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Morphological and Functional Characteristics of Short-Term and Long-Term Bone Marrow Cultures in Chronic Myelogenous Leukemia

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Clonogenic capacity of bone marrow progenitors and stromal layers established from bone marrow of 12 patients with CML and 13 healthy controls were evaluated. The initial BFU-E and CFU-GM contents were slightly higher in the CML patients ($p > 0.05$) in contrast to CFU-GEMM. CFU-GEMM was lower in the patients compared to healthy controls ($p < 0.001$). In long-term cultures, the number of non-adherent cell population and total clonogenic progenitor cell content decreased gradually in both groups. Weekly evaluation of stromal confluency of adherent cells revealed that establishment of adherent stromal layer was slower in CML patients than in control samples ($p < 0.05$). At the end of fourth week, the number of samples presenting confluency was 41.7% in the CML group compared with 92.3% in the controls. The initial CD34 positive cell content of the bone marrow samples was similar in both groups. Although CD34 positive cell number in the adherent stromal layer was well preserved in the control group at the end of 4 weeks, this figure decreased significantly in the CML group. The numbers of total adherent cells as well as the total clonogenic progenitor content of adherent layer were also lower in the CML group (3.03% vs 98.2%). When normal CD34+ cells were cultured on IFN-α-treated stromal layer followed by the assessment of the long-term culture initiating cells, a reduced capacity to support hemopoietic growth was observed with IFN-α-treated normal stroma. This reduction was even higher when CML stroma was treated with IFN-α followed by the seeding of the normal CD34+ cells on this stromal layer (26.9% vs 42.8%). These findings show that stromal cells are abnormal in CML patients as well as the progenitor cells, and IFN-α treatment causes further defects of the stromal cells. Am. J. Hematol. 62:212–220, 1999. © 1999 Wiley-Liss, Inc.

Key words: chronic myelogenous leukemia; interferon-α; long-term bone marrow culture; clonogenic assay

INTRODUCTION

Chronic myelogenous leukemia (CML) is a malignant disorder originating from hematopoietic stem cell, and it is characterized by a reciprocal translocation between chromosome 9 and chromosome 22 [1,2]. This translocation-related hybrid bcr-abl gene encodes a protein with 210 kDa (p210) molecular weight, which has several properties, including increased tyrosine kinase activity, induction of the expansion of myeloid lineage, and inhibition of apoptosis [3,4].

Chronic phase of CML is characterized by expansion of myelopoiesis at both progenitor and mature cell levels. The colony-forming capacity of these cells shows no significant abnormality [5–9]. However, the progenitor cell content of the peripheral blood and the proliferation rate of these cells were reported to be increased [9], which was suggested to be due to generalized amplification of all progenitor compartments, decreased retention of progenitor cells in the bone marrow due to adhesion defects, and suppression of apoptosis at the progenitor level [3,4,9,10].

It has also been suggested that abnormal expansion of CML progenitors are partly caused by their unresponsiveness to normal regulatory control of marrow stroma

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[10,11]. Adherence to stroma usually lead normal primitive progenitors to retain in quiescent state [12]. However, CML progenitors continue to proliferate while in contact with stroma [11,13]. This unresponsiveness may be caused by their adhesion defect through leukocyte adhesion molecules, LFA-3 in particular [14]. Verfaille et al. have also shown that β_1-integrin-dependent fibronectin adhesion was also abnormal in CML progenitors [15].

Cells capable of initiating long-term culture (LTC-IC) are lineage marker negative, CD34 positive, and do not express HLA-DR and CD33 antigens [16–18]. Verfaille et al. have shown that bone marrow samples obtained from CML patients contains two different hematopoietic progenitor populations. The first group is CD34+ but HLA-DR and bcr-abl negative normal progenitors which have an ability to adhere to the normal stroma [17,18]. The number of these cells is approximately one-tenth of the normal controls [17–19]. However, the progenitors of the malignant clone express both CD34 and HLA-DR and are bcr-abl hybrid gene positive and defective in stromal adhesion [17,18].

Bone marrow microenvironment is also defective in CML, and the stromal layers established from patients were found to have less ability to support normal hematopoiesis [20,21]. Alteration in the stromal function could not be attributed to the stroma-derived cytokines since cytokine production was found to be comparable to the normal stroma [21,22]. Although relative proportions of different cell types were also observed to be similar in both CML and normal stroma, CD14+ stromal macrophages were found to be derived from bcr-abl positive malignant clone [21]. The defect of CML stroma in supporting the normal hematopoietic progenitors was shown to be related with the presence of those bcr-abl positive macrophages [21].

Interferon-α (IFN-α) is widely used in the treatment of patients with CML, and it has been proposed to have multiple effects on the normal hematopoietic progenitors, stromal cells, as well as the malignant clone [13,14,18, 23–33]. IFN-α has an direct anti-proliferative effect on both normal and malignant hematopoiesis [32,33]. It also restores adhesion of the malignant progenitors to the bone marrow stroma by either altering extracellular carbohydrate moieties of the stroma and restoring LFA-3 expression on malignant clone, or by altering the function of β_1-integrin molecules [13,14,23]. Restoration of stromal adhesion might subsequently lead to the contact inhibition of previously uncontrolled proliferation of the CML clone. IFN-α also suppress GM-CSF and G-CSF levels in the CML patients [26]. Induction of interleukin-1 (IL-1) antagonists and class II antigen expression by IFN-α also contributes to the suppression of the malignant clone [18,19,31]. Despite the beneficial effects of IFN-α in CML, Beelen et al. has suggested that prolonged administration of IFN-α prior to allogeneic bone marrow transplantation might have adverse affect on transplant outcome in CML patients [34]. Transplant-related mortality and engraftment failure in matched-unrelated transplantation was found to be increased in patients who received prolonged IFN-α treatment [34]. Although Giralt et al. could not confirm these findings, the adverse effect of IFN-α treatment on transplant outcome reported by Beelen et al. might indicate altered stromal function by this agent [35].

TABLE I. Clinical Characteristics of CML Patients

Median age	42.5 years (32–59)
Sex, F/M	8/4
Disease Stage	
Chronic phase	10
Accelerated phase	2
Blast crisis	0
Treatment	
None	1
Busulphan	3[a]
Hydroxyurea	11
Interferon-α	11[b]
Response to Treatment	
Hematological remission	5
Cytogenetic remission	1
Bone Marrow Fibrosis	
None	6
Minimal	3
Mild–moderate	2

[a]They were not receiving busulphan during the last 4 months prior to the study.
[b]Five patients were treated with IFN-α treatment at the time of the study, and the remaining 6 patients did not receive IFN-α at least 3 months prior to the study.

The aim of this study was to investigate clonogenic capacity of stroma obtained from patients with CML and normal controls and possible effect of IFN-α on the stromal function.

MATERIALS AND METHODS

Bone Marrow Samples

Twelve patients with CML and 13 normal healthy volunteers were studied. Informed consents were obtained from all patients. Patient characteristics are summarized in Table I. In CML group, ten patients were in chronic phase and two patients were in accelerated phase. Four patients out of 10 patients in chronic phase were in hematological remission, and one patient was in cytogenetic remission following IFN-α treatment. These four patients and additional two patients were still using IFN-α at the time of the study. Five patients had been treated with IFN-α in the past, but they were not using IFN-α at least for 3 months. Three patients had been treated with busulphan initially but they were off busul-

phan treatment for more than 4 months prior to evaluation. Eleven patients were being treated with hydroxyurea at the time of the study. Only one patient was a newly diagnosed CML and had no history of any treatment.

Bone marrow was obtained at the same time as the routine bone marrow cytogenetic samples. Chronic and accelerated phases of CML and hematological and cytogenetic remissions were classified according to the criteria described by Kantarjian et al. [36].

Nine of the healthy volunteers were donors for allogeneic bone marrow transplantation, and bone marrow samples were obtained for routine examination. Three samples were obtained from volunteers who were undergoing orthopedic surgery and the samples were collected during operation. One patient was performed bone marrow aspiration for iron deficiency anemia.

Anti-coagulated bone marrow samples were obtained by aspiration from the posterior iliac crest, and mononuclear cells were isolated by Ficoll-Hypaque (Sigma Diagnostics) density gradient separation (specific gravity, 1.077). The mononuclear cells were used in immunophenotyping, progenitor cell assay, and long-term culture. During long-term cultures non-adherent cells were collected every week for cell count, immunophenotyping, and progenitor cell assay. At the end of 4 weeks, adherent stromal cells were harvested by trypsin treatment, and similar determinations were performed.

Immunophenotyping

Separated bone marrow mononuclear cells or non-adherent cells collected from long-term cultures were stained with FITC conjugated monoclonal antibodies (anti-CD34, HPCA-1, and anti-CD45, Leukogate, Becton-Dickinson) and isotype-matched, FITC-coupled mouse IgG1 control. Mononuclear cells (1×10^6 cells/100 μl) in phosphate-buffered saline containing 1% bovine serum albumin (PBS/BSA) were incubated with 20 μl of the monoclonal antibody for 30 min at 4°C, washed two times, resuspended in the same buffer, and analyzed by flow cytometer (FACSort, Becton-Dickinson). Following a "live gate" CD34+ cells in low side scatter region was subgated and positivity obtained by isotypic control at the same region was subtracted for determining the number of true CD34+ cells [37].

CD34 Positive Cell Selection

Normal bone marrow sample, collected from 24 year-old healthy allogeneic bone marrow donor was used for CD34 positive cell selection. Isolex 50 system (Baxter, Germany) was used for this purpose, and CD34+ cells were isolated as recommended by the manufacturer. Mononuclear cells were isolated by Ficoll-Hypaque gradient centrifugation, washed, and re-suspended in PBS containing 1% human serum albumin (PBS/HSA) and 5% acid citrate dextrose-adenine (ACD-A) (Baxter). The number of nucleated cells was determined by using cell counter (Coulter, USA), and the amount of CD34+ cells in the sample was determined by flow cytometry. The initial CD34+ cell number was 1.98%.

Mononuclear cells were sensitized with a type I anti-CD34 monoclonal antibody, 9C5 (0.5 μg/10^6 cells) (Baxter), for 30 min at 4°C under end-over-end rotation. Sensitized cells were washed three times with PBS/HSA and re-suspended in PBS/HSA containing 0.5% human immunoglobulin (PBS/HSA/Ig). Paramagnetic microspheres coated with sheep anti-mouse IgG_1 (SAM beads, Dynal) were washed three times in PBS/HSA and suspended in PBS/HSA/Ig. Sensitized mononuclear cells and washed SAM beads were mixed at a ratio of 0.5 beads/cell and incubated for 30 min at 4°C by slow end-over-end rotation to allow rosette formation. Beads and attached cells were collected by the help of a magnet (Isolex 50, Baxter) and washed 3 times with PBS/HSA. Unbound cells were collected by gravity flow through the outlet tubing. Rosette-forming CD34 positive cells were released from the beads by incubation with chymopapain (200 pKat/ml) (Baxter) for 15 min at room temperature. CD34+ cells and unbound cells were concentrated by centrifugation, counted, and immunophenotyped. The final CD34+ cell ratio was 56.2% with 98% viability.

Short term culture [38]. Mononuclear cells from bone marrow samples, non-adherent cells and adherent cells collected from long-term cultures were cultured in methylcellulose media. For this purpose 5×10^4 cell/ml was plated to 0.8% methylcellulose in IMDM medium (Sigma Chemicals, UK) supplemented with 30% fetal calf serum (FCS; Gibco, UK), 10% phythohaemagglutinin, leukocyte-conditioned medium, antibiotics, 2 mM L-glutamine, 25 mM HEPES, 5×10^{-5} M 2-mercaptoethanol (Sigma, UK), 4 IU recombinant erythropoietin (Eprex, Cilag), 1% deionized bovine serum albumin (Sigma, UK), and 1×10^{-6} M methylprednisolone (Sigma, UK). Cultures were incubated for 14–18 days in a humidified atmosphere at 37°C containing 5% CO_2. They were then assessed for the presence of colony forming unit granulocyte macrophage (CFU-GM), burst-forming unit–erythrocyte (BFU-E), and colony-forming unit granulocyte–erythrocyte macrophage-megakaryocyte (CFU-GEMM).

Long term bone marrow culture (LTBMC) [38]. Bone marrow stromal layers were established by bone marrow mononuclear cells obtained from CML patients and normal controls. For this purpose, $(8–10) \times 10^6$ bone marrow mononuclear cells were cultured in a T-25 flask containing IMDM medium supplemented with 10% FCS, 10% horse serum (Gibco, UK), 2 mM L-glutamine, penicillin (1,000 U/ml), streptomycin (100 U/ml; Sigma, UK), and 1×10^{-6} M hydrocortisone (Sigma, UK).

Flasks were first incubated at 37°C for 3 days and then transferred to 33°C in a humidified 5% CO_2 atmosphere and cultured for 4 weeks. Every week cultures were demipopulated and non-adherent cells were aspirated and suspended in medium. Non-adherent cells were then counted, immunophenotyped, and plated to methylcellulose medium for progenitor cell assay.

The confluency of the stromal layers was checked under inverted microscope and assessed by a semiquantitative scoring system: score 0, no adherent cell; score 1, few adherent cells which did not form foci; score 2, presence of less than 10 foci; score 3, 10 foci or more without connection (sub-confluent layer); score 4, continuous network of adherent cells covering entire bottom of the flask (confluent layer) [39].

At the end of 4 weeks of culture, adherent cells of the established stromal layers were harvested by 0.25% trypsin (Sigma Chemicals, UK) and subcultured in 24-well plates (30,000 stromal cells/cm^2). Half of the wells from each stromal layer was fed by LTBMC medium containing IFN-α at a concentration of 50 U/ml, and the other half was fed with only LTBMC medium. After 1 week, wells were washed with LTBMC medium for three times and then irradiated at 1.5 Gy by ^{137}Cs γ blood cell irradiator (IRL, France) to eliminate hematopoietic progenitors.

Stroma contact cultures. Purified normal CD34+ cells (2.5×10^4) obtained from a healthy donor was added onto irradiated stromal layers obtained from patients with CML and normal controls and subcultured. Only the stromal layers established from five CML patients who were not receiving IFN-α were used for this group of assay. Cobblestone areas were counted under a phase-contrast inverted microscope after 4 weeks. Cell groups consisting of 15 or more cells were quantitated as one cobblestone area [38].

Statistical Analysis

Results were reported as the mean ± 1 standard error of the mean (SEM). The Mann–Whitney *U* test was used to compare differences between cell counts and colony numbers of two groups. The significance of time-dependent changes of non-adherent cell counts and colony numbers within groups were determined by non-parametric Friedman's statistics. Hotelling's *T*2 test was used to compare the time-dependent changes between groups [40].

RESULTS

Progenitor Cell Numbers in the Bone Marrow

Bone marrow samples obtained from patients with CML had higher CD34 positive cell content compared with the healthy donors (3.64% ± 1.9% vs 1.77% ± 0.43), but the difference between two groups was not statistically significant ($P > 0.05$) (Table II).

TABLE II. Comparison of the Initial Cell Populations

	CML group[a]	Control group[a]
CD34+ cell ratio	3.64% ± 1.9%	1.77% ± 0.43%
CFU-GM number per 5×10^4 MNC	177.8 ± 32.9	135.3 ± 11.8
BFU-E number per 5×10^4 MNC	106.7 ± 17.9	67.7 ± 15.1
CFU-GEMM number per 5×10^4 MNC[b]	2 ± 0.38	7.4 ± 1.92

[a]Mean ± SEM.
[b]$P < 0.01$.

Initial bone marrow progenitor cell counts (mean number for per 5×10^4 cultured mononuclear cells) were also slightly higher in CML patients compared with those from normal donors (CFU-GM, 177.8 ± 32.9 vs 135.3 ± 11.6; BFU-E, 106.7 ± 17.9 vs 67.7 ± 15.1) but again differences were not statistically significant ($P > 0.05$). However, the number of CFU-GEMM appeared to be significantly low in CML patients (2 ± 0.38 vs 7.4 ± 1.92) ($P < 0.001$) (Table II).

CML patients were also compared according to their treatment modalities (IFN-α and hydroxyurea) but no significant difference in terms of CD34 positive cell number and CFU-GM content could be found ($P > 0.05$ for both).

CFU-GM Production in LTBMC

At the end of first week of long-term culture, total CFU-GM count of the non-adherent cells per flask was $(6.09 \pm 8.6) \times 10^4$ in the control group, and this was significantly higher than the CML group ($(2.8 \pm 0.7) \times 10^4$) ($P < 0.01$). CFU-GM production for per 5×10^4 cultured mononuclear cells found to be increased 1.7 times in the control group, whereas it was slightly lower compared to the initial number in the CML group ($P < 0.01$) when these figures were presented as a percentage of the initial CFU-GM counts (Fig. 1). In contrast to the burst observed at the first week, the CFU-GM content decreased considerably in the control group during the following weeks. This decline was comparatively slower in the CML group (Fig. 1), and at the end of third and fourth weeks, CFU-GM numbers for per 5×10^4 non-adherent cells in CML group were approximately two times higher than that of the CFU-GM number of the control group ($P < 0.01$ and $P < 0.001$, respectively).

Adherent Cell Population in LTBMC

Confluent stromal layer formation capacity was found to be decreased in CML group and at the end of 4 weeks total confluency was observed in only 41.7% of the CML patients, in comparison with 92.3% of normal donors. As seen in Fig. 2, semiquantitative analysis of stromal layer formation showed lower scores throughout 4 weeks culture in the CML group ($P < 0.05$) (Fig. 2).

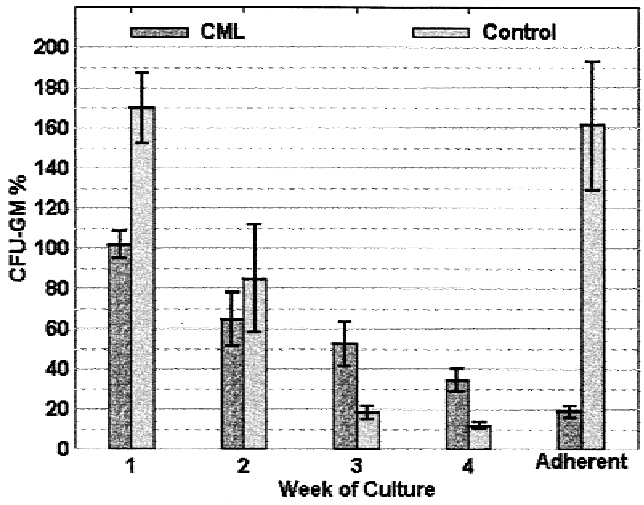

Fig. 1. Change of CFU-GM content in controls and CML patients during the long-term culture. The solid bars represent CFU-GM content in CML patients, and the gray bars represent CFU-GM content in controls. CFU-GM content per 5×10^4 cells has been expressed as a percent of the initial CFU-GM content of the bone marrow samples. Values shown are the means ± SEM of controls and CML patients, assessed individually for non-adherent cell populations for each week of culture and for the adherent cell population.

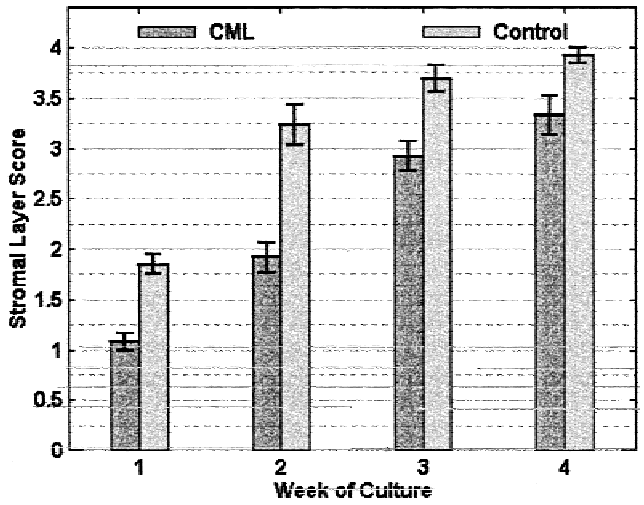

Fig. 2. Stromal layer confluency scores of the controls and CML patients during the long-term culture. The solid bars represent scores of CML patients, and the gray bars represent the controls. The bars express the means ± SEM of the controls and CML patients, assessed individually for each week.

At the end of 4 weeks, stromal layers established from patients with CML and normal controls were also morphologically different. Control stromal layers consisted of a confluent adherent layer containing reticular fibroblasts, macrophages, fat cells, and numerous cobblestone areas, whereas CML stroma was apparently less cellular, irregular, and less confluent and had cobblestone areas with lower cell numbers (Figs. 3 and 4). Giant cells were

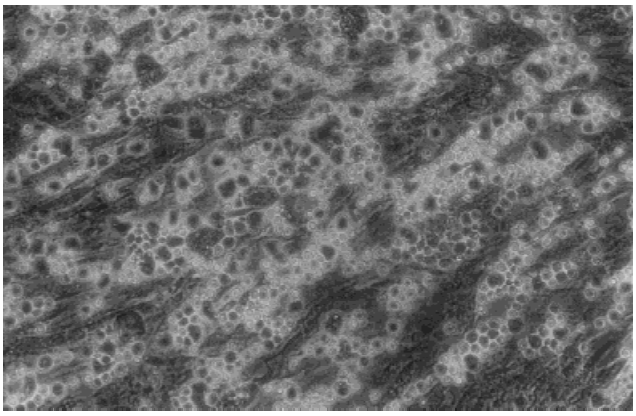

Fig. 3. Confluent stromal layer established from bone marrow sample of a control in long-term bone marrow culture at the end of 4 weeks (phase contrast, ×20). Cobblestone areas of hematopoietic cells, stromal macrophages, and vacuolated fat cells are readily apparent.

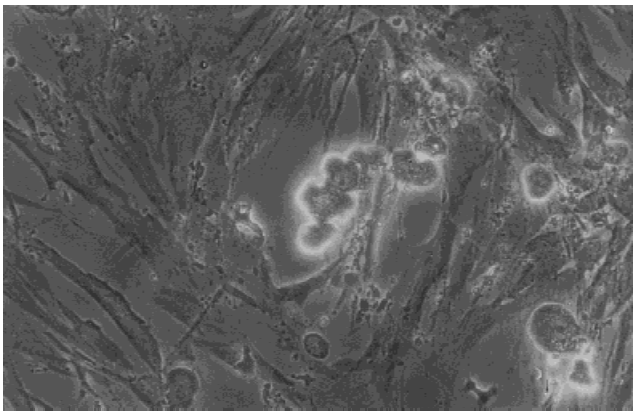

Fig. 4. Stromal layer established from bone marrow sample of a CML patient in long-term bone marrow culture at the end of four weeks of culture (phase contrast, ×20). An apparently less cellular, irregular adherent layer with prominent stromal macrophages.

easily distinguishable in the CML stroma cultures, under inverted microscope. They resembled pseudo-Gaucher's cells, and May-Grünwald Giemsa-stained cytospin preparation confirmed this observation (Figs. 5 and 6).

Total adherent cell count in per flask in CML group ($(1.66 \pm 0.32) \times 10^6$) was significantly lower in the CML group compared with that of the control group ($(3.38 \pm 0.47) \times 10^6$) ($P < 0.001$) (Table III). Total CD34+ cell number in the adherent cell population was also lower in the CML group (2.33% ± 1.05%) compared with the normal controls (10.6% ± 2.8%). CD34+ cells in the adherent cell population were well preserved in the control group (final count being 113% of the initial CD34+ cell number), whereas the final CD34+ cell number was only 13.6% of the initial CD34 positive cells in the CML group ($P < 0.05$).

Evaluation of progenitor cell contents of adherent cells also revealed that CML stromal layers had significantly

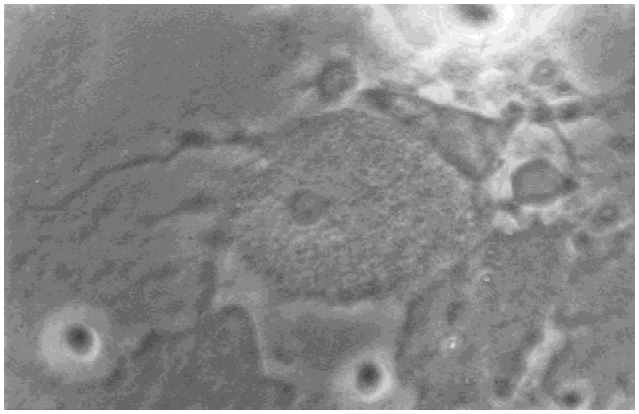

Fig. 5. Pseudo-Gaucher's cell in the stromal layer of a CML patient under phase contrast microscope (×40).

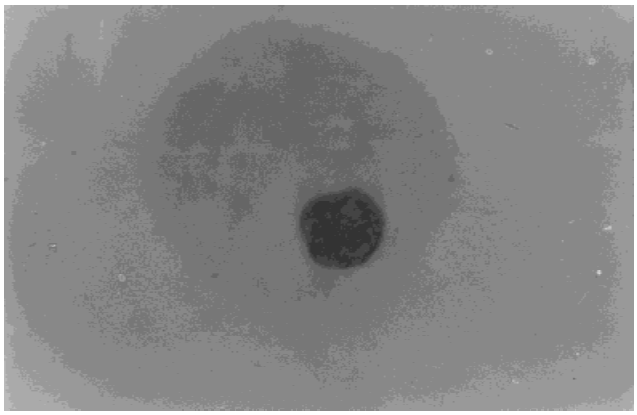

Fig. 6. May-Grünwald Giemsa-stained cytospin preparation of a trypsinized stromal layer of a CML patient. Pseudo-Gaucher's cell under light microscope (×100).

lower capacity to generate myeloid colonies for per 5×10^4 cells (28.4 ± 4.2 vs 207.3 ± 29.9) ($P < 0.0001$) (Table III). In control group, total CFU-GM content of the adherent cell population was 98% ± 24.2% of the initial CFU-GM number, but CML stromal layers could preserve only 3.03% ± 0.62% of the initial CFU-GM content ($P < 0.0001$) (Table III).

The patients who were receiving IFN-α at the time of the study had higher adherent CFU-GM count ($(1.6 \pm 0.5) \times 10^3$) than the remaining CML patients' ($(0.63 \pm 0.13) \times 10^3$) ($P < 0.03$), but this value was still significantly lower than the control group ($P < 0.05$).

Stroma-Contact Cultures and the Effect of IFN-α

To assess the supportive capacity of the stromal layers obtained from patients with CML and normal controls, CD34+ cells which were obtained from a normal donor were plated onto CML and normal stromas and cocultured for 4 weeks (stroma contact culture), and cobble-stone areas were assessed. Half of the established stromal layers were incubated with IFN-α for 1 week, followed by stroma contact cultures, and cobble-stone areas were determined after 4 weeks.

The number of cobble-stone areas induced by CML stroma (51.7 ± 3.4) was significantly lower than that induced by the normal stroma (94.6 ± 3.2). Treatment of stroma by IFN-α caused reduction of cobble-stone area formation in both CML (29.4 ± 2.4) and control (68 ± 7.34) groups. However, the reduction in CML group (43.6% ± 4.1%) was more pronounced than the reduction observed in control group (26.7% ± 6.1%) ($P < 0.03$).

DISCUSSION

Interactions between hematopoietic stem cells and the bone marrow microenvironment have an important role in the regulation of hematopoiesis. Stroma has dual function of both supporting continued hematopoiesis and of maintaining hematopoietic stem cell in a quiescent state until appropriately stimulated [12]. Direct adhesive interactions between primitive stem cells (long-term culture-initiating cells, LTC-IC) and stromal components have been proposed to be one of the important control mechanisms of the hematopoietic stem cell proliferation [12,41–43].

Malignant CML clone has intrinsic defects, which may lead to abnormal interactions with the bone marrow microenvironment. Eaves et al. reported defective adherence of CML progenitors to marrow stroma, and this was later confirmed by others [10,11,13–15]. Bone marrow mononuclear cells obtained from CML patients on the stromal layer have been reported to cause depletion of Philadelphia-chromosome positive clone and predominance of normal hematopoiesis [17–19,44]. Reduced expression of the surface adhesion molecules such as lymphocyte function-associated antigen (LFA-3) and abnormal β-integrin receptor function were also defined in CML progenitors [14,15].

Altered stromal composition and function have also been reported in addition to abnormal progenitors in CML [20,21]. Growth of normal hematopoietic progenitors on stroma obtained from CML patients was found to be significantly reduced in comparison with normal stroma, and this was proposed to be related with bcr-abl positive stromal macrophages [21]. Adherent layer formation in in-vitro long-term cultures established from CML patients has also been reported to be defective [20,38].

Although a number of abnormalities were defined in CML, the extent of these abnormalities in different progenitor compartments is still debatable. In newly diagnosed or even treated chronic-phase patients, the presence of normal progenitor cells was reported [17–19,44]. It is generally accepted that colony-forming progenitors are high in the peripheral blood circulation but not in the bone marrow of the CML patients [5–9].

TABLE III. Comparison of Some Features of the Adherent Cell Populations After Four Weeks of Culture

	CML group[a]	Control group[a]
Total adherent cell number[b]	$(1.66 \pm 0.32) \times 10^6$	$(3.38 \pm 0.47) \times 10^6$
CD34+ cell ratio[b]	2.33% ± 1.05%	10.65% ± 2.85%
Total CD34+ cell number[b]	$(4.8 \pm 2.07) \times 10^4$	$(35.3 \pm 9.04) \times 10^4$
CFU-GM number per 5×10^4 MNC[b]	28.4 ± 4.25	207.3 ± 29.93
Total CFU-GM content[b]	1017 ± 258	20662 ± 3626
Preserved clonogenic capacity[b]	3.03% ± 0.62%	98.2% ± 31.4%

[a]Mean ± SEM.
[b]$P < 0.05$.

No significant difference for CD34+ cell and erythroid progenitor contents of the bone marrow samples could be found between patients and normal controls in this study. Although CFU-GM counts of both groups were similar, the CFU-GEMM count of the CML group was found to be significantly reduced. Similar findings were reported previously by some authors, but the cause and the importance of this finding could not be explained [9]. Although CFU-GEMM frequency was low in CML, this could be compensated by significantly increased cellularity and absolute number of CFU-GEMM may still be in normal limits in these patients.

Stromal layers established from CML patients presented a lower confluency rate, and at the end of four weeks culture only 41.3% of the patients had confluent stroma (Fig. 3). Poor adherent layer formation or failure to establish long-term hematopoiesis have been previously reported, especially in patients under treatment. Dilution of mesenchymal precursors by the significantly increased neoplastic clone, abnormalities in stromal cell function, and variable effects of treatment on normal and malignant hematopoiesis have been considered as underlying causes of this observation [20,38].

Interestingly, we have observed a burst in CFU-GM after 1 week of culture in the control group. This may be attributable to the lack of negative control of bone marrow stroma on normal hematopoietic progenitors in in vitro conditions, since stromal layers did not reach confluency during the first or in some cases second weeks of culture. However, there was no change in CFU-GM number at first week compared with the initial counts in CML group. This may be explained by CML progenitors escaping from stromal inhibition. Formation of stromal layers in the following weeks might have inhibited proliferation of the progenitors in both groups, but CFU-GM number in the CML samples decreased more slowly, which again may suggest a defect in stromal control.

This study showed a decreased number of total stromal cells in CML adherent layers, and a relative increase in stromal macrophages laden with cellular debris, resembling pseudo-Gaucher cells was observed. CML stromal layers also had a low CD34+ cell number and colony forming progenitor cell contents. CML stromal layers were found to have only 3.03% of the initial CFU-GM content, which was significantly lower than the normal controls (98.2%). It has been previously reported that only 4–8% of the total progenitors accounts Ph′-negative clone in most cases of CML [17–19]. Although the number of Ph′-negative progenitors was not determined, the very low number of CFU-GM detected in our patients (3.03%) may be regarded as a sign of significantly suppressed normal hematopoietic progenitors in CML patients.

IFN-α has proven to be beneficial in the treatment of CML patients and a number of different mechanisms have been proposed to explain its effects. IFN-α was found to have a more prominent anti-proliferative effect on the Ph′-positive clone than on normal hematopoiesis [13,14,18,23–31], and also it induced MHC class II antigen expression on malignant clone. The latter may subsequently cause increased cytotoxicity against the malignant clone [18]. IFN-α was also shown to improve interactions between hematopoietic progenitors and bone marrow derived stromal cells by normalization of adhesive features. It has been shown that IFN-α overrides the defective adhesion by changing the neuroaminic acid composition in the stroma and by restoring impaired β1 integrin receptor function [14,23]. IFN-α treatment could also restore the deficient expression of LFA-3 which is thought to be a critical molecule in the adhesion between CML progenitors and stroma [13]. These findings indicates an improved stromal control on malignant clone by IFN-α treatment. In this study IFN-α treatment also seemed to be effective for some improvement in the quality of the adherent layers. Stromal layers established from IFN-α receiving patients had more CFU-GM and CD34 cell content than the patients who were not receiving IFN-α ($P < 0.05$). The adhesion of progenitor cells to stromal layers, however, was still poor in this group in comparison with the normal control group ($P = 0.004$).

We have observed significantly less cobble-stone area formation on CML stroma (51.7 ± 3.4) than that induced by normal stroma (94.6 ± 3.2). Prior treatment of stromal layers with IFN-α for 1 week caused decreased hematopoietic reconstitution on both CML and normal stromal layers. However, cobble-stone area formation by CML

stromal layers was found to be more severely affected by the IFN-α treatment (43.6% decrease).

Although our patient group was small and heterogeneous, our findings have demonstrated that both CML progenitors and stroma have a number of abnormalities compared with the normal controls. The adverse effects of IFN-α treatment on the bone marrow microenvironment may have implications with respect to unfavorable bone marrow transplant outcome (34).

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