Research Article   **Open Access**

Properties of the Bone Marrow Stromal Microenvironment in Adult Patients with Acute Lymphoblastic Leukemia before and After Allogeneic Transplantation of Hematopoietic Stem Cells

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Abstract

The bone marrow stromal microenvironment that regulates normal hematopoiesis suffers during leukemia development and its treatment. In this study, we examined Multipotent Mesenchymal Stromal Cells (MMSCs) and fibroblast colony-forming units (CFU-F) derived from the Bone Marrow (BM) of 15 adult patients with acute lymphoblastic leukemia (ALL) before and after allogeneic hematopoietic stem cell transplantation (allo-HSCT). The time points assessed after allo-HSCT were defined by the treatment protocol. The analogous cells obtained from the BM of 64 healthy donors were used as controls. The ability of MMSCs to proliferate, concentration of CFU-F in BM, and gene expression in both cell types were assessed. The data indicate that MMSCs of ALL patients before allo-HSCT did not differ from MMSCs of healthy donors either in cumulative cell production or in gene expression except for SDF1. The SDF1 expression was decreased 2-fold in the MMSCs of ALL patients. The MMSC cumulative cell production from ALL patients was significantly decreased during 1 year after allo-HSCT. The expression level of SDF1 was also downregulated during the observation period. We identified changes in the FGF2 and PDGF signaling pathways. The CFU-F analysis revealed that its concentration in the BM of ALL patients had been profoundly decreased for the whole year after allo-HSCT. This decrease was accompanied by the downregulation of FGFR1 and slight upregulation of differentiation marker gene expression. Thus the number of stromal precursor cells decreased and their ability to regenerate was depressed after allo-HSCT. These changes were accompanied by an increase of more mature precursor cells with reduced proliferative potential.

Keywords: Acute lymphoblastic leukemia (ALL); Allogeneic hematopoietic stem cell transplantation (allo-HSCT); Stromal microenvironment; Multipotent mesenchymal stromal cells (MMSCs); Fibroblast colony-forming units (CFU-F); Gene expression

Introduction

Hematopoiesis is supported in adults by a stromal microenvironment consisting of mesenchymal stem cells (MSCs) and their descendants that include fibroblast Colony-Forming Unit (CFU-F) progenitors of intermediate maturity and specialized differentiated cells. MSCs are stem cells with the capacity to differentiate into all elements of the stromal microenvironment [1]. More mature descendants of MSCs such as CFU-F are able to maintain hematopoiesis by differentiating into osteogenic and adipogenic lineages [2,3]. The stromal microenvironment has higher radio- and chemoresistance than hematopoietic cells because of its low self-renewal frequency [4].

The stromal microenvironment is often damaged in patients with hematological diseases [5-8]. The following changes in the stromal microenvironment of acute leukemia patients were previously described: disturbances of signaling pathways [9], genetic abnormalities [10-12] and functional changes [13-15]. Alterations in the stromal microenvironment were also observed in chronic leukemia patients [16,17], myelodysplastic syndrome patients [18,8,12,19], and multiple myeloma patients [7]. However, other investigators have not found pathological changes in the stromal microenvironment of acute and chronic leukemia patients [20,21]. The treatments for patients with hematological malignancies include high doses of cytotoxic drugs and allogeneic hematopoietic stem cell transplantation (allo-HSCT). Both chemotherapy and pretransplant conditioning affect stromal progenitor cells [22-25]. A damaged stromal microenvironment may impair hematopoiesis in patients after allo-HSCT [26-28].

Acute lymphoblastic leukemia (ALL) is the most common neoplastic disease in children [29] and is well characterized in this age group. Stromal precursor cells of ALL patients have also been studied and their functional changes were previously identified [14,15]. However, the state of the stromal microenvironment in adult ALL patients has not been studied in detail.

This study examines the elements of the MSCs compartment including the multipotent mesenchymal stromal cells (MMSCs) [30] and CFU-F in patients with ALL before and after allo-HSCT.

Materials and Methods

ALL patients and healthy donors

This study included 15 ALL patients (Table 1) with the following immunophenotypes: Pro-B (1 patient), Pre-B (4 patients), B common (5 patients), Mature B (2 patients), T (1 patient), and Pro -T (2 patients). The age of the patients ranged from 18 to 39 years (median age 28.6 years). Bone marrow (BM) was obtained during routine diagnostic aspiration after receiving the patients' informed consent. The routine

Patient	Age/gender	Diagnosis	Treatment before allo-HSCT	Conditionoing regimen
1 Sch	36/female	Pre-B*	ALL-2009+ glivek	Busulfan+cyclophosphan
2 Elm	22/male	T	ALL-2009	Busulfan+cyclophosphan
3 Gor	35/male	T	ALL-2009	Busulfan+cyclophosphan
4 Nic	39/male	B *	ALL-2009+ glivek	Busulfan+cyclophosphan
5 Vas	24/male	B com	ALL-2009	Busulfan+cyclophosphan
6 Zey	37/male	B com	ALL-2009	FLAMSA mod
7 Tes	18/male		ALL-BFM-90m	Busulfan+flurad+ATG
8 Bor	29/male	Pre-B	ALL-2009	Busulfan+cyclophosphan
9 Che	28/male	Pre-B	ALL-2009	Busulfan+cyclophosphan
10 Isi	22/male	Pro-B	ALL-2009	Busulfan+cyclophosphan
11 Chr	33/female	Pre-B	ALL-2009	Busulfan+cyclophosphan
12 Bul	24/male	B com*	Hoeltzer95	Busulfan+cyclophosphan +ATG
13 Kor	25/female	B with myeloid markers	ALL-2009	Busulfan+cyclophosphan
14 Rak	38/male	Pro-T	ALL-2009	Busulfan+cyclophosphan
15 Vor	24/male	B com	ALL-2009	Busulfan+cyclophosphan

Table 1: Characteristics of acute lymphoblastic leukemia (ALL) patients
*Indicate Ph+ positive patients; FLAMSA (consisting of one dose of fudarabine, amsacrine and ara-C for four days), ATG (Anti-thymocyte globulin), Flurad (fluradabine).

diagnostic aspiration was performed prior to conditioning and at +30, 60, 90, 120, 180 and 365 days after allo- HSCT.

BM samples were analyzed from 64 healthy donors and included 34 men and 30 women aged from 18 to 59 years (median-34 years). All BM samples were obtained during exfusion for allo-HSCT in the FGBU Hematological Scientific Center after receiving the patients' signed informed consent. These samples were used as controls. Characteristics of ALL patients are shown in (Table 1).

MMSCs cultivation

MMSCs were derived from 5-10 ml of the BM cells. To obtain mononuclear cells the BM was mixed with an equal volume of alpha-MEM (ICN, Costa Mesa, USA) media containing 0.2% methylcellulose (1500 cP, Sigma-Aldrich, St. Louis, USA). After 40 min, the erythrocytes and granulocytes had precipitated and the mononuclear cells remained in suspension. The suspended (upper) fraction was aspirated and centrifuged for 10 minutes at $450 \times g$.

The cells from the sediment were resuspended in a standard cultivation medium composed of alfa-MEM supplemented with 10% fetal calf serum (Hyclone, Waltham, USA), 2 mM L-glutamine (ICN), 100 U/ml penicillin (Ferein, Moscow, Russia) and 50 mg/ml streptomycin (Ferein). The cells were cultured at 3×10^6 cells per T25 cm^2 culture flask (Corning-Costar, Tewksbury, USA). When a confluent monolayer of cells had formed (passage 0), the cells were washed with 0.02% EDTA (ICN) in a physiologic solution (Sigma-Aldrich) and then trypsinized (ICN) with 0.25% trypsin (ICN). The cells were seeded at 4×10^3 cells per cm^2 in culture flasks. The cultures were maintained at 37°C in 5% CO^2. MMSCs were cultured until the second passage.

All MMSCs were immunophenotyped as described [31] with the following markers: CD105, CD73, CD45, CD34, CD14, and HLA-DR using standard protocols. The antibodies were purchased at BD Pharmingen, Franklin Lakes, USA (CD105, CD59, CD73, CD90, CD31, CD34 and CD14), Sigma, St. Louis, USA (CD45, FSP) and DAKO, Glostrup, Denmark (HLA-DR). The level of nonspecific antibody binding was analyzed using a mouse immunoglobulin IgG1 isotype control (BD Pharmingen, Franklin Lakes, USA). To assess viability the cells were stained with 7 -aminoactinomycin D (7-AAD), (Sigma). The analysis was performed on a FACSCanto II (BD Biosciences, Franklin Lakes, USA) and the results were analyzed with BD FACSDiva 6.1.3 software (Franklin Lakes, USA).

Analysis of CFU-F

Mononuclear BM cells were seeded at 10^6 and 5×10^5 per T25 flask in alpha -MEM with 20% fetal calf serum (Hyclone), 2 mM L- glutamine (ICN), 100 U/ml penicillin (Ferein), 50 mg/ml streptomycin (Ferein) and analyzed after 14 days. The colony count was performed using a microscope (Opton, Oberkochen , Germany) after staining the cells with 1% crystal violet in 20% methanol.

RNA isolation and quantitative reverse transcription-polymerase chain reaction

Total RNA was extracted from the MMSCs at passage 1 using the standard guanidine isothiocyanate method [32]. The cDNA was synthesized using a mixture of random hexamers and oligo(dT) primers. Gene expression levels were quantified by real-time quantitative PCR (qRT-PCR) using hydrolysis probes (Taqman) on a Rotor-Gene 6000 (Corbett Research, Concorde, USA). The gene-specific primers were designed by the authors and synthesized by Syntol R&D (Moscow, Russia). The primers and probes are provided in (Table 2). The relative gene expression levels were determined by normalizing the expression of each target gene to the levels of *BACT* (beta- actin) and *GAPDH* (glyceraldehyde -3- phosphate dehydrogenase) and calculated using the $\Delta\Delta C_t$ method [33] for each MMSC sample. The reaction was conducted using the following PCR protocol: initial denature at 95 ºC for 10 min, cyclic denaturation at 95 ºC for 20 sec, hybridization with primers and template extension at 60 °C for 60 sec.

Statistical analysis

All values are expressed as the means ± SEM. The data were analyzed using independent Student's t-tests in Microsoft Excel.

Results and Discussion

Cell production in MMSC cultures from ALL patients

The total cell production of MMSCs derived from ALL BM prior to allo-HSCT conditioning was not significantly different than BM from healthy donors (Figure 1). At the moment of allo-HSCT, 14 of 15 studied patients were in remission after treatment with the standard protocol ALL-2009 and 1 patient was in relapse. There were 2 patients with Ph+ ALL that received additional tyrosine kinase inhibitor therapy. Thus, the treatment protocol used did not inhibit MMSC proliferation. Notably the total MMSCs cell production from the patient in relapse

Gene		Sequence
b-ACTIN	Forward primer	CAA CCG CGA GAA GAT GAC C
	Reverse primer	CAG AGG CGT ACA GGG ATA GC
	Probe	ROX-AGACCTTCAACACCCCAGCCATGTACG-RTQ2
GAPDH	Forward primer	GGT GAA GGT CGG AGT CAA CG
	Reverse primer	TGG GTG GAA TCA TAT TGG AAC A
	Probe	ROX CTC TGG TAA AGT GGA TAT TGT TGC CAT CA RTQ2
BMP-4	Forward primer	ACAGCACTGGTCTTGAGTATC
	Reverse primer	TGGGATGTTCTCCAGATGTTC
	Probe	FAM- AACACCGTGAGGAGCTTCCACCA -RTQ1
IL6	Forward primer	ACCTGAACCTTCCAAAGATG
	Reverse primer	CTCCAAAAGACCAGTGATGA
	Probe	FAM-ATTCAATGAGGAGACTTGCCTGGTG-RTQ1
CFH	Forward primer	TTACCCTTACAGGAGGAAATGT
	Reverse primer	GCTGTCACTGGTAAACACTTC
	Probe	FAM-CTTCACATATAGGAATATCATTGGTCCAT-RTQ1
IDO1	Forward primer	AGCGTCTTTCAGTGCTTTG
	Reverse primer	GGATTTGACTCTAATGAGCACA
	Probe	FAM-ACATGCTGCTCAGTTCCTCCAGG-RTQ1
PTGES	Forward primer	CTGGTCATCAAGATGTACGTG
	Reverse primer	CTCCGTGTCTCAGGGCAT
	Probe	FAM- CTTCTTCCGCAGCCTCACTTGG-RTQ1
CSF1	Forward primer	AGGAACTCTCTTTGAGGCTG
	Reverse primer	CATTCTTGACCTTCTCCAGCA
	Probe	FAM-CTTGTCATGCTCTTCATAATCCTTGG-RTQ1
FABP4	Forward primer	ATGATAAACTGGTGGTGGAAT
	Reverse primer	TCAATGCGAACTTCAGTCC
	Probe	FAM-TGGCTTATGCTCTCTCATAAACTCTCG-RTQ1
PPARG	Forward primer	TACTGTCGGTTTCAGAAATGC
	Reverse primer	CAACAGCTTCTCCTTCTCG
	Probe	FAM-CCATCAGGTTTGGGCGGATGCC-RTQ1
SPP1	Forward primer	ATAGTGTGGTTTATGGACTGAG
	Reverse primer	ATTCAACTCCTCGCTTTCC
	Probe	FAM-CCAGTACCCTGATGCTACAGACGAG-RTQ1
BGLAP	Forward primer	GCAGCGAGGTAGTGAAGAG
	Reverse primer	GAAAGCCGATGTGGTCAG
	Probe	FAM-CTCCCAGCCATTGATACAGGTAGC-RTQ1
JAG1	Forward primer	ATAAAGTCCTTCCCGCTG
	Reverse primer	TTATCTTCTCCCATCATTAAG
	Probe	FAM-AGACAACAGACAAATCACCATTCGT-RTQ1
FGFR1	Forward primer	CAGAATTGGAGGCTACAAGG
	Reverse primer	TGATGCTGCCGTACTCATTC
	Probe	FAM- CATCATAATGGACTCTGTGGTGC-RTQ1
FGFR2	Forward primer	GCAGCGAGGTAGTGAAGAG
	Reverse primer	GAAAGCCGATGTGGTCAG
	Probe	FAM-CTCCCAGCCATTGATACAGGTAGC-RTQ1
LGALS1	Forward primer	CCAGCAACCTGAATCTCA
	Reverse primer	CGAAGCTCTTAGCGTCAG
	Probe	FAM-CACTCGAAGGCACTCTCCAGGT-RTQ1
Il1B	Forward primer	ATTCTCTTCAGCCAATCTTCA
	Reverse primer	AAGGAGCACTTCATCTGTTTA
	Probe	FAM-AGAACAAGTCATCCTCATTGCCAC-RTQ1
IL1R1	Forward primer	CTAATGAGACAATGGAAGTAGAC
	Reverse primer	AGCACTGGGTCATCTTCATC
	Probe	FAM- CAGTTGAGTGACATTGCTTACTGGAA -RTQ1
PDGFRA	Forward primer	TGGCTAAGAATCTCCTTGGA
	Reverse primer	ACCAGGACAATAAGTGAGATG
	Probe	FAM-CAATCACCAACAGCACCAGGACT-RTQ1
PDGFRB	Forward primer	CTCCCTTATCATCCTCATCA
	Reverse primer	TCCACGTAGATGTACTCATG
	Probe	FAM-TCACAGACTCAATCACCTTCCATC-RTQ1

|		
IL8	Forward primer	ACCATCTCACTGTGTGTAAAC
	Reverse primer	GTTTGGAGTATGTCTTTATGC
	Probe	FAM-CAGTTTTGCCAAGGAGTGCTAAAG-RTQ1
SOX9	Forward primer	AGCAAGACGCTGGGCAAG
	Reverse primer	GTTCTTCACCGACTTCCTC
	Probe	FAM-CTGGAGACTTCTGAACGAGAGC-RTQ1
VEGFA	Forward primer	AGG CGA GGC AGC TTG AGT TA
	Reverse primer	ACC CTG AGG GAG GCT CCT T
	Probe	FAM-CCT CGG CTT GTC ACA TCT GCA AGT ACG T-RTQ1
FGF2	Forward primer	GAAGAGCGACCCTCACATCAAG
	Reverse primer	TCCGTAACACATTTAGAAGCCAGTA
	Probe	FAM-TCATAGCCAGGTAACGGTTAGCACACACTCCT-RTQ1
SDF	Forward primer	CTACAGATGCCCATGCCGAT
	Reverse primer	TAGCTTCGGGTCAATGCACA
	Probe	FAM-CAGTTTGGAGTGTTGAGAATTTTGAG-RTQ1

Table 2: Primers and probes.
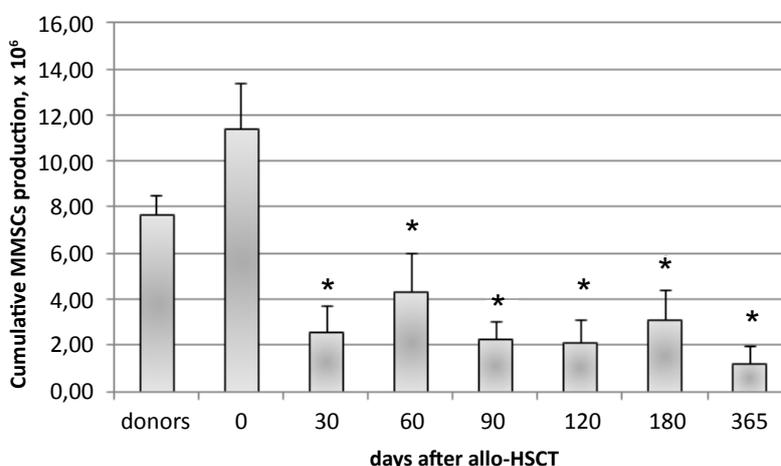
Figure 1: Alterations in cumulative cell production in MMSC cultures from ALL patients before and after allo-HSCT. Cumulative MMSC production after 3 passages is presented as the mean ± SEM. The data summarize the results of MMSCs production from 64 BM donors and 15 patients with ALL before and after allo-HSCT. *indicates a significant difference (p<0.05) between MMSC production after allo-HSCT and MMSC production both from donors and patients before allo-HSCT. Time point "0" indicates MMSC samples obtained before the start of pretransplant conditioning, and time points "30-365" indicate samples of MMSCs derived from BM of ALL patients obtained on corresponding days after allo-HSCT.

Number of passage	Donors	ALL patients, days after allo-HSCT						
		0	30	60	90	120	180	365
Po	12.9 ± 0.9	13.9 ± 1.3	18.6 ± 1.5*	19.2 ± 1.2*	17.7 ± 1.1*	17.0 ± 1.8	19.1 ± 1.9*	16.4 ± 0.6*
P1	4.1 ± 0.2	5.0 ± 0.7	8.7 ± 2.1	4.5 ± 0.5	6.1 ± 0.7*	7.3 ± 1.7	6.5 ± 1.0	5.8 ± 1.1
P2	4.3 ± 0.1	4.4 ± 0.6	4.0 ± 0.5	7.4 ± 0.3*	6.4 ± 0.9	4.0 ± 0.5	5.7 ± 1.1	7.0 ± 0.7
P3	4.5 ± 0.4	5.5 ± 0.8	7.3 ± 0.9	8.0 ± 1.0	7.5 ± 1.3	3.8 ± 0.1	5.5 ± 0.8	5.7 ± 0.6
P4	5.4 ± 0.4	5.5 ± 0.6	6.0 ± 0. 5	7.0 ± 0.6	7.5 ± 1.3	6.0 ± 0.5	9.5 ± 1.6	4.7 ± 0.5
P5	5.4 ± 0.5	4.7 ± 0.4	4.3 ± 0.6	6.0 ± 0.5	6.7 ± 0.7	5.0 ± 0.5	5.0 ± 0.4	5.7 ± 0.6

Table 3: Time needed to achieve confluence of the layer of MMSCs
P_0 is the time since seeding of 3×10^6 BM cells in a flask to confluent monolayer. P1 – P5 is the time between passages. (*) significant differences with donor MMSCs (p<0.05) Data presented as M ± SEM.

was substantially reduced. These results are consistent with the data on cell production in MMSC cultures from newly diagnosed ALL patients [34]. However, studies of ALL in children at the time of diagnosis [35] and after chemotherapy [14,15] demonstrated that MMSCs were strongly suppressed. This result might occur because in children the disease developed simultaneously with the formation and growth of the hematopoietic microenvironment. In adults with acute myeloid leukemia (AML) [36], chronic myeloid leukemia (CML) [20] and B-cell chronic leukemia (B-CLL) [13] growth characteristics of MMSCs were not changed.

The pretransplant conditioning significantly reduced the proliferative potential of MMSCs (Figure 1). There was no recovery of MMSCs proliferation observed during the year after allo-HSCT. There was a significant increase in the time needed to reach a confluent monolayer of MMSCs from ALL patients compared with cultures of control MMSCs. However, in the subsequent passages there were no significant differences in the time to confluence (Table 3).

These data suggested that in the BM of ALL patients the number of MMSCs had been reduced after allo-HSCT. It was previously

shown that the total cell production of MMSCs was also reduced in patients with various hematological malignancies after allo-HSCT [31]. Therefore, the observed decline in proliferative potential of MMSCs was not associated with the disease itself and was caused by the pretransplant conditioning. The standard conditioning protocol included busulfan and cyclophosphamide. Studies using an animal model demonstrated that busulfan impaired stromal progenitor cells

(CFU-F) and their function was not subsequently restored. However, the mesenchymal stem cells were insensitive to cytostatic treatments [23]. The data indicating that human MMSCs were sensitive to high doses of cytotoxic drugs used in transplantation once again confirmed that the population of MMSCs was not the population of true stem cells. Thus, MMSCs consist of more mature stromal precursor cells [37]. The growth characteristics of MMSCs from ALL patients in remission
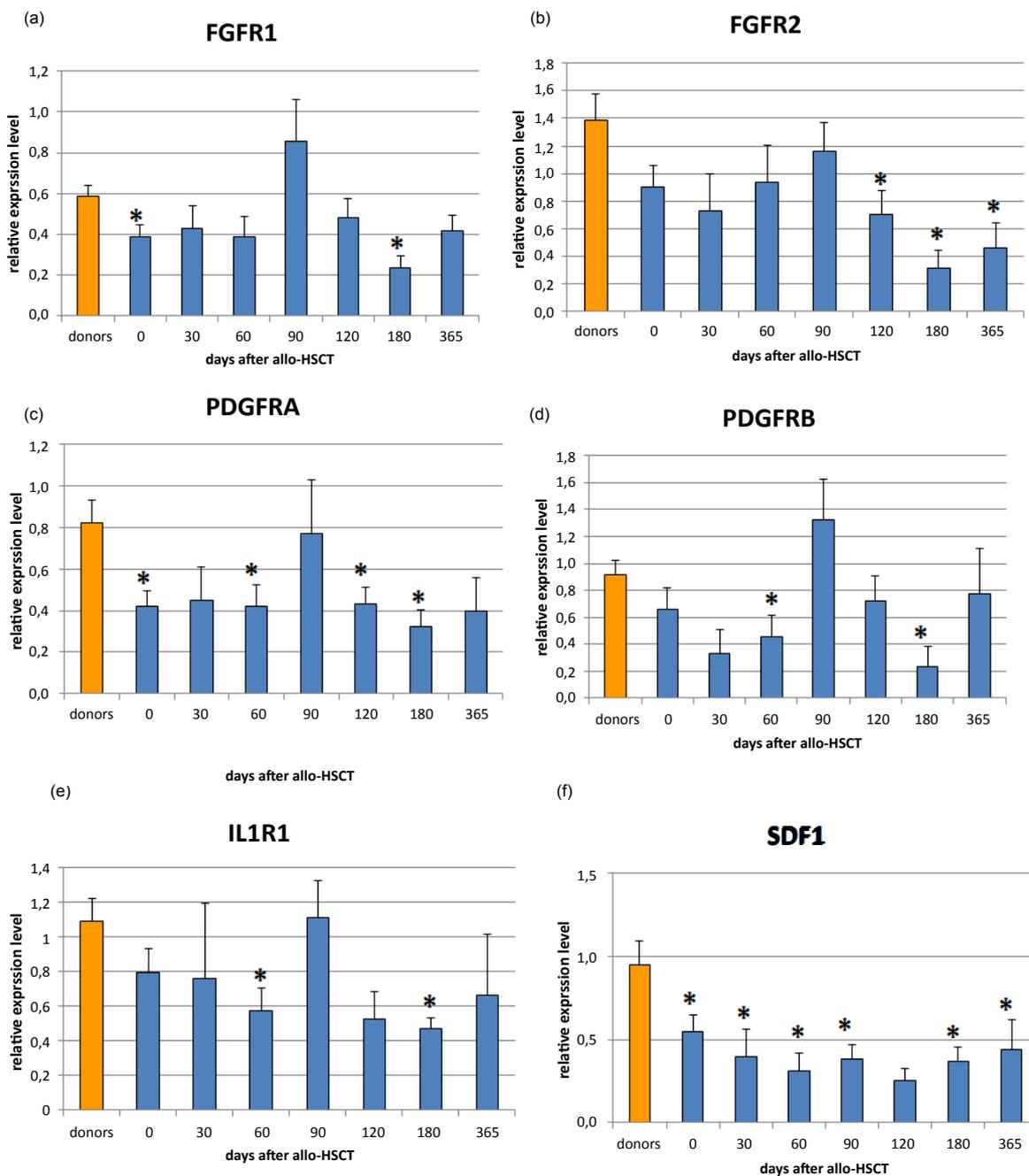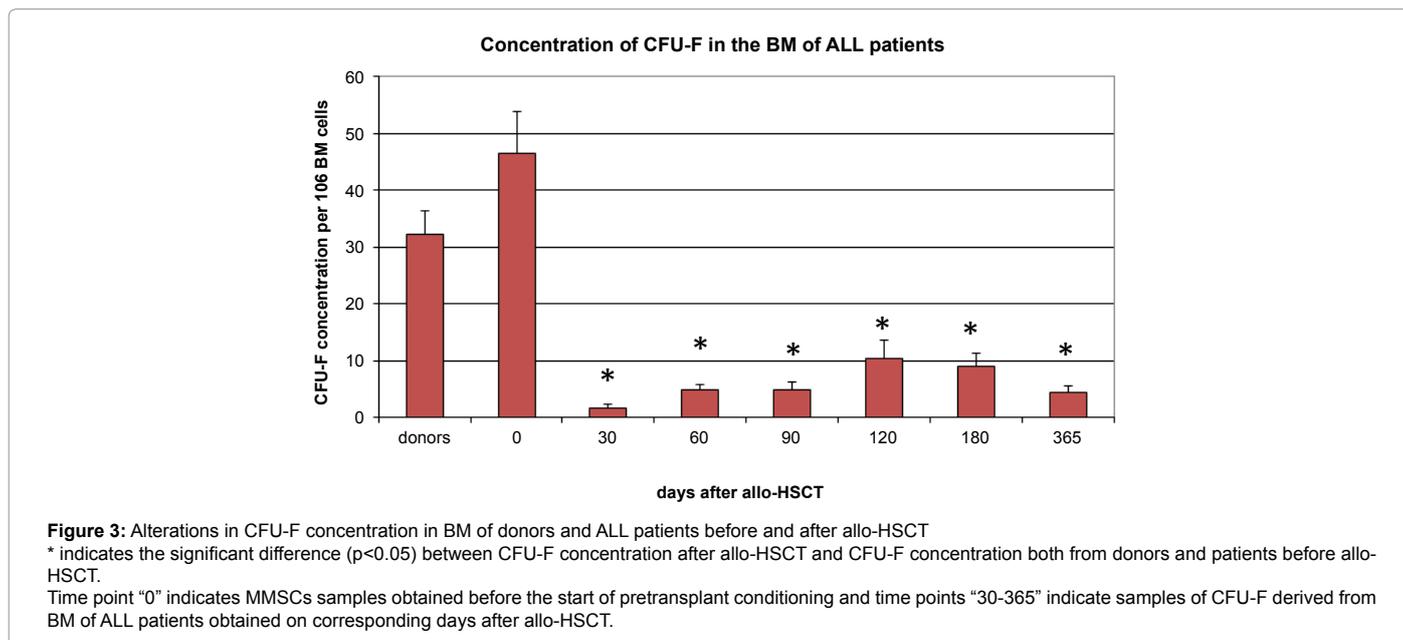

Figure 2: a,b,c,d,e,f: Relative expression level of genes in MMSCs from donors and ALL patients before and after allo-HSCT. Gene expression was analyzed by qRT-PCR with TaqMan probes. The relative expression level was calculatedusing the ΔΔCt method. The results were normalized according to the expression of BACT and GAPDH. Genes analyzed are shown as follows: A-*FGFR1*, B-*FGFR2*, C- *PDGFRA*, D-*PDGFRB*, E-*IL1R1*, F-*SDF1*.
* indicates a significant difference (p<0.05) between MMSC production before and after allo-HSCT and MMSC production from donors.
Time point "0" indicates MMSC samples obtained before the start of pretransplant conditioning, and time points "30-365" indicate samples of MMSCs derived from BM of ALL patients obtained on corresponding days after allo-HSCT.

Genes	ALL patients, days after allo-HSCT						
	0	30	60	90	120	180	365
IL1b	0.8 ± 1.0	30.2 ± 56.1	1.5 ± 2.6	0.4 ± 0.6	6.3 ± 10.8	7.0 ± 12.1	0.4 ± 0.6
VEGF	1.3 ± 0.4	2.5 ± 1.4	1.4 ± 0.6	2.1 ± 0.9	0.7 ± 0.3	1.2 ± 0.5	1.1 ± 0.5
FGF2	0.7 ± 0.4	4.1 ± 4.1	1.1 ± 0.8	0.8 ± 0.5	1.2 ± 0.9	1.9 ± 1.6	0.8 ± 0.5
TGFB1	0.9 ± 0.2	0.9 ± 0.3	0.8 ± 0.3	0.6 ± 0.2	0.6 ± 0.2	1.1 ± 0.4	1.4 ± 0.6
TGFB2	2.0 ± 1.2	4.9 ± 4.4	2.0 ± 1.8	1.5 ± 1.3	2.7 ± 2.8	2.0 ± 1.6	0.6 ± 0.5

Table 4: Alterations of relative expression level of gene expression in MMSCs of ALL patients compared to donor's MMSCs.

Figure 3: Alterations in CFU-F concentration in BM of donors and ALL patients before and after allo-HSCT
* indicates the significant difference (p<0.05) between CFU-F concentration after allo-HSCT and CFU-F concentration both from donors and patients before allo-HSCT.
Time point "0" indicates MMSCs samples obtained before the start of pretransplant conditioning and time points "30-365" indicate samples of CFU-F derived from BM of ALL patients obtained on corresponding days after allo-HSCT.

before allo-HSCT were slightly changed. So one can conclude that the pretransplant conditioning led to prolonged damage to MMSCs proliferation and reduced MMSCs number in the BM of patients.

Gene Expression

We next examined the expression levels of several genes regulating stromal cell division in the MMSCs of ALL patients because of the observed changes in their ability to proliferate. Prior to allo-HSCT, we observed reduced expression levels of *FGFR1, FGFR2, PDGFRA* and *PDGFRB* genes (Figure 2 A,B,C,D). However, in some cases the reduction was not significant. After allo-HSCT the expression level of these genes remained significantly reduced. These genes encode receptors for growth factors regulating the proliferation of MMSCs. It has been shown that the inhibition of at least one of these signaling pathways leads to MMSC growth reduction [38]. The reduction of gene expression in MMSCs of ALL patients indicates a possible dysregulation of signal transduction in the *FGF2* and *PDGF* pathways. This result may explain the observed decline in total cellular production of MMSCs. The expression level of *FGF2* did not vary significantly between the donors and ALL patients before and after allo-HSCT (Table 4). It is known that IL-1 beta is involved in the growth regulation of stromal progenitor cells [39-41]. Moreover, IL-1 beta is expressed by fibroblasts [42]. The expression level of the IL-1 beta receptor in MMSCs from ALL patients was reduced during the entire observation period. However, the changes were significant only at 60 and 180 days after allo-HSCT (Figure 2E). The expression of IL -1 beta itself was not altered significantly (Table 4).

A reduction in gene expression of receptors for growth factors, but not growth factors themselves, was identified. This result suggested that autocrine secretion of these growth factors did not determine the proliferative ability of MMSCs from ALL patients. These pathways could be important for the proliferation of MMSCs due to the coincident reduction in receptor expression and cells proliferation. Conversely, the expression of all these genes tended to decrease in MMSCs before allo-HSCT, while total cell production in these cultures was not reduced. Probably the genes we examined were not vital for the proliferation of MMSCs, and there still were some other regulatory mechanisms of their division that had not been included in this study.

The expression level of the chemokine *SDF1* was significantly reduced in MMSCs of ALL patients before and after allo-HSCT (Figure 2F). A similar effect was also described in patients with AML [43]. This chemokine plays a key role in the homing of hematopoietic cells to the BM niche [44]. Following autologous and allogeneic HSCT both hematopoietic dysfunction and long-term cytopenia are often observed in patients [45–47]. We predict that these disorders are associated with the changes in the interaction between hematopoietic stem cells and the elements of the stromal microenvironment. These changes may be specifically related to the decrease in *SDF1* expression.

Characteristics of CFU-F in ALL patients

The concentration of CFU-F in the BM of ALL patients before allo-HSCT slightly but insignificantly increased (p=0.15) compared to donors (Figure 3). This result might be caused by the mild differences in the mean age as ALL patients which ranged from 18 to 39 years

(median - 28.6 years), whereas the donors ranged from 18 to 59 years (median - 34 years). It is known that the CFU-F concentration decreases significantly with the age of the donor [48,49]. Several authors have analyzed BM before chemotherapy and did not find differences in CFU-F concentration between patients and healthy donors [50,51]. Other studies have revealed a decline or complete exhaustion in CFU-F concentration in the BM of patients with acute leukemia [52-54,24]. The same result was observed in mice injected with acute leukemia cells [55,56]. However, it is unclear whether chemotherapy treatment occurred in the human studies and the protocol followed was not

described. This information is crucial when analyzing the changes in the stromal microenvironment [25,57,58].

After allo-HSCT the concentration of CFU-F in the BM ALL patients was reduced by more than 10-fold and was not restored during the subsequent year (Figure 3). The results were consistent with the changes in the concentration of CFU-F caused by the transplantation protocol and are likely not reparable in patients more than 5 years old [48]. The effect could be associated with the significant decrease in the expression level of *FGFR1* in CFU-F of ALL patients after allo-
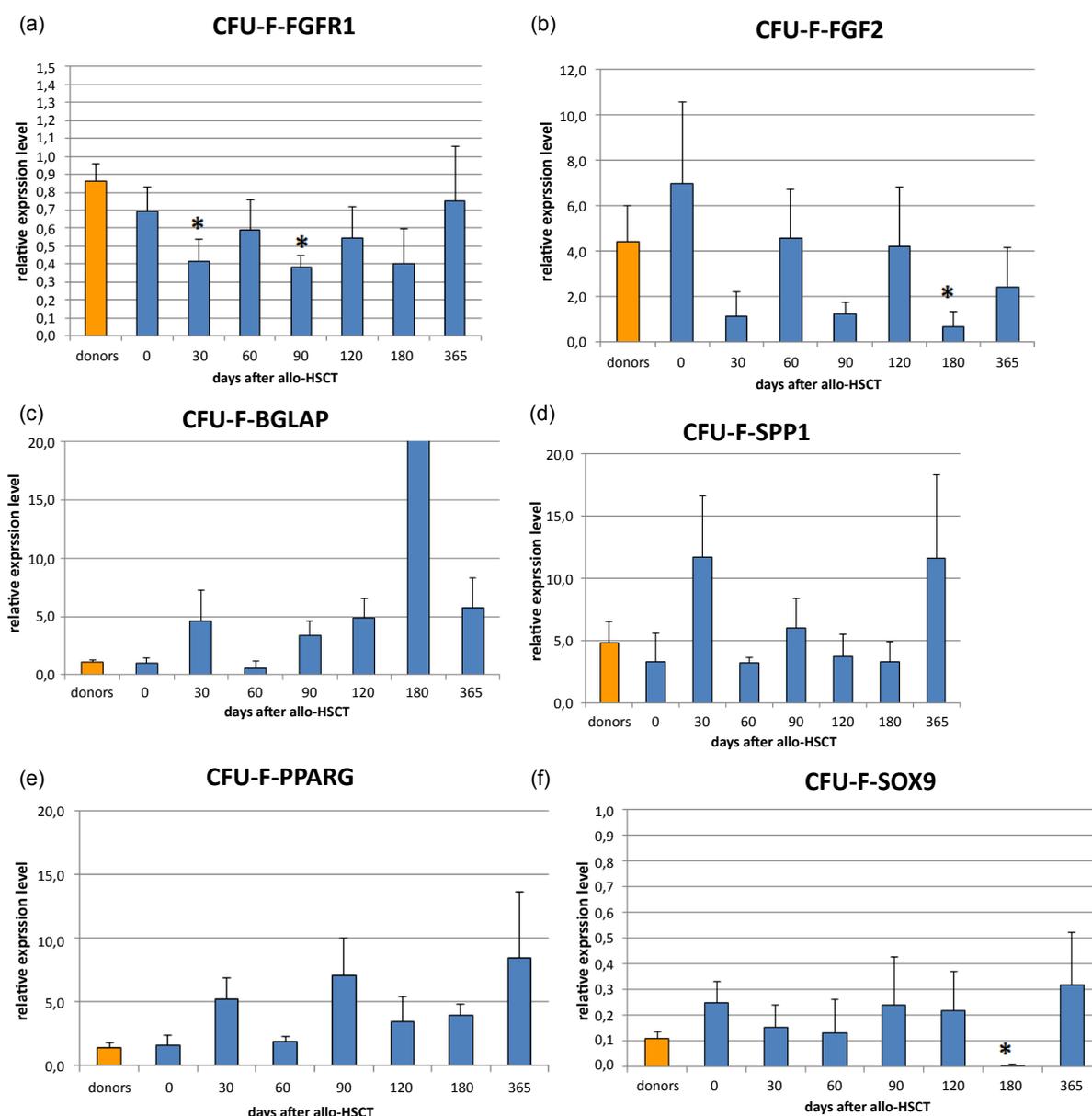
Figure 4: a,b,c,d,e,f: Relative expression level of genes in CFU-F derived colonies from donors and ALL patients before and after allo-HSCT. Gene expression was analyzed by qRT-PCR with TaqMan probes. The relative expression level was calculated using the ΔΔCt method. The results were normalized by the expression of BACT and GAPDH.
Genes analyzed are shown as follows: A-FGFR1, B-FGF2, C- BGLAP, D-SPP1, E-PPARG, F-SOX9.
*indicates a significant difference (p<0.05) between gene expression levels in CFU-F colonies before and after allo-HSCT and in CFU-F from donors.
Time point "0" indicates CFU-F samples obtained before the start of pretransplant conditioning, and time points "30-365" indicate samples of CFU-F derived from BM of ALL patients obtained on corresponding days after allo-HSCT.

HSCT (Figure 4A). The autocrine expression of *FGF2* was significantly upregulated before allo-HSCT, but it was not reduced significantly after transplantation (Figure 4B). We found a range of expression levels for this gene in the MMSCs from different patients. Due to the low total number of patients we cannot make a straightforward conclusion. The observed effect was consistent with the finding that *FGF2* was described as the growth factor for stromal progenitor cells [59,60]. We found the simultaneous decrease in the expression of *FGF2* and its receptors in CFU-F cells. This result suggests the reduced concentration of CFU-F in the BM after allo-HSCT was associated with impaired *FGF2* signaling. The reduced number of stromal precursor cells could be caused by the changes in their proliferative potential. The decrease in proliferative capacity reflected the loss of undifferentiated status of precursor cells and was accompanied by increased expression of differentiation-associated genes. In this study, we analyzed 2 markers for osteogenic differentiation (*BGLAP*, *SPP1*) and one for each of adipogenic (*PPARG*) and chondrogenic (*SOX9*) lineages. The expression of *SOX9* only was changed in CFU-F from BM of ALL patients before allo-HSCT. We found that *SOX9* was increased, but the changes were not significant (Figure 4F). The expression level of this gene remained slightly and non-significantly increased up to 120 days after allo-HSCT. The expression then decreased to nearly zero before returning to baseline one year after allo-HSCT. The expression level of osteogenic and adipogenic markers of differentiation tended to increase with time after allo-HSCT (Figure 3 C, D, E). The non-significant changes were associated with a range of values in CFU-Fs from ALL patients. Nevertheless, we suppose a shift towards more differentiated cells had occurred in the population of CFU-F derived colonies after allo-HSCT. It was shown that the conditioning before allo-HSCT was accompanied by the accumulation of reactive oxygen species in the cells of the organism [61,62], which could cause premature "aging" of the cells and tissues [63]. The reduction of the MMSCs and CFU-F number in BM of ALL patients after allo-HSCT suggest the damage to those stromal precursor cells that were attributed to the top of the mesenchymal stem cells hierarchy. It is known that the HSCT patients suffer from disorders of the musculoskeletal system including bone loss and osteoporosis [45,46,64]. The results of this study revealed possible mechanisms of bone marrow stroma damage after allo-HSCT in ALL patients. The number of stromal precursors was reduced and their growth and regeneration were inhibited after allo-HSCT. These changes were accompanied by an increased number of more differentiated progenitors with decreased proliferative potential. The damage to stromal cells by different protocols for treatment of leukemia should be taken into consideration.

Acknowledgement

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