Karolinska Institutet, Stockholm, Sweden

BIOSYNTHESIS AND BIOLOGICAL ROLE OF LEUKOTRIENES IN B LYMPHOCYTES

Yilmaz Mahshid

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Copied and printed by Yilmaz Mahshid
Scheelesv. 2, SE-171 77 Stockholm, Sweden

ABSTRACT

Leukotrienes (LT) are potent lipid mediators, synthesized from arachidonic acid (AA) upon cell activation. The essential enzyme for leukotriene biosynthesis is 5-lipoxygenase (5-LO), converting AA, through a two step process to LTA_4. The activity of 5-LO is dependent on 5-lipoxygenase activating protein (FLAP). Two other enzymes can further metabolize LTA_4. Leukotriene A_4 hydrolase converts LTA_4 to LTB_4, a potent pro-inflammatory and chemotactic compound. Leukotriene B_4 exerts its biological action through two receptors known as BLT1 and BLT2. The second enzyme able to convert LTA_4 is LTC_4 synthase, which converts LTA_4 to LTC_4. Leukotriene C_4 is the first metabolite in the group of cysteinyl containing LTs (cys-LT), where LTD_4 and LTE_4 are the other two. Cysteinyl LTs are generally believed to be involved in asthmatic and allergic diseases and exert their biological actions through three receptors, CysLT1, CysLT2 and the newly GPR17.

The BLT1 expression, the activity of 5-LO and the inhibitory action of leukotriene biosynthesis inhibitors were investigated in chronic B lymphocytic leukemia cells (B-CLL). Chronic B lymphocytic leukemia cells produce LTB_4 in similar amounts as neutrophils when stimulated with azodicarboxylic acid bis(dimethylamide) (diamide), AA and calcium ionophore (A23187). The mechanism of diamide is not known, but it is believed to change the cellular redox status of the cell. FACS analysis revealed the expression of BLT1 in all B-CLL clones investigated. Cultivation of B-CLL cells with CD40-ligand (CD40L) expressing cells for 96 hours induced DNA synthesis and antigen expression of CD23, CD54 and CD150. MK-886 (a specific FLAP inhibitor) and BWA4C (a specific 5-LO inhibitor) markedly reduced the DNA synthesis and the expression of antigens. Exogenously LTB_4 reversed the effects of inhibitors. These results demonstrate the intrinsic and exogenous role of LTB_4 in B-CLL cells.

To better understand the expression of enzymes and receptors of the leukotriene biosynthesis cascade in B lymphocyte biology, subsets of B cells were purified and analyzed. Immunohistochemical analysis of tonsils revealed a high expression of 5-LO in mantle zone B cells. Western blot analysis was in agreement with the immunohistochemical findings. In addition, the western blot result also indicated that memory B cells expressed similar amounts of 5-LO as mantle zone B cells. RT-PCR demonstrated the expression of 5-LO, FLAP and LTA_4 hydrolase in memory B cells and mantle zone B cells. Mantle zone B cells and memory B cells also expressed the CysLT1 transcript. The transcripts of LTC_4 synthase, CysLT2 and BLT2 were not detected and a very low amount of BLT1 was observed. Mantle cell lymphoma (MCL) cell lines and cyclin D1 positive B cell prolymphocytic leukemia (B-PLL) (which is considered to be leukemic form of MCL) were all able to produce LTB_4 upon stimulation with diamide, AA and A23187. Cysteinyl leukotrienes induced a robust calcium response which was in agreement with the PCR data. The calcium response could be abrogated with a selective CysLT1 antagonist. Immunohistochemical staining of MCL tumor biopsies stained positive for 5-LO.

In conclusion, the present thesis demonstrates the potential involvement of leukotrienes in B cell malignancies and that 5-LO in normal B cells is primarily expressed in mantle zone and memory B cells.

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II. **Yilmaz Mahshid**, Marcus-René Lisy, Xiao Wang, Rainer Spanbroek, Jenny Flygare, Birger Christensson, Birgitta Sander, Magnus Björkholm, Andreas JR Habenicht, Hans-Erik Claesson.
The expression of 5-lipoxygenase and cysteinyl leukotriene receptor 1 in mantle zone B cells. Manuscript.

CONTENTS

LIST OF ABBREVIATIONS

12(S)-HETE	12(S)-hydroxy-5,8,14-cis-10-*trans*-eicosatetraenoic acid
12(S)-HPETE	12(S)-hydroperoxy-5,8,14-cis-10-*trans*-eicosatetraenoic acid
15(S)-HETE	15(S)-hydroxy-5,8,11-cis-13-*trans*-eicosatetraenoic acid
5,6-DHETE	5(S),6(R,S)-dihydroxy-7,9,11,14-eicosatetraenoic acid
5-HPETE	5(S)-hydroperoxy-8,11,14-cis-6-trans-eicosatetraenoic acid
5-HETE	5(S)-hydroxy-8,11,14-cis-6-trans-eicosatetraenoic acid
5-LO	5-lipoxygenase
AA	Arachidonic acid
ALCL	Anaplastic large-cell lymphoma
ARDS	Acute respiratory distress syndrome
B-CLL	Chronic B lymphocytic leukemia cells
B-PLL	B cell prolymphocytic leukemia
CMKRL1	Chemoattractant receptor-like 1
COPD	Chronic obstructive pulmonary disease
cPLA2	Cytosolic phospholipase A2
diamide	Azodicarboxylic acid bis(dimethylamide)
FACS	Fluorescence activated cell sorting
fMLP	f-Met-Leu-Phe, N-formyl-methionyl-leucyl-phenylalanine
GPCR	G-protein coupled receptor
HUVEC	Human umbilical vein endothelial cells
IBD	Inflammatory bowel disease
IL-1	Interleukin-1
IL-4	Interleukin-4
MAPEG	Membrane-associated proteins in eicosanoid and glutathione metabolism
LPS	Lipopolysaccharide
LT	Leukotriene
LTA_4	Leukotriene A_4, 5,6-epoxy-7,9-trans-11,14-cis-eicosatetraenoic acid
LTB_4	Leukotriene B_4, 5(S),12(R)-dihydroxy-6,14-cis-8,10-trans-eicosatetraenoic acid
LTC_4	Leukotriene C_4, 5(S)-hydroxy-6(R)-S-glutathionyl-7,9-trans-11,14-cis-eicosatetraenoic acid
LTD_4	Leukotriene D_4, 5(S)-hydroxy-6(R)-S-cysteinylglycyl-7,9-trans-11,14-cis-eicosatetraenoic acid
LTE_4	Leukotriene E_4, 5(S)-hydroxy-6(R)-S-cyteinyl-7,9-trans-11,14-cis-eicosatetraenoic acid
MS	Multiple sclerosis
NK cell	Natural killer cell
PAF	Platelet-activating factor
PBL	Peripheral blood leukocytes
PTX	*Bordetella pertussis* toxin
RA	Rheumatoid arthritis
TNF-α	Tumor necrosis factor-α
UDP	Uridine diphosphate

1 INTRODUCTION

1.1 HISTORICAL BACKGROUND

In the first half of the last century several physiological observations were made of extracts from prostate gland and human semen. In 1913 Battez and Boulet[1] found that injection of extracts from prostate gland into dogs lowered the blood pressure and contracted the urinary bladder. In the 1930s, Kurzrok and Lieb[2] found in *in vivo* and *in vitro* experiments that human semen affected human uterus by contraction and relaxation. Further investigations were carried out by Goldblatt[3,4] and von Euler[5-7] who were able to perform biochemical characterizations of these unknown substances. Von Euler's observations led to the name "prostaglandin"[6], substances from the prostate gland. Bergström and Sjövall managed in late 50´s and early 60´s to isolate and elucidate the structures of prostaglandin E and F[8-10]. In 1964, van Dorp's and Bergström's groups demonstrated the enzymatic formation of prostglandin E_2 from arachidonic acid[11,12].

At the same time other researchers were interested in biologically active compounds from the lung. Harkavy reported in 1930[13] that sputum from patients suffering from asthma contained a compound that led to contraction of intestinal smooth muscle cells. In 1938, Feldberg and Kellaway published a study about the effects of cobra venom on perfused lungs. The effluent from the lungs induced a slow and long lasting contraction of guinea-pig intestinal smooth muscle cells[14]. This observation led to the creation of the name slow-reacting substance (SRS). The biological action of SRSs were further demonstrated between 1940[15] to 1960[16]. During this period SRS were renamed as SRS-A (slow-reacting substance of anaphylaxis) since most observations were made in experiments were anaphylactic shock was induced. In the 70's it was shown that SRSs contained conjugated double bonds[17], sulfur[18] and could be released upon stimulation with calcium ionophore[19]. Samuelsson and coworkers finally resolved the structures of SRSs. It was shown to be a mixture of three compounds, leukotriene C_4, D_4 and E_4, later named as cysteinyl containing leukotrienes (cys-LTs)[20].

1.2 EICOSANOIDS

The word eicosanoid is derived from the greek word eicosi, meaning 20. It is a common name for the classification of compounds derived from polyunsaturated fatty acids containing 20 carbons. Eicosanoids are biologically active mediators acting in a paracrine and autocrine manner *in vivo*. Due to the inability of the human being to introduce double bonds beyond carbon nine, some of the eicosanoid precursors can only be intaken dietary. The polyunsaturated fatty acids linoleic acid and α-linolenic acid are therefore regarded as essential fatty acids. Both linoleic acid and α-linolenic acid are 18 carbon long and can further be metabolized through elongation to eicosastetraenoic acid or eicosapentaenoic acid, respectively (figure 1)[21]. Eicosatetraenoic acid also called arachidonic acid is an ω-6 fatty acid, (denoted by calculating the first double bond from the carbon on the opposite side of the

carboxylgroup), and can be metabolized to prostaglandins and thromboxanes of the 2-series and leukotrienes and lipoxins of the 4-series. This is the main pathway by which prostaglandins and leukotrienes are produced in the human body. Accordingly, eicosapentaenoic acid is an ω-3 fatty acid and can also be metabolized to prostaglandins and thromboxanes of the 3-series and leukotrienes and lipoxins of the 5-series. ω-3 fatty acids are common in fish oil and investigations have shown diets in high ω-3 gives rise to less potent eicosanoids[22-25].

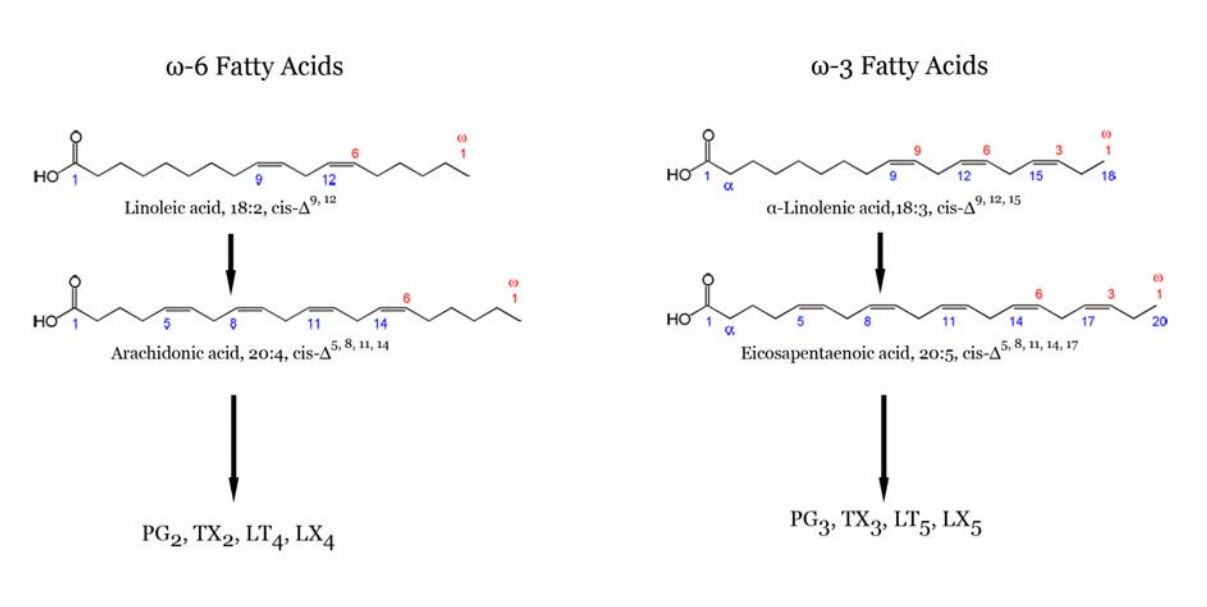

Figure 1. Precursors of eicosanoids. PG – Prostaglandins, TX – Thromboxane, LT – Leukotriene and LX – Lipoxin.

2 LEUKOTRIENES

2.1 BIOSYNTHESIS OF LEUKOTRIENES

Biosynthesis of leukotrienes requires free AA. Arachidonic acid is found in the sn-2 position of membrane phospholipids and $cPLA_2$ translocates upon cell activation from the cellular cytosol to the nuclear membrane and liberates AA[26,27]. Conversion of free AA to leukotrienes is catalyzed by 5-lipoxygenase (5-LO). This 78 kDa monomeric enzyme contains a non-heme iron atom pivotal for enzyme activity[28,29]. In the first catalytic step 5-LO converts AA to 5-HPETE (figure 2) and 5-HPETE can further be enzymatically or non-enzymatically converted to 5-HETE. Alternatively 5-HPETE can be enzymatically converted to LTA_4. To enable cellular 5-LO activity, the presence of 5-LO activating protein (FLAP) is necessary. This 18 kDa membrane associated protein is thought to support the leukotriene biosynthesis by presenting free AA to 5-LO[30,31]. Leukotriene A_4 is an unstable intermediate in the leukotriene biosynthesis and can either enzymatically or non-enzymatically be converted to 6-trans-LTB_4, 12-epi-6-trans-LTB_4, 5,6-DHETE, LTB_4 and LTC_4. To convert LTA_4 to LTB_4 (figure 2) the enzyme LTA_4 hydrolase is necessary, this enzyme acts by hydrolyzing the epoxide of LTA_4[32]. Leukotriene C_4 synthase conjugates a glutathione molecule to LTA_4 thereby producing LTC_4[33].

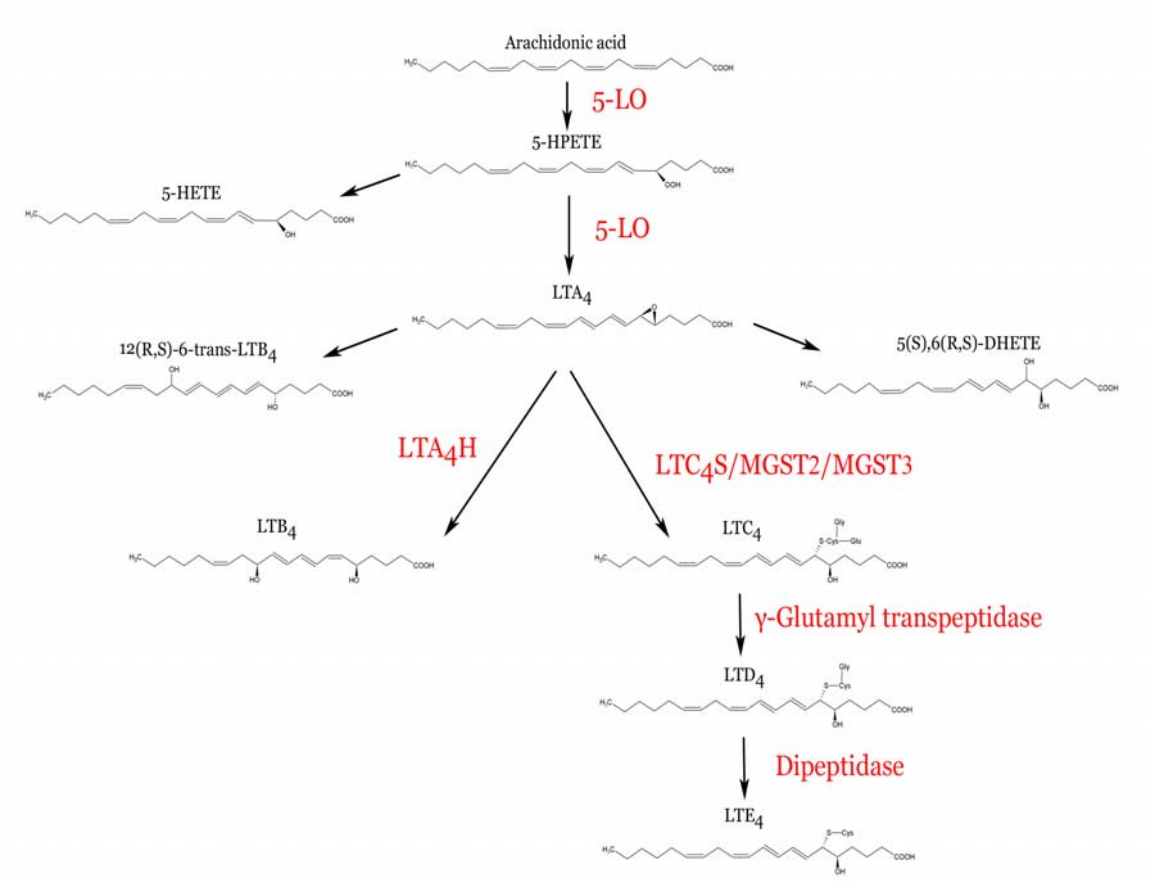

Figure 2. Biosynthesis of leukotrienes. LTA_4H – LTA_4 hydrolase, LTC_4S – LTC_4 synthase, MGST2 – microsomal glutathione S-transferase 2, MGST3 - microsomal glutathione S-transferase 3.

The molecular weight of LTC_4 synthase is 18 kDa and the protein is a member of the membrane-associated proteins in eicosanoid and glutathione metabolism (MAPEG) superfamily[34]. Two other MAPEG proteins, MGST2 and MGST3, have also been shown to convert LTA_4 to LTC_4[34-36]. To yield LTD_4 from LTC_4, the action of γ-glutamyl transpeptidase is required[37,38], and LTE_4 is formed from LTD_4 catalyzed by a dipeptidase[39-41].

2.2 ACTIVATION OF LEUKOTRIENE BIOSYNTHESIS IN HUMAN CELLS

In 1976 it was found that rabbit polymorphonuclear cells could transform AA to 5-HETE[42]. Three years later it was found that AA could give rise to LTB_4 in the same cells[43]. Today we know that 5-LO is primarily expressed in human mature myeloid cells e.g. monocytes, macrophages, neutrophils, eosinophils, mast cells and B lymphocytes[44,45]. Myeloid cells can be triggered to produce LTs for example with bacteria, fMLP or calcium ionophore (A23187)[46]. To trigger the production of LTB_4 in intact B lymphocytes a redox active agent, exogenous AA and A23187 is required[45]. Leukotriene A_4 hydrolase is expressed in most tissues even in cells lacking 5-LO e.g. endothelial cells, erythrocytes, fibroblasts and T cells. Despite the lack of 5-LO these cells can produce LTB_4 when provided with LTA_4 from another cell, so called transcellular metabolism[47-51]. Other cells lacking 5-LO and have been described to produce LTC_4 are those expressing LTC_4 synthase, MGST2 or MGST3, e.g. platelets, endothelial cells, vascular smooth muscle cells, lung tissue and kidney tissue[36,46,47,52-57].

2.3 LEUKOTRIENE B_4

Leukotriene B_4 is a potent inflammatory mediator and exerts its biological effects primarily on leukocytes. Neutrophils are affected by LTB_4 at nano- to micromolar concentrations by chemotaxis, adherence to the endothelial walls, aggregation, production of superoxide anions, degranulation and release of lysozomal enzymes[58-63]. Upon LTB_4 treatment neutrophil survival is prolonged by preventing apoptosis[64]. Mice lacking the 5-LO gene required exogenously added LTB_4 to activate neutrophils and alveolar macrophages for effective phagocytosis and killing of bacteria[65,66]. These mice respond to endotoxin shock in the same way as wild type mice but they do not suffer lethal effects from PAF-induced shock and have less severe symptoms from ear inflammation induced by AA[67,68].

Leukotriene B_4 has been shown to be an agonist for the nuclear transcription factor PPARα, implicating a feedback mechanism of LTB_4 and/or lipidmetabolism[69]. Monocytes treated with LTB_4 affects the production of IL-6, IL-1 and TNF-α[70,71]. A positive effect of LTB_4 in myelopoiesis and immunoregulation has been reported[72-75]. Moreover, LTB_4 is considered to affect proliferation and differentiation of B cells and also the expression of CD23 and secretion of immunoglobulins[74,75]. It has also been proposed to induce NK cell activity, IL-2Rβ expression and sensitivity to IL-2[70,76,77].

Treatment of $CD8^+$ T cells with LTB_4 increases the proliferation rate, and IL-2Rβ expression. $CD4^+$ T cells respond to LTB_4 by increased IL-2 production. LTB_4 not only activate human leukocytes but also carcinoma cell lines and endothelial cells[78-81]. Treatment of endothelial cell monolayers with LTB_4 increases their binding of neutrophils, and promotes neutrophils transendothelial migration[79,80]. Human carcinoma cell lines and human pancreatic cell lines treated with LTB_4 stimulated the proliferation rate[78,82]. Furthermore, LTB_4 has been implicated in several inflammatory and autoimmune diseases, e.g. cystic fibrosis[83,84], chronic obstructive pulmonary disease (COPD)[85,86], asthma[46], acute respiratory distress syndrome (ARDS)[87], multiple sclerosis (MS)[88], rheumatoid arthritis (RA)[89], psoriasis[90,91], inflammatory bowel disease (IBD)[92,93], and chronic B lymphocytic leukemia (B-CLL)[94]. Human diseases where LTB_4 has been implicated is summarized in table 1.

Disease	Reference
Cystic fibrosis	83,84
Chronic obstructive pulmonary disease	85,86
Asthma	46
Acute respiratory distress syndrome	87
Multiple sclerosis	88
Rheumatoid arthritis	89
Psoriasis	90,91
Inflammatory bowel disease	92,93
Chronic B lymphocytic leukemia	94

Table 1. LTB_4 and human diseases.

2.4 CYSTEINYL LEUKOTRIENES

Cysteinyl leukotrienes (cys-LT) are produced by mast cells, macrophages and eosinophils but also through transcellular metabolism between PMNLs and endothelial cells/platelets[46]. These substances are potent constrictors of human bronchial smooth muscle cells both *in vitro* and *in vivo*[95-102]. On concentration basis cys-LTs are 100-1000 times more potent than histamine[103]. Elevated levels of LTE_4 is found in the urine of atopic asthmatics after allergen challenge and in the urine of aspirin intolerant asthmatics after lysine-aspirin challenge. These and previously presented data support the concept of cys-LTs being mediators of asthma[46,104-107]. Cysteinyl LTs also increase vascular permeability in postcapulary venules, stimulate mucus secretion of epithelial cells and recruit inflammatory cells[108-110]. Cysteinyl leukotrienes have also been implicated in the pathogenesis allergic rhinitis and psoriasis[46]. There is also evidence that cys-LTs stimulate myelopoiesis[73].

2.5 RECEPTORS OF LEUKOTRIENE B_4

In 1996 two independent groups cloned an orphan G-protein coupled receptor (GPCR) that was named R2 and chemoattractant receptor-like 1 (CMKRL1)[111,112]. One year later the LTB_4 receptor was fully cloned from differentiated HL60 cells[113]. The receptor was first named BLTR and renamed to BLT1 when the second LTB_4 receptor was discovered. The K_d was found to be between 0.39-1.5 nM for LTB_4[114-116]. Northern blot experiments have demonstrated the expression of BLT1 primarily to leukocytes but was also found in a much lower extent in spleen, thymus, bone marrow, lymph nodes, heart, skeletal muscle, brain and liver[117]. The BLT1 affinity for different ligands relative to LTB_4 is, LTB_4 >12-oxo-LTB_4 = 20-OH-LTB_4 >> 20-COOH-LTB_4[113].

The second LTB_4 receptor BLT2 was cloned year 2000 by four independent groups[118-121]. Like BLT1, BLT2 is a GPCR receptor. The similarity between the two human receptors is 45.2%, similar to the homology in mice, 44.6%. The similarity between BLT2 in human and mice is high as 92.7%. BLT2 is referred to as the low affinity receptor of LTB_4 due to the 20-fold higher K_d[118]. The lower affinity of BLT2 is also confirmed by intracellular calcium measurements and in chemotaxis assays[118]. Many other eicosanoids have also been described as ligands for BLT2; LTB_4> 12(S)-HETE> 12(S)-HPETE> 15(S)-HETE> 20-OH-LTB_4[122]. BLT2 expression in human is significantly different from BLT1. Most human tissues express BLT2, with expression being highest in spleen, liver, ovary, and peripheral leukocytes. There is evidence that the receptors couple through different G proteins depending on cell type (both PTX-sensitive and –insensitive G proteins) but also within the same cell different signaling cascades occur[117]. Several pharmacological active antagonists have been developed against the receptors, some are BLT1 or BLT2 specific and some binding both receptors[117].

BLT1 expression is primarily involved in inflammation and evidence is presented mostly in mouse models. Resting neutrophils and macrophages in mice barely express BLT1 but sodium casein injection into the peritoneum dramatically induce BLT1 in these cells[123]. Stimulation of RAW 264.7 macrophage cell line by IFNγ and human peripheral neutrophils by dexamethasone also induced BLT1 expression[123,124]. Treatment of endothelial cells with a LTB_4 receptor antagonist almost completely abolished LTB_4-induced neutrophil transmigration[125]. Reduced phagocytosis was observed in human neutrophils pretreated with a LTB_4 receptor antagonist. Upregulation of BLT1 has been demonstrated to be one mechanism through which glucocorticoids prolong neutrophil survival[124]. Several human pancreatic cancer cell lines have been shown to induce apoptosis *in vitro*, upon treatment with an LTB_4 receptor antagonist[126]. *In vivo* treatment of athymic mice receiving human pancreatic carcinoma xenografts with an LTB_4 receptor antagonist also induced apoptosis of these tumors[126]. Recently it was shown that BLT1 and BLT2 are differentially expressed in activated HUVEC. LPS or LTB_4 treatment of HUVEC led to a specific induction of BLT1 while TNF-α treatment primarily induced BLT2 expression[127].

In BLT1 deficient mice neutrophil chemotaxis, activation and LTB_4-induced calcium flux is abrogated[128,129], indicating that these functions are mediated through BLT1. Furthermore, edema and protein extravasation in response to topically applied AA is reduced and female BLT1 deficient mice have reduced mortality in a model of immediate hypersensitivity induced by intravenous injection of PAF[129]. The observed sex differences in this model have not yet been elucidated. BLT2 deficient mice have

yet not been created, therefore little is known about the specific biological effects mediated through this receptor. The high degree of similarity across species of BLT2 suggests that this receptor have an important biological function. Recently a double BLT1/BLT2 mice knock out was created. In an arthritis model with collagen these mice were equally good protected from disease development as the single BLT1 knock out mice[130].

LTB_4 receptor antagonists have been used with success in several inflammatory animal models i.e. amelioration or complete improvements has been observed. The BLT2 antagonist LY255283 significantly improved systemic arterial hypotension, pulmonary arterial hypertension, pulmonary edema and arterial hypoxemia in a porcine model of shock and ARDS[131]. In a study with anaplastic large-cell lymphoma (ALCL), LY293111 (non-specific BLT antagonist) treatment inhibited proliferation and induced G1-S cell cycle arrest[132]. Significant improvements with CP-105696 (BLT1 antagonist) were seen in collagen induced arthritis, experimental allergic encephalitis (model of MS), airway hyperresponsiveness (model of Asthma), cardiac allograft rejection (model of cardiac transplantation) and atherosclerosis[133-137]. ONO4057 (non-specific BLT antagonist) significantly prolonged allograft survival in a rat model of transplantation[138]. SC-53228 improved the disease outcome in a murine model of IBD[139].

2.6 RECEPTORS OF CYSTEINYL LEUKOTRIENES

The biological response of cys-LTs has been believed to be elicited through two receptors, CysLT1 and CysLT2[140-144]. Recently, a third receptor for cys-LTs was identified, GPR17[145]. All three receptors are, as LTB_4-receptors, G-protein coupled receptors.

Human CysLT1 receptor mRNA has been found in several cells and tissues e.g. spleen, PBL, lung, placenta and colon[143,144,146,147]. The receptors affinity for cys-LTs is between 2.5 nM – 240 nM, with highest affinity for LTD_4 and lowest for LTE_4[143]. In addition to cys-LTs, acetyl choline and UDP have also been reported as ligands for CysLT1 and homology with P2Y has been identified[148,149].

CysLT2 receptor mRNA seems to be ubiquitously expressed with highest expression in heart, brain, spleen, PBL, placenta, lymph node, and adrenal gland[141,142,144]. The affinity of CysLT2 is similar for LTC_4 and LTD_4 with EC50 values ranging from 2-10 nM. As for CysLT1, LTE_4 have the lowest affinity for the receptor with EC50 values between 50-330 nM[142,144].

The latest member of the cys-LT receptor family is GPR17. This GPCR was deorphanized in the beginning of 2006 and determined by phylogenic analysis to be at intermediate position, between P2Y receptor and cys-LT receptors. It was shown to have nanomolar affinity for cys-LTs and micromolar affinity to uracil nucleotides. GPR17 is expressed at mRNA levels in rat and human brain, heart and kidney[145].

Implications of cys-LTs and their receptors have primarily been in the pathogenesis of allergic and asthmatic diseases[46,150].

3 B LYMPHOCYTES

3.1 NORMAL B LYMPHOCYTES

B lymphocytes are developed from the B progenitor cell in the bone marrow. They proliferate and reach a certain maturation stage in the bone marrow. During this maturation process the B cells first rearrange their immunoglobulin heavy chain. Secondly they rearrange their immunoglobulin light chain and enter the blood stream as B lymphocytes expressing surface immunoglobulins. Immature B cells get activated in the lymph nodes. During the activation process B cells go through somatic mutations, class switching and differentiate either into memory B cells or into immunoglobulin producing plasma cells.

3.2 CHRONIC B LYMPHOCYTIC LEUKEMIA

Chronic B lymphocytic leukemia (B-CLL) is the cancer of small B lymphocytes in the bone marrow, peripheral blood and lymph nodes. The disease is characterized by a slow accumulation of malignant B cells, which escapes apoptosis via a variety of mechanisms[151]. B-CLL is a disease that is most common in individuals above 50 years of age with a median age of approximately 65 years. It is the most common leukemia in the western part of the world. The disease is two times more common in men than in woman. The incidence in Sweden is about 5 cases per 100,000 individuals.

Chronic B lymphocytic leukemia is diagnosed by morphological examinations and analysis of cell specific antigens like, CD5, CD19, CD20, CD23 and surface membrane immunoglobulin[152].

The disease is very heterogeneous with variable clinical outcome. Classification of the different clinical stages are made according to Rai and Binet[153,154] and is the basis for therapeutic decisions. Approximately one third of the patients display symptoms and require immediate treatment. Another third have an indolent course which can progress. The remaining third never require treatment and die from other causes[155]. Recently many new prognostic features have been identified which may in the feature be helpful when deciding a therapeutic approach[156].

4 AIMS OF THE PRESENT INVESTIGATION

The aim of this thesis was to understand the biological role and biosynthesis of leukotrienes in B lymphocytes:

Malignant B cells have been used to study the role of leukotrienes in B cell malignancies and as a tool to study the enzymes and receptors involved in leukotriene biosynthesis in order to better understand the biology of normal B lymphocytes.

5 RESULTS AND DISCUSSION

5.1 PAPER I

Leukotriene B_4 plays a pivotal role in CD40-dependent activation of chronic B lymphocytic leukemia cells

The biological role of leukotrienes in B lymphocytes is poorly understood in comparison to myeloid cells. B lymphocytes express 5-LO as myeloid cells, but do not produce leukotrienes upon challenge with calcium ionophore (A23187). To activate the biosynthesis of leukotrienes in intact B cells stimulation with diamide, a thiol-reactive reagent, is needed together with AA and A23187[70]. It has been reported that LTB_4 affect B cells in different ways e.g. activation, proliferation and increased antibody production[70,75,157]. Microarray studies have shown 5-LO to be one of the most abundant expressed genes in chronic B lymphocytic leukemia (B-CLL)[158]. It was therefore of interest to investigate whether B-CLL cells had the capacity to produce leukotrienes and which role 5-LO plays in the function of these cells.

The capacity of B-CLL cells to produce leukotrienes was investigated. When cells were challenged with AA or A23187 alone, leukotrienes could not be detected. Activation of cells with AA and A23187 in combination led to the formation of LTB_4 (mean 2.6 pmol / 10^6 cells). High amounts of LTB_4 (mean 33.5 pmol / 10^6 cells) was produced when intact cells where treated with diamide (as described above) and also in sonicated cells incubated with AA, ATP and calcium (mean 34.8 pmol / 10^6 cells).

B-CLL cells were subjected to flow cytometry analysis to investigate if they expressed the BLT1 receptor. Cells from six patients with B-CLL and two patients with B cell prolymphocytic leukemia (B-PLL) were investigated; average expression was 42% and 74% of the cells, respectively. As a comparison 30-50% of the normal B lymphocytes did also express the BLT1 receptor.

In order to determine whether leukotrienes could affect the proliferation of B-CLL cells, CD40-ligand expressing L cells (CD40L-L) were used to activate the B-CLL cells in combination with LTB_4 and two leukotriene biosynthesis inhibitors. The proliferation rate was determined with thymidine incorporation assay. When B-CLL cells were cultivated with control L cells (96 hrs) only minimal thymidine incorporation was observed in contrast to cultivation with CD40L-L cells (96 hrs) where a significant increase was observed. In the presence of 100 nM MK-886 (a specific FLAP inhibitor)[159] a significant inhibition of thymidine incorporation was seen. BWA4C, a specific 5-LO inhibitor, also significantly inhibited the proliferation at 10 nM. Exogenously added LTB_4 (150 nM) almost completely reversed the inhibitory action of MK-886 and BWA4C. LTB_4 alone did not further amplify the CD40-induced thymidine incorporation.

FACS analysis demonstrated that CD40-CD40L interactions caused an increased expression of CD23, CD54 and CD150. MK-886 (100 nM) and BWA4C (100 nM) markedly counteracted the antigen expressions. Addition of exogenous LTB_4 (150 nM) almost completely reversed the effects of MK-886 and BWA4C. LTB_4 alone did not further induce CD23, CD54 and CD150 expression.

In this paper we provide evidence that 5-LO in B-CLL cells can, as in normal B lymphocytes, be activated during certain conditions[45,160]. CD40-CD40L interaction

caused a pronounced increased proliferation of B-CLL cells detected by thymidine incorporation assay and FACS analysis of antigen expressions. MK-886 (a specific FLAP inhibitor) and BWA4C (a specific 5-LO inhibitor) markedly inhibited the proliferation both on DNA synthesis and antigen expression. Interestingly, exogenous LTB_4 reversed the effects of the inhibitors. These results are in agreement with earlier findings that LTB_4 affects the activation and proliferation of B cells[70]. It is known that normal B cells barely express CD54, while B cells from CLL patients have an increased expression[161]. High expression is associated with poor prognostic features, including increased tumor burden and sometimes a short lymphocyte doubling time[161,162]. CD23 has been proposed to be involved in cell viability and proliferation[163]. High serum levels of soluble CD23 was associated with high tumor burden and shorter time to progression in B-CLL[164,165]. It has also been reported that LTB_4 stimulate the expression of CD54 on endothelial cells and CD23 on B cells[74,157,166]. In summary, this study demonstrates that LTB_4 plays an important role in the activation of B-CLL cells. Inhibitors of leukotriene synthesis have so far only been used for treatment of asthma. The present report indicates that leukotriene biosynthesis inhibitors, LTA_4 hydrolase inhibitors, or BLT1 antagonists, alone or in combination with conventional therapy, might also be useful in the treatment of B-CLL cells.

5.2 PAPER II

The expression of 5-lipoxygenase and cysteinyl leukotriene receptor 1 in mantle zone B cells

In this paper we investigated the expression of leukotriene biosynthesis enzymes and receptors in different subsets of B lymphocytes.

Three different subpopulations of B cells were analyzed in tonsils. The expression of 5-LO was characterized immunohistochemically in mantle zone B cells (IgD^+), germinal center (GC) B cell ($CD38^+$) and plasma cells ($CD38^{++}$ or $CD138^+$). Mantle zone B cells displayed high expression of 5-LO, GC B cells displayed low 5-LO expression and plasma cells (both inside and the GC) did not express 5-LO.

The expression of 5-LO in different subpopulations of B cells was further investigated with western blot. Mantle zone B cells, GC B cells, plasma cells and memory B cells were separated from tonsils. Western blot results were in agreement with the immunohistochemical analysis of 5-LO expression in tonsils. In addition to previous finding the western blot result indicate that memory B cells contain similar amounts of 5-LO as mantle zone B cells.

RT-PCR was performed on isolated total RNA of mantle zone B cells, GC B cells and memory B cells. Following leukotriene related genes were analyzed, 5-LO, FLAP, LTA_4 hydrolase, LTC_4 synthase, BLT1, BLT2, CysLT1 and CysLT2. Memory B cells, GC B cells and mantle zone B cells all expressed the transcripts for 5-LO, FLAP and LTA_4 hydrolase. No subpopulation of B cells expressed LTC_4 synthase. Mantle zone B cells and memory B cells expressed the transcript of CysLT1. The transcript of CysLT2 was undetectable, the transcript of BLT2 was barely detectable and BLT1 exhibited very low expression.

In order to investigate the capacity of mantle zone B cells to produce leukotrienes, cyclin D1 positive prolymphocytic B cell leukemia cells (B-PLL, which is considered to be a leukemic form of mantle cell lymphoma, MCL) and MCL cell lines were investigated. B-PLL cells stimulated with diamide, AA and A23187 produced similar amounts of LTB_4 (mean 30 pmol / 10^6 cells) as human neutrophils[167]. Sonicated cells also readily produced LTB_4 (mean 25.8 pmol / 10^6 cells). Stimulation with A23187 alone resulted in about 1 pmol LTB_4 / 10^6 cells, indicating the low contamination of neutrophils in these preparations. The MCL cell lines Granta519, Jeko1 and Rec1 did also produce LTB_4, 6.2 pmol, 14.5 pmol and 7.0 pmol / 10^6 cells, respectively, after diamide, AA and A23187 stimulation. The 5-LO expression in MCL cell lines was confirmed with western blot, showing the 5-LO protein in all three cell lines.

To study the existence of a functional CysLT1 in MCL cell lines, intracellular calcium measurements were made. Stimulation of all three cell lines with LTD_4 (100 nM) led to a robust calcium signal. This response was completely abrogated when cells were pre-treated for 15 minutes with Zafirlukast (10 nM), a selective CysLT1 antagonist. The other two cysteinyl leukotrienes, LTC_4 and LTE_4 resulted also in a calcium response but not as robust as the signal from LTD_4. In contrast LTB_4 (1-1000 nM) did not result in any calcium signal.

Tumor biopsies (one lymph node, one spleen) obtained from two patients with MCL were analyzed. Virtually all cells stained for CD20 and 5-LO in both biopsies, indicating a strong 5-LO expression in mantle cell lymphoma B cells.

It has been known for 15 years that B lymphocytes express 5-LO, but the mechanism of activation of the enzyme is still not clarified. B lymphocytes do not readily produce LTB_4 after calcium ionophore challenge as myeloid cells. To achieve similar amounts of LTB_4 by intact B lymphocytes as myeloid cells, a change in the cellular oxidative status is required[45,168,169]. It is also known that LTB_4 affect the proliferation, differentiation and Ig production of B lymphocytes[74]. In this paper we identify and dissect the expression of four known leukotriene receptors and four proteins and enzymes of the leukotriene cascade in subsets of B lymphocytes. Western blot and immunohistochemical analysis identify the expression of 5-LO primarily in mantle zone and memory B cells. RT-PCR analysis confirm the 5-LO expression but also identifies the expression of FLAP and LTA_4 hydrolase in mantle zone B cells, memory B cells and GC B cells. RT-PCR analysis also identifies the expression of CysLT1 in mantle zone and memory B cells. The CysLT1 expression is confirmed with intracellular calcium measurements in MCL cell lines. Investigation of samples from B-PLL patients and MCL cell lines resulted in detection of LTB_4, showing that these cells have active LTB_4 producing enzymes. Immunohistochemical analysis of tumor biopsies from MCL further confirm our observation that 5-LO is expressed in mantle zone B cells. Taken together, this study and earlier reports demonstrate that 5-LO is expressed in relatively immature B cells, peripheral B cells, various malignant B cells, mantle zone B cells and memory B cells but not in plasma cells and the most immature B cells[45,168,170]. The production and release of LTB_4 by B cells is still up to debate. No physiological stimulus that activates the leukotriene biosynthesis cascade has yet been identified. LTB_4 is perhaps exerting its biological effect within the B cell. Another idea is that 5-LO has a role in the differentiation of B cells, since most immature and mature B cells do not express 5-LO. Why mantle zone B cells express 5-LO and CysLT1 is at

this moment difficult to speculate. It might be involved in the progression of the disease or differentiation of B cells.

6 METHODOLOGY

Methodologies used in this thesis are established methods within the fields of biochemistry, immunology and molecular biology. They are listed and described in the respective paper.

7 ACKNOWLEDGEMENTS

Now, finally, I've managed to put together a small licentiate thesis. This means that I'm officially halfway through, to reach my PhD. I hope the next half will not be as hard as the first one, just more efficient (a wish that hopefully will become true).

According to tradition you should acknowledge people around you, people who have contributed to the work, people who have helped you with techniques and so on. But I feel that this thesis is just a small stop before I reach my final destination. If I write all my appreciations now, there would not be much left for my next thesis. Therefore I just shortly want to thank my Professor, Hans-Erik Claesson for giving me the opportunity to do my licentiate thesis under his supervision. It's been a pleasure. Thanks also to my co-authors in the first paper and in the manuscript. Now I will instead share some of my thoughts about everything and anything.

Science has its ups and downs. Mostly you work and work ending up with negative results and thinking that you've wasted your time for nothing. Sometimes your experiments are positive and you are happy, until it turns out that it was an artifact. In rare cases you get positive results that can be repeated (I should point out that this last example seldom strikes me, positive results seems to be my enemy, it has just occurred a couple of times during the last 3.8 years). But when you get those results you become euphoric, sometimes so euphoric that you want to go for bungee jumping, until you realize that you appreciate living too much.

Now I just described some moments in science, but being a part of Biolipox gives you other memorable moments. Actually it can be called science but on the other hand it is not. I would call it human biology. I can admit with my hand on my heart and without exaggerating that I have never seen so many pregnant women at the same time in the same place. The funniest thing is that it doesn't end, as soon as a baby has plopped out another one is on its way and it seems like a bottom less well. It does of course work men here, who also appreciate having a child, but they are a minority. Congratulations to all the first time, second time and third time mothers and fathers. Without any names mentioned I would also like to congratulate the fourth time mother. When writing the next thesis I will probably see some of the mothers with a new big, round thing pointing in the walking direction. You will be acknowledged again, then.

One thing my department where I'm registered as a PhD student doesn't have in common is the above mentioned phenomenon. Memorable moments from Kemi 2 are instead all the things that end with a p-word and starts with Christmas, crayfish, birthday or just the p-word. Many thanks to Anders who always is a key player in this, without you probably none of the p-things would have happened. Thank you SCP for reading and commenting this document.

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I

Leukotriene B_4 plays a pivotal role in CD40-dependent activation of chronic B lymphocytic leukemia cells

Gudmundur Runarsson, Anquan Liu, Yilmaz Mahshid, Stina Feltenmark, Annika Pettersson, Eva Klein, Magnus Björkholm, and Hans-Erik Claesson

Biosynthesis of leukotrienes (LTs) occurs in human myeloid cells and B lymphocytes. However, the function of leukotrienes in B lymphocytes is unclear. Here, we report that B-cell chronic lymphocytic leukemia (B-CLL) cells produce leukotriene B_4, and that specific leukotriene biosynthesis inhibitors counteracted CD40-dependent activation of B-CLL cells. Studies on the expression of the high-affinity receptor for LTB_4 (BLT1) by flow cytometry analysis showed that the receptor was expressed, to a varying degree, in all investigated B-CLL clones. At a concentration of 100 nM, the drugs BWA4C (a specific 5-lipoxygenase inhibitor) and MK-886 (a specific 5-lipoxygenase activating protein inhibitor) markedly inhibited CD40-induced DNA synthesis (45% and 38%, respectively) and CD40-induced expression of CD23, CD54, and CD150. Addition of exogenous LTB_4 (150 nM) almost completely reversed the effect of the inhibitors on DNA synthesis and antigen expression. Taken together, the results of the present study suggest that leukotriene biosynthesis inhibitors may have a therapeutic role in B-CLL. (Blood. 2005; 105:1274-1279)

Introduction

Leukotrienes (LTs) are biologically active metabolites of arachidonic acid.[1] Once liberated by phospholipase A_2, arachidonic acid can be converted to prostaglandins, thromboxanes, and leukotrienes. The key enzyme in leukotriene biosynthesis is 5-lipoxygenase (5-LO), which in a 2-step reaction catalyzes the formation of LTA_4 from arachidonic acid.[1] Leukotriene A_4 can be further metabolized into LTB_4, a reaction catalyzed by LTA_4 hydrolase. Cellular leukotriene biosynthesis is dependent on 5-LO activating protein (FLAP), a membrane protein that binds arachidonic acid and facilitates the 5-LO reaction.[1]

In contrast to prostaglandins, which are produced by almost all type of cells, formation of leukotrienes from arachidonic acid is restricted to a few cell types in the human body. Biosynthesis of leukotrienes occurs mainly in myeloid cells and B lymphocytes.[1] The production of LTB_4 and the biologic effects of this compound on myeloid cells are well characterized, and LTB_4 stimulates neutrophil trafficking and activation at very low concentrations.[1] However, the biosynthesis and function of leukotrienes in B lymphocytes are much less characterized. In contrast to myeloid cells, intact B cells do not produce LTB_4 after challenge with calcium ionophore A23187 only.[2,3] The mechanism of activation of leukotriene biosynthesis in intact B cells is unclear, but there is accumulating evidence that the cellular oxidative status is of importance for biosynthesis of leukotrienes.[3-6] Furthermore, the p38 mitogen-activated protein kinase appears also to be involved in stress-induced leukotriene synthesis in B cells.[7] There is no convincing report demonstrating that T lymphocytes contain 5-LO and can produce leukotrienes. T lymphocytes do express FLAP, however, but the function of this protein in T cells is not known.[3]

The actions of LTB_4 on leukocytes are mainly mediated by BLT1, a high-affinity G-protein coupled LTB_4 receptor expressed on neutrophils and monocytes.[8-10] BLT1 is also expressed on activated T lymphocytes, both $CD8^+$ cells and $CD4^+$ cells,[11-13] and weakly on peripheral human nonactivated B lymphocytes.[14] A second LTB_4 receptor, BLT2, with lower ligand affinity and wider tissue distribution, has also been characterized.[15,16]

Leukotriene B_4 is an immunomodulator, and this compound activates B cells, T cells, and natural killer (NK) cells.[17] Several reports indicate that LTB_4 enhances activation, proliferation, and antibody production in tonsillar B lymphocytes,[18-20] and stimulates various T-cell functions.[21,22] Leukotriene B_4 is a very potent chemotactic compound for activated T lymphocytes, and BLT1-receptor–deficient mice have an impaired trafficking of activated $CD8^+$ cells and $CD4^+$ cells.[11-13]

The B-cell surface protein CD40 belongs to the tumor necrosis factor/nerve growth factor receptor family, and plays an important role in T-cell–dependent B-cell activation. Ligation of this receptor with antibodies or with CD40-ligand (CD40L) generates an intracellular signal that induces a variety of stimulatory events in both normal and malignant B lymphocytes. Stimulation of B cells

From the Departments of Medicine and Medical Biochemistry and Biophysics, and the Microbiology and Tumor Biology Center, Karolinska Institutet, Stockholm, Sweden; Karolinska University Hospital, Stockholm, Sweden; and the Division of Molecular Neurobiology, Wallenberg Neuroscience Center, Lund University, Lund, Sweden.

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G.R. and A.L. contributed equally to this work.

Reprints: Hans-Erik Claesson, Department of Medical Biochemistry and Biophysics, Division of Chemistry II, Karolinska Institutet, 17177 Stockholm, Sweden; e-mail: hans-erik.claesson@mbb.ki.se.

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with CD40L and interleukin-4 (IL-4) leads to homotypic adhesion, proliferation, and differentiation into immunoglobulin (Ig)–producing cells.[23-26] The expression of CD23 is a marker of activation of B cells, and CD54 (intercellular adhesion molecule-1 [ICAM-1]) is an important adhesive molecule expressed to various extents on many B-cell chronic lymphocytic leukemia (B-CLL) clones. CD150 is an antigen involved in the bidirectional stimulation of T and B cells and is up-regulated on activated B cells.[27-29]

Microarray studies have demonstrated an abundant expression of *5-LO* in certain B-cell malignancies.[30-33] In fact, *5-LO* was one of the most abundantly expressed genes of 1024 selected "lymphocyte" genes in B-CLL samples compared with a mixture of normal human tissues used as the reference sample.[33] Certain types of diffuse large B-cell lymphomas also have a comparatively high expression of *5-LO*.[30]

In light of these studies, it was of interest to explore the biosynthesis of leukotrienes in B-CLL cells and the effects of specific leukotriene biosynthesis inhibitors on the activation of B-CLL cells.

Patients, materials, and methods

Reagents and cell lines

The calcium ionophore A23187 was purchased from Calbiochem-Behring (La Jolla, CA). High-performance liquid chromatography (HPLC) solvents were obtained from Rathburn Chemicals (Walkerburn, United Kingdom), and the synthetic standards of LTB_4 and prostaglandin (PG) B_2 were from Biomol (Plymouth, PA). BWA4C was a kind gift from Lawrie G. Garland (Wellcome Research Laboratories, Beckenham, United Kingdom) and MK-886 from Jilly F. Evans (Merck Research Laboratories, Rahway, NJ,). Azodicarboxylic acid bis(dimethylamide) (diamide) was purchased from Sigma (Stockholm, Sweden). Mouse fibroblastic L cells transfected with the human CD40L (CD40L-L) were used for activation, and nontransfected L cells (L) as control.[34]

Patients

Cell samples were studied from 6 patients with B-CLL. There were 3 women and 3 men with a median age of 66 years. The median time since diagnosis was 73 months, and 2 patients were chemotherapy naive. The remaining 4 patients had received 1 to 6 different treatments.

Isolation of cells

B cells were isolated from patients suffering from B-CLL or B-prolymphocytic leukemia (B-PLL) who had not received chemotherapy in the previous 6 weeks. Peripheral blood samples were obtained after informed consent and with local ethics committee (Karolinska University Hospital) approval. Blood samples were purified by Ficoll-Paque (Amersham Biosciences, Uppsala, Sweden) and washed twice in phosphate buffered saline (PBS) and either frozen in PBS with 50% human blood type AB serum and 10% dimethylsulfoxide, or analyzed while fresh. Frozen cell samples were thawed and washed in ice-cold fetal calf serum (FCS) and subsequently suspended in PBS before analysis. Cells from 2 patients were used twice: both freshly isolated cells and cells after freezing. However, similar results were obtained (data not shown). The purity of the isolated cells was estimated by flow cytometric analysis with FACS Calibur (Becton Dickinson, Mountain View, CA). Morphologic analysis was performed after staining with May-Grunewald/Giemsa solution. The purity of B-CLL and B-PLL cells was greater than 98%.

Incubation of intact B-CLL cells

Freshly isolated cells (10^7) were suspended in 1 mL PBS and incubated for 2 minutes with or without diamide (100 μM) prior to stimulation with arachidonic acid (40 μM) and/or calcium ionophore A23187 (1 μM). The cells were stimulated for 5 minutes at 37°C, and the incubations were terminated with 1 mL methanol.

Incubation of sonicated B-CLL cells

Freshly isolated cells (10^7) were suspended in 1 mL calcium-free PBS including EDTA (ethylenediaminetetraacetic acid; 2 mM) and sonicated 3 times for 5 seconds. The cells were incubated for 2 minutes in the presence of adenosine triphosphate (ATP; 1 mM) prior to addition of calcium chloride (2 mM) and arachidonic acid (40 μM). The reaction was terminated with 1 mL methanol after 5 minutes of incubation at 37°C.

HPLC analysis of leukotrienes

After addition of 0.5 mL PBS and the internal standard PGB_2 (100 pmol) to the samples, the cells were centrifuged (800*g*, 5 minutes). The supernatant was subsequently subjected to solid-phase extraction on Sep-Pak Vac C_{18} columns (100 mg; Waters, Stockholm, Sweden). The methanol eluate was analyzed on Waters Alliance 2695 reverse-phase HPLC and detected with Waters PDA 996. Methanol plus water plus trifluoroacetic acid (70:30:0.007, vol/vol) was used as mobile phase at a flow rate of 1.2 mL/min. Qualitative analysis was performed by comparison of retention times of synthetic standards and online analysis of ultraviolet (UV)–spectra of eluted compounds. Quantitative determinations were performed by computerized integration of the area of eluted peaks.

Flow cytometry analysis of BLT1

Fresh blood samples from healthy donors and fresh samples from patients were separated by Ficoll-Paque and washed in PBS. For analysis of whole-blood leukocytes (including granulocytes) from healthy donors, cells were washed in PBS and lysed with fluorescence-activated cell-sorting (FACS) lysing solution (Becton Dickinson, Mountain View, CA). Frozen patient samples (B-CLL and B-PLL) were thawed as described in "Isolation of cells" and washed in PBS. After resuspending cells in 100 μL PBS, antibodies were added according to manufacturer's instructions and incubated at room temperature for 10 minutes. The cells were washed in 2 mL PBS and fixed in 1% paraformaldehyde before analysis with FACS Calibur (Becton Dickinson) using CellQuest software (Becton Dickinson). In this study, all the antibodies used for flow cytometry were directly conjugated with either fluorescein isothiocyanate (FITC), phycoerythrin (Pe), or peridinin chlorophyll protein (PerCP). The BLT1 antibody 7B1 FITC was raised in-house.[35] IgG1-FITC, IgG1-Pe, IgG1-PerCP, CD4-Pe, CD5-Pe, CD8-PerCP, CD14-FITC, CD14-Pe, CD19-FITC, CD19-Pe, CD20-PerCP, CD22-Pe, CD33-FITC, CD33-Pe, and IgG2a-FITC were purchased from Becton Dickinson.

Measurement of DNA synthesis

Purified B-CLL cells were treated with MK-886 (1 nM-1 μM) or BWA4C (1 nM-100 nM) and/or LTB_4 (150 nM) in RPMI 1640 medium for 30 minutes. The B-CLL cells (4×10^5) were thereafter seeded in 200 μL culture medium in precoated 96-well plates with irradiated (150 Gy [15 000 Rad]) CD40L-L cells or control L cells. The culture medium contained RPMI 1640 medium, supplemented with 10% FCS, 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin. The cells were incubated at 37°C in an atmosphere of 5% CO_2 for 96 hours. ^{3}H-thymidine (0.037 MBq [1 μCi]) was present in the wells for the final 8 hours of the incubation period. The cells were harvested onto a glass fiber filter and radioactivity was measured in a liquid scintillation counter. Each sample was represented by triplicates.

Flow cytometry analysis of CD23, CD54, and CD150 expression

Purified B-CLL cells were treated with MK-886 (1 nM-1 μM) or BWA4C (1 nM-100 nM) and/or LTB_4 (150 nM) in RPMI 1640 medium for 30 minutes. The B-CLL cells (6×10^6) were thereafter seeded in 3 mL culture medium in precoated 12-well plates with irradiated (150 Gy [15 000 Rad]) CD40L-L cells or control L cells. The culture medium contained RPMI 1640 medium supplemented with 10% FCS, 2 mM L-glutamine, 100 U/mL

penicillin, and 100 μg/mL streptomycin. The cells were incubated at 37°C in an atmosphere of 5% CO_2 for 96 hours. B-CLL cells were collected (without the plastic attached L cells) and used for FACS detection. Surface marker expression was detected by indirect immunofluorescence. The cells (10^6/sample) were washed in cold PBS containing 1% FCS and 0.1% sodium azide and then exposed to the relevant antibodies. The cells were washed and incubated with the R-phycoerythrin (RPE)–conjugated secondary antibody. All incubations were done at 4°C. Samples were run on a FACScan flow cytometer (Becton Dickinson). Each sample represents 10 000 collected events, and CellQuest software was used both for acquisition and analysis. Only the viable cells were considered for analysis based on their light scatter (forward scatter/side scatter [FSC/SSC]) characteristics. The following antibodies were used: monoclonal antibody (MAb) MHM-6 (anti-CD23; from Dr M. Rowe, University of Wales, Cardiff, United Kingdom), MAb LB-2 (anti-CD54; from E. A. Clark, University of Washington, Seattle), MAb IPO-3 (anti–signaling lymphocytic activation molecule [SLAM]; kind gift from S. Sidorenko, Academy of Science of Ukraine, Kiev), and RPE-conjugated rabbit anti–mouse Ig $F(ab')_2$ (Dako, Copenhagen, Denmark) were used as secondary antibodies.

Results

Biosynthesis of leukotrienes in B-CLL cells

The capacity of B-CLL cells to produce leukotrienes was investigated. The cells were challenged with calcium ionophore A23187, arachidonic acid, or calcium ionophore A23187 plus arachidonic acid. No cell clones produced detectable amounts of leukotrienes after challenge with either calcium ionophore A23187 or arachidonic acid alone (Figure 1). Activation of the cells with calcium ionophore A23187 and arachidonic acid led to the formation of LTB_4 (mean, 2.6 ± 0.8 pmol/10^6 cells). The B-CLL cells did not produce LTC_4 (data not shown). Preincubation of intact cells with the thiol-reactive agent diamide, prior to the addition of calcium ionophore and arachidonic acid, led to a markedly increased production of LTB_4 (mean, 33.5 ± 1.2 pmol/10^6 cells) compared with untreated intact cells. Similar amounts of LTB_4 (mean, 34.8 ± 1.7 pmol/10^6 cells) were produced in sonicated cells, incubated with arachidonic acid. There was no obvious correlation between the capacity to produce leukotrienes and the clinical stage of the disease (data not shown). Taken together, the results demonstrated that all investigated B-CLL clones had the capacity to produce LTB_4 and that all B-CLL clones contained substantial amounts of 5-LO, which could be activated under certain conditions.

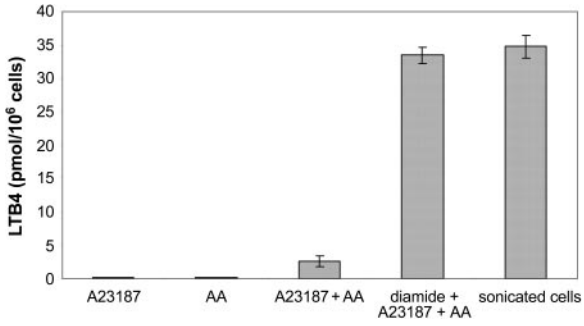

Figure 1. Biosynthesis of LTB_4 by B-CLL cells. Intact B-CLL cells (10×10^6) were incubated for 5 minutes at 37°C with calcium ionophore A23187 (1 μM), arachidonic acid (AA; 40 μM) or A23187 (1 μM) plus AA (40 μM). The cells were preincubated for 2 minutes at 37°C, in the presence or absence of diamide (100 μM), prior to addition of indicated compound(s). Sonicated cells were preincubated with ATP (1 mM) for 2 minutes at 37°C and then incubated with calcium chloride (2 mM) and AA (40 μM) for 5 minutes. The results show the mean ± SD from 6 B-CLL patients.

BLT1 expression

Peripheral blood leukocytes from healthy donors were analyzed with FACS for the expression of BLT1. Gates for granulocytes, lymphocytes, and monocytes were set on the basis of forward and side scatter. Virtually all cells gated as granulocytes ($CD33^+$) expressed BLT1 (Figure 2A). Cells in the monocyte gate ($CD14^+$) showed the same pattern of BLT1 expression (data not shown). In the lymphocyte gate, no expression of BLT1 was observed on peripheral nonactivated $CD4^+$ or $CD8^+$ T lymphocytes (Figure 2B-C). These results are in agreement with the observation that naive nonactivated mouse T lymphocytes do not express BLT1.[13] In contrast, 30%-50% of the peripheral B lymphocytes ($CD19^+$, $CD20^+$, and $CD22^+$) stained positively for BLT1 (Figure 2D). The BLT1 expression on peripheral B lymphocytes was weaker than that on granulocytes and monocytes.

B cells from 6 patients with B-CLL and 2 patients with B-PLL were analyzed with FACS for BLT1 expression. BLT1 expression varied from about 15% to 85% in the investigated B-CLL clones (average expression, 42%; Figure 2E). In the B-PLL group, the average expression of BLT1 was 74% in the 2 investigated clones (Figure 2F).

Effects of leukotriene synthesis inhibitors on DNA synthesis in B-CLL cells

In order to determine whether leukotrienes are of importance for proliferation of B-CLL, the cells were cultivated in the presence of leukotriene biosynthesis inhibitors. B-CLL cells were cultivated together with CD40L-L cells or control L cells for 96 hours in the absence or presence of MK-886 (a specific FLAP inhibitor)[36] or BWA4C (a specific 5-LO inhibitor).[37] CD40-CD40L interactions activated B-CLL cells and resulted in an increased DNA synthesis, measured as ^{3}H-thymidine incorporation in the final 8 hours of a 4-day culture (Figure 3). MK-886, at a concentration of 100 nM, markedly inhibited DNA synthesis induced by CD40L stimulation (Figure 3A). Because of the relatively high binding of MK-886 to serum proteins,[36] the effect of 1 μM MK-886 on DNA synthesis was also investigated in 3 experiments. This concentration of the inhibitor caused only a slightly more pronounced inhibition of DNA synthesis. The inhibitory action of 1 μM and 100 nM MK-886 on thymidine incorporation was 46% and 38%, respectively. Leukotriene B_4 (150 nM) alone did not amplify CD40-induced thymidine incorporation. However, exogenously added LTB_4 almost completely reversed the inhibitory effect of MK-886 on thymidine incorporation. The specific 5-LO inhibitor BWA4C was an even more potent inhibitor than MK-886 in blocking DNA synthesis (Figure 3B). Significant inhibitory effect of BWA4C on thymidine incorporation was observed at 10 nM. In line with the results obtained with MK-866, exogenous LTB_4 (150 nM) almost completely reversed the inhibitory action of 100 nM BWA4C on thymidine incorporation (Figure 3B). The cell survival after 4 days of cultivation was about 80% in all B-CLL cultures stimulated with CD40L-L, both in the absence and presence of inhibitor or LTB_4 (data not shown). Taken together, these data demonstrate that specific inhibitors of leukotriene synthesis cause a pronounced inhibition of DNA synthesis that could be reversed by addition of exogenous LTB_4.

Effects of leukotriene biosynthesis inhibitors and LTB_4 on CD23, CD54, and CD150 expression in B-CLL cells

FACS analysis demonstrated that CD40-CD40L interactions caused an increased expression of CD23, CD54, and CD150

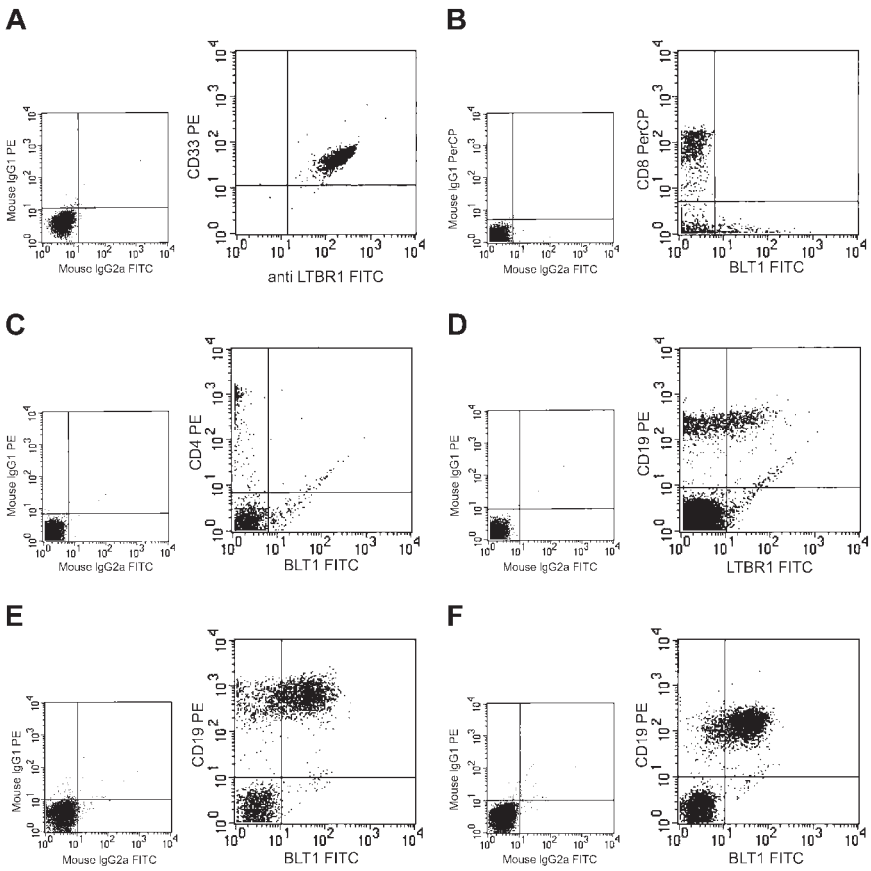

Figure 2. Expression of BLT1 on human leukocytes. The expression of BLT1 was analyzed in various leukocytes by FACS. PMNL (A), peripheral $CD8^+$ T cells (B), peripheral $CD4^+$ T cells (C), normal peripheral B cells (D), B-CLL cells (E), and B-PLL cells (F). Large panels show expression of BLT1 and the cell-specific antigen. Small panels show results with negative control antibodies. The figure depicts 1 typical experiment out of 6 except for B-PLL (2 experiments).

(Figure 4). MK-886 and BWA4C, at concentrations of 100 nM, markedly counteracted this CD40-induced expression of CD23, CD54, and CD150. Leukotriene B_4 alone did not cause any significant effect on the expression of the investigated antigens. However, addition of exogenous LTB_4 (150 nM) almost completely reversed the inhibitory effect of the inhibitors on antigen expression (Figure 4). These results show that LTB_4 is involved in the expression of these antigens, which are associated with activation of B-CLL cells.

Discussion

The enzyme 5-LO is abundantly expressed in B-CLL cells,[4,31-33] and the cells have the capacity to produce LTB_4 (Figure 1). The biosynthesis of LTB_4 by B cells seems not to occur in low differentiated malignant B lymphocytes because the most immature B-cell phenotypes do not have the capacity to produce leukotrienes.[38] The cellular events that activate the endogenous formation of LTB_4 is not yet known. However, although the B-CLL clones produced comparatively low amounts of LTB_4 after activation with calcium ionophore A23187 and arachidonic acid (compared with calcium ionophore–activated granulocytes), sonicated and thiol-activated B-CLL cells produced markedly more LTB_4 (Figure 1), which is in agreement with earlier reports.[3,4] Thus, B-CLL cells have under certain conditions the capacity to produce and release LTB_4 in similar amounts to that of myeloid cells. Furthermore, it is possible that the 5-LO pathway in B-CLL may generate LTB_4 both for export and as a messenger in an intrinsic signal transduction system.

The monoclonal antibodies used in this study for analysis of the BLT1 expression have previously been used to demonstrate BLT1 expression on granulocytes and differentiated HL-60 cells.[35] Here, we have demonstrated the expression of BLT1 in normal peripheral B lymphocytes, B-PLL cells, and B-CLL cells (Figure 2). The degree of BLT1 expression varied between different B-CLL clones but all investigated cell clones expressed BLT1 to some extent. BLT1 is very important for the trafficking of T lymphocytes.[11-13] The role of BLT1 on migration of B-CLL, and other types of B cells, remains to be seen. The presence of BLT1 on B-CLL cells suggests that LTB_4 might influence the function of B-CLL cells in an autocrine and/or paracrine manner.

In order to understand the function of the 5-LO pathway in B-CLL cells, we investigated the effects of specific leukotriene biosynthesis inhibitors on the activation of these cells. For that purpose, the CD40-CD40L model system, which imitates T-cell–dependent activation of B cells, was used. MK-886 and BWA4C are inhibitors of the 5-LO pathway that inhibit the synthesis of leukotrienes by a completely different mechanism of action (ie, MK-886 is a specific FLAP inhibitor and BWA4C is a specific 5-LO inhibitor). At a concentration of 100 nM, both inhibitors markedly inhibited DNA synthesis in B-CLL cells (Figure 3). In addition, exogenous LTB_4 completely reversed the effects of the drugs, indicating that the effects of the inhibitors did not reflect a nonspecific effect of the drugs (Figure 3). However, LTB_4 alone did not further stimulate CD40-induced DNA synthesis,

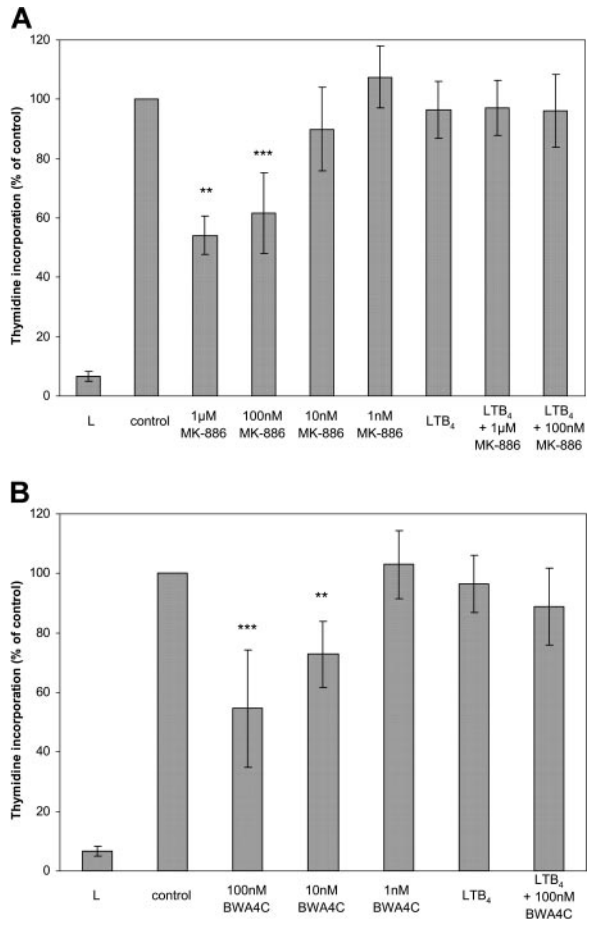

Figure 3. Effects of leukotriene biosynthesis inhibitors on CD40L-induced thymidine incorporation in B-CLL cells. B-CLL cells (4×10^5) were cultivated together with either irradiated L cells alone (L) or irradiated CD40L-L cells plus indicated compound(s) for 96 hours. When inhibitors and/or LTB_4 were used, B-CLL cells were pretreated with the indicated compound(s) for 30 minutes in a serum-free medium. ^{3}H-thymidine (0.037 MBq [1 μCi]) was present for the final 8 hours of the incubation period. (A) MK-886 (1 μM-1 nM) or (B) BWA4C (100 nM-1 nM) with or without LTB_4 (150 nM). Control represents B-CLL cells cultured together with irradiated CD40L-L cells alone. Activation of B-CLL cells with CD40L-L treatment led to between 3580 and 15 369 cpm (^{3}H-thymidine) incorporation in the different experiments (control). This was set as 100% in each experiment. Each sample was represented by triplicates. The results show the mean ± SDs from 8 separate experiments (B-CLL cells from 2 patients were analyzed 2 times). The highest concentration of MK-886 (1 μM) was only used in 3 experiments. Student *t* test was used to calculate statistics (ie, control vs control plus indicated compound(s) [$^{**}P < .01$, $^{***}P < .001$]).

suggesting that endogenous LTB_4 caused maximal effects. Leukotriene B_4 has also been reported to stimulate proliferation of myeloid cells.[39,40] MK-886, at a concentration of 100 nM, has an antiproliferative effect and induces apoptosis in HL-60 cells.[41] Addition of exogenous LTB_4 could reverse the effect of the inhibitor on these cells. Furthermore, MK-866 has been found to be a potent inhibitor of DNA synthesis in a subset of acute myeloid leukemia cells.[42]

CD54/ICAM-1 is a single-chain membrane glycoprotein, which is expressed on many types of cells such as leukocytes, endothelial cells, and epithelial cells. Normal peripheral B lymphocytes barely express CD54, while B lymphocytes from CLL patients have an increased expression of CD54.[28] High expression is associated with poor prognostic features, including increased tumor burden and sometimes a short lymphocyte doubling time.[27,28] Soluble CD54 (sICAM-1) levels are high in patients with an advanced clinical stage/high tumor burden.[43] Both the FLAP inhibitor and the 5-LO inhibitor counteracted the stimulatory action of CD40-CD40L interaction on the expression of CD54 (Figure 4). In these experiments, exogenous LTB_4 (150 nM) also reversed these effects of the inhibitors (Figure 4).

CD23 is a low-affinity receptor for IgE and is involved in the feedback regulation of IgE synthesis. CD23 has been proposed to be involved in cell viability and proliferation.[44] High serum levels of soluble CD23 (sCD23) was associated with high tumor burden and shorter time to progression in B-CLL.[45,46] The expression of CD23 on B-CLL cells was inhibited by the leukotriene biosynthesis inhibitors and reversed by LTB_4 (Figure 4). In agreement with these findings, LTB_4 has been reported to stimulate the expression of CD54 on endothelial cells and CD23 on B cells.[18,19,47]

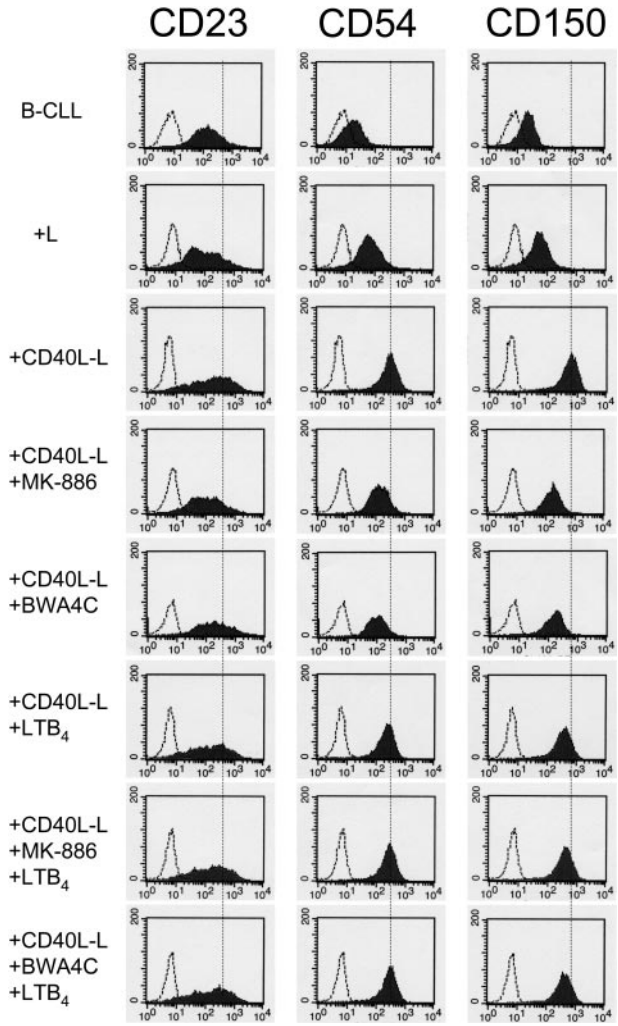

Figure 4. Effects of leukotriene biosynthesis inhibitors on the expression of CD23, CD54, and CD150 in CD40L-activated B-CLL. Purified B-CLL cells were cultivated together with either L cells or CD40L-L cells in the absence or presence of MK-886 (100 nM), BWA4C (100 nM), and/or LTB_4 (150 nM) for 96 hours. When inhibitors and/or LTB_4 were used, B-CLL cells were pretreated with the compound(s) for 30 minutes in a serum-free medium prior to cultivation together with L cells or CD40L-L cells. B-CLL cells were collected and analyzed by FACS with antibodies against CD23, CD54, or CD150. The figure depicts 1 typical experiment out of 6. In order to more clearly demonstrate the different degree of expression of indicated antigen in the various samples, the inserted dotted line represents the expression of the indicated antigen in B-CLL cells stimulated with CD40L-L only. The shaded histograms show the expression of CD23 (left column), CD54 (middle column), or CD150 (right column). The open histograms represent the fluorescence of the cells stained with isotype-matched control mAbs of irrelevant specificity.

In summary, this study demonstrates that LTB_4 plays an important role in the activation of B-CLL cells. Inhibitors of leukotriene synthesis have so far only been used for treatment of asthma. The present report indicates that leukotriene biosynthesis inhibitors, LTA_4 hydrolase inhibitors, or BLT1 antagonists, alone or in combination with conventional therapy, might also be useful in the treatment of B-CLL.

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II

The expression of 5-lipoxygenase and cysteinyl leukotriene receptor 1 in mantle zone B cells

Yilmaz Mahshid[1], Marcus-René Lisy[2], Xiao Wang[3], Rainer Spanbroek[2], Jenny Flygare[3], Birger Christensson[3], Birgitta Sander[3], Magnus Björkholm[4], Andreas JR Habenicht[2], Hans-Erik Claesson[1, 5]

[1]Department of Medical Biochemistry and Biophysics, Karolinska Institutet, Stockholm, Sweden, [2]Institute of Vascular Medicine, Friedrich-Schiller-University, D-07743 Jena, Germany, [3]Department of Laboratory Medicine, Division of Pathology, Karolinska Institutet, Huddinge University Hospital, Stockholm, Sweden, [4]Department of Medicine, Karolinska Institutet, Stockholm, Sweden, [5]Biolipox AB, Berzeliusv. 3, 171 65 Solna

Corresponding author: Hans-Erik Claesson
MBB, Division of Chemistry II
Karolinska Institutet
S-171 77 Stockholm
Telephone: +46 8 728 76 14
E-mail: hans-erik.claesson@ki.se

Abstract

Human B lymphocytes have similar capacity to produce leukotriene (LT) B_4 as myeloid cells but the biological function of the 5-lipoxygenase (5-LO) pathway in B cells is unclear. In order to better understand the role of 5-LO and LTB_4 in B cells we investigated which subsets of normal B cells from tonsils that expressed 5-LO. Based on immunostaining with a polyclonal antibody raised against 5-LO and Western blot analysis, high expression of 5-LO was found in mantle zone cells from tonsils. In contrast, only a weak expression of 5-LO was detected in germinal center cells and plasma cells in tonsils. Therefore, it was of great interest to characterize the 5-LO pathway in mantle zone B cells. Due to the difficulty to culture normal B lymphocytes in vitro we used cell lines from mantle cell lymphoma (MCL) which morphologically share many features with normal mantle zone B cells. MCL cell lines also expressed 5-LO and readily produced LTB_4 after activation. In frozen sections of primary MCL we found high expression of 5-LO. This enzyme is active in primary MCL since the leukemic form of MCL, B-PLL, produced leukotrienes upon stimulation. RT-PCR analysis of different subsets of B cells from tonsils demonstrated that mantle zone B cells expressed CysLT1 receptor but not CysLT2 and BLT2 and only weak BLT1. In agreement with this finding, three different MCL cell lines were found to respond to LTD_4 (100 nM) with a robust calcium signal, which was completely abrogated with a specific CysLT1 antagonist, Zafirlukast (10 nM). Taken together, the present report indicates that 5-LO and the CysLT1 receptor have functions in normal and malignant mantle zone B cells and other B cell subsets with exception of plasma cells.

Introduction

Arachidonic acid can be converted to leukotrienes which mediate inflammatory and immunological reactions[1]. The key enzyme in leukotriene biosynthesis is 5-lipoxygenase (5-LO), which upon activation and interaction with 5-LO activating protein (FLAP) converts arachidonic acid, via a two step process, to leukotriene (LT) A_4. This compound can easily be transformed to LTB_4, through the action of LTA_4 hydrolase, or to LTC_4, catalyzed by LTC_4 synthase[1]. Leukotriene C_4 can be further converted to LTD_4 and LTE_4. Leukotriene C_4 and its metabolites are collectively named cysteinyl-containing leukotrienes (cys-LT). The biological effects of leukotrienes are dependent on receptor interaction. There are five leukotriene receptors that have been identified and characterized [1-4,26]. The receptors for LTB_4 are named BLT1 and BLT2. The high affinity receptor, BLT1, is mainly expressed in leukocytes, whereas BLT2 is a low affinity receptor ubiquitously expressed. Cysteinyl-containing leukotrienes are ligands for three high affinity receptors named CysLT1, CysLT2 and GPR17[1,26]. The major effects of cysteinyl-leukotrienes in human airways are mediated by the CysLT1 receptor but certain responses in human vasculature tissues are mediated by CysLT2 receptor[1,5]. The third cys-LT receptor has recently been identified and more characterization is needed to define the biological role[26].

Leukotriene B_4 is a very potent chemotactic compound for myeloid cells and T lymphocytes[6-9]. Several reports have demonstrated a function of LTB_4 in the immune system as a stimulator of monocytes, T lymphocytes and B lymphocytes[10-12]. The cysteinyl-containing leukotrienes are potent bronchoconstrictors and mediate vascular oedema[1]. Biosynthesis of leukotrienes is restricted to a few cell types in the human body. Myeloid cells are the main source of leukotriene formation but B lymphocytes have also the capacity to produce LTB_4. The activation of leukotriene synthesis in B cells is quite different in comparison to myeloid cells. Granulocytes and monocytes readily produce leukotrienes upon stimulation with calcium ionophore A23187. B cells, however, do not produce LTB_4 after challenge with calcium ionophore but the cells can produce similar amounts of LTB_4 as myeloid cells after changing the cellular oxidative status[13-15]. The 5-lipoxygenase activity in B cells appears to be cryptic and the mechanism of activation of the enzyme under physiological conditions is not known yet. The leukotriene pathway, however, has an endogenous role in the activation of chronic B lymphocytic leukaemia cells[16].

Mantle cell lymphoma (MCL) is a heterogeneous disease with estimated incidence of 5% of all non-Hodgkin's lymphomas. It is a disease with an aggressive course

and a poor clinical outcome[17]. The current hypothesis is that the tumour cells are derived from the mantle or marginal zone of the B cell follicles. Microarray data of MCL have revealed high expression of 5-LO in these cells in comparison to control lymphoid tissue[18].

The enzyme 5-LO has been in found in immature B cells, peripheral B cells, tonsillar B cells and various types of malignant B cells[13-14,19]. However, it is not known which subsets of mature B lymphocytes that express 5-LO and can produce LTB_4. Therefore, in order to encircle the function of the leukotriene pathway in B cells, the present report deals with the expression of 5-LO and receptors of leukotrienes in different subsets of B lymphocytes. For that purpose, the relevant malignant variants of B cells have also been used in this study.

Material and Methods

Reagents and cell lines

The calcium ionophore A23187 was purchased from Calbiochem-Behring (La Jolla, CA, U.S.A.). HPLC solvents were obtained from Rathburn chemicals (Walkerburn, U.K.) and the synthetic standards of LTB_4 and prostaglandin (PG) B_1 and LTD_4 were from Biomol (Plymouth, PA, U.S.A.). Azodicarboxylic acid bisdimethylamide (Diamide) was from sigma-aldrich, Sweden and arachidonic acid (AA) from NU-CHEK PREP Inc., MN, U.S.A. Granta519, Jeko1 and Rec1 were all purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Germany).

Calcium mobilization

The fluorophore used was Calcein3 fluorescence dye solution, and the assay was performed according to the manufacturer's protocol (Molecular Devices, CA, USA). The fluorescence reader used was a FLEX station (Molecular Devices, CA, USA), emission wavelength: 485 nm, excitation wavelength: 525 nm, cut-off: 515 nm. Cells (3×10^5 cells / 100 µl) were washed twice in PBS, and dispensed into wells of a black, poly-D-lysine coated 96-well plate (Costar 3667, Corning, NY, USA). The fluorescence was measured for a total time of 77 seconds. After 17 seconds, the indicated leukotriene (100 nM, final concentration) was added. Zafirlukast (10 nM, final concentration) was added 15 minutes prior to the addition of leukotrienes.

Immunofluorescence analysis of tonsillary B cells

Tonsils were obtained from patients undergoing routine tonsillectomies (Friedrich-Schiller-University Hospital, Jena, Germany). Confocal laser scan microscopy was performed with 7 µm cryostat sections embedded in Tissue Tec (Sakura, Japan). Specimens were placed on glass slides and fixed with acetone for 10 min at 4°C. After rehydration in PBS supplemented with FCS (2%) for 20 min at RT, specimens were incubated for 1h at room temperature with rabbit anti 5-LO antiserum and for double immunofluorescence analyses with unlabeled primary antibodies against IgD (Pharmingen), CD38 (Becton Dickinson), CD19 (Coulter Immunotec) or CD138 (Serotec). Nuclei were counterstained with DAPI. Secondary antibodies (goat anti mouse IgG F(ab')2-Cy2 and donkey anti rabbit IgG F(ab')2-Cy3; Dianova) were applied for 30 min at room temperature. Sheets were mounted on coverslips

with Permafluor (Beckman, Munich, Germany) and viewed on a Zeiss Axiovert 200M microscope equipped with a confocal laser scanning head (LSM510). Pictures were taken and analyzed using LSM510 Image Examiner software (Zeiss, Jena, Germany).

Incubation of B-PLL cells and MCL cell lines

Intact cells (10×10^6) cells were suspended in 1 ml phosphate buffered saline (PBS) and pre-incubated for 2 minutes with azodicarboxylic acid bisdimethylamide (Diamide) (100 μM) prior to stimulation with arachidonic acid (40 μM) and calcium ionophore A23187 (1 μM). The cells were stimulated for 5 minutes and were terminated with 1 ml methanol. Alternatively, cells (10×10^6) were re-suspended in 1 ml calcium free PBS including EDTA (2 mM) and sonicated 3 x 5 s. The cells were pre-incubated for 2 minutes in the presence of ATP (1 mM) prior addition of calcium chloride (2 mM) and arachidonic acid (40 μM). The reaction was terminated after 5 minutes of incubation with 1ml methanol. Incubations were performed at 37°C.

Analysis of leukotrienes

After addition of 0.5 ml PBS and the internal standard PGB_1 (50 pmol), the samples were centrifuged (1250 x g, 5 min). The supernatant was subsequently subjected to solid phase extraction on Oasis Extraction Cartridges (10 mg, Waters, Sweden). The methanol fraction was analyzed on Waters Alliance 2695 reverse phase HPLC and detected with Waters PDA 996. Methanol:water:trifluoroacetic acid (70:30:0.007, v / v) was used as mobile phase at a flow rate of 1.2 ml / min. Qualitative analysis was performed by comparison of retention times of synthetic standards and quantitative determinations were performed by computerized integration of the area of eluted peaks.

Isolation of subpopulations of B cells

Isolated cells from tonsils were incubated with CD19 micro beads (Miltenyi, Germany) and loaded in a magnetic field on a MACS separation column LS (Miltenyi). Cells were washed three times and eluted (4-6 ml) with PBS / EDTA (2 mM) / FCS (5%). Isolated cells were concentrated to 10^7 / 50 μl and incubated with anti CD38-PE (Pharmingen) and anti IgD-FITC (Pharmingen) for 20 min at 4°C. Cells were washed twice, set to 2×10^6 cell / ml in PBS/EDTA/FCS and sorted with FACSVantage SE cell sorting instrument (Beckton Dickinson, Germany).

Western blot

Tonsillary B cell samples were washed in PBS (2x) and re-suspended to 4 x 10^4 cells / µl in 5x sample buffer (250 mM Tris/HCl pH 6.8; 30% (v/v) Glycerol, 10% (w/v) SDS, 5% (v/v) β-mercaptoethanol; 0.02% (w/v) Bromphenolblue; complete mini protease inhibitor (Roche)). The suspension was heated to 95°C, 5-10 minutes and run on a regular 10% SDS-PAGE with SDS running buffer (25 mM Tris/HCl; 190 mM Glycine; 0.1% (w/v) SDS). HybondC nitrocellulose membrane (GE Healthcare, Germany) and semi-dry blotting apparatus (LMS labortechnik, Germany) was used for western blot transfer. The detection was performed with ECL kit (GE Healthcare, Germany). Primary antibody used for the immunoblot detection was anti 5-LO polyclonal rabbit antiserum[20] and secondary antibody was an anti rabbit IgG coupled with HRP (GE Healthcare, Germany).

MCL cell lines were washed in PBS (w/o calcium and magnesium) and suspended in PBS (w/o calcium and magnesium) supplemented with complete mini protease inhibitor (Roche). Cells were sonicated on ice (3 x 5 s) and centrifuged (1 x 10^5 x g, 60 min, 4°C). Supernatant was collected and protein concentration was determined with Bradford.

Isolation of total RNA and RT PCR

Total RNA was separated according to manufacturer's protocol with RNeasy mini kit (Qiagen, Germany). Concentration and quality of RNA was determined with a Bioanalyzer2100 (Agilent, Germany). The reversed transcription reaction was performed on 1.8 µg of total RNA in a RT-PCR mixture (1x RT buffer, 167 µM dNTP, 0.7 U/µl RNasin, 0.1 µg/µl BSA, 0.35 U/µl AMV reverse transcriptase (Roche)).

PCR

PCR was performed with 60 ng of cDNA for each reaction. Primers used are listed in table X. The PCR reaction mixture contained; PCR buffer, dNTP mix (0.2 mM each), BSA (0.1 µg/µl), SybrGreen (1:50) (Roche), $MgCl_2$ (1.8 mM), Platinum Taq DNA polymerase (1.5 U) (Invitrogen) and primer mix (0.2 µM each) (table 1).

Gene	Annealing temp.	Size of PCR Product	sequence
5-LO	72°C	486 bp	5´ACCATTGAGCAGATCGTGGACACGC 3´GCAGTCCTGCTCTGTGTAGAATGGG
FLAP	71°C	352 bp	5´GGCCATCGTCACCCTCATCAGCG 3´GCCAGCAACGGACATGAGGAACAGG
LTA4H	72°C	464 bp	5´GCAGTCACGGGATGCATGCTTGCT 3´GCCTGGCTCTACTCTCCTGGACTG
BLT1	71°C	374 bp	5´CTCCCGACGGCCATGAACACTAC 3´CGGGCCACCGCCAGTGAGCGG
BLT2	67°C	152 bp	5´CGTCTTCACCGCTGGAGATCTGC 3´CCTGCCCCACCACTTTCAGCTG
CysLT1	68°C	545 bp	5´GTGCCGCCTCAGCACCTATGC 3´CGGACTTCTGCATTCTAAGGACAG
CysLT2	72°C	385 bp	5´GATCTCCTGTTCATAAGCACGCTTC 3´GCAGGCAGCCCACCACCAAGGC
GAPDH	66°C	520 bp	5´TCGGAGTCAACGGATTTGGTCGTA 3´ATGGACTGTGGTCATGAGTCCTTC

Table 1. List of primers used in the PCR reactions.

Immunohistochemistry

Tumor biopsies (one lymph node, one spleen) obtained from two patients with MCL were analyzed. The biopsies were snap-frozen upon arrival to the Department of Pathology and subsequently stored at -70°C. Sections (µm thick) were placed on glass slides and fixed with acetone for 10 minutes at RT. After rehydration in TBS for 10 minutes at RT, specimens were stained on an Autostainer Plus (DAKO, Denmark), using Dako REAL™ EnVision™ Detection System, Peroxidase/DAB, Rabbit/Mouse kit as recommended by the manufacturer. Primary rabbit anti 5-LO antiserum was diluted to 1:300 in Dako REAL™ Antibody Dilutent and incubated for 1 hour at RT. Sheets were mounted with EUKITT mounting medium (VWR International, USA).

Results

Immunohistochemical analysis of 5-LO expression in subsets of B cells from tonsils

Tonsils were obtained from patients undergoing routine tonsillectomies and prepared for immunohistochemical analysis. To characterize the expression of 5-LO in subpopulations of tonsillar B cells, a panel of antibodies were used to identify mantle zone B cells (IgD^+), germinal center (GC) B cells ($CD38^+$) and plasma cells ($CD38^{++}$ or $CD138^+$). Figure 1A shows high expression of 5-LO in mantle zone B cells (IgD^+). In contrast, a very weak expression of 5-LO was observed in GC cells ($CD38^+$) (Fig 1B). Figure 1C demonstrates no expression of 5-LO in plasma cells ($CD38^{++}$) within the GC. In agreement with this finding, no expression of 5-LO was observed in plasma cells ($CD138^+$) outside the GC (Fig. 1D).

Western blot analysis of 5-LO expression in subsets of B cells

Subpopulations of B cells from tonsils were separated by FACS and analyzed by Western blot in order to further characterize the expression of 5-LO. The supernatant of sonicated cells were submitted to SDS/PAGE followed by Western blotting using a polyclonal anti-human 5-LO antibody. A strong immunoreactive band was observed in mantle zone B cells and memory B cells, and a weak band was detected in GC B cells (Fig. 2). In contrast, no band was detected in samples from plasma cells. These results are in agreement with the immunohistochemical analysis of 5-LO expression in tonsils (Fig. 1). In addition, these results also indicate that memory B cells contain similar amounts of 5-LO as mantle zone B cells.

PCR analysis of enzymes/proteins and receptors in the leukotriene biosynthesis pathway

RT-PCR was performed on isolated total RNA of subsets of B cells from tonsils to elucidate the gene expression of receptors and enzymes (other than 5-LO) involved in the leukotriene cascade. Memory B cells, mantle zone B cells and GC B cells all expressed the transcripts for 5-LO, FLAP and LTA_4 hydrolase (Fig. 3A) indicating that these cells have the capacity, in various degree, to produce LTB_4. Germinal center cells, however, had low expression of the 5-LO protein (figs. 1 and 2), indicating that the transcript signal did not reflect the amount of 5-LO protein to the same extent as for mantle zone B cells and memory B cells. No subpopulation of B cells expressed LTC_4 synthase. Figure 3B depicts the gene expression of four leukotriene receptors. Cysteinyl leukotriene receptor 1 (CysLT1) was expressed in mantle zone B cells and memory B cells. Neither CysLT2 nor LTB_4 receptor 2 (BLT2) were

expressed in B cells from tonsils. Very low expression of the transcript for BLT1 was detected in the B cells. In contrast, B cells isolated from patients with chronic B lymphocytic leukaemia and a subpopulation of peripheral B lymphocytes stained positively for BLT1[16].

Biosynthesis of LTB_4 in mantle cell lymphoma

Mantle cell lymphoma (MCL) cells were investigated in order to more extensive study the biosynthesis of LTB_4. For that purposes, both B-prolymphocytic leukaemia (B-PLL) cells (which are MCL cells found in peripheral blood) and MCL cell lines were investigated. Figure 4 depicts the capacity of B-PLL cells and MCL cell lines to produce leukotrienes. Challenge of B-PLL cells with ionophore A23187 plus arachidonic acid and the thiol-active compound diamide led to the formation of similar amounts of LTB_4 (mean 30 ± 6 pmol / 106 cells) as produced by human neutrophils[21]. This cocktail of compounds is known to induce LTB_4 production by B lymphocytes[14]. Sonicated cells also readily produced LTB_4 (mean 25.8 ± 4.7 pmol / 106 cells) (Fig.4A). About 1 pmol LTB_4 / 10^6 cells was produced after incubation of B-PLL cells with calcium ionophore only, showing that there are minimal amounts of myeloid cells contaminating these preparations of B-PLL cells. Figure 4B shows that the MCL cell lines Granta519, Jeko1 and Rec1 produced 6.2 pmol, 14.5 pmol and 7.0 pmol LTB_4 / 10^6 cells, respectively, after stimulation with ionophore A23187 plus arachidonic acid and diamide. Sonicated MCL cells produced similar amounts of LTB_4. Taken together, these results demonstrate that both B-PLL cells and MCL cell lines have the capacity to produce LTB_4 and that the cells contained substantial amounts of 5-LO, which could be activated under certain conditions.

Expression of 5-LO in MCL cell lines

To further characterize the expression of 5-LO in MCL, western blot analysis was performed on MCL cell lines with an anti-5-LO antibody. The 5-LO protein was detected in all three investigated MCL cell lines (Fig. 5). In comparison, no expression of 5-LO was detected in the T cell lines Jurkat and Molt4.

LTD_4 triggers an increase in intracellular calcium via CysLT1 in MCL cell lines

Analysis of normal mantle zone B cells demonstrated that these cells expressed CysLT1 at transcriptional level (Fig 3). Thus, it was of interest to study if MCL cells expressed a functional CysLT1 receptor. The calcium response upon challenge with leukotrienes was investigated with a fluorophore based assay. Stimulation of all three investigated MCL cell lines with LTD_4 (100 nM) led to a robust calcium signal (Figure 6). This response was completely abrogated when the cells were pre-treated for 15 minutes with Zafirlukast (10 nM), a selective cysteinyl leukotriene receptor 1 antagonist. The addition of LTC_4 and LTE_4 resulted also in a calcium response but not as robust as the signal from LTD_4 (data not shown). In contrast, the addition of LTB_4, (1-1000 nM), did not result in any calcium signal (data not shown). The experiment was performed in triplicates and repeated three times. Taken together, these results show that MCL cells have high expression of a functional CysLT1 receptor which is in agreement with the finding in normal mantle zone B cells (Figure 3).

Immunohistochemical analysis of 5-LO in mantle cell lymphoma tumor biopsies

Tumor biopsies (one lymph node, one spleen) obtained from two patients with MCL were analyzed. Anti CD20 antibody was used to detect B cells and a rabbit polyclonal 5-LO antibody was used for the detection of 5-LO. Virtually all cells stained for CD20 and 5-LO in both biopsies, indicating a strong expression of 5-LO in mantle cell lymphoma B cells.

Discussion

The biological function of 5-LO in human B lymphocytes is unclear although the capacity of B cells to produce LTB_4 are similar to myeloid cells[14,15]. In order to better understand the role of 5-LO and LTB_4 in B cells we investigated the expression of 5-LO in subsets of normal B cells from tonsils. Polyclonal 5-LO antibody used in immunohistochemical and western blot analysis showed high expression in mantle zone cells from tonsils (Figs.1, 2). In contrast, only a weak expression of 5-LO was found in germinal center B cells and plasma cells in tonsils (Figs.1, 2). In addition, memory B cells were also found to express similar amounts of 5-LO as mantle zone cells (Fig.2). The results of RT-PCR analysis of different subsets of B cells from tonsils were in agreement with these findings (fig.3). Thus, it was of great interest to characterize the 5-LO pathway and receptor expression in mantle zone cells. Since the amount of mantle zone B cells from tonsils that can be separated by FACS is limited, we investigated B-PLL cells and cell lines derived from patients with MCL. B-PLL is in fact a heterogenous disease and those cases that are positive for cyclin D1 and carry the t(11:14) translocation involving CCND1 are considered to be leukemic forms of MCL[22,23].

Three different MCL cell lines were used in this study and all were found to express 5-LO and to have the capacity to produce LTB_4 (figs 4, 5). Also B-PLL cells could produce similar amounts of LTB_4 as MCL cell lines and myeloid cells. B-PLL cells and MCL cell lines produced LTB_4 after challenge with calcium ionophore plus arachidonic acid and diamide but not after stimulation with calcium ionophore only, showing that the mechanism of activation of the leukotriene pathway in MCL cells is similar as other investigated B cells[16,19,24]. Also, immunostained biopsies from MCL patients expressed 5-LO in virtually all B cells. Taken together, this study and earlier reports demonstrate that 5-LO is expressed in relatively immature B cell, peripheral B cells, various malignant B cells, mantle zone B cells and memory B cells but not in plasma cells and the most immature B cells[13,14,19]. The question if B cells can release LTB_4 is still open. There are several possible explanations for this; 1) The role of the 5-LO pathway is quite different in B cells than in myeloid cells and the pathway has only an endogenous function in B cells. Thus, the cells will never release LTB_4 although they can respond to LTB_4, produced by myeloid cells. Our recently reported study on the function of 5-LO and LTB_4 in chronic B lymphocytic leukaemia does not contradict this hypothesis[16]; 2) The physiological conditions which activate the 5-LO pathway in B cells has not yet been elucidated but the cellular oxidative status of the cell seem to be of importance[13-15]. Studies are ongoing on this issue; 3) The major role of 5-LO in B cells is in

the final stage of B cell differentiation or apoptosis. Thus, the enzyme is "silent" until that stage in the B cell life. The finding that the enzyme is not expressed in germinal center or plasma cells could indicate that the enzyme might be activated and thereafter degraded in the cell. It is known that caspases can degrade 5-LO under certain conditions[24]. For that purpose, we will now study the role of 5-LO in apoptosis in B cells.

RT-PCR analysis of different subsets of B cells from tonsils demonstrated that mantle zone B cells expressed CysLT1 receptor but not CysLT2 and BLT2 and only weak BLT1 (fig 3). In agreement with this finding, MCL cells were found to express CysLT1, but not the other leukotriene receptors, and a robust calcium signal was observed after stimulation with LTD_4 (100 nM) (Fig. 6). This signal was completely abrogated with Zafirlukast (10 nM) which is a specific CysLT1 antagonist. Thus, the MCL cell lines used in this study seem to be a good model system to study the leukotriene cascade in mantle zone cells since similar enzymes and receptors were detected in normal mantle B zone cells and MCL cells.

Recently it was shown that CysLT1 can be induced in peripheral B cells by exposing peripheral B cells for IL-4 and CD40-ligand[25]. Furthermore, LTD_4 was also found to induce immunoglobulin secretion. Another recently published report showed that LTD_4 stimulated the expression of early pro-inflammatory genes e.g. IL-8, CXCL2 and COX2, in endothelial cells which express CysLT2, indicating that cys-LTs may participate in complex inflammatory processes[5]. At this moment, it is difficult to speculate about the biological role of CysLT1 receptor in mantle zone cells. It might be coupled to the immunoglobulin production or the initiation of an inflammatory cascade. However, it seems likely that LTC_4 and its metabolites, produced by macrophages, dendritic cells or mast cells, play a role in the function of B cells. Ongoing microarray studies might give further insight in this issue.

In summary, the present study shows that the expression of 5-LO in B cells is highly expressed in mantle zone B cells and memory cells but not in plasma cells indicating a role of the 5-LO pathways in B cells before the cells reach the final stage of differentiation. The expression of CysLT1 in mantle zone B cells and MCL might also lead to novel knowledge about the effect of cysteinyl-containing leukotrienes on normal B cells and MCL cells.

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LEGENDS TO FIGURES

Figure 1. 5-lipoxygenase expression in B cells from tonsils

Tonsils were prepared for immunofluorescence analyses and stained with anti-IgD, anti-CD38, anti-CD138 and DAPI. **A)** Mantle zone B cells were stained with anti-IgD (green, A1); anti-5-LO (red, A2) and DAPI (blue, A3). The combined picture (A4) depicts high expression of 5-LO in IgD^+ cells. **B)** Germinal center B cells were stained with anti-CD38 (green, B1); anti-5-LO (red, B2) and DAPI (blue, B3). The combined picture (B4) shows very weak expression of 5-LO in $CD38^+$ cells. **C)** Plasma cells within the germinal center were stained with anti-CD38 (green, C1); 5-LO (red, C2) and DAPI (blue, C3). The combined picture (C4) demonstrates no expression of 5-LO in $CD38^{++}$ cells. **D)** Plasma cells outside the germinal center were stained with anti-CD138 (green, D1); 5-LO (red, D2) and DAPI (blue, D3). The combined picture (D4) depicts no expression of 5-LO in $CD138^+$ cells.

Figure 2. 5-lipoxygenase expression in B cells from tonsils

Immunoblot analysis of 5-LO. Western blot was performed on different subpopulation of B cells isolated from tonsils (4 µg protein/ sample). Lane 1 – total B cells from tonsils, lane 2 – mantle zone B cells, lane 3 – germinal center B cells, lane 4 – plasma B cells, lane 5 – memory B cells and lane 6 – HL60 cells differentiated with DMSO (positive control).

Figure 3. Semiquantitative RT-PCR analysis of enzymes and receptors in the leukotriene pathway in subpopulations of B cells from tonsils

Semiquantitative RT-PCR analysis was performed on purified non-fractioned B cells (tonsillary), memory B cells, mantle zone B cells and germinal center B cells. Panel A depicts the expression of 5-LO, FLAP, leukotriene A_4 hydrolase (LTA4H) and leukotriene C_4 synthase (LTC4S). Panel B shows the expression of cysLT1, cysLT2, BLT1 and BLT2. The values are presented as ´Transcripts / GAPDH 10^3 transcripts`.

Figure 4. Biosynthesis of LTB_4 by B-PLL cells and MCL lines

Intact cells (10×10^6) were pre-incubated for 2 minutes at 37°C, in the presence of diamide (100 µM), prior to addition of A23187 (1 µM) plus arachidonic acid (AA) (40 µM) for 5 minutes. Sonicated cells (homogenate) were preincubated with ATP (1 mM) for 2 minutes at 37°C and then incubated with calcium chloride (2 mM) and arachidonic acid (40 µM) for 5 minutes. Panel A depicts the formation of LTB_4 in B-PLL cells isolated from four different donors (mean ± SD). Panel B depicts the formation of LTB_4 in three different MCL lines i.e. Granta519, Jeko1 and Rec1.

Figure 5. Western blot analysis of 5-LO in various MCL cell lines

Immunoblot analysis of 5-LO. Western blot was performed on different three different MCL cell lines (Granta519, Jeko1 and Rec1) and two different T cell lines (Jurkat and Molt4). Lane 1 – Granta519, lane 2 – Jeko1, lane 3 – Jurkat, lane 4 – Molt4 and lane 5 – Rec1.

Figure 6. Effects of LTD_4 on calcium response in MCL cell lines

MCL cells were washed twice in PBS and dispensed into wells of a black, poly-D-lysine coated 96-well plate (3×10^5 cells / well). The fluorescence was measured for a total time of 77 sec and LTD_4 (final concentration 100 nM) was added after 17 sec (indicated with arrow). The selective CysLT1 receptor antagonist Zafirlukast (final concentration 10 nM), was added 15 min prior to the addition of LTD_4. The graph depicts the average relative fluorescence units (RFU) of the calcium response with standard deviation of A) Granta519, B) Jeko1 and C) Rec1 cells. The experiment was performed in triplicates and repeated three times.

Figure 7. Immunohistochemical staining of mantle cell lymphoma

Immunohistochemical staining on frozen sections of mantle cell lymphoma involving a lymph node. The tumor cells are positive for the pan-B cell marker CD20 (a) and for 5-lipoxygenase (b) and (c). Negative control without a primary antibody is shown in (d).

FIGURES

Figure 1.

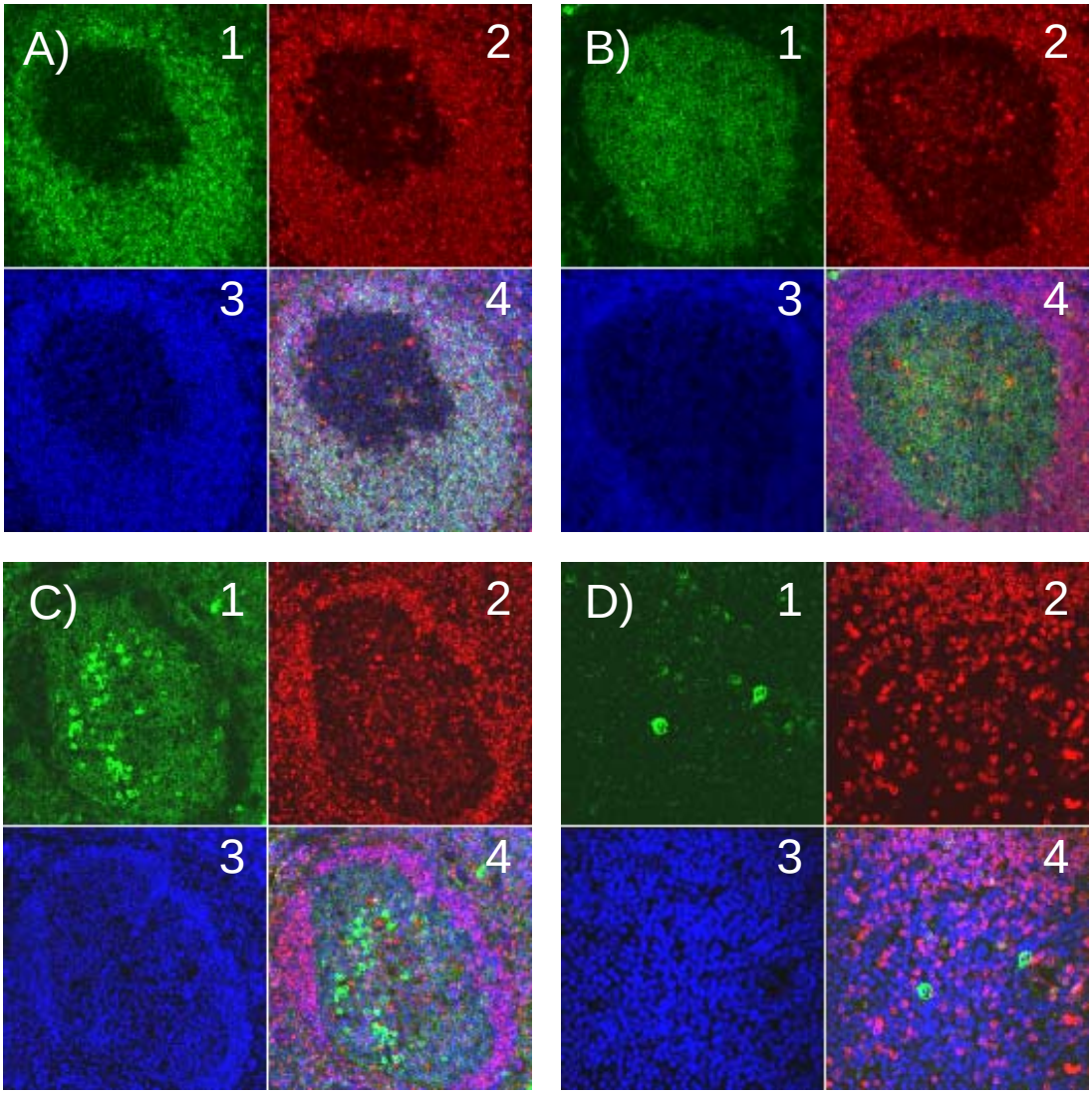

Figure 2.

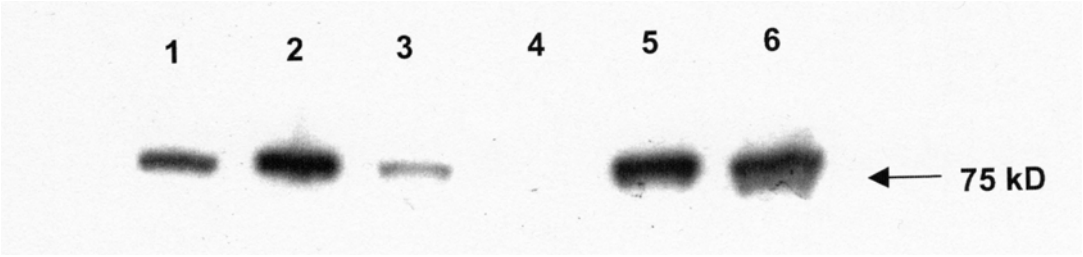

Figure 3.

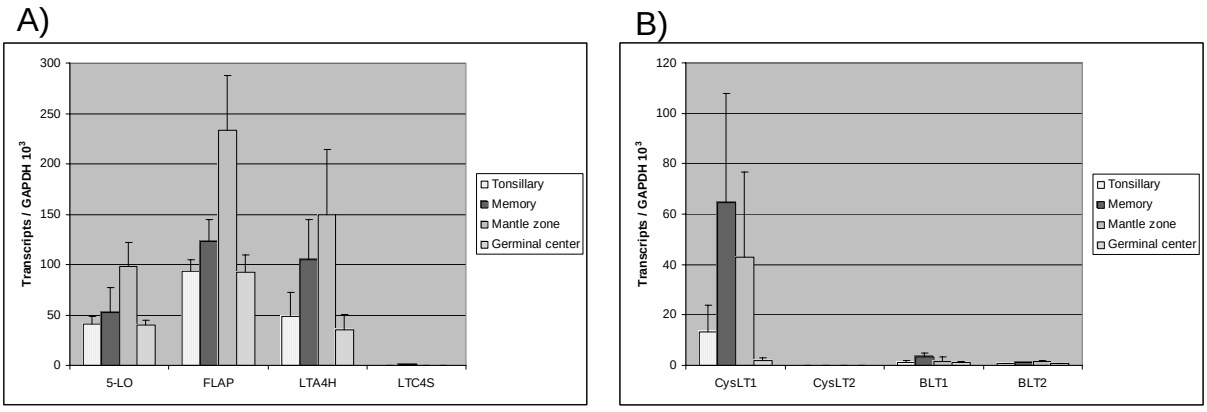

Figure 4.

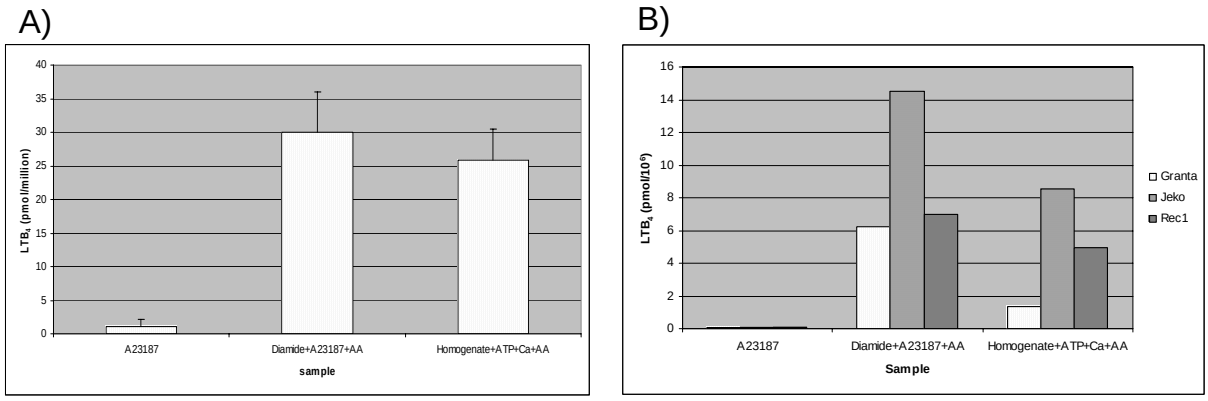

Figure 5.

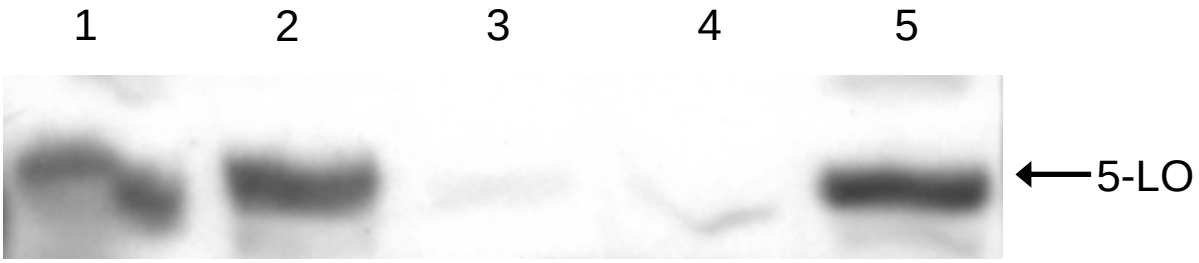

Figure 6.

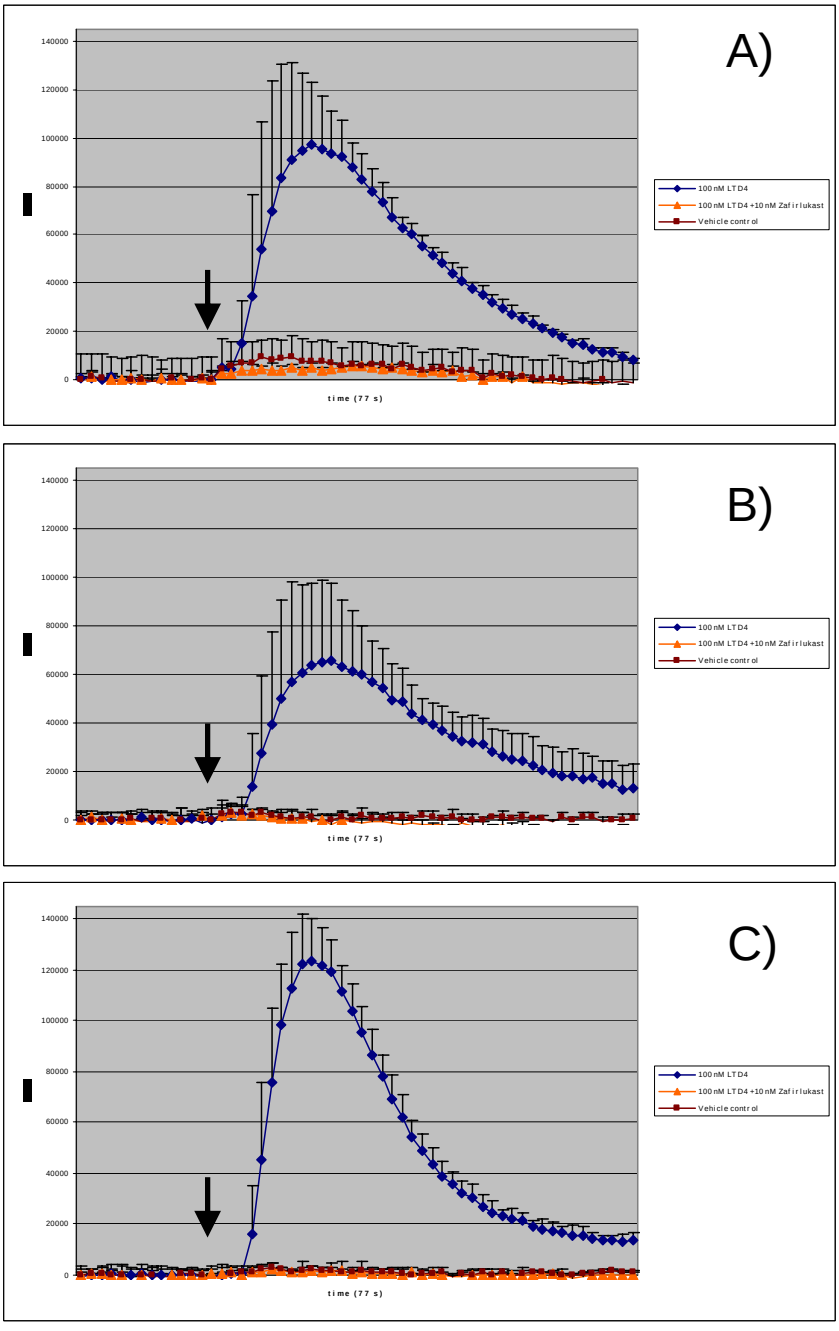
A)
B)
C)
140000
120000
100000
80000
60000
40000
20000
0
time (77 s)
100 nM LTD4
100 nM LTD4 +10 nM Zafirlukast
Vehicle control

Figure 7.