Absence of c-kit Receptor and Absent Proliferative Response to Stem Cell Factor in Childhood Burkitt’s Lymphoma Cells

By Jörg Tomeczowski, Andreas Beilken, Detlef Frick, Britta Wieland, Andrea König, Martin H. Falk, Alfred Reiter, Karl Welte, and Karl-Walter Sykora

The cytokine stem cell factor (SCF) synergizes with interleukin-7 (IL-7) to enhance the proliferation of pre-B cells. To examine the role of SCF and its receptor, c-kit, in the pathogenesis of pediatric Burkitt’s lymphomas (BL), we investigated the expression of SCF and c-kit in BL cells and the mitogenic activity of SCF on BL cells. A panel of 13 BL cell lines and 7 fresh biopsy tumors was investigated. BL cells were stimulated either by Epstein-Barr virus (EBV) infection or by different reagents and cytokines, and expression of SCF and c-kit was studied on the mRNA level by Northern blot analysis and reverse-transcriptase polymerase chain reaction (RT-PCR), followed by Southern blotting. c-kit expression was also studied by fluorescence-activated cell sorting and by crosslinking of digoxigenin-labeled recombinant human SCF to the cell surface. Proliferation of BL cell lines was measured by 3H-thymidine incorporation. Low-level expression of c-kit mRNA was detected in 2 of 13 unstimulated BL cell lines and in 1 fresh BL tumor. One cell line showed upregulation of c-kit mRNA with A23187 and downregulation with phorbol myristate acetate. Neither c-kit nor SCF could be detected in any other cell line under any condition of stimulation as analyzed by Northern blot analysis, RT-PCR followed by Southern blot analysis, crosslinking, and immunofluorescence. No response to SCF was seen in 3H-thymidine incorporation assays. We conclude that most BL cells express neither SCF nor c-kit and that the low-level expression of c-kit in some BL cells most likely has no biologic significance.

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Submitted September 12, 1994; accepted March 30, 1995.

Supported by Deutsche Krebshilfe Grant No. W88/91/Syl.

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0006-4971/95/8604-0001/$3.00/0

differentiated to B220<sup>+</sup> cells. Murine pro-B—cell and pre-B—cell clones express c-kit, the tyrosine kinase receptor of SCF,<sup>25,29</sup> The finding that c-kit is involved in the growth regulation of pre-B cells makes this receptor a candidate for oncogenic involvement in the development of B-cell—lineage lymphomas.

For that reason and for the beginning of clinical use of SCF, we examined the direct effect of recombinant human SCF (rhSCF) on pediatric BL cells to elucidate whether SCF is a cytokine that is involved in the growth regulation of these malignancies. We also examined the production of SCF, the presence of the receptor for SCF on BL cells, and the inducibility of SCF and c-kit under different conditions of stimulation.

**MATERIALS AND METHODS**

**Fresh lymphoma cells.** The fresh lymphoma cells were isolated for flow cytometry by Ficoll-Paque centrifugation (Pharmacia, Uppsala, Sweden), washed, and stored in BL medium (see below) containing 10% dimethylsulfoxide (DMSO) and 20% fetal calf serum were again isolated by Ficoll-Paque centrifugation, washed, and re-suspended in phosphate-buffered saline (PBS). The percentage of L<sub>3</sub> morphology blast cells was determined in each final cell preparation by Wright Giemsa staining. The age and sex of the patient as well as the source and percentage of blast cells in the cell preparation are summarized in Table 1.

Fresh peripheral B and T cells were isolated by Ficoll-Paque centrifugation and subsequent separation over a nylon wool column.<sup>40</sup>

**Cell lines.** Cell lines were established from 1 patient’s ascites and from 1 patient’s bone marrow. The cells were isolated by Ficoll-Paque centrifugation, washed, and re-suspended in BL culture medium and maintained at 37°C in humidified 5% CO<sub>2</sub> with half replacement of medium twice weekly. The BL medium consisted of RPMI 1640 (GIBCO BRL, Eggenstein, Germany) with 10% FCS, supplemented with 20 nmol/L bathocuprine disulfonic acid (Sigma, Deisenhofen, Germany), 50 μmol/L α-thioglycerol (Sigma), 1 mmol/L pyruvate (GIBCO BRL), 10 mmol/L HEPES (Seromed Biochrom, Berlin, Germany), and 0.25 μg/mL antibiotic-antimycotic solution (Sigma). Lots of FCS were tested to support optimal growth of B-lymphoid cell lines. The BL cell lines that could be established from ascites cells were termed MHH-BL-1 (CD10<sup>+</sup>, CD23<sup>+</sup>, surface μ<sup>+</sup>) and from bone marrow cells MHH-BL-2. In addition, fresh cells from MHH-BL-1 were investigated.

All other BL cell lines initially established from Epstein-Barr virus (EBV)-positive and -negative BL biopsies<sup>43-45</sup> at the International Agency for Research on Cancer (IARC; Lyon, France) were kindly provided by Dr Georg W. Bornkammar (Forschungszentrum für Umwelt und Gesundheit GmbH, Munich, Germany). Two of the EBV-negative BL cell lines, BL-2 and BL-41, had previously been infected in vitro by two different EBV strains<sup>46</sup> to study mechanisms of EBV-induced phenotypic alterations of BL cells.<sup>43</sup> The EBV status, karyotype, and Ig expression are summarized in Table 2. To detect mycoplasma contamination, all cell lines were screened with the Gen-Probe test kit (Biermann, Bad Nauheim, Germany).

The megakaryoblastic cell line MOLT<sup>46</sup> was used as a positive control for c-kit, whereas the endothelial cell line ECV 304<sup>46</sup> was used as a positive and the monocytic cell line U937<sup>46</sup> as a negative control for SCF mRNA transcripts.

**<sup>3</sup>H-thymidine incorporation assays.** The mitogenic activity of rhSCF was determined under serum-free conditions in a <sup>3</sup>H-thymidine incorporation assay. BL cell lines were washed three times with serum-free medium consisting of BL medium without FCS, supplemented with Iscove’s Supplement (GIBCO BRL) and insulin-transferrin-sodium selenite supplement (Boehringer Mannheim, Mannheim, Germany) to a final concentration of 20 μg/mL soy bean lipids,<sup>47</sup> 1 mg/mL bovine serum albumin (BSA) fraction V, 21 μg/mL transferrin, 1 μg/mL insulin, and 1 ng/mL sodium selenite. The cells were then plated at a concentration of 5 × 10<sup>4</sup> cells/mL in 96-well flat-bottom microtiter plates in 200 μL serum-free BL medium containing various concentrations (0, 50, and 500 ng/mL) of rhSCF. After a culture period of 68 hours at 37°C, 5% CO<sub>2</sub>, the cells were exposed to a 4-hour pulse of 0.5 μCi <sup>3</sup>H-thymidine (25 Ci/mmol, New England Nuclear, Burlington, Mass.)

<table>
<thead>
<tr>
<th>Cell Lines</th>
<th>Sex/Age (yr)</th>
<th>Ethnicity</th>
<th>EBV</th>
<th>Cyto genetic</th>
<th>Ig</th>
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<tr>
<td>MHH-BL-1</td>
<td>F/14</td>
<td>White</td>
<td>Positive BL</td>
<td>ND</td>
<td>μα</td>
</tr>
<tr>
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<td>ND</td>
<td>μα</td>
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<tr>
<td>IARC-BL-2</td>
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<td>t(8;22)</td>
<td>μα</td>
</tr>
<tr>
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<td>μα</td>
</tr>
<tr>
<td>IARC-BL-2P3HR-1</td>
<td>M/7</td>
<td>White</td>
<td>Negative BL</td>
<td>t(8;22)</td>
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</tr>
<tr>
<td>IARC-BL-16</td>
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<tr>
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<td>μα</td>
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<tr>
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Abbreviation: ND, not done.

<table>
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<tr>
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<th>L3 Cells (%)</th>
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<td>F/14</td>
<td>Ascites</td>
<td>45</td>
</tr>
<tr>
<td>2</td>
<td>M/10</td>
<td>Ascites</td>
<td>ND</td>
</tr>
<tr>
<td>3</td>
<td>F/11</td>
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<td>87</td>
</tr>
<tr>
<td>4</td>
<td>M/7</td>
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</tr>
<tr>
<td>7</td>
<td>M/9</td>
<td>Ascites</td>
<td>61</td>
</tr>
</tbody>
</table>

Abbreviation: ND, not done.
Amersham, Braunschweig, Germany). Finally, the cells were harvested on glass fiber strips with an automated cell harvester and the incorporated radioactivity was determined by scintillation counting. All assays were performed in triplicate.

**Stimulation of BL cell lines.** To assess induced expression of c-kit or SCF mRNA in BL cell lines, the appropriate cytokine or all assays were performed in triplicate.

**Nort hern blot analysis.** Total cellular RNA from BL cell lines and fresh BL cells was extracted using the single-step acid guanidinium thiocyanate-phenol-chloroform extraction described by Chomczynski and Sacchi.\(^9\) Fifteen micrograms of RNA per lane was formaldehyde and transferred to an agarose gel containing 17% dilution into 25 \(\mu\)L of PCR buffer containing 7.5 pmol of the upstream and downstream primers. The final MgCl\(_2\) concentration was 1.5 mmol/L. The set of SCF primers was designed to include exon 6 and 72°C for 70 s.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Location</th>
<th>Strand</th>
<th>Sequence (5'-3')</th>
<th>35 Cycles</th>
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</thead>
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<td>SCF (Martin et al(^3))</td>
<td>388-407</td>
<td>Sense</td>
<td>TGATAACGCAGATGGTAGTG</td>
<td>55°C for 85 s, 72°C for 70 s, 94°C for 65 s</td>
</tr>
<tr>
<td>SCF (Martin et al(^3))</td>
<td>1125-1144</td>
<td>Antisense</td>
<td>TGGTAGACAGAAAGGCTATTTT</td>
<td>50°C for 85 s, 72°C for 70 s, 94°C for 65 s</td>
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<tr>
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<td>653-673</td>
<td>Sense</td>
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<td>66°C for 85 s, 72°C for 70 s, 94°C for 65 s</td>
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<tr>
<td>c-kit (Yarden et al(^3))</td>
<td>141-163</td>
<td>Antisense</td>
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<tr>
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<td>561-583</td>
<td>Antisense</td>
<td>GGCAGGCGATCGGCTGAGA</td>
<td>50°C for 85 s, 72°C for 70 s, 94°C for 65 s</td>
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<td>16S mt-rRNA (Eperon et al(^3))</td>
<td>3077-3099</td>
<td>Antisense</td>
<td>GGGAGGATTGAAAGGATAG</td>
<td>94°C for 65 s</td>
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**Fig 1.** RT-PCR of SCF transcripts. Using the exon 6-encapsulating primer pair, exon 6-containing transcripts generated a 757-bp product, whereas exon 6-negative transcripts generated a 670-bp fragment. Amplified transcripts were separated on a 1.8% agarose gel and stained with ethidium bromide (middle panel). After Southern transfer, the membrane was probed with a Dig-labeled SCF antisense RNA (upper panel). The aldolase gene was amplified as positive control (lower panel). The length of size markers (M) is indicated in base pairs.
ers and the PCR conditions are shown in Table 3. PCR reactions were started by the addition of 1 U Taq polymerase (Perkin Elmer Cetus, Überlingen, Germany) in a DNA thermal cycler (Landgraf, Hannover, Germany).

**Southern blot analysis.** Products from the RT-PCR were separated by electrophoresis in a 1.8% agarose gel in TBE-buffer. DNA was denatured twice in a buffer containing 0.5 mol/L NaOH, 1.5 mol/L NaCl and subsequently neutralized twice in a buffer containing 0.5 mol/L Tris•HCl, 3 mol/L NaCl, pH 7.5. The gel was checked for the pH and blotted overnight onto positively charged nylon membrane (Boehringer Mannheim) using 20× SSC as the transfer buffer. After transfer, the DNA was immobilized using an UV crosslinker (Stratalinker; Stratagene) as described in the Northern blot protocol. The membranes were washed briefly in H2O and air dried. Prehybridization and hybridization were performed using a nonradioactive labeling and detection system (Boehringer Mannheim). Briefly, the membranes were washed for 5 minutes in buffer 1 containing 0.1% SDS for 5 minutes and two times with 0.1× SSC, 0.1% SDS at 56°C for 2 minutes. Chemiluminescent detection was performed according to the manufacturer's instructions (Boehringer Mannheim). Brieﬂy, the membranes were washed for 5 minutes in buffer 1 (0.5 mol/L maleic acid, 0.15 mol/L NaCl, pH 7.5), blocked with 1% blocking reagent (Boehringer Mannheim) diluted in buffer 1 (buffer 2) for 30 minutes, and incubated for another 30 minutes with anti–Dig-AP antibody (Boehringer Mannheim) diluted 1:10,000 in buffer 2. The membranes were washed twice for 15 minutes in buffer 1 containing 0.3% Tween 20 (Sigma) and equilibrated with buffer 3 (0.1 mol/L Tris•HCl, pH 9.5, 0.1 mol/L NaCl, 0.05% MgCl2). AMPPD stock solution (10 mg/mL) or CPD-star stock solution (25 mmol/L; Tropix, Heidelberg, Germany) was diluted 1:100 in buffer 3. Membranes were incubated for 30 minutes with 1 mL of diluted AMPPD solution or CPD-star solution at 37°C in a plastic bag and after removing excess buffer exposed to Cronex DDS 100 autoradiography ﬁlms (Du Pont, Hamburg, Germany).

**Labeling of SCF and chemical crosslinking with Dig-rhSCF.** A total of 1 mL of rhSCF (2.2 mg/mL) in buffer pH 8.5 was labeled by incitation for 2 hours with 5.97 mg of digoxigenin-3-O-methylcarbonyl-c-aminocaproinsäure-N-hydroxy-succinimidester (Dig; Boehringer Mannheim) dissolved in 150 μL ethanol at room temperature. Free Dig was removed by passage of Dig-rhSCF with PBS through a desalting column (Sephadex G-25). The labeled rhSCF

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**Fig. 2.** RT-PCR of c-kit transcripts. Amplified 534-bp c-kit transcripts were separated on a 1.8% agarose gel and stained with ethidium bromide (middle panel). The gel was blotted and hybridized with a Dig-labeled c-kit–specific internal oligonucleotide (upper panel). Mitochondrial 16S rRNA was amplified as positive control (lower panel). The length of size markers (M) is indicated in base pairs. Negative control reactions (−RNA and −cDNA) were performed by omitting RNA or cDNA template, respectively.
was found to have retained mitogenicity for M07e cells comparable to unlabeled rhSCF of the same concentration. M07e and BL cells (5 x 10^6 in 100 μL) were incubated with 15 μL (0.13 mg/mL) Digi-nhSCF for 1 hour on ice in the presence or absence of a 100-fold excess of unlabeled rhSCF. Cells were washed twice with ice-cold PBS to remove unbound SCF. Subsequently, the cells were treated with 0.5 mmol/L of the bifunctional crosslinker disuccinimidylsuberate (DSS; Pierce, Geisenheim, Germany). The reaction was stopped after 45 minutes with 100 μL of 2 mol/L glycine, pH 6.8, and the cells were lysed with ice-cold PBS with 1% Triton X-100 (Serva). The soluble proteins were denatured for 15 minutes at 50°C in a buffer containing 0.06 mol/L Tris, 3% SDS wt/vol, 30% glycerol, 1 mmol/L phenylmethylsulfonyl fluoride (PMSF), and 15% β-mercaptoethanol and separated in a 7.5% SDS-polyacrylamide gel. The gel was blotted for glycerol, 50°C in a buffer containing 0.06 mol/L Tris, 3% SDS wt/vol, 30% methanol, 0.0375% SDS, pH 9.5, as the transfer buffer. The membranes were washed twice in 20 mmol/L Tris containing 0.1% Tween 20, 0.5 mol/L NaCl, pH 7.5, and incubated overnight in blocking buffer containing 100 mL Hank’s solution (8 g/L NaCl, 0.4 g/L KCl, 0.121 g/L Na2HPO4, × 2 H2O, 0.1 g/L MgSO4, × 7 H2O, 1 g/L glucose × 1 H2O, 0.1 g/L MgCl2 × 6 H2O, 0.35 g/L NaHCO3, 0.3 g/L CaCl2 × 6 H2O, 0.01 g/L phenol red), 0.1% Tween 20, and 0.2 g/L 1-Block (Tropix), pH 7.5. The membranes were equilibrated two times with a buffer containing 20 mmol/L Tris and 0.05% Tween 20, pH 7.5. Subsequently, the anti-Dig-AP antibody (Boehringer Mannheim) was diluted 1:10,000 in blocking buffer and the membranes were incubated for 1 hour; washed 7 to 10 times for 5 minutes in 20 mmol/L Tris containing 0.1% Tween 20, 3% dry milk; and equilibrated two times with assay buffer (1% vol/vol diethanolamin, 1 mmol/L MgCl2, pH 10.0). CSPD stock solution (11.6 mg/mL; Tropix) was diluted 1:100 in assay buffer. Membranes were incubated for 30 minutes with 1 mL of diluted CSPD solution at 37°C in a plastic bag. Excess buffer was removed completely and membranes were exposed to Cronex DDS 100 autoradiography films (Du Pont).

Flow cytometry. Cell samples were incubated for 20 minutes on ice with saturating amounts of monoclonal antibody (MoAb) in 1× PBS, 0.5% BSA, 0.1% sodium azide and were washed twice in 1× PBS, 0.1% BSA, 0.1% sodium azide. The MoAbs were stained by fluorescein isothiocyanate (FITC)-conjugated F(ab’2); rabbit antimouse IgG (Dako, Hamburg, Germany) used as a second-step reagent. All samples were analyzed on a FACScan flow cytometer (Becton Dickinson, San Jose, CA). Primary MoAbs used were anti-CD10 (Calla; Becton Dickinson), anti-CD117 (c-kit; supernatant from clone 57A5D8B1 kindly provided by Dr H.-J. Bühring, Tübingen, Germany) and FITC-conjugated rabbit antihuman IgG, IgA, and IgM heavy and light chains (Behring, Marburg, Germany).

RESULTS

RT-PCR of either aldolase mRNA or 16S RNA. The quality of the generated cDNA was judged by amplification of the cDNA with aldolase specific primers that generate a 443-bp product or with 16S RNA specific primers that generate a 752-bp product. The amplification led to comparable amounts of DNA from all cell lines.
RT-PCR and Southern blot analysis of SCF mRNA. SCF exists in both cell surface bound and soluble forms. SCF that is missing 28 amino acids in the extracellular domain is not protease cleavable and therefore remains bound to the cell surface. The 84 DNA basepairs that encode the 28 amino acids correspond precisely to the sixth exon of the human gene.\textsuperscript{6-8} The primer pair used in the present experiments discriminates between exon 6-positive and exon 6-negative transcripts. With exon 6-positive transcripts, it generates a 757-bp product. With exon 6-negative transcripts, it generates a 670-bp product. The amplification products were resolved in a 1.8% agarose gel, and the specificity of the products was determined by Southern blot analysis. Only the positive control cell line ECV 304 showed specific SCF transcripts. In all other cell lines, SCF transcripts remained undetectable using RT-PCR after 35 rounds of amplification followed by ethidium bromide staining, as well as using Southern blot analysis (Fig 1). MHH-BL-2 and BL-16 cells showed nonspecific low molecular weight PCR products for SCF using Southern blot analysis that were not seen on ethidium bromide-stained gels (data not shown).

RT-PCR and Southern blot analysis of c-kit mRNA. Expression of c-kit receptor mRNA was investigated using RT-PCR followed by Southern blot analysis. The positive control cell line MO7e showed detectable amounts of c-kit mRNA by RT-PCR on an ethidium bromide-stained agarose gel, whereas MHH-BL-1 and BL-18 cells showed low-level expression of c-kit mRNA only detectable by subsequent Southern blot analysis (Fig 2). These two cell lines were always positive. On repeat PCR examination, the fresh MHH-BL-1 cells and the cell lines BL-2 P3HR-1, BL-16, BL-18, BL-41, BL-41 B95-8, and BL-41 P3HR-1 were only in some experiments weakly positive, indicating a very low level of expression (data not shown). c-kit mRNA was never detectable in the negative control cell line HL-60\textsuperscript{20} (data not shown). No c-kit mRNA was detected in freshly isolated T and B cells and all other BL cell lines tested (Fig 2). The question of whether c-kit mRNA was inducible and not expressed constitutively was answered by the addition of PMA or A23187 to the BL cell cultures. Only the cell line MHH-BL-1 that already expressed c-kit mRNA showed a gradual upregulation of c-kit mRNA over the time of 48 hours in the presence of A23187 (Fig 3), whereas the addition of PMA led to a rapid downregulation after 1 hour (Fig 4). c-kit transcripts in unstimulated MHH-BL-1 cells are visible in Figs 2 and 4, in contrast to Fig 3, because of the longer exposure time of the blot.

Northern blot analysis of SCF and c-kit mRNA. Northern blot analysis of SCF mRNA expression showed the presence of 5.6-kb and 3.6-kb specific transcripts in the endothelial cell line ECV 304 and a 5.0-kb c-kit specific transcript in the megakaryoblastic cell line M07e used as positive controls. SCF and c-kit mRNA were not detected in the BL cell lines tested. To investigate the possibility that SCF and c-kit mRNA expression is not constitutive but can be induced to a higher level than that found using Southern blot analysis, we stimulated the cell lines with different reagents. Figures 5 and 6 show that the addition of IL-1β, TNF-α, antihuman IgM antibodies, PMA, A23187, and SAC cells to the cell lines for 5 hours before RNA extraction did not result in the induction of SCF and c-kit mRNA.

Receptor expression on BL cells. The SCF binding was determined by chemical crosslinking of Dig-rhSCF to its receptor. The experiment was performed with the cell line M07e used as a positive control and the cell lines MHH-BL-1 and BL-2 P3HR-1, which showed detectable c-kit mRNA expression using RT-PCR and Southern blot analysis. A cytokine-receptor complex of 165 to 170 kD was found only in MO7e cells, corresponding to the expected molecular weight of the c-kit receptor of approximately 150 kD. Specific binding of labeled SCF to its receptor could be shown by the addition of 100-fold molar excess of unlabeled SCF. BL cell lines showed no receptor-ligand complex after chemical crosslinking (Fig 7). Seven fresh BL cell preparations and the cell line MO7e were analyzed by FACS for the presence of c-kit receptors. All BL cells were gated for the CD10 antigen and found to express surface Igs. The CD117 antigen, the c-kit receptor, was found only on MO7e cells used as a positive control but on none of the BL cells (Fig 8). To determine whether A23187 treatment, which upregulates c-kit mRNA expression in MHH-BL-1 cells, would lead to the expression of c-kit receptors on the protein level, we treated MHH-BL-1 cells for 48 hours with A23187 before FACS analysis. CD117 protein remained undetectable in these stimulated cells (data not shown).

Mitogenic activity of rhSCF on BL cell lines. We tested
rhSCF on 13 BL cell lines and used the megakaryoblastic leukemia cell line M07e as a positive control in 1H-thymidine incorporation assays. A significant dose-dependent proliferative response to rhSCF was only detected in the M07e cell line. None of the BL cell lines tested responded to rhSCF (Fig 9).

**DISCUSSION**

The aim of this study was to determine the role of c-kit and SCF in childhood BL. Because of the potential clinical use of SCF in patients undergoing autologous bone marrow harvest, we investigated SCF and c-kit expression in childhood BL cell lines and fresh tumor biopsy cells. We detected spontaneous specific c-kit mRNA expression on a low level in 2 of 13 BL cell lines by RT-PCR followed by Southern blot analysis. Other cell lines showed low-level expression of c-kit mRNA only with the highly sensitive RT-PCR method followed by Southern blotting and even then not in all experiments (data not shown). In only 1 of the cell lines, MHH-BL-1, a c-kit-specific PCR product was visible after 35 cycles of PCR in an ethidium bromide-stained gel. None of the BL cell lines showed hybridization to a c-kit-specific probe by Northern blot analysis with total RNA. This finding indicates absent or only very low level
spontaneous expression of c-kit on the mRNA level in the majority of the BL cells tested.

The possibility, that c-kit is not expressed spontaneously but requires induction by other cytokines or factors present in vivo was explored by in vitro stimulation experiments. BL cells in vivo are exposed to many other factors and cytokines that theoretically could serve as costimulatory factors for SCF by inducing its receptor, especially because low-level expression of c-kit mRNA was detected in some of the BL cells. In our experiments, the cytokines IL-1β and TNF-α did not induce the expression of c-kit and SCF mRNA as detected by Northern blot analysis. Both cytokines had been found to be highly expressed in lymph nodes from lymphoma patients. These locally produced cytokines were considered most likely to be biologically relevant, because BL cells were also known to express the receptors for IL-1 and TNF-α. In endothelial cells, we previously used these cytokines to induce the expression and showed regulation of SCF and c-kit mRNA by IL-1. In the experiments with BL cells, the expression of c-kit could not be induced as detected by Northern blot analysis in BL cells treated with the IgM receptor crosslinking reagents anti-IgM and SAC cells but not by the protein kinase C activator PMA and the calcium ionophore A23187. In addition, 2 of the EBV-negative BL cell lines infected in vitro by the B95-8 immortalizing strain and the nonimmortalizing variant of EBV, strain P3HR-1, showed no induction of c-kit and SCF mRNA.

To detect c-kit regulation on a low level, we performed RT-PCR followed by Southern blot analysis. Only MHH-BL-1 cells showed an upregulation of c-kit mRNA in the presence of A23187 and a downregulation in the presence of PMA, c-kit mRNA could also be upregulated by IL-7 in some IL-7 receptor-positive BL cell lines (unpublished observation).

To further investigate the biologic significance of this mRNA expression, we examined c-kit expression on the protein level using FACs analysis and crosslinking experiments and examined the proliferative effect of SCF on BL cells using "thymidine incorporation. The receptor was undetectable on 7 freshly isolated BL cells using FACs analysis and using crosslinking experiments in two BL cell lines with low-level c-kit expression. rhSCF showed no mitogenic effect on the 13 cell lines tested. To find out whether the absence of a proliferative response to exogenous rhSCF could be explained by the autocrine production of SCF or by binding of endogenous SCF to intracellular c-kit receptors leading to its constitutive activation, we performed RT-PCR from unstimulated BL cells and Northern blot analysis from unstimulated and stimulated BL cells to detect SCF transcripts. Specific SCF mRNA transcripts were not detectable in stimulated and unstimulated BL cell lines using Northern blot analysis or RT-PCR followed by Southern blotting (data from stimulated cells investigated by RT-PCR and Southern blot analysis not shown). From these results we conclude that the low-level c-kit expression detected on the mRNA level has little biologic significance, because c-kit mRNA is neither expressed nor inducible in the majority of BL cells and because no protein expression or changes in proliferation were observed on addition of rhSCF.

Some cytokines involved in normal lymphopoiesis can also be involved in the growth of B-cell malignancies. Whether SCF acts synergistically with IL-6 and IL-7 in malignant lymphopoiesis has not been investigated in detail. The autocrine stimulation by IL-6 has been shown in a small number of B-cell lymphomas. The distribution of both IL-7 receptor and c-kit receptor has been investigated in acute lymphoblastic leukemia cells. Although IL-7 receptors could be detected on the surface of these malignant cells, no c-kit mRNA or surface c-kit protein was expressed. Expression of both c-kit and SCF was detected in neoplastic cells from human colon carcinomas, leading to the suggestion that the autocrine production of SCF plays a role in the growth of some human solid tumor cell lines.

BL cells appear to be derived from more mature B cells that express Igs on their surface. If they behaved like their normal counterparts, they would not be expected to express the c-kit receptor. Normal B cells that had progressed in their differentiation to light-chain rearrangement were found not to express the receptor for SCF and IL-7 any more. Consistent with these findings in normal cells, we were not able to detect c-kit mRNA in freshly isolated normal
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Fig 8. Surface marker analysis of freshly isolated BL cells. Purified BL cells were first stained with MoAbs against the indicated cell surface antigens and then with an FITC-conjugated F(ab')2 rabbit antimouse IgG and analyzed by flow cytometry.

B cells by 35 cycles of RT-PCR. However, it is not clear whether the process of malignant transformation leads to the persistence of less mature properties in BL cells. Support of this assumption is given by the recent findings showing detection of IL-7 receptor mRNA in BL cell lines. Moreover, whereas IL-7 receptor-positive BL cells are able to respond to rhIL-7, SCF did not act as a costimulatory factor in these cells (unpublished observation).

Fig 9. Effect of rhSCF on proliferation of BL cell lines. Cultures were maintained for 72 hours in serum-free medium in the presence of increasing amounts of SCF, pulsed for 4 hours with 0.5 μCi 3H-thymidine, and harvested for liquid scintillation counting. Data are the means and standard errors of three independent samples. The megakaryoblastic leukemia cell line M07e was used as a positive control.
There are several observations that suggest that SCF is not mandatory for B-lineage expansion in vivo. First, in normal mice treated with a neutralizing antibody against c-kit, almost all hematopoietic progenitor cells disappeared from the bone marrow, leading to a failure to produce normal numbers of erythroid and myeloid cells, whereas B-cell lymphopoiesis was not significantly affected. Second, adult W locus anemic mice, which have functional mutations at the c-kit locus, maintain normal numbers of pre-B and B cells throughout postnatal life. These experiments show that B lymphopoiesis is possible in the absence of functional SCF or c-kit. Nevertheless, SCF can be a costimulatory factor for IL-7 in the proliferation of a subpopulation of early B cells. Another subpopulation of B-lineage cells does not require SCF and proliferates in response to IL-7 alone.

From our data we conclude that the lack of c-kit expression on the protein level in BL cells is consistent with their more mature B-cell phenotype. Also, during the process of malignant transformation, the expression of c-kit as a marker of an immature phenotype was not maintained, as apparently happened in the case of the IL-7 receptor. Pinto et al. found that expression of c-kit receptor in human lymphomas is restricted to Hodgkin’s disease and anaplastic large-cell lymphomas (ALCL). No c-kit expression was found in lymphoid cells of reactive lymph nodes, normal tonsils, and in tumor cells from 24 cases of non-Hodgkin’s lymphoma other than ALCL.

From our studies we conclude that c-kit and SCF play no role in the process of malignant transformation and in the growth of the pediatric BL cells investigated. Based on these results, we do not expect a direct negative influence of rhSCF on chemotherapeutic treatment outcome in pediatric BL. We also think that a direct stimulation of childhood BL cells or SCF-induced direct mobilization of BL cells during an autologous peripheral stem cell harvest primed by SCF is unlikely. Secondary cytokine effects induced by the treatment of a patient with SCF, such as activation of endothelial cells and stromal elements, cannot be excluded based on our results. Whether a relevant influence on growth of the lymphoma and on treatment outcome arises from the administration of this cytokine can finally be shown only in clinical treatment studies.

ACKNOWLEDGMENT

We thank Dr G.W. Bornkamm (GSF, Munich, Germany) for discussion about growth conditions of lymphoma cells, Dr K.M. Zsebo (Amgen) for the supply of rhSCF, and Dr H.-J. Bühring (Tübingen, Germany) for the supply of CD117 MoAb.

REFERENCES

21. Zsebo KM, Wypych J, McNiece IK, Lu HS, Smith KA, Karkare SB, Sachdev RK, Yuschenkoff VN, Birkett NC, Williams LR,


38. Steel CM, Hutchins D: Soluble factors and cell-surface mole-
the kit ligand growth factor is determined by alternative splicing and is missing in the Sid mutant. Cell 64:1025, 1991
68. Buhring HJ, Ullrich A, Schaudt K, Muller CA, Busch FW: The product of the proto-oncogene c-kit (P145c-kit) is a human bone marrow surface antigen of hematopoietic precursor cells which is expressed on a subset of acute non-lymphoblastic leukemic cells. Leukemia 5:854, 1991
Absence of c-kit receptor and absent proliferative response to stem cell factor in childhood Burkitt's lymphoma cells

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