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)

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Abstract. PBMNC from patients with CML and healthy control persons were separated into plastic-adherent and nonadherent cell fractions. A colony assay in semisolid 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 (Szczylwik et al., 1991; De Fabritiis et al., 1998), or cytotoxic T and NK cells, which may possibly eliminate CML cells from the autograft.

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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
PBMCNC for colony-forming progenitor cells in their
plastic-adherent and nonadherent fractions. An approxi-
mately 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 PBMCNC 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 com-
parative purposes. All donors gave their informed con-
sent according to the Declaration of Helsinki.

Cell fractions

PBMCNC 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 con-
centration was adjusted to 5–6 × 10^6 viable cells in 6–8
ml per T-25 tissue culture flask (Greiner, Köl n, Ger-
many). One to three T-25 flasks per experiment were
incubated for 90 min at 37°C in a CO₂ incubator. Adher-
ent 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,
much 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 CO₂ incubator (Heraeus, Hanau, Germany), colonies con-
sisting of at least 50 cells were counted and the theoretical
number of colonies was calculated per 10^6 PBMCNC 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-macro-
phage (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 sus-
pension 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 MgCl₂ (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 CaCl₂
and a second dehydration step, hybridization was per-
fomed 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-dihy-
drochloride [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
PBMCNC fractions from CML patients in stable
chronic phase

For comparison, the proportion of adherent and non-
adherent 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