Alterations in Hematopoietic Microenvironment in Patients with Aplastic Anemia

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Abstract

Mechanisms of hematopoietic failure in patients with aplastic anemia (AA) are obscure. We investigate alterations in the hematopoietic microenvironment in AA patients. We present the results of studying mesenchymal stromal cells (MSC), fibroblastic colony-forming units (CFU-F), and adherent cell layers (ACL) of long-term bone marrow cultures (LTBMC) from bone marrow (BM) samples of AA patients. MSC of AA patients proliferated longer than those of donors. In half of the patients' MSC cultures, adipogenesis was impaired. Osteogenic differentiation was not achieved in 36% of AA MSC. CFU-F formed enlarged colonies, and their concentration in the BM of AA patients was significantly increased. Our data suggest that the physiological activation of the stromal microenvironment is characteristic of AA. We detected a decrease in the expression of the angiopoetin-1 (*ANG-1*) and vascular cell adhesion molecule-1 (*VCAM-1*) genes, together with an increase in the expression of vascular endothelial growth factor (*VEGF*) in ACL of AA patients. This indicates abnormal regulatory patterns in both osteoblastic and vascular contexts. Addition of AA patients' serum to donors' LTBMC for 3 weeks induced similar gene expression alterations. The addition of parathyroid hormone (PTH) resulted in the expression levels of analyzed genes returning to normal, in both AA LTBMC and donor cultures treated with AA serum. The physiologic status of the BM stromal microenvironment (MSC, CFU-F, and ACL of LTBMC) of AA patients was altered.

Keywords: aplastic anemia, mesenchymal stromal cells, CFU-F, gene expression, PTH

Introduction

Aplastic anemia (AA) is an acquired disease characterized by low blood cell production due to chronic depression of hematopoiesis in the bone marrow (BM). AA has the following characteristics: decreased hematopoietic stem cell (HSC) number,[1–3] impaired HSC function,[4–7] and increased HSC apoptosis level.[8–10] The pathogenesis of AA remains unclear. Normal hematopoiesis needs appropriate interactions between HSC and the stroma of hematopoietic organs,[11] and any abnormality may result in impaired hematopoiesis.[4,12–14] However, the presence of blood-borne autoantibodies in certain patients to kinectin,[15] moesin,[16] and diazepam-binding protein 1[17] has been noted. This fact, together with the nature of the clinical response to immunosuppressive therapy in 70% of patients, may suggest an autoimmune origin of AA.[18] This statement is also supported by recent studies that showed a reduced fraction of natural killer T cells[19] and regulatory T cells,[20] together with an increased level of interleukin (IL)-17 and a consequent elevation of IL-6, IL-8, and tumor necrosis factor (TNF)-α production[21] in AA patients. It would appear that individual patients exhibit different disease etiologies.

Analysis of mesenchymal stromal cells (MSC) from patients with various hematopoietic disorders, in particular myelodysplastic syndrome (MDS), has revealed functional incompetence of the stromal cells.[22,23] As AA can subsequently progress into paroxysmal nocturnal hemoglobinuria or MDS,[24,25] it seems likely that MSC in most AA patients must be altered by the disease, at least from a functional perspective. Some investigators have equated MSC progenitors—fibroblastic colony-forming units (CFU-F)—with MSC itself.[26–28] The CFU-F concentration in the BM varies among patients with different hematologic diseases,[29–31] and it may also depend on the treatment or phase of the disease.[7,32,33] The characteristics of CFU-F could therefore potentially be used for monitoring the function of the stromal microenvironment over the course of the disease.

The hematopoietic BM microenvironment consists of osteoblastic (including MSC, CFU-F, and mature stromal cells) and vascular niches. The main component of the osteoblastic niche that regulates quiescent HSC are spindle-shaped osteoblasts. Control of self-renewal is accomplished through the interactions of Notch-1 and Tie-2 molecules at the cell surface of early hematopoietic precursors with Jagged-1 and angiopoetin-1 (ANG-1) molecules expressed on the osteoblasts.[34] Spindle-shaped osteoblasts themselves can be activated and stimulated to proliferate by parathyroid hormone (PTH).[35,36] Proliferating HSC are situated in the BM sinuses and are bound up with endothelial cells that express the VCAM-1 adhesion molecule[37] and vascular endothelial growth factor (VEGF).[38] Thus, during normal hematopoiesis, the early HSC are located in the osteoblastic niches, whereas the more mature, proliferating, and migrating HSC are located in the vascular niches. The functional status of HSC might be deduced from the expression of various regulatory molecules. Altered expression of these crucial proteins is likely to play a role in the pathogenesis of different diseases. Analysis of the expression levels of the genes that regulate HSC in the niches could help to define more clearly the pathogenic mechanisms of AA.

The objective of this study was to investigate the functional characteristics of the hematopoietic microenvironment in AA patients. The following three approaches were used: (1) analyses of long-term bone marrow culture (LTBMC), in which the adherent cell layer (ACL) included various stromal elements (MSC, CFU-F, and their differentiated progeny—adipocytes, osteoblasts, fibroblasts, and endothelial cells); (2) cultivation and differentiation of MSC and (3) determination of CFU-F concentration. We describe alterations in the physiological status of the stromal microenvironment of AA patients. Impaired differentiation of MSC derived from patients with AA has been revealed. CFU-F formed enlarged colonies as compared with

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donors, and CFU-F concentration significantly increased in AA BM. Our research examines the sera of multiple AA patients and provides insight into factor(s) that represses the normal expression of the genes responsible for HSC regulation.

Methods

Patients

BM samples from 26 AA patients were analyzed. The age varied from 16 to 37 years (median 22 years), and our study included 12 females and 14 males. Of these, 10 patients had AA and 16 suffered from severe AA (SAA). Nine patients were newly diagnosed, 12 were in remission, and 5 were in relapse. Donor BM obtained for BM transplantation was used as a control in all experiments. There were 54 BM samples from 25 males and 29 females. Donor age varied from 11 to 54 years (median 27 years). All BM samples were collected after informed consent and approval from the local ethics committee.

MSC culturing

BM mononuclear cells were cultivated in α-MEM with 10% fetal bovine serum (FBS) (HyClone, South Logan, UT, USA), 2 mM L-glutamine (ICN, Solon, OH, USA), and antibiotics (penicillin–streptomycin mixture; Ferein, Moscow, Russia). The normal plating concentration was 120×10^3 cells/cm^2 of culture ware area. When a confluent monolayer formed, the cells were trypsinized and seeded at a concentration of 4,000 cells/ cm^2. To examine adipogenic differentiation, the following components were added to α-MEM with 10% FBS, L-glutamine, and antibiotics: 0.5 mM 3-isobutyl-1-methylxanthine (Sigma, St. Louis, MO, USA), 1 µM dexametasone (Sigma), 0.2 µM indomethacin (Sigma), and 10 µg/mL insulin (Sigma). Adipogenic differentiation was confirmed by oil red O staining (Sigma). For osteogenic differentiation, the following components were added to α-MEM with 10% FBS, L-glutamine, and antibiotics: 0.1 µM dexamethasone, 0.15 mM ascorbate-2-phosphate, and 3 mM NaH_2PO_4. Osteogenic differentiation was confirmed by alizarin red staining (DiaM).

CFU-F analysis

In total, $0.25–1.0 \times 10^6$ mononuclear cells were plated into a 25-cm^2 culture flask in α-MEM with 20% FBS (previously selected lot), L-glutamine and antibiotics. For 2 weeks, the cells were cultivated without media change at 37°C and 5% CO_2. The resulting colonies were then stained with 0.1% crystal violet in 20% methanol and counted using an inverted microscope. The size of the colony was estimated using the digital images by means of Scion Image software (Scion Corporation, Frederick, MD, USA).

Long-term bone marrow culture

LTBMC were established from six patients in remission. One million of BM nucleated cells were plated in a single well of a 24-well plate, as previously described.[39] Half of the media was replaced weekly.

Human PTH (1-34) (GenScript Corp., Piscataway, NJ, USA) was added to LTBMC from the very first day and with every media change. The final concentration of PTH was 5×10^{-8} M.

Analysis of AA patients' blood serum and its influence on ACLs of donor LTBMC

Serum samples from 19 patients were analysed. Each serum sample was tested on 3–4 LTBMC from different donors. The tests were performed by adding the individual serum sample to eight wells of donor LTBMC. These cultures were established from the BM samples of 8 donors (4 females, 4 males, aged 16–51 years, median 28 years). For the first 3 weeks of culturing, 10% of the AA serum was added weekly to the culture media. Subsequently, from one part of the cultures, total RNA was isolated from the adherent cells, whereas another part of the cultures was cultured further without any addition of the AA serum. A mixture of donors' serum (blood group AB) was used as a control. After a further 3 weeks of culturing, all cultures were terminated, and RNA was extracted from the adherent cells.

Gene expression analysis

Gene expression level was analyzed in ACLs of AA patients' LTBMC (4 females and 2 males, aged 16–31 years, median 23 years; 3 patients with AA, 3 patients with SAA). The same analysis was performed with donors' LTBMC (2 females and 5 males, aged 21–54 years, median 37 years).

Gene expression was estimated using semiquantitative PCR with prior reverse transcription (RT-PCR). A denaturing solution was added to the culturing wells, then collected and proceeded according to the standard protocol.[40] Following hybridization of poly-T primers to the mRNA, the first DNA strand was synthesized with reverse transcriptase enzyme

Passage number	Donors	AA patients
1	17.2 ± 2.2	15.3 ± 0.9
2	11.3 ± 3.9	5.7 ± 0.5
3	6.3 ± 1.2	5.4 ± 0.5
4	5.5 ± 0.9	5.1 ± 0.5
5	7.5 ± 1.8	5.5 ± 0.5
6	8.0 ± 1.6	6.3 ± 0.5

Table 1. Time to each passage of MSC from donors and AA patients (days).

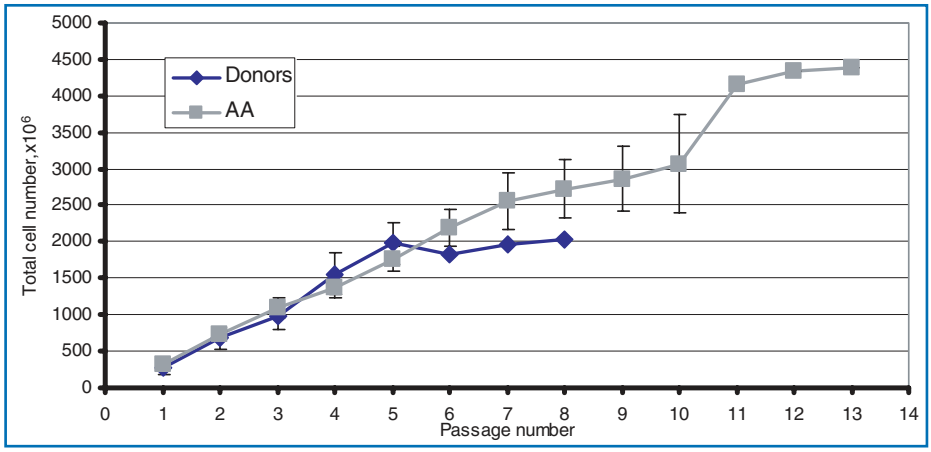


Figure 1. Total cell production in MSC cultures from donors and AA patients. MSC were obtained and passaged as described in the Methods section. Total cell production figures represent averaged data from 8 donors and 21 AA patients.

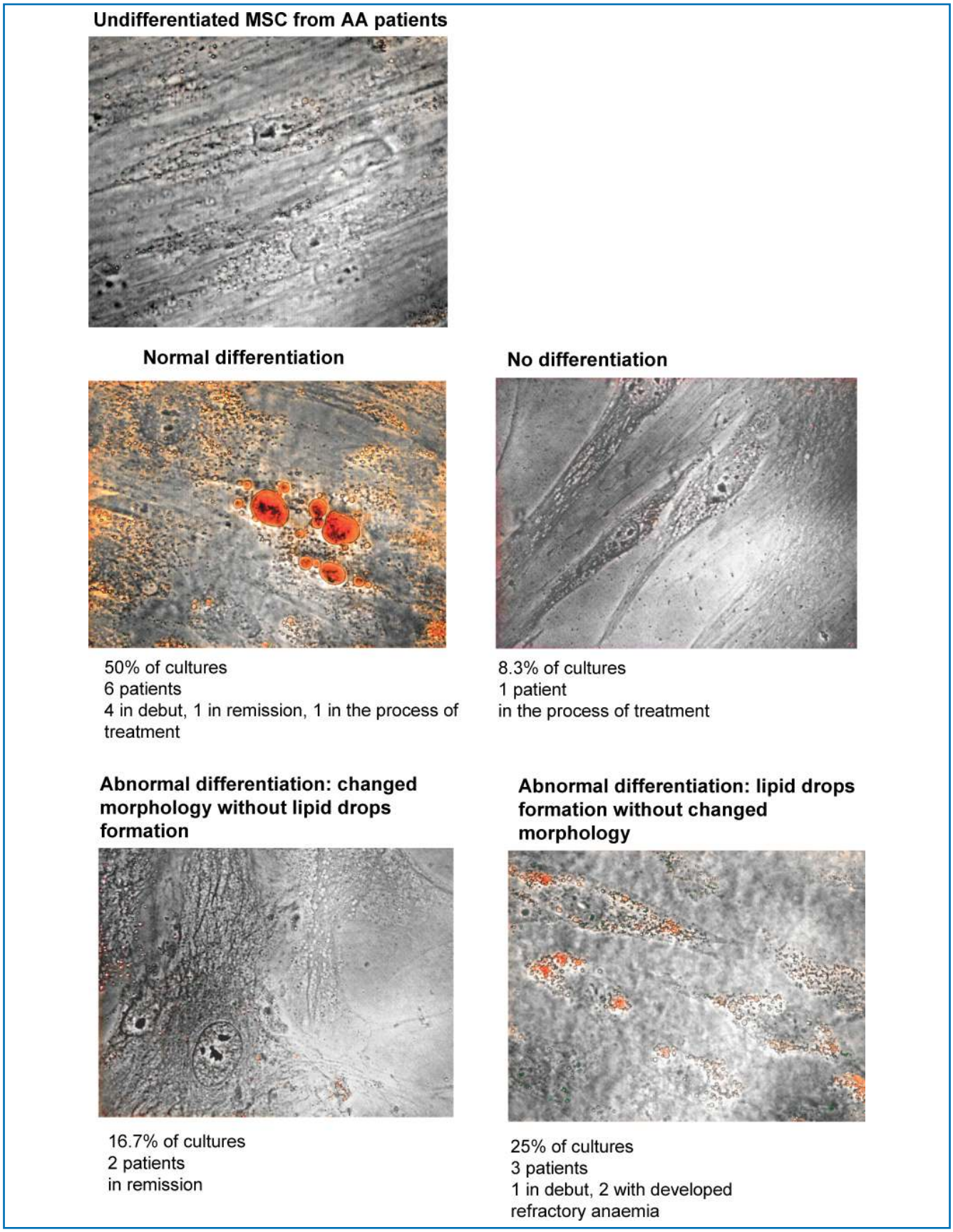


Figure 2. Alterations in adipogenic differentiation of MSC from AA patients. Adipogenic differentiation was induced as described in the Methods section and was confirmed by oil red O staining. For evaluation of adipogenic differentiation, undifferentiated MSC from AA patients was used as a negative control. The data represent 16 donors and 16 AA patients.

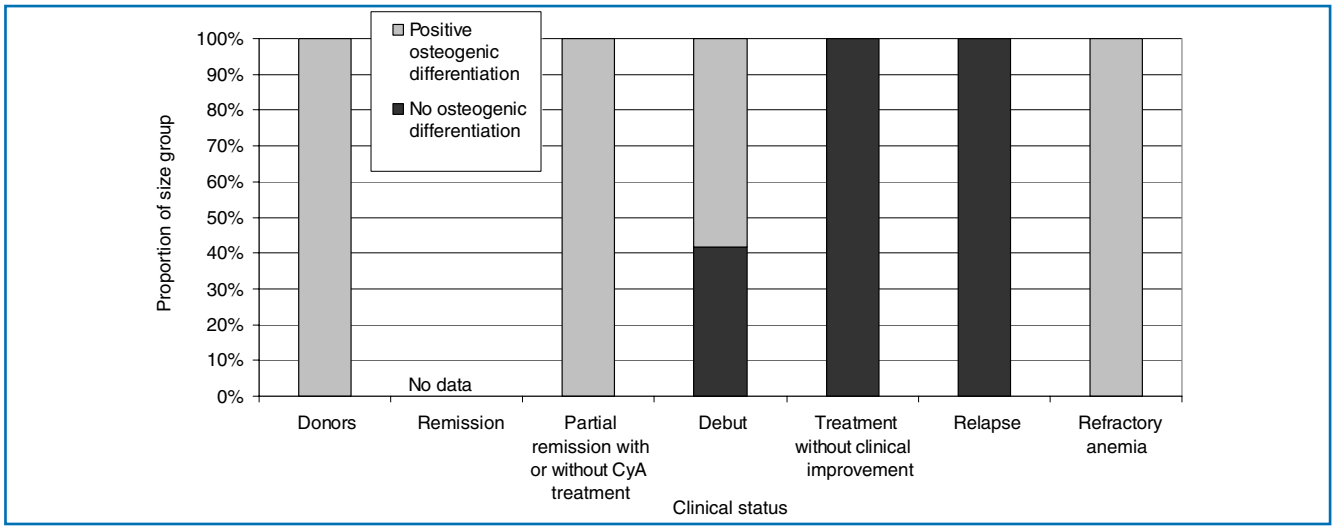


Figure 3. Alterations in osteogenic differentiation of MSC from AA patients. Osteogenic differentiation was induced as described in the Methods section and was confirmed by alizarin red staining. The data represent 13 donors and 16 AA patients.

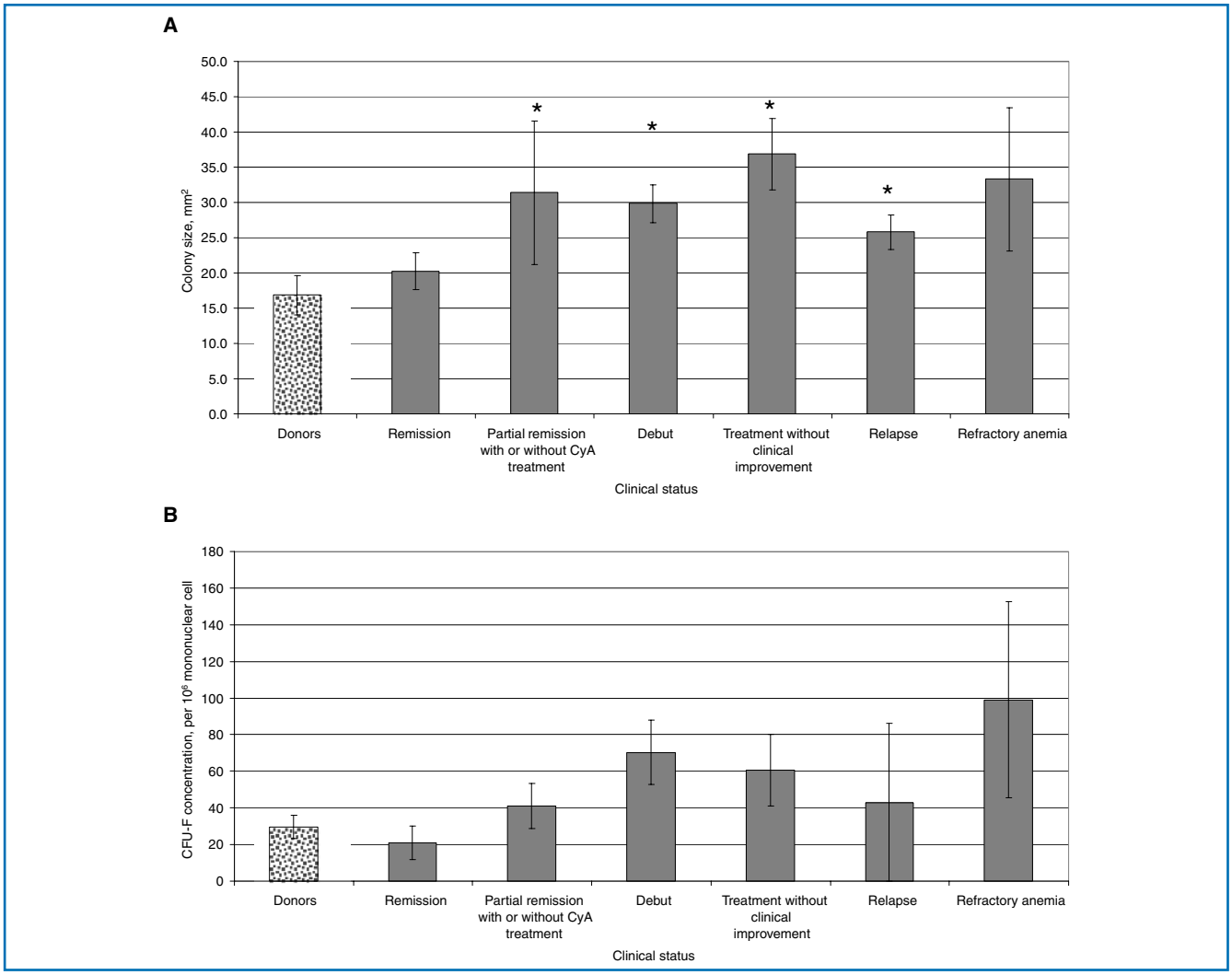


Figure 4. Alterations in CFU-F from BM of donors and AA patients. (**A**) AA patients' CFU-F colony size enlargement. Colony area was assessed on a digital image by means of the Scion Image software. AA patients were divided into groups on the basis of clinical status. * indicates $p \leq 0.05$. The data represent 27 donors and 26 AA patients. (**B**) Concentration of CFU-F in BM of donors and AA patients. AA patients were divided into groups on the basis of clinical status. The data represent 27 donors and 26 AA patients. (**C**) Dynamics of CFU-F concentration in AA patients during the treatment. The data from 5 individual patients describe the changes in CFU-F concentration. Patient 30 at the end of observation had a remission, patients 32 and 37 were diagnosed as having a stabilized disease status, patient 39 exhibited no hematological improvement and patient 38 had developed refractory anemia that was accompanied by a dramatic increase in CFU-F concentration.

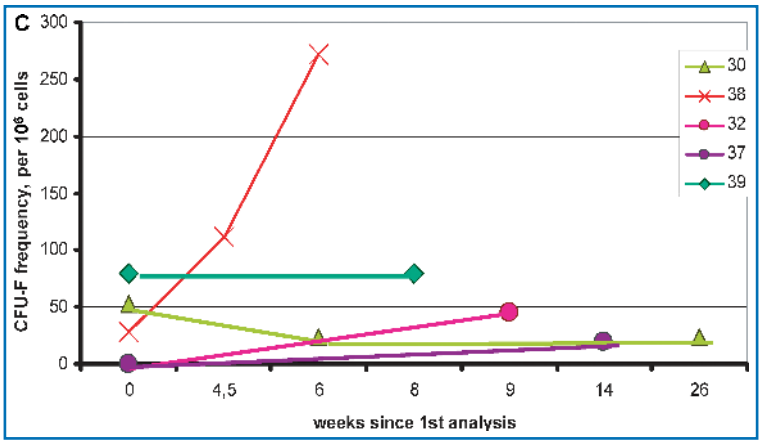


Figure 4. Continued.

(M-MLV; Promega, Madison, WI, USA). The presence of the gene of interest was determined by the specific primers β-actin, basic fibroblast growth factor (*bFGF*), *ANG-1*, *VCAM-1, VEGF,* bone morphogenetic protein (*BMP*), and insulin-like growth factor-1 (*IGF-1*).[41] To compare relative expression levels between different samples, the synthesized PCR fragments during PCR were hybridized with specific probes labeled with ^{32}P.[42] The amount of PCR product was estimated during the exponential phase of PCR (20–30 cycles). Three repetitions with two-cycle deviations were usually analyzed for each probe. The expression levels were estimated using the phosphoimager Cyclon (Packard Bell, Nijmegen, The Netherlands). The manufacturer's software was used to analyze the images. Gene expression was normalized by comparison with β-actin expression level.

Results

Culturing of MSC from donor and AA BM

The efficiency of establishing MSC cultures did not differ between AA and donor BM groups. The time taken to form a confluent monolayer from initially seeded nucleated BM cells, denoted passage zero (P_0), was approximately the same for AA and donor cultures, and the time taken to increment between passages did not differ significantly (*Table 1*). Cumulative cell production was also similar in both types of cultures for the first six passages (*Figure 1*). At that point, donor MSC growth almost ceased, whereas AA MSC continued to proliferate and was able to undergo at least six more passages, thus doubling total cell production. This phenomenon could be a consequence of increased *bFGF* expression in AA MSC as compared with donors (relative expression level in cultures from AA patients was 0.78 ± 0.15, in cultures from donors was 0.55 ± 0.09).

MSC from AA patients differed from donors' MSC in the ability to differentiate along an adipogenic lineage (*Figure 2*). The relative expression levels of *IGF-1* in cultures that failed to change their morphology was less than those in MSC cultures that were able to exhibit true adipogenic differentiation (0.31 ± 0.14 vs. 0.91 ± 0.15, $p < 0.05$).

Osteogenic differentiation was also impaired in AA MSC (*Figure 3*). Among patients whose MSCs failed to perform osteogenic differentiation, 80% had SAA. The expression level of *BMP-4* in those MSC samples that did not undergo osteogenic differentiation did not differ from that in donor cells (0.87 ± 0.16 in donor MSC and 0.99 ± 0.2 in AA MSC), whereas *BMP4* expression was downregulated in those MSC that achieved osteogenic differentiation (0.43 ± 0.17, $p < 0.05$). It is worth noting that the only patient whose cells failed to undergo adipogenic differentiation did not successfully perform osteogenic differentiation either.

Thus, MSC samples from AA patients probably exhibit stronger and more stable proliferative activity; however, the mechanisms of adipogenic and osteogenic differentiation are impaired in certain patients.

CFU-F from AA patients

MSC progenitors—clonal polypotent stromal precursors—were analyzed for their ability to form fibroblast colonies in culture (CFU-F). Among 20 patients analyzed for the presence of CFU-F in the BM, 3 SAA patients failed to form such colonies, 4 patients (3 AA and 1 SAA) formed colonies of normal size and 13 patients (3 AA and 10 SAA) formed colonies larger (in terms of area) than those of donors (*Figure 4A*). Those BM samples where normal-sized fibroblast colonies formed also featured normal CFU-F concentrations. Patients that presented enlarged colony sizes also increased their CFU-F concentration (correlation coefficient 0.7; *Figure 4B*). There was no correlation between colony number and *bFGF* expression level (correlation coefficient 0.49) and no difference in the expression level of this factor in the colonies and in the MSC derived from the same patients (*bFGF* relative expression level in the fibroblast colonies is 0.9 ± 0.35, in the MSC 0.9 ± 0.27).

Five patients were analyzed repeatedly for their CFU-F concentration. This allowed for the observation that normalization of CFU-F concentration was followed by an improvement in the patients' conditions (1 had a remission, 2 were stabilized) (*Figure 4C*). One patient had no positive changes in CFU-F concentration and clinical status. One patient developed refractory anemia (MDS) that was accompanied by a dramatic increase in CFU-F concentration.

Thus, changes in CFU-F size and concentration accompanied changes in the patient disease status.

Alteration of gene expression in the adherent cells from AA patients

To analyze the expression of those genes that are important for maintaining hematopoiesis in the stromal microenvironment of AA patients, LTMBC was used as a model. We demonstrated that after 3 weeks of culture, the expression levels of *ANG-1* and *VCAM-1* decreased significantly ($p < 0.01$ and $p < 0.02$, correspondingly), whereas the expression of *VEGF* increased when compared with donor cultures ($p < 0.05$) (*Figure 5A*). An additional 3 weeks of culturing led to normalization of expression levels of these genes (*Figure 5B*). One might suggest that this implies AA patients express factors that negatively affect stromal regulation of HSC. This would also explain the normalization of gene expression because culturing is an *in vitro* technique, and as such, all organism-derived influences are removed. To confirm this hypothesis, donor cultures were cultured with an additional 10% of blood serum from AA patients.

Influence of serum of AA patients on gene expression in the adherent cells of donor cultures

The addition of the AA serum to donor LTBMC for 3 weeks led to a decrease in the expression levels of *ANG-1* and *VCAM-1* and an increase in *VEGF* expression in ACLs in the same manner as had been observed in AA LTMBC. When AA serum addition was subsequently discontinued for 3 weeks, we observed a

normalization in the expression levels of these genes. This was observed in 9 out of 12 cultures for *ANG-1* expression, in 7 out of 13 for *VEGF* and in 11 out of 14 for *VCAM-1* (*Figure 5C*). This phenomenon implies the existence of a substance that negatively affects the expression of genes important for the maintenance of hematopoiesis in serum of AA patients.

Role of PTH in normalization of gene expression in AA and donor ACLs following the addition of AA serum

The ability of PTH to normalize gene expression level in AA ACLs and donors after the addition of AA serum was investigated. Addition of PTH to AA patients' LTBMC led to partial normalization of the expression level of *ANG-1*, *VCAM-1*, and *VEGF*, even after 3 weeks of culturing (*Figure 6A*). As in the experiments without PTH described above, 6 weeks in culture led to a complete restoration in the expression levels of these genes (*Figure 6B*). Analogously, the addition of both PTH and AA serum to the donor cultures decreased the negative effect of the sera on gene expression (*Figure 5C*). The number of cultures that exhibited disturbed gene expression decreased 2.5 to 3.0 times. In response to PTH, cultures' relative gene expression levels almost completely returned to normal. Thus, PTH served to normalize gene expression level in the cells of the stromal microenvironment of LTBMC of both AA patients and donors treated with AA serum.

Discussion

Obtaining short-lived stromal cultures from the BM of newly diagnosed AA patients was successful in only 30% of cases.[43] The efficiency of establishing LTBMC from the BM of AA patients is also low.[7] So being able to obtain MSC cultures with equal efficiency from donors and AA patients seems important. Equal time to reach P_0, together with equal cumulative cell production during the first six passages, seems sufficiently convincing to conclude that possible variability in BM stromal cell number in donors' and AA patients' marrow samples did not significantly affect MSC growth. MSC samples from newly diagnosed patients tend to proliferate more actively than MSC samples from patients in remission ($p < 0.05$, data not shown). The relative expression level of *bFGF* in MSC samples from AA patients is increased as compared with donors. The hematopoietic territories in an organism are strongly regulated; however, the enlargement of these territories in AA patients seems to be an attempt to compensate for noneffective hematopoiesis.

Impaired adipogenic differentiation of CFU-F from the BM of chronic myeloid leukemia (CML) patients in the accelerated phase and blast crisis but not in the chronic phase or polycythemia vera or essential thrombocytopenia has been previously demonstrated.[32] Taken together with our observation of impaired adipogenic differentiation of AA MSC including morphological abnormalities and decreased level of *IGF-1* expression, the data hint that adipogenic differentiation is impaired at times that correlate with the worst phases of hematopoietic depression. Fat cells are necessary for the adequate maintenance of hematopoiesis, and their dysfunction in the worst phase of the disease points to the existence of secondary alterations in MSC of AA patients.

Correlation of osteogenic differentiation with clinical status is also shown in our results. In patients with severe hematopoetic impairment, MSC samples lost their ability to differentiate. Other researchers have shown that *BMP-4* induces osteogenic differentiation in a human osteoblastic cell line.[44] However, the expression level of this gene in AA MSC, which resulted in a failure to differentiate, was identical to that in MSC of donors. Moreover, in AA, MSC samples that underwent osteogenic differentiation expressed *BMP-4* at a

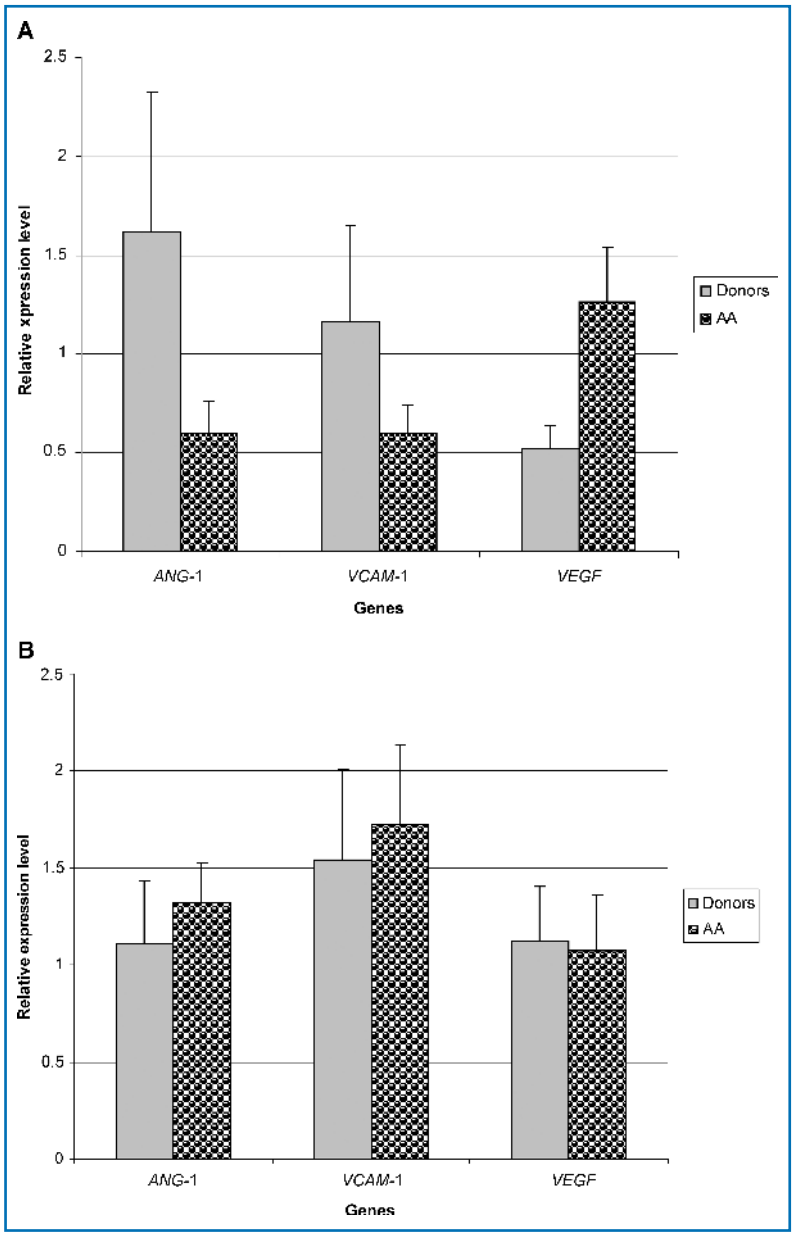
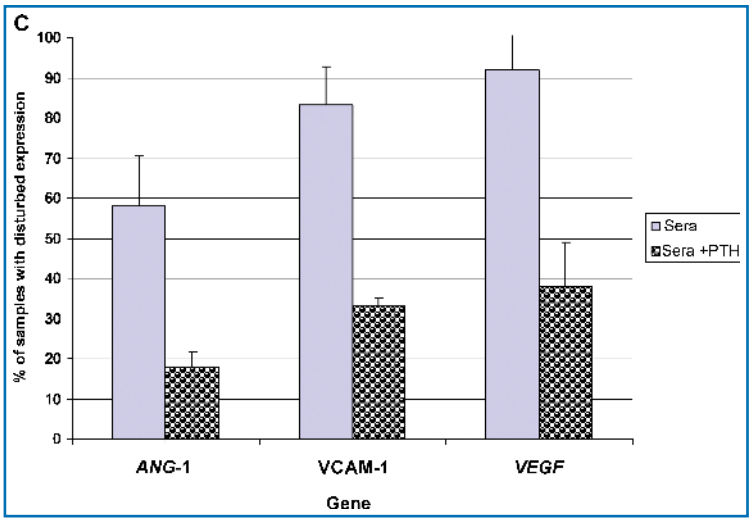


Figure 5. Relative expression level of *ANG-1*, *VCAM-1*, and *VEGF* in adherent cell layers of LTBMC. (**A**) Three weeks in culture. (**B**) Six weeks in culture. The data are presented as a mean of 7 donors and 6 AA patients. The data from the phosphoimager for each gene were normalized, consistent with the β-actin expression level. All experiments were performed in triplicate. (**C**) Donor LTBMC treated with sera of AA patients with and without addition of PTH for 3 weeks. The data represent the proportion of cultures with decreased expression level of *ANG-1* and *VCAM-1* and increased expression level of *VEGF*. Adherent cell layers from the cultures of 8 donors were treated independently with serum samples from 19 AA patients. All experiments were performed in triplicate.



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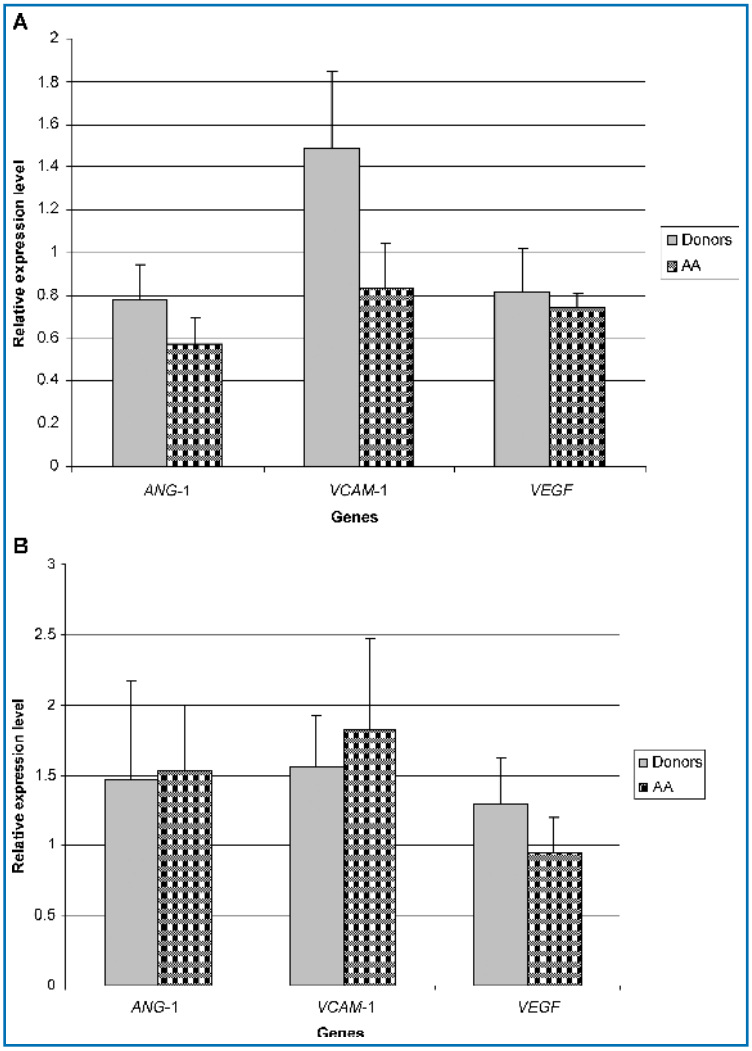

Figure 6. Relative expression levels of *ANG-1*, *VCAM-1*, and *VEGF* in adherent cell layers of long-term BM cultures in the presence of PTH. (**A**) Three weeks in culture. (**B**) Six weeks in culture. The data are presented as the mean of 7 donors and 6 AA patients. The data from the phosphoimager for each gene were normalized, consistent with the β-actin expression level. All experiments were performed in triplicate.

significantly lower level. These paradoxical data constitute another piece of evidence for the existence of stromal alterations in AA patients. Taking into consideration the leading role of osteoblasts in the regulation of early HSC function, this defect may be critical for the inhibition of hematopoiesis observed in AA. However, the absence of osteogenic differentiation was demonstrated in only 36% of all cases, and this fact is probably due to the heterogeneous etiology of the disease.

The size of CFU-F-derived colonies depends on the clinical status of AA patients. The data show that *bFGF* does not regulate the size and number of CFU-F colonies. Enlargement of CFU-F colonies and their increased concentration might be a result of additional mitosis, that is, due to the activation of stromal precursor cells that support MSC. Analysis of the changes in CFU-F concentration demonstrated that, for patients with a more severe condition, CFU-F may not be present at all or, on the contrary, the concentration of these precursor cells may be increased up to 10-fold in comparison with donors' BM. With improvement in patient health status, the concentration of CFU-F in the BM tends to reach donor's level, and it subsequently stabilizes when the patient reaches remission. Probably, there is a certain interdependence between the quality of hematopoiesis and the activity of stromal precursors in AA patients.

In stromal ACLs from LTBMC of AA patients, for the first 3 weeks of culturing, the levels of *ANG-1* and *VCAM-1* were decreased, whereas *VEGF* expression level increased. This is evidence for abnormal regulation processes in the osteoblastic (altered expression of *ANG-1*) and vascular (altered expression of *VCAM-1* and *VEGF*) niches in the BM of AA patients. Culturing for another 3 weeks normalized the expression level of these genes. It has been suggested that this effect may have been due to the disappearance of some organism-derived factor during culturing. This explanation was confirmed with experiments that involved the addition of sera from AA patients to donor LTBMC. Three weeks of culturing the cells from the BM of healthy donors in the presence of sera from AA patients led to alterations in the expression levels of the genes in a manner that was similar to the expression observed in AA patients' cells. Culturing for three additional weeks without any AA sera resulted in normalization of gene expression. These data reveal the existence in AA patients' serum of certain factors that effect gene expression. This effect could not be explained with the cellular component of blood alone, and accordingly, one could not make an analogy with an inhibiting effect of tumor cells on the function of the stromal microenvironment.[29,30] The observed inhibition could be related to autoantibodies within the blood.

PTH is known to activate osteoblasts in the niches where its presence improves the maintenance of hematopoiesis and adhesion of HSC to the stromal cells.[45] Accordingly, it was hypothesized that PTH may affect gene expression in LTBMC from AA patients or donors after treatment with AA serum. Our results show that the addition of PTH to these cultures normalized the pathological alterations. However, although PTH treatment improved the ability of donors' ACL to maintain hematopoietic precursor cells, it had no impact on AA patients' ACL (data not shown).

Thus, normalization of gene expression did not lead to a functional restoration of AA stromal microenvironment.

It was hypothesized that PTH may affect gene expression in LTBMC from AA patients or donors after treatment with AA serum. Our results suggest that the addition of PTH to these cultures normalized the pathological alterations, indicating that the negative effect of AA sera is directly linked to the action of stromal regulatory molecules and is not dependent on autoantibodies and patient transfusion history.

Conclusions

AA patients express alterations with respect to certain components of the stromal microenvironment, including MSC, CFU-F, and cells that form ACL in LTBMC. Simultaneously, in AA patients, a certain factor has been revealed to inhibit normal expression of the genes that are responsible for the regulation of HSC in the osteoblastic and vascular niches. The alterations in the stromal microenvironment of AA patients could either contribute to the pathogenesis of AA or be the result of impaired hematopoiesis. Accordingly, we suggest that further investigations into the stromal component in AA patients would be necessary for better understanding the mechanism of this disease.

Acknowledgement

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