Signaling and regulation of cysteinyl leukotriene receptors in intestinal epithelial cells and colon cancer

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Signaling and regulation of cysteinyl leukotriene receptors in intestinal epithelial cells and colon cancer

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Abstract
Colorectal cancer is the third most common type of cancer, and the second leading cause of cancer-related deaths in the Western world. Chronic inflammation, such as inflammatory bowel diseases (IBD), increases the risk of cancer. Cysteinyl leukotrienes are pro-inflammatory lipid mediators that are involved in IBD. They are ligands to the G-protein coupled receptors CysLT1R and CysLT2R, of which CysLT1R is associated with poor prognosis and CysLT2R with good prognosis for colorectal cancer patients. Signaling through CysLT1R results in increased proliferation, survival and migration of intestinal epithelial cells, whereas signaling through CysLT2R leads to differentiation of colorectal cancer cells.
My main findings have been that 1) Leukotriene D4 (LTD4) induces intestinal epithelial cell proliferation by activating the mitogenic JNK/AP-1 pathway; 2) The mitogen EGF, involved in cancer progression, down-regulates CysLT2R expression and is inversely correlated with CysLT2R in a colorectal cancer tissue array, while 3) the anti-tumorigenic agents all-trans retinoic acid and interferon α up-regulate CysLT2R expression in colorectal cancer cells; 4) LTC4, a ligand for CysLT2R, inhibits migration of colorectal cancer cells; 5) Intestinal epithelial and colorectal cancer cells express enzymes and receptors of the cysteinyl leukotriene and prostaglandin pathways; and 6) The pro-tumorigenic cytokine TNFα up-regulates CysLT1R while down-regulating CysLT2R.
These studies provide more evidence that CysLT1R and CysLT2R have opposite functions in colon cancer progression, and we now know how to regulate their expression. This thesis provides a basic understanding on the role and regulation of cysteinyl leukotriene receptors, which is useful for designing future therapies of colorectal cancer.

Key words: Leukotriene; CysLT1R; CysLT2R; colorectal cancer; inflammation; EGF; all-trans retinoic acid; interferon; COX-2; TNF alpha

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II. Cecilia Magnusson, **Astrid M Bengtsson**, Jian Liu, Roy Ehrnström, A. Yvonne Olsson Ceder and Anita Sjölander. EGF down-regulates the expression and functional response of the tumor-suppressing cysteinyl leukotriene 2 receptor in colon cancer cells. *Submitted manuscript*

III. **Astrid M Bengtsson**, Cecilia Axelsson, Cecilia Magnusson, Gunilla Jönsson and Anita Sjölander. The cysteinyl leukotriene 2 receptor is involved in all-*trans* retinoic acid-induced differentiation of colon cancer cells. *Manuscript*

IV. Yulyana Yudina, Ladan Parhamifar, **Astrid M Bengtsson**, Maria Juhas and Anita Sjölander. Regulation of the eicosanoid pathway by tumour necrosis factor alpha and leukotriene D_4 in intestinal epithelial cells. Prostaglandins Leukot Essent Fatty Acids. 79 (2008):223-31.

ABBREVIATIONS

AA	Arachidonic acid
AP-1	Activator protein 1
5-ASA	5-aminosalicylic acid
APC	Adenomatosis polyposis coli
APL	Acute promyelocytic leukemia
ATRA	All-*trans* retinoic acid
Bcl-2	B-cell lymphoma 2
CDX2	Caudal-type homeobox 2
COX-2	Cyclooxygenase-2
$cPLA_2\alpha$	Cytosolic phospholipase $A_2\alpha$
CRBII	Cellular retinol binding protein II
CRC	Colorectal cancer
CRE	cAMP-responsive element
CREB	cAMP-responsive element-binding protein
CysLT	Cysteinyl leukotriene
$CysLT_1R$	Cysteinyl leukotriene receptor 1
$CysLT_2R$	Cysteinyl leukotriene receptor 2
DR	Direct repeat
E-box	Enhancer box
ECM	Extracellular matrix
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EMT	Epithelial to mesenchymal transition
ERK1/2	Extracellular signal-regulated kinase 1 or 2
FAP	Familial adenomatous polyposis
FLAP	5-lipoxygenase activating protein
5-FU	5-fluorouracil
GPCR	G-protein coupled receptor
HAT	Histone acetyltransferases
HDAC	Histone deacetylase
HIF	Hypoxia-inducible factor
5-HPETE	5-hydroperoxyeicosatetraenoic acid

HUVEC	Human umbilical vein endothelial cells
IAP	Intestinal alkaline phosphatase
IBD	Inflammatory bowel disease
IFN	Interferon
IFNAR	type 1 interferon receptor
IκB-α	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor alpha
IKK	IκB kinase
IL	Interleukin
iNOS	Inducible nitric oxide synthase
IRF	Interferon regulatory factor
ISRE	Interferon-stimulated response element
JAK	Janus kinase
JNK	c-Jun N-terminal kinase
5-LO	5-lipoxygenase
15-LO	15-lipoxygenase
LPS	Lipopolysaccharide
LT	Leukotriene
LTC_4S	Leukotriene C_4 synthase
MAPK	Mitogen-activated protein kinase
MRP	Multidrug resistance-associated protein
MMP	Matrix metalloprotease
MUC2	Mucin 2
NaBT	Sodium butyrate
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NIK	NF-κB-inducing kinase
NLS	Nuclear localization sequence
N-CoR	Nuclear receptor corepressor
NSAID	Non-steroidal anti-inflammatory drug
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
PG	Prostaglandin
PGE_2	Prostaglandin E_2
PI3K	Phosphatidylinositol-3-kinase
PKC	Protein kinase C
PPAR	peroxisome proliferator-activated receptor
p90RSK	90-kDa ribosomal S6 kinase
Raf-1	v-raf-1 murine leukemia viral oncogene homolog 1
RANTES	Regulated on activation T cell expressed and secreted

RAR	Retinoic acid receptor
RARE	Retinoic acid response element
ROR	RAR-related orphan receptor
ROS	Reactive oxygen species
RTK	Receptor tyrosine kinase
RXR	Retinoid X receptor
SMRT	Silencing mediator of retinoid and thyroid receptors
STAT	Signal transducer and activator of transcription protein
TAM	Tumor-associated macrophage
TBP	TATA-binding protein
TF	Transcription factor
TGF-β	Transforming growth factor β
T_H	T helper cell
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor α
UDP	Uridine diphosphate
VEGF	Vascular endothelial growth factor

INTRODUCTION

The gastrointestinal tract is one of the fastest dividing organs in the body. In the mucosal lining of the gut, epithelial cells constitute a barrier against pathogens while at the same time the cells facilitate digestion of and provide transport of nutrients and water. The colon is colonized by commensal bacteria, which live in symbiosis with the host. Colorectal cancer afflicts individuals primarily in the Western world and is a multi-factorial disease, where genetic predisposition, chronic inflammation and environmental factors all have been shown to play a role in the pathogenesis of this cancer. One of the environmental factors modulating the risk of colorectal cancer is our diet.

We have learned that the essential unsaturated omega-3 fatty acids are protective, while omega-6 counterparts are unfavorable in, e.g., heart diseases and colorectal cancer. However, that omega-6 fatty acids are harmful is a truth with modification. Omega-6 derivatives of arachidonic acid can give rise to both beneficial and harmful signaling pathways, depending on which enzymes and receptors are activated.

The most important omega-6 fatty acid is arachidonic acid, an essential constituent of the plasma membrane. In response to certain stimuli, arachidonic acid is released from the plasma membrane and metabolized into eicosanoids. These are potent lipid mediators, some of which encompass pro-inflammatory properties while others have anti-inflammatory modes of action. Leukotrienes are a family of pro-inflammatory eicosanoids, first discovered in the 1930s as a slow-reacting substance of anaphylaxis. Since then, they have been found to be involved in the pathogenesis of several inflammatory disorders and in cancer. Antagonists of enzymes and receptors of the leukotriene pathway are successfully used as treatment for asthma.

In my thesis, I have examined how intracellular signaling mediated by leukotrienes affects intestinal epithelial cells and colorectal cancer cells. The work has been focused on the role of cysteinyl leukotriene receptors (CysLTR) 1 and 2 in colorectal cancer. In a cancer setting, $CysLT_1R$ mediates pro-tumorigenic pathways while $CysLT_2R$ mediates

differentiation. Previous studies from our group have shown that high expression of $CysLT_1R$ while low expression of $CysLT_2R$ in colon tumor tissue correlate with poor patient survival. I have elucidated the mechanisms behind how leukotriene D_4, a pro-inflammatory eicosanoid, induces proliferation of intestinal epithelial cells. Moreover, I have investigated how $CysLT_2R$ may be modulated by the anti-cancer agents interferon α and all-*trans* retinoic acid and the pro-tumorigenic epidermal growth factor. Colorectal cancer is a disease treated by surgery in combination with chemotherapy. Pharmacologic fine-tuning of the eicosanoid pathways may be a way to meet the existing needs for pharmaceutical treatment in the future.

BACKGROUND

Anatomy of the intestines

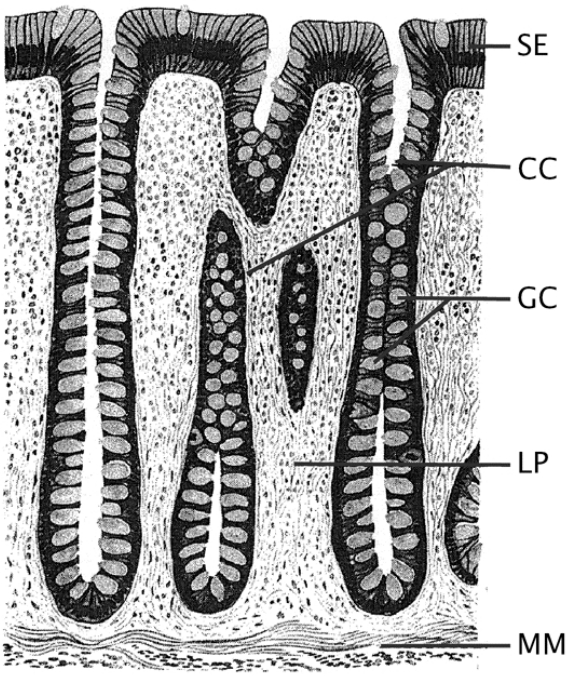
The intestines under normal conditions

The mucosal lining of the colon is formed into crypts and is composed of simple columnar epithelial cells, mucus-secreting goblet cells and Paneth cells [2]. The epithelial cells are renewed about every 5 days from stem cells residing in the bottom of the crypts. The progenitor cells generate rapidly proliferating cells, which undergo differentiation while migrating up the crypt [3]. One human crypt contains roughly 2,000 cells and is believed to harbor about 19 stem cells [4]. The apical surface of the epithelial cells has 1-µm-long microvilli, also called the brush border, that increase the surface area for the digestion and transport of molecules from the intestinal lumen [5]. Goblet cells are bell-shaped cells of endocrine origin whose main function is to secrete mucins [6,7]. Paneth cells are a part of the innate immune system and release granules rich in β-defensins into the intestinal lumen as a defense against pathogens [8]. The cells adhere to a basement membrane consisting of a network of collagen IV fibrils, laminins, nidogen and proteoglycans that attaches the cells to the underlying submucosa [9].

Differentiation of colon epithelial cells

The transcription factor caudal-type homeobox 2 (CDX2) is important in governing embryonic development of the colon and in maintenance of the epithelial lining of both the small and the large intestine [10]. CDX2 is most active in differentiated epithelial cells above the crypts and regulates transcription of genes representative of differentiated colonocytes, including lactase and carbonic anhydrase-1 [11]. The Wnt signaling pathway is perhaps the most dominant in controlling cell fate along the crypt–villus axis. Nuclear β-catenin, a key component in Wnt signaling, is observed in the intestinal crypts [12]. Differentiated colon epithelial cells can be characterized morphologically by the presence of apical brush borders and basolateral junctional complexes, and biochemically by the

presence of brush border enzymes, e.g., intestinal alkaline phosphatase (IAP), aminopeptidase, lactase, and sucrase-isomaltase [13]. Sodium butyrate (NaBT), a natural metabolite from intestinal microflora fermentation, is a strong inducer of differentiation of colonocytes and colon cancer cells via a mechanism of histone hyperacetylation [14,15], allowing induction of expression of brush border enzymes such as IAP [13]. IAP is important in digestion in that this phosphatase hydrolyses monophosphate-esters from food [16]. Recently, IAP was also found to protect the gut against bacterial invasion across the gut barrier by detoxifying bacterial lipopolysaccharides (LPS) through blocking the transcription factor nuclear factor kappa-light-chain enhancer of activated B cells (NF-κB) [17]. IAP has been found to be down-regulated in tissue from patients with inflammatory bowel disease [18].



Similar to goblet cells, the epithelial cells of the colon are also able to produce mucus, which is composed of secreted mucins. Mucins are high-molecular-weight glycoproteins that act as a barrier of the intestinal wall, to protect the mucosa from harmful pathogens [19]. Mucins 1-6 are expressed by the colorectal epithelium [19]. Mucin 2 (MUC2) has been found down-regulated in tissue samples from patients with Crohn's disease [20] and colorectal cancer [21].

Figure 1. Colon tissue morphology. Labels show surface epithelium (SE), colon crypts (CC), goblet cells (GC), lamina propria (LP), and muscularis mucosa (MM). From Frank, 2007 [22].

The intestines under pathological conditions

Inflammatory bowel diseases

The gastrointestinal tract is a major site for pathogen entry. During homeostatic conditions, the intestines are in a state of controlled inflammation. In the colon, antigen-presenting cells such as dendritic cells and macrophages reside just beneath the surface epithelium where they are exposed to antigens, and in lymphoid follicles together with B and T cells [23]. The dendritic cells have protrusions extending through the epithelium and are mainly responsible for the tolerance to pathogens [24]. However, homeostasis is disrupted in chronic inflammatory conditions such as inflammatory bowel diseases (IBDs). IBD is a common name for several chronic pathologies affecting the gastrointestinal tract, including Crohn's disease and ulcerative colitis. The highest incidence rates and prevalence of Crohn's disease and ulcerative colitis have been reported from the Western world (3 to 7 per 100,000 individuals) [25]. What causes IBD is not known, but the epithelial layer has increased permeability in both Crohn's disease and ulcerative colitis [26].

Crohn's disease manifests as ulcers and chronic inflammation that can occur anywhere along the gastrointestinal tract [27]. The pathogenesis depends on a combination of genetic susceptibility factors, the composition of the enteric microflora and immune cell-mediated tissue damage [28]. In Crohn's disease, antigen-presenting cells such as macrophages and dendritic cells produce e.g. interleukin-12 (IL-12), resulting in an excessive T helper cell type 1 (T_H1)-type of immune response [29]. In addition, LTE_4, a metabolite of the cysteinyl leukotrienes (CysLTs) has been detected in urine from patients with Crohn's disease [30]. Recently, the role of IL-23-driven T_H17 cells has also been discussed, and polymorphisms in the gene encoding the IL-23 receptor have been linked to both Crohn's disease and ulcerative colitis [31,32]. T_H1 and T_H17 cells release mediators such as interferon-γ (IFN-γ), tumor necrosis factor α (TNF-α) and IL-6, which enhance the inflammation [27,31]. These processes trigger the recruitment of neutrophils, change the epithelial barrier and activate matrix metalloproteases (MMPs) that degrade the stromal extracellular matrix (ECM). Upon chronic exposure to these cytokines and other factors, tissue damage is eventually induced. Apoptosis-resistant T cells have also been suggested to take part in the chronicity of Crohn's disease [33], but more recent evidence proposes that

defective recruitment of neutrophils results in inadequate clearance of bacteria and initiation of disease [34].

Chronic exposure to inflammatory mediators induces the epithelial cells to become more inflammatory-like. In addition, cytokines such as TNF-α sustain the inflammation by inducing the production of other pro-inflammatory mediators. As an example, we found that TNF-α induces expression of the leukotriene-converting enzyme 5-lipoxygenase (5-LO) and up-regulates the LTD_4 receptor $CysLT_1R$ in intestinal epithelial cells [35]. Moreover, inducible nitric oxide synthase (iNOS) and cyclooygenase-2 (COX-2) are increased in epithelial cells in active inflammatory foci of inflammatory bowel disease [36,37]. COX-2 is an enzyme responsible for generating prostaglandins (PGs) and is linked to carcinogenesis, and will be discussed more in detail below [38,39]. About 10-15% of patients with Crohn's disease carry a mutation in the gene for the intracellular pattern recognition receptor NOD2, presumably resulting in altered Toll-like receptor (TLR) signaling [24].

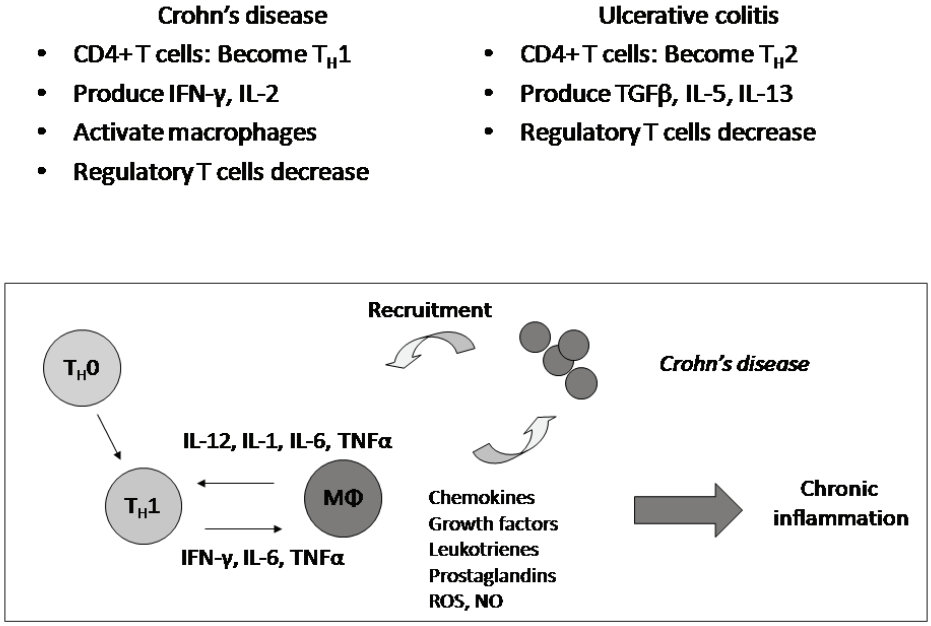


Figure 2. Cells and mediators of inflammatory bowel diseases.

In contrast to Crohn's disease, ulcerative colitis has a T_H2-type cytokine profile, where IL-5 and IL-13 are secreted by natural killer T cells in increased amounts [24]. The inflamed mucosa releases not only cytokines but also eicosanoids such as prostaglandins and leukotrienes [40,41]. High levels of leukotriene B_4 (LTB_4) and LTD_4 have been detected in inflamed colonic mucosa from patients with ulcerative colitis [42,43]. Ulcerative colitis is a relapsing non-transmural inflammatory disease that is restricted to the colon [44].

Conventional treatment of IBD consists of sulfasalazine, mesalazine (5-ASA [5-aminosalicylic acid], which inhibits eicosanoid production [45]) and immunosuppressive drugs such as corticosteroids. However, all of these drugs have drawbacks such as toxicity and high relapse rates [28]. Lately, treatment also includes the monoclonal antibody infliximab, which antagonizes the effects of TNF-α [46].

The link between inflammation and cancer

In 1863, Rudolf Virchow observed leukocytes in tumor tissue. He proposed a theory that the origin of cancer was at sites of chronic inflammation. Since then, much evidence has been presented that supports his theory. Infiltrating leukocytes in the tumor microenvironment participate in the neoplastic process [47]. During tissue injury and subsequent wound healing, cell proliferation is enhanced. In a normal situation, inflammation and proliferation discontinue once the tissue has been repaired. In the case of chronic inflammation, proliferation is sustained, the stroma is activated and releases reactive oxygen and nitrogen species which induce DNA damage. Thus, the features of a tumor resemble those of a wound that does not heal. These factors together promote neoplastic risk [47]. Furthermore, released inflammatory mediators trigger the epithelial-derived tumor cells to acquire properties of inflammatory cells. The mediators utilize adhesion molecules, cytokines, chemokines and chemokine receptors to proliferate and spread to other sites of the body [48]. The strongest association of chronic inflammation with malignant disease is in individuals with IBD, where colorectal cancer accounts for 15% of all deaths of IBD patients [49]. After 30 years of ulcerative colitis, the risk of developing colon cancer is 18%, which corresponds to a 2.6- to 5.4-fold greater risk than in the average population [50]. Interestingly, alteration in the p53 gene has been found in ulcerative colitis, and is considered an early event in colitis-associated dysplasia [51].

Inflammatory component	Associated neoplasms
Papillomavirus	Cervical carcinoma
H. Pylori induced gastritis	Gastric adenocarcinoma, MALT lymphoma
Bronchitis	Lung carcinoma
Inflammatory bowel disease	Colorectal carcinoma
Hepatitis virus B and C	Hepatocellular carcinoma
Chronic pancreatitis	Pancreatic carcinoma
Bladder inflammation	Bladder carcinoma
Chronic cholecystitis	Gall bladder cancer
Human herpesvirus type 8	Kaposi's sarcoma
Barrett's oesophagus	Oesophageal carcinoma
Epstein-Barr virus	Burkitt's lymphoma, B-cell non-Hodgkin's lymphoma
Gonnorrhoea, chlamydia	Ovarian carcinoma
Schisostomiasis	Bladder, liver, rectal carcinoma, follicular lymphoma of the spleen

Table 1. Inflammation-associated cancers. MALT, mucosa-associated lymphoid tissue. Modified from Balkwill, 2001 and Coussens, 2002 [47,48].

The connection between inflammation and cancer is also evident in other malignancies, as cancers of the liver, cervix and bladder often have an inflammatory component involved (see Table 1). It is estimated that 15-20% of cancers globally arise from chronic inflammatory conditions [47]. Convincingly, long-term use of non-steroidal anti-inflammatory drugs (NSAIDs) including aspirin reduces colon cancer risk by 40-50% [52,53]. The inflammatory infiltrate consists primarily of tumor-associated macrophages (TAMs) [54]. In contrast to classically activated macrophages, which often have an M1 phenotype rendering them capable of killing microbes and tumor cells, TAMs have been polarized into the M2 phenotype and are as a consequence poor antigen-presenting cells and produce immunosuppressive factors [55]. Dendritic cells in tumors have an immature phenotype, defective in mounting a proper T-cell response [56].

In addition, eosinophils, mast cells and lymphocytes are present, all of which may secrete cytokines, reactive oxygen species and MMPs. The latter degrade the extracellular matrix, which facilitates metastasis of malignant cells [47]. TAMs are key producers of the factors just mentioned. In addition, TAMs are a major source of angiogenic and growth factors and cytokines including transforming growth factor β (TGF-β), platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), TNF-α and IL-1 [57]. The broad capacity of TAMs makes them powerful mediators of tumor cell proliferation, metastasis and angiogenesis [55]. Evidence from several mouse models indicates that TNF-α acts as a tumor promoter [58]. TNF-α mediates its effects intracellularly primarily via NF-κB, which is a major inducer of proliferation and anti-apoptotic genes [1]. The role of NF-κB will be discussed in more detail below.

How tumors arise and progress

Extra- and intracellular signaling are highly complex and diverse processes that in addition are context-dependent. During homeostasis, there is a delicate balance between tumor suppressors inducing cell cycle arrest, apoptosis and differentiation and factors that promote cell cycle initiation, survival, angiogenesis and migration of the cells. In addition, the interplay between cells and the cells-extracellular matrix is of importance in regulating intracellular events. The more than 100 distinct types of cancer tells us that there is a great diversity in the signals that lead to neoplasia [59].

Tumors arise as a consequence of multiple events. Tumors may originate from spontaneous or familial mutations in tumor suppressor genes such as p53 [60] or in oncogenes such as Ras [61] or, as in the case of familial adenomatous polyposis (FAP), the adenomatosis polyposis coli (APC) gene [62]. For an individual tumor to develop, an accumulation of different mutations must occur [63]. Carcinogens may be the triggering factors behind mutagenesis, and these include chemicals from fossil fuel, cigarette smoke and pesticides, radiant energy and microbial agents such as oncogenic viruses [25].

Accumulating evidence indicates that tumors arise from cancer stem cells [64]. Cancer stem cells have been detected in leukemic, brain, breast and colon cancers [65]. It has been suggested that normal colonic stem cells might be the targets for early carcinogenesis events and

that dysregulation of the tightly regulated process of stem cell self-renewal might give rise to colon cancer stem cells [4].

Tumors are characterized by a high grade of proliferation. The reason may be a combination of excessive activation of proliferative pathways and a disrupted action of growth-suppressive signals. In contrast to normal cells that require a mitogenic signal for cell division to occur, tumors do not rely on exogenous stimuli but use oncogenic proteins to substitute for these actions. An example of self-sufficient growth of tumors is autocrine generation of the mitogen PDGF within glioblastoma tumors [59].

Another property of cancer cells is that they exhibit enhanced survival. The mechanisms behind can be either a disruption of pro-apoptotic signaling (e.g., the Fas death receptors of Bax), or by overexpression or mutation of anti-apoptotic proteins in the phosphatidylinositol-3 (PI3) kinase-AKT pathway or Bcl-2 (B-cell lymphoma 2), all of which have been found activated by LTD_4 in intestinal epithelial cells [59,66,67]. The most common way cancer cells can evade apoptosis is by a loss-of-function mutation in the tumor suppressor gene p53 [68].

Once tumors grow large, they become hypoxic in the interior. Hypoxic tumors are generally more aggressive, presumably because these cells to a higher extent need to migrate to more oxygenated areas. The transcription factor family hypoxia-inducible factors (HIFs) orchestrate the up-regulation of VEGF, for example, which induces endothelial cells to promote angiogenesis, resulting in the sprouting of new blood vessels [69,70].

Furthermore, VEGF is a growth factor also capable of changing the properties of the extracellular matrix (ECM), and causing endothelial cells to switch to express more invasive-competent integrins [59]. Activation of integrin α2β1, a receptor for laminin and collagen present in the basement membrane, induced COX-2 expression in intestinal epithelial cells, which in turn resulted in elevated generation of reactive oxygen species (ROS) and increased cell migration [71]. Invasive cancer cells also switch their integrins from integrin α2β1 to, e.g., αVβ3, which enables the cell to migrate on almost any matrix protein the cell may encounter [72]. In this way, tumor cells can bind to the underlying stroma that has been loosened up by matrix metalloproteases such as MMP-2, MMP-7 and MMP-9, thereby facilitating metastasis [73]. Another feature of tumor invasion and metastasis is the phenomenon called epithelial to

mesenchymal transition (EMT). During this process, phenotypic changes occur where immotile epithelial cells are converted to motile mesenchymal cells. EMT requires a loss of cell-cell adhesion and apical-basal polarity, along with acquisition of a stromal cell phenotype [74]. Loss of function of the cell-cell junction protein E-cadherin is a frequent characteristic of epithelial cancers [75].

Loss of E-cadherin function can generate de-differentiation, where expression of embryonic markers increases, while markers of differentiated cells (in the case of colon cancer, e.g., MUC2) decrease [21]. Many tumors encompass overexpression of the oncogene c-myc, a transcription factor that is suppressed during development of normal cells. Hence, differentiation is impaired, and growth is promoted [59].

Often, more aggressive cancer cells evade the immune system, which has a tumor surveillance function. Tumor cells, including colon cancer cells, often up-regulate co-inhibitory B7 molecules that are used to dampen T-cell responses. These B7 family members have immune-suppressive capacities. Aberrant expression of these coinhibitory molecules might therefore negatively interfere with the host immune response, leading to disease progression. Indeed, they are associated with poor prognosis [76].

Colorectal cancer

Colorectal cancer (CRC) is the third most common form of cancer, and the second leading cause of cancer-related death in the Western world with 639,000 deaths per year worldwide. About 5-10% of the cases of colorectal cancer are hereditary and due to a germline mutation in the tumor suppressor gene adenomatosis polyposis coli (APC), which typically gives rise to 500-2,500 polyps along the colon, resulting in familial adenomatous polyposis (FAP) [77]. The resulting 90-95% of CRC cases result from a combination of a series of mutational events and environmental factors. APC mutations occur as often as in 60% of the cases of sporadic CRC [78]. This protein is part of a complex that binds β-catenin. In the absence of binding and degradation of β-catenin by the proteasome, β-catenin translocates to the nucleus, where it activates the TCF/LEF transcription factors [79]. The constitutive β-catenin–TCF complex drives expression of genes involved in proliferation, such as cyclin D1 and c-myc, in colorectal cancer cells, a process identical to the Wnt cascade in crypt stem cells [12]. Mutations in APC typically give rise to COX-2 induction, which is

overexpressed in many colorectal tumors [80]. Ras mutations occur in about 50% of the cases of CRC [77]. Risk factors are smoking, a diet high in red meat or cholesterol, heavy alcohol intake, a history of IBD and a family history of CRC. The mean age of onset for IBD-associated CRC is lower than that for sporadic CRC (45 versus 60 years) [81]. Fewer than 20% of cases of sporadic CRC occur before the age of 50 years. Males are 20% more often affected than females [25].

Dukes stage	TNM stage		Description
Dukes A	T0	No primary tumor	Invasion into but not through the bowel wall
	T1	Invasion through submucosa	
	T2	Invasion into muscularis propria	
Dukes B	T3	Invasion through subserosa	Invasion through the bowel wall but not involving lymph nodes
	T4	Invasion through other organs	
	N0	No lymph node involvement	
Dukes C	N1	1-3 lymph nodes involved	Involvement of lymph nodes
	N2	4 or more lymph nodes involved	
	N3	Involvement of lymph nodes along the vascular trunk	
	M0	No distant metastasis	
Dukes D	M1	Distant metastasis	Widespread metastases

Table 2. Colorectal tumor classification.

Current treatment

Surgical resection is the general treatment for colorectal cancer patients. However, approximately 30% of postoperative patients have a recurrence within 5 years [82]. To reduce this risk, patients receive irinotecan or 5-fluorouracil (5-FU) adjuvant chemotherapy. Additional treatment includes the VEGF inhibitor bevacizumab, which blocks angiogenesis, and the epidermal growth factor (EGF) receptor inhibitor cetuximab, which targets

metastasis and tumor cell growth [83]. The COX-2 selective inhibitor Rofecoxib (Vioxx) was taken off the market in 2004 due to the risk of severe cardiovascular side effects. Meta-analyses show that cardiovascular risk increases significantly upon long-term high-dose (>400 mg) exposure to NSAIDs [84]. However, another COX-2 inhibitor, Celecoxib (Celebra) is used in the treatment of FAP.

Classification of colorectal cancer

Colorectal tumors have been traditionally assessed according to Dukes' classification [85]. The TNM staging system is more commonly used today where the primary tumor (T), regional nodes (N) and metastasis (M) are followed by a number, where 0 indicates undetectable, and 1-4 indicate a progressive severity (see Table 2). Thus, a tumor may be described as T1, N2, M0 [86]. Recently, a gene expression profiling study of large cohorts of colorectal cancers showed that immunological data (the type, density and location of immune cells within the tumor samples) were a better predictor of patient survival than the histopathological methods described above [87]. Perhaps these findings may lead to revision of the current indicators of clinical outcome in colorectal cancer.

The role of epidermal growth factor in cancer

Epidermal growth factor (EGF) is a growth factor for variety of cells of both ectodermal and mesodermal origin. For example, EGF is involved in normal stem cell renewal in colon crypts [88]. EGF binds specifically to the EGF receptor (EGFR), a receptor tyrosine kinase (RTK) of the ErbB family [89]. Apart from EGFR (also known as ErbB-1/HER1), ErbB-2 (neu, HER2), ErbB-3 (HER3) and ErbB-4 (HER4) also belong to this family. EGF and EGFR are often overexpressed in carcinomas, and amplification of the EGFR gene and mutations of the EGFR tyrosine kinase domain have been found in carcinoma patients. Upon ligand binding, homo- or heterodimerization of EGFR leads to tyrosine kinase activation and intrinsic trans-phosphorylation. This results in activation of multiple signaling pathways including the Ras/Raf/MEK/mitogen-activated protein kinase (MAPK) pathway through either Grb2 or Shc adaptor proteins, PLCγ and PI3 kinase activation. These mediators activate various transcription factors such as c-fos, c-Jun, c-myc, NF-κB or the transcriptional repressors SNAI1 (Snail), SNAI2 (Slug) and Twist [88]. These repressors bind to target

sequences called E-boxes (Enhancer boxes) in the promoters of genes, which are conserved elements with the consensus sequence CANNTG. As mentioned above, EMT is a major event in tumor metastasis and is characterized by loss of E-cadherin, which is a crucial protein in the maintenance of adhesion and polarity of epithelial cells. The transcriptional repressors SNAI1, SNAI2 and Twist drive EMT by repressing E-cadherin [74,90,91]. The expression of SNAI1 is associated with distant metastasis, and SNAI2 is a marker of poor prognosis in CRC [90]. Interestingly, SNAI1 expression can be induced by the COX-2–PGE_2 pathway, which highlights the importance of inflammatory components present in the tumor microenvironment, on the induction of EMT [92].

The role of NF-κB in cancer

Bacterial invasion in the gut or presence of inflammatory mediators such as IL-1 and TNF-α in the intestinal wall is a common feature in IBD. These mediators converge intracellularly in the NF-κB signaling pathway. Expectedly, NF-κB is active in the colonic mucosa of ulcerative colitis patients [93]. The transcription factor NF-κB targets a vast number of genes, among which many are involved in inflammation (IFN-γ, IL-6, IL-8), others in tumor promotion (COX-2, iNOS), proliferation (Cyclin D1), survival (Bcl-2), angiogenesis (VEGF) and metastasis (ICAM-1, VCAM-1, MMP-9) [1,94,95]. This array of evidence points to a dual role of NF-κB in pathogenesis, where one is to sustain inflammation and another is to promote tumor development. Indeed, deletion of the NF-κB signaling machinery, such as in the IκB kinase β (IKK-β) null mouse, has resulted in inhibition of colitis-associated colon cancer growth and failure of tumor progression in an experimental model of inflammation-induced hepatocellular carcinoma [96,97]. NF-κB thus plays an important role not only in inflammatory responses but also in cancer, and has been postulated to be “the link between inflammation and cancer” [1].

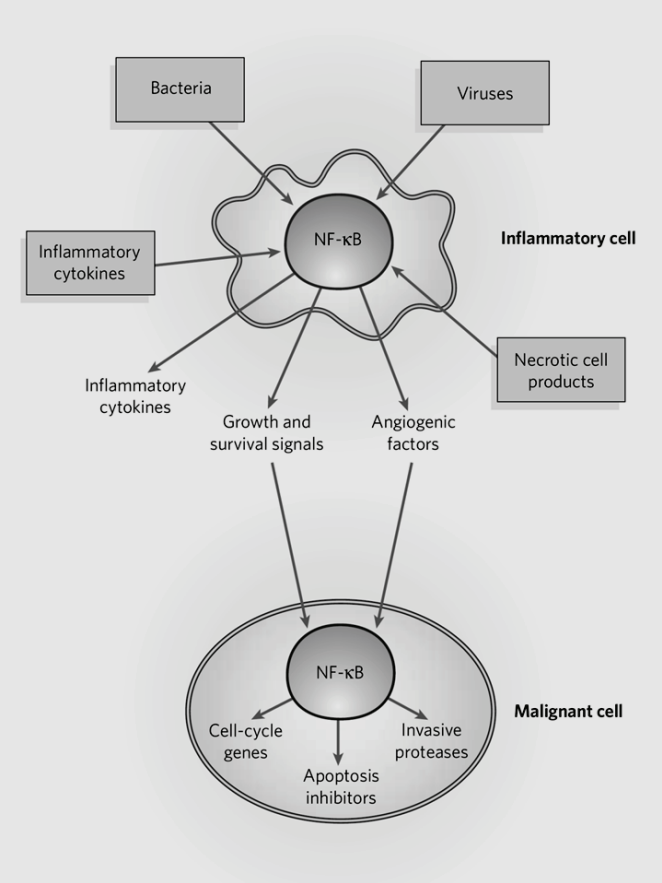


Figure 3. Model of NF-κB as a link between inflammation and cancer. Adapted from Karin, 2006 [1].

NF-κB was first discovered in B lymphocytes, where it was shown to play a role in B-cell differentiation in response to LPS [98]. The mechanisms of NF-κB signaling have been extensively studied throughout the years, and more than 20 different mouse models targeting proteins in the NF-κB pathway have been developed [99]. Two pathways leading to NF-κB activation are known, the classical, which includes activation of the IKK complex, and the alternative spathway. The classical pathway is activated by a number of stimuli, including viruses, pro-inflammatory cytokines, UV irradiation, hypoxia and oxidative stress [95]. The activation initiates a cascade of events through phosphorylation of the IKK complex, which is composed of two catalytic subunits (IKK-α and IKK-β) and a regulatory subunit (IKK-γ/NEMO). The IKK complex in turn phosphorylates the inhibitory protein IκB-α, whose role is to retain the NF-κB subunits inactive in the cytoplasm. Upon phosphorylation, IκB-α becomes ubiquitinated and consequently becomes degraded in the proteasome [94]. Thereby, the NF-κB subunits are able to enter the nucleus to bind to target genes in homo- or heterodimer formation of five known subunits: RelA (p65), c-Rel, RelB, NF-κB1 (p105/p50) and NF-κB2 (p100/p52) [100]. The alternative pathway is induced by LPS and

lymphotoxin, and activates NF-κB-inducing kinase (NIK) to phosphorylate the precursor forms of NF-κB1 (p105) and NF-κB2 (p100). This results in proteolytic cleavage and the generation of the DNA-binding p50 and p52 subunits [101,102]. The p50/p52-dependent transcription of genes is induced by transactivation of this NF-κB dimer by Bcl-3, a proto-oncogene and a member of the IκB family [103,104].

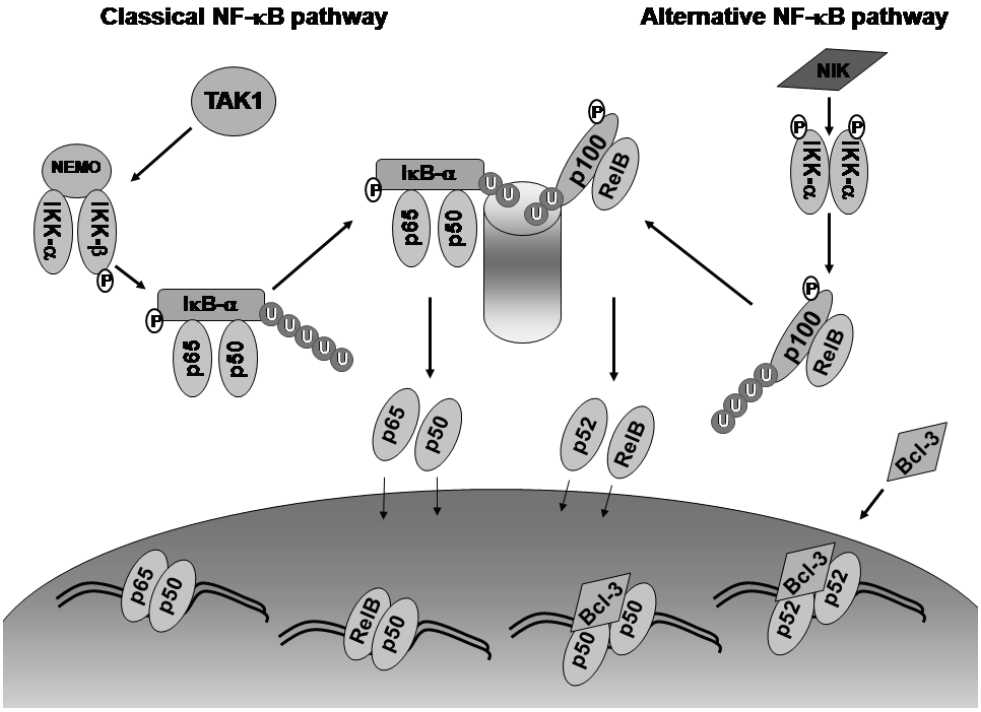


Figure 4. NF-κB signaling pathways.

The role of AP-1 in cancer

Activator protein-1 (AP-1) was one of the first transcription factors to be discovered [105]. It can be activated by a wide range of stimuli, including growth factors, cytokines, neurotransmitters, cell-matrix interactions, bacteria, viruses, UV light and chemical stress. These stimuli activate the MAPK pathways, of which extracellular signal-regulated kinases (ERKs) respond to growth factors, whereas pro-inflammatory cytokines and stress induce activation of c-Jun N-terminal kinase (JNK) and p38. Upon

activation, the MAPKs translocate to the nucleus where they phosphorylate AP-1 proteins [106]. AP-1 is a family of transcription factors composed of homo- or heterodimers of Jun (c-Jun, JunB, JunD), Fos (c-Fos, FosB, Fra1 and Fra2), Maf (c-Maf, MafA, MafB, MafG/F/K and Nrl) or ATF (ATF2, ATF3, B-ATF, JDP1 and JDP2) subunits, of which c-Jun and c-Fos were originally described as proto-oncogenes and homologs of the retroviral oncoproteins v-Fos and v-Jun [106-108]. While ERK phosphorylates c-Fos, JNK can activate c-Jun and ATF2, and p38 induces activation of ATF2. The AP-1 proteins are basic region-leucine zipper dimers that bind to TPA- or cAMP-responsive elements (CREs) in promoter regions of genes [106].

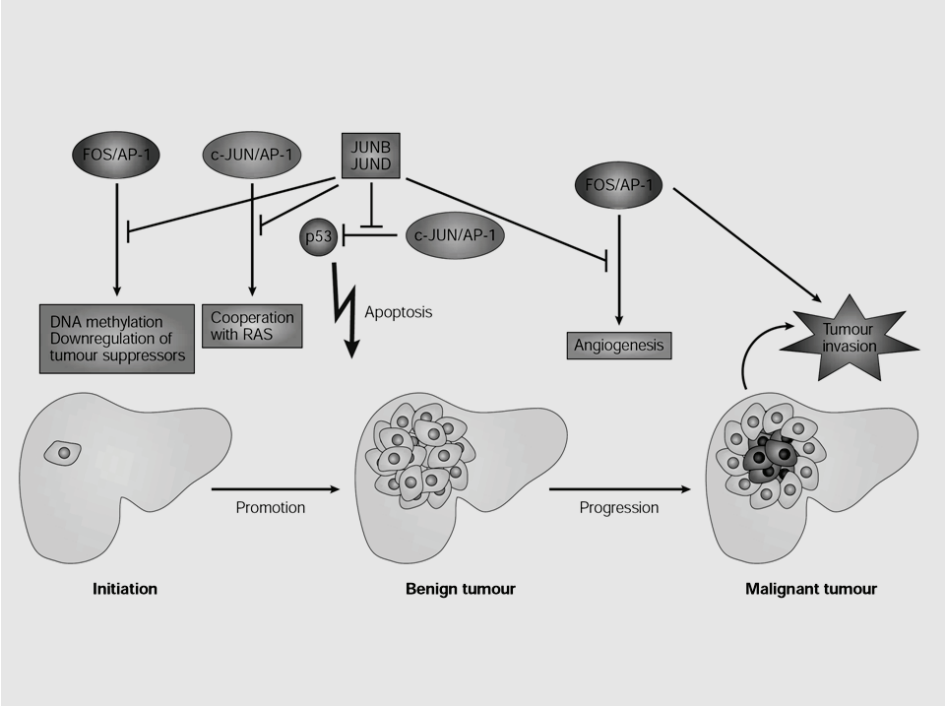


Figure 5. AP-1 in liver tumorigenesis. Adapted from Eferl, 2003 [108].

Depending on the context, i.e., the local environment or cell type, AP-1-induced transcription of genes can result in responses involved in proliferation, survival and differentiation. In general, it seems that c-Jun and c-Fos drive tumorigenesis, while JunB and JunD mediate apoptosis

[108]. c-Jun, c-Fos and FosB can transform cells in culture, whereas JunB and JunD lack this capacity [109]. c-Jun induces Cyclin D1 expression and down-regulates p53 expression, thereby contributing to proliferation and survival [106]. Moreover, c-Fos and c-Jun can induce EMT, and up-regulate CD44 and matrix metalloproteases, which promotes invasiveness [108].

There are many examples linking AP-1 to carcinogenesis. In the liver, c-Jun is required for early stages of tumorigenesis in a mouse model of hepatocellular carcinoma. c-Jun's mechanism of action was found to be through inhibiting apoptosis by antagonizing p53 [110]. In the APC^{Min} mouse model of intestinal cancer, c-Jun cooperates with the transcription factor TCF4 of the Wnt signaling pathway in driving tumorigenesis [111]. Overexpression of c-Jun, JunB, Fra-1 and Fra-2 was found in a study with human colorectal cancer tissue from 75 donors [112].

Leukotrienes can induce AP-1 activation. Previous studies show that LTB_4 can induce c-Fos mRNA in human monocytes, and cysteinyl leukotrienes can induce c-Jun phosphorylation in fibroblasts [113,114]. Moreover, 5-LO is required for EGF-mediated induction of JunB expression in human squamous carcinoma cells [115].

Eicosanoids in inflammation and cancer

Arachidonic acid (AA) is a key component of the plasma membrane and is cleaved off upon activation of the enzyme cytosolic phospholipase A_2 ($cPLA_2$). As shown in Figure 6, AA can be converted to prostaglandins, lipoxins or leukotrienes.

Prostaglandin biosynthesis

Prostaglandins are generated in response to cytokines, growth factors, thrombin or mechanical trauma. AA is metabolized into the intermediate prostaglandin PGH_2 by the cyclooxygenases COX-1 or COX-2, where COX-1 is constitutively expressed and COX-2 is induced during inflammation or cancer. The subsequent generation of thromboxane, prostacyclins and prostaglandins by multiple enzymes gives rise to various biological actions in different organs. For example, thromboxane A_2 aggregates platelets and mediates vasoconstriction, whereas prostacyclin is produced in endothelial cells and counteracts these functions [116].

Prostaglandin E_2 (PGE_2) is a ligand to four G-protein coupled receptors (GPCRs) termed EP1-4 and mediates numerous effects, including uterus contraction during labor, fever and proliferation of tumors overexpressing COX-2.

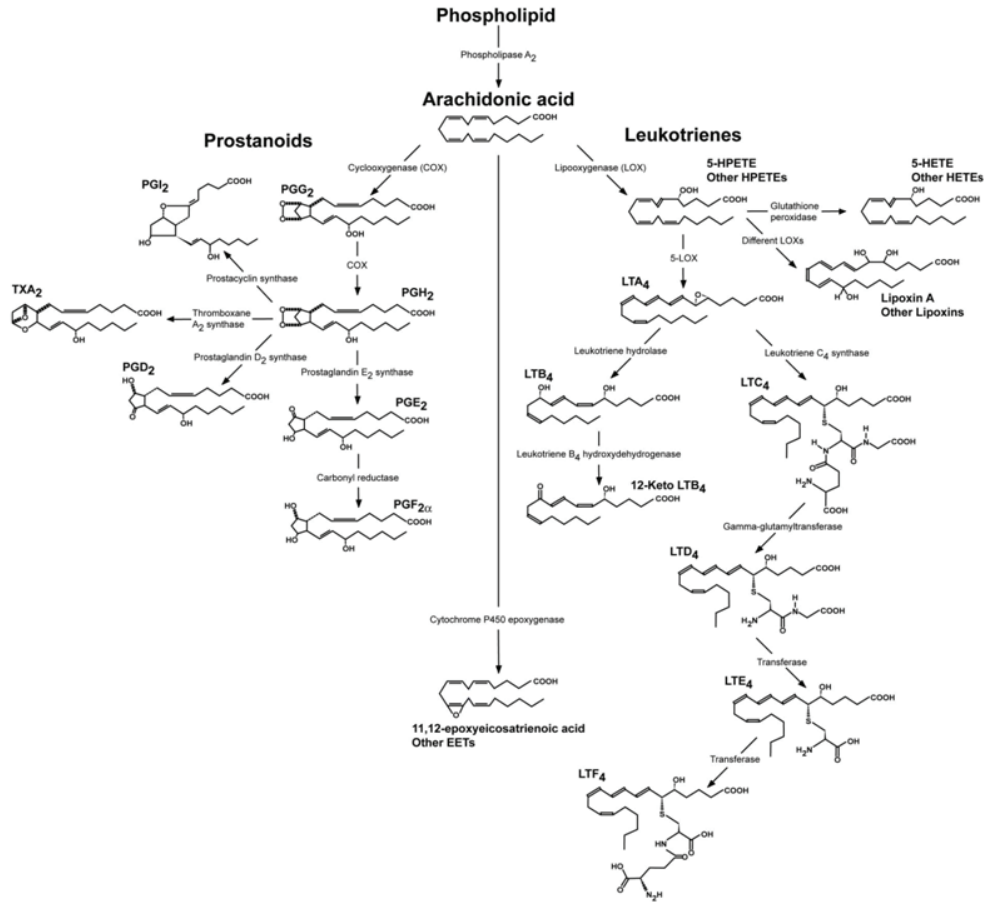


Figure 6. Eicosanoid biosynthesis. EET, epoxyeicosatrienoic acids; HETE, hydroxyeicosatetraenoic acids; HPETE, hydroperoxyeicosatetraenoic acids. Adapted from Heckmann, 2008 [117].

Leukotriene biosynthesis

For the generation of leukotrienes, AA is converted to 5-hydroperoxyeicosatetraenoic acid (5-HPETE) and subsequently to the unstable intermediate LTA_4 through the action of 5-lipoxygenase (5-LO). 5-LO translocates from the cytosol to the outer nuclear membrane where 5-

LO is activated by 5-LO-activating protein (FLAP) [118]. LTA_4 is converted to the chemotactic LTB_4 via the action of LTA_4 hydrolase, or to cysteinyl leukotrienes (CysLTs), which will be described below [116]. LTB_4 binds to the GPCR BLT_1 and BLT_2. BLT_1 is a high-affinity receptor specific for LTB_4, whereas BLT_2 is a low-affinity receptor that also binds other eicosanoids [119]. LTB_4 causes adhesion and chemotaxis of leukocytes and stimulates aggregation, enzyme release and generation of superoxide in neutrophils [120]. The BLTRs play a role in neutrophil recruitment, as demonstrated in the $BLTR^{-/-}$ mouse model [121]. Enhanced levels of LTB_4 occur in a number of diseases such as cystic fibrosis, glomerulonephritis, psoriasis, rheumatoid arthritis and IBD [122].

LTC_4 synthase

LTC_4S is an 18 kDa enzyme that resides in a trimer formation in the outer nuclear membrane [123]. LTC_4S's function is to conjugate LTA_4 and glutathione to form LTC_4 [124]. The LTC_4S gene is located on chromosome 5q and has five exons. Binding sites for the Kruppel-like and Sp-1 transcription factors have been found in LTC_4S's promoter [125]. Studies on $LTC_4S^{-/-}$ mice have focused on its role in inflammation, and these mice have reduced vascular permeability and reduced passive cutaneous anaphylaxis in an *in vivo* inflammation model [126]. In a model of bleomycin-induced pulmonary fibrosis, $LTC_4S^{-/-}$ mice have less macrophage and neutrophil recruitment, fibroblast accumulation and collagen deposition [118]. Clearly, LTC_4S seems to play a role in inflammation.

The cysteinyl leukotrienes and their receptors

After export out of the cell via an energy-dependent step that requires multidrug resistance-associated proteins (MRP) 1 and 4, LTC_4 is metabolized by cleavage removal of glutamic acid and then glycine to provide LTD_4 and LTE_4, respectively [116]. The CysLTs are ligands of the GPCRs $CysLT_1R$, $CysLT_2R$ and GPR17 and have been implicated in asthma, allergic rhinitis, atopic dermatitis, stroke, cardiovascular diseases, rheumatoid arthritis, inflammatory bowel diseases and cancer (see above) [127,128]. CysLTs have many modes of action, including bronchoconstriction, to increase vascular permeability in postcapillary

venules, to induce proliferation and to stimulate mucus secretion in the bronchi [120].

CysLT$_1$R

$CysLT_1R$ was molecularly and functionally characterized by two independent groups in 1999 [129,130]. It responds to cysteinyl leukotrienes with a calcium mobilization response, and the binding affinities are LTD_4 >> LTE_4 = LTC_4 >> LTB_4. Several $CysLT_1R$ antagonists exist, e.g., montelukast (Singulair), zafirlukast (Accolate) and pranlukast (Onon), and these are used as therapy for asthma [127]. The $CysLT_1R$ gene is localized on the X chromosome [129,130]. The human and mouse $CysLT_1R$ share 87% homology [131]. Only one isoform exists in humans whereas the mouse gene has two splice variants, giving rise to one short and one long isoform [118]. $CysLT_1R$ is expressed in a large number of cells, including leukocytes, smooth muscle cells of the bronchi and heart, endothelial cells and epithelial cells of the small intestine, colon and lung. In addition,$CysLT_1R$ is expressed in organs such as the prostate, ovary, brain and thymus and in skeletal muscle [132]. $CysLT_1R$ can be induced by IL-4 (through STAT6 binding to its promoter) and IL-13 in macrophages, by IL-5 in eosinophils, by IL-13, IFN-γ and TGF-β in airway smooth muscle, and by IL-1β in human umbilical vein endothelial cells (HUVEC), respectively [132-134].

$CysLT_1R^{-/-}$ mice show reduced vascular permeability, but neutrophil recruitment was unaffected in a zymosan-induced peritonitis model [118]. Somewhat surprising, in a model of bleomycin-induced pulmonary fibrosis, $CysLT_1R^{-/-}$ mice showed features of exaggerated fibrosis and secreted more CysLTs in the bronchoalveolar lavage fluid than wild-type mice [118]. The functions of $CysLT_1R$ seem in part to depend on the cell type, but proliferation is a recurrent feature of LTD_4 signaling through $CysLT_1R$ observed in different contexts. As an example, LTD_4 induces astrocyte proliferation and mediates brain edema through the $CysLT_1R$ [135,136]. In addition, $CysLT_1R$ signaling results in ERK-induced proliferation of mast cells [137]. LTD_4 induces proliferation, survival and migration of colorectal cancer cells by signaling via $CysLT_1R$, and the mechanisms behind this will be described in detail below [66,67,116,138].

CysLT$_2$R

The CysLT$_2$R gene is mapped to chromosome 13q14, a region linked to atopic asthma [139]. CysLT$_2$R belongs to the same family of GPCRs as CysLT$_1$R, although they have only 38% homology in humans. The human and mouse CysLT$_2$R are 74% identical [140]. Two splice variants exist in mice whereas humans have only a single isoform [118]. Similar to CysLT$_1$R, the low affinity receptor CysLT$_2$R responds to cysteinyl leukotrienes with a calcium mobilization response, and the binding affinities are LTC_4=LTD_4>>LTE_4 [139]. Until recently, no specific CysLT$_2$R antagonist was available. For this reason, many studies have used the dual CysLT$_1$R and CysLT$_2$R antagonist Bay-u9773 [141]. The expression pattern of CysLT$_2$R in cells and organs is in principle identical to that of CysLT$_1$R [132]. IFN-γ can up-regulate CysLT$_2$R in eosinophils [142] and in endothelial cells [143]. CysLT$_2$R expression can also be augmented by IL-4 in mast cells and by TNF-α in HUVEC [132].

The CysLT$_2$R gene has a TATA-less promoter with several transcription start sites. It has six variably spliced exons that were found variably spliced in eight alternative transcripts identified in endothelial cells, monocytes, and monocytic cell lines. This gene contains several binding sites for transcription factors such as Sp-1 and GATA. The gene also has putative interferon-regulatory sites. Although IFN-γ stimulation induced CysLT$_2$R mRNA, no reporter gene activity could be detected [143].

Similar to CysLT$_1$R, signaling through CysLT$_2$R also results in calcium release. However, the functional role of CysLT$_2$R seems to depend on the cell type in which CysLT$_2$R is expressed. CysLT$_2$R mediates the death of astrocytes after ischemic damage *in vitro*, whereas in endothelial cells, which hardly express any CysLT$_1$R at all, CysLT$_2$R is thought mainly to act pro-inflammatory during vascular injury [135,144,145]. That CysLT$_2$R mediates a pro-inflammatory phenotype has also been observed in CysLT$_2$R$^{-/-}$ mice exposed to bleomycin-induced pulmonary fibrosis and in a passive cutaneous anaphylaxis model [146]. CysLT$_2$R seems to have a function that is distinguished from that of CysLT$_1$R. In mast cells, CysLT$_2$R promotes a cytokine profile different from that induced by CysLT$_1$R [147]. Moreover, it was recently shown that CysLT$_2$R antagonizes CysLT$_1$R-driven proliferative signaling in mast cells [137].

Other cysteinyl leukotriene receptors

The CysLT receptors belong to the purine receptor cluster of the rhodopsin family [127]. The P2Y receptors are also members of this cluster. Ligands for the P2Y are extracellular purinergic or pyrimidinergic nucleotides and, through P2Y receptors, regulate a variety of functions, including development, differentiation, proliferation and immune responses. $CysLT_1R$ and P2Y receptors are co-expressed in a number of inflammatory cells. Functional crosstalk has been observed between the nucleotide and the CysLT systems in the monocytic cell line U937 [148]. Moreover, $CysLT_1R$ antagonists inhibit P2Y receptor signaling in the same cell line [149]. Likewise, crosstalk between P2Y1 and $CysLT_1R$/$CysLT_2R$ has been suggested in rat microglia cells [150]. It has also been reported that $CysLT_1R$ expressed in mast cells also responds with calcium flux to uridine phosphate (UDP) [151]. The human orphan receptors GPR17 and GPR23 are situated at the intermediate phylogenetic position between P2Y and CysLT receptors, and have 33-35% homology to $CysLT_2R$ [139]. Recently, it was discovered that GPR17 is specifically activated by both uracil nucleotides and CysLTs. Inhibition of GPR17 function reduced ischemic damage in a rat focal ischemia model, suggesting that GPR17 mediates brain damage caused by nucleotides and CysLTs [128]. Another recent study proposes the existence of a new receptor with preference for LTE_4 that mediates vascular leakage in mice. The authors have named the receptor $CysLT_ER$ [152].

The role of eicosanoids in cancer

Epidemiologic studies show that aspirin and other NSAIDs have an established protective effect against colon cancer [25]. Inhibition of COX-2 is likely responsible for the observed effect of NSAIDs. As mentioned above, COX-2 is an enzyme responsible for the generation of prostaglandins. Of particular importance is PGE_2, which has tumor-promoting properties, in that it favors epithelial proliferation and angiogenesis and inhibits apoptosis [25]. COX-2 mRNA and protein, and PGE_2 are elevated in colorectal cancers [153]. Up to 90% of colorectal carcinomas overexpress COX-2 [25].

Patients with ulcerative colitis who take 5-aminosalicylic acid (5-ASA) reduce their cancer risk by as much as 75% [50]. 5-ASA inhibits the 5-LO enzyme [154]. In accordance with this observation, 5-LO and its products leukotrienes have been found to play a role in tumor-associated

events. 5-LO metabolites promote proliferation, and 5-LO antagonists inhibit lung, breast, colon and prostate cancer development [155,156]. 5-LO and 12-LO are highly expressed in human bladder cancer [157]. Previous members of our group showed that 5-LO expression is elevated in colorectal cancer and correlates with poor prognosis [158].

LTB_4 is endogenously produced by colon epithelial cells and promotes proliferation of colon and pancreatic cancer cells, and its receptors BLT_1 and BLT_2 are overexpressed in pancreatic cancer [159-162]. Our group has shown that elevated expression of $CysLT_1R$ in colorectal cancer tissue is associated with poor prognosis [158]. High $CysLT_1R$ expression has also been detected in high-grade prostate cancer, transitional cell carcinoma of the bladder, neuroblastoma, astrocytoma and classical Hodgkin's lymphoma [163-167].

The studies performed by our group will be summarized here. LTD_4 induces proliferation via activation of $CysLT_1R$. The pathway includes activation of 90-kDa ribosomal S6 kinase (p90RSK) as well as cytosolic phospholipase A_2 α ($cPLA_2$-α). The activation of p90RSK is a PKCε/Raf-1/ERK1/2-dependent process while $cPLA_2$-α activation can be blocked by inhibitors against PKC (protein kinase C), ERK1/2, p38 MAPK and NF-κB [168].

Moreover, LTD_4 increases cell viability and prevents apoptosis by up-regulating the anti-apoptotic protein Bcl-2 and decreasing caspase-3 activity in non-transformed intestinal epithelial cells [66]. The LTD_4-mediated survival in intestinal epithelial cells seems to rely on PKC-α and cAMP-responsive element-binding protein (CREB) [138]. In the same cells, LTD_4 activates the β-catenin–TCF/LEF pathway, and induces an association of β-catenin with Bcl-2 [169].

In addition to the above properties, LTD_4 has a capacity of promoting colorectal cancer cell migration. It was shown that LTD_4 via $CysLT_1R$ augmented adhesion to collagen I via the ECM binding integrin α2β1, in the colorectal adenoma cell line Caco-2 [170]. Furthermore, LTD_4 induces migration of non-transformed intestinal epithelial cells by a PI3-kinase (PI3K) and Rac-dependent mechanism [67].

Our group has shown that both non-transformed intestinal epithelial cells and colorectal cancer cells are capable of producing CysLTs. Moreover, treatment with $CysLT_1R$ antagonists significantly reduced proliferation, but had no effect on apoptosis in several colorectal cancer cell lines [171]. Based on this and previous findings, we conclude that it is

likely that constitutive $CysLT_1R$ signaling mediates both survival and proliferation in colorectal cancer cells.

Eicosanoids with anti-inflammatory / anti-tumorigenic functions

Lipoxins, generated by the action of 5-LO, were discovered in 1984 as arachidonic acid-derived anti-inflammatory mediators. They take part in the resolution phase of inflammation by controlling neutrophil entry to sites of inflammation and reducing vascular permeability. The switch from pro- to anti-inflammatory eicosanoids is an active process that is vital for the inflammation to terminate [172,173]. Lipoxin A_4 (LXA_4) has been shown to decrease pro-inflammatory cytokine production and disease symptoms in mouse models of inflammatory bowel disease, asthma and cystic fibrosis, to name a few [174].

Some studies suggest that 15-lipoxygenase (15-LO) might have anti-tumorigenic effects, particularly by antagonizing other LO products. 15-LO has been shown to counteract the pro-tumorigenic effects of 5-LO by inhibiting cellular responses to LTB_4 [175]. 15-LO converts linoleic acid and arachidonic acid to form 15-S-HETE. 15-S-HETE inhibits proliferation of prostate cancer cells, and induces apoptosis of colorectal cancer cells [176]. Prostate carcinomas often display reduced expression of 15-LO-2 [175,176]. In addition, colorectal tumor specimens from FAP patients exhibit decreased levels of the 15-LO-1 enzyme in adenoma tissue compared to non-neoplastic tissue [176].

Decreased expression of $CysLT_2R$ is a feature of many colorectal tumors, and this is associated with poor prognosis. Signaling via $CysLT_2R$ in colon cancer cells has recently been found to lead to cellular differentiation [177]. Interestingly, LTC_4 was unable to mimic the LTD_4-induced effect on cell adhesion and survival of colorectal cancer cells and non-transformed intestinal epithelial cells, respectively [66,170]. To summarize the findings in colon cancer and the effects on the cell types described under the *$CysLT_2R$* section, it seems that $CysLT_2R$ may antagonize the proliferative pathways of $CysLT_1R$ regardless of the cell type where $CysLT_2R$ is expressed.

Antitumor agents and tumor suppression

Interferons

Interferons (IFNs) were discovered 50 years ago [178]. These are widely expressed cytokines that take part in the first line of defense against viral infections and have important roles in the immune surveillance of malignant cells [179]. The human type I interferons are IFN-α, IFN-β, IFN-ε, IFN-κ and IFN-ω, and they bind to the type I interferon receptor (IFNAR), which is composed of IFNAR1 and IFNAR2 [180]. IFN-γ is the only type II interferon, and binds the type II receptor IFNGR [179]. Less is known about the type III interferons, which are IFN-λ1-3, also known as IL-29, IL-28A and IL-28B, respectively. They also have antiviral properties, but bind a different receptor, which is a dimer of IL-28Rα and IL-10Rβ [181].

Type I interferons

Type I interferons are a part of the innate immune defense and are rapidly secreted in response to viruses and TLR signals. In addition to the antiviral role, type I interferons display anti-tumorigenic properties [182]. IFN-α acts anti-proliferatively and is capable of inducing apoptosis [183]. Recombinant IFN-α is used in therapy for metastatic myeloma, Kaposi's sarcoma, cervical cancer, renal cell carcinoma, head and neck tumors and melanoma [178,183]. Signaling through the IFNAR results in receptor dimerization and phosphorylation of receptor-associated Janus kinases (JAKs). Sequentially, signal transducers and activators of transcription protein (STATs) become activated and translocate to the nucleus [180]. As indicated above, the biological effects of type I interferons are many. For example, IFN-α augments the cytotoxic response of NK cells against tumor cells. In the colon cancer cell lines SW480 and HT-29, IFN-α has an anti-proliferative effect by inducing p21 activation [184]. Of interest for our studies is that IFN-α treatment can give rise to fever, which can be ameliorated by COX-2 inhibition. The likely cause behind this is that IFN-α is able to mediate activation of phospholipase A_2 *in vitro* and as a consequence arachidonic acid release [178]. Combined 5-FU and IFN-α treatment of CRC patients has shown promising effects in some, but not other, clinical trials [185-187].

Interferon regulatory factors (IRFs)

Interferon regulatory factors (IRFs) are a family of nine transcription factors, IRF-1-9, which can interact with STATs or with each other [188]. The complex that IRFs form bind to interferon-stimulated response element (ISRE) where it initiates gene transcription [189]. Viral infection induces the activation of IRF-3, which results in rapid formation of IFN-α and IFN-β. The induced IFN cause a transcription of the IRF-7 gene. Sustained infection activates IRF-7 to translocate to the nucleus, to mediate a second induction of IFN genes and the cytokine RANTES (regulated on activation, normal T expressed and secreted). The phenomenon of an early and a late phase of IFN induction may be a way to amplify the response to viral infection. Transcription of IRF-7 can also be induced by phorbol esters, LPS and sodium butyrate, but not by IFN-γ [188].

Retinoids

In our diet, liver, carrot, spinach and broccoli leaves are major sources of vitamin A (retinol). Vitamin A and its metabolites constitute the family of retinoids. All-*trans* retinoic acid (ATRA) is one of the most potent derivatives of retinol. By binding to the retinoic acid receptor (RAR), ATRA influences the process of embryonal development, vision and inflammation (see review [190]). In addition, ATRA acts as a tumor suppressor and a differentiating agent, and can promote apoptosis and growth inhibition of different cell types. Individuals with a low dietary intake of vitamin A are at higher risk of developing cancer [190].

ATRA (tretinoin) is successfully used as a treatment of acute promyelocytic leukemia (APL). APL patients lack mature myelocytes in the peripheral blood due to a chromosomal translocation t(15;17) resulting in the PML-RAR-α fusion protein, which induces a blockage of myeloid differentiation. This inhibition of maturation of myelocytes is efficiently restored by ATRA [191].

Retinoic acid has been shown to be promising in the treatment of other diseases as well. A combination therapy of IFNα- and 13-*cis*-RA in a clinical trial of metastatic renal carcinoma showed longer progression-free and overall survival of patients compared to those who received IFN-α alone [192]. A combination of human IFN-β and ATRA inhibited growth *in vitro* of human breast carcinoma, ovarian carcinoma and malignant

melanoma cell lines to a greater extent compared to IFN-β or ATRA alone. The same study showed that established ovarian carcinomas in nude mice underwent regression when treated with the combination of IFN-β and ATRA but not with single-agent therapy [193]. Several synthetic analogues of retinoic acid are currently being tested in clinical trials of glioblastoma, neuroblastoma, non-Hodgkin's lymphoma, ovarian and prostate cancers [192].

RAR-α and RAR-β mediate ATRA-induced growth inhibition of colon carcinoma cells [194,195]. RAR-β is decreased or down-regulated in a number of human tumors, including colon, lung, esophageal, breast and head and neck cancers [196-199]. Retinoids suppress tumorigenesis in a many animal models, including those of the skin, breast, oral cavity, lung, prostate, bladder, liver, bladder and pancreas [199]. The growth inhibitory effects of ATRA may be due to its repression of β-catenin-TCF/LEF signaling, induction of E-cadherin expression and reduction of AP-1 and Cyclin D1, shown both in colon cancer and in breast cancer cell lines [200-202]. ATRA induces apoptosis of keratinocytes by up-regulating p53 and caspase-3 [203]. Moreover, ATRA is capable of inhibiting breast cancer cell invasion, proposedly through the inhibition of MMPs [204]. Similarly, ATRA inhibits the migratory capacity of rat invasive prostate adenocarcinoma cells and of squamous cell carcinoma cell lines by inhibition of MMP-2 and MMP-9 activity [205,206].

Colorectal cancer cells can be made to differentiate upon ATRA treatment. Upon stimulation with ATRA, colon cancer cells increased their alkaline phosphatase activity [207]. Colorectal cancer cells with a mutation in APC have reduced levels of the enzymes necessary for retinol metabolism into ATRA. The same study reported that APC and CDX2 are responsible for inducing a retinoid-mediated program of colonocyte differentiation [208]. Moreover, ATRA is able to induce RAR-β and the retinol transport protein cellular retinol binding protein II (CRBPII) in colon cancer cells, suggesting that ATRA treatment can restore its own synthesis in cancer cells [209,210].

ATRA has been shown to act on the eicosanoid pathway as well. ATRA stimulated LTC_4S promoter activity, increased LTC_4S mRNA and protein and LTC_4 production, resulting in differentiation of rat basophilic leukemia myeloid cells [211-213]. In addition, ATRA causes inactivation of the pro-tumorigenic LTB_4 by inducing its breakdown [214]. Interestingly, treatment of human carcinoma cell lines with ATRA reduces COX-2 expression [215]. Similarly, induction of RAR-β suppresses COX-2

in esophageal cancer cells [216]. As mentioned above, mutations in APC and overexpression of COX-2 occur frequently in colorectal cancers. One recent study explained the COX-2 overexpression in APC mutant cells depends on the lack of ATRA biosynthesis [215]. In summary, ATRA may have the capability of shifting the eicosanoid balance from pro-tumorigenic to anti-tumorigenic properties.

ATRA-resistance is unfortunately a feature of some solid tumors [217]. Strategies to overcome resistance include combination therapy and the use of non-classical retinoids. Another option is to identify target genes that mediate ATRA's beneficial effects.

It is not clear what mechanisms lie behind ATRA resistance, but it has been suggested that it may occur through increased P450 catabolism, decreased production of ATRA-converting enzymes, decreased RAR expression through promoter methylation, mutations in RAR, or by alteration in coactivator or corepressor complexes, by persistent histone deacetylation [217]. Retinol can, independently of RAR, to some extent compensate for ATRA in that retinol inhibits growth and migration, but retinol cannot induce differentiation or apoptosis in ATRA-resistant colon cancer cells [218,219]. There are also reports on the existence of non-classical ATRA pathways that are independent of the RAR/retinoid X receptor (RXR). In this way, ATRA can activate CREB bronchial epithelial cells, induce extracellular signal-regulated kinase 1 or 2 (ERK1/2) and AP-1 in Sertoli cells and activate another nuclear receptor, PPARβ/δ, which instead results in pro-survival gene activation [220-222].

Retinoic acid receptors (RARs)

ATRA and its synthetic analogs bind two families of nuclear receptors: RAR-α, -β, -γ and RXR-α, -β, -γ, which heterodimerize and bind to DNA on retinoic acid-responsive elements (RARE) in promoters of genes. There are several splice variants of RAR: two α, four β and two γ. RXRs can, in addition to RAR, heterodimerize with PPARs (peroxisome proliferator-activated receptors) and other members of the nuclear hormone receptor class II family. Novel retinoic acid receptors are the RAR-related orphan receptors (RORs) [223].

RARs and RXRs activate or repress transcription. In the absence of ligands, RARs are in complex with corepressors such as nuclear receptor corepressor (N-CoR), preventing gene transcription (see below). The RAR/RXR dimers bind constitutively to retinoic acid response

elements (RARE) in promoters of genes; these are characterized by two consensus half sites AGGTCA generally arranged as direct repeats (DRs), but can also occur in the reverse orientation, and are most often separated by 2 to 5 nucleotides [217,224]. Receptor selectivity depends on the arrangement of, and the spacing between, the direct repeats.

Transcription factors and cancer

Transcription factors (TFs) are proteins that bind to specific parts of DNA using DNA binding domains. TFs control the initiation of transcription of a specific target gene. They are often in a complex with an activator, activating, or a repressor, preventing, the presence of RNA polymerase, which induces the transcription of genes.

TFs have at least three domains. First, a DNA binding motif recognizes a DNA sequence, often referred to as a response element. Second, a trans-activating domain is pivotal for the transcription or repression to occur; this part enables interaction with the TATA-binding protein (TBP) and accordingly the RNA polymerase complex [225]. Third, a protein interaction domain is often present, allowing modulation by TBP-associated factors such as histone acetyltransferases (HAT) or by other TFs [226]. The majority of the known transcription factors recognize short DNA sequences (5-15 bp) called response elements [227]. Binding of one transcription factor to a response element is rarely sufficient to induce transcription. The combination and orientation of transcription factors are crucial. Transcription factors often bind to genes in homo- or heterodimer formation, and the corresponding response elements can be direct repeats or palindromic sequences.

Many TFs reside in the cytoplasm and need a ligand to go to the nucleus. Some of these, such as NF-κB and β-catenin, carry a nuclear localization sequence (NLS), and are retained in the cytoplasm by an inhibitory complex. They are released upon upstream signal activation. Other TFs, such as AP-1 and STAT, require a ligand-induced phosphorylation for to be able to bind DNA or cofactors.

Most promoters of genes contain at least three features: the transcription start site, the TATA box and the sequences bound by transcriptional regulators. The transcriptional regulators include activators, enhancers, repressors and silencers [228]. While activators and repressors

bind at close proximity to the transcription start site, enhancers and silencers can be situated as far away as 85 kb.

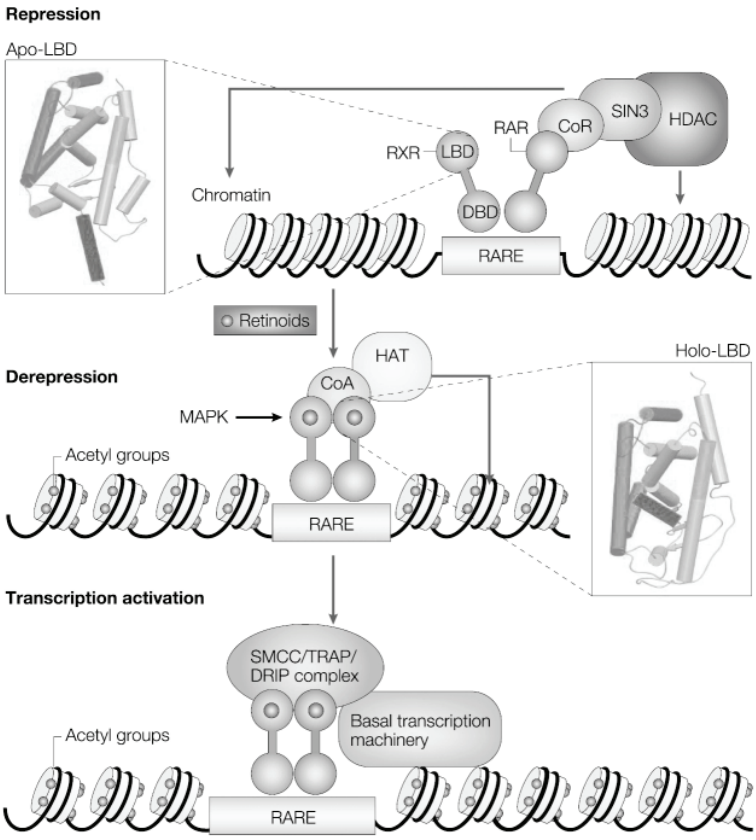


Figure 7. Mechanisms of transcriptional repression and activation by RAR–RXR heterodimers. DBD, DNA-binding domain; apo-LBD, ligand-binding domain; CoRs, corepressors; HDACs, histone deacetylases; HATs, histone acetyltransferases; TRAP, thyroid-hormone-receptor associated protein; DRIP, vitamin D receptor-interacting protein; SMCC, Srb and mediator protein-containing complex; RAR, retinoic-acid receptor; RARE, retinoic-acid response element; RXR, rexinoid receptor. Adapted from Altucci, 2001 [192].

Transcriptional activation

As described above, TFs such as IRF and RAR induce activation of genes by binding to consensus sequences in the genome, most often in the promoters of genes. However, coactivators are also needed for successful gene transcription to occur. They do not have a DNA binding domain but instead bind to the TF. The same coactivator can act on many different genes, since it is the TF that provides the specificity. Enhancers are coactivating binding sites for TFs that can be at great distance from the start site and regulate the cell-type specific expression of genes [229,230]. Gene activation can also be regulated on an epigenetic level, by histone acetyltransferases (HAT). HAT, as exemplified by the p300/CBP protein, destabilize nucleosomes so that TFs and the RNA polymerase complex can bind the DNA [231].

Transcriptional repression

Transcriptional repression can be mediated by corepressors [228]. Similar to coactivators, corepressors bind transcription factors, unable to bind DNA by themselves. Their role is to down-regulate gene expression. The first corepressors identified were the nuclear receptor corepressor (N-CoR) and the silencing mediator of retinoid and thyroid receptors (SMRT). Both the N-CoR and SMRT were discovered as corepressors for retinoid and thyroid-hormone receptors [232,233]. Upon ligand binding, the corepressor complex is removed, and replaced by a coactivator complex, allowing gene transcription to occur (see Figure 7) [192]. On certain elements, the N-CoR remains associated with RAR/RXR heterodimers even in the presence of RAR ligands, resulting in constitutive repression [234]. An example of such repression is RAR-mediated promoter inhibition on the EGFR gene, by binding to a 36 bp fragment 5' of the gene [235]. Silencers are another tool to repress gene transcription. Similar to enhancers, silencers may act from a vast distance in an orientation-independent manner [228]. Histone deacetylases (HDACs) block access to genes by causing the DNA to wrap more tightly around the histones. In turn, HDACs themselves can be inhibited, e.g., by the colonocyte differentiating agent butyrate [15]. Yet another means of inhibiting gene transcription is by epigenetic silencing. This is achieved through promoter hypermethylation at CpG sites in the genome, or by micro-RNAs binding to mRNA to inhibit translation, both of which are known to often be involved in cancer.

PRESENT INVESTIGATION

Aim

The main objective of this thesis has been to explore the role of pro-inflammatory cysteinyl leukotrienes and their receptors in colorectal cancer. Whereas $CysLT_1R$ is up-regulated in colorectal cancer and promotes mitogenic pathways, $CysLT_2R$ seems to play a more protective role in human colorectal cancer. The specific aims have been the following:

To examine the role of pro-tumorigenic transcription factors in leukotriene D_4-mediated proliferation in intestinal epithelial cells

To determine the expression of enzymes and receptors of the eicosanoid pathway in intestinal epithelial and colon cancer cells

To investigate the function and regulation of $CysLT_2R$ in intestinal epithelial and colon cancer cells

Paper I

Background

The transcription factor NF-κB is a powerful mediator of inflammation-driven pathways, and has been linked to tumorigenesis. Leukotrienes are potent pro-inflammatory mediators involved in many inflammatory processes, and LTD_4 promotes proliferation, survival and migration of intestinal epithelial and colorectal cancer cells via $CysLT_1R$. LTD_4 has previously been shown to be able to activate NF-κB in HEK293 cells and in lung mononuclear cells of BALB/c mice [236,237]. Since we observed that LTD_4-induced $cPLA_2\alpha$ activation could be repressed by an NF-κB inhibitor, we hypothesized that NF-κB would be a pathway that is activated by LTD_4 in intestinal epithelial cells. Similar to NF-κB, AP-1 is a transcription factor that is linked to tumorigenesis. We therefore sought to investigate whether LTD_4 in addition could activate the AP-1 pathway and if this is one of the means by which LTD_4 induces proliferation. We used the intestinal epithelial cell line Int 407.

Results and discussion

We analyzed NF-κB activation by the following means. By using Western blot, we evaluated the protein expression of IκB-α, an inhibitory protein in the classical NF-κB cascade. Upon activation, IκB-α is phosphorylated, ubiquitinated and targeted for the proteasome for degradation, to allow the release the p50 and p65 subunits of NF-κB. These are then free to enter the nucleus, where they bind as a transcription factor heterodimer to target genes. We used TNF-α as a positive control for activation of the classical pathway. Upon TNF-α treatment, IκB-α was completely degraded after 30 minutes, whereas no degradation occurred after LTD_4 treatment irrespective of the time-point studied (5 minutes up to 2 hours). Further, we studied activation of NF-κB by means of a luciferase reporter construct containing an NF-κB consensus sequence. Upon TNF-α treatment, NF-κB activity was induced, resulting in a luciferase luminescence signal, but no signal was induced upon LTD_4 treatment. We also analyzed microscopically the translocation of the NF-κB subunit p65 by immunofluorescent labeling. TNF-α, but not LTD_4, induced a translocation of p65 from the cytosol to the nucleus of the cells. To exclude that NF-κB activation did not occur

through an alternative pathway, which can be induced by bacterial lipopolysaccharide triggering of TLR and MyD88, we analyzed translocation of the alternative NF-κB subunit p52, by means of immunofluorescent labeling and by Western blot of nuclear and cytosolic fractions. LPS, but not LTD_4, was able to induce p52 translocation. We then investigated activation of the AP-1 pathway. Five minutes of LTD_4 treatment induced phosphorylation of the MAPK JNK, which is a known activator of AP-1. Moreover, LTD_4 induced activation of AP-1, as examined by using a luciferase reporter construct containing a consensus AP-1 sequence. Furthermore, we were able to repeat what had been observed before, that LTD_4 was able to induce proliferation. This was determined by the Alamar blue method. The LTD_4-induced proliferation could be reduced by adding a JNK inhibitor prior to LTD_4 stimulation. Taken together, these results indicate that the JNK-AP-1 pathway mediates LTD_4-induced proliferation of intestinal epithelial cells, but that the NF-κB pathway is not induced by LTD_4 in these cells. The result from this paper sheds light onto how LTD_4 exerts its proliferative effects in intestinal epithelial cells.

Paper II

Background

In this study, we focused on how the expression of $CysLT_2R$ can be regulated in colorectal cancer cells. In contrast to $CysLT_1R$, which is implicated in the progression of colon cancer, high $CysLT_2R$ expression in colorectal tumors is associated with a better survival prognosis. Upon stimulation with LTC_4, $CysLT_2R$ was found to induce differentiation of colon cancer cells. When performing a bioinformatic search of the $CysLT_2R$ promoter region, we found a response element for IRF-7, an IFN-α-induced transcription factor. We also found E-box elements in the promoter region. These findings led us to investigate the role of IFN-α and EGF in the regulation of $CysLT_2R$ expression in colorectal cancer cells. We also investigated whether $CysLT_2R$ can regulate cell migration of colorectal cancer cells. As mentioned above, previous results indicate that $CysLT_2R$ has an antagonizing function to that of $CysLT_1R$, which induces cell migration [67,137,177].

Results and discussion

We described four major findings as follows: 1) IFN-α up-regulates promoter activation, mRNA and protein expression of $CysLT_2R$ in the intestinal epithelial cell line Int 407, but not in the colorectal cancer cell line Caco-2, which expresses low basal levels of $CysLT_2R$. We observed IFN-α-induced differentiation of colon cancer cells, but it cannot be attributed to $CysLT_2R$. IFN-α-induced $CysLT_2R$ promoter activation could be reduced by deleting the known IRF-7 response element. However, additional elements are also required for IFN-α-induced $CysLT_2R$ promoter activation. 2) EGF induces phosphorylation of Snail, which represses genes by binding to E-boxes. Moreover, EGF represses $CysLT_2R$ promoter activity, mRNA and protein expression in both Int 407 and in SW480 colorectal cancer cells. The repression is to some extent E-box dependent, although other factors are also needed. 3) LTC_4 inhibits colorectal cancer cell migration, including both basal migratory capacity and EGF-induced migration. 4) We found that the expression of EGFR, which frequently is overexpressed in carcinomas and drives tumorigenic processes, is negatively correlated to $CysLT_2R$ expression in a tissue array from colorectal cancer patients ($p<0.001$). There was no statistically significant correlation between IFN-α/β-R1 and $CysLT_2R$ expression in the colorectal tumor material. We can speculate that $CysLT_2R$, in addition to, e.g., p21 and TRAIL up-regulation, is one of the mechanisms by which IFN-α exerts its anti-tumor effects [178,238].

We showed for the first time that LTC_4 has the capability to inhibit migration of colorectal cancer cells. Moreover, the mitogen EGF represses $CysLT_2R$, and there is an inverse correlation between its receptor EGFR and $CysLT_2R$. The reduced expression of $CysLT_2R$ in tumors that overexpress EGFR could perhaps be a result of aberrant EGF signaling, resulting in repression of the $CysLT_2R$. In summary, these findings draw attention to ways to sustain $CysLT_2R$ function in tumors.

Paper III

Background

All-*trans* retinoic acid (ATRA) is a mediator of differentiation, apoptosis and growth inhibition in many cell types and cancers. ATRA is an effective treatment of acute promyelocytic leukemia. We found a retinoic acid

response element (RARE) in the $CysLT_2R$ promoter. As mentioned above, $CysLT_2R$ promotes differentiation of colorectal cancer cells.

Results and discussion

Stimulation with ATRA up-regulates $CysLT_2R$ mRNA and protein within 3-24 hours in the two colorectal cancer cell lines Caco-2 and SW480. The effect persisted, and continued to increase during long-term treatment even after treatment with ATRA. ATRA induced MUC2 mRNA expression and alkaline phosphatase activity, and this could be reduced by pre-treatment with a $CysLT_2R$ inhibitor. This indicates that ATRA-induced differentiation is to a certain extent mediated through $CysLT_2R$. ATRA did not induce apoptosis or growth inhibition, suggesting that the main function of ATRA in these cells is to induce differentiation. The ATRA-induced up-regulation of $CysLT_2R$ could not be confirmed in the ATRA-resistant colorectal cancer cell line HCT-116, which has a dysfunctional RAR. This finding, together with a preliminary indication that ATRA induces $CysLT_2R$ promoter activity, suggests that ATRA-induced $CysLT_2R$ expression is mediated through RAR binding to the RARE in its promoter. In addition to $CysLT_2R$, ATRA up-regulates mRNA expression of LTC_4S, the enzyme responsible for generation of the $CysLT_2R$ ligand LTC_4. On the contrary, ATRA did not up-regulate $CysLT_1R$ mRNA or protein expression. ATRA thus provides both a ligand and a receptor for an eicosanoid pathway that can induce differentiation of colorectal cancer cells. ATRA can also modulate other mediators of the eicosanoid pathway. ATRA up-regulates the anti-tumorigenic 15-LO enzyme, and down-regulates the pro-tumorigenic enzymes 5-LO and COX-2 [239,240].

In conclusion, we found that ATRA up-regulates expression of LTC_4S and $CysLT_2R$, and that the $CysLT_2R$ pathway is partially responsible for ATRA-induced differentiation of colorectal cancer cells. ATRA is able to shift the balance from pro- to anti-tumorigenic eicosanoids, and this knowledge can be used therapeutically.

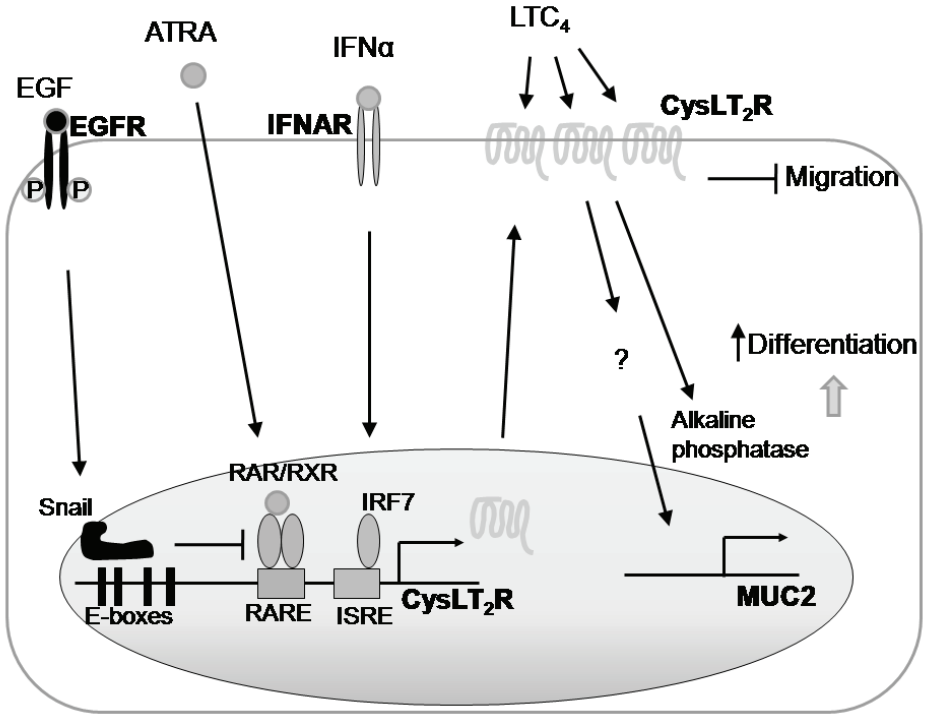


Figure 8. A hypothetical model based on the findings in paper II and III.

Paper IV

Background

The aim of this study was to determine whether intestinal epithelial cells have the capability of expressing enzymes and receptors of the eicosanoid pathways. We exposed the cells to TNF-α and LTD_4, inflammatory mediators present during IBD. We analyzed mRNA and protein expression of the eicosanoid biosynthesis enzymes 5-LO, LTC_4S and COX-2 and cysteinyl leukotriene receptors $CysLT_1R$ and $CysLT_2R$ by real-time quantitative PCR (polymerase chain reaction) and Western blot. In addition, we analyzed cysteinyl leukotriene production.

Results and discussion

In this study, we showed that intestinal epithelial cells, as well as colorectal cancer cells, express the enzymes and receptors necessary for endogenous

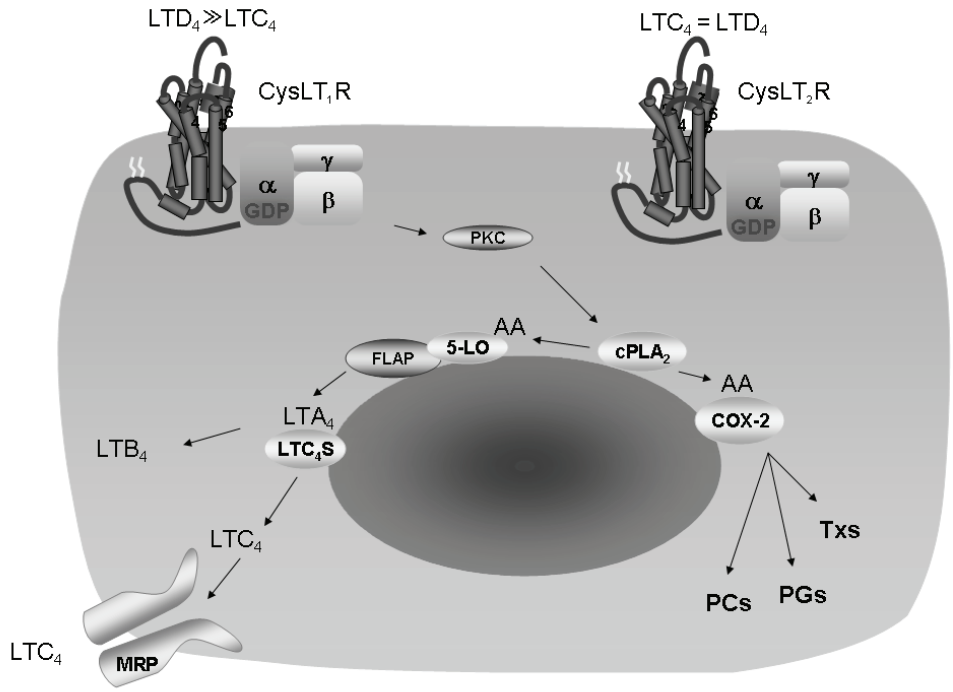


Figure 9. Schematic illustration of cysteinyl leukotriene receptors and eicosanoid biosynthesis in an intestinal epithelial cell. $cPLA_2$, cytosolic phospholipase A_2; PKC, phospholipase C; AA, arachidonic acid; COX-2, cyclooxygenase-2; 5-LO, 5-lipoxygenase; FLAP, 5-lipoxygenase activating protein; LTC_4S, LTC_4 synthase; MRP, multidrug resistance-associated protein.

eicosanoid production and signaling, along with CysLT production. We thereby confirmed earlier findings of an autocrine loop of CysLT production [171]. LTD_4 up-regulates all of the studied enzymes and receptors in intestinal epithelial and colon cancer cells. TNF-α induces expression of 5-LO, LTC_4S, $CysLT_1R$ and COX-2 in both non-transformed intestinal epithelial cells and in colorectal cancer cells. TNF-α was unable to induce $CysLT_2R$ mRNA and protein in intestinal epithelial cells, and down-regulated $CysLT_2R$ mRNA in colorectal cancer cells.

Taken together, inflammation, once induced by pro-inflammatory and mitogenic mediators such as TNF-α and LTD_4, can lead

to autocrine production of eicosanoids in intestinal epithelial cells. With the knowledge that COX-2, 5-LO and their products and $CysLT_1R$ are powerful mediators of tumor progression [241,242], once triggered by pro-inflammatory mediators, the endogenous production of eicosanoids by epithelial cells could be a mechanism of inflammation-induced cancer.

Concluding remarks and future perspectives

The four studies in this thesis have paved way for an understanding of the regulation and function of cysteinyl leukotriene receptors in colorectal cancer. We have observed that $CysLT_1R$ and $CysLT_2R$ play different roles in colorectal cancer. Whereas $CysLT_1R$ promotes proliferation, here shown via the JNK/AP-1 pathway, $CysLT_2R$ mediates differentiation and inhibits migration. The expression of $CysLT_1R$ is enhanced by TNF-α and LTD_4, which both mediate proliferation of cancer cells. The expression of $CysLT_2R$ is suppressed by the mitogenic stimuli EGF, and enhanced by ATRA and IFN-α, which are anti-tumorigenic agents. These studies provide more evidence that $CysLT_1R$ and $CysLT_2R$ have opposite functions in colon cancer progression, and we now know how to regulate their expression.

As our clinical data in paper II showed a negative correlation between $CysLT_2R$ and EGFR, it would be interesting to investigate whether this correlation has functional consequences. For example, we could analyze $CysLT_2R$ levels in tumor samples from patients who undergo treatment with an EGFR antagonist such as cetuximab. In that way, we would find an answer to if EGF represses $CysLT_2R$ in patients, as indicated by our *in vitro* data.

Furthermore, it would be of interest to study the role of $CysLT_2R$ in tumor progression, differentiation and migration *in vivo*. This could be achieved by establishing a model of colitis-induced colorectal cancer in $CysLT_2R^{-/-}$ mice (e.g., by dextran sodium sulphate and azoxymethane). We would analyze if $CysLT_2R^{-/-}$ mice were more prone to developing tumors, and whether these mice displayed more metastases compared to wild-type mice. The knock-out mouse exists, but has not previously been used in cancer research.

$CysLT_1R$ antagonists are successfully used in asthma treatment, and show few side effects. It would also be of importance to investigate whether $CysLT_1R$ antagonists can reduce tumor development in the model described above. Considering the successful combination of

retinoids and IFN-α in cancer therapy, it would be worthwhile in the future to evaluate a combinatorial approach with $CysLT_1R$ antagonists as treatment of colorectal cancer.

In summary, we have shown the following:

- The JNK/AP-1 pathway mediates LTD_4-induced proliferation in non-transformed intestinal epithelial cells, whereas the NF-κB pathway is not involved
- IFNα and ATRA are able to up-regulate $CysLT_2R$ in colorectal cancer cells
- LTC_4 suppresses migration of colorectal cancer cells
- ATRA-induced differentiation of colorectal cancer cells is in part mediated through $CysLT_2R$
- EGF is able to down-regulate $CysLT_2R$ in colorectal cancer cells
- EGFR and $CysLT_2R$ expression is negatively correlated in a tissue array from colon cancer patients
- TNF-α and LTD_4 can up-regulate eicosanoid enzymes and receptors in intestinal epithelial and colorectal cancer cells

POPULÄRVETENSKAPLIG SAMMANFATTNING

Tjocktarmscancer är den tredje vanligaste cancerformen, och den näst vanligaste orsaken till cancer-relaterad död. Att kosten är viktig för hälsan är välkänt. Fettsyror av typen omega-3 skyddar mot hjärt-kärlsjukdom och cancer i tjocktarmen medan omega-6-fettsyror kan spjälkas till både metaboliter som skyddar mot cancer och sådana som kan påskynda cancer. Likaså finns det ett känt samband mellan intag av vitamin A och minskad risk för cancer.

Arakidonsyra är den viktigaste av omega-6-fettsyrorna, och kan spjälkas till bland annat s.k leukotriener. Leukotriener är molekyler med inflammatoriska egenskaper och bidrar till sjukdomsprocessen i bland annat astma. En av dessa, leukotrien D_4, har även visats stimulera cancerprocesser såsom ökad celldelning, ökad cellöverlevnad och metastasering. Leukotrien D_4 binder till en receptor på cellytan som kallas $CysLT_1$-receptorn. Patienter med tjocktarmscancer som har hög förekomst av denna receptor i tarmen har sämre chans till överlevnad. Forskning i vår grupp har tidigare visat att leukotrien C_4, som är förstadiemolekyl till leukotrien D_4, har motsatt effekt. Till skillnad från leukotrien D_4 ger den i cellodlingskultur en utmognad av cancerceller från tjocktarmen till att likna mer normala tarmceller. Leukotrien C_4 binder framförallt till $CysLT_2$-receptorn, vilken i motsats till $CysLT_1$-receptorn har goda effekter. Patienter med tjocktarmscancer som har låg förekomst av $CysLT_2$-receptorn har sämre chans till överlevnad. Låga nivåer av $CysLT_1$-receptorn men höga nivåer av $CysLT_2$-receptorn ger alltså bättre prognos vid tjocktarmscancer.

I min första studie har jag visat att leukotrien D_4 orsakar en ökad celldelning av tarmceller i cellodlingskultur, och att detta sker genom att ett protein inuti cellen kallat aktivatorprotein-1, känt för att vara aktivt i cancer, aktiveras. I nästa studie visade vi att tillsats av en tillväxtfaktor kallad EGF, som är känd för att kunna bidra till den ökade celldelningen i cancer, ger en minskning av antalet $CysLT_2$-receptorer i tarmceller. Vi har

kartlagt att EGF gör detta bland annat genom att aktivera s.k. E-boxar som sitter framför genen för $CysLT_2$-receptorn. Den troliga mekanismen för hur EGF minskar $CysLT_2$-receptorn är att E-boxarna hindrar aktivering av genen för $CysLT_2$-receptorn. I samma studie fann vi dessutom att patienter med hög förekomst av EGF-receptorn hade låg förekomst av $CysLT_2$-receptorn. En annan molekyl, interferon alfa, som redan används som läkemedel mot vissa cancerformer, visade sig öka förekomsten av $CysLT_2$-receptorn i tarmceller. Vi såg också att tillsats av interferon alfa i cellodlingskultur ger utmognad av tarmcancerceller till att likna normala tarmceller. I min tredje studie har jag funnit att tillsats av vitamin A ökar förekomsten av $CysLT_2$-receptorn i tarmcancerceller i cellodlingskultur. Vidare har jag funnit att vitamin A ger en utmognad av cancerceller från tjocktarmen till att likna mer normala tarmceller och även att detta verkar vara en process som delvis sker via $CysLT_2$-receptorn. I den fjärde studien undersökte vi om tarmceller kan producera leukotriener av sig själva och om de har de enzymer som krävs för detta. Tidigare har det förutsatts att infiltrat av vita blodkroppar i tarmväggen står för leukotrienproduktionen. Vi fann att tarmcellerna producerar leukotriener i cellodlingskultur och även att de har de enzymer och receptorer som krävs. Vidare fann vi att tillsats av de inflammatoriska molekylerna TNF alfa och leukotrien D_4, som båda visats viktiga för utvecklingen av inflammations-orsakad cancer, bidrar till en förhöjd halt av dessa enzymer och receptorer. Sammantaget visar den studien att vid tarminflammation kan tarmceller programmeras till att i detta avseende bete sig likt vita blodkroppar och att tarmcellerna har verktygen att själva kan fortsätta inflammationen oberoende av om de vita blodkropparna är närvarande eller inte.

Tjocktarmscancer behandlas primärt kirurgiskt (undantaget patienter med den ärftliga varianten, vilka utgör 5-10 %). Därför finns ett stort behov av nya läkemedel mot tjocktarmscancer. Den här avhandlingen visar på möjligheter att förändra den störda balansen mellan leukotrien-receptorerna $CysLT_1$ och $CysLT_2$ som observerats i material från tjocktarmscancer. Dessa studier tyder på att bättre överlevnad kan nås om $CysLT_1$-receptorn hämmas och $CysLT_2$-receptorn ökas. Specifika $CysLT_1$-receptorhämmare finns redan på marknaden och är effektiva som astmabehandling. Dessutom används interferon alfa och vitamin A med gott resultat vid behandling av flera cancerformer. I framtiden skulle därför en kombinationsbehandling med dessa befintliga läkemedel lämpa sig väl för kliniska prövningar av patienter med tjocktarmscancer, vilket kan leda till mer effektiva behandlingsmetoder.

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