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High expression of the chemokine receptor CXCR4 predicts extramedullary organ infiltration in childhood acute lymphoblastic leukaemia

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Summary. Childhood acute lymphoblastic leukaemia (ALL) is a malignancy with the potential to infiltrate the liver, spleen, lymph nodes and brain. Such extramedullary presentation is important for understanding the biology of childhood ALL and also for developing new prognostic parameters. A potential mechanism in the trafficking of leukaemia cells is the interaction of the chemokine receptor CXCR4, which is expressed on ALL cells, and its ligand stromal cell-derived factor-1 (SDF-1), produced by stromal cells in bone marrow and extramedullary organs. Functionality of CXCR4 was demonstrated by a high correlation between cell surface density of CXCR4 and transendothelial migration of leukaemia blasts towards a gradient of SDF-1 ($r = 0{\cdot}73$, $P = 0{\cdot}001$). Inhibition of SDF-1-induced migration by an anti-CXCR4 monoclonal antibody ($78{\cdot}33 \pm 23{\cdot}86\%$ inhibition) evidenced the specificity of CXCR4 to SDF-1. In order to evaluate clinical significance of CXCR4 expression, lymphoblasts from the bone marrow of 73 patients with and without extramedullary organ infiltration were compared. Multiparameter flow cytometry revealed that lymphoblasts from patients with high extramedullary organ infiltration, defined as ultrasonographically measured enlargement of liver or spleen, expressed the CXCR4 receptor at higher fluorescence intensity (median $66{\cdot}12 \pm 66{\cdot}17$) than patients without extramedullary organ infiltration (median $17{\cdot}56 \pm 19{\cdot}29$; $P < 0{\cdot}001$). Consequently, high expression of CXCR4 was strongly predictive for extramedullary organ involvement, independently of the peripheral lymphoblast count. Highest CXCR4 expression was seen in mature B ALL (median $102{\cdot}74 \pm 92{\cdot}13$; $P < 0{\cdot}003$), a disease characterized by a high incidence of extramedullary bulky disease. As high expression of the chemokine receptor CXCR4 predicts extramedullary organ infiltration in childhood ALL, we suggest that CXCR4 and its ligand play an essential role in extramedullary invasion.

Keywords: CXCR4, SDF-1, ALL, childhood, organ infiltration.

Acute lymphoblastic leukaemia (ALL) is the most common childhood malignancy (Young *et al*, 1986). Although marrow replacement is the major cause of symptoms of leukaemia, many important syndromes result from extramedullary invasion. Such infiltration may easily be clinically apparent: at initial diagnosis, 30% to 50% of children have enlargement of liver or spleen (Reiter *et al*, 1994). Although fewer than 5% of children with acute lymphoblastic leukaemia have clinical central nervous system involvement at the time of diagnosis (Bleyer & Poplack, 1985; Kreuger *et al*, 1991), this remains the most common site of clinically apparent extramedullary leukaemia. The involvement of extramedullary organs during bone marrow remission is of great importance, both therapeutically and prognostically. Occult leukaemia cells from extramedullary sites may seed other sites, including the bone marrow, and may result in haematological or extramedullary relapse. Although extramedullary relapse may occur at any site, the central nervous system (CNS) is the most common location (Mathe *et al*, 1966; Baum *et al*, 1979; George *et al*, 1985). Therefore, prognostic features at diagnosis, as well as recognition and investigation of the mechanism of extramedullary dissemination, continue to be crucial issues in the management of affected patients.

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Generally, it is thought that the degree of organ infiltration correlates with peripheral blast cell count, thus reflecting the total leukaemia mass (Reiter *et al*, 1994). Although many studies have been performed to elucidate the pathophysiology of leukaemia, trafficking of lymphoblastic leukaemia cells is not well understood. Mechanisms for the extravasation of lymphoblastic cells may resemble processes that are widely used by normal lymphocytes for trafficking and homing between the blood and lymphoid tissues. Recent studies reveal a multistep process that requires the sequential engagement of adhesion molecules and the activation through chemokine receptors (Peled *et al*, 2000). These steps are thought to be critical for extravasation and homing to distinct lymphoid-tissue microenvironments that provide supportive growth and regulatory factors. There is substantial evidence from *in vitro* and *in vivo* experiments that the stromal cell-derived factor-1 (SDF-1) plays an important role in leucocyte trafficking. It belongs to the CXC chemokine family, which is characterized by an intervening residue separating the first two cysteine residues within a conserved motif (Tashiro *et al*, 1993; Bleul *et al*, 1996a,b; Oberlin *et al*, 1996; Nagasawa *et al*, 1998). In contrast to other members of the CXC chemokine family that are produced upon cytokine stimulation (e.g. increased interleukin 8 expression during inflammation), SDF-1 is constitutively produced by stromal cells. SDF-1 is not only restricted to the bone marrow, but is also found in other tissues such as lymph nodes, liver, spleen and brain in mouse (Aiuti *et al*, 1997) and human organs (Mueller *et al*, 2001). SDF-1 signals through a G protein-coupled receptor termed CXCR4. The chemokine receptor CXCR4 has been shown to be expressed on different leucocytes including thymocytes, naive and activated T cells, Th1 and predominantly Th2 cells, B-cell progenitors, naive and activated B cells (Aiuti *et al*, 1999). SDF-1/CXCR4 probably contribute to the extravasation of leucocytes in the absence of inflammation, which is important for lymphocyte trafficking.

In contrast to other chemokines, the interaction of SDF-1 and CXCR4 appears to be specific without cross-reactivity with other chemokines or chemokine receptors, resulting in a similar phenotype of SDF-1- and CXCR4-deficient mice (Nagasawa *et al*, 1996; Ma *et al*, 1998; Zou *et al*, 1998). Mice lacking the gene encoding SDF-1 or CXCR4 have dramatically reduced bone marrow haematopoiesis, suggesting that the release of SDF-1 by bone marrow stromal cells and the expression of CXCR4 on haematopoietic stem cells support the tropism, particularly for the bone marrow. In addition to haematopoietic stem cell homing, SDF-1/CXCR4 are also involved in embryogenesis, including heart development, neuronal cell migration and vascular development (Nagasawa *et al*, 1996; Ma *et al*, 1998; Tachibana *et al*, 1998; Zou *et al*, 1998).

Recently we have shown that CXCR4 is expressed on $CD34^+$ haematopoietic progenitor cells including primitive, multipotential progenitors (Möhle *et al*, 1998), and also variably in malignant $CD34^+$ myeloid precursor cells (e.g. acute myeloid leukaemia, AML), adult acute and chronic lymphoblastic leukaemia (CLL) (Möhle *et al*, 1998, 1999, 2000). CXCR4 expressing $CD34^+$ progenitor cells as well as leukaemia cells respond to SDF-1 with increased transendothelial migration.

As the chemokine receptor CXCR4 is variably expressed on malignant leukaemia cells and SDF-1 is constitutively produced by fibroblastic stromal cells, one might speculate that this chemoattractant might contribute to the tropism of malignant leukaemia cells to appropriate microenvironments depending on the expression intensity of CXCR4 on malignant lymphoblasts.

In order to evaluate the importance of CXCR4 expression in leukaemia cell development and trafficking, we analysed CXCR4 receptor expression in a large series of lymphoblasts from paediatric ALL cases and compared the data with clinical different presentations.

PATIENTS AND METHODS

Patients. Seventy-three patients with newly diagnosed childhood ALL treated according to Berlin–Frankfurt–Munster trials ALL-BFM-90, ALL-BFM-95, and NHL-BFM-90 and NHL-BFM-95 were included in the study following informed consent. The patient characteristics (Table I) are in accordance with previously published data (Reiter *et al*, 1994). According to concepts of normal liver and spleen size in children, significant organ infiltration at initial diagnosis was defined by the ultrasonographic extension of the liver edge $\geqslant 2$ cm below the right costal margin in the right midclavicular line or the spleen $\geqslant 2$ cm below the left costal margin (Balistreri, 2000).

Leukaemia cells. The diagnosis of leukaemia was based on routine morphological evaluation and on cytochemical smears, as well as on immunophenotyping according to the criteria of the European Group for the Immunological Characterization of Leukaemia (EGIL) (Bene *et al*, 1995) (Table I). Within the B-immunophenotype, leukaemia blasts were classified into four subtypes based on the expression of a pattern of lineage-associated antigens: early CD10-negative pre-B blasts (κ^-/λ^-, $CD19^+$, $CD10^-$), early CD10-positive pre-B blasts (κ^-/λ^-, $CD19^+$, $CD10^+$), pre-B blasts (κ^+/λ^+, $CD19^+$, IgD^-) and mature B-blasts (κ^+/λ^+, $CD19^+$, IgD^+). Primary ($n = 73$) acute leukaemia cells were obtained from bone marrow samples, stored in liquid nitrogen, fine needle biopsy of thymus ($n = 1$), testes ($n = 1$) and lumbar puncture of cerebrospinal fluid ($n = 1$).

Peripheral blood mononuclear cells. Control peripheral blood mononuclear cells (PBMCs) were obtained after informed consent from healthy children ($n = 10$) undergoing surgical treatment and separated by Ficoll density gradient centrifugation.

Cell lines. The T-ALL cell line Jurkat (a kindly gift from Kofler Reinhard) was cultured in Roswell Park Memorial Institute (RPMI)-1640 medium (Life Technologies, Paisley, UK) supplemented with 10% fetal calf serum (FCS). The cells were incubated at 37°C in a fully humidified atmosphere containing 5% CO_2 in air and passaged weekly.

Cell counts. Cell numbers and concentrations were assessed using a haemocytometer or automated cell

Table I. Patient characteristics of children with newly diagnosed ALL.

	Early pre-B CD10$^-$	Early pre-B CD10$^+$	Pre-B	Mature B	Pre-T/T
Number of patients	11	18	20	7	17
Sex (% male)	27·3	44·4	40·0	71·4	88·2
Age (years)					
$<$ 1 (%)	45·5	5·5	5·0	42·9	5·9
1–6 (%)	36·4	72·2	65·0	42·9	35·3
$>$ 6 (%)	18·1	22·2	30·0	14·3	58·8
White blood cell: median ($\times 10^9$/l)	143·49	55·36	55·44	67·14	110·4
Platelets $\leqslant 100 \times 10^9$/l (%)	80·0	77·7	80·0	100	83·3
Haemoglobin $\leqslant 8$ g/dl (%)	60·0	61·1	62·5	20·5	50·0
Splenomegaly* (%)	72·7	44·4	35·0	85·7	52·9
Hepatomegaly* (%)	63·6	61·1	55·5	85·7	52·9
Mediastinal mass (%)	0	5·5	0	0	52·9
Lymphadenopathy (%)	54·5	61·1	25·0	85·7	64·7
Central nervous system disease (%)	18·2	11·1	0	14·3	11·8

*Ultrasonographic extension of the liver edge $\geqslant$ 2 cm below the right costal margin in the midclavicular line or the spleen $\geqslant$ 2 cm below the left costal margin.

counter. The viability of the cells was determined by Trypan Blue dye exclusion.

Human cytokines. Recombinant human SDF-1β was obtained from R & D Systems (Vienna, Austria) and used at a final concentration of 500 ng/ml for chemotaxis assay.

Flow cytometry analysis. A total of 1–2 $\times 10^5$ cells were saturated with purified normal mouse Ig (MOPS; Sigma-Aldrich, Austria) at room temperature for 10 min The cells were then incubated for 30 min at 4°C with the fluorescence isothiocyanate (FITC) or phycoerythrin (PE)-conjugated monoclonal antibodies (mAb) CD7-FITC (clone 4H9), CD10-FITC (clone W8E7), CD19-FITC (clone SJ25C1) (all from Becton Dickinson, Vienna, Austria), and CXCR4-PE (clone 12G5; Pharmingen, Hamburg, Germany). Isotype-identical antibodies served as controls (IgG_1 and IgG_{2a}, FITC-/PE-conjugated; Becton-Dickinson). Cell-associated immunofluorescence was measured using a FACSCAN flow cytometer (Becton Dickinson, San Diego, CA, USA) and analysed using the CELL QUEST software package. To calculate the percentage of CXCR4-positive cells, a proportion of 1% false positive events was accepted in the negative control. The median fluorescence intensity was calculated from the fluorescence histogram and expressed in arbitrary units. To discriminate lymphoblasts from the remaining non-malignant cells, co-expression analysis of lineage-associated antigens such as CD19, CD10 and CD7 was used. The flow cytometer was calibrated with CaliBRITE-3 beads (Becton Dickinson Immunocytometry Systems) and FACSCOMP Software (Becton Dickinson Immunocytometry Systems).

Immunocytochemistry. Cytospin preparations of cerebrospinal fluid (CSF) and bone marrow aspirates were put into Tris buffer for 5 min, and refixed with 1% paraformaldehyde for 10 min. After rinsing in Tris buffer, the slides were incubated with CXCR4 antibody at 4°C for 21 h. After rinsing in Tris buffer, the detection system Histo Cons (Consortia Laboratories, Verona, Italy) was applied according to the product protocol.

Transendothelial migration. Transendothelial migration across endothelium *in vitro* was analysed as described previously (Möhle *et al*, 1998). All assays were performed in quadruplicate. The human microvascular endothelial cell line 5A32 was cultivated in RPMI-1640 medium supplemented with 10% FCS. For the transmigration assays 5×10^5 cells from the endothelial cell line 5A32 were seeded on 3-μm transwell microporous membranes (Transwell, Corning-Costar, Bodenheim, Germany), forming a confluent monolayer after 3–4 d. The transwell inserts were then placed in a six-well tissue culture plate, thus separating an upper from a lower chamber in each well. To perform the migration assay, conditioned medium from the SDF-1-producing murine bone marrow stromal cell line MS-5 (0·2 ml medium per cm^2 confluent MS-5 layer incubated for 24 h) was added to the lower chamber. Leukaemia cells (5×10^5) were added to the upper chamber and incubated for 10 h at 37°C in 5% CO_2 and 95% air. The upper chamber was then carefully removed and cells in the bottom chamber recovered for counting or flow cytometry. In additional experiments, recombinant SDF-1 (rhSDF-1β, Biomedica, Vienna, Austria) was added to the lower chamber of the transmigration system at a final concentration of 500 ng/ml. Furthermore, the effect of a partially blocking CXCR4 antibody (clone 12G5, Pharmingen, Los Angeles, CA, USA) on transendothelial migration in

Table II. Classification of the 73 patients according to the expression of CXCR4 and the peripheral lymphoblast count.

Group	1	2	3
CXCR4 median fluorescence intensity	> 54·63	< 54·63	< 54·63
Peripheral lymphoblast count ($\times 10^9$/l)	Independent	< 1·14	> 1·14
Number of patients	27	7	36
Patients with significant organ infiltration* (number)	26	0	20
Patients with significant organ infiltration* (%)	96·29	0	55·56

*Ultrasonographic extension of the liver edge ≥ 2 cm below the right costal margin in the midclavicular line or the spleen ≥ 2 cm below the left costal margin.

High expression of CXCR4 (> 54·63 median fluorescence intensity; group 1) predicts for 96·33% of the patients extramedullary organ infiltration, whereas no organ infiltration has been diagnosed in patients with low expression (< 54·63 median fluorescence intensity) and low peripheral lymphoblast count (< $1{\cdot}14 \times 10^9$/l; group 2). Low expression of CXCR4 as well as peripheral lymphoblast count were not predictive for tumour cell dissemination (group 3).

response to either MS-5-conditioned medium or rhSDF-1β was assessed. The antibody was incubated with the leukaemia cells at a final concentration of 10 ng/ml before performing the transmigration assays.

Statistics. Statistical analyses were performed with SPSS FOR WINDOWS 95. The statistical significance of the data obtained was analysed using the Mann–Whitney test. A *P*-value $< 0{\cdot}05$ was considered significant. Box-plots were used for graphic demonstration. The box gives the interquartile range (50% of all values were within this range). With the method of classification trees, the 73 patients were divided into three groups according to the expression of CXCR4 (median fluorescence intensity) and the peripheral lymphoblast count (Table II).

RESULTS

Variable amounts of CXCR4 are expressed on primary childhood ALL blasts isolated from bone marrow

Primary leukaemia blasts from bone marrow of children with ALL expressed variable amounts of CXCR4. Representative fluorescent profiles for CXCR4 expression are shown in Fig 1 as ALL1–9 respectively. The majority (91·9%) of ALL patients showed expression of the chemokine receptor with an average median fluorescence intensity of 33·08 ± 60·37(± SD). Highest percentage of CXCR4 expressing cells was noted on blasts derived from mature B-ALL (Fig 2) with 97·90% ± 3·60 of cells being positive ($P < 0{\cdot}002$) and an average 102·74 ± 92·13 median fluorescence intensity ($P < 0{\cdot}003$). No statistical difference in chemokine receptor expression was observed between the remaining immunophenotypes of Pre-B ALL and T-ALL (data not shown). Comparing expression of CXCR4 on lymphoblasts from mature B-ALL with B-lymphocytes from healthy donors showed higher expression on mature B-blasts ($P < 0{\cdot}07$).

SDF-1 supports transendothelial migration of childhood ALL blasts in vitro

In order to evaluate receptor function, the ability of SDF-1 to induce migration through an endothelial cell layer *in vitro*

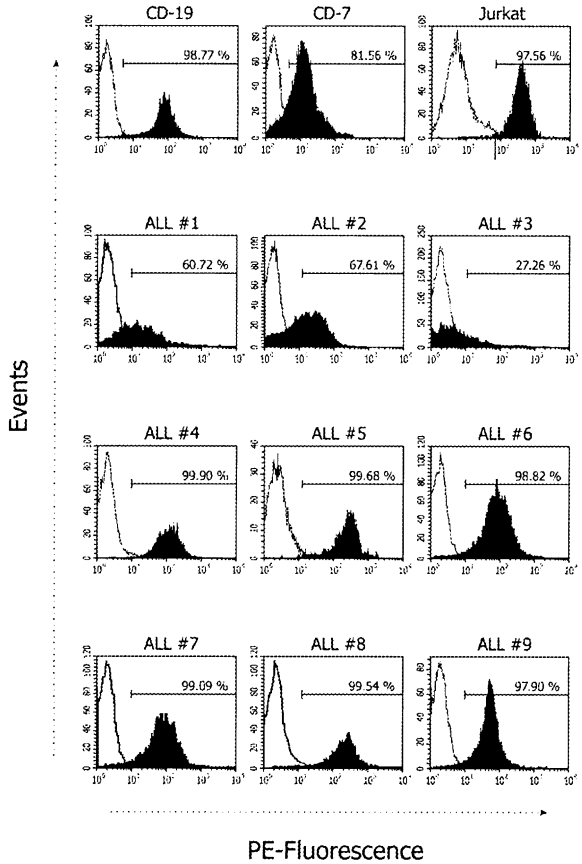

Fig 1. Selected examples of the analysis of CXCR4 expression using flow cytometry. The results are presented as fluorescence histograms (line, immunoglobulin G control; shading, CXCR4 expression). Panel 1: receptor expression on $CD19^+$ cells and $CD7^+$ cells of healthy children and on the T-leukaemia cell line Jurkat; Panel 2: bone marrow blasts of childhood ALL with low CXCR4 expression: ALL #1: early $CD10^-$ pre-B; ALL #2: early $CD10^+$ pre-B; ALL #3: pre-B (ALL#1–3 without significant organ infiltration at initial diagnosis); Panel 3: bone marrow blasts of childhood ALL with high expression: ALL #4: pre–B; ALL #5: mature B; ALL #6: T-ALL (ALL#4–6 with significant organ infiltration at initial diagnosis). Panel 4: blasts of childhood ALL isolated from extramedullary tissues with high expression: ALL #7 from testes, ALL #8 from thymus and from bone marrow of a child with first bone marrow relapse (ALL #9).

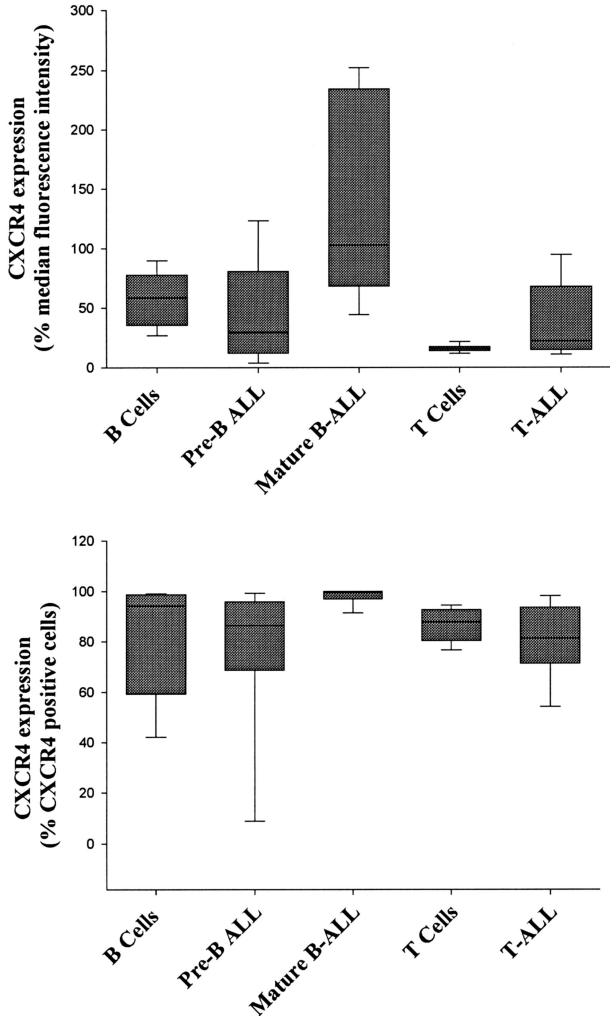

Fig 2. CXCR4 expression on peripheral mononuclear cells of healthy children (B Cells: $n = 10$; T Cells: $n = 10$) and lymphoblasts of ALL patients (Pre-B ALL: $n = 49$; Mature B-ALL: $n = 7$; T-ALL: $n = 17$). The median fluorescence intensity of each group is given as mean ± standard deviations.

was examined. The average proportion of ALL blasts with high expression of CXCR4 (median fluorescence intensity $> 54{\cdot}63$) that migrated to the lower chamber was $7{\cdot}69\% \pm 3{\cdot}11$ of input cells (mean ± SD, $n = 13$ patients) compared with the average proportion of migrating ALL blasts with low expression of CXCR4 (median fluorescence intensity $< 54{\cdot}63$; migration index: $3{\cdot}58\% \pm 2{\cdot}20$, $n = 13$ patients). In contrast, the proportion of input cells that migrated to control chambers without SDF-1 was $1{\cdot}27\% \pm 0{\cdot}76$. (Fig 3: ALL#1–ALL#4: representative examples of SDF-1-induced transendothelial migration). According to the higher expression of CXCR4 on the control T-ALL cell line Jurkat, these cells migrated more avidly in response to SDF-1 compared with childhood ALL blasts (Fig 3: Jurkat). Results of SDF-1-induced transendothelial migration of Jurkat cells were similar to those of previously published data (Möhle *et al*, 1998).

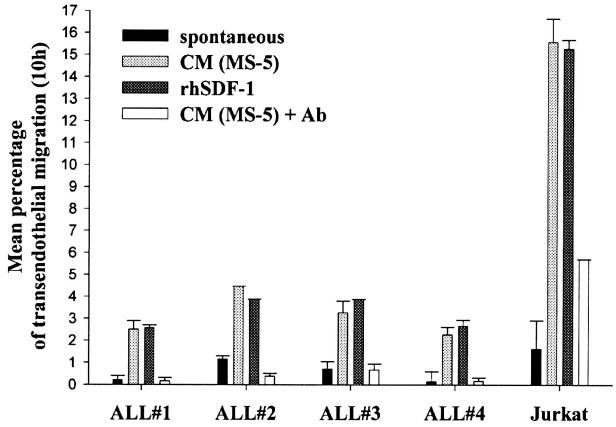

Fig 3. Selected examples of transendothelial migration of ALL-lymphoblasts (ALL#1–4) and T-leukaemia cell line Jurkat. A total of 5×10^5 cells were added to the upper chamber of the transmigration system. After 10 h, transmigrated cells were recovered from the lower chamber and counted. Spontaneous migration (black) was compared with migration induced by MS-5-conditioned medium (light grey), rhSDF-1β induced migration without (grey) and with preincubation with the CXCR4-blocking antibody 12G5 (white).

The chemotactic effect of MS-5-conditioned medium on ALL blasts is owing to SDF-1

Lymphoblasts migrated across endothelium in response to SDF-1-containing conditioned medium from the murine stromal cell line MS-5 almost as efficiently as in response to recombinant human SDF-1β (Fig 3: ALL#1–ALL#4, Jurkat). To prove the specificity of the effects of rhSDF-1β and of the MS-5-conditioned medium, blasts were incubated with a blocking monoclonal antibody against CXCR4 (12G5) before performing the chemotaxis assays. Figure 3 shows that the chemotactic response of blasts to SDF-1 could be efficiently inhibited by 12G5, indicating that CXCR4 is the relevant receptor for SDF-1 and that CXCR4 is functionally active.

The expression level of CXCR4 correlates with transmigration in response to SDF-1

A positive correlation ($r = 0{\cdot}73$, $P < 0{\cdot}001$) between the SDF-1-induced transendothelial migration (percentage of cells transmigrating in response to MS-5-conditioned medium) and the cell surface density of CXCR4 (as reflected by the median fluorescence intensity) on various immunophenotypes of ALL indicated that the chemokine receptor was functionally active in malignant ALL blasts (Fig 4).

Organ infiltration in paediatric ALL patients is associated with higher CXCR4 expression on leukaemia blasts

For investigating the influence of CXCR4 expression on ALL blasts on organ infiltration, patients were divided into two groups according to the degree of organ enlargement at the time of diagnosis. Significant leukaemia organ infiltration of liver or spleen ($n = 49$) was associated with a higher CXCR4 receptor expression density (median fluorescence intensity = MFI) ($P < 0{\cdot}001$) and a higher percentage of CXCR4-positive cells ($P < 0{\cdot}001$) on lymphoblasts (Fig 5A

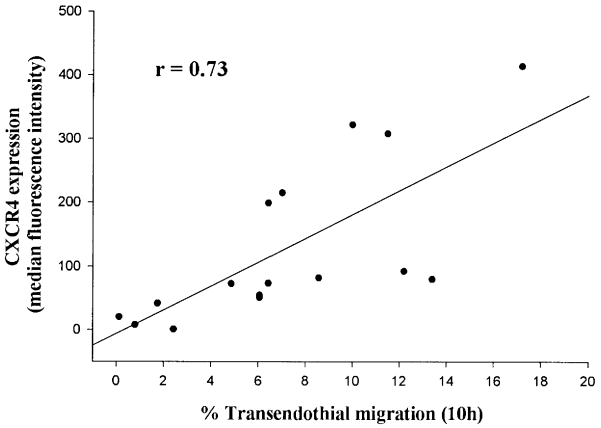

Fig 4. Correlation between CXCR4 expression and the SDF-1-induced transendothelial migration of ALL lymphoblasts. A positive correlation of $r = 0{\cdot}73$ ($P = 0{\cdot}001$) was found between the expression level of CXCR4 (measured as median fluorescence intensity) and the percentage of ALL lymphoblasts transmigrating in response to SDF-1 (MS-5 conditioned medium in the lower chamber of the transmigration system) ($n = 17$).

and B) compared with insignificant organ infiltration ($n = 24$). The same results were obtained when pre-B-ALL and T-ALL were compared separately for CXCR4 expression intensity and percentage of CXCR4 positive blasts [pre-B-ALL: $P < 0{\cdot}009$ (MFI), $P < 0{\cdot}01$ (%); and T-ALL: $P < 0{\cdot}02$ (MFI), $P < 0{\cdot}8$ (%)]. Interestingly no statistical difference between patients with significant versus insignificant organ infiltration was noted regarding the peripheral lymphoblast count (Fig 5C). With the method of classification, high expression of CXCR4 (median fluorescence $> 54{\cdot}63$) was highly predictive for extramedullary organ infiltration independently of peripheral lymphoblast count (Table II). Interestingly, patients with low CXCR4 expression (median fluorescence $< 54{\cdot}63$) and low peripheral lymphoblast count ($< 1{\cdot}14 \times 10^9$ cells/l) had no dissemination of ALL cells in extramedullary organs. Within patients with low expression of CXCR4 and differing peripheral lymphoblast counts, significant organ infiltration could not be further predicted by the above-mentioned parameters (Table II).

Expression of CXCR4 on blasts isolated from extramedullary tissues

For further investigation of the role of CXCR4 in extramedullary leukaemia, blasts isolated from extramedullary tissues were investigated for CXCR4 expression. For this purpose blasts from the testes and thymus of two different patients were investigated. CXCR4 was found to be expressed in significant amounts on the blasts from the testes and thymus with MFI/percentage of positive cells of 207·2/99·5% and 110·0/98·53% respectively (Fig 1). Immunocytochemistry analysis of cytospin preparations of cerebrospinal fluid from four patients with CNS relapse (defined on the base of more than

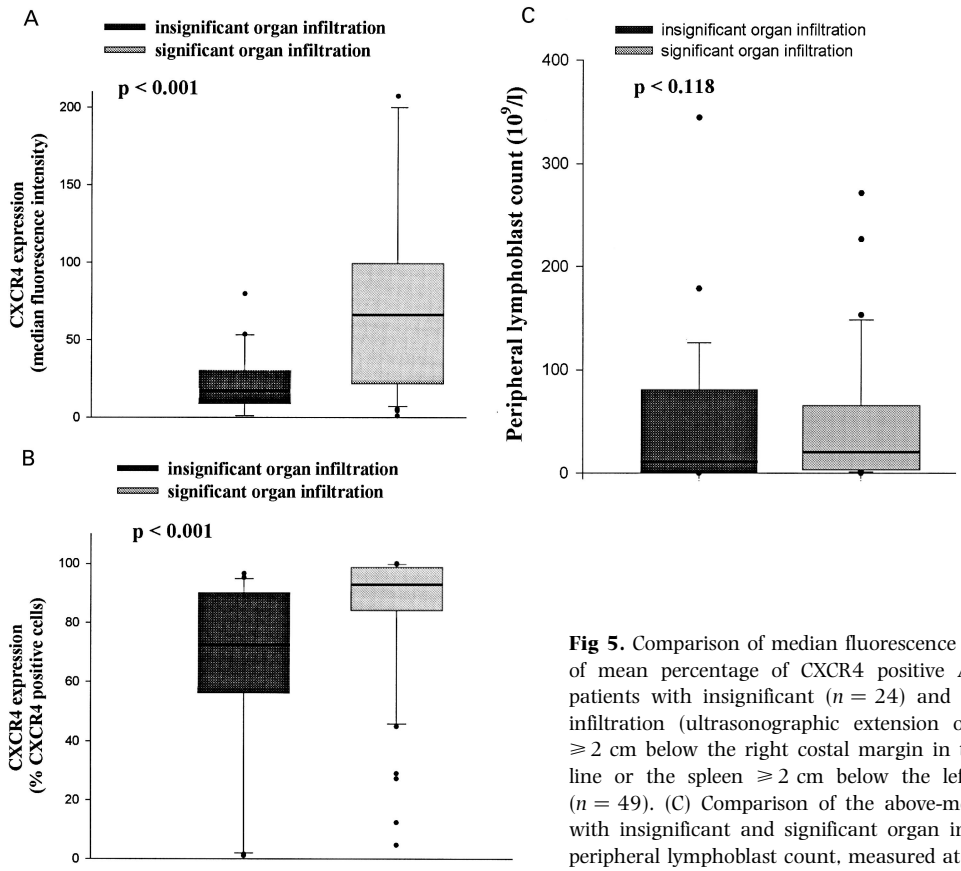

Fig 5. Comparison of median fluorescence intensity (A) and of mean percentage of CXCR4 positive ALL-blasts (B) of patients with insignificant ($n = 24$) and significant organ infiltration (ultrasonographic extension of the liver edge ≥ 2 cm below the right costal margin in the midclavicular line or the spleen ≥ 2 cm below the left costal margin) ($n = 49$). (C) Comparison of the above-mentioned patients with insignificant and significant organ infiltration for the peripheral lymphoblast count, measured at diagnosis.

5 leucocytes/μl with morphologically unequivocal lymphoblasts) depicted strong CXCR4 expression by tumour cells, indicating high levels of CXCR4 expression for lymphoblasts migrated to extramedullary sites (data not shown).

DISCUSSION

Research on chemokines has progressed rapidly during the last 3 years and the list of new chemoattractants capable of supporting the *in vitro* migration of malignant lymphoblasts is growing (Legdeur *et al*, 1997; Möhle *et al*, 1998; Tanaka *et al*, 1998; Burger *et al*, 1999; Trentin *et al*, 1999). Limited information, however, is available with regard to the biological and clinical importance for the locomotion of leukaemia cells. For example, chronic as well as acute lymphocytic leukaemia B cells have been reported to express the chemokine receptor CXCR4 and to migrate after stimulation with the stromal cell-derived factor-1 (Möhle *et al*, 1999, 2000; Nishii *et al*, 1999; Burger *et al*, 1999; Bradstock *et al*, 2000).

Here, we report for the first time that overexpression of the chemokine receptor CXCR4 on malignant acute leukaemia cells is associated with extramedullary organ infiltration. This conclusion is supported by an analysis of lymphoblasts obtained from the bone marrow from a wide series of 73 clinically different childhood ALL patients. Functionality of the chemokine receptor CXCR4 was confirmed *in vitro* through SDF-1-induced transendothelial migration.

Childhood acute lymphoblastic leukaemia is a malignant lymphoproliferative disease, which arises from the deregulated clonal expansion of immature lymphoid progenitor cells that are blocked at a particular stage of differentiation (Dow *et al*, 1985). The presumed normal counterparts of malignant acute lymphoblastic B cells, i.e. developing bone marrow B cells, have recently been studied on the expression and function of CXCR4 (Fedyk *et al*, 1999; Honczarenko *et al*, 1999). Expression of the chemokine receptor was found to be sinusoidal: highest on pre-B cells, decreased as cells developed into immature B cells, and then increased again upon transition to the mature B-cell stage. Interestingly, the migration responses induced by the corresponding ligand SDF-1 was disproportionate to the level of CXCR4 expression, showing highest responsiveness in the pre-B cell subtype. In our study we found a similar CXCR4 expression pattern on lymphoblastic subpopulations. Highest expression was noted in the mature-B immunophenotype, low expression in pre-B ALL. In contrast to the findings in normal bone marrow cells, our data revealed a functional chemokine receptor on lymphoblasts, even on mature B-ALL blasts, which showed high migration indices, accounting for the strong correlation between CXCR4 expression and SDF-1-induced transendothelial migration. SDF-1-induced migration of CXCR4 expressing lymphoblasts was specific, as a monoclonal antibody against CXCR4 could efficiently block chemotaxis. This confirms that, despite malignant transformation of the lymphoblasts, the chemokine receptor maintains its function. In this regard, our findings suggest a major role for the function of CXCR4 in ALL compared with CXCR4 expression on normal B cells in the bone marrow, in which function has been associated with distinct anatomical location in the bone marrow microenvironments.

Given the fact that SDF-1 is constitutively produced by stromal cells from the bone marrow and other tissues (Tashiro *et al*, 1993; Aiuti *et al*, 1999), one might speculate that it contributes to marrow and extramedullary tissue infiltration of leukaemia blasts. Indeed, infiltration of non-haematopoietic tissues such as gum or skin is most often observed in acute myeloid leukaemia (AML) with monocytic differentiation (AML FAB M4 and M5), which expresses the greatest level of CXCR4 among AML subtypes (Möhle *et al*, 1999). However, quantitative data on SDF-1 production in different extramedullary organs are not available, therefore it is unknown whether typical sites of leukaemia infiltration are characterized by a greater expression and production of SDF-1 compared with other tissues that are not invaded by the blasts. However, expression of SDF-1 has also been detected in endothelial cells (Pablos *et al*, 1999). As the presentation of SDF-1 on endothelial cells stimulates the integrin-mediated adhesion of circulating cells on the vascular endothelium (Peled *et al*, 2000), expression of CXCR4 by leukaemia blasts might facilitate leukaemia tissue infiltration. Recent data for the role of CXCR4 and SDF-1 in non-Hodgkin's lymphoma further confirms the speculation, that CXCR4 plays a role in the dissemination of malignant lymphoproliferative cells *in vivo* (Corcione *et al*, 2000). At diagnosis, enlarged lymphoid follicles of uniform size with a prominent germinal centre and a mantle zone of variable thickness, containing malignant B lymphocytes, are usually observed. The interfollicular tissue containing fibroblastic reticular cells is compressed among the expanding follicles, but can also be found in follicular centre lymphoma lymph nodes, suggesting that stromal cells may favour the spread of malignant B lymphocytes from one follicle to the adjacent ones by generating a chemotactic gradient of SDF-1. The locomotory responsiveness of follicular centre lymphoma B cells to SDF-1 could be an explanation for the widespread lymph node involvement seen for this disease at diagnosis. In agreement with the postulated function of CXCR4 and SDF-1 in non-Hodgkin's lymphoma, *in vivo* studies of breast cancer metastasis demonstrated that neutralizing the chemokine receptor CXCR4 by antibodies significantly impaired metastasis of breast cancer cells to organ sites such as regional lymph nodes and lung in mice (Mueller *et al*, 2001).

As SDF-1 is not only produced in lymph nodes, but constitutively also in other extramedullary tissues such as liver, spleen, thymus, lung, kidney and brain (Aiuti *et al*, 1997; Mueller *et al*, 2001), one might argue that the high CXCR4 expression seen on acute lymphoblastic cells, especially on the mature subtype, might contribute to the widespread extramedullary organ infiltration often seen in childhood ALL. Generally it is thought that the degree of organ infiltration correlates with peripheral blast count, thus reflecting the leukaemia mass (Reiter *et al*, 1994). Our study in a smaller series of patients, however, does not confirm that high blast cell counts are associated with

extramedullary tissue infiltration. More important, however, we demonstrated higher levels of CXCR4 expression and higher percentages of CXCR4-positive cells in children with significant extramedullary organ infiltration compared with patients without significant organ infiltration. Consistent with this observation, the strongest CXCR4 expression was detected in mature B-cell ALL, which is generally characterized by a high incidence of extramedullary bulky disease or CNS involvement (Price & Johnson, 1973), whereas the lowest expression was found on early B-ALL. Moreover, we demonstrated, that high CXCR4 expression on lymphoblasts at diagnosis was highly predictive for extramedullary organ infiltration. Further evidence for the link between CXCR4 expression and a function in extramedullary organ infiltration was shown by receptor expression on blasts isolated from extramedullary tissues. Blasts isolated from cerebrospinal fluid, thymus and testes showed similar expression levels seen on the mature B immunophenotype. It is therefore conceivable that expression of CXCR4 in a functionally active form on malignant haematopoietic cells contributes to the tropism for extramedullary microenvironments. With this possibility in mind, the data presented here may give further insights in the pathophysiology of ALL.

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