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Autografting

Impaired bone marrow hematopoietic progenitor cell function in rheumatoid arthritis patients candidated to autologous hematopoietic stem cell transplantation

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Summary:

We have evaluated bone marrow morphology, percentage of bone marrow CD34$^+$ cells, proliferative activity of bone marrow precursors, clonogenic assay (BFU-E and CFU-GM) in short-term bone marrow cultures, and bone marrow cell apoptosis, together with serum TNF-α and IL-6, in 16 chronic, refractory RA patients, as well as in five healthy controls. Of 16 RA patients (68.7%), 11 showed a reduced bone marrow cellularity, while it was normal in all the controls. In RA patients, the median percentage of CD34$^+$ bone marrow cells, the median percentage of proliferating bone marrow myeloid precursors, and the median number of both BFU-E and CFU-GM colonies were significantly lower than observed in the controls. As far as TNF-α and IL-6 titers is concerned, the latter did not significantly differ from controls' values, while TNF-α titers were significantly lower in healthy controls. Finally, the median apoptotic index of early bone marrow myeloid cells of RA patients was significantly higher compared with controls. These observations may identify the biological risk factors for impaired mobilization and/or engraftment when RA patients are candidates for autologous hematopoietic stem cell grafting.
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Rheumatoid arthritis (RA) is the most common systemic autoimmune disease, affecting 0.5–1% of the whole world population;[1] despite being a chronic evolving disease, RA can shorten life by 5–10 years;[2] furthermore, variables reflecting disease severity have been demonstrated to negatively impact on RA patients' survival.[3]

Although, on the one hand, a number of different drugs (including immunosuppressive and cytotoxic agents) may suppress disease activity and/or palliate disease-related symptoms, on the other hand, they also require chronic administration, do not cure the autoimmune disease itself, cannot prevent irreversible end organ damage, often cause severe toxicity, and have considerable economic costs for the individual as well as the community.[4]

For all these reasons, autologous hemopoietic stem cell transplantation (aHSCT) has been proposed for the treatment of both severe refractory RA and other life-threatening autoimmune diseases;[5–15] indeed, such therapeutic procedures are increasingly performed in autoimmune disease patients, according to the *European Bone Marrow Transplantation* (EBMT) registry data.[16,17]

Several abnormalities in the hemopoietic and immune systems in patients with RA have been documented and include: ineffective erythropoiesis,[18] inhibition of granulomonocytopoiesis,[19] T-cell hyporesponsiveness or anergy,[20] B-cell functional abnormalities (especially decreased *in vitro* immunoglobulin production),[21] and functional deficiency of antigen-presenting cells, at least in the synovial fluid.[22] Furthermore, an increased number of bone marrow CD34$^+$ cells in response to an abnormally high production of G-CSF by bone marrow stroma has been recently described by Papadaki *et al*.[23]

Since the complex immune deregulation present in autoimmune diseases may affect bone marrow progenitor cell development at several stages of differentiation,[24] Ikehara[25] *et al* suggested that autoimmune diseases should be considered as primary stem cell disorders.

Furthermore, patients with long-lasting and/or severe RA, who are ideal candidates for aHSCT, may have been exposed for years to myelosuppressive and/or myelotoxic drugs. Thus, impaired or altered bone marrow function, partially intrinsic and partially iatrogenic, may have an influence on the functional quality of circulating progenitor cells (CPCs) to be mobilized and then autografted.

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In this *in vivo* study, we investigated the biologic and functional characteristics of bone marrow hematopoietic progenitors in severe refractory RA patients who were candidates for aHSCT.

Patients and methods

Patients

After obtaining informed consent, 16 caucasian female patients with severe refractory RA were enrolled into the study.

In all patients, RA was diagnosed according to the *American College of Rheumatology* criteria.[26]

The average age was 55 years (median 57; range 29–65); on the average, RA duration in these patients was 112.4 months (median 84, range 23–348), that is, more than 9 years.

All patients chronically received steroids, at a median cumulative dose of 8640 mg of prednisone equivalent (mean 20 491.71, range 350–178 290); all were still receiving steroids at the time of enrollment into this study.

All patients but two had received methotrexate (MTX) at a median cumulative dose of 732.5 mg (mean 1122.5, range 200–2730), 11 still under MTX treatment at the time of their enrollment into this study.

Finally, seven patients also received cyclosporine A (CyA), at a median cumulative dose of 78 000 mg (mean 90 050, range 9100–172 750), with six still under CyA.

None of the above RA patients was treated with anti-TNF-α or anti-IL-1 antibodies before the study.

As a parameter of disease activity, C-reactive protein (CRP),[27] measured both at the time of study entry as well as every 3 weeks during the course of the disease, that is, cumulative CRP, was used.

Patient characteristics are reported in Table 1.

Controls

As controls, five healthy females, adequately chosen within the age range of RA patients (average age 52 years, median 54, range 30–70), were studied after informed consent was obtained; all controls presented with a normal baseline hematologic profile and none was known to have evident diseases, or were under pharmacological treatment that could interfere with hematopoiesis.

Bone marrow cytology

Bone marrow samples were obtained from posterior iliac crest aspirates; differential cell count was assessed by evaluating 1000 nucleated cells from different areas of May–Grünwald–Giemsa-stained preparations. Scoring included erythroblasts, nonproliferating (metamyelocytes, plus band forms, plus neutrophils) and proliferating (myeloblasts, plus promyelocytes, plus myelocytes) myeloid cells, as previously described.[28]

Bone marrow $CD34^+$ cell evaluation

The percentage of $CD34^+$ progenitor cells in bone marrow samples was evaluated on a Coulter Epics-XL (Coulter International Co., Miami, FL, USA) flow cytometer, using the HPCA-2 anti-CD34 monoclonal antibody (Becton Dickinson, Mountain View, CA, USA), according to the 'Milan protocol'[29] (Figure 1).

Flow-cytometric (FCM) analysis of proliferative activity (S-phase) of bone marrow myeloid precursors

Single-cell suspensions were obtained from bone marrow particles that were gently washed in saline solution, placed into a 5 ml tube containing 1 ml of PBS (Sigma Chemicals, St Louis, MO, USA), and finally disrupted by pipetting.

Light-density mononuclear cells (LDMNCs) were collected after centrifugation on a Ficoll-Hypaque (Pharmacia

Table 1 Patients' characteristics

No.	*Sex*	*Age (years)*	*Disease duration (months)*	*Present ESR (mm)*	*Present CRP (mg/dl)*	*Actual DMARDs*	*Total steroid dose (mg)*	*Cumulative CRP*
1	F	65	23	69	15.2	GS, HCQ	350	23.85
2	F	49	36	76	6.4	MTX	7920	12.3
3	F	60	216	19	11.3	MTX	1825	n.a.
4	F	57	48	53	1	HCQ, MTX, CyA	12 562.5	84.55
5	F	57	240	41	1.8	SSZ, HCQ	178.290	56.1
6	F	58	24	96	10.9	MTX, HCQ	6275	66.3
7	F	50	216	44	9.6	GS	2190	9.6
8	F	54	96	51	2.7	MTX, SSZ	19 530	42.03
9	F	29	72	6	0.7	MTX, HCQ, CyA	12 915	63.5
10	F	50	108	70	0.6	MTX, HCQ, CyA	16 680	83.69
11	F	47	48	6	7.2	MTX, HCQ, CyA	4500	44.7
12	F	65	108	71	9.3	MTX	34 620	85.58
13	F	64	48	5	0.3	HCQ, CyA	9360	0.5
14	F	46	132	60	1	GS	11 670	n.a.
15	F	64	36	63	13.7	MTX, HCQ, CyA	4560	106.5
16	F	65	348	24	1.8	MTX	4620	21.6

ESR = erythrocyte sedimentation rate; CRP = C-reactive protein; DMARDs = disease-modifying antirheumatic drugs; MTX = methotrexate; HCQ = hydroxychloroquine; CyA = cyclosporine A; SSZ = sulfasalazine; GS = gold salts.

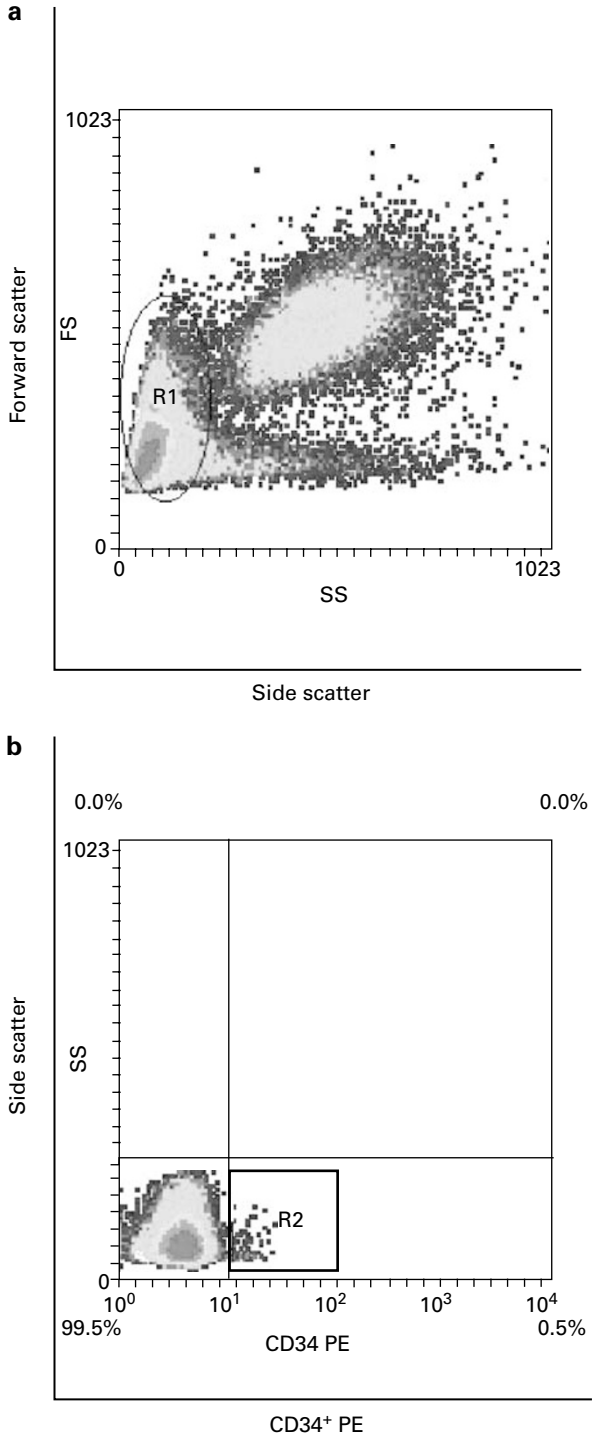

Figure 1 Identification of bone marrow $CD34^+$ cells by flow cytometry. Panel shows representative dot plot data illustrating the analytical method utilized to identify $CD34^+$ cells in bone marrow (see *Materials and methods*). (**a**) Mononuclear cells analysis region applied to FS × SS data acquired for exclusion of granulocytes and debris (region R1). (**b**) The same identified cell population after labelling with CD34 anti-MoAb (region R2).

Fine Chemicals, Piscataway, NJ, USA) density gradient ($1.077\,g/cm^3$) in Iscove's modified Dulbecco's medium (IMDM, Sigma Chemicals, St Louis, MO, USA).

The cells were further purified by removing adherent cells and T lymphocytes as follows: a 5 ml suspension of LDMNCs at a concentration of 5×10^6 cells/ml in IMDM plus 15% FCS (Sigma Chemicals, St Louis, MO, USA) was incubated in $25\,cm^2$ tissue culture flasks (Falcon Co., Germany) for 60 min at 37°C, and nonadherent cells were carefully collected; this procedure was repeated twice.

T-lymphocyte-depleted LDMNCs were obtained by rosetting mononuclear cell suspensions (5×10^6 cells) with 2-aminoethylisothiouronium bromide (AET, Sigma Chemicals, St. Louis, MO, USA)-treated sheep red blood cells in a 5% suspension with IMDM; nonrosetting cells were finally separated using a second Ficoll–Hypaque density centrifugation.

The viability after cell separation was evaluated by the Trypan blue (Sigma Chemicals, St Louis, MO, USA) dye-exclusion test.

Cells were washed twice in PBS, filtered through a 35-μm pore nylon filter, resuspended in PBS and used for FCM analyses.

The DNA staining and FCM techniques we used have been extensively described and discussed elsewhere.[30,31]

Briefly, cells were suspended in PBS, and drawn through needles of decreasing diameters; the obtained suspension was stained with propidium iodide (PI, Calbiochem, Behring Co., San Diego, CA, USA) at a concentration of 50 μg/ml in PBS plus 0.1% Nonidet P40 (Calbiochem, Behring Co., San Diego, CA, USA), 0 and 0.05% RNAse (Sigma Chemicals, St Louis, Mo, USA), for 30 min at room temperature. Cell cycle analysis was carried out using a DAKO/Partec PAS III flow cytometer (Dako, Glostrup, Denmark) under the following conditions: argon ion laser excitation power 50 mW at 488 nm, 610 nm long pass filter for the red fluorescence (PI) detector. The percentage of cells in the S-phase of the cell cycle was thus recorded.

Clonogenic assay

Clonogenic assays were performed as described elsewhere,[32] with minor modifications. Briefly, 2×10^4 LDMNCs were plated in 35-mm Petri dishes in 1 ml aliquots of IMDM containing 30% FBS (HyClone, Logan, UT, USA), 5×10^{-5} mol/l β-mercaptoethanol, 0.9% (w/v) methylcellulose (both from Sigma Chemicals, St Louis, MO, USA), 10 ng/ml GM-CSF (PeproTech Inc., Rocky Hill, NJ, USA), 10 ng/ml IL-3 (Sandoz International, Basel, Switzerland), 50 ng/ml SCF (PeproTech Inc., Rocky Hill, NJ, USA), and 3 IU/ml erythropoietin (Boehringer-Mannheim, Mannheim, Germany).

Since it has been reported that patients suffering from severe rheumatoid arthritis have increased serum levels of cytokines, such as interferon-γ or TNF-α,[33,34] which are known to exert an inhibitory effect on the growth of clonogenic progenitor cells, we also performed clonogenic assays in which FBS was replaced by patient serum.

Cultures were incubated at 37°C and 5% CO_2 for 14 days; at the end of incubation, the number of BFU-E and CFU-GM was scored using an inverted microscope.

Serum TNF-α and IL-6 titration by flow cytometry

For the quantitative titration of two serum cytokines TNF-α and IL-6 by flow cytometry, a commercial *kit*

(Quantiflow, Bio Ergonomics, Centerville Road, MN, USA) was used, according to the manufacturer's instructions (Figure 2a–d).

Briefly, venous blood derived from venipuncture of an antecubital vein of the forearm was centrifuged at 2000 r.p.m. and then stored at −20°C for subsequent titrations; 500 μl of blood was then vortexed for 60′ together with 10 μl of TNF-α or IL-6 capture beads; following two washings with IFA Buffer 10 ×, 10 ml of PE-conjugated TNF-α or IL-6 reporter antibody were added; following incubation and washings, the samples were analyzed using a Coulter Epics-XL (Coulter International Co., Miami, FL, USA) equipment.[35]

Evaluation of bone marrow cell apoptosis

Apoptosis was measured on early bone marrow myeloid precursors using a terminal-deoxynucleotidyl-transferase (TdT)-mediated deoxyuridine triphosphate (dUTP) nick end labeling (TUNEL) technique[36] (Figure 3).

Briefly, the In situ Cell Death Detection Kit, AP (Boehringer-Mannheim, Germany), which contains calf thymus TdT, fluorescein-dUTP, and an alkaline phosphatase antifluorescein sheep Fab fragment, was used according to the manufacturer's instructions, improved by the fixation of smears with cold acetone for 10 min, and by the omission of cell permeabilization. After the alkaline phosphatase reaction, counterstaining was performed with Mayer's hematoxylin. A negative control was performed by omitting TdT from the labelling mixture.

The apoptotic index (AI) was expressed as the percentage of positive nuclei for 500 counted cells at a magnification of × 1000, evaluated on the same sample by two different experienced hematologists unaware of sample origin.

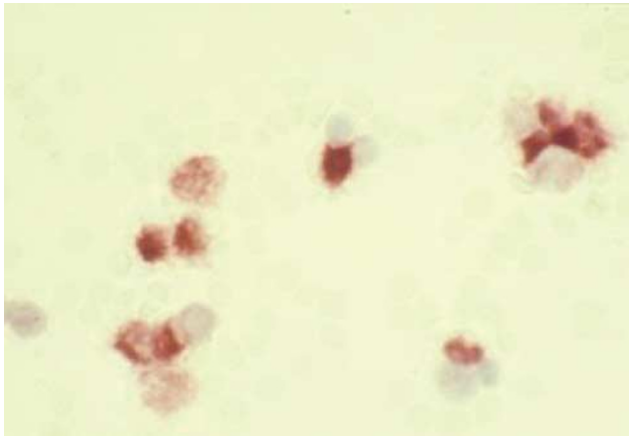

Figure 3 Bone marrow smear from a RA patient; TUNEL technique. A high percentage of immature cells shows strong nuclear reativity (× 480).

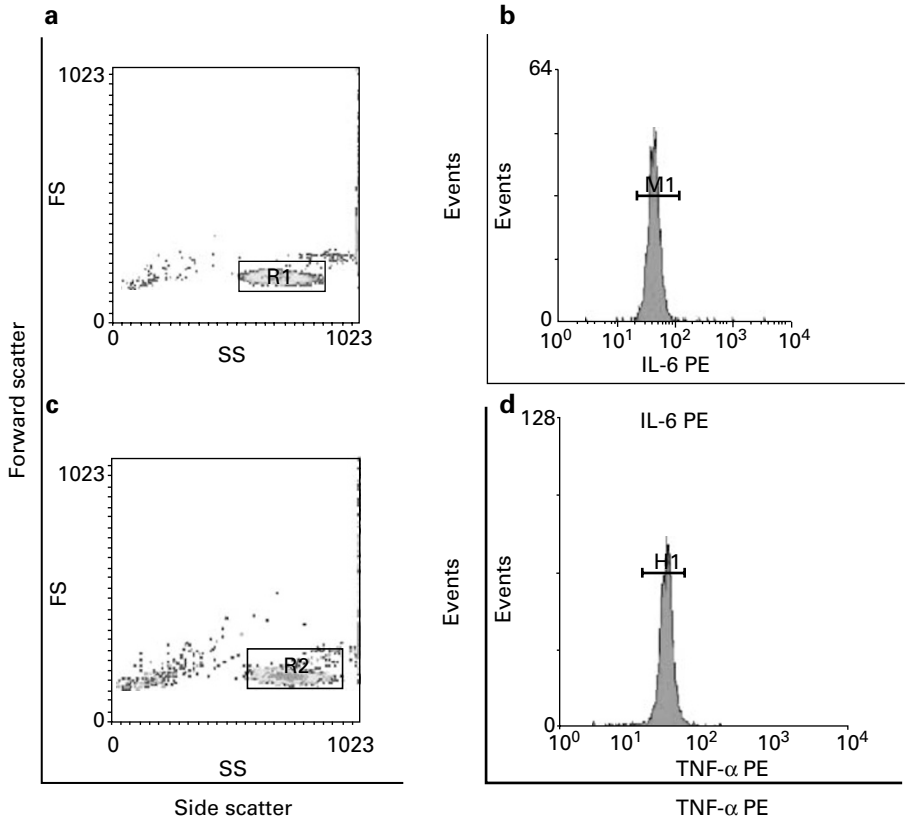

Figure 2 Flow cytometric determination of serum IL-6 and TNF-α. Panel shows representative data illustrating the analytical method (see *Materials and methods*). (**a,c**) FS × SS analysis of PE-conjugated capture beads for both cytokines. (**b,d**) Corresponding fluorescent signals of beads (the mean channel is determined by gating on the main peak of the beads).

Statistics

Traditional statistics and correlations between variables were performed using the MS Excel software, run on an iMac computer (Apple Computers, Cupertino, CA, USA); differences between patients and controls were analyzed using the Student's *t*-test.

Results

Bone marrow cytology

Out of 16 patients, 11 (68.7%) showed reduced bone marrow cellularity by direct light microscope examination of bone marrow smears; in five, the reduction was minimal though evident, while in six it was more marked.

In the remaining five patients, as well as in all the five healthy controls, a normal bone marrow cellularity was present.

Bone marrow CD34$^+$ cells

The mean percentage of CD34$^+$ bone marrow cells in our RA patients was 0.25 ($\pm$0.13 s.d., median 0.28, range 0.1–0.5). This figure was significantly ($P=0.003$) lower than that observed in the five healthy controls (mean 1.87$\pm$0.59 s.d., median 1.89, range 0.99–2.0).

Proliferative activity (S-phase) of bone marrow myeloid precursors

The mean percentage of proliferating bone marrow myeloid precursors, that is, those myeloid cells in the S-phase of the cell cycle, was 4.03 ($\pm$3.28 s.d., median 2.85, range 1.5–12.5) in our RA patients. Also, in this case, the above figure was significantly lower ($P=0.05$) than that observed in the five healthy controls (mean 9.2$\pm$4.36 s.d., median 7.0, range 5.1–14.9).

Clonogenic assay

The median number of BFU-E in the RA patients was 14.5 ($\pm$16.5 s.d., mean 21.2, range 3–54), a number significantly lower ($P=0.01$) than that observed in the five healthy controls (median 48$\pm$13.7 s.d., mean 43.2, range 21–56).

The mean number of CFU-GM in RA patients was 21 ($\pm$17 s.d., median 24, range 1–59), a figure that did not significantly differ ($P=$n.s.) from that observed in the five healthy controls (mean 38$\pm$4.47 s.d., median 38, range 36–44). These data are summarized in Figure 4.

Furthermore, no statistically significant differences were observed between clonogenic assays with FBS or with patient's serum (data not shown).

Serum TNF-α and IL-6 titers

Both TNF-α and IL-6 were detected in the serum of our RA patients; indeed, the mean TNF-α titers were 9.68 pg/ml ($\pm$11.02 s.d., median 7, range 2–50) and IL-6 146.63 ($\pm$406.05 s.d., median 14.75, range 2.8–1600), respectively.

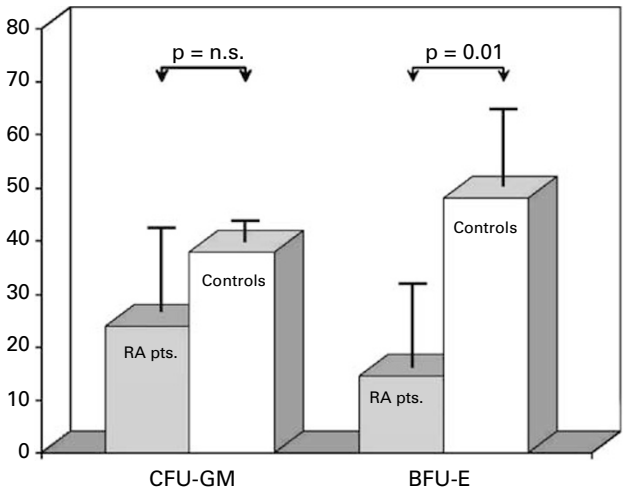

Figure 4 Clonogenic assay. In RA patients, the median number of BFU-E was significantly lower than that observed in the controls, while the median number of CFU-GM in RA patients did not significantly differ from that of controls.

TNF-α titers were significantly lower ($P=0.01$) in the five healthy controls (mean 2.26$\pm$0.25 s.d., median 2.2, range 2.1–2.7) than in RA patients. IL-6 titers were lower (mean 4.2$\pm$1.12 s.d., median 3.6, range 3.3–5.9), but not significantly different, from the above patients' values. A wide dispersion of IL-6 titers was found in patients with RA, according to the different disease activity score.

Bone marrow cell apoptosis

The mean apoptotic index of early bone marrow myeloid cells of our RA patients was 43 ($\pm$11.76 s.d., median 41.65, range 23–60), a figure that was significantly ($P=0.0005$) higher compared with results in the five healthy controls (mean 11.7$\pm$1.09 s.d., mean 12, range 10–13).

Parameters of bone marrow function according to disease history

No statistically significant correlations were found when bone marrow function parameters, that is, number of CD34$^+$ cells, proliferative activity of myeloid precursors, CFU-GM and BFU-E *in vitro* growth, as well as bone marrow apoptosis, were plotted against disease duration and against parameters of disease activity, including cumulative CRP and disease activity score. When patients under MTX treatment were compared to RA patients who were not receiving MTX, no significant differences were found in the bone marrow function parameters (Table 2).

Discussion

Although RA is a systemic autoimmune disease characterized mainly by a chronic destructive polyarthritis, growing evidence suggests that the bone marrow may also be actively involved in its pathophysiology, so that RA can be considered as a bone marrow,[37] or a primary stem cell, disorder. [25] This has reinforced the rationale, initially based

Table 2 Bone marrow function parameters in MTX-treated or untreated patients

Bone marrow function parameter	*RA patients under MTX treatment (mean±s.d., median, range)*	*RA not treated with MTX (mean±s.d., median, range)*	*P*
$CD34^+$	0.24 (±0.13, 0.3, 0.1–0.4)	0.3 (±0.13, 0.2, 0.2–0.5)	n.s.
S-phase	2.38 (±0.96, 2.3, 1.5–3.8)	4.8 (±3.7, 3.0, 2.2–12.5)	n.s.
Apoptosis	45.4 (±13.74, 45, 26–64)	42.5 (±13.43, 44, 23–64)	n.s.

Of the five patients not under treatment with MTX, three of them never reiceved it, while two experienced MTX-related serious toxicity, leading to its withdrawal.

only on animal data and anectodical case reports, for the use of hematopoietic stem cell transplantation in the treatment of RA.[8,17,25]

Thus, it is clearly important to ascertain if the chronic inflammatory process,[38] and/or the sustained immunosuppressive therapy commonly employed in these patients,[39] could negatively impact either on mobilization of stem cells into the blood or, especially, on their engraftment after transplantation.

Indeed, besides their absolute number,[40] the functional quality of the autologous CPCs mobilized into the peripheral blood, including their cell cycle status,[41–43] apoptosis,[44,45] and phenotype,[46,47] is a key element in the success of the autografting procedure, as already demonstrated in both solid and hematologic malignancies.

For all these reasons, we have studied the quantitative and qualitative characteristics of bone marrow hematopoietic progenitor cells in 16 RA patients who were candidates for autologous stem cell transplantation.

From a morphological viewpoint, the majority of our patients showed a markedly reduced bone marrow cellularity with respect to healthy controls.

Similarly, flow cytometry showed a significantly lower percentage of $CD34^+$ bone marrow cells and suppressed proliferative activity (evaluated through the determination of DNA S-phase) of bone marrow myeloid precursors, compared with controls. Furthermore, the median apoptotic index of early bone marrow myeloid precursors (evaluated by TUNEL analysis) was significantly higher compared to controls. These results are in agreement with recent observations by Papadaki *et al*.[48] In contrast to the observations of these authors using the clonogenic assay,[48] we were able to identify only suppression of BFU-E, and not of CFU-GM, in our RA patients, when either FBS or patients' serum was used. Indeed, our observation could explain the high incidence of anemia in RA patients, in contrast to neutropenia.[49]

We studied IL-6[50] and TNF-α[51] as the possible mediators of inflammation, potentially able to contribute to bone marrow function suppression and apoptosis. Both cytokines were detected by a FCM technique in the serum of patients and controls. No significant differences were found in IL-6 serum levels between patients and controls, probably due to the limited number of cases studied and a wide variation related to disease activity. Nonetheless, significantly higher serum TNF-α titres were found in RA patients independently of the disease activity score. The latter observation is in agreement with the results of Papadaki *et al*, who showed high TNF-α levels in the supernatant of cultured bone marrow stromal cells.[48]

The chronic use of an immunosuppressive drug such as MTX could also explain the biological alterations reported above. We compared the bone marrow function parameters of patients receiving MTX treatment at the time of the study, with those of patients who were not receiving MTX. Since no differences were observed between these two groups, we can conclude that the observed alterations are not only due to MTX treatment.

Taken together, these observations may allow the identification of 'biological risk factors' for impaired mobilization and/or a delayed engraftment when RA patients with the above clinical characteristics, that is, long disease duration, active disease, and heavy pretreatment, are candidates for high-dose chemotherapy and autologous stem cell grafting.

As far as mobilization is concerned, available data are controversial; indeed, even though CPCs harvested from patients with severe RA have already been demonstrated to significantly differ in terms of composition from those of normal individuals, *in vitro* studies supported normal CPC function,[52] and at least one *in vivo* study reported no mobilization impairment in RA patients.[53] Furthermore, we have previously demonstrated that CD34 mobilization by G-GSF alone is effective in RA but lower than in healthy donors, while the combined use of an intermediate dose of cyclophosphamide and G-CSF as mobilization regimen appears to be more effective.[54]

We conclude that the combined use of chemotherapy, hematopoietic growth factors, and antagonists of myelosuppressive cytokines, for example, anti-TNF-α monoclonal antibody, warrants further investigation.

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