Prostaglandins, Leukotrienes and Essential Fatty Acids 79 (2008) 223–231

Contents lists available at ScienceDirect

Prostaglandins, Leukotrienes and Essential Fatty Acids

journal homepage: www.elsevier.com/locate/plefa

Regulation of the eicosanoid pathway by tumour necrosis factor alpha and leukotriene D_4 in intestinal epithelial cells ☆

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ARTICLE INFO

Article history:
Received 25 July 2008
Received in revised form
20 September 2008
Accepted 30 September 2008

Keywords:
Leukotriene D_4
LTD_4
$CysLT_1R$
$CysLT_2R$
LTC4S
TNF-α
Intestinal epithelial cells
5-LO
COX-2

ABSTRACT

In this study the mRNA and protein levels of the key enzymes involved in eicosanoid biosynthesis and the cysteinyl leukotriene receptors ($CysLT_1R$ and $CysLT_2R$) have been analysed in non-transformed intestinal epithelial and colon cancer cell lines. Our results revealed that tumour necrosis factor alpha (TNF-α), and leukotriene D_4 (LTD_4), which are inflammatory mediators implicated in carcinogenesis, stimulated an increase of cyclooxygenase-2 (COX-2), in non-transformed epithelial cells, and 5-lipoxygenase (5-LO) in both non-transformed and cancer cell lines. Furthermore, these mediators also stimulated an up-regulation of LTC_4 synthase in cancer cells as well as non-transformed cells. We also observed an endogenous production of CysLTs in these cells. TNF-α and LTD_4, to a lesser extent, up-regulate the $CysLT_1R$ levels. Interestingly, TNF-α also reduced $CysLT_2R$ expression in cancer cells. Our results demonstrate that inflammatory mediators can cause intestinal epithelial cells to up-regulate the expression of enzymes needed for the biosynthesis of eicosanoids, including the cysteinyl leukotrienes, as well as the signal transducing proteins, the CysLT receptors, thus providing important mechanisms for both maintaining inflammation and for tumour progression.

1. Introduction

Arachidonic acid is metabolised mainly by two major pathways, involving either the cyclooxygenases (COX) or the lipoxygenases (LO). The metabolites catalysed by these enzymes are important mediators of acute and chronic inflammation. Both enzymes and their metabolites are well known to be involved in cancer development and progression [1].

In addition to cytokines, such as tumour necrosis factor alpha (TNF-α) [2], many lipid mediators, in particular prostaglandins (PG) and leukotrienes (LT), are involved in the innate immune response. LTs, released predominantly by inflammatory cells such as polymorphonuclear leukocytes, macrophages and mast cells, represent a group of essential mediators in the inflammatory milieu. Overproduction of these substances is associated with chronic inflammation [3,4].

LTs are derived from arachidonic acid via the 5-LO pathway [5], and leukocytes are the richest source of these eicosanoids. However, cells of epithelial and endothelial origin are also able to produce these mediators [6]. Archidonic acid is liberated from membrane phospholipids via calcium-dependent cytosolic phospholipase A_2 and then converted into the unstable intermediate LTA_4 by the actions of 5-lipoxygenase (5-LO) and 5-LO activating protein (FLAP) [7]. LTA_4 in turn is conjugated to a reduced glutathione by the integral nuclear membrane protein, leukotriene C_4 synthase (LTC_4S), and thereby converted to LTC_4 [8]. Furthermore LTC_4 is converted extracellularly to LTD_4 by a γ-glutamyl leukotrienase [9].

PG are formed from arachidonic acid by COX and PG synthases [10–12]. PGs and LTs are involved in a variety of homeostatic biological functions and inflammatory and allergic conditions [10,13–15].

LTD_4 is the most potent of the three cysteinyl leukotrienes (LTC_4, LTD_4, LTE_4,), and it exerts its effects through specific cell-surface receptors belonging to the G-protein-coupled receptor family, namely the $CysLT_1$ and $CysLT_2$ receptors [16]. Whereas $CysLT_2$ receptor is implicated in cellular differentiation and its reduced expression in colon cancer is associated with poor prognosis [17], most of the pro-inflammatory and pro-carcinogenic effects of LTD_4 are mediated through its high-affinity $CysLT_1$ receptor.

LTD_4 is associated with the pathogenesis of respiratory, cardiovascular and gastrointestinal inflammatory diseases, in particular inflammatory bowel diseases (IBD) [18]. Patients suffering from IBD have a 30–50% increased risk of developing

☆ This work was supported by grants awarded to the author A.S. from the Swedish Cancer Foundation, the Swedish Medical Research Council, the Foundations at Malmö University Hospital, the Ruth and Richard Juhlin Foundation, Gunnar Nilsson Foundation and the Österlund Foundation.

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0952-3278/$ - see front matter © 2008 Published by Elsevier Ltd.
doi:10.1016/j.plefa.2008.09.024

colon cancer [19]. One of the most well-recognised pro-inflammatory mediators involved in the pathogenesis of type 1 T helper cell (Th1) dependent IBDs is TNF-α [20]. Among the different kinds of treatment for IBD, substances that antagonise TNF-α, such as the monoclonal antibody infliximab, are often used. However, during long-term treatment some groups of patients relapse [21]. Here, we show the importance of these mediators that are present in the inflammatory milieu, particularly LTs, in the maintenance of inflammation.

Since LTD_4 could play an essential role in cancer progression, the goal of this study was to investigate how inflammatory mediators might affect cysteinyl leukotrienes (cysLTs) biosynthesis and their receptors in intestinal epithelial cells.

2. Materials and methods

2.1. Materials

LTD_4, the rabbit polyclonal anti-human $CysLT_1R$ and $CysLT_2R$ antibodies were obtained from Cayman Chemicals Co. (Ann Arbor, MI), human recombinant TNF-α was purchased from R&D systems (Minneapolis, MN). Hybond polyvinylidene fluoride (PVDF) membranes and hyperfilm were from Amersham Biosciences (Little Chalfont, Bucks, UK). The rabbit polyclonal anti-human 5-lipoxygenase antibody and MK886 were a kind gift from Merck. The rabbit polyclonal anti-human 2-cyclooxygenase antibody was from Abcam (Cambridge, UK). The mouse anti-actin antibody was purchased from ICN (Aurora, OH). The secondary peroxidase-conjugated goat anti-rabbit and anti-mouse antibodies were purchased from Dako Cytomation (Glostrup, Denmark). EZ-ECL Luminol enhancer solution was from Santa Cruz Biotechnology (Santa Cruz, CA). TaqMan primers and master-mix for real-time PCR were purchased from Applied Biosystems (Cambridge, UK). Coomassie blue was obtained from Pierce (Rockford, IL). RNeasy MinElute Spin Columns were from Qiagen GmbH (Hilden, Germany). All other reagents were of analytical grade and purchased from Sigma Chemicals Co. (St. Louis, MO).

2.2. Cell culture

Human non-transformed intestinal epithelial cells, Int-407, which exhibit typical epithelial cell morphology were cultured in Eagle's Basal Medium (Invitrogen, Carlsbad, CA) supplemented with 15% newborn calf serum [17]. Four different colon cancer cell lines were used, HT-29, SW-480, Caco-2 and HCT-116 cells. HT-29 cells were cultured in McCoy's 5A medium supplemented with 10% foetal bovine serum. SW-480 and HCT-116 cells were cultured in RPMI 1640 supplemented with 15% foetal bovine serum. Caco-2 cells were cultured in Dulbecco's modified Eagle medium supplemented with 10% foetal bovine serum and 1% non-essential amino acids. All media were supplemented with 55 μg/ml streptomycin, 55 IU/ml penicillin and fungizone. The cells were kept at 37 °C in a humidified atmosphere of 5% CO_2. All cell lines were cultured for 5 days as a monolayer to approximately 70% confluence. The cells were kept in medium supplemented with 1.5% foetal bovine serum overnight and then stimulated with 80 nM LTD_4 or 10 ng/ml TNF-α for the indicated time points. All cells were kept in low serum medium (1.5%) for total 36 h. The cells were regularly tested to ensure the absence of mycoplasma contamination.

2.3. Quantitative PCR

Cells were harvested on ice in PBS and homogenised 10 times with a 20G needle. The cells were centrifuged for 2 min at 11,000 rpm and the pellet was resuspended in 1 ml TRIzol (Invitrogen, Carlsbad, CA) and immediately frozen at −80 °C. RNA was isolated using the phenol–chloroform extraction method. The RNA was dissolved in RNase-free H_2O and purified on RNeasy MinElute Spin Columns (Qiagen, Hilden, Germany). cDNA synthesis was performed using Superscript™ II reverse transcriptase (Invitrogen, Carlsbad, CA). Exactly 2 μg of cDNA was mixed with 0.9 μM TaqMan primers and master mix and amplified at 60 °C in a GeneAmp 7900HT Fast system thermocycler (Applied Biosystems, Foster City, CA). The following primers were used: $CysLT_1R$: Hs00929113_m1; $CysLT_2R$: Hs00252658_s1; cyclooxygenase-2: Hs01573475_g1; 5-lipoxygenase: Hs01095327_g1; LTC_4 synthase: Hs00168529_m1; GAPDH: Hs00266705_g1. The samples were analysed and normalised against a housekeeping gene (GAPDH) using the SDS 2.3 software (Applied Biosystems, Foster City, CA).

2.4. Gel electrophoresis and immunoblotting

Harvesting of cells was performed by scraping them on ice in lysis buffer (50 mM TRIS, 1 mM EDTA, 1 mM EGTA, 50 mM sodium fluoride, 5 mM sodium pyrophosphate, 10 mM β-glycerophosphate [pH 7.5], supplemented with 4 μg/ml Leupeptin, 60 μg/ml PMSF, 2 mM Na_3VO_4 and 1% (v/v) Triton X-100), homogenised 10 times with a Dounce and centrifuged at 200g for 10 min followed by 1000g for 5 min to remove cell debris. The samples were adjusted for equal protein content by the Bradford method using Coomassie blue Pierce (Rockford, IL). Samples were boiled for 10 min in sample buffer (0.5 M TRIS-base, 10% SDS, glycerol, bromophenol blue, 15 mg/ml DTT) and then frozen at −20 °C. Cell lysates were boiled for 2 min before application on an 8% or 12% polyacrylamide gel in the presence of SDS and aliquots (25 μg protein) of samples in loading buffer were subjected to electrophoresis. The separated proteins were transferred to a PVDF membrane. Membranes were blocked with 3% non-fat dry milk or 3% gelatine in TBS-T (Tris–HCl pH 7.5; containing 100 mM NaCl and 0.05% Tween-20) for 3 h, followed by incubation overnight at 4 °C with respective antibodies (1:500 dilution for COX-2 and $CysLT_2$; 1:1000 for 5-LO; and 1:250 for $CysLT_1$). The membranes were thereafter washed and incubated with horseradish-peroxidase linked secondary antibody for 1 h at room temperature. After washing, the membrane was incubated with ECL detection reagents and exposed to hyperfilm ECL. Films were scanned in a GS-800 Calibrated Densitometer and bands were quantified and normalised to beta-actin by using Quantity One (Bio-Rad, Hercules, CA).

2.5. CysLT enzyme immunoassay

Production of CysLTs was measured with an enzyme immunoassay performed according to the manufacturer's instructions (Cayman Chemicals Company). In short, cells (2×10^7) were either stimulated or not with ionophore A23187 (5 μM) for 30 min, and FLAP inhibitor, MK886 for 24 h. The cell media were collected, and LTs were separated by solid-phase extraction Sep-Pak Vac RC (C18-500 mg) catridges (Water Corp., Milford, MA). The samples were transferred to a 96-well plate for the enzyme immunoassay and absorbance was measured at 405 nm with a BMG plate reader (Offenburg, Germany).

2.6. Statistical analysis

Diagrams are presented as means ± standard deviation of at least three independent experiments. A P-value of $\leqslant 0.05$ was