Establishment of Two Permanent Human Bone Marrow Stromal Cell Lines With Long-term Post Irradiation Feeder Capacity

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We describe the establishment of two permanent Simian virus 40-transformed human stromal cell lines, designated L87/4 and L88/5, derived from the bone marrow of a hematologically normal male patient. Both cell lines show a fibroblastic morphology and do not express hematopoietic cell markers. L87/4 but not L88/5 expresses the macrophage marker CD68. The most remarkable feature of these new stromal cell lines is their ability to persist as growth-arrested adherent feeder cells after ionizing-irradiation at doses up to, and exceeding 20 Gy (L87/4). This renders them particularly useful for studying aspects of feeder dependence of hematopoietic cell development in long-term culture. Both cell lines are able to function as feeder cells, supporting the long-term proliferation of CD34+ human cord blood cells as well as the clonogenic growth of the human Burkitt lymphoma B-cell line BL70.

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MATERIALS AND METHODS

Bone Marrow

BM cells for long-term culture and transfection experiments were obtained from freshly resected ribs of hematologically normal patients. All specimens were obtained by informed consent and according to protocols approved by institutional ethics committees.

Long-term Culture

BM cells were isolated from the rib by aspiration in phosphate-buffered saline (PBS). They were plated without any further purification step at a density of 2 × 10^6 cells/ml in 75-cm² flasks (Nunc, Roskilde, Denmark) in Dextrose-type LTBMC medium (McCoy's 5a medium supplemented with 12.5% preselected fetal calf serum (FCS), 12.5% preselected horse serum, 1% sodium bicarbonate, 1% sodium pyruvat, 0.4% modified Eagle's medium (MEM) nonessential aminoacid solution, 0.8% MEM essential aminoacid solution, 1% vitamin solution, 1% L-glutamine (200 mmol/L), 1% penicillin-streptomycin solution (all solutions from GIBCO, Grand Island, NY), 10⁻² mol/L α-thioglycerol, 10⁻⁶ mol/L hydrocortisone). Cultures were incubated at 37°C in a humidified atmosphere at 5% CO₂ and fed weekly by half-medium change.

Establishment of Human BM Stromal Cell Lines

BM cells were cultured for 2 to 3 weeks in LTC medium until the stromal layers reached subconfluency. Adherent stromal cells were collected by trypsinization and replaced in 25-cm² culture flasks at a density of 5 × 10^6 cells/mL. CaCl₂ gradient-purified plasmid vectors pSVIN-1 (origin-defective SV40 genome cloned in pBR322) and pUCIN-1 (origin-defective SV40 genome cloned in pUC12, late genes partially deleted) were used for the transfection experiments. Both plasmid vectors were gifts from Dr Ellen Fanning (Department of Biochemistry, University of Munich, Munich, Germany).

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Transfection using liposomes was performed essentially as described in the producer's (Serva, Heidelberg, Germany) transfection protocol. Briefly, semiconfluent adherent cells were washed once with PBS, once with McCoy's 5A, and incubated for 5 to 18 hours with a freshly prepared plasmid/transfectam mixture in 8 mL serum-free LTC medium in 25-cm² flasks. After transfection cells were washed twice with PBS/10% FCS and cultured in 10 mL LTC medium to confluence. After a latency period of about 6 weeks, transfected cells started overgrowing primary stromal cells and were passaged continuously at a ratio of 1:2. Transfected cells were maintained in LTC medium at 37°C in a humidity incubator at 5% CO₂.

**Southern Blot Experiments**

After at least six cell passages, DNA of transfected cells was purified by CsCl gradient centrifugation and electrophoresed on a 0.8% agarose gel. DNA was blotted on Hybond N filters (Amersham Buchler, Braunschweig, Germany) and hybridized with a radiolabeled BamHI fragment encoding SV40 large T-Ag.

**Northern Blot Experiments**

Total RNA of transfected cells was isolated by the guanidinium-isothiocyanate extraction method, digested with selected enzymes, and electrophoresed on a 0.8% agarose gel. Northern blots were hybridized with a radioactively labeled BamHI fragment of the plasmid pSVIN-1 coding for the SV40 large T-Ag.

**Radiosensitivity Assay**

Transfected cells were plated at a density of 5 × 10⁴/mL in 75-cm² flasks in LTC medium and grown for 18 hours. Subsequently they were irradiated with 5 to 20 Gy using a cesium-137 γ-ray source (Atomic Energy of Canada, Ontario, Canada). After irradiation the medium was changed completely and the cells were incubated for 7 days (37°C, 5% CO₂) in LTC medium. On day 8, adherent and nonadherent cells were harvested by trypsinization, cell numbers were counted, and the colony-forming potential of the irradiated cells was measured by counting day-14 colonies in agar.

** Colony-forming Assay**

To examine the clonogenic potential of transformed stromal cell lines, adherent cells were harvested by trypsinization (0.25% trypsin, Gibco) and plated in semisolid agar cultures as reported. In brief, stromal cells were plated in triplicate at a concentration of 1 × 10⁵ cells/mL using equal amounts of 0.6% Bactoagar (DIFCO, Detroit, MI) and double-strength Iscove's modified Dulbecco's medium (IMDM; Gibco) containing 40% preselected FCS. Colony growth was stimulated by 10% (vol/vol) giant cell tumor-conditioned medium (GCT-CM; American Type Culture Collection, Rockville, MD). Cultures were incubated for 14 days at 37°C in a humidified atmosphere and 5% CO₂ in air. Fibroblast colonies were scored on day 14 using an inverted microscope (32-fold magnification).

**Immunofluorescence Staining**

*Indirect immunofluorescence staining.* L88/5 and L87/4 cells were cultured on glass slides washed with calcium-free PBS (PBsd) and fixed for 10 minutes in a 1:1 mixture of ice-cold methanol and acetone. After rinsing the slides with PBsd-diluted rabbit antiserum against Factor VIII-related antigen (Behringwerke, Marburg, Germany, 100 µL antiserum + 1.5 mL PBsd) was layered on the slides. The slides were then incubated at 37°C for 30 minutes in a humidity chamber, rinsed with PBsd, and overlaid with a fluorescein isothiocyanate (FITC)-labeled secondary antirabbit antibody. After incubation at 37°C for 30 minutes, the slides were washed with PBsd, overlaid with a mixture of glycerol and PBsd (1:1), and covered with a coverslip.

**Immunofluorescence staining for FACS analysis.** Adherent stromal cells were detached from the culture flask by incubation with collagenase (0.1 U/mL)/dispase (0.8 U/mL) for 15 minutes at 37°C. Cells were washed with PBsd, suspended in IF-buffer (PBsd with 0.1% sodium azide and 2% FCS), and labeled for 30 minutes at 4°C with the first antibody, which was either FITC-conjugated, phycocerythrin (PE)-conjugated, or unlabeled (Table 1). Cells were then washed twice with 1 mL IF-buffer, and in the case of unlabeled first antibody, treated as described above with a second FITC- or PE-conjugated antibody (Table 1). Stained cells were suspended in 1 mL IF-buffer and analyzed by a FACScan flow cytometer (Becton Dickinson).

**Cytochemical Staining**

Cells were cultured on glass slides washed with PBsd air-dried and stained with chloracetate-esterase or α-naphthyl acetet esterase as described by the producer's instructions (Sigma Chemical Co, St Louis, MO).

**Limiting Dilution**

Adherent feeder cells were seeded in 96-well plates, grown to confluency, and irradiated (MRC5, 50 Gy; BM feeder, 50 Gy; L88/5, 15 Gy; L87/4, 20 Gy) in a Ca137 source (Atomic Energy of Canada, Ltd, Ontario). After 24 hours, BL70 cells were washed twice in serum-free medium and added at the indicated cell densities in at least 24-well plates. Plates were fed twice weekly, and outgrowth of colonies was monitored up to day 40. All limiting dilution experiments were performed in RPMI 1640 supplemented with 5% FCS, 2 mmol/L L-glutamine, and antibiotics. BL70 cells were kept in the same medium in the presence of irradiated (50 Gy) MRC5 cells. MRC5 cells were cultured in Dulbecco's MEM supplemented with 10% FCS, 2 mmol/L L-glutamine and antibiotics.

**Coculture Experiments With CD34⁺-Enriched Human Cord Blood Cells**

Percoll-separated mononuclear cord blood cells were either stained with anti-CD34 monoclonal antibody (MoAb) (Dianova,
Hamburg, Germany) directly conjugated to FITC and then sorted on a FACStarPlus (Becton Dickinson) for high CD34 expression or the CD34+ cells were isolated by using Dynabeads M-450 (Dynal, Hamburg, Germany) directly coated with the MoAb BI-3C5. CD34+ cord blood cells were plated in 24-well plates (5 × 10^3 cord blood cells/well) on irradiated semiconfluent L87/4 (20 Gy) and L88/5 (15 Gy) stromal cells in LTC medium. Cultures were maintained at 37°C for 5 weeks with half-medium changes weekly after culture week 2. Nonadherent cells were assayed in semisolid medium for the presence of erythroid burst forming unit (BFU-E) and myeloid (granulocyte-macrophage colony-forming cells [GM-CFC]) progenitor cells. Cultures were plated with 10^7 nonadherent cells/mL in IMDM, 30% FCS, 1% bovine serum albumin, 10^-3 M a-thioglycerol, 5% phytohemagglutinin-LCM, 0.98% methylcellulose, 3 U/mL erythropoietin (all substances from the Terry Fox Laboratories) and 100 ng/mL kit-Ligand (gift from Dr Mailhammer, Hämato logikum der GSF, Munich, Germany). Cultures were plated in 1-mL volumes in 35-mm tissue culture plates in 5% CO₂ at 37°C and colonies were counted on day 14.

RESULTS

Establishment and Characteristics of Early Passage Human BM Stromal Cell Lines

BM cells of a 70-year-old hematologically normal male patient were cultured in 25-cm² flasks in conventional Dexter-type LTBMC for 3 weeks. Confluent stromal layers were passaged once and transfected with either pSVIN-I or pUCIN-I plasmid vectors by lipofection. Both plasmids contain the sequences coding for the SV40 T-Ag known to be a transforming factor. Ten passageable cell lines, selected by their growth advantage over primary stroma, were obtained after lipofection. Spontaneous outgrowth of nonadherent Epstein Barr virus-immortalized B cells was observed in 20% of the culture flasks. Five of the 10 cell lines designated L87/4, L88/5, L90/7, L91/8, and L87/12 were selected for further studies. All cell lines display fibroblastoid morphology, harbor a stably integrated SV40 construct (Fig 1), and express SV40 large T-Ag as determined by Northern blotting (data not shown). The integration site of the plasmid vector is different in each cell line. Three of the five cell lines were clonal after six passages (Fig 1), whereas two were oligoclonal. The SV40-transformed cell lines grew at a comparatively high rate for 25 to 30 cell passages and then entered a crisis. Two of the five lines were rescued subsequently (lines L88/5 and L87/4). They have now been maintained in continuous culture for more than 70 passages (18 months). All further experiments were performed with clonal postcrisis L88/5 and L87/4 cell lines.

Characterization of the Stromal Cell Lines L88/5 and L87/4

By phase-contrast morphology both stromal postcrisis cell lines exhibit fibroblastoid morphology (Fig 2). Cells divide rapidly with doubling times of one day for L88/5 and two days for L87/4. The cells show contact inhibition and do not

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![Southern blot analysis of precrise stromal cell lines. Genomic DNAs of the SV40-transformed human stromal cell lines L87/4 passage 14 (lane 1), L88/5 passage 14 (lane 2), L90/7 passage 12 (lane 3), L91/8 passage 15 (lane 4), and L87/12 passage 12 (lane 5) were digested with different restriction enzymes as indicated and hybridized with a 32P-labeled SV40 T-Ag fragment. Genomic DNA of primary human BM was used as a negative control (lane 6).](image-url)
form foci in liquid culture. They grow into fibroblastoid colonies when plated in semisolid agar in the presence of 1% GCT-CM (Fig 2).

Both cell lines are positive for SV40 T-Ag expression (Fig 3) and display the same genomic SV40 integration sites as previously observed in the precrisis L87/4 and L88/5 cells. As shown by immunofluorescence, L87/4 and L88/5 express the stromal cell surface markers CD10 and CD13, whereas they do not express a variety of hematopoietic cell markers (Table 2). Nevertheless, L87/4 can be dis-
T-Antigen

1 2 3 4 5 6

Fig 3. Northern blot analysis of postcrisis stromal cell lines L87/4 and L88/5. Total RNAs of L87/4 cells, L88/5 cells, primary stromal cells, and MRC-SV2 cells were hybridized with a 32P-labeled SV40 T-Ag probe. Lanes: 1, L87/4 passage 47; 2, L88/5 passage 51; 3, L87/4 passage 59; 4, L88/5 passage 62; 5, primary stroma (negative control); and 6, MRC-SV2 (positive control).

L88/5 and L87/4 Cells Support Long-term Hematopoiesis of Human Cord-Blood Progenitors

Semiconfluent irradiated L87/4 (20 Gy) and L88/5 (15 Gy) cells seeded in 24-well plates were charged with CD34+ human cord blood cells (5 x 10^5 CD34+ cells/well) and cultured for 5 weeks in LTC medium. Up to culture-week 5, both stromal layers supported the proliferation of nonadherent cord blood cells with a maximum of cell numbers observable 2 to 3 weeks after initiation of the cultures. The number of nonadherent cells is amplified about 200-fold during the culture period compared with the number of input cord blood cells. Large numbers of myeloid (GM-CFC) and erythroid (BFU-E; data not shown) progenitor-derived colonies were present in the nonadherent cell fraction up to 5 weeks after culture initiation as determined by methylcellulose colony-formation assays (Fig 6).

Table 2. Phenotypes of the Postcrisis Stromal Cell Lines L87/4 and L88/5

<table>
<thead>
<tr>
<th>Phenotypes</th>
<th>L87/4</th>
<th>L88/5</th>
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<tbody>
<tr>
<td>c-kit</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>HLA-DR</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>CD10</td>
<td>+</td>
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<td>CD11a, CD11b, CD14</td>
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<td>CD25, CD32, CD33</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>CD34, CD36, CD38</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>CD56, CD61, CD64</td>
<td>–</td>
<td>–</td>
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<tr>
<td>CD68</td>
<td>+</td>
<td>+</td>
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<tr>
<td>CD71</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Factor VIII-related antigen</td>
<td>–</td>
<td>–</td>
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<tr>
<td>Chloracetate esterase</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>α-Naphyl acetate esterase</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>SV40 T-Ag</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Smooth muscle type actin</td>
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<td>–</td>
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<tr>
<td>Laminin</td>
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<td>–</td>
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<tr>
<td>Fibronectin</td>
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<td>Vitronectin</td>
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low cell densities under low serum conditions. At high cell densities they do not require feeder cell layers. Feeder function in this system can be provided by a series of primary human and rodent fibroblast cells. However, several available SV40-immortalized human fibroblast cell lines constantly failed to support the proliferation of a model BL line, BL70. Therefore, we asked whether our SV40-immortalized stromal cells function as feeder cells in the BL70 system. As shown in Fig 5, irradiated (15 Gy) L88/5 cells as well as irradiated (20 Gy) L87/4 cells support the clonogenic growth of BL70 cells even better than primary irradiated BM stromal cells and human MRC5 fibroblasts. BL70 cells dying within 2 days in the absence of feeder cells can be maintained on irradiated L87/4 and L88/5 feeder layers for more than 5 weeks. The graphical representation of BL70 limiting dilution assays (Fig 5) indicates a single-hit kinetic. This shows that L88/5 cells and L87/4 cells (as well as MRC5 cells or primary BM stroma) provide all factors necessary for optimum proliferation of BL70 cells.

L87/4 and L88/5 Cells Substitute for Fibroblast and BM Feeder Cells

A number of Burkitt lymphoma (BL) cell lines depend on a feeder layer of irradiated human fibroblasts if grown at distinguished from L88/5 by expressing the macrophage marker CD68.

Radiosensitivity of L88/5 and L87/4 cells

L88/5 and L87/4 cells were cultivated and irradiated as indicated in Fig 4 and described in Materials and Methods. Both cell lines can be irradiated with up to 15 Gy without detachment. Proliferation and colony formation of L88/5 ceases at doses exceeding 15 Gy, whereas L87/4 cells retain their ability to grow and form colonies in soft agar. L87/4 must be irradiated with 20 Gy to abolish proliferation in suspension culture and clonal growth in soft agar (Fig 4).

We have also observed that the more irradiation-resistant cell line L87/4 can be irradiated at even higher doses without cell detachment if irradiated cell layers are recharged within 24 hours with low numbers (5 x 10^3/mL) of CD34+ umbilical cord blood cells. Cultivation of L87/4 irradiated at higher doses (greater than 20 Gy) without recharging inevitably leads to cell detachment and cell death.

L88/5 and L87/4 Cells Substitute for Fibroblast and BM Feeder Cells

A number of Burkitt lymphoma (BL) cell lines depend on a feeder layer of irradiated human fibroblasts if grown at
Fig 4. Radiosensitivity of the stromal cell lines L87/4 and L88/5. Cells were plated at a density of 5 $\times$ 10^5/mL in 75-cm^2 flasks in LTC medium and irradiated with 5 to 20 Gy. After irradiation, the medium was changed completely and the cells were incubated for 7 days (37°C, 5% CO_2) in LTC medium. On day 8, adherent and nonadherent cell numbers were determined (A, B) and the cells plated in agar containing GCT-CM. Day-14 agar colonies were counted in (C) and (D).

DISCUSSION

Maintenance of hematopoiesis and the differentiation of cells into one of several hematopoietic lineages depends on a variety of high and low molecular weight substances acting on these cells in a framework of particular sequences and concentrations. BM stromal cells, in conjunction with their extracellular matrix, form the so-called hematopoietic inductive microenvironment that maintains the functional integrity of this complex system. The major cellular components of this microenvironment are thought to comprise macrophages, fibroblasts, adipocytes, and endothelial cells. Whereas some progress has been made in the mouse system, the exact role of individual stromal subpopulations still has to be elucidated.

The long-term culture of BM cells using stromal cell layers provides an experimentally accessible model of hematopoiesis. Several attempts have been made in the past to replace functional capacities of the relatively undefined stromal cell layers by distinct human stromal cell lines.
ress has long been hampered by the inability of human stromal cells to transform spontaneously into cells that can be cultivated indefinitely. This problem has been overcome, at least in part, by establishing adenovirus- and SV40-transformed stromal cell lines.\textsuperscript{17,18} The known cell lines apparently have two disadvantages, namely, they are being used only in early cell passages\textsuperscript{29,30} and they cannot be maintained as adherent cells after growth arrest by irradiation.\textsuperscript{31}

We now have established two permanent cell lines, designated L88/5 and L87/4, from BM of a hematologically normal 70-year-old male patient. Both cell lines have survived crisis occurring at passage 30 and have now been maintained for more than 70 passages (18 months). Several parameters including site of SV40 integration, SV40 T-Ag expression, CD68 expression in L87/4, and response to irradiation are stably maintained in precrisis and postcrisis cells. In postcrisis cells, they are now stable for more than 30 passages. Our cell lines can also be maintained as adherent growth-arrested

A. Experiment 1

![Graph A](image1)

B. Experiment 2

![Graph B](image2)

Fig 6. Support of cord-blood GM-CFCs by the stromal cell lines L87/4 and L88/5. Nonadherent cord-blood cells produced on the stromal cell lines L87/4 and L88/5 were harvested weekly after culture week 2 and assayed in methylcellulose cultures for myeloid progenitors. Colonies (greater than 50 cells) were counted 14 days after plating. The results represent two representative and independent experiments. (\(\boldsymbol{-\; -\; -\; -\; -}\)), L88/5; (\(\boldsymbol{-\; @\; -\; -\; -}\)), L87/4; (\(\boldsymbol{-\; @\; -\; -}\)), plastic.
cell layers after irradiation with doses of up to 15 Gy. It is noteworthy that even higher doses of ionizing radiation are tolerated, at least by L87/4 cells, if these cells are rechallenged in time with CD34-enriched umbilical cord blood cells at low densities.

Although the two cell lines have been established from the BM of the same patient they do show differences. Both lines appear to be of marrow fibroblast origin as suggested by the expression of CD10 and CD13 and the nonexpression of hematopoietic markers (see Table 2). However, at least L87/4 differs from L88/5 and also from other known human stromal cell lines in that it also expresses the macrophage marker CD68. Figure 2 further illustrates that L88/5 displays more typically the phenotype of fibroblasts in vitro than L87/4.

We have shown that our two permanent cell lines also possess feeder capacity in postcrisis passages (passage 60). This is shown by their ability to maintain clonogenic cell proliferation of the stroma cell-dependent BL cell line BL70 for more than 5 weeks. The BL70 assay clearly shows that the two cell lines produce all factors necessary for malignant B-cell proliferation. This does not preclude the possibility that they produce further cytokines supporting the growth and differentiation of other cell types as well. As shown by PCR analysis the two cell lines appear to produce a variety of hematopoietic growth factors including interleukin-6 (IL-6), IL-7, IL-8, IL-10, IL-11, kit ligand, leukemia inhibitory factor, granulocyte-macrophage colony-stimulating factor (GM-CSF), G-CSF and M-CSF (data not shown; Thalmeier et al, manuscript in preparation). This cytokine profile would suggest that L87/4 and L88/5 should be capable of supporting the development of GM-CFCs (Fig 6) and BFU-Es from CD34+-enriched cord blood cell cultures. In conclusion, the feeder capacities and the remarkable and unique adherence properties of our cell lines unaffected by ionizing radiation suggest that they constitute a particularly useful system for studying interactions between feeder and hematopoietic cells in long-term culture.

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Establishment of two permanent human bone marrow stromal cell lines with long-term post irradiation feeder capacity

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