Blast colony-forming cell binding from CML bone marrow, or blood, on stromal layers pretreated with G-CSF or SCF

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Abstract. Blast colony-forming cells (CFU-BL) represent a specific subpopulation of special primitive progenitors characterized by colony formation only in close contact with a preformed stromal layer. CFU-BL derived from bone marrow of chronic myeloid leukaemia (CML) patients have been proved to adhere poorly to bone marrow derived stromal layers suggesting that the appearance of progenitors and precursors in the circulation is due to a defective adhesion of these cells to the bone marrow micro-environment. In the present experiments the effect of short-term incubation of preformed normal bone marrow stroma on the adherence of CML derived CFU-BL was studied. For stroma cultures bone marrow cells were cultured in microplates in the presence of hydrocortisone. Cultures were used when stromal layers became confluent and no sign of haemopoiesis could be observed. CFU-BL were studied by panning plastic non-adherent mononuclear (PNAMNC) bone marrow or blood cells. 8.9 ± 2.4 colonies/ 10^3 PNAMNC (six experiments) were formed from normal bone marrow on stromal layers and 4.8 ± 2.1 colonies/ 10^3 PNAMNC (five experiments) from CML bone marrow. Colony formation from normal bone marrow was not increased if stromal layers were incubated with 100 ng/mL granulocyte colony-stimulating factor (G-CSF) or stem cell factor (SCF). Incubation of stroma with G-CSF or SCF, however, increased the colony formation of PNAMNC from CML bone marrow or blood significantly. These findings suggest that local concentration of haemopoietic growth factors at the time of panning may influence the attachment of CML progenitors to the stroma.

INTRODUCTION

Chronic myeloid leukaemia (CML) is a clonal malignant disease of the pluripotent stem cell, characterized by the rearrangement between the BCR gene (breakpoint cluster region) and the ABL gene resulting in a characteristic chromosome aberration, an abnormally small chromosome: Philadelphia (Ph) chromosome. Clinically, CML is characterized by a massive expansion of immature precursors, which leave the bone marrow prematurely resulting in a very high level of granulocytes and their precursors in the peripheral blood.

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In vitro studies from the 1980s suggested that CML progenitors, especially a primitive progenitor (blast colony-forming cells: CFU-BL, characterized by colony formation *only* in close contact with a pre-established haematopoiesis supporting stromal layer, Gordon *et al.* 1985) adhere poorly to bone marrow derived stromal layers (Gordon *et al.* 1987). Later Bhatia *et al.* (1994) proved that CML progenitors show reduced attachment to extracellular matrix elements, as well. These findings suggested that the appearance of progenitors and precursors in the circulation of CML patients is caused by a defective adhesion of these cells to the bone marrow micro-environment. Others proved that even more primitive haemopoietic cells (long-term culture initiating cells: LTC-IC) from CML bone marrow also adhere defectively to the stroma (Eaves *et al.* 1986; Udomsakdi *et al.* 1992; Verfaillie *et al.* 1997).

This reduced adherence of CML progenitors to stroma or fibronectin can be explained by the finding that although the number of expressed $\alpha_4\beta_1$, $\alpha_5\beta_1$ and CD-44 adhesion receptors on CD34⁺ HLA-DR⁺ progenitors of CML patients is normal, these receptors might be in a low affinity state (Verfaillie *et al.* 1997).

Adhesion may be critical for the behaviour of stem and progenitor cells (Wilson 1997). It has been proved in numerous experiments that cells in close contact with stroma are in a quiescent state (Hurley *et al.* 1995). Ph positive CML colony forming cells and LTC-IC, however, are not subject to normal growth inhibitory signals (e.g. macrophage inhibitory protein- α = MIP-1 α) (Eaves *et al.* 1993; Dürig *et al.* 1999). Although increased proliferation of bone marrow stem cells preceding mobilization was reported, increased proliferation *per se* does not induce mobilization (Brasel *et al.* 1997; Liu *et al.* 1997; Papayannopopou 2000).

In clinical practice, in chronic phase CML patients, cells for autologous transplantation are collected after granulocyte colony-stimulating factor (G-CSF) mobilization. We supposed that the binding of CFU-BL from CML patients to bone marrow stromal layers that have been pre-treated with various growth factors may give some insight into the attachment of these cells to the stroma and their mobilization into the circulation.

MATERIALS AND METHODS

Stromal layers

Stromal layers were cultured by the technique of Gordon *et al.* 1985, modified in our laboratory. Briefly, 1×10^4 mononuclear bone marrow cells were placed in a flat-bottom 96-well tissue culture plate (Greiner, Nürtingen, Germany). Tissue culture medium consisted of α -minimum essential medium (MEM, Gibco Invitrogen Life Technologies, Glasgow, UK) supplemented with 15% fetal calf serum (FCS, GIBCO). Cultures were grown in the presence of 10^{-6} M hydrocortisone hemisuccinate (Sigma-Aldrich, Milwaukee, WI, USA) and were maintained at 37 °C in 5% CO₂-air. Medium was changed completely at weekly intervals. The time for obtaining a confluent stromal layer was usually four weeks. Cultures were used when haemopoiesis (round cells on the surface) ceased to be observed.

Panning of bone marrow cells (CFU-BL assay)

Adherent cells were removed from mononuclear fractions of bone marrow or peripheral blood by incubation in plastic tissue culture flasks for 2 h. After having removed the supernatant containing plastic non-adherent mononuclear cells (PNAMNC) they were centrifuged, counted and added to the established stromal layer at a concentration of 1×10^3 /well (bone marrow) or 1×10^4 /well (peripheral blood). After 2 h of cocultivation, the supernatant was removed and the

stromal layer was carefully washed 3 times to remove non-attached cells. The stromal layer was then covered with 100 μ L of 0.3% agar in α -MEM supplemented with FCS. Twelve parallel wells were used for each CFU-BL assay (on control or treated stromal layers). Colonies growing on the surface of the stromal layer were counted by inverted microscope on day 7. Stromal layers with no panned cells produced no colonies. Colony count was referred to the panned plastic non-adherent cells and expressed as CFU-BL/ 10^3 PNAMNC (bone marrow) or CFU-BL/ 10^4 PNAMNC (blood). Normal bone marrow cells were obtained from the remnants of cells harvested for bone marrow transplantation (granulocyte-macrophage colony-forming cells: CFU-GM assay), CML cells were the remnants of bone marrow or blood samples collected for cytogenetic assays with the informed consent of the donors/patients.

Cytokines

Cytokines (G-CSF; stem cell factor SCF; Amersham International, Amersham, UK) were added to the stromal cultures in a standard dose (10 ng/well = 100 ng/mL) 20 h before panning the PNAMNC. As a control, conditioned medium of a bladder carcinoma cell line (5637, containing SCF, interleukin (IL)-1, IL-6, granulocyte-macrophage colony-stimulating factor (GM-CSF), G-CSF, Kaashoek *et al.* 1991) was also used in the experiments at a final concentration of 10%.

Statistical analysis

Linear regression and two paired *t*-test was calculated by Statgraphics computer program.

RESULTS

Using established stromal layers from normal bone marrow cultured in the presence of hydrocortisone a strict linear correlation was observed between panned PNAMNC and colony formation ($r = 0.9646$). Although the absolute number of CFU-BL/ 10^3 PNAMNC bone marrow cells varied, an average of 8.8 ± 2.4 colonies were formed on the stroma.

If stromal layers were incubated with G-CSF or SCF for 20 h before panning, CFU-BL colony formation did not change significantly and incubation with conditioned medium did not alter

Figure 1. The effect of incubation of stromal layers with growth factors on the adherent blast colony formation of plastic non-adherent mononuclear cells from normal bone marrow. Bars represent mean $\pm$ SD of six experiments. CFU-BL: blast colony forming cell/ 10^3 PNAMNC. HC⁻: stromal cells cultured without hydrocortisone, HC⁺ stromal layers cultured with 10^{-6} M hydrocortisone. Control: stromal layer not incubated with growth factors, CM: 10% conditioned medium of 5637 cell line, G-CSF: 10 ng/well, SCF: 10 ng/well.

Figure 2. The effect of incubation of stromal layers with growth factors on the adherent blast colony formation of plastic non-adherent mononuclear cells from CML bone marrow. Bars represent mean $\pm$ SD of 5 experiments. HC⁻: stromal cells cultured without hydrocortisone, HC⁺ stromal layers cultured with 10^{-6} M hydrocortisone. For growth factor incubation of the stroma see legend to Fig. 1. * $P < 0.05$ compared to CFU-BL growth on untreated (control) stroma.

Table 1. CFU-GM and CFU-BL in the peripheral blood of CML patients

Patient	Phase of CML ^a	CFU-GM/10 ⁵ MNC Mean $\pm$ SD	CFU-BL/10 ⁴ PNAMNC Mean $\pm$ SD
1 (A.V.)	AP	18.0 $\pm$ 2.1	6 $\pm$ 3.0
2 (B.I.)	Newly diagnosed	130.8 $\pm$ 2.0	11.6 $\pm$ 4.2
3. (N.Z.)	CP, IF treatment	2.0 $\pm$ 1.0	6.8 $\pm$ 2.9
4. (B.B.)	CP, IF treatment	1.3 $\pm$ 1.0	0
5. (K.G.)	CP, IF treatment	0.8 $\pm$ 0.7	0
6. (V.G.)	CP, IF treatment	1.7 $\pm$ 0.2	1.2 $\pm$ 0.7
7. (K.G.)	CP, IF treatment	0.3 $\pm$ 0.1	0
8. (B.E.)	Newly diagnosed	23.0 $\pm$ 4.0	7.5 $\pm$ 4.0

^aNewly diagnosed: untreated CML, AP: accelerated phase of the disease, CP: chronic phase of the disease, IF: interferon treatment.

blast colony formation either. Cluster/colony formation on stroma cultured without hydrocortisone was not altered either by preincubation with cytokines or conditioned medium (Fig. 1).

PNAMN *bone marrow* cells obtained from five Philadelphia positive CML patients formed 4.83 ± 2.1 blast cell colonies/ 1×10^3 PNAMN bone marrow cells, and the same strict linear correlation between panned cell count and colony formation was observed as for normal bone marrow. The patients involved were: three newly diagnosed, untreated patients, one in relapse after rejection of bone marrow graft and one interferon-treated patient in chronic phase at the time of bone marrow harvest for autologous transplantation. If stromal layers were incubated for 20 h with G-CSF, SCF or conditioned medium, a statistically significant increase of blast cell colony formation was observed. G-CSF and SCF could also increase blast colony formation of CML derived bone marrow cells on stromal layers cultured in the absence of hydrocortisone, i.e. consisting only of fibroblasts (Fig. 2).

In four of eight CML patients PNAMN *blood cells* formed only clusters on the stroma. Only four patients (no. 1: CML in accelerated phase of the disease, no. 2, no. 3 and no. 8 newly diagnosed, untreated patients) showed evaluable blast colony formation. Although granulocyte-macrophage colony formation and blast cell colony formation by PNAMN bone marrow cells from CML patients did not show a close correlation (our unpublished observations), a rather good correlation between CFU-GM and CFU-BL was found for CML blood cells (Table 1).

Figure 3. The effect of incubation of stromal layers with growth factors on the adherent blast colony formation of plastic non-adherent mononuclear cells from CML blood. Bars represent mean $\pm$ SD of 4 experiments. Patients producing only clusters under the same conditions were omitted. Stromal layer was cultured with hydrocortisone. For growth factor incubation of the stroma see legend to Fig. 1. $P < 0.05$ compared to CFU-BL growth on untreated (control) stroma.

In those cases where CFU-BL formation could be obtained, incubation of the stroma with G-CSF or SCF induced a significantly higher colony formation from blood PNAMNC (Fig. 3).

DISCUSSION

CFU-BL belong to a primitive subpopulation of clonogenic progenitors of bone marrow characterized by their strong attachment to haematopoiesis maintaining bone-marrow-derived stroma (Gordon *et al.* 1985). As a result of this feature they differ from those primitive colony-forming cells originally described by Nakahata & Ogawa (1982), that form colonies in soft gel in the absence of stroma but are able to form primitive colonies if replated. Under normal conditions they are not present in the circulation but can be detected in blood of CML patients (Gordon *et al.* 1987). This is well in agreement with the findings that (a) an elevated number of predominantly Ph positive LTC-IC circulate in the blood of CML patients, and (b) an increased number of CFU-GM can be observed in blood of CML patients, especially in patients with a high leucocyte level (Eaves *et al.* 1986; Petzer *et al.* 1996, our unpublished observation).

The fact that primitive CML progenitors adhere poorly to pre-established bone marrow derived stroma *in vitro* (Gordon *et al.* 1987; Verfaillie *et al.* 1992) suggests that this deficiency could lead to the increased release of progenitors and immature cells into the circulation in CML patients *in vivo*. Since primitive CML progenitors are not subject to growth inhibitory signals when in close contact with the stroma (Verfaillie *et al.* 1997; Cashman *et al.* 1998), these phenomena suggest that at least one of the defects in CML haematopoiesis is the defective adherence of the progenitors. The most attractive assumption is that, though CD34⁺ HLA-DR⁺ CML progenitors express normal numbers of various adhesion receptors ($\alpha_4\beta_1$, $\alpha_5\beta_1$ integrins, CD-44), they may be abnormal either structurally or functionally (Verfaillie *et al.* 1997).

In the present experiments CML-derived bone marrow non-adherent to plastic mononuclear cells showed a reduced capacity to bind to bone marrow stromal layers when compared to their normal counterparts. Very few CFU-BL were observed in the peripheral blood of CML patients except for newly diagnosed, untreated CML, or a CML patient in accelerated phase of the disease, but a correlation was found with the actual CFU-GM concentration.

G-CSF, SCF or a cocktail of growth factors (conditioned medium of 5637 cell line containing SCF, IL-1, IL-6, GM-CSF and G-CSF, Kaashoek *et al.* 1991), could increase significantly the binding and colony formation of bone marrow or blood derived CFU-BL from CML patients. Co-existence of normal and malignant haematopoiesis in CML bone marrow was first documented by Coulombel *et al.* (1983). Although we presume that the majority of the induced blast cell colony formation on cytokine-treated stroma derived from malignant clones, to date we have no direct proof whether these blast cell colonies were of Ph positive origin. The circumstantial evidence that the increase of local growth factor concentration increased the binding and/or colony formation of progenitors from CML patients without affecting normal bone marrow derived CFU-BL, however, supported our hypothesis that these colonies derived from Ph positive clones. We assumed, consequently, that the actual local growth factor concentration tends to normalize the adherence of CML progenitors to the microenvironment. The fact that this effect could also be observed on stroma not supporting haematopoiesis (cultured without hydrocortisone) supported our belief that the local concentration of growth factors may be critical to the adhesion of CML cells.

Although in the present experiments only the adhesion of CML CFU-BL to stromal layers was studied, these data together with the previously observed defective adhesion of CML progenitors to fibronectin (Bhatia *et al.* 1994) may suggest that decreased attachment to stroma or extracellular matrix is a general feature of these cells.

C-kit receptor has already been proved to play a decisive role in the adhesion of stem cells to stromal cells in a murine setting (Kodama *et al.* 1994). In long-term cultures completely deficient of SCF, clonogenic cell output of CML marrow at the earlier culture period was lower than in cultures with (soluble or transmembrane) SCF. The percentage of malignant progenitor output (bcr/abl transcript positive individual soft gel colonies) was lower if cultured on SCF-deficient stroma suggesting that SCF may specifically favour the growth of malignant progenitors (Agarwal *et al.* 1995).

In the present experiments a short-term coincubation with either SCF or G-CSF had no effect on the binding of *normal* bone-marrow-derived CFU-BL to normal stroma. An attractive assumption is that under normal conditions normal bone marrow derived stroma can produce an adequate amount of regulatory factors for those cells that are attached to the microenvironment. For attachment of CML cells, however, increased local growth factor concentration is required, emphasizing the impaired adhesive features of CML cells.

Whether a normal sensitivity to growth inhibitory signals will follow this presumed normalized attachment of CML cells to microenvironment needs to be studied in further experiments.

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