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BCR/ABL-mediated downregulation of genes implicated in cell adhesion and motility leads to impaired migration toward CCR7 ligands CCL19 and CCL21 in primary BCR/ABL-positive cells

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The mechanism underlying $p210^{BCR/ABL}$ oncoprotein-mediated transformation in chronic myelogenous leukemia (CML) is not fully understood. We hypothesized that $p210^{BCR/ABL}$ suppresses expression of genes which may explain at least some of the pathogenetic features of CML. A subtractive cDNA library was created between BCR/ABL-enhanced-green-fluorescent-protein (GFP)-transduced umbilical cord blood (UCB) $CD34^+$ cells and GFP-transduced UCB $CD34^+$ cells to identify genes whose expression is downregulated by $p210^{BCR/ABL}$. At least 100 genes were identified. We have confirmed for eight of these genes that expression was suppressed by quantitative real-time-RT-PCR (Q-RT-PCR) of additional $p210^{BCR/ABL}$-transduced $CD34^+$ UCB cells as well as primary early chronic phase (CP) bone marrow (BM) CML $CD34^+$ cells. Imatinib mesylate reversed downregulation of some genes, to approximately normal levels. Several of the genes are implicated in cell adhesion and motility, including L-selectin, intercellular adhesion molecule-1 (ICAM-1), and the chemokine receptor, CCR7, consistent with the known defect in adhesion and migration of CML cells. Compared with GFP UCB or normal (NL) BM $CD34^+$ cells, p210 UCB and CML $CD34^+$ cells migrated poorly towards the CCR7 ligands, CCL19 and CCL21, suggesting a possible role for CCR7 in the abnormal migratory behavior of CML $CD34^+$ cells.

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Introduction

Chronic myelogenous leukemia (CML) is a malignancy of the hematopoietic stem cell[1] caused by the Philadelphia chromosome[2] and the resulting $p210^{BCR/ABL}$ cytoplasmic oncoprotein.[3] Clinically, CML is characterized by increased proliferation,[4,5] increased resistance to apoptosis[6–8] and altered adhesion and migration.[9–12] The increased Abl tyrosine kinase activity caused by its association with BCR, leads to phosphorylation of Crk-L, RAS-GAP, Paxillin, Fak, phosphoinositide 3-kinase (PI3K), as well as $p210^{BCR/ABL}$ itself, which creates binding sites for a number of adaptor proteins. As a result, $p210^{BCR/ABL}$ activates mitogenic and antiapoptotic signals through RAS, mitogen-activated protein (MAP) kinase, Janus kinase-signal transducers and activators of transcription (Jak-Stat), PI3K and myc. BCR/ABL also induces multiple abnormalities of cytoskeletal function,[13,14] which in cell lines leads to increased adhesion to fibronectin,[15] but in primary human CML progenitor cells decreases adhesion to bone marrow stroma and extracellular matrix.[9,10] A causal role for the abnormal adhesion and migration has been shown for β1-integrins.[10,16,17] The role of other adhesion molecules, such as, selectins, sialomucins and chemokines in the aberrant adhesion and migration properties observed in CML has been less clarified.

Aside from affecting protein function, there is also evidence that presence of $p210^{BCR/ABL}$ may affect more global gene expression patterns. Deregulated gene expression has been studied in BCR/ABL-transformed cell lines,[18–21] and we have recently reported on the increased expression of a number of genes in primary $CD34^+$ cells as a result of $p210^{BCR/ABL}$.[22,23] We here hypothesized that presence of $p210^{BCR/ABL}$ may also downregulate expression of known and yet to be identified genes that may contribute to BCR/ABL-mediated malignant transformation in CML.

Materials and methods

Cell lines and materials

K562 cells were obtained from American Type Culture Collection (ATCC) (CCL-243) and maintained in Iscove's modified Dulbecco's medium (IMDM; Invitrogen, Carlsbad, CA, USA) supplemented with 10% FCS (HyClone, Logan, UT, USA) and 1% L-glutamine (Invitrogen). The 293 cell line was obtained from ATCC (CCL-86) and maintained in Dulbecco's minimal essential medium (DMEM) with high glucose (Invitrogen) with 10% FCS. Antibodies used in this study were directed against, β-actin (monoclonal IgG1; Santa Cruz Biotech., Santa Cruz, CA, USA), ABL (monoclonal IgG1; Santa Cruz Biotech.), phosphotyrosine (mouse monoclonal IgG1; UBI, Lake Placid, NY). Imatinib was a generous gift from Novartis (Basel, Switzerland).

Primary $CD34^+$ cells

Bone marrow (BM) was obtained from nine patients with early CP BCR/ABL-positive CML and nine NL healthy volunteers. In addition, we obtained 35 UCB samples from healthy term deliveries. All samples were obtained after informed consent using guidelines approved by the Committee on the Use of Human Subjects at the University of Minnesota. $CD34^+$ cell-enriched populations were selected from mononuclear cells using immunomagnetic columns (Miltenyi Biotec, Sunnyvale, CA, USA). Purity of $CD34^+$ cells after two passes over the immunomagnetic columns was $>90\%$.

Transduction of UCB $CD34^+$ cells

UCB $CD34^+$ cells were transduced with an MSCV-based retroviral vector containing either GFP cDNA alone (M-GFP)

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or the BCR/ABL cDNA upstream from an IRES-GFP sequence (M-p210-GFP) as described.[24]

Primary CD34$^+$ cell culture

After fluorescence-activated cell sorting (FACS) and before lysis for RNA or protein extraction, CD34$^+$ cells were maintained for 12–16 h in serum-free medium with low-dose cytokines consisting of BIT-9500 (Stem Cell Technologies, Vancouver, British Columbia, Canada) supplemented with IMDM, 50 μM 2-mercaptoethanol (Sigma; St. Louis, MO, USA), 40 μg/ml low-density lipoprotein (Sigma), 250 pg/ml granulocyte colony stimulating factor (G-CSF; Amgen; Thousand Oaks, CA, USA), 10 pg/ml granulocyte–macrophage colony stimulating factor (GM-CSF; Immunex; Seattle, WA, USA), 1 ng/ml interleukin-6 (IL-6; R&D Systems; Minneapolis, MN, USA), 50 pg/ml leukemia inhibitory factor (LIF; R&D Systems), 200 pg/ml macrophage inflammatory factor (MIP-1α; R&D Systems), and 200 pg/ml stem cell factor (SCF; Amgen). For imatinib mesylate (Imatinib) studies, CD34$^+$ cells were maintained for 48 h in serum-free medium with low-dose cytokines in the presence of 1 μM Imatinib.

Subtractive library

A cDNA library created as described[22] was used to identify downregulated genes. Differential screening was performed by high-throughput dot blot analysis using the PCRSelect differential screening kit according to the manufacturer's protocol (Clontech) and as reported.[22] Clones from the REVERSE subtracted library (present in GFP$^+$ cells but not in BCR/ABL$^+$GFP$^+$ cells) were blotted simultaneously on four nylon membranes (Hybond N+; Amersham, Arlington Heights, IL, USA), hybridized with ^{32}P-labeled FORWARD (present in BCR/ABL$^+$GFP$^+$ cells but not GFP$^+$ cells) and REVERSE subtracted cDNA probes, as well as unsubtracted cDNA from GFP$^+$ and BCR/ABL$^+$GFP$^+$ cells. Only those clones that hybridized with the REVERSE subtracted probe but not the FORWARD subtracted probe were selected for sequencing.

Sequencing

cDNA clones that were confirmed downregulated in BCR/ABL$^+$GFP$^+$ cells in the high throughput dot blot screen were sequenced using the dye Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA). Sequences were characterized by BLAT (human genome working draft), BLAST algorithm to SwissProt, GenBank protein and nucleotide collections, murine and human EST contigs. The sequences were considered part of known genes if they shared >95% homology over at least a 100-bp DNA sequence by BLAST search.

Q-RT-PCR

Total RNA from GFP or p210 UCB CD34$^+$ cells, or from NL or CML BM CD34$^+$ cells was isolated using the RNeasy procedure (Qiagen) and used for Quantitative RT-PCR using the SYBR green method, as described.[22] Primers used are listed in Table 1.

Table 1 Primers used for Q-RT-PCR experiments

Primer	*Name*	*Primer sequence*
NF1	Forward	TTCCCTTGTAGCTGTGAGCA
NF1	Reverse	AAGCGTCAGCCTAGAAGCAA
SELL	Forward	CTGGGGGTTGAGGGATAAAT
SELL	Reverse	GGCACCTCCTACGTCAAACA
BIRC1	Forward	TGGATCCTGTGTTAAACCCG
BIRC1	Reverse	ACAGCAAACCCTCCATTGTC
MX1	Forward	CTGGTGATAGGCCATCAGGT
MX1	Reverse	GTGCATTGCAGAAGGTCAGA
HCK	Forward	ATCCCTTACCCAGGGATGTC
HCK	Reverse	CTGTGGCCGTGTAGAAGTCA
ICAM1	Forward	TGAGGCCTTATTCCTCCCTT
ICAM1	Reverse	CATATTCCCTGGGCACTCAT
β-actin	Forward	TACCTCATGAAGATCCTCA
β-actin	Reverse	TTCGTGGATGCCACAGGAC
CCR7	Forward	AAGGGTCAGGAGGAAGAGGA
CCR7	Reverse	GGCTGGTCGTGTTGACCTAT
SORL1	Forward	TCCCAGAGGGCTGCTAGTAA
SORL1	Reverse	TGGATATCTGGTGGTGGGAT
ABL	Forward	CTTCAGCGGCCAGTAGCATCT
ABL	Reverse	GTGATTATAGCCTAAGACCCGG
BCR/ABL	Forward	CGTGTGTGAAACTCCAGACTGTCA
BCR/ABL	Reverse	AGATGCTACTGGCCGCTGAAG

All primers are given in the 5′→3′ direction.

Western blotting

A total of 5×10^4 GFP or p210 UCB CD34$^+$ cells, and CML or NL BM CD34$^+$ cells were used to assess the presence of p210$^{BCR/ABL}$, and to assess global phosphorylation patterns, using methods as previously described.[22]

Flow cytometry

Expression of L-selectin (PE-conjugated anti-CD62L, eBioscience), ICAM-1 (PE-conjugated anti-CD54, eBioscience) CD34 (APC-conjugated anti-CD34, BD Biosciences) was analyzed by FACS-Calibur (Becton-Dickinson) using the CellQuest Pro software. To study CCR7 expression, cells were labeled sequentially with an anti-CCR7 mouse monoclonal antibody (R&D Systems) and rat-anti-mouse-PE (BD-Biosystems).

Chemotaxis assay

Chemokine-dependent chemotaxis of GFP or p210 CD34$^+$ UCB cells, and NL or CML BM CD34$^+$ cells was determined using Costar Transwells (6.5-mm, 5-μm pore size, polycarbonate membrane).[25] The transwell inserts were coated with 100 μg/ml fibronectin (FN) or bovine serum albumin (BSA) (both from Sigma) for 12 h at 4°C. The solution was then removed and inserts were blocked with 2% BSA for 30′ at room temperature and washed with IMDM. Prior to use in chemotaxis assay, cells were preincubated for 12 h in X-VIVO 15 (Bio Whittaker) media supplemented with 2.5 ng/ml G-SCF, 100 pg/ml GM-CSF, 10 ng/ml IL-6, 2 ng/ml MIP1α, 2 ng/ml SCF and 500 pg/ml LIF. After washing, $2–4 \times 10^5$ GFP or p210 UCB CD34$^+$ cells, or NL BM or CML CD34$^+$ cells were resuspended in 100 μl of X-VIVO15 without cytokines and placed in duplicate in the transwell inserts. The same media with or without 1 μg/ml chemokine ligand 19 (CCL19) (R&D Systems) or 2 μg/ml CCL21 (R&D Systems) was added in the bottom chamber. In selected

experiments, cells were preincubated for 15′ at 37°C and 5% CO_2 with anti-human-CCR7 blocking antibody (R&D Systems) or IgG-isotype control (R&D Systems) prior to plating in the transwell inserts. In all samples, 2×10^5 polystyrene microbeads (Polyscience, Inc.) were added to the cell solution to allow enumeration of migrated cells. Cell migration was allowed for 4–4.5 h at 37°C and 5% CO_2. Cells that migrated through the transwell were harvested from the bottom chamber and the number of migrated $CD34^+$ cells determined by FACS-Calibur (Becton-Dickinson). Cells were labeled with a PE-conjugated anti-$CD34^+$ antibody (BD Biosciences) and gated on $eGFP^+CD34^+$ (transduced UCB samples) or $CD34^+$ (primary BM samples) cells. The percent migration was calculated using polystyrene beads (Polyscience, Inc.) as an internal control.

Statistics

The significance of differences between mRNA levels and migration percentage GFP and p210 UCB $CD34^+$ samples was determined using a paired Student's *t*-test. The significance of the difference between mRNA levels and migration percentage between NL and CML BM $CD34^+$ cells was analyzed by Student's *t*-test with unequal variance.

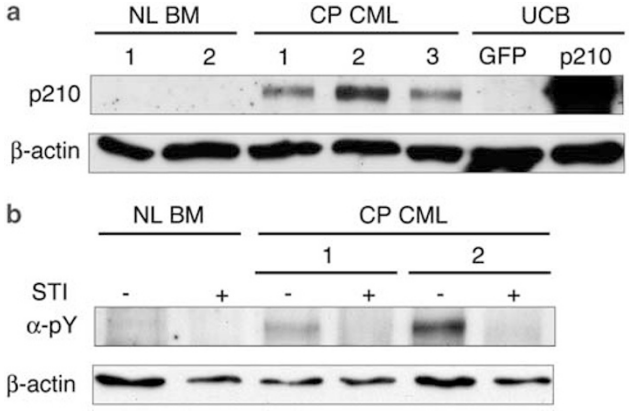

Figure 1 $p210^{BCR/ABL}$ mRNA levels and $p210^{BCR/ABL}$-mediated phosphorylation in the presence and absence of Imatinib. (a) Western blots were performed using lysates from 5×10^4 GFP or p210 UCB $CD34^+$ cells, and NL or early CP CML $CD34^+$ cells. Membranes were probed with Abs against ABL and *β*-actin. Representative blot of two experiments is shown. (b) Western blots were performed using lysates from 5×10^4 GFP or p210 UCB $CD34^+$ cells, and NL BM or early CP CML $CD34^+$ cells that were cultured with or without 1 μM Imatinib for 48 h. Membranes were probed with Abs against phosphotyrosine (α-pY) and *β*-actin. Representative blot of two experiments is shown.

Results

Genes downregulated in p210 UCB and in ECP CML $CD34^+$ cells

We used the cDNA library previously generated[22] to identify genes that are downregulated as a result of introduction of the BCR/ABL cDNA in UCB $CD34^+$ cells. Of the 1000 clones identified by subtractive hybridization, 13% were confirmed lower expressed in p210 than GFP UCB $CD34^+$ cells after performing a high-throughput dot blot analysis. These cDNA clones were sequenced and characterized by comparison to available on-line databases. To confirm differential expression, we generated additional GFP and p210 UCB $CD34^+$ samples. Expression of BCR/ABL mRNA transcripts in p210 UCB $CD34^+$ cells quantified by Q-RT-PCR was between 10 and 60% of that in K562 cells and levels of BCR/ABL mRNA in CML BM $CD34^+$ cells were between 10 and 20% of K562 cells (not shown). Presence of the $p210^{BCR/ABL}$ protein in p210 UCB $CD34^+$ cells was confirmed by Western blotting (Figure 1a).

Q-RT-PCR, using sequence specific primers listed in Table 1, demonstrated that eight genes identified in the subtractive screen were downregulated in an additional 5–6 samples of

Table 2 Q-RT-PCR confirmation of significantly downregulated genes in p210-UCB CD34 cells and CP $CD34^+$ cells

Gene family	*Gene name*	*p210-UCB*[a]	*CP CML*[a]
Adhesion/motility	ICAM1	−2.1 (0.1)	−3.4 (1.2)
	L-selectin	−15.3 (4.3)	−3.3 (0.3)
	CCR7	−5.6 (1.2)	−31.5 (8.9)
Protein modification	SORL1	−19.5 (0.9)	−2.2 (0.5)
	HCK	−4.7 (0.9)	−4.2 (1.2)
Apoptosis	BIRC1	−7.7 (1.3)	−2.6 (0.8)
	MX1	−4.4 (0.5)	−6.3 (3.1)
Proliferation	NF1	−6.9 (1.7)	−3.5 (1.0)

[a]Mean fold change (standard error) compared with BCR/ABL-negative sample (GFP-UCB $CD34^+$ cells, or NL BM $CD34^+$ cells).

Table 3 Imatinib in part reverses decreased mRNA levels of several genes in p210 UCB $CD34^+$ cells and CP $CD34^+$ cells

Gene family	*Gene name*	*p210-UCB*[a]		*CP CML*[a]	
		Imatinib−[a]	*Imatinib+*[b]	*Imatinib−*[a]	*Imatinib+*[b]
Adhesion/motility	ICAM1	−2.1 (0.1)	+2.2 (0.2)	−3.4 (1.2)	+3.2 (0.5)
	L-selectin	−15.3 (4.3)	+15 (5.7)	−3.3 (0.3)	+2.0 (0.4)
	CCR7	−5.6 (1.2)	+3.0 (0.4)	−31.5 (8.9)	+3.8 (0.8)
Protein modification	SORL1	−19.5 (0.9)	+3.2 (0.1)	−2.2 (0.5)	+3.3 (0.7)
	HCK	−4.7 (0.9)	+2.0 (0.2)	−4.2 (1.2)	+3.0 (1.1)
Apoptosis	BIRC1	−7.7 (1.3)	+2.7 (0.2)	−2.6 (0.8)	+2.8 (0.7)
	MX1	−4.4 (0.5)	+0.9 (0.2)	−6.3 (3.1)	+1.3 (0.1)
Proliferation	NF1	− 6.9 (1.7)	+2.5 (0.1)	−3.5 (1.0)	+3.8 (1.4)

[a]Mean fold change (standard error) compared with BCR/ABL-negative sample (GFP-UCB $CD34^+$ cells, or NL BM $CD34^+$ cells).
[b]Mean fold change (standard error) between imatinib− and imatinib+.

independently BCR/ABL-transduced UCB CD34$^+$ as well as early CP CML BM-derived CD34$^+$ cells (Table 2). These included ICAM1, L-selectin, CCR7, Sortilin (SORL1), hematopoietic cell kinase (HCK) baculoviral IAP repeat-containing protein 1 (BIRC1), myxovirus resistance 1 (MX1) and NF1.

Imatinib treatment restores normal levels of mRNA of some but not all genes

We next evaluated whether downregulation of these genes could be specifically attributed to the increased Abl tyrosine kinase activity in CML cells. We treated GFP and p210 UCB CD34$^+$ cells, or CML and NL BM CD34$^+$ cells with 1 μM Imatinib for 48 h. This resulted in decreased overall protein phosphorylation (Figure 1b). In p210 UCB CD34$^+$ cells (Table 3), Imatinib reversed transcript levels of 7/8 of the downregulated genes to approximately normal levels (ICAM1, L-selectin, CCR7, SORL1, HCK, BIRC1 and NF1), but did not affect mRNA levels of MX1. For CML CD34$^+$ cells, Imatinib significantly increased levels of mRNA for all genes except for MX1 (Table 3).

Decreased protein expression of L-selectin and ICAM1 on p210 containing CD34$^+$ cells

We next tested whether results seen at the mRNA level held true at the protein level. For these studies, we focused on gene products involved with cell adhesion and migration. In accordance with mRNA results, we found that a lower number of p210 UCB and CML BM CD34$^+$ cells expressed L-selectin and ICAM1 (Figure 2a) compared with GFP UCB or NL BM CD34$^+$ cells. Upon treatment of p210 UCB CD34$^+$ cells with imatinib the fraction of cells expressing L-selectin or ICAM1 was unchanged, while for early CP CML CD34$^+$ cells, expression of L-selectin but not ICAM1 increased (Figure 2b). We were unable to detect CCR7 protein expression on CD34$^+$ cells, even though the antibody did stain normal T-lymphocytes (data not shown).

CCR7 ligands fail to induce migration of p210 UCB and early CP CML CD34$^+$ cells

We used the CCR7 ligands, CCL19 and CCL21, in two-chamber migration assay to assess the function of CCR7 in p210 containing cells. Migration of UCB CD34$^+$ cells towards CCL19 was blocked by 100 μg/ml CCR7 blocking antibody (not shown), supporting the notion that migration driven by this ligand was specific for the CCR7 receptor.

We observed lower spontaneous migration of p210 UCB and early CP CML CD34$^+$ cells over fibronectin-coated membranes as compared to GFP$^+$ UCB and NL BM CD34$^+$ cells (Figure 3). Both, CCL19 and CCL21 increased migration of GFP-CD34$^+$ UCB cells (Figure 3a) and NL BM CD34$^+$ cells (Figure 3b). However, these ligands did not enhance migration of p210 UCB (Figure 3a) or CML BM CD34$^+$ cells (Figure 3b). Treatment of p210 UCB cells and CML CD34$^+$ cells with STI571 did not restore migration to CCL19 or CCL21 (Figure 3c–f).

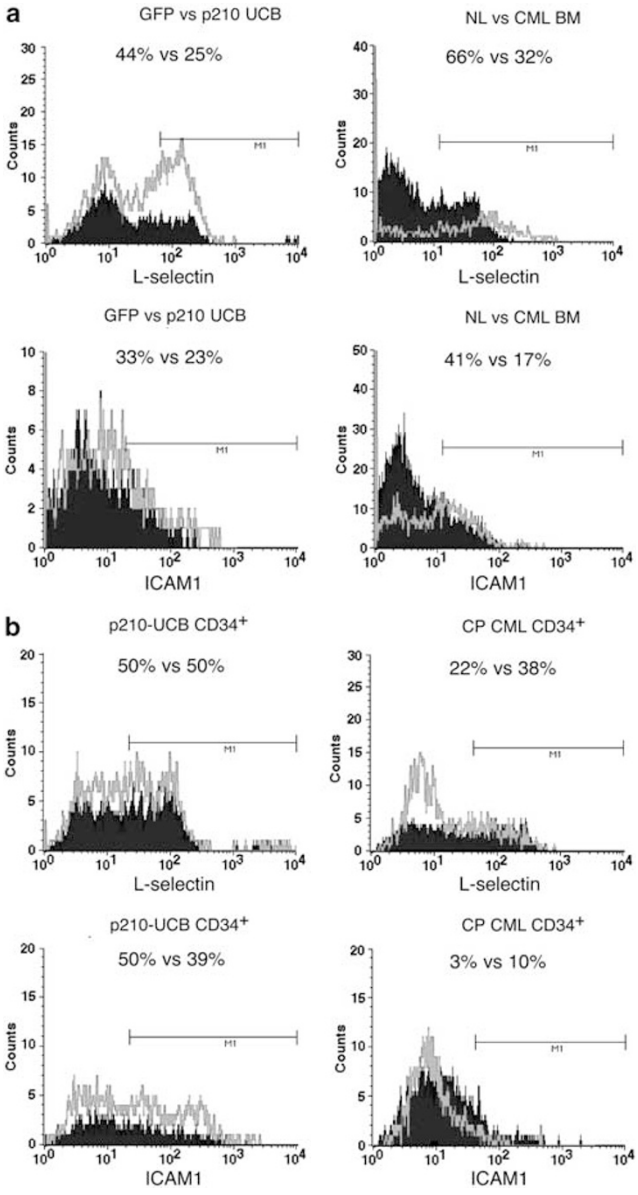

Figure 2 L-selectin and ICAM-1 protein expression in GFP *vs* p210 UCB and NL *vs* CML CD34$^+$ cells treated with or without imatinib. (a) NL (gray line) and CML (black line) CD34$^+$ cells, as well GFP (gray line) and p210 (black line) UCB CD34$^+$ cells were maintained for 12 h in serum-free medium with low-dose cytokines. Fluorescence intensity of L-selectin and ICAM1 was measured on CD34$^+$ gated cells by flow cytometry. (b) NL and CML CD34$^+$ cells, as well GFP and p210 UCB CD34$^+$ cells were maintained for 48 h in serum-free medium with low-dose cytokines in the presence of 1 μM imatinib. Fluorescence intensity of L-selectin and ICAM-1 was measured on CD34$^+$ gated cells of p210 and GFP UCB cells as well as early CP CML and NL CD34$^+$ cells by flow cytometry. Imatinib treated CD34$^+$ (black line) and untreated (gray line) cells. Plots shown are a representative example of three individual studies.

Discussion

We have previously demonstrated that the model of CML used here, BCR/ABL-transduced UCB CD34$^+$ cells, recreates all important features of early CP CML, namely altered adhesion, increased proliferation and inhibited apoptosis,[24] and is suitable to study molecular mechanisms through which BCR/ABL causes functional defects in CML CD34$^+$ cells. One benefit compared to using CD34$^+$ cells from primary early CP CML patients is that transduction of BCR/ABL into UCB CD34$^+$ cells allows study of the effect of BCR/ABL on cell behavior without possible interference from other genetic abnormalities due to the long-standing presence of BCR/ABL, even in early CP CML. We show

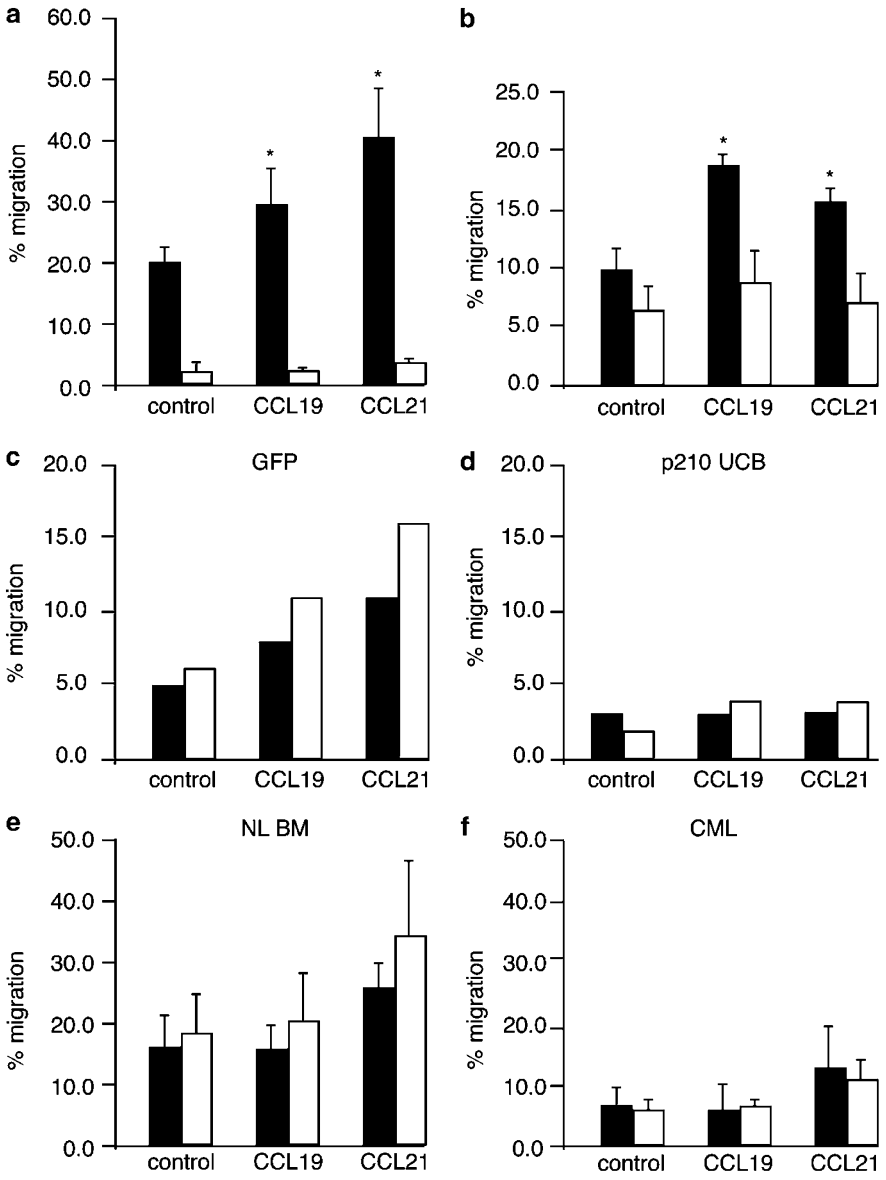

Figure 3 Reduced migration of p210 UCB cells upon stimulation with CCR7 ligands, which is not corrected by imatinib. (a, b) GFP (black bars) and p210 (white bars) UCB cells (a) and NL (black bars) and CP CML (white bars) $CD34^+$ cells (b) were plated in the upper chamber of transwell transmigration assays. The percent migrated $CD34^+$ cells to CCR7 ligands (CCL19 and CCL21) in the lower chamber was calculated as described in Materials and methods. Data are presented as mean $\pm$ s.d. of three independent experiments. $^*P<0.05$ compared to the control (medium without ligand). (c–f) GFP (c) and p210 (d) UCB cells and NL (e) and CP CML (f) $CD34^+$ cells were treated with (white bars) or without (black bars) 1 μM imatinib and plated in the upper chamber of transwell transmigration assays. The percent migrated $CD34^+$ cells to CCR7 ligands (CCL19 and CCL21) was calculated as described in Materials and methods.

here, and previously, that levels of BCR/ABL mRNA are similar when comparing the transduction model with primary CML cells. Using this model, we recently identified by subtractive hybridization increased expression of multiple known and novel genes that may contribute to the pathogenesis of CML.[22]

Using a similar strategy, we here identified multiple genes that are downregulated upon introduction of BCR/ABL in primary $CD34^+$ cells derived from UCB. Furthermore, we confirmed decreased expression of these genes in $CD34^+$ cells of patients with early CP CML as compared to NL BM $CD34^+$ cells. Three of the eight downregulated genes, L-selectin, ICAM1 and CCR7, are implicated in cell adhesion and motility but their role in CML is not yet known. There is a significant body of evidence that abnormal expression and/or function of cell adhesion molecules contributes to the pathophysiology of CML.[26,27] For instance, we and others have shown abnormal β1-integrin function in CML,[9,10,17,28] which could be reversed by interferon-α[28] or BCR/ABL-tyrosine kinase inhibitors,[11] two drugs used to treat CML.

We demonstrate here that mRNA and protein levels of ICAM1 are decreased in $CD34^+$ cells expressing the $p210^{BCR/ABL}$ oncoprotein, and that mRNA levels of this adhesion molecule are only moderately increased following treatment with Imatinib. The role of ICAM1 in hematopoiesis has not been extensively studied. ICAM1-homozygous deficient animals have moderate granulocytosis,[29] possibly consistent with the pronounced granulocytosis seen in CML. Baron *et al*[30] recently reported that presence of BCR/ABL in UT-7 cell is associated

with increased ICAM1 expression, which appears at odds with our results. However, differences in target cell (cell line *vs* primary human CD34$^+$ cells) may play a role in the discrepancy with our results, similar to differences reported for the role of β1-integrins in normal compared with CML hematopoiesis when studied in cell line models[15] and primary CML cells.[11]

Selectins (L-, P-, and E-selectin) are a family of glycoproteins that mediate adhesive interactions with their ligands under shear. L-selectin is expressed on early hematopoietic progenitors and there is evidence from cell migration experiments, clonogenic assays, and clinical studies that L-selectin may play a role in the trafficking of hematopoietic progenitors and hematopoiesis.[31–37] Decreased mRNA levels for L-selectin in p210 UCB and early CP CML CD34$^+$ cells is consistent with published reports demonstrating decreased expression of L-selectin on BM CD34$^+$ cells derived from CML patients.[38–40] We observed populations of CD34$^+$ cells with higher and lower levels of L-selectin protein expression in primary cells as well as in the UCB transduction model possibly based on subpopulations. Hence, it is possible that the decreased levels of ICAM1 and L-selectin in CML samples is due to skewing of the population away from the population expressing higher numbers of these adhesion molecules. However, as this was also seen in the transduced UCB model, we believe that skewing is less likely, as cells were evaluated within 48 h following transduction.

Fruehauf *et al*[40] also showed that imatinib restores expression of L-selectin in BCR-ABL-positive cell lines, a phenomenon we could not confirm in CD34$^+$ cells. Whether lack of L-selectin and/or ICAM1 play a role in the premature release of CD34$^+$ cells from the marrow in patients with CML will still need to be elucidated.

Chemokines are small chemotactic proteins that mediate their effect by binding to seven-transmembrane-spanning G protein coupled receptors.[41] They are implicated in migration of leukocytes, inflammatory responses and regulation of tumor growth, as well as hematopoiesis.[42–44] SDF1α and its receptor CXCR4 play important roles in migration of HSC from the fetal liver to the fetal BM[45,46] as well as in postnatal life.[47,48] In CML, chemotaxis towards SDF1α is impaired,[49,50] which may contribute to the premature release of leukemic progenitors from the BM into the circulation. An other chemokine MIP1α, also affects the adhesive behavior of CML CD34$^+$ cells, even though a specific role for MIP1α in the retention of CD34$^+$ in the BM and/or homing to the BM has not been shown.

We here demonstrate that mRNA levels of CCR7, an other member of chemokine-receptor family, is downregulated in p210 UCB CD34$^+$ and CML BM CD34$^+$ cells, and hypothesize that CCR7 may a role in altered migration of p210 UCB CD34$^+$ cells. CCR7 is an important organizer of the primary immune response, regulating trafficking of T and B lymphocytes as well as DC to or within lymphoid organs.[51] CCL19 (ELC) and CCL21 (MIP-3beta) are ligands for CCR7 expressed within lymph nodes, Peyer's patches, spleen, endothelial cells of HEVs.[51–54] Kim *et al*[55] showed that both CCR7 ligands, CCL19 and CCL21 have chemotactic activity for NL CD34$^+$ cells and suppress their proliferation. Although we attempted to evaluate the expression of CCR7 at the protein level, we were unable to detect significant levels of this chemokine receptor on either NL or leukemic CD34$^+$ cells with commercially available antibodies. However, as blocking antibodies against CCR7 inhibit migration of NL BM CD34$^+$ cells to its ligands, CCR7 must be expressed on NL CD34$^+$ BM cells and GFP$^+$ UCB CD34$^+$ cells. Using a two-chamber migration system, we found that although CCR19 and CCR21 significantly increased migration of NL UCB and BM CD34$^+$ cells, they had no effect on BCR/ABL-containing CD34$^+$ cells. Whether the decreased levels of CCR7 in CML can explain the abnormal trafficking of CML progenitors, and may contribute to the abnormal proliferative behavior seen in CML needs to be further determined.

Of note, treatment with imatinib restored levels of mRNA for all three adhesion related proteins, but did not result in restoration of ICAM1 or L-selectin protein levels, nor the function of CCR7. The reason for this is not clear at this time. One possibility is that duration and/or concentration of imatinib treatment was insufficient to affect protein levels. However, we show that exposure to 1 μM imatinib reversed the abnormal protein phosphorylation pattern seen in CML, and that the enhanced phosphorylation of Crk-L was reversed.[22] Another possibility might be that the half-life of these adhesion molecules may be longer than 24 h, and that prolonged exposure of CD34$^+$ cells to imatinib may reverse the decreased protein expression. Obviously, it is also possible that the intracellular machinery needed for migration to occur is not affected by imatinib.

A possible role in the pathogenesis of CML has been described for two additional genes found suppressed in BCR/ABL$^+$ CD34$^+$ cells. NF1, encodes the protein neurofibromin, which is a negative regulator of the RAS signaling pathway.[56] Mutations in NF1 lead to juvenile myelomonocytic leukemia (JMML).[57,58] Low levels of NF1 in CML CD34$^+$ cells may therefore contribute to deregulation of the RAS pathway as observed in CML. The second gene thought to play a role in CML pathogenesis, is HCK, the expression of which is restricted to myeloid cells[59] and couples BCR/ABL to STAT5 activation in murine 32Dcl3 myeloid cells.[60]

A possible role in leukemia or hematopoiesis has not been identified for the last three genes suppressed as a result of expression of BCR/ABL, namely SORL1, MX1, and BIRC1.The structure of the sorLa-1 protein encoded by SORL1 suggests that this is an endocytic receptor possibly implicated in uptake of lipoproteins and proteases.[61] Whether this protein is important in hematopoiesis has not been addressed. The protein encoded by the BIRC1 gene contains regions of homology to two baculovirus inhibitors of apoptosis, and suppresses apoptosis induced by various signals.[62,63] As CML is characterized by increased resistance to apoptosis, this result may seem contradictory to the pathophysiology of CML. However, apoptosis regulation is mediated by a number of different effectors, and the balance between multiple pro- and antiapoptotic genes decides the fate of the cell, rather than expression of one single gene product. The MX proteins belong to the family of large GTPases. In the mouse, the interferon-regulated MX protein is responsible for a specific antiviral state against influenza virus infection. Li and Youssoufian[64] reported that altered MX1 gene expression may play a role in Fanconi anemia. Whether MX1 play a role in pathogenesis of CML or not is not known.

Conclusion

We demonstrate downregulation of L-selectin and ICAM1 in BCR/ABL$^+$ CD34$^+$ cells, consistent with a role of these genes in altered adhesion and migration properties of CML cells.[9–11,49] Furthermore, we show that CCR7 expression is impaired and that this is associated with impaired chemotaxis towards its ligands CCL19 and CCL21. What the role of loss of this ligand/receptor interaction might be in CML will need to be evaluated further by *in vivo* homing studies using either human cells in a xenogeneic transplant model, or knockout mouse models.

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