BCR/ABL-Negative Clonogenic Hematopoietic Cells Do Not Accumulate in the Plastic-Adherent Fraction of Peripheral Blood Mononuclear Cells from Patients with Chronic Myeloid Leukemia in Stable Chronic Phase

(chronic myeloid leukemia / *BCR/ABL* sequence / fluorescence *in situ* hybridization / colony-forming cell / plastic-adherent fraction)

B. SCHULTHEIS, G. PASTERNAK, R. HEHLMANN

III. Medizinische Klinik, Fakultät für Klinische Medizin der Universität Heidelberg, Mannheim, Germany

Abstract. PBMNC from patients with CML and healthy control persons were separated into plastic-adherent and nonadherent cell fractions. A colony assay in semi-solid medium was used to estimate the number and lineage commitment of CFC in each of the fractions. The CML blood-derived colonies were isolated and analyzed by FISH for *BCR/ABL* sequences. Thus, we were able to test the hypothesis whether a selective enrichment is possible of normal progenitor cells in the blood of CML patients in stable chronic phase after HU and/or IFN. Although the number of leukocytes differed considerably between patients at diagnosis and in stable chronic phase, the proportion of adherent and nonadherent cells was about the same in all preparations tested. There were also only minor differences of adherence between MNC of CML and normal origin. Furthermore, *BCR/ABL*-positive and negative colonies were equally distributed among unseparated, adherent, and nonadherent PBMNC fractions. In conclusion, an accumulation of *BCR/ABL*-negative CFC was not found in any of the PBMNC fractions. CFC from PBMNC of the same lineage commitment were simultaneously present in plastic-adherent and nonadherent cell fractions, indicating that their surface charges might be different and, on the other hand, that different lineage commitment precursors can be present in either of the fractions irrespective of CML or blood origin.

Several approaches have been described of chronic myeloid leukemia (CML), bone marrow (BM) or peripheral blood mononuclear cells (PBMNC) purging *in vitro* to enrich normal hematopoietic progenitors for autologous transplantation (Dunbar and Stewart, 1992; Daley and Goldman, 1993; McGlave et al., 1993; Carella et al., 1995; Reiffers et al., 1996). Autologous transplantation is first of all carried out when compatible allogeneic grafts are not available, and particularly in risk groups and in blast crises, to reproduce the initial stage of the disease. Most promising results were obtained even with unselected cell preparations. Although an increased frequency of cytogenetic remissions could be observed after transplantation, recurrence of the disease is only temporarily delayed (Haines et al., 1984; Reiffers et al., 1991). Cytogenetic remissions in CML patients can be observed after effective interferon (IFN) and/or chemotherapy when nonclonal normal, hematopoietic cells reappear in BM and peripheral blood. Improvement of autografting is expected if efficient separation techniques *in vitro* can be elaborated allowing the enrichment of normal or elimination of CML progenitor cells. There are a number of experimental efforts aiming at the autograft improvement. *Ex vivo* marrow culture may cause a selective decrease or loss of Philadelphia positive (Ph^+) progenitors, giving rise to predominantly nonclonal normal progenitors (Coulombel et al., 1983; Dubé et al. 1984; Hogge et al., 1987; Udomsakdi et al., 1992; Verfaillie et al., 1992; Agarwal et al., 1995). In our hands CML progenitor cells from peripheral blood survived long-term culture even when the blood donor had circulating *BCR/ABL*-negative colony-forming cells (CFC) (Heissig et al., 1997). Other techniques *in vitro* favoring selection of normal progenitor cells include isolation procedures by antibodies to phenotypic markers (Verfaillie et al., 1992), CML marrow purging with cyclophosphamide derivatives (Carlo-Stella et al., 1991) or IFN-γ (McGlave et al., 1987; McGlave et al., 1990), use of *BCR/ABL* antisense oligonucleotides (Szczylik et al., 1991; De Fabritiis et al., 1998), or cytotoxic T and NK cells, which may possibly eliminate CML cells from the autograft.

Obviously, certain CML progenitor cells differ from normal hematopoietic cells in terms of their adhesive properties (Gordon et al., 1987; Verfaillie et al., 1992). According to Grand et al. (1997), *BCR/ABL*-negative cells are enriched in the fraction of $CD34^+$ cells that

Received July 14, 2000.

Corresponding author: Beate Schultheis, III. Medizinische Klinik Mannheim, Universität Heidelberg, Wiesbadener Str. 7-11, 68305 Mannheim, Germany.

Abbreviations: BFU-E – burst-forming unit erythroid, BM – bone marrow, CFC – colony-forming cells, CFU-E – colony-forming unit erythroid, CML – chronic myeloid leukemia, DAPI – 4,6-diamino-2-phenylindole-dihydrochloride, GEMM – granulocyte-erythroid/macrophage-monocyte, GM – granulocyte-macrophage, FISH – fluorescence *in situ* hybridization, HU – hydroxyurea, IFN – interferon, LTC-IC – long-term culture initiating cells, MNC – mononuclear cells, PBS – phosphate-buffered saline, Ph status – Philadelphia status, PBMNC – peripheral blood mononuclear cells.

adhere to tissue culture plastic. The conclusion was that adhesion to plastic is a property of very primitive normal hematopoietic cells. We have tested normal and CML PBMNC for colony-forming progenitor cells in their plastic-adherent and nonadherent fractions. An approximately similar distribution expressed as a percentage was found of adherent and nonadherent cells in normal and CML blood. As demonstrated by fluorescence *in situ* hybridization (FISH)-plucked colonies for analysis, an accumulation was not found of *BCR/ABL*-negative CFC in the adherent fraction of PBMNC from CML patients in stable chronic phase.

Material and Methods

Patients and control persons

Eighteen patients with CML were included in the study, 15 of them being in stable chronic phase of their disease under treatment with HU and/or IFN, five out of them being examined at initial diagnosis, and three of them suffering from advanced disease, defined by a lack of hematologic response to therapy. Blood samples and BM from 18 healthy volunteers were used for comparative purposes. All donors gave their informed consent according to the Declaration of Helsinki.

Cell fractions

PBMNC were isolated from heparinized blood by gradient centrifugation on histopaque (Sigma Chemical Co., Irvine, UK) after 1 : 1 dilution in PBS. After washing and centrifugation in IMDM (Gibco BRL, Paisley, UK), the mononuclear cells (MNC) were suspended in the same medium, trypan blue was added, and the cells were counted using a Neubauer chamber. For separation into plastic-adherent and nonadherent fractions, the cell concentration was adjusted to 5–6 $\times 10^6$ viable cells in 6–8 ml per T-25 tissue culture flask (Greiner, Köln, Germany). One to three T-25 flasks per experiment were incubated for 90 min at 37°C in a CO_2 incubator. Adherent and nonadherent fractions were prepared using largely constant mechanical conditions. The flasks were slowly placed in an upright position and the medium on the bottom containing the nonadherent cells was carefully removed by a transfer pipette. Adherent cells were detached by a cell scraper (Falcon 3085, Becton Dickinson, Lincoln Park, NJ) after the cells that were not tightly fixed had been washed off twice with 5 ml each of medium. Cells in the washing medium were discarded. Thus, cell loss was relatively high, more than 50% of input numbers.

Colony assay

A routine colony 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 CO_2 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-macrophage (CFU-GM).

FISH

Heparinized BM samples or peripheral blood samples were subjected to Ficoll gradient centrifugation (density 1.077 g/ml, Pharmacia) (Boyum, 1976). Buffy coats were washed once with phosphate-buffered saline (PBS). Part of the specimen was used for morphological analysis by Wright-Giemsa staining; the remainder of the cell suspension was brought onto slides, fixed in methanol/acetic acid 3 : 1 and stored at −70°C. Dual-color fluorescence *in situ* hybridization was performed following standard protocols with slight modifications (Lichter and Cremer, 1992). Briefly, slides were destained in methanol for 10 min, digested 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 $MgCl_2$ (Merck, Darmstadt, Germany) in PBS. After dehydration in 70%, 90% and 100% ethanol for 10 min each, slides were denatured in 70% formamide (Merck) in 2 x SSC for 2 min at 72°C. After incubation in 100 mM ice-cold $CaCl_2$ and a second dehydration step, hybridization was performed for 12 to 16 hours. Commercially available DNA probes for *BCR/ABL* (Vysis, Stuttgart, Germany) were used according to the instructions of the manufacturer. Slides were counterstained in Vectashield anti-fading medium containing 4,6-diamino-2-phenylindole-dihydrochloride [DAPI] (1 μg/ml). Evaluation was performed by analyzing at least 200 interphase nuclei per sample using fluorescence microscopy.

Statistical analysis

Samples were analyzed for statistical significance using Student's t-test according to standard procedures.

Results

Distribution of BCR/ABL-positive clonogenic cells in plastic-adherent and nonadherent PBMNC fractions from CML patients in stable chronic phase

For comparison, the proportion of adherent and nonadherent cell fractions was measured in blood from healthy control persons and CML patients in stable chronic phase. Although the number of MNC isolated per ml CML blood was only 1/3 of that of the controls, the