may provide a signal for expansion of more mature cell subsets after transplant.[31,32]

We found that the deficiency in early hematopoietic cells after BMT is less evident at the level of $CD34^+$ and committed progenitor cells (measured as CFU-GM) than at the level of more immature progenitors (measured as LTC-IC). Indeed, the number of circulating $CD34^+$ cells did not differ from that measured in normal controls. As circulating $CD34^+$ cells constitute only a minor fraction of the total $CD34^+$ cell pool, this finding may indicate redistribution or a shift of these cells into the periphery, possibly in response

to a higher demand and elevated growth factor levels. Our results also imply that the number of $CD34^+$ cells cannot be used as a direct measure of the reconstituting ability of hematopoietic progenitors (although it is the most frequently used parameter). An alternative hypothesis is that BM and PB $CD34^+$ cells may derive from two different stem cell pools. This hypothesis is supported by functional differences detected between marrow and circulating $CD34^+$ cells, as well as by a shorter time required for repopulation when peripheral stem cells are used for transplant.[33,34] At variance to $CD34^+$ cells, LTC-IC were comparably depleted in marrow and peripheral blood, suggesting that they represent members of a uniform stem cell pool. Based on the correlation between the number of PB and BM LTC-IC, it appears that enumeration of LTC-IC in peripheral blood provides sufficient information for the analysis of the stem cell compartment.

In conclusion, our data confirm a previous study on a larger number of patients with a longer follow-up, showing that the bone marrow stem cell compartment, as measured by the LTC-IC assay, is deeply and permanently depressed following allogeneic bone marrow transplant.[9] In addition, our study shows that circulating LTC-IC can be used to measure the stem cell compartment after BMT. All data indicate that a long-lasting deficiency of the most immature progenitors does not preclude normal functioning of the hematopoietic system, at least in a steady state condition. It remains to be verified whether in conditions of increased demand (infections, injuries, immunologic complications) a depleted stem cell pool may lead to a secondary bone marrow failure syndrome.

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