EXPERIMENTAL and MOLECULAR MEDICINE, Vol. 37, No. 1, 45-50, February 2005

Leukotriene B_4 pathway regulates the fate of the hematopoietic stem cells

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Accepted 31 January 2005

Abbreviations: FL, Flt-3 ligand; HSC, hematopoietic stem cell; LTB_4, leukotriene B_4; RT-PCR, reverse transcription PCR; TPO, thrombopoietin; UCB, umbilical cord blood

Abstract

Leukotriene B_4 (LTB_4), derived from arachidonic acid, is a potent chemotactic agent and activating factor for hematopoietic cells. In addition to host defense *in vivo*, several eicosanoids have been reported to be involved in stem cell differentiation or proliferation. In this study, we investigated the effect of LTB_4 on human cord blood $CD34^+$ hematopoietic stem cells (HSCs). LTB_4 was shown to induce proliferation of HSC and exert anti-apoptotic effect on the stem cells. Blockade of interaction between LTB_4 and its receptor enhanced self-renewal of the stem cells. Effect of LTB_4 on differentiation of $CD34^+$ HSCs were confirmed by clonogenic assays, and induction of the expression of BLT2 (the low-affinity LTB_4 receptor), during the *ex vivo* expansion was confirmed by reverse transcription-PCR. Our results suggest that LTB_4-BLT2 interaction is involved in the cytokine-induced differentiation and *ex vivo* expansion of hematopoietic stem cells.

Keywords: BLT_2; CD34; apoptosis; cell differentiation; hematopoietic stem cells; leukotriene B_4; receptors

Introduction

Hematopoietic stem cells (HSCs) have ability to self renew while functionally repopulating the cells of the blood and lymph for the life of an individual. These cells are thought to retain a high capacity for plasticity that would contribute to their ability to differentiate into not only hematopoietic cells but also non-hematopoietic tissues such as brain, heart, skeletal muscle, liver and endothelial cells, following injury or stress (Heike *et al.,* 2004). Such capacity of these cells gives rise to the hope that hematopoietic stem cells may regenerate various damaged tissues. In that regard, *ex vivo* expansion of hematopoietic stem cells is a promising technology for many potential applications from marrow reconstitution to gene therapy. Despite of considerable progresses gained during the past ten years in understanding the biology of hematopoietic stem cells and its *ex vivo* expansion, the success was limited (Piacibello *et al.,* 1997; Shih *et al.,* 1999; Lewis *et al.,* 2001). Self-renewal of stem cells depends on several critical factors, including the hematopoietic microenvironment, interactions with supporting stromal cells (Charbord, 2001), features of the extracellular matrix (Williams *et al.,* 1991), hematopoietic growth factors (Feugier *et al.,* 2002; Terada *et al.,* 2003) and cytokines (Flores-Guzman *et al.,* 2002).

Leukotriene B_4 (LTB_4), derived from arachidonic acid, is a potent chemotactic agent and activating factor for hematopoietic cells. Until recently, two cell surface receptors for LTB_4 have been identified; BLT1, a high affinity receptor, and BLT2, a low-affinity receptor. BLT2 is expressed ubiquitously, in contrast to BLT1 expressed predominantly in leukocytes. The differences in the distribution and pharmacological characteristics of BLT1 and BLT2 suggest distinct role(s) for these receptors *in vivo* (Toda *et al.,* 2002). Based on the previous observations, LTB_4 seems to play important roles in inflammation in addition to host defense *in vivo* (S.W. Crooks *et al.,* 1998). Recently, it has been reported that cysteinyl leukotriene receptor is involved in differentiation of HSCs into eosinophils (Hashida *et al.,* 2001). In addition, several eicosanoids have been reported to be involved in stem cell differentiation or proliferation (Desplat *et al.,* 2000; Braccioni *et al.,* 2002). In this study, we have investigated the effect of LTB_4, one of the potent lipid mediators, on cell proliferation, differentiation, and self-renewal capacity during the long-term culture of HSCs.

Materials and Methods

Cell source and $CD34^+$ cell purification

Umbilical cord blood was obtained at the end of full-term deliveries. Mononuclear cells were isolated using a Ficoll-Hypaque (density, 1.077; Pharmacia Biotech, Upsala, Sweden) density gradient centrifugation. After two cycles of plastic adherence for 60 min, the cells were washed and suspended in PBS (phosphate buffered saline) (pH 7.4), which contained 0.1% bovine serum albumin. During these procedures, all adherent cells were removed and only nonadherent cells were recovered. The $CD34^+$ cell fraction was isolated with a superparamagnetic microbead selection using monoclonal antibody (QBEND10) and miniMACS columns (Miltenyi Biotec; Bergisch Gladbach, Germany) (Kim, 2003). The efficiency of purification and immunophenotype were verified with flow cytometry and counterstained with a FITC-anti-human CD34 (HPCA-2; Becton Dickinson; Mountain View, California) as previously described (Yoo *et al.,* 1999).

Ex vivo expansion of $CD34^+$ hematopoietic stem cells

$CD34^+$ cells were placed into 24-well plates (Falcon; Becton Dickinson Biosciences) at a concentration of 1×10^3 cells per well. Each well contained 0.5 ml liquid medium plus the growth factors of interest. The culture medium consisted of Iscove's modified Dulbecco's medium (IMDM; Gibco BRL) supplemented with 10% fetal bovine serum. The cytokines included recombinant human G-CSF (100 U/ml), TPO (10 U/ml), and FL (50 ng/ml). The cultures were incubated at 37°C in a humidified atmosphere containing 5% CO_2. On day 14, cells were harvested from the culture and the number of viable cells was determined by trypan blue exclusion. Cell counts were also performed using a hemocytometer. The cells from these aliquots were assayed for the number of hematopoietic progenitor cells. Apoptosis assay was performed using Annexin-V-Flous staining kit (Roche) following the manufacturer's protocol.

Clonogenic assays

Clonogenic assays were performed as follows: 1×10^3 $CD34^+$ UCB cells were cultured at two plates per point in complete methylcellulose medium (HCC-4434; Methocult; Stem Cell Technologies, Vancouver, Canada). It was supplemented with 50 ng/ml stem cell factor (SCF), 10 ng/ml interleukin-3, GM-CSF, and 1 U/ml recombinant human erythropoietin (EPO) at 37°C in a humidified atmosphere at 5% CO_2. After 14 days of incubation, colonies were enumerated with the aid of an inverted microscope and counted by morphologic criteria.

Reverse transcription (RT)-PCR

Total cellular RNA was extracted from HSCs and dissolved in DEPC (diethylpyrocarbonate)-treated water. One microgram of the extracted RNA was reverse transcribed into cDNA and amplified to determine both BLT2 and GAPDH mRNA expression according to manufacturer's protocol (Reverse-iTTM One-Step RT-PCR Kit; ABgene). Primers for human BLT2 are 5'-AGCCTGGAGACTCTGACCGCTTTCG-3' (forward) and 5'-GACGTAGAGCACCGGGTTGACG-CTA-3' (reverse) (Tong *et al.,* 2002). Primers for GAPDH are 5'-CTGCACCACCAACTGCTTAGC-3' (forward) and 5'-CTTCACCACCTTCTTGATGTC-3' (reverse). The mixture for BLT2 was heated at 94°C for 20 s, annealed at 68°C for 30 s and extended at 72°C for 30 s for 30 repetitive cycles. The mixture for GAPDH was heated at 94°C for 20 s, annealed at 58°C for 30 s and extended at 72°C for 30 s for 25 repetitive cycles. GAPDH was used as an internal control. The final PCR products were separated on a 1.2% agarose gel with ethidium bromide and visualized under UV light.

Data analysis and statistics

The results are presented as the mean ± SD of the data obtained from three or more experiments performed in duplicates. Statistical comparisons between groups were made with the Student's *t*-test. Values of $P < 0.05$ were considered significant.

Results

LTB_4 induces proliferation of $CD34^+$ HSCs

The effect of LTB_4 on the *in vitro* proliferation of $CD34^+$ hematopoietic progenitor cells from freshly purified UCB $CD34^+$ cells was assessed using trypan blue exclusion assay. Earlier report of optimal culture condition for hematopoietic stem cells with combined supplementation of G-CSF, TPO and FL (Yoo *et al.,* 1999) was used as a control culture and compared with the additional supplementation of LTB_4. In the absence of exogenous cytokines, no viable cells were observed after 2 weeks of culture, regardless of whether or not LTB_4 was present (data not shown). When mixture of G-CSF, TPO, and FL was added to the medium, the number of total cells was increased approximately 350 folds during the culture as expected (data not shown). Addition of LTB_4 (300 nM) significantly enhanced the growth of $CD34^+$ HSCs approximately by 1.5 fold when compared to the condition where no LTB_4 was added (Figure 1A). The effect of LTB_4 was abolished by the addition of CP105696 (10 μM), an antagonist of LTB_4 receptors (BLT) (Figure 1A), suggesting the specific effect of LTB_4 on the proliferation of $CD34^+$ hematopoietic stem cells.

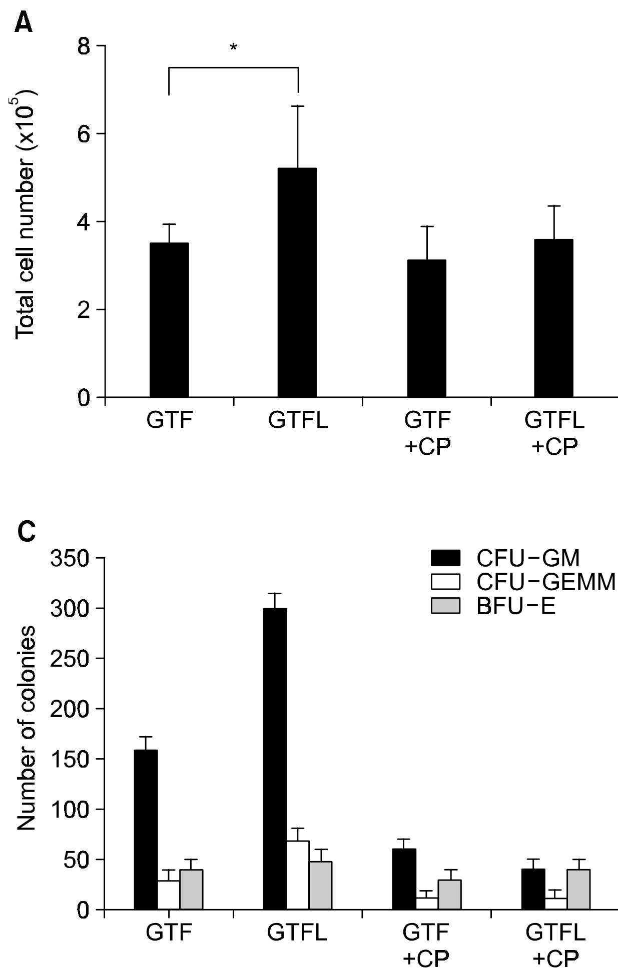

Figure 1. Effects of LTB4 on differentiation of $CD34^+$ hematopoietic stem cells. (A) Total number of mononuclear cells in liquid culture. Freshly isolated $CD34^+$ cells were cultured in 10% IMDM with G-CSF (10 U/ml, G), TPO (T) and FL (F) in the absence or the presence of LTB_4 (300 nM) and/or CP105696 (10 uM). On day 14, cells were harvested from each culture and the viable cells were counted using trypan blue. Values are the means ± SD of 3 separate experiments in duplicate cultures (*$P < 0.05$). (B) Purity of $CD34^+$ HSCs after long term culture. The cells obtained after 2-week culture were stained with FITC-anti-CD34 (HPCA-2) antibody, and analyzed by flow cytometry. Data are presented as means of fluorescence (*$P < 0.05$). (C) Effects of LTB_4 on *in vitro* clonal growth of hematopoietic progenitor cells from isolated human UCB $CD34^+$ cells. Freshly isolated $CD34^+$ cells (1×10^3 cells/ml) were incubated in a liquid culture as described in the legend of (A). On day 14, cells harvested from each culture were plated into a methylcellulose culture supplemented with SCF, IL-3, GM-CSF, G-CSF and EPO. The culture was incubated for 14 days. Values are the means ± SD of three separate experiments in duplicated cultures.

LTB_4 exerts anti-apoptotic effect on $CD34^+$ hematopoietic stem cells

LTB_4 was reported to be involved in cell survival by its anti-apoptotic effect on various cells such as neutrophil (Hebert *et al.,* 1996; Zhang *et al.,* 2002). We performed Annexin-V staining to determine whether LTB_4 exerts anti-apoptotic effect also on $CD34^+$ HSCs in a long term culture. As seen in Figure 2, addition of LTB_4 decreased apoptosis when compared to the control and CP105696 treatment inhibited the anti-apoptotic effect of LTB_4 statistically significantly ($P < 0.05$). These results suggest that the growth advantage of LTB_4 on HSC is, at least partly, due to its anti-apoptotic effect.

LTB_4 induces differentiation of $CD34^+$ HSCs

After 2 weeks of *ex vivo* expansion, purity of $CD34^+$ HSCs was assesed by flow cytometry using CD34 antibody. Interestingly, the results were opposite to the growth experiments, where treatment of LTB_4 showed lowest purity of HSCs in terms of retention of CD34 (Figure 1B). Accordingly, addition of BLT antagonist showed highest CD34-positive populations (Figure 1B). The fact that the specific antagonist increased the purity of $CD34^+$ HSCs even in the absence of LTB_4 might be due to the blockade of interaction between BLT and the endogenously produced LTB_4 during differentiation of $CD34^+$ HSCs (Spanbroek *et al.,* 2000). These results suggest that LTB_4 induces differentiation of HSCs through its receptor during *ex vivo* expansion by autocrine or paracrinic manner, since the cells that lost CD34 might be differentiated or at least differentiating cells. Thus, we next performed clonogenic assays with methylcellulose cultures to confirm the involvement of LTB_4 in HSC differentiation. In the methylcellulose cultures, addition of LTB_4 yielded approximately two-fold increase in colony formations of CFU-GM and CFU-GEMM, when compared to the condition where no LTB_4 was added (Figure 1C). Again, this effect was inhibited by the addition of BLT antagonist, CP105696 (10 nM). In contrast, no significant differences in BFU-E-derived colony formation were observed in the presence or the absence of LTB_4 or CP105696 (Figure 1C). Taken together, these results suggest that LTB_4 may induce differentiation of $CD34^+$ HSCs by its receptor-mediated interaction.

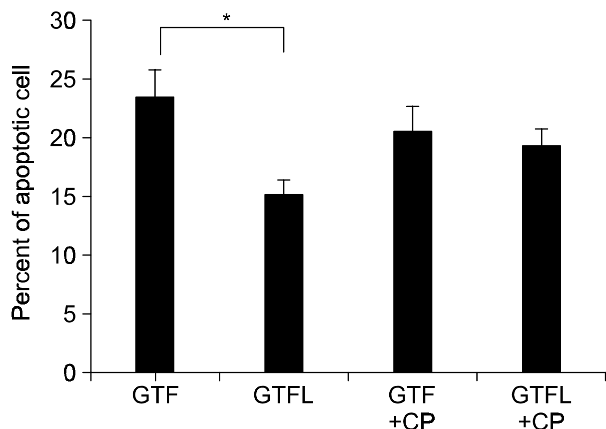

Figure 2. Anti-apoptotic effect of LTB_4 on human cord blood CD34+ cells. Human cord blood stem cells were isolated and cultured in the same condition described in the legend of Figure 1 (A). After two-week culture, cells were harvested and subjected to Annexin V-staining and rate of apoptosis was calculated following the manufacturer's protocol. Values are the means ± SD of 3 separate experiments in duplicate cultures (*$P < 0.05$).

Blockade of LTB_4 pathway induces self-renewal of $CD34^+$ HSCs

From the above results, we assumed that it might be possible that self-renewal of $CD34^+$ HSC can be increased by blocking LTB_4-BLT interaction. Thus, we next determined the effect of BLT antagonist on self-renewal of $CD34^+$ HSCs. After two weeks of *ex vivo* expansion, the cultures were collected and run through the MACS column as mentioned above. After purification of $CD34^+$ cells, number of the cells was assessed by trypan blue exclusion. As expected, treatment of CD105696 (10 μM) increased number of $CD34^+$ HSCs by approximately two-fold, compared to the control, whether LTB_4 was added or not (Figure 3A). Even though there might be loss of a small portion of the cells during the purification, this result showed the similar tendency with absolute number of $CD34^+$ cells (Figure 3B) calculated from total number of the cells (Figure 1A) and the purity of $CD34^+$ HSCs (Figure 1B), confirming blockade of BLT induces proliferation of $CD34^+$ HSCs. Since these $CD34^+$ cells retained differentiating capacity as measured in clonogenic assays using methyl cellulose medium (data not shown), blockade of BLT may induce not only the proliferation but also the self-renewal of $CD34^+$ HSCs.

Expression of BLT2, a receptor for LTB_4, is induced during differentiation of $CD34^+$ HSCs

The induced expression of BLT1 during the cytokine-induced differentiation of $CD34^+$ stem cells was reported by Pettersson *et al.* (2003). We next performed RT-PCR with BLT2-specific primers to investigate whether expression of BLT2, another receptor for LTB_4 is also related to *in vitro* differentiation of $CD34^+$ HSCs. As seen in Figure 4, BLT2 expression was increased during the long term culture when the cytokines were added in the presence or absence of LTB_4. However, the expression was decreased when the LTB_4 receptor antagonist was treated. Taken together with previous report, these results show the expression of BLT maybe directly or at least indirectly

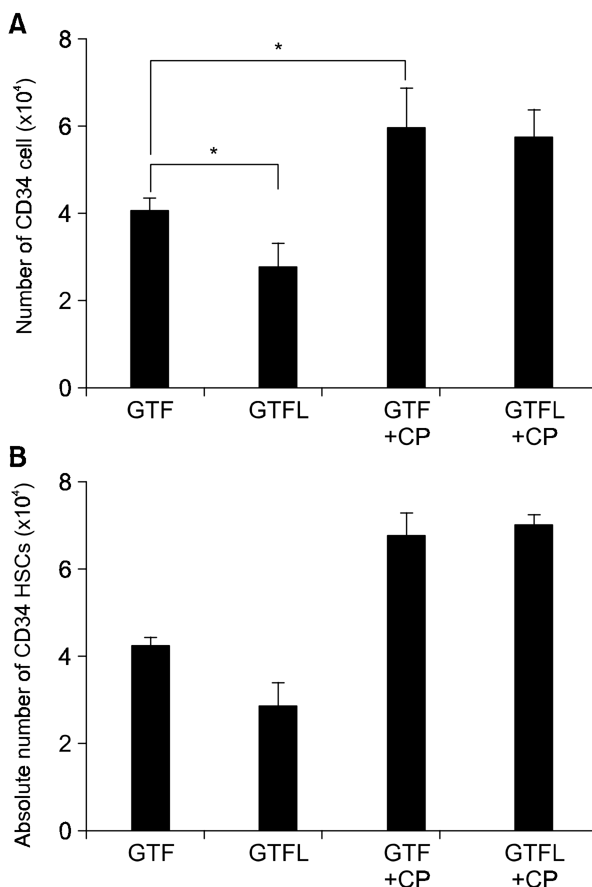

Figure 3. Effects of BLT-antagonist on self-renewal of human UCB $CD34^+$ cells. A. Freshly isolated $CD34^+$ cells (1×10^3 cells/ml) were *ex vivo* expanded in a liquid culture as described in the legend of Figure 1 (A). On day 14, total cells in each well were harvested and subjected to MACS purification. Numbers of $CD34^+$ cells in each well were determined under microscope, and the values were expressed as the means ± SD (*$P < 0.05$). B. Absolute number of $CD34^+$ HSCs calculated from Figure 1A and 1B.

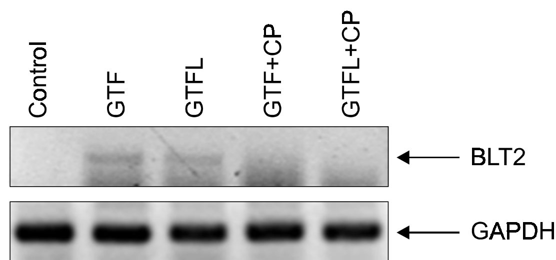

Figure 4. Expression of BLT2 in $CD34^+$ HSCs during long term culture. Total RNAs were isolated from *ex vivo* expanded HSCs and subjected to RT-PCR as described in the Material and Methods.

related to the effect of LTB_4 on HSCs' proliferation, survival, differentiation and self-renewal.

Discussion

Maintaining self-renewal and differentiation potential of hematopoietic stem cell (HSC) is a crucial component and a major challenge in stem cell research and potential clinical applications for HSC *ex vivo* expansion including tumor cell purging, gene therapy, and stem cell transplantation. In the present study, effects of LTB_4 and its receptor on *ex vivo* expansion, differentiation and self-renewal of hematopoietic stem cells were determined.

LTB_4 has been reported to enhance growth of a variety of cells including human pancreatic cancer cells (Tong *et al.,* 2002), and fibroblast (Woo *et al.,* 2002). In addition, various cytokines, evaluated for their ability to support human HSC long-term *ex vivo* expansion, share many common characteristics with LTB_4, such as proinflammatory effects, chemotactic activities and growth-stimulating factors, and suggests that LTB_4 might also be involved in the proliferation phase of HSCs.

In our study, LTB_4 itself was unable to induce proliferation of HSC in the absence of other cytokines or even in the presence of any one or two combinations of three cytokines used in this study (data not shown). This is consistent with previous report where LTB_4 had no effect on proliferation of $CD34^+$ HSCs in the absence of any cytokines (Desplat *et al.,* 2000). However, when LTB_4 was added to the culture medium supplemented with all three cytokines including G-CSF, TPO and FL, the growth of HSCs was increased by approximately 50%, compared to the control. Thus, mechanism of LTB_4 in HSC proliferation is not solely due to self-mediated action but due to the concerted actions of other cytokines. For example, the cytokines might activate LTB_4 signaling pathway or induce the expression of the proteins involved in LTB_4 signaling. Certain cykokines such as IL-13 upregulates leukotriene receptor in human lung fibroblast (Chibana *et al.,* 2003). Indeed, the expression of BLT2, a LTB_4 receptor, was induced during *ex vivo* expansion of HSCs in the presence of the cytokines (Figure 4), suggesting the cytokines might up-regulate the expression of BLT2, directly or indirectly. Interestingly, the treatment of BLT2 antagonist induced down-regulation of the BLT2 itself expression implicating autocrine regulation of the receptor expression.

Several eicosanoids, including prostaglandins and leukotrienes, have been reported to regulate cellular differentiation and apoptosis (Rudolph *et al.,* 2001). Our results also showed anti-apoptotic effect of LTB_4 on HSCs, suggesting the increase of the number of HSCs in the long term culture by LTB_4 is, at least in part, due to the retardation of apoptosis. Even though the total cell number was increased, population of CD34 positive cells in LTB_4-treated cells was lower than that of control. However, when the LTB_4 receptor antagonist was treated, retention rate of CD34 was even higher than that of control, suggesting LTB_4 induces differentiation of HSCs during *ex vivo* expansion. This was confirmed by the clonogenic assay, where LTB_4 treatement enhanced differentiation of HSC into various hematopoietic progenitors including CFU-GM and CFU-GEMM. The fact that absolute number of CD34-positive cells was higher when the HSCs were treated with BLT antagonist, even though the total cell number was relatively lower, suggests that the regulation of LTB_4 signaling pathway may contribute to the self-renewal as well as differentiation of HSCs. Taken together, LTB_4-BLT interaction is closely related to the cytokines-induced hematopoeitic stem cell proliferation, differentiation, and self-renewal, and it might be possible to determine the stem cell fate as needed, i.e. towards self-renewal or differentiation, during *ex vivo* expansion by controlling LTB_4-BLT2 signaling pathway.

Stem cells can be used to treat a variety of diseases and several recent studies in animal models demonstrate the potential of bioengineering strategies targeting adult and embryonic stem cells. In order to obtain the desired cells for transplantation, stem cell bioengineering approaches involve the manipulation of environmental signals influencing cell survival, proliferation, self-renewal and differentiation. In that regard, even though the exact mechanisms are still to be elucidated, our findings will facilitate the studies and the clinical applications in the field of the stem cell-based regenerative therapies, as treatment accessibility will depend on the development of powerful technologies to produce sufficient cell numbers.

Acknowledgement

This work was supported by grants from Korea Research Foundation (KRF-2003-003-C00110).

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