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Leukotriene signaling via ALOX5 and cysteinyl leukotriene receptor 1 is dispensable for in vitro growth of CD34+CD38− stem and progenitor cells in chronic myeloid leukemia

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1. Introduction

Chronic myeloid leukemia (CML) is characterized by the expression of the BCR-ABL fusion protein, a constitutively active tyrosine kinase that promotes growth of leukemic stem and progenitor cells by multiple signaling pathways involved in cell survival, proliferation and differentiation [1]. The leukemic stem cells (LSC) in chronic phase CML reside in a phenotypically similar stem and progenitor cell population (CD34+CD38−) as normal bone marrow (BM) stem and progenitor cells [1,2] and differentiate predominantly into an expanding population of granulocytes, thrombocytes and their precursors, whereas in the terminal phase, blast crisis, immature lymphoid or myeloid progenitors accumulate as leukemic blasts.

Tyrosine kinase inhibitors (TKI) including imatinib, targeting the BCR-ABL oncoprotein, have been remarkably effective in inducing deep molecular remission in most CML patients. However, TKI are less effective to eradicate the leukemic stem cells (LSC), resulting in disease persistence. Therefore, there is great need to develop novel therapeutic strategies to specifically target the LSC. In an experimental mouse CML model system, the leukotriene pathway, and specifically, the expression ALOX5, encoding 5-lipoxygenase (5-LO), has been reported as a critical regulator of the LSC. Based on these results, the 5-LO inhibitor zileuton has been introduced in clinical trials as a therapeutic option to target the LSC although its effect on primary human CML LSC has not been studied. We have here by using multiplex single cell PCR analyzed the expression of the mediators of the leukotriene pathway in bone marrow (BM) BCR-ABL+CD34+CD38− cells at diagnosis, and found low or undetectable expression of ALOX5. In line with this, zileuton did not exert significant overall growth inhibition in the long-term culture-initiating cell (LTC-IC) and colony (CFU-C) assays of BM CD34+CD38− cells from 7 CML patients. The majority of the single leukemic BCR-ABL+CD34+CD38− cells expressed cysteinyl leukotriene receptors CYSLT1 and CYSLT2. However, montelukast, an inhibitor of CYSLT1, also failed to significantly suppress CFU-C and LTC-IC growth. These findings indicate that targeting ALOX5 or CYSLT1 signaling with leukotriene antagonists, introduced into the clinical practice primarily as prophylaxis and treatment for asthma, may not be a promising pharmacological strategy to eradicate persisting LSC in CML patients.

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less effective in eradicating the LSC, resulting in disease persistence, most often necessitating lifelong pharmacological therapy and carrying a risk for development of drug resistance and progression to blast crisis [3]. Therefore, there is a great need to identify additional therapeutic strategies to specifically target LSC in CML.

Leukotrienes (LT) are biologically active lipid mediators involved in inflammatory processes and in malignant diseases [4,5]. The first step in LT biosynthesis is conversion of arachidonic acid by 5-lipoxygenase (5-LO). In an experimental mouse CML model, the gene encoding 5-LO, ALOX5 was overexpressed in stem cells transfected with BCR-ABL [6], and loss of ALOX5 in the BCR-ABL LSC impaired the development of leukemia. Treatment with zileuton, an inhibitor of 5-LO, specifically inhibited the development of leukemia but did not inhibit the non-leukemic stem cells in this mouse model [6]. Based on these results, zileuton was proposed to be an option to eradicate the human LSC and to offer potential to improve the treatment of CML [7]. However, low levels of ALOX5 transcript were found at diagnosis in CD34+ progenitors of patients with chronic phase CML as compared to normal CD34+ progenitors [8]. Its expression and function in the more primitive leukemic BCR-ABL+ CD34+ CD38+ cells of CML patients has not been explored.

During LT biosynthesis from arachidonic acid, LTA4, an unstable intermediate product, is converted by LTA4 hydrolase (LTA4H) to LTB4 or by LTC4 synthase (LTC4S) to cysteinyl-containing LTC4, which is further metabolized to cysteinyll LTD4 and LTE4. The expression and activity of LTC4S has been shown to be increased in myeloid cells in patients with CML [9,10]. While LTB4 binds to receptors BLT1 and BLT2, the cysteinyll LT act on target cells mainly through the G-protein coupled receptors CYSLT1 and CYSLT2 [5]. Recently, the expression of the CYSLT receptors in CML cell lines was reported [11], and montelukast, an inhibitor of CYSLT1, was shown to inhibit the development of leukemia in this mouse model [6]. Based on these results, zileuton was proposed to be an option to eradicate the human LSC and to offer potential to improve the treatment of CML [7]. However, low levels of CYSLT2 receptors, montelukast failed to signiﬁcantly target LSC in CML.

2. Materials and methods

2.1. Human sample collection and CD34+ CD38− cell sorting by FACS

BM aspirates collected from healthy adult volunteers and newly diagnosed patients with chronic phase CML (n = 10, Table S1) were used in accordance with approved protocols (LU-195-00, LU-826-2004, 2012/4:10 and 2013/3:1). The WHO criteria for chronic phase CML were employed [12]. Mononuclear cells were isolated by Ficoll-Hypaque (Lymphoprep, Axis-Shield PoC AS, Oslo, Norway), the CD34+ cells were enriched by using CD34 MicroBead Kit (Miltenyi Biotec, Bergisch Gladbach, Germany). The CD34+ or CD34+CD38− cells were further sorted by FACS Ariall Cell Sorter (BD Biosciences) after staining with monoclonal antibodies (BD Biosciences, San Jose, CA, USA), anti-CD34-FITC or APC (8G12) and anti-CD38-PECY7 or PE (HB7). The gates were set according to fluorescence-minus-one controls. The dead cells were excluded by propidium iodide (PI; Invitrogen) staining.

2.2. Reagents

Imatinib and leukotriene antagonists were provided by Cayman Chemical Company (Ann Arbor, MI, USA). Stock solutions were prepared with DMSO (imatinib 10 mM, montelukast 50 mM and zileuton 100 mM). The final concentrations used in cultures were imatinib 1 or 5 μM, and zileuton and montelukast 20 μM.

2.3. Colony assays

The purified CD34+CD38− or CD34+ cells were cultured at a density of 150 cells/dish (for CD34+ cells) or 50 cells/dish (for CD34+CD38− cells) in Methocult H4435 (Stem Cell Technologies) in duplicate. The colony-forming units in culture (CFU-Cs) were counted at day 14.

2.4. LTC-IC assay

LTC-IC assay was performed as described [13,14]. The CD34+CD38− cells were sorted directly and plated in 200 μL Myelocult H5100 (Stem Cell Technologies), 10−6 M hydrocortisone (Sigma) in collagen I coated or plain 96-well microplates (BD Biosciences) on irradiated (8000 cGy) murine stromal cell line M2-10B4 (10000 cells/well), engineered to express human cytokines, IL-3 and G-CSF [13]. The cells were seeded at 3, 10, 30 and 100 cells/well or 1, 3, 10 and 30 cells/well, 10−18 replicates except for 30 or 100 cells/well where a small total cell number in some cases restricted the number of replicates. Imatinib and/or leukotriene antagonists or DMSO in the corresponding final concentration as in the inhibitors, were added at the initiation of the cultures, and the cultures were maintained at 37 °C and 5% CO2 for 6 weeks with weekly half-medium change. Thereafter 140 μL of the medium was carefully removed and the wells were overlaid with 150 μL Methocult H4435/well. The wells were scored as positive or negative after 14 days, and the frequencies of LTC-IC were calculated using the L-Calc software (StemCell Technologies).

2.5. Multiplex single-cell RT-PCR analysis

Single-cell RT-PCR analysis was performed as previously described [15]. The CD34+CD38− CML cells were deposited directly at 1 cell/well in 4 μL of lysis buffer (dNTPs, DT, 10% NP40, RNase inhibitor) per well in 96-well plates (Thermo Scientific, #AB-0800). Cell lysates were reverse transcribed using multiple pairs of gene-specific reverse primers and 500 nM MMLV-RT per reaction in the buffer provided by the supplier (200 U/ml, Life technologies). The first-round PCR with 35 cycles was performed by addition of 40 μL PCR buffer and 1.25 U Taq polymerase (TaKaRa kit, CloneTech). One microliter aliquots of first-round PCR products were further amplified using fully nested gene-specific primers. Aliquots of second-round PCR products were subjected to gel electrophoresis and visualized by GelRed™ staining (Invitrogen) on 1.5–3% agarose gel. The expression of BCR-ABL and the leukotriene pathway genes (CYSLTR1, CYSLTR2, ALOX5, and LTA4H) were analyzed simultaneously with HPRT, a reference gene. The wells were defined as positive for containing one cell if they were positive for HPRT gene product, or
for two of other genes in the GelRed™ stained agarose gel if negative for HPRT. The primer sequences are listed in Table S2.

2.6. Statistics

Statistical differences were evaluated by Wilcoxon matched-pairs signed rank test. The two-sided p-value was considered to be significant when p < 0.05. See supplemental materials for additional methods.

3. Results

3.1. Expression of LT signaling molecules in single CML CD34⁺CD38⁻ BCR-ABL⁺ cells

To specifically analyze the expression of LT signaling molecules in LSC, we determined the co-expression of BCR-ABL with LT pathway mediators ALOX5, CYSLTR1, CYSLTR2 and LTA4H mRNA in single BM CD34⁺CD38⁻ cells from 8 CML patients (Table S1) by using nested RT-PCR (Fig. 1). The frequencies of the BCR-ABL⁺ cells were 38%–70% of the CD34⁺CD38⁻ cells (Figs. 1C and 2A). Notably, ALOX5 was undetectable in one BM sample and was expressed only in a small fraction of leukemic BCR-ABL⁺ CD34⁺CD38⁻ cells in most BM samples with a median frequency of 10.6% (Fig. 2). The median frequency of residual nonleukemic BCR-ABL⁺ cells expressing ALOX5 was similarly low (16.0%). The expression of LTA4H was overall high with median frequencies of 95.6% and 79.6% in the BCR-ABL⁺ and BCR-ABL⁻ cells, respectively (Fig. 2B). The cysteinyl LT receptors CYSLTR1 and CYSLTR2 were also expressed in most BCR-ABL⁺ LSC (median frequency of 64.5% and 57.3%, respectively) and BCR-ABL⁻ CD34⁺CD38⁻ cells (median frequency of 71.7% and 81.7%, respectively) (Fig. 2B).

3.2. Growth response of CML CD34⁺CD38⁻ BCR-ABL⁺ cells to imatinib and LT signaling inhibitors in LTC-IC assay

To test the functional response of the primitive leukemic stem and progenitor cells to the LT inhibitors zileuton or montelukast alone and in combination with imatinib, we performed limiting dilution LTC-IC assay of BM CD34⁺CD38⁻ cells from 7 CML patients and 5 healthy donors (Fig. 3). The median frequencies of LTC-IC in BM CD34⁺CD38⁻ cells from CML patients and normal BM donors were 0.38 and 0.40, respectively, in agreement with a previous report [17]. Imatinib induced a reduction of LTC-IC frequencies in CD34⁺CD38⁻ cells from 6 out of 7 tested CML patient samples, representing a significant overall growth inhibition (median of 0.12, p = 0.03) (Fig. 3A, C). In contrast to imatinib, neither zileuton nor montelukast were able to induce significant overall inhibition of LTC-IC growth of the 7 tested CML BM samples (Fig. 3A), although some reductions were observed in CML5 by 38.2% and in CML8 by 53% compared to the DMSO control. Similarly, no significant additive inhibition could be detected when the LT inhibitors were combined with imatinib (Fig. 3A,C). However, in a BM sample (CML8) from a patient who progressed to blast crisis soon after diagnosis (Table S1), zileuton in combination with imatinib seemed to further suppress LTC-IC activity of the CD34⁺CD38⁻ cells with the LTC-IC frequency reduced from 0.031 in zileuton alone to 0.018 in zileuton plus imatinib (Fig. 3A). Cytopenias are known off-target effects of imatinib, and inhibition of LTC-IC activity by imatinib was seen in some healthy donor BM CD34⁺CD38⁻ cells. Although some inhibition by zileuton and montelukast, comparable to that observed in few CML samples, was seen in the LTC-IC assays of few normal BM samples, there was no significant overall decrease in LTC-IC frequencies in the normal BM in cultures with imatinib, LT inhibitors or the combination (Fig. 3B, D).

Fig. 1. Multiplex single cell RT-PCR analysis showing expression of leukoetsi signaling molecules in BCR-ABL⁺ and BCR-ABL⁻CD34⁺CD38⁻ cells of CML patients.
(A) Experimental setup for nested-PCR and in vitro function inhibition assays of CML stem and progenitor cells to LT inhibitors. The CD34⁺CD38⁻ cells were sorted for single cell PCR analysis and for LTC-IC assay. For single cell PCR, the cells were deposited directly in 96-well plates at 1 cell/well and subjected to cDNA synthesis followed by two-round multiplex nested PCR to amplify the genes. The electrophoresis was run to identify the specific RT-PCR products.
(B) FACS profile for sorting of CML CD34⁺CD38⁻ cells. The cells were gated for live PI⁻ CD34⁺ cells and subsequently gated for CD34⁺CD38⁻ cells.
(C) The frequencies of BCR-ABL⁺ cells within the CD34⁺CD38⁻ cells in the CML patients detected by single cell PCR.
(D) Representative image of RT-PCR products for CYSLTR1, CYSLTR2, ALOX5, LTA4H and HPRT.
3.3. No overall significant inhibition of CFU-C from CML CD34<sup>+</sup> cells by zileuton or montelukast

CFU-C assays were performed on CD34<sup>+</sup> cells from 5 CML patients (Fig. 3E). Not surprisingly, total CFU-Cs were dramatically suppressed by imatinib in all 9 assays of 6 patient samples (p = 0.03), in agreement with the reported more efficient suppression by imatinib of less primitive leukemic progenitor cells [18]. Inhibition of CFU-C growth by imatinib was similar at 1 μM and 5 μM (Fig. S1). There was no significant inhibition in the colony growth by montelukast or zileuton.

3.4. Residual BCR-ABL<sup>+</sup> cells were detected in single colonies from LTC-IC cultures

In order to determine whether the LT inhibitors could specifically target the LSC in the LTC-IC cultures, we analyzed cells from these cultures by FISH for the BCR-ABL fusion gene. We could detect residual BCR-ABL<sup>+</sup> cells after all treatments (Fig. 4), showing that even when total LTC-IC frequencies were reduced by imatinib (Patients CML3, 7, and 9, Fig. 3), LSCs were not fully eradicated.

4. Discussion

Signaling pathways mediated by leukotrienes have been identified as important regulators of acute and chronic inflammation, but are also involved in the pathogenesis in several types of cancers, including colon, lung, prostate and breast cancers. In line with this, studies using in vivo murine colon cancer xenograft models have demonstrated notable potential of both zileuton and montelukast as anti-cancer agents, suggesting that inhibiting LT signaling may be a promising additional pharmacological strategy to target these tumors [19].

The role of LT signaling in human normal hematopoiesis and leukemia remains elusive. The receptor for LTB4, BLT1 is expressed in CD34<sup>+</sup> cells in CML at diagnosis and its expression appeared to be correlated with the clinical response to imatinib [8]. In addition, the other LTB4 receptor BLT2 was found to be upregulated by BCR-ABL in primary CML blast crisis CD34<sup>+</sup> progenitors, and inhibition of this receptor suppressed proliferation and induced apoptosis of the leukemic cells [20]. High expression of CYSLTR1 was reported in chronic lymphocytic leukemia, and in vitro inhibition of it induced apoptosis of the leukemic cells, suggesting this receptor as a potential therapeutic target [21].

Based on these reports suggesting a role for 5LO/ALOX5 and/or cysteinyl LT signaling in solid cancers and in leukemia, we have here focused on the expression of the target molecules of these two respective inhibitors zileuton and montelukast in primary BCR-ABL<sup>+</sup> CD34<sup>+</sup> CD38<sup>−</sup> BM cells from 7 patients and blood cells from 1 patient (patient 2) with CML.
Fig. 3. Functional response of BM hematopoietic stem and progenitor cells from patients with CML and healthy donors to imatinib and leukotriene inhibitors in LTC-IC and CFU-C assays.

(A-D) The growth response of CML (A, C) and normal BM (B, D) CD34⁺CD38⁻ cells to imatinib (IM), zileuton (ZIL), montelukast (MON) and combination of IM with leukotriene inhibitors in LTC-IC assay. The data are from 7 newly diagnosed CML patients and 5 normal BM donors.

(E) The growth response of CML CD34⁺ cells to IM, ZIL and MON in CFU-C colony assays. Data are the numbers of CFU-C per 150 CD34⁺ BM (circle) and PB (square) cells of 5 indicated CML patients. Each dot represents mean of duplicate measurements in the colony assays.

*p < 0.05 Wilcoxon matched-pairs signed rank test, compared to DMSO control. Horizontal bars show the median values.

Fig. 4. FISH analysis of BCR-ABL fusion gene expression in the cells derived from the LTC-IC assay shows the residual BCR-ABL⁺ cells after treatment with imatinib and leukotriene inhibitors zileuton and montelukast.

The cells from individual LTC-IC culture wells were picked and transferred to microscopic slides by cytospin for the FISH analysis. (A) The representative images of the BCR-ABL⁺ cells derived from the colonies in culture wells. (B) The frequencies of the BCR-ABL⁺ cells in wells after LTC-IC cultures from 4 patients. At least 55 cells in total in each sample were counted for calculating the frequency of the BCR-ABL⁺ cells.
unclear.

By single cell PCR analysis we detected a very low overall frequency of ALOX5 expression in the primitive BCR-ABL+ CD34+ CD38− LSC and BCR-ABL+ CD34+ CD38− nonleukemic cells of CML patients, suggesting distinct regulation and functional importance of 5-LO/ALOX5 in primitive human CML LSC and in the mouse BCR-ABL− LSC. However, the expression of downstream LT4AH in most leukemic and nonleukemic cells may indicate transcellular biosynthesis of LT downstream of 5-LO in the LSC, as reported in other cell types [24].

In the in vitro functional stem and progenitor cell assays of primary CD34+ CD38− cells of CML patients, imatinib significantly inhibited the overall LTC-IC activity in leukemic, but not in normal donor BM samples, supporting a specific inhibitory effect on BCR-ABL+ LSC in these culture systems. Imatinib also significantly inhibited CFU-C growth of the leukemic BM progenitors. In contrast, zileuton, did not induce any significant overall inhibition in the LTC-IC activity of CD34+ CD38− cells or the CFU-C activity of CD34+ cells of the CML patient BM samples. Our data suggest that targeting the ALOX5 gene product, 5-LO using zileuton to treat CML may not be as promising as expected from previously reported mouse studies.

In contrast to the reported inhibition of the growth of CML blast crisis cell lines by montelukast in a short term liquid culture assay [11], we did not find significant overall growth suppression of BM CD34+ CD38− or CD34+ stem and progenitor cells of CML patients by montelukast in LTC-IC or CFU-C assays, suggesting that pharmacological inhibition of CYSLTR1 may not either be a promising therapeutic strategy for eradicating CML LSC.

In summary, our findings indicate that the LT signaling pathway via ALOX5 or CYSLTR1 may be less important for survival of primary human leukemic LSCs than that of mouse BCR-ABL+ cells or CML blast cell lines. Consequently, inhibiting 5-LO or CYSLTR1 by zileuton and montelukast may not be a promising option to eradicate CML LSC.

Conflict of interest

M.E., L.S., S.M., U.O.S. have participated as investigators in several international clinical CML studies sponsored by pharmaceutical companies (Novartis, BMS, Pfizer and Ariad), generating remuneration to these individuals or to their respective institutions/departments. Other authors declare no competing financial interest regarding this study.

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MD, AP, ME and HQ have participated in conception and design, performing experiments, collection and assembly of data, data analysis and interpretation and manuscript writing. EG, WMW, JK and ASJ performed experiments, collected data. PX, LS, SM, EYK and UOS, contributed to data analysis and interpretation. All authors have approved the final version of the manuscript.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2017.06.051.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2017.06.051.

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