

Interferon-alpha-Treated Patients with Chronic Myelogenous Leukemia Show *BCR/ABL*-Positive Peripheral Blood Progenitor Cells Surviving Long-Term Culture

(chronic myelogenous leukemia / peripheral blood progenitor cells / *BCR/ABL* status / long-term culture / fluorescence *in situ* hybridization)

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Abstract. Several groups have shown that Ph-progenitors reappear in LTC of CML bone marrow or PBMNC when the cell preparations were derived from newly diagnosed Ph-positive patients or after induction chemotherapy.

We have tested the hypothesis whether LTC may further decrease CML progenitors if the cells to be cultured were from IFN-treated patients. In our experiments, PBMNC were cultured from 7 IFN- and 5 HU-treated patients in stable chronic phase of the disease, and from 9 patients at diagnosis. Progenitor cells in PBMNC were quantitatively analyzed before and after 35 days of LTC by combining the clonogenic assay in semisolid medium with dual-color interphase FISH for identification of the *BCR/ABL* status of colony-forming progenitor cells. A median of 22 colonies (range 7–88) before and 30 colonies (5–71) after LTC were analyzed per patient. Our results show that the number of *BCR/ABL*-positive CFC before and after LTC was approximately the same. This was independent of IFN or HU therapy. In the IFN group there were 58% (median) *BCR/ABL*-positive CFC before and 54% (median) after LTC of PBMNC. In the HU group, 80% of CFC were *BCR/ABL*-positive before and 85% after LTC. A complete elimination of *BCR/ABL*-positive cells was not achieved. We conclude that CML early progenitors in PBMNC of IFN-treated CML patients may survive LTC.

During recent years, autologous stem cell transplantation has become a promising approach for treatment of chronic myelogenous leukemia (CML) (Brito-Babapulle et al., 1989; Dexter, 1989; Daley and Goldman, 1993; Barnett et al., 1994; Carella, 1995). Autografts have been

performed when compatible allogeneic bone marrow (BM) cells were not available. When harvested at the beginning of the disease, autografts may reproduce the initial stage when transplanted during progression. The quality of the grafts certainly depends on the proportion of normal stem cells, which may be still high initially and during an effective treatment regimen.

Improvement of autograft quality is expected to result from effective purging techniques allowing proliferation of isolated normal stem cells in the absence of leukemia cells. As a precondition, a cell preparation is required that contains a high number of normal hematopoietic stem cells to be isolated from leukemic BM or blood. In CML, this situation is given in those patients responding to interferon (IFN) treatment. Philadelphia positive (Ph⁺) cells may apparently disappear after a period of 3–4 weeks on stromal cells *in vitro*, and normal hematopoietic cells begin to dominate (Coulombel et al., 1983; Udomsaki et al., 1992, Verfaillie et al., 1992a, Verfaillie et al., 1992b). However, long-term culture (LTC) as an effective purging technique for autologous grafts is still in an experimental stage, not only because of conflicting results concerning long-term persistence *in vitro* of CML stem cells.

Since reliable methods are required to measure quantitatively the proportion of normal stem cells after purging, an exact estimation of CML and normal colony-forming cells (CFC) has become possible by a combination of the clonogenic assay with fluorescence *in situ* hybridization (FISH). In CML, FISH has first been introduced by Tkachuk et al. (1990) for single-cell analysis. A number of studies have been done since to identify *BCR/ABL*-positive cells by FISH (Arnoldus et al., 1990; Benty et al., 1994). The combined techniques of colony assay and FISH, which allow the quantification of normal and leukemic CFC, have the advantage that the nonspecific background is negligible. All or none of the cells are positive or negative, because cells of a colony are the descendants of a single progenitor cell. Long-term survival of *BCR/ABL*-positive cells *in vitro* up to more than 100 days has already been found in our previous studies (Pasternak and Pasternak, 1994). However, reverse-

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Abbreviations: BM – bone marrow, CFC – colony-forming cells, CFU-GM – colony-forming unit granulocyte-macrophage(s), CML – chronic myelogenous leukemia, FISH – fluorescence *in situ* hybridization, HU – hydroxyurea, IFN – interferon, LTC – long-term cultures, PBMNC – peripheral blood mononuclear cells, PBS – phosphate-buffered saline, Ph⁺, Ph⁻ – Philadelphia positive, negative, RT-PCR – reverse-transcriptase polymerase chain reaction.

transcriptase polymerase chain reaction (RT-PCR) analyses of the whole tissue-culture samples used at this time did not allow identification of the cell type involved. Not until the application of fluorescence *in situ* hybridization for hematopoietic colonies it has become possible to quantitatively and qualitatively measure CML progenitors.

In our experiments, we have tested the hypothesis whether IFN treatment supporting re-emergence of Philadelphia negative (Ph⁻) hematopoietic progenitors in peripheral blood of chronic-phase CML patients is producing a condition that may further suppress CML and favor normal stem cells by *in vitro* LTC.

Material and Methods

Isolation of peripheral blood mononuclear cells (PBMNC)

PBMNC from CML were isolated following standard protocols (Boyum, 1976). Briefly, about 5 to 10 ml of heparinized venous blood underwent gradient centrifugation on Histopaque (Sigma, Deisenhofen, Germany). After washing in phosphate-buffered saline (PBS), the cells were resuspended in IMDM + 2% fetal calf serum (FCS) (Eurobio, Les Ulis, France). Viable cells were estimated by the trypan blue dye-exclusion test.

Patients

For FISH experiments and colony assays, blood samples were used from 18 CML patients in chronic phase of their disease, nine out of them at initial diagnosis before any cytoreductive treatment, seven out of them treated with interferon, five out of them treated with hydroxyurea (HU). One patient could be followed at initial diagnosis and under therapy with IFN, another one at initial diagnosis and under therapy with first HU and then IFN. Heparinized venous blood samples were obtained after informed oral consent of the patients. Additional samples from healthy volunteers served as controls.

Clonogenic assay

A routine clonogenic assay in semisolid medium (MethoCult H4431, Stem Cell Technologies Inc., Vancouver, Canada) was performed as described (Coulombel et al., 1983; Hörner et al., 1997). Between 0.15×10^4 to 30×10^4 cells were seeded in triplicates in 35 mm petri dishes (Falcon, Becton Dickinson, Heidelberg, Germany). After 10–12 days in a CO₂ incubator (Heraeus, Hanau, Germany), colonies consisting of at least 50 cells were counted and the theoretical number of colonies was calculated per 10^6 PBMNC input. Colonies, which were classified according to their erythroid, monocyte-macrophage, and mixed morphology, were listed as a whole. They were plucked with glass capillaries, smeared onto lysin-coated slides, Wright-Giemsa stained and described morphologically. The majority of colonies (about

80%) were colony-forming unit granulocyte-macrophages (CFU-GM).

Stromal layer

Fresh allogeneic BM cells isolated by Histopaque gradient centrifugation as described above were cultured in MethoCult H5100 medium (Stem Cell Technologies Inc., Vancouver, Canada) at a concentration of 10^6 cells per ml in T-75 and T-25 tissue culture flasks (Falcon, Becton Dickinson, Heidelberg, Germany). After 3–4 weeks, when a confluent stromal layer was found, cells were trypsinized, washed and irradiated with 16 Gy using a ¹³⁷Cs source. Irradiated cells were seeded into the wells of 24-well plates (Falcon, Becton Dickinson, Heidelberg, Germany). Each well contained 7.5×10^4 cells which were adherent after 24 h at 37°C. Adherent cells were used as feeders in LTC.

LTC of PBMNC

Generally, 10^6 PBMNC per well were seeded into 24-well plates containing irradiated feeder cells as described. Cultures were fed weekly by replacing half of the medium. Almost 50% of nonadherent cells were removed each time by this procedure. After 35 days of culture, the adherent cells underwent the clonogenic assay.

Fluorescence in situ hybridization

Double-color *in situ* hybridization was performed following standard protocols with slight modifications (Lichter and Cremer, 1992). Briefly, Wright-Giemsa-stained cells on the slides were destained in methanol for 10 min, digested for 13 min in 0.01 N HCl containing 3–5 mg/100 ml pepsin (Serva, Heidelberg, Germany), washed twice in PBS, and fixed with 1% paraformaldehyde (Sigma, Deisenhofen, Germany) and 5 mM MgCl₂ (Merck, Darmstadt, Germany) in PBS. After dehydration in 70%, 90% and 100% ethanol for 10 min each, slides were denatured in 50% formamide (Merck) in 2× SSC for 2 min at 72°C. After incubation in 100 mM ice-cold CaCl₂ and a second dehydration step, hybridization was performed for 12 to 16 hours. In a first set of experiments, *BCR*- and *ABL*-DNA probes were isolated from the YAC clone D107F9 and the cosmid clone cos-abl-8, respectively (kindly provided by Dr. Th. Cremer, Heidelberg, Germany, and Dr. N. Heisterkamp, Los Angeles, USA). Labeling by *in situ* nick translation with either biotin-16-dUTP (cos-abl-8) (Boehringer Mannheim, Mannheim, Germany) as well as hybridization and detection of the DNA probes were performed as described previously (Lichter and Cremer, 1992; Bentz et al., 1995).

Secondly, a set of commercially available DNA probes (LSITM *BCR/ABL*, Vysis, Stuttgart, Germany) was used; washing steps were performed following the instructions of the manufacturer. Slides were counterstained in Vectashield anti-fading medium (Vector,