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EICOSANOIDS IN CANCER: NEW THERAPEUTIC TARGETS IN NEUROBLASTOMA

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ABSTRACT

Cancer is one of the most common causes of death for both children and adults in developed countries. Neuroblastoma is a cancer of the sympathetic nervous system that affects infants and young children. Neuroblastoma tumors are the most common solid extracranial tumors in children and are also the most deadly. About half of the patients diagnosed are classified as high-risk, and despite an intensive multimodal treatment, the survival rate for these patients is only 55%. The overall survival for all neuroblastoma patients is about 70%. A better biological understanding is required to develop novel targeted therapy that may improve the outcome for cancer patients. Therefore we need to search for additional targets for therapeutic interventions in neuroblastoma.

Inflammatory cells and mediators are important constituents of the local tumor environment that drives tumor progression. Treatment that inhibits inflammation can reduce tumor growth. Neuroblastoma cells are enriched in arachidonic acid, a pro-inflammatory omega-6 fatty acid, but deficient in anti-inflammatory omega-3 fatty acids. We have previously shown that neuroblastoma expresses high levels of cyclooxygenase-2 (COX-2), which converts arachidonic acid into prostaglandins and that treatment with COX inhibitors reduces neuroblastoma tumor growth. In this thesis, I have investigated the role of arachidonic acid-derived metabolites in cancer, with a special focus on neuroblastoma.

To understand the significance of a high COX-2 expression in neuroblastoma we investigated the role of prostaglandin E_2 (PGE_2), its pro-inflammatory metabolite. Neuroblastoma cells produce PGE_2 and express all four PGE_2 receptors. PGE_2 stimulates neuroblastoma cell growth, and inhibition of PGE_2 receptor signaling reduces cell survival. We also evaluated the potential of a more specific targeting of the COX-2 pathway to reduce PGE_2 production by inhibition of microsomal prostaglandin E synthase-1 (mPGES-1). Downregulation of mPGES-1 expression reduces the clonogenic capacity and the tumorigenic potential of prostate and lung cancer cells.

Another route for arachidonic acid metabolism is the 5-lipoxygenase (5-LO) pathway and the production of pro-inflammatory leukotrienes. We detected expression of all the enzymes that are required for leukotriene biosynthesis in neuroblastoma tumors and cell lines. Neuroblastoma cells produce leukotrienes that promote cell growth and survival. Treatment with 5-LO pathway inhibitors or leukotriene receptor antagonists induced apoptosis of neuroblastoma cells.

Very little is known about the role of inflammation in childhood cancers. We show here a progressive inflammatory tumor microenvironment that parallels neuroblastoma tumor growth in the transgenic TH-MYCN model of neuroblastoma. Treatment with a low dose of anti-inflammatory acetylsalicylic acid, aspirin, modulates inflammatory parameters and significantly reduces tumor growth.

In summary, this thesis shows that PGE_2 and the leukotrienes act as autocrine and/or paracrine growth and survival factors in neuroblastoma. This suggests that inhibition of PGE_2 and leukotriene signaling may represent novel targeted therapy for neuroblastoma.

LIST OF PUBLICATIONS

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LIST OF ABBREVIATIONS

15-PGDH	15-hydroxyprostaglandin dehydrogenase
ASA	Acetylsalicylic acid, aspirin
ATL	Aspirin-triggered 15-epi-lipoxin
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
$cPLA_2$	Cytosolic phospholipase A_2
CTL	Cytotoxic T lymphocyte
CysLT	Cysteinyl leukotriene
EIA	Enzyme immunoassay
Erk	Extracellular signal-regulated kinase
FACS	Fluorescence activated cell sorting
GD2	Disialganglioside 2
GPCR	G-protein coupled receptor
HPRT	Hypoxanthine-guanine phosphoribosyl transferase
HRP	Horseradish peroxidase
IFNγ	Interferon γ
IL	Interleukin
LC-MS/MS	Liquid chromatography – tandem mass spectrometry
LO	Lipoxygenase
LT	Leukotriene
LTA_4H	Leukotriene A_4 hydrolase
LTC_4S	Leukotriene C_4 synthase
MDSC	Myeloid derived suppressor cell
MHC	Major histocompatibility complex
MMR	Macrophage mannose receptor
MRP	Multidrug resistance-associated protein
NSAID	Nonsteroidal anti-inflammatory drug
PBS	Phosphate buffered saline
PFA	Paraformaldehyde
PG	Prostaglandin
PGT	Prostaglandin transporter
PI3K	Phosphatidyl-inositol 3 kinase
PMA	Phorbol 12-myristate 13-acetate
ROS	Reactive oxygen species
TAM	Tumor associated macrophage
TGF-β	Tumor growth factor β
Th2	T helper 2
TMRE	Tetramethylrhodamine ethyl ester
TNFα	Tumor necrosis factor α
Treg	T regulatory cell
TXA	Thromboxane
VEGF	Vascular endothelial growth factor
Wnt	Wingless

1 INTRODUCTION

1.1 CANCER

In Sweden, over 50.000 adults and approximately 300 children are diagnosed with cancer every year. For both patient groups, cancer is the second most common cause of death in Sweden (www.Barncancerfonden.se; Cancerfondsrapporten, 2009). Cancer is the common name for a highly heterogeneous group of diseases that are all named after the tissue from which they arise, and a tumor may originate from almost any of the different cell types in the body. The common features for all solid tumors are uncontrolled cell growth, formation of new blood vessels, an ability to invade adjacent tissues, and metastatic potential (Hanahan and Weinberg, 2011).

Children and adults develop different types of cancer. Adults predominantly develop cancers from mature tissues of an epithelial origin. The tumors develop over a long time period and acquire many genetic aberrations that result in uncontrolled cell growth. Childhood tumors have a much shorter latency and result from a disruption of normal development. These tumors arise in an immature tissue microenvironment, and they have fewer genetic aberrations and a different biology (Scotting *et al.*, 2005; Grimmer and Weiss, 2006; Johnsen *et al.*, 2009). The current treatment modalities for both children and adults include radiation, surgery, and chemotherapy, and the importance of each of these treatments varies depending on the tumor type and stage of the disease.

1.1.1 Neuroblastoma

Neuroblastoma is a childhood tumor of the sympathetic nervous system, and in Sweden, 10-20 children are diagnosed with a neuroblastoma every year (Gustafsson *et al.*, 2007b; Träger, 2009). The median age at diagnosis is 18 months, and almost all patients are diagnosed before the age of four (Gustafsson *et al.*, 2007b). The cause of neuroblastoma is largely unknown, and a family history of the disease exists in only 1-2% of the cases. Germline mutations have been discovered in two different genes, the paired-like homeobox 2B gene (*PHOX2B*; Trochet *et al.*, 2004) and the anaplastic lymphoma kinase gene (*ALK*; Mosse *et al.*, 2008), and these mutations explain most of the hereditary cases.

Neuroblastoma tumors arise from sympathetic neural pre-cursor cells, the neural crest cells. The neural crest is a transient multipotent population of cells derived from the ectoderm that forms at the top of the neural tube, the structure that will become the spinal cord. The neural crest cells migrate throughout the embryo and differentiate to give rise to most of the cells of

the peripheral nervous system, the enteric nervous system, and cells of the craniofacial skeleton, as well as pigment cells, Schwann cells, and adrenal medullary cells (Jiang *et al.*, 2011). Neuroblastoma tumors can arise anywhere along the sympathetic axis, but most primary tumors arise in the abdomen, and they most frequently occur in the adrenal medulla (Figure 1; Brodeur, 2003).

The clinical behavior of neuroblastoma is very heterogeneous. A small subset of tumors spontaneously regresses or matures without any treatment, whereas others do not respond to intensive multimodal treatment. A number of biological and clinical features are used to predict both the behavior of the tumor and the patient outcome. These factors include local or metastatic disease, age at diagnosis, histology, grade of tumor differentiation, amplification of the oncogene MYCN, genetic loss of chromosome 11q, and the tumor cell DNA ploidy. Using these factors, patients are classified into different risk groups according to the International Neuroblastoma Risk Group Staging System (INRGSS; Cohn *et al.*, 2009). Patients are categorized into low-, intermediate-, or high-risk groups, and receive different treatments depending on their risk group. High-risk patients are treated with an intensive therapy that includes induction chemotherapy, surgery, myeloblative chemotherapy, stem cell rescue, and irradiation, followed by maintenance

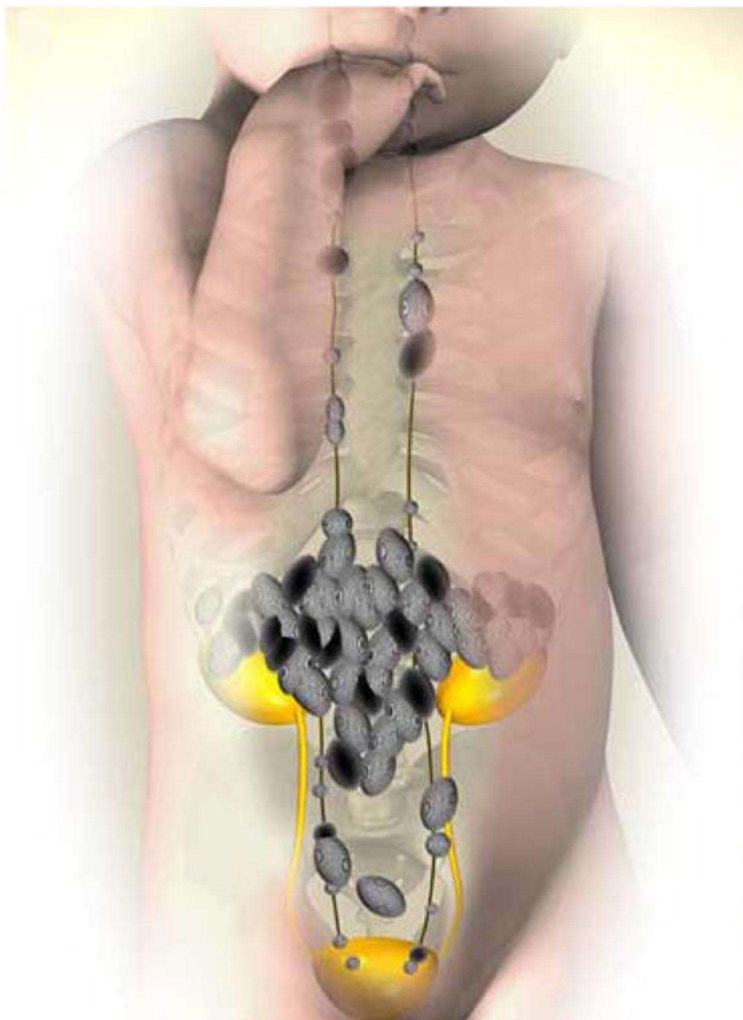

Figure 1. Neuroblastoma primary tumors derived from the neural crest arise in the sympathetic nervous system including the adrenal medulla, sympathetic ganglia and paraganglia. Neuroblastomas mainly metastasize to lymph nodes, bone and bone marrow, and in infants also spread to liver and subcutaneous tissue.

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therapies consisting of retinoic acid in combination with immunotherapy, with monoclonal antibodies targeting disialganglioside 2 (GD2). Patients in the intermediate-risk group receive a milder therapy consisting of limited chemotherapy and surgery, and some of these patients also receive treatment with irradiation and retinoic acid. Low-risk patients may be cured by local surgery alone, and for the special form of metastatic neuroblastoma that can spontaneously regress, no treatment may be needed (Brodeur, 2000). Despite advances in the treatment of neuroblastoma, the overall survival rate for neuroblastoma patients in Sweden today is 74%, with a survival rate of only 55% for patients in the high-risk group (Träger, 2009)

1.2 EICOSANOIDS AND ARACHIDONIC ACID

Eicosanoids are small, short-lived, fatty acid-derived signaling molecules. *Eicosa* is the Greek word for 20, and all eicosanoids consist of a 20-carbon backbone with different numbers and positions of the carbon-carbon double bonds and various side chains. Due to its high abundance, the predominant precursor of the eicosanoids is the polyunsaturated fatty acid arachidonic acid (20:4, ω6). Arachidonic acid is formed from the essential fatty acid linoleic acid (18:2, ω6) by a multi-stage process that includes desaturation by Δ6 and Δ5 desaturases, which remove hydrogen atoms and create double bonds, and an elongation of two more carbon atoms by elongase (Schmitz and Ecker, 2008). Arachidonic acid is stored in an esterified form in the phospholipids of cellular membranes (Schmitz and Ecker, 2008). The concentration of free non-esterified arachidonic acid is very low in the cell (Irvine, 1982), but is increased when arachidonic acid is released by cytosolic phospholipase A_2 ($cPLA_2$; Haeggstrom and Funk, 2011; Murakami *et al.*, 2011). Due to its importance in eicosanoid biosynthesis, the activity of $cPLA_2$ is strictly regulated. It is activated by a calcium-dependent translocation from the cytoplasm to the perinuclear membrane (Clark *et al.*, 1991), and at the membrane the hydrolytic activity is regulated by phosphorylation and by phosphatidylinositol-4,5-bisphosphate, PIP_2 (Tucker *et al.*, 2009). Free arachidonic acid is rapidly converted to eicosanoids by the enzymes cyclooxygenase (COX), lipoxygenase (LO), and cytochrome p450 (Figure 2). The metabolism of arachidonic acid by these pathways generates eicosanoids including prostaglandins (PGs), leukotrienes (LTs), lipoxins, hydroxyeicosatetraenoic acids (HETEs), hydroperoxyeicosatetraenoic acids (HpETEs), and epoxyeicosatrienoic acids (EETs).

Arachidonic acid is not the only substrate for eicosanoid biosynthesis. Generally, eicosanoids formed from arachidonic acid are pro-inflammatory, while those formed from the omega-3 fatty acids, docosahexaenoic acid (DHA) or eicosapentaenoic acid (EPA), are anti-inflammatory. However, a

notable exception to this simplistic view is the anti-inflammatory lipoxins, which are formed from arachidonic acid.

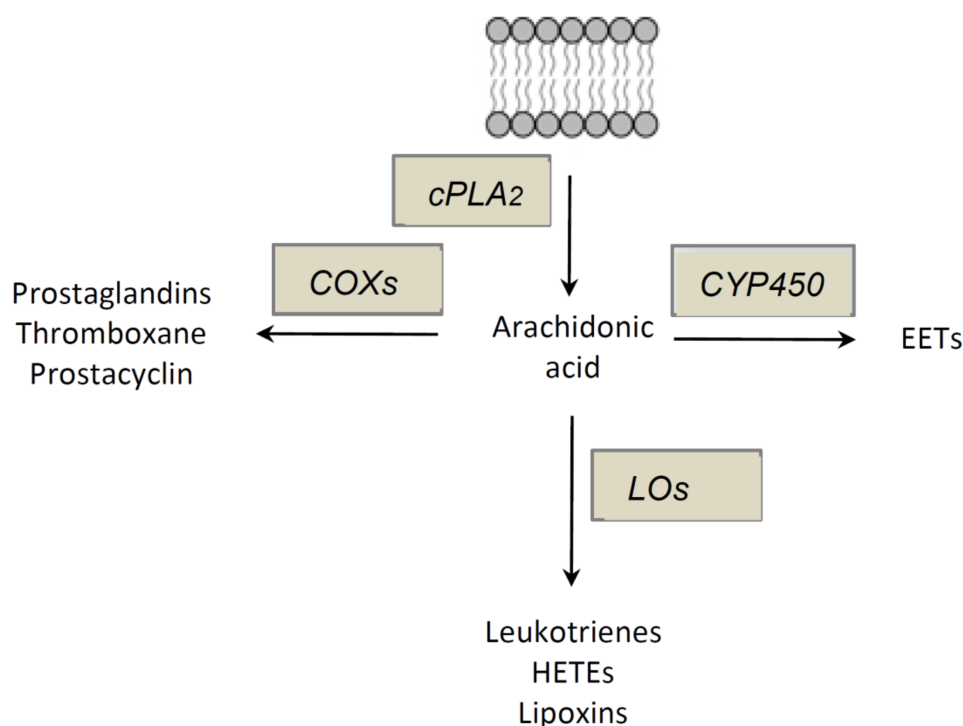

Figure 2. Schematic illustration of arachidonic acid metabolism. Arachidonic acid is released from membrane phospholipids by cytosolic phospholipase A_2 ($cPLA_2$). Free arachidonic acid is rapidly converted by cyclooxygenases (COXs) into the prostaglandins, thromboxanes and prostacyclin, by the lipoxygenases (LOs) to leukotrienes, hydroxyeicosatetraenoic acids (HETEs) and lipoxins, or by cytochrome p450 into epoxyeicosatrienoic acids (EETs).

1.3 THE CYCLOOXYGENASE PATHWAY

Cyclooxygenases catalyze the conversion of arachidonic acid to (PGs) of the 2-series, thromboxane A_2 (TXA_2), and prostacyclin (PGI_2). PGs are involved in many processes in the body, such as pain, fever, and inflammation. PGs can be formed by most cells in the body. They are very short-lived and cannot be stored, but are synthesized *de novo* upon activation (Curtis-Prior, 2004).

1.3.1 Cyclooxygenases and prostaglandin biosynthesis

Two cyclooxygenase isoforms exist, COX-1 (DeWitt and Smith, 1988; Merlie *et al.*, 1988) and COX-2 (Xie *et al.*, 1991), both of which catalyze the same reaction, though they differ in tissue distribution and function. COX-1 is constitutively expressed in many tissues to maintain housekeeping functions

and homeostasis. In contrast, COX-2 is not expressed in most organs under basal conditions, except for the brain, kidney, colon, testis, and female reproductive organs, but the expression can be induced by mitogenic and inflammatory stimuli (Zidar *et al.*, 2009). The conversion of arachidonic acid by COX actually consists of two sequential activities in the same enzyme but at two distinct sites. In the first step, the cyclooxygenase active site removes two molecules of oxygen from arachidonic acid, leading to the formation of the cyclic endoperoxide PGG_2, which in the next step is reduced to PGH_2 by the peroxidase active site (Curtis-Prior, 2004). PGH_2 is further metabolized into PGE_2, PGD_2, $PGF_{2\alpha}$, TXA_2, and PGI_2 by specific synthases (Figure 3).

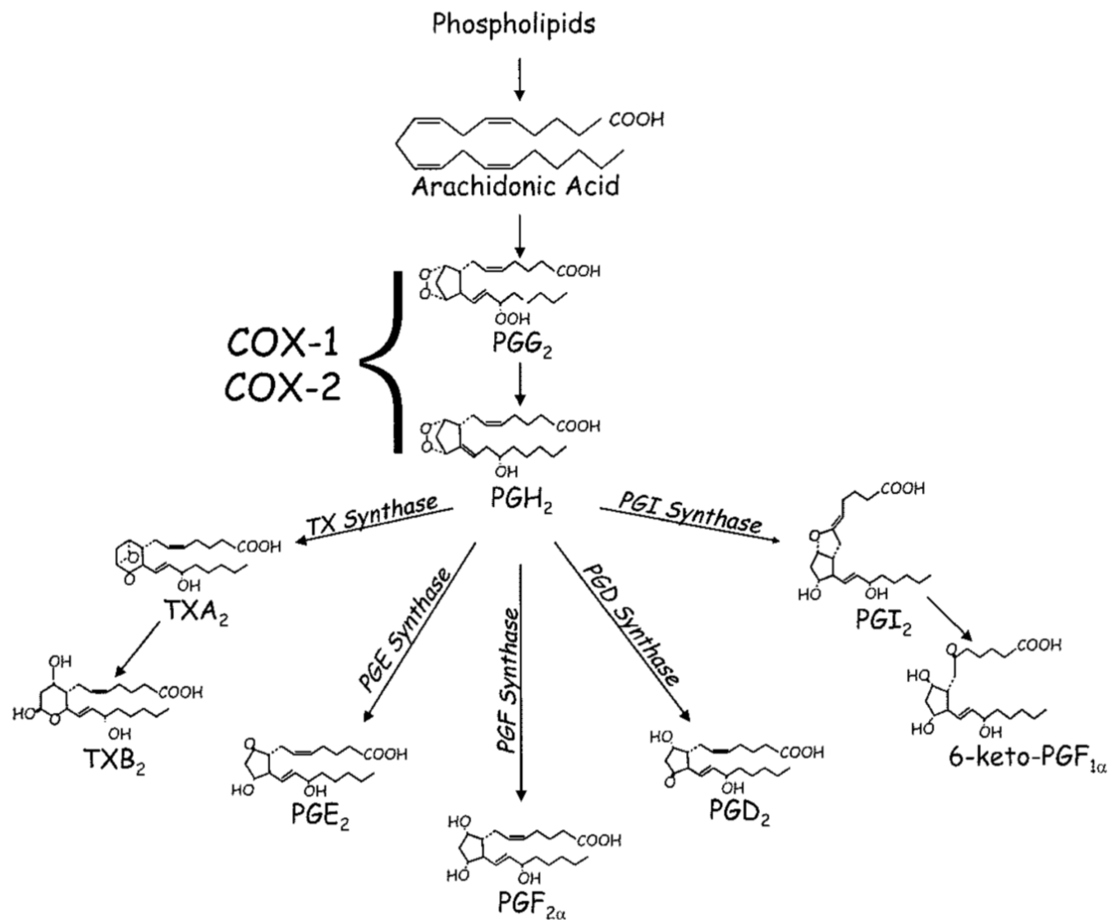

Figure 3. The COX pathway. COX-1 and COX-2 oxygenate arachidonic acid to form PGG_2, which is further reduced to PGH_2. PGH_2 is highly unstable and is rapidly converted by specific synthases to PGE_2, $PGF_{2\alpha}$, PGD_2, PGI_2 and TXA_2. PGI_2 and TXA_2 have a very short half-life and are rapidly hydrolyzed to the inactive compounds 6-keto-$PGF_{1\alpha}$, and TXB_2, respectively. Reprinted with permission from The FASEB Journal. Modified from (Romano and Claria, 2003).

PGE_2 synthases catalyze the isomerization of the endoperoxide moiety of PGH_2 into a keto group at carbon 9 and a hydroxyl group at carbon 11 to form PGE_2. Three enzymes with PGE_2 synthases activity have been

described in the literature; two membrane-bound enzymes and one cytosolic enzyme. Microsomal prostaglandin E synthase 1 (mPGES-1) is a glutathione-dependent, membrane-bound enzyme (Jakobsson *et al.*, 1999) that is suggested to predominantly couple with COX-2 (Murakami *et al.*, 2000; Thoren and Jakobsson, 2000). mPGES-1 is constitutively expressed in the placenta, prostate, testis, mammary gland, and bladder, but is normally absent or expressed at low levels in other tissues (Jakobsson *et al.*, 1999). As for COX-2, the expression can be induced by inflammatory stimuli (Jakobsson *et al.*, 1999; Murakami *et al.*, 2000). Mice deficient in mPGES-1 confirm the importance of this enzyme for the *in vivo* production of PGE_2, and indicate a role for this enzyme in both acute and chronic inflammatory reactions (Trebino *et al.*, 2003). The two other synthases, mPGES-2 (Tanikawa *et al.*, 2002) and cycosolic PGE synthase (cPGES; Tanioka *et al.*, 2000), can convert PGH_2 into PGE_2 *in vitro*, but studies using knockout mice indicate that these synthases may not be essential for production *in vivo* (Lovgren *et al.*, 2007; Jania *et al.*, 2009). However, the production of PGE_2 can still be detected in mice lacking mPGES-1, thus supporting the hypothesis that additional pathways are involved in the production.

Specific enzymes that catalyze the conversion of PGH_2 to PGD_2, $PGF_{2\alpha}$, TXA_2, and PGI_2 have also been identified. The chemical structure of PGD_2 is very similar to PGE_2, with the only difference being that the keto group and the hydroxyl group are in opposite positions. Two enzymes are responsible for the isomerization of PGH_2 to PGD_2, lipocalin-type and haematopoetic PGD synthase. $PGF_{2\alpha}$ can be formed by the reduction of PGH_2 by 9,11-endoperoxide reductase or by a reduction of PGE_2 or PGD_2 by keto reductases. In addition, thromboxane synthase and prostacyclin synthase catalyze the synthesis of TXA_2 and PGI_2, respectively (Curtis-Prior, 2004).

Once the PGs have been synthesized, they are transported out of the cell into the extracellular matrix. Studies have shown that PGs can be effluxed by simple diffusion (Chi *et al.*, 2006) and/or actively transported by multidrug resistance-associated proteins (MRP; Fletcher *et al.*, 2010). However, the contribution and relative importance of each of these two routes is not yet known.

1.3.2 Prostaglandin E_2 signaling and biological effects

PGs, TXA_2, and PGI_2 are potent hormone-like, short-lived mediators that act near their site of synthesis in a paracrine and autocrine manner. They mediate their signals by binding to specific G-protein coupled receptors (GPCRs) that are named after the ligand bound into EP, DP, FP, IP, and TP (Woodward *et al.*, 2011). GPCRs are found in cellular membranes, and they all share the structure of seven-transmembrane α-helices that weave in and out of the membrane. When a ligand binds to the receptor, it causes a conformational

change that activates a G-protein trimer. The G-protein trimer consists of an α, β, and γ subunit. Upon activation, the $G\alpha$ subunit dissociates from the $G\beta\gamma$ subunit complex, which can then stimulate downstream effectors. Further signal transduction is primarily dependent on the type of the $G\alpha$ subunit (Dorsam and Gutkind, 2007).

PGE_2 is an important actor in and regulator of both pro- and anti-inflammatory immune responses, though it also has a broad range of other biological activities (Hirata and Narumiya, 2011). PGE_2 exerts its actions by binding to four different receptor subtypes, designated EP1, EP2, EP3, and EP4. One single cell can express one or more of these receptors. PGE_2 binding to the EP1 receptor leads to an increased intracellular calcium level. The EP1 receptor has a prominent role in pain perception and stress, but is also expressed and participates in the physiological functions of the lungs, stomach, and kidneys. Activation of both the EP2 and EP4 receptors results in elevated cyclic adenosine monophosphate (cAMP) levels. The two receptors can act in concert, but also mediate receptor-specific effects. The EP2 receptor is widely distributed in the body and mediates a broad range of actions, including bronchodilatory and tocolytic effects, neuroprotection, and neurotoxicity. Similarly, the EP4 receptor exhibits a wide tissue distribution, and is primarily involved in cardiovascular biology and bone metabolism. The EP3 receptor has several splice variants capable of coupling to different G-proteins, thereby contributing to the wide spectrum of EP3 actions; however, the majority of the isoforms act to inhibit cAMP production. This receptor mediates the fever response, and is involved in pain perception and labor (Woodward *et al.*, 2011).

1.3.3 Termination of prostaglandin E_2 signaling

The catabolism and termination of PGE_2 signaling occur in two steps (Nomura *et al.*, 2004). In the first step, PGE_2 is actively transported from the extracellular milieu into the cytoplasm by the prostaglandin transporter (PGT; Chi *et al.*, 2006). In the second step, PGE_2 is rapidly inactivated through oxidation of the hydroxyl group at carbon 15 to 15-keto PGE_2 by 15-hydroxyprostaglandin dehydrogenase (15-PGDH). Both PGT (Lu *et al.*, 1996) and 15-PGDH (Tai *et al.*, 2002) are ubiquitously expressed in mammalian tissues.

1.3.4 The cyclooxygenase pathway in cancer

The expression of COX-2 is upregulated in pre-malignant lesions such as gastric dysplasia (Thiel *et al.*, 2011) colorectal adenoma, prostatic intraepithelial neoplasia, carcinoma *in situ*, chronic hepatitis, and Barrett's esophagus (de Groot *et al.*, 2007). COX-2 is also overexpressed in malignant

tissues of various origins, in invasive cancers, and at metastatic sites (Harris, 2009). Increased PGE_2 levels are frequently found in pre-malignant and malignant tissues (McLemore *et al.*, 1988; Pugh and Thomas, 1994; Freedman *et al.*, 2007) and in the urine of cancer patients (Johnson *et al.*, 2006; Kekatpure *et al.*, 2009). Moreover, a high expression of COX-2 (Harris, 2009; Thiel *et al.*, 2011) and increased PGE_2 levels (Rolland *et al.*, 1980; Kekatpure *et al.*, 2009) have been associated with a poor prognosis. In the pediatric age group, COX-2 is upregulated in neuroblastoma (Johnsen *et al.*, 2004), brain tumors (Patti *et al.*, 2002; Baryawno *et al.*, 2008), and sarcomas (Dickens *et al.*, 2002).

Most research has focused on the role of COX-2 in cancer, although COX-1, which is thought to mainly be involved in housekeeping functions, has also been shown to be overexpressed in breast (Hwang *et al.*, 1998), ovarian (Gupta *et al.*, 2003; Freedman *et al.*, 2007) and cervical tumor tissues (Sales *et al.*, 2002).

Many mechanisms underlie the tumor-promoting effects of PGE_2 (Wang and Dubois, 2010). First, PGE_2 stimulates cell proliferation, increases cell survival, and inhibits apoptosis through the activation of pro-survival pathways such as phosphatidyl-inositol 3 kinase (PI3K)-Akt, extracellular signal-regulated kinase (Erk), canonical wingless (Wnt), transactivation of the receptor for epidermal growth factor (EGFR), and the upregulation of anti-apoptotic Bcl-2 (Wang and Dubois, 2010). Second, PGE_2 stimulates cell migration (Sheng *et al.*, 2001) and invasion by upregulation of the chemokine receptor CCR7 (Pan *et al.*, 2008), activation of Rap signaling (Wu *et al.*, 2011), and transactivation of EGFR (Buchanan *et al.*, 2006). Furthermore, PGE_2 increases adhesion by enhancing the expression of integrin $\alpha3\beta1$ (Mitchell *et al.*, 2010), and mediates breakdown of the extracellular matrix by increasing matrix metalloproteinase-2 production (Ito *et al.*, 2004). PGE_2 supports angiogenesis by inducing the expression of vascular endothelial growth factor (VEGF; Sonoshita *et al.*, 2001; Chang *et al.*, 2004) and the chemokine (C-X-C motif) ligand 1 (Wang *et al.*, 2006). In addition, PGE_2 is a key mediator in the crosstalk between tumor cells and stromal cells in the tumor microenvironment, and PGE_2 contributes to the creation of a tumor promoting-inflammatory microenvironment and suppresses activation of the immune system (Wang and Dubois, 2010).

1.3.5 Nonsteroidal anti-inflammatory drugs (NSAIDs)

Nonsteroidal anti-inflammatory drugs (NSAIDs) are widely used in the treatment of pain, fever, and inflammation. Their basic mode of action is to inhibit the COX enzymes, thereby reducing the production of PGs that are critically involved in these reactions. NSAIDs are a structurally diverse group of compounds that include salicylates, proprionic acids, pyrazoles, acetic

acids, oxicams, fenamates, naphthyl-alkanones, and coxibs (Reuben, 2007). They are called nonsteroidal to distinguish them from steroids, which also reduce eicosanoid production and have anti-inflammatory properties.

Traditional NSAIDs are dual inhibitors of both COX-1 and COX-2. With the increasing use of these medications, serious adverse gastrointestinal side effects became evident. These effects mainly occurred due to the inhibition of PGs produced by COX-1 that protect the gastric mucosa (Ong *et al.*, 2007). To avoid the adverse effects of traditional NSAIDs, specific COX-2 inhibitors, called coxibs, were developed. The existence of an extra side pocket in the active site of COX-2 made this possible. However, although the use of coxibs reduced the gastrointestinal complications, these compounds proved to have other side effects—most seriously, an increased risk for cardiovascular events—that lead to the withdrawal of rofecoxib (Vioxx) in 2004 (Bresalier *et al.*, 2005). Coxibs are thought to shift the balance between TXA_2 and PGI_2 that maintains cardiovascular homeostasis. Specifically, coxibs inhibit the production of anti-thrombotic PGI_2 by COX-2 in vascular endothelial cells without changing the synthesis of pro-thrombotic TXA_2 from COX-1 activity in platelets (Cheng *et al.*, 2002).

1.3.5.1 Acetylsalicylic acid

The history of acetylsalicylic acid (ASA), or aspirin, goes back to early herbal folklore and the use of willow bark and meadowsweets to relieve pain and fever; however, its molecular mechanism was not uncovered until 1971 (Vane and Botting, 2003). ASA belongs to the salicylate group of traditional NSAIDs. In contrast to the other NSAIDs, ASA irreversibly inhibits the COX enzymes by covalent modifications (Curtis-Prior, 2004). ASA acetylates COX-1 at serine 530, which prevents arachidonic acid from entering the active site and thus inhibits PG biosynthesis. In addition, ASA acetylates COX-2 at homologous serine 516, which changes the enzymatic activity so that arachidonic acid is instead converted to 15-hydroxyeicosatetraenoic acid (15*R*-HETE). 15*R*-HETE can further be converted to anti-inflammatory lipoxins and resolvins (Claria and Serhan, 1995; Chiang *et al.*, 2004; Serhan *et al.*, 2008).

1.3.6 NSAIDs in cancer therapy

In 1988, an epidemiologic study revealed that colorectal cancer patients were less likely than controls to have used aspirin in the past (Kune *et al.*, 1988). Ever since, numerous studies have evaluated the effect of NSAIDs in cancer prevention. These studies show an indisputable promise in using NSAIDs as chemopreventive agents in various adult cancers, though they have also raised serious concerns about NSAIDs' adverse side effects. Most of these adverse complications are likely due to on-target effects; however, these

effects also depend on dosage. Epidemiologic studies show that a low-dose (75-300 mg) ASA is as effective as a higher dose (≥500mg) ASA and reduces the incidence of colorectal (Rothwell *et al.*, 2010) and prostate cancer (Salinas *et al.*, 2010), while also reducing the risk of cancer-related death (Rothwell *et al.*, 2011).

NSAIDs can also reduce tumor growth of already established tumors by inhibiting the tumor-promoting actions of PGE_2. Treatment with NSAIDs induces apoptosis (Johnsen *et al.*, 2004), reduces angiogenesis and metastatic spread (Masferrer *et al.*, 2000; Kakiuchi *et al.*, 2002), and abrogates PGE_2-induced immunosuppression (Stolina *et al.*, 2000; Lonnroth *et al.*, 2008). NSAIDs can also potentiate the toxic effect of cytostatic drugs and protect against chemotherapy-associated side effects (de Groot *et al.*, 2007). Even so, many NSAIDs and coxibs have off-target effects that may contribute to their cytotoxic and anti-tumorigenic potential (Fischer *et al.*, 2011).

In neuroblastoma, treatment with NSAIDs results in reduced tumor growth by the induction of apoptosis through modulation of the expression of p73 isoforms (Lau *et al.*, 2009) and cleavage of caspase-9 and -3 and PARP (Johnsen *et al.*, 2004; Parashar and Shafit-Zagardo, 2006). Moreover, NSAIDs inhibit angiogenesis (Ponthan *et al.*, 2007) and potentiate the effect of cytostatic drugs on neuroblastoma (Lau *et al.*, 2007; Ponthan *et al.*, 2007).

1.4 THE 5-LIPOXYGENASE PATHWAY

Lipoxygenases (LO) are a family of iron-containing enzymes that catalyze the peroxidation of polyunsaturated fatty acids to hydroperoxy derivatives. They are named after the carbon atom of arachidonic acid to which oxygen is added. Three main types of LOs exist in humans: 5-LO, 12-LO, and 15-LO (Haeggstrom and Funk, 2011). The oxygenation of arachidonic acid by the LOs leads to the production of 5-, 12- and 15-HpETE, respectively, which can be further metabolized into LTs, hepoxilins, lipoxins, and HETEs (Samuelsson *et al.*, 1987). 5-LO catalyzes the initial steps in the formation of LTs and lipoxins (Figure 4).

1.4.1 5-Lipoxygenase and the leukotrienes

5-LO is primarily expressed in various leukocytes such as B-lymphocytes, dendritic cells, monocytes/macrophages, mast cells, neutrophils, and eosinophils (Radmark and Samuelsson, 2009). In resting cells, 5-LO resides in the cytoplasm or nucleoplasm and translocates upon calcium activation to the perinuclear membrane. The nuclear redistribution of 5-LO is regulated by phosphorylations (Radmark *et al.*, 2007), and *in vitro*, 5-LO is phosphorylated by the MAPK-activated protein kinase 2 at Ser-271 (Werz *et al.*, 2002b), by

Erk2 at Ser-663 (Werz *et al.*, 2002a) and by protein kinase A at Ser-523 (Luo *et al.*, 2004). At the nuclear membrane, 5-LO co-localizes with $cPLA_2$ and 5-lipoxygenase activating protein (FLAP; Dixon *et al.*, 1990). FLAP is a small integral nuclear membrane protein that is required for LT biosynthesis, but does not have enzymatic activity and was recently suggested to act as a scaffold protein for 5-LO (Mandal *et al.*, 2008). Subsequently, in a two-step reaction, 5-LO catalyzes the oxygenation of arachidonic acid at C-5 to 5-hydroperoxytetraenoic acid (5-HpETE) and the further dehydration to

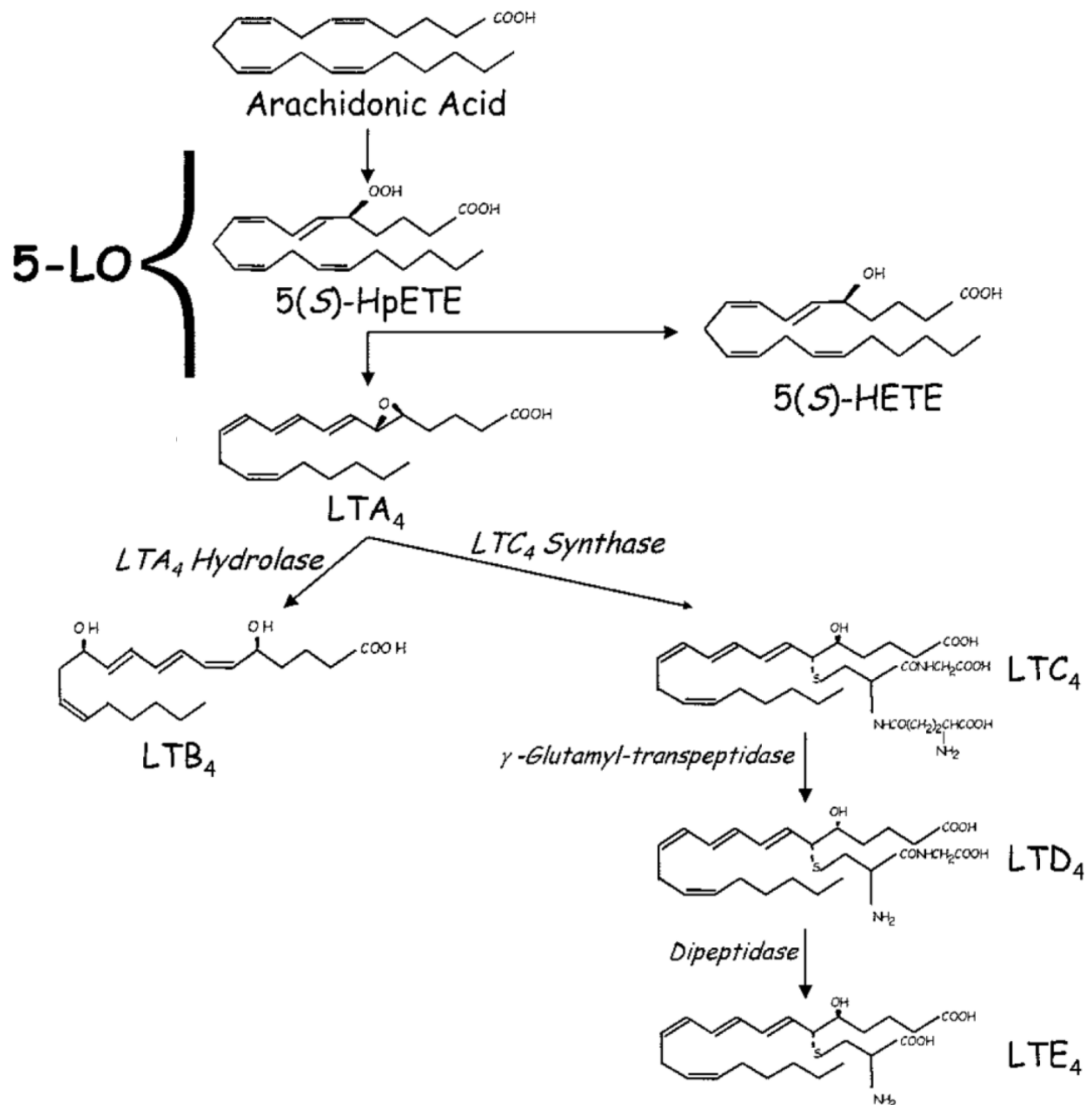

Figure 4. The 5-LO pathway. 5-LO catalyzes the oxygenation of arachidonic acid to 5(*S*)-HpETE. 5(*S*)-HpETE can be reduced to 5(*S*)-HETE or subjected to the stereospecific removal of the pro-R hydrogen at carbon-10 to generate the highly unstable LTA_4. LTA_4 is either hydrolyzed to LTB_4 by LTA_4 hydrolase or converted into LTC_4 by addition of the peptide glutathione by LTC_4 synthase. LTC_4 can undergo further metabolism through a series of peptidic cleavage reactions to yield LTD_4 and LTE_4. Reprinted with the permission from FASEB journal. Modified from (Romano and Claria, 2003).

the epoxide LTA_4 (Samuelsson *et al.*, 1987). An alternative fate for 5-HpETE is to be metabolized to 5-HETE, 5-oxo-ETE, and lipoxins (Radmark *et al.*, 2007). The instable intermediate LTA_4 can be metabolized by two routes: enzyme leukotriene A_4 hydrolase (LTA_4H) to LTB_4 or leukotriene C_4 synthase (LTC_4S) to LTC_4.

LTA_4H is a soluble protein that resides in the cytosol, although a nuclear localization has also been reported (Brock *et al.*, 2001). In contrast to 5-LO, LTA_4H has a wide tissue and cellular distribution, and is particularly expressed in the intestine, spleen, lung, and kidney, in addition to its expression in various blood cells (Haeggstrom, 1999). LTA_4 can also be conjugated with glutathione by LTC_4S to form the cysteinyl containing LTC_4, which is the parent compound of the cysteinyl leukotrienes (CysLTs) LTD_4 and LTE_4. LTC_4S is an integral membrane protein that is located in the outer nuclear membrane (Christmas *et al.*, 2002), and the expression of LTC_4S is restricted to basophils, eosinophils, mast cells, monocytes/macrophages, and platelets (Lam and Austen, 2002). LTC_4 is secreted from the cell by MRP1 and -4 (Leier *et al.*, 1994; Wijnholds *et al.*, 1997). Once transported to the extracellular space, γ-glutamyl transdipepdidase cleaves the glutamic moiety to form LTD_4, which can be further metabolized by cleavage of the glycine moiety by a dipepdidase enzyme to LTE_4 (Hammarstrom *et al.*, 1985).

LTs can also be produced by transcellular metabolism. In that case, the LTA_4 that is produced in one cell is transferred to another cell that further metabolizes LTA_4 into LTs (Dahinden *et al.*, 1985; McGee and Fitzpatrick, 1986; Fabre *et al.*, 2002; Zarini *et al.*, 2009). This process explains the evidence that LTA_4H and LTC_4S are expressed in many cell types that do not express 5-LO.

1.4.2 Leukotriene signaling and biological effects

The name "leukotriene" comes from the words "leukocyte" and *treien*: "leukocyte" because they were first found in leukocytes, and *treien* because they all contain three conjugated double bonds in their chemical structure. LTs are predominantly produced by leukocytes, and they mediate their effects by binding the G-protein coupled LT receptors, which are subdivided into the LTB_4 receptors (BLT) and the CysLT receptors. LTs are very potent pro-inflammatory molecules that can mediate both short-term effects and long-term effects on transcription.

1.4.2.1 Leukotriene B_4

The effect of LTB_4 is mediated through activation of two different receptors: a high-affinity receptor named BLT1 (Yokomizo *et al.*, 1997), and a lower-

affinity receptor named BLT2 (Yokomizo *et al.*, 2000). Human BLT1 and BLT2 are structurally similar, with a 45% amino acid sequence identity, though they differ in tissue and cell distribution (Yokomizo *et al.*, 2000). BLT1 is primarily expressed on leukocytes, and is present at a low level in thymus and spleen (Yokomizo *et al.*, 1997). The BLT2 receptor is more ubiquitously expressed, with the highest expression in the spleen, ovary, liver, and leukocytes (Yokomizo *et al.*, 2000). In addition, ligands other than LTB_4 can bind to and activate the BLT2 receptor (Okuno *et al.*, 2008). Both BLT1 and BLT2 couple to intracellular G-proteins that mediate an intracellular increase in calcium and a reduced level of cAMP (Yokomizo *et al.*, 2000; Toda *et al.*, 2002). In addition to BLT1 and BLT2, LTB_4 also binds to a nuclear receptor, peroxisome proliferator-activated receptor α, or PPARα. This receptor activates the transcription of genes involved in lipid metabolism and inflammation (Devchand *et al.*, 1996; Narala *et al.*, 2010).

LTB_4 is a very potent chemoattractant factor for leukocytes. It promotes leukocyte recruitment to sites of inflammation by stimulating migration and enhancing the adherence of leukocytes to the blood vessel wall, which is a prerequisite for migration into the tissue (Ford-Hutchinson *et al.*, 1980; Smith *et al.*, 1980; Goodarzi *et al.*, 2003; Tager *et al.*, 2003; Lundeen *et al.*, 2006; Del Prete *et al.*, 2007). Once the leukocytes reach the inflamed site, LTB_4 promotes leukocyte activation and proliferation and cell survival (Peters-Golden *et al.*, 2005; Kim and Luster, 2007). In addition to its chemotactic properties, LTB_4 plays an important role in the body's defense against pathogens by augmenting neutrophil and macrophage phagocytosis and bactericidal activity (Peters-Golden *et al.*, 2005). LTB_4 has also been implicated in various inflammatory diseases, autoimmune disorders and cardiovascular disease (Peters-Golden and Henderson, 2007).

1.4.2.2 Cysteinyl leukotrienes, LTC_4, LTD_4, and LTE_4

Pharmacological studies have revealed the existence of two CysLT receptors, designated CysLT1 (Lynch *et al.*, 1999) and CysLT2 (Heise *et al.*, 2000). They are both high-affinity receptors that upon activation lead to an intracellular calcium mobilization and/or reduction in cAMP levels, but they differ in rank order potency of agonist activation. The rank order for CysLT1 is $LTD_4 >> LTC_4 = LTE_4$ (Lynch *et al.*, 1999), whereas for CysLT2 the rank order is, $LTD_4 = LTC_4 > LTE_4$ (Heise *et al.*, 2000). Nonetheless, significant evidence points to the existence of additional CysLT receptor subtypes that need further characterization (Singh *et al.*, 2010; Haeggstrom and Funk, 2011). Both receptors are expressed in leukocytes, the spleen, and the lungs, although CysLT2 is also abundantly expressed in the heart, adrenal glands, and brain (Singh *et al.*, 2010).

CysLTs are potent mediators of bronchoconstriction (Dahlen *et al.*, 1980), they increase vascular permeability (Dahlen *et al.*, 1981) and promote mucous

secretion (Marom *et al.*, 1982). They also mediate functions of the immune system, including the recruitment of eosinophils and monocytes/ macrophages towards sites of inflammation, the stimulation of cytokine and chemokine production, and the promotion of activation and maturation of eosinophils and dendritic cells (Kim and Luster, 2007). CysLTs also play a prominent role in asthma, and LT inhibitors are currently used in asthma treatment (Peters-Golden and Henderson, 2007).

1.4.3 5-Lipoxygenase signaling pathway in cancer

The importance of LOs in cancer has not been studied as extensively as that of the COX enzymes, and their role is more complex and includes both pro- and anti-tumorigenic LO isoforms. In general, 5-LO and 12-LO have pro-tumorigenic effects, whereas 15-LO-2 has anti-tumorigenic effects and the role of 15-LO-1 is controversial (Wang and Dubois, 2010). Increasing evidence indicates that the pro-tumorigenic isoforms are preferentially expressed during cancer development, while the expression of anti-tumorigenic isoforms is lost (Furstenberger *et al.*, 2006).

5-LO is expressed in early pre-malignant lesions of the pancreas (Hennig *et al.*, 2005), colon (Melstrom *et al.*, 2008), and esophagus (Chen *et al.*, 2004), as well as in malignant tissues of various origin such as the brain (Boado *et al.*, 1992; Ishii *et al.*, 2009), lungs (Avis *et al.*, 1996), prostate (Gupta *et al.*, 2001), pancreas (Hennig *et al.*, 2002), colon (Ohd *et al.*, 2003) and esophagus (Chen *et al.*, 2004). A concomitant increased concentration of 5-LO products can be detected in cancer tissues (el-Hakim *et al.*, 1990; Simmet *et al.*, 1990; Larre *et al.*, 2008), and many cancer cell lines are capable of producing LTs (Avis *et al.*, 1996).

Mechanisms underlying the tumor-promoting effects of LTs are not fully understood. LTs have been shown to increase the proliferation of cancer cells through activation of the MEK/Erk and PI3K/Akt pathways (Tong *et al.*, 2005). Direct evidence that LTB_4 promotes tumor growth comes from a study that shows that LTB_4 increased the incidence, number, and volume of macroscopic oral tumors of DMBA-induced hamster cheek pouch carcinomas (Sun *et al.*, 2006). 5-LO has also been documented to contribute to the production of reactive oxygen species (ROS) from arachidonic acid that can induce DNA damage and increase cell proliferation (Catalano *et al.*, 2005). Furthermore, the upregulation of 5-LO protects cancer cells from apoptosis induced by treatment with cytotoxic drugs and by the inhibition of p53 (Catalano *et al.*, 2004).

Therapeutic inhibition of key enzymes in the 5-LO pathway leads to an induction of apoptosis by downregulation of anti-apoptotic bcl-2, upregulation of pro-apoptotic bax (Avis *et al.*, 2001; Tong *et al.*, 2002; Lim *et*

al., 2010), inhibition of cell proliferation, and reduced tumor growth (Avis *et al.*, 2001; Gunning *et al.*, 2002; Chen *et al.*, 2003; Melstrom *et al.*, 2008). Similar to the NSAIDs, many of the compounds used to inhibit the 5-LO pathway and LT signaling have off-target effects at high doses that may contribute to anti-tumor activity (Fischer *et al.*, 2010). One study has shown that the genetic deletion of 5-LO in the transgenic $APC^{\Delta 468}$ mice model of colon polyposis resulted in a reduced number and size of intestinal polyps, with a reduced proliferation and an increased apoptosis (Cheon *et al.*, 2011). The study observed an even more pronounced effect when 5-LO was deleted only in hematopoietic cells, suggesting a paracrine effect wherein leukocytes produce LTs that potentiate the proliferation of intestinal epithelial cells (Cheon *et al.*, 2011).

1.5 CANCER-RELATED INFLAMMATION

Smoldering inflammation is an essential component of the tumor microenvironment, independent of the trigger for development that can contribute to all the hallmarks of cancer (Mantovani *et al.*, 2008; Hanahan and Weinberg, 2011). It supplies the tumor cells with growth and survival factors that sustain proliferation and inhibit cell death. It promotes angiogenesis, invasion, and metastasis. Smoldering inflammation suppresses the initiation of adaptive immune responses that could potentially carry out surveillance and eradicate nascent tumors, and it also affects the tumor's response to therapy (Mantovani *et al.*, 2008; Hanahan and Weinberg, 2011).

An inflammatory tumor microenvironment can be initiated by two different pathways: an extrinsic pathway or an intrinsic pathway (Mantovani *et al.*, 2008). The extrinsic pathway is driven by chronic inflammation and infections that increase the risk of developing certain cancers, while the intrinsic pathway is driven by oncogenes that activate transcriptional programs involved in inflammation.

1.5.1 Tumor-infiltrating immune cells

Various leukocytes of both the innate and adaptive immune system are frequently present within tumors (Mantovani *et al.*, 2008). Many of these cells have an intrinsic ability to exert both pro- and anti-tumorigenic activities (Biswas and Mantovani, 2010; Grivennikov *et al.*, 2010). The inflammatory tumor milieu attracts and induces both suppressive and pro-inflammatory immune cell phenotypes and guides their activities in favor of tumor growth and progression (Mantovani *et al.*, 2008; Grivennikov *et al.*, 2010).

Key players in normal inflammatory responses and in cancer-related inflammation are the macrophages. Tumor-associated macrophages (TAM)

generally have a tolerant M2 phenotype that promotes tumor progression (Mantovani *et al.*, 2008). TAM influences nearly all aspects of carcinogenesis and tumor growth, including increased genomic instability and cell proliferation, the promotion of angiogenesis and lymphangiogenesis, the degradation and remodeling of the extracellular matrix that facilitates migration, as well as invasion, metastasis, and suppression of adaptive immune responses (Biswas and Mantovani, 2010). The inflammatory tumor microenvironment also accumulates and activates myeloid-derived suppressor cells (MDSCs) and regulatory T cells (Treg) that strongly suppress innate and adaptive anti-tumor immunity, thereby facilitating tumor growth (Ostrand-Rosenberg and Sinha, 2009; DeNardo *et al.*, 2010). Additionally, tumors often contain small numbers of dendritic cells that are essential for the activation of T cells and adaptive immune responses against tumors. The dendritic cells within tumors usually have an immature phenotype that is unable to stimulate an anti-tumor response (Gabrilovich, 2004). Another important mechanism by which tumors subvert an effective anti-tumor response of the adaptive immune system is by inducing a T helper 2 (Th2)-type of response that suppresses cytotoxic T lymphocytes (CTL) responses in combination with an increased humoral immunity (Johansson *et al.*, 2008).

1.5.2 Inflammatory signaling molecules

Cytokines, chemokines, and eicosanoids are important mediators involved in the crosstalk and reciprocal interactions between cancer cells and immune cells. These mediators are critically involved in the evolution of an inflammatory tumor microenvironment.

1.5.2.1 Cytokines

Cytokines are proteins that are involved in the regulation of immune responses. By binding to cell surface receptors, they can alter the behavior and properties of immune cells. Different cytokines can have pro-inflammatory or anti-inflammatory effects, depending on the type of cell interaction and other coexisting signaling molecules (Lin and Karin, 2007). The tumor microenvironment is often dominated by pro-inflammatory, immunosuppressive Th2 cytokines such as interleukin (IL)-1β, IL-4, IL-5, IL-6, IL-10, IL-13, and tumor necrosis factor α (TNFα; Dranoff, 2004) that induce programs promoting tumor progression.

1.5.2.2 Eicosanoids

PGE_2 is a very powerful inflammatory mediator that contributes to the buildup of an inflammatory tumor microenvironment, and it is the only PG or LT that has a clear role in tumor immunosuppression (Wang and Dubois, 2010). PGE_2 can be produced by cancer cells and also by various immune cells such as macrophages, dendritic cells, and Treg cells (Harris *et al.*, 2002).

One important function of PGE_2 is the modulation of cytokine production. PGE_2 enhances the production of Th2-type cytokines while inhibiting the production of anti-inflammatory cytokines (Harris *et al.*, 2002).

PGE_2 can also exert a direct effect on various immune cells. It inhibits the anti-tumor activity of macrophages and favors an M2 polarization of TAM (Harris *et al.*, 2002; Eruslanov *et al.*, 2009). PGE_2 attracts MDSCs and increases their suppressive activity (Sinha *et al.*, 2007), and, by upregulation of the transcription factor FOXP3, PGE_2 enhances the inhibitory function of Treg cells (Baratelli *et al.*, 2005). PGE_2 also decreases the number of tumor-infiltrating dendritic cells (Ahmadi *et al.*, 2008), prevents the differentiation and maturation of these cells, and downregulates antigen presentation and cross-presentation pathways (Yang *et al.*, 2003; Ahmadi *et al.*, 2008), resulting in an abrogated anti-tumor-specific CTL response (Ahmadi *et al.*, 2008). Moreover, PGE_2 directly inhibits the anti-tumor activity of CTLs (Zeddou *et al.*, 2005) and natural killer cells (Joshi *et al.*, 2001). PGE_2 has also been demonstrated to promote the expansion of Th17 cells, which may contribute to inflammation and tumor growth (Wang and Dubois, 2010).

The role of LTs in cancer-related inflammation has not been thoroughly studied, though as discussed above, LTs play an important role in normal inflammatory processes by increasing vascular permeability and attracting and activating leukocytes, and could potentially exert the same effects if present in the tumor microenvironment. In fact, one study shows that LTB_4 attracts MDSCs to the tumor and increases their suppressive activity (Cheon *et al.*, 2011).

Because of limitations in technology, very few studies have investigated the eicosanoid profile of the tumor microenvironment. One study measured the concentration of six different eicosanoids in ovarian cancer tissues, and only PGE_2 was significantly more abundant in malignant tissues than in benign tissues (Freedman *et al.*, 2007). The study also reported that the combined level of PGE_2, 12-HETE, 5-HETE, and LTB_4 increased during tumor progression. In contrast, no significant change was observed for 15-HETE and 13-HODE, which might suppress tumor growth. Another study examined the profile of LO-derived products during colorectal tumorigenesis (Shureiqi *et al.*, 2010) and found a significant decrease in 13-HODE during tumor progression, though no change was found in 12-HETE, 15-HETE, or LTB_4 levels.

1.5.3 Neuroblastoma tumor microenvironment

Neuroblastoma tumors contain infiltrating monocytes/macrophages that contribute to cell proliferation and growth. Expression of TAM markers such as CD14, CD16, IL-6, the IL-6 receptor, and TGF-β in primary high-risk

neuroblastoma tumors are inversely correlated with a five-year, event-free survival (Song *et al.*, 2009). TAM stimulate neuroblastoma proliferation, at least in part by the production and release of IL-6 (Song *et al.*, 2009). The cytokine IL-6 appears to play an important role in neuroblastoma (Pistoia *et al.*, 2011). IL-6 helps to promote neuroblastoma growth, protects cells from drug-induced apoptosis, and has been implicated in bone invasion and metastasis (Ara *et al.*, 2009; Song *et al.*, 2009). High-risk neuroblastoma patients with bone marrow metastasis show an increased level of IL-6 in both serum and bone marrow compared to normal patients (Ara *et al.*, 2009). In comparison with other cytokines, IL-6 is expressed at moderate levels in primary neuroblastoma tumors that express large amounts of IL-8, IL-13, interferon γ (IFNγ), and TNFα, but relatively low levels of the typical Th2 cytokines IL-4, IL-5, and IL-10 (Carlson, 2011). In addition, neuroblastoma cells ubiquitously express high concentrations of the ganglioside GD2, which directly inhibits dendritic cell differentiation and activation (Redlinger *et al.*, 2004).

Quite a bit of research has focused on the potential of immunotherapy in neuroblastoma (Seeger, 2011). Recently, a phase III study showed an improved outcome for high-risk neuroblastoma patients when an anti-GD2 monoclonal antibody, in combination with IL-2 and granulocyte-macrophage colony-stimulating factor GM-CSF, was added to the standard retinoic acid maintenance therapy (Yu *et al.*, 2010).

2 AIMS

Neuroblastoma cells are enriched in arachidonic acid (Reynolds *et al.*, 2001) and overexpress COX-2 (Johnsen *et al.*, 2004), but are deficient in the anti-inflammatory fatty acid DHA. The purpose of this thesis was to further investigate the role of arachidonic acid-derived eicosanoids in cancer, particularly in neuroblastoma.

The specific aims of this thesis were:

- To determine whether the COX metabolite PGE_2 and its receptors are important for neuroblastoma cell survival and proliferation.

- To determine whether mPGES-1 is expressed in prostate cancer and whether knockdown of mPGES-1 could prevent tumor growth.

- To determine whether neuroblastoma cells express 5-LO and the leukotriene receptors and investigate the importance of leukotrienes for neuroblastoma cell survival.

- To characterize the tumor microenvironment of transgenic TH-MYCN neuroblastoma tumors to determine the composition and dynamics of immune cells during tumor progression.

- To investigate the expression of COX-1 and COX-2 and the effect of a low-dose ASA treatment on the composition of immune cells in TH-MYCN tumors.

3 METHODS

3.1 PATIENT MATERIAL

3.1.1 Human tissue samples

3.1.1.1 Neuroblastoma tissue samples

All neuroblastoma tissue samples were collected at the Astrid Lindgren Children's Hospital. The diagnoses were established in standard of care, which includes careful histological and biological assessments. Patient and tumor characteristics are summarized in table I in **paper III**. Ethical approval was obtained from the Karolinska University Hospital research ethics committee and the Stockholm regional ethics committee.

3.1.1.2 Prostate tumor tissue samples

The prostate tumor tissue samples were collected at the Karolinska University Hospital. The diagnoses were established in standard of care, and the patient and tumor characteristics are summarized in table I in **paper II**. Ethical approval was obtained from the Karolinska University Hospital research ethics committee and the Stockholm regional ethics committee.

3.1.2 Human cell lines

The neuroblastoma cell lines used are all derived from high-risk patients with tumors having different genetical and biological characteristics. The panel of cell lines used is as follows: IMR-32, SK-N-AS, SK-N-BE(2), SK-N-DZ, SK-N-FI, SK-N-SH, and SH-SY5Y. The myelocytic cell line U-937 was used for control purposes. In **paper II,** prostate cancer cell lines DU145, PC3, and LNCaP and the non-small lung cancer cell line A549 were used. Cells were cultured in Eagle´s MEM (SH-SY5Y), Ham´s F-12 (A549), or RPMI 1640 (all others) medium containing 10% heat-inactivated fetal bovine serum, 2 mmol glutamine, 100 IU/mL penicillin, and 100 μg/mL streptomycin. The cells were grown at 37ºC in a humidified 5% CO_2 atmosphere, split twice weekly, and harvested in log-phase for experimental use.

3.2 EX VIVO

3.2.1 Immunohistochemistry

Formalin-fixed and paraffin-embedded tissue sections were used for detection of proteins. Tissue sections on glass slides were deparaffinized and rehydrated before microwave-citrate antigen retrieval. After blocking in bovine serum albumin (BSA) sections were incubated with primary antibody

at 4ºC overnight. The next day, endogenous peroxidases were blocked using hydrogen peroxide, and then a SuperPicTure Polymer detection kit (Invitrogen, Paisley, UK) was used with the appropriate secondary antibody coupled to horseradish peroxidase (HRP) together with a 3,3´-diaminobenzidine tetrahydrochloride substrate-chromogen system to visualize immunopositivity. All slides were counter-stained with hematoxylin and photographed in a light microscope. As a control for non-specific binding, matched isotype controls and antibody-specific blocking peptides were used. Tissue sections were also stained with hematoxylin and eosin (H&E) to show the tumor architecture.

3.3 IN VITRO

3.3.1 Immunoblotting

Cells were harvested and pelleted and the proteins were extracted using RIPA buffer (Cell Signaling, Beverly, MA, USA) or M-PER buffer (Thermo Fisher Scientific, Göteborg, Sweden) supplemented with a cocktail of protease inhibitors (Roche Diagnostics, GmbH, Mannheim, Germany). For extraction of proteins from tissue, the samples were also sonicated. The protein concentration was determined using Bradford reagent (Bio-Rad, Sunbyberg, Sweden), and equal amounts were separated by SDS-PAGE and transferred to nylon or nitrocellulose membranes. The membranes were blocked in 5% BSA or dry milk before the primary antibody was added and were then incubated overnight at 4ºC. After washing, the membranes were incubated with an appropriate secondary antibody conjugated with HRP. Pierce Super Signal (Pierce, Rockford, IL, USA) or ECL plus (GE Healthcare, Uppsala, Sweden) were used for detection. As a control for equal protein loading, all membranes were probed with an anti-β-actin antibody.

For detection of phosphorylated proteins, all solutions added after trypsinization were cold and the samples were kept on ice. In addition, two phosphatase inhibitors, NaF and NaO_2V_4, were added to the RIPA buffer.

3.3.2 Immunofluorescence

Cells were grown on chamber slides, then washed and fixed in cold methanol or in 2% paraformaldehyde (PFA) followed by 70% methanol. After washing, slides were incubated overnight at 4ºC with the primary antibody. Thereafter, a matched fluorescent secondary antibody was added and samples were analyzed in a fluorescence microscope. To show the cell nucleus, a mounting medium containing DAPI was used.

3.3.3 Fluorescence-activated cell sorting analysis (FACS)

3.3.3.1 Analysis of BLT1 expression

Cells were harvested, washed, and pelleted before a phycoerythrin (PE)-conjugated BLT1 antibody or a PE-conjugated isotype matched control was added. After incubation, cells were washed twice and analyzed on the FL2 channel on a FACS Calibur flow cytometer using Cell Quest software (BD Biosciences, San Jose, CA, USA).

3.3.3.2 Measurement of the mitochondrial transmembrane potential

After treatment in six-well plates, cells were incubated in tetramethylrhodamine ethyl ester (TMRE; Molecular Probes, OR, USA) for 30 min prior to harvesting. Cells were harvested using the standard protocol with reagents containing 25 nM TMRE. The mitochondrial transmembrane potential was measured directly on the FL2 channel on a FACS Calibur flow cytometer using Cell Quest software (Becton-Dickinson).

3.3.3.3 Analysis of cell DNA content

After treatment in six-well plates, both detached and attached cells were harvested. The cells were fixed in 4% PFA at room temperature overnight, pelleted, and suspended in 95% ethanol. Thereafter samples were rehydrated in water prior to treatment with subtilisin Carlsberg solution (Sigma Aldrich, Stockholm, Sweden) and staining with DAPI-sulforhodamine solution. The samples were analyzed above 435 nM on a PAS II flow cytometer (Partec, Münster, Germany), and the multicycle program for cell cycle analysis (Phoenix Flow Systems, San Diego, CA) was used for histogram analysis.

3.3.3.4 Analysis of TH-MYCN tumor single-cell suspensions

Tumor tissue was mechanically digested in 0.1% phosphate buffered saline (PBS)/BSA using 100 μm cell strainers to get single-cell suspensions. The cells were washed and pelleted and the red blood cells were removed by incubation in a red blood cell lysis buffer (BD PharmLyse, BD Biosciences). After washing, 300.000 cells were incubated in a mouse-specific Fc receptor block (BD Biosciences) before fluorescently-labeled antibodies were added. For intracellular stainings, fixation and permeabilization was performed using cytofix/cytoperm (BD Biosciences) according to the manufacturer's instructions. Cells were stained for 30 min, washed, and subsequently analyzed on an LSR II flow cytometer (BD Biosciences) using Flow Jo software (Treestar, Ashland, OR, USA). Prior to FACS analysis, an aqua dead cell marker (Invitrogen) was added to all samples. As a control for unspecific staining, isotype matched controls or fluorescence minus one was used.

Ex vivo stimulation was performed in 12-well culture plates. Two million cells from tumor-single cell suspensions were incubated in RPMI medium

containing 5 ng/mL phorbol 12-myristate 13-acetate (PMA) and 10 μM ionomycin. After one hour of incubation, GolgiStop (BD Biosciences) was added and the cells were further incubated for five hours. Thereafter, the cells were washed and subjected to antibody staining.

3.3.4 Polymerase chain reaction (PCR)

3.3.4.1 PCR

Pups were earmarked and biopsied at 14 days of age. DNA was extracted from the tissue using the DNeasy Blood and Tissue kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. After extraction, DNA concentration and purity was determined using a nano-drop. Two PCR reactions were prepared for each DNA sample, one containing primers detecting the wild type allele, and one containing primers to detect the transgenic allele. The two PCR reactions were run in a GeneAmp PCR System 2400 (Applied Biosystems, Branchburg, NJ) and amplified using the same temperatures and cycle numbers. The PCR products were loaded in separate wells on a 1.5% agarose gel containing ethidium bromide or GelRed (Biotium, Hayward, CA, USA) and photographed under UV light.

3.3.4.2 Reverse transcription-PCR

Cells were harvested and pelleted and total RNA was extracted using Trizol reagent according to the manufacturer's protocol (Life Technologies Inc., Carlsbad, CA, USA). For cDNA synthesis, 2 μg of RNA was reverse transcribed using a SuperScript Preamplification kit (Life Technologies). PCR was performed using different amounts of cDNA, and the PCR conditions were optimized for each primer pair. The PCR was performed in a PTC-200 Peltier Thermal Cycler (MJ Research Inc., Waltham, MA, USA), and the final products were analyzed using agarose gel electrophoresis and photographed under UV light.

3.3.4.3 Real-time reverse transcriptase-PCR

Tumor tissue was collected and stored at -80ºC. RNA was prepared from 30 mg of tissue by mechanical dissociation using 100 μm cell strainers and an RNeasy mini-kit (Qiagen) according to the manufacturer's instructions. 1 μg of total RNA was used for cDNA synthesis using a High Capacity RNA-to-cDNA kit (Applied Biosystems). The PCRs were performed in MicroAmp optical 96-well plates, in a total volume of 25 μL of reaction mixture containing TaqMan Universal PCR Master Mix, TaqMan Gene Expression Assay (Applied Biosystems), and cDNA. 10 μL of cDNA was used for detection of the target gene, and 2 μL of cDNA was used for detection of the reference gene, hypoxanthine-guanine phosphoribosyl transferase (HPRT). All samples were run in triplicate and all experiments included a no template

control. PCR amplification was performed in an ABI PRISM 7500 sequence detection system (Applied Biosystems) and analyzed using 7500 software v2.0.4 (Applied Biosystems). From standard curves using a pooled cDNA sample, the efficiency of amplification for each primer was calculated. Each sample was corrected for the efficiency and the ΔCT value between the target gene, and HPRT was calculated as $\Delta CT = CT_{target} - CT_{HPRT}$.

3.3.5 Cell viability assays

3.3.5.1 MTT assay

To evaluate the impact of PGs and LTs on neuroblastoma cell viability, cells were seeded in 96-well plates and cultured overnight to attach. The next day, the medium was changed to RPMI medium without serum, and the cells were starved for 24 hours. Then the medium was changed to a fresh serum-free RPMI medium containing LTD_4, LTB_4, or 16,16-dimethyl PGE_2 ($dmPGE_2$), a stable PGE_2 analogue. Plates were incubated for 24-96 hours and the medium was changed every second day. At final time points, cells were subjected to MTT assay, previously described by Mosmann (Mosmann, 1983). The cell medium was removed and a serum-free medium containing 0.5 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoleum bromide (MTT, Sigma Aldrich) was added. The plates were incubated for 2-3 hours at 37°C, after which acidified isopropanol was added to dissolve the crystals. The plates were covered in aluminum foil and stored at 4°C overnight before absorbance was measured using a microplate reader at 595 nm. The viability ratio between control and treated cells was calculated.

To investigate the effect of enzyme inhibitors and receptor antagonists on cell growth, neuroblastoma cells were incubated in the selected compounds or drug combinations, diluted in OptiMEM medium, as indicated. The cells were treated for 48 hours before they were subjected to MTT assay.

3.3.5.2 WST-1 assay

Prostate cancer cells were seeded in 96-well plates and cultured overnight. The next day, different concentrations of PGE_2 were added just before the addition of 300 nM of adriamycin. PGE_2 was added again after 12 hours of incubation. After 24 hours, cells were washed in PBS and 2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium (WST-1, Roche) was added. The plates were incubated at 37 °C for two hours and the absorbance was measured at 450 nM and 620 nM. The values are given as absorbance at 450 nM (the formazan product) minus the absorbance at 620 nM (reference).

3.3.6 Clonogenic assay

One hundred and fifty cells per well were seeded in 50 mm^2 Cell+ Petri dishes. After 12 days of incubation, plates were rinsed with PBS, and the cells were fixed in PFA and stained with Giemsa (Gibco BRL, Sundbyberg, Sweden). Colonies with more than 75 cells were counted manually using a colony counter.

3.3.7 Apoptosis assay

Prostate cancer cells were seeded in 96-well plates and allowed to attach overnight. The following day, the medium was changed to a fresh medium containing 300 nM adriamycin. After 24 hours of incubation, 10% NP40 was added to lyse the cells. The amount of apoptotic cells was determined by measuring the amount of soluble caspase-cleaved fragments of cytokeratin 18, using the M30 Apoptosense ELISA according to the manufacturer's instructions (Peviva, Bromma, Sweden).

3.3.8 Gene silencing

3.3.8.1 Small interfering RNA (siRNA)

Neuroblastoma cells were seeded in six-well plates and 15 cm Petri dishes and cultured to a 30-50% confluence. Cells were transfected with target or non-targeting siRNA (Santa Cruz Biotechnology, Santa Cruz, CA, USA) using Lipofectamin 2000 (Invitrogen) according to the manufacturer's instructions. Seventy two hours after the initial transfection, cells were harvested and subjected to counting using tryphan blue exclusion, western blotting, or measurement of leukotriene production using enzyme immune assay (EIA), as described below. To measure the transfection efficiency, cells were transfected with a fluorescein-labeled non-targeting siRNA. These cells were harvested, washed, and analyzed on the FL1 channel on a FACS Calibur flow cytometer using Cell Quest software (BD Biosciences).

3.3.8.2 Small hairpin RNA (shRNA)

Prostate and lung cancer cells were seeded in six-well plates. Cells were transfected with vector plasmid (MISSION pLKO.1-puro, Sigma Aldrich) encoding mPGES-1 specific shRNA or non-targeting shRNA, using Lipofectamin 2000 (Invitrogen) according to the manufacturer's protocol. By culturing the cells in 8 μg/mL puromycin, stably transfected clones could be isolated. As a control for successful downregulation, expression of mPGES-1 was evaluated using western blotting and real-time RT-PCR, and the mPGES-1 enzyme activity was measured as described below.

3.3.9 Intracellular calcium mobilization

Cells were seeded on cover slips in Petri dishes and cultured overnight. The medium was changed to a Krebs-Ringer solution containing 5 μM of the calcium fluorescent dye Fluo-4/AM (Invitrogen), and the cells were incubated for 30 min at 37°C. Thereafter, cells were washed with Krebs-Ringer solution and examined with a confocal laser scanning microscope (Zeiss LSM 510 META). A 5-min baseline, with or without 2 mM of ethylene glycol tetraacetic acid (EGTA), was determined for each sample before 1 μM of dmPGE$_2$ was added.

3.3.10 Intracellular concentration of cAMP

Cells were seeded in Petri dishes and cultured for 48 hours. The medium was changed to a serum-free RPMI medium and the plates were incubated overnight before, the indicated compounds were added. After treatment, cells were rinsed with PBS and lysed in 0.1 M HCl at room temperature. The plates were scraped, the lysate was collected and centrifuged and the supernatant was used for intracellular cAMP determination using EIA, according to the manufacturer's protocol (Cayman Chemical, Ann Arbor, MI, USA).

3.3.11 Lipid measurements

3.3.11.1 Liquid chromatography – tandem mass spectrometry (LC-MS/MS)

Cells were cultured in clear OptiMEM medium with or without 40 μM of arachidonic acid and 10 ng/mL of IL-1β for 12-48 hours. At the end of incubation, cells were harvested, washed in PBS, and counted using tryphan blue exclusion. 8-20 million cells were suspended in PBS supplemented with protease inhibitors, sonicated on ice, and further incubated in 80 μM of arachidonic acid for 30 min at 37°C. Awaiting further analysis samples were stored at -80°C. To extract the lipids from the cell homogenates, a liquid-liquid extraction method described previously (Kempen *et al.*, 2001) was used. Briefly, internal standard PGE$_2$-d$_4$ was added to each sample before an addition of 1:1 v/v hexane:ethyl acetate. Samples, standards, and controls were mixed and centrifuged, and the upper organic phase was collected, whereas the lower water phase was extracted twice more. Stock solutions of PGE$_2$ and PGD$_2$ were prepared in methanol and further diluted in PBS for preparation of standards. The chromatic system consisted of Waters Acquity UPLC system (Waters Corp., Milford, MA, USA) and liquid chromatography was performed on a 2.1 x 100 mm Waters Acquity HSS T3 (C$_{18}$) UPLC column maintained at 50°C with a programmed gradient from solvent A (methanol/water/ammonium acetate of 10/90/2 mM, v/v/concentration) to

B (methanol/water/ammonium acetate 90/10/2 mM, v/v/concentration) at 0.4 ml/min to resolve PGE_2 from PGD_2.
Mass spectrometry was performed using a Waters Quattro Premier XE tandem quadrupole mass spectrometer (Waters Corp). The system was controlled by MassLynx version 4.1 and the ionization was achieved using electrospray in a negative ionization mode. The injection volume was 15 µl and the injection interval was 7 min. LC-MS/MS was performed with an autosampler temperature of 5°C, a desolvation gas temperature of 340°C, a source temperature of 120°C, a desolvation gas flow of 900 L/h, a cone gas flow of 40 L/h, a collision gas pressure of 3.5×10^{-3} mBar (argon), and ion energies of 0.9 V for both quadrupoles. Quantitative analysis was performed in the multiple reaction monitoring (MRM) mode with the following transitions: m/z 351→315 (quantification ion), 351→271 (qualifier ion), and 351→315 (qualifier ion). For the internal standards, MRM transitions m/z 355→319 and 355→275 were used. The dwell time was set to 20 ms for each transition. The method demonstrated a good linearity and reproducibility with a correlation coefficient (r^2) of >0.99 and a coefficient of variation of <10%.

3.3.11.2 High-performance liquid chromatography (HPLC)

The enzymatic activity of mPGES-1 was determined using a method previously described (Thoren and Jakobsson, 2000). Harvested and pelleted cells were resuspended in a 0.1 M potassium phosphate buffer, supplemented with a cocktail of protease inhibitors, and sonicated three times on ice. Homogenates were subjected to differential centrifugation at 10.000 x *g* for 15 min and at 100.000 x *g* for two hours, at 4°C. Microsomal fractions were resuspended in a potassium buffer containing 10% v/v glycerol, and an aliquot of 25 µg protein was removed and incubated with 10 µM of PGH_2 for 10 min on ice. PGH_2 was produced by stirring 1 mM of arachidonic acid in a suspension of the microsomal fraction of whole homogenates of sheep vesicular glands, together with L-tryptophan. After 2 min, PGH_2 was extracted with diethyl ether and purified by normal-phase HPLC. The solvent system consisted of 2-propanol-hexane-acetic acid (2.5/97.5/0.002; v/v/v) and afforded a purity of >95%. PGH_2 was dissolved in dry aceton and stored at -80 °C for further use. During the experiment, PGH_2 was kept on dry ice. The reaction was stopped by addition of a iron (II) chloride solution supplemented with 11-β-PGE_2 as an internal standard to estimate the extraction efficiency. The samples were further subjected to solid-phase extraction using Oasis HLB columns (Waters Corp). The samples were separated by reversed-phase HPLC on a Waters 2790 HPLC system equipped with a NovaPak C18 column (3.9 mm x 150 mm x 4 µm) and eluated with water/acetonitrile/formic acid (62/38/0.0025; v/v/v) at a flow rate of 0.3 mL/min. The products were monitored using UV light on a Waters 996 diode array detector at 195 nm. Standards of different

concentrations were processed in the same way, and the product was quantified by integration of the peak areas.

3.3.11.3 Enzyme immunoassay (EIA)

For measurement of LT production, tumor tissue and cells were suspended in Ca^{2+}- and Mg^{2+}-free PBS supplemented with a cocktail of protease inhibitors (Roche). Samples were homogenized and sonicated three times on ice prior to the addition of 2 mM ATP, 2 mM Ca^{2+}, and 80 μM of arachidonic acid. After incubation for 10 min at 37°C, the reaction was quenched with an equal volume of methanol and acidified to pH 3-4 for detection of LTB_4 or to pH 5-6 for detection of CysLTs. Thereafter, samples were purified and concentrated by solid-phase extraction (Supelclean LC-18, Sigma Aldrich), eluted in methanol, and dried under nitrogen. The residue was reconstituted in an EIA buffer and subjected to EIA for determination of the concentration of LTB_4 and CysLTs according to the manufacturer's protocol (Cayman Chemicals).

3.4 IN VIVO

3.4.1 Xenograft mouse model

Female athymic mice NMRI nu/nu (Taconic Laboratories, Ejby, Denmark), four to eight weeks old, were used for *in vivo* xenograft experiments. Mice were housed and maintained under special pathogen-free conditions with access to sterile water and food *ad libitum*. DU145 and A549 xenografts were established by injection of 2 $x10^6$ or 5 $x10^6$ cells, respectively, subcutaneously in both flanks under general anaesthesia (2-4% Isofluran). For each cell line, wild type, mPGES-1 knocked down and non-targeting shRNA control cells were injected into different mice. Tumor development and growth was monitored every day. The size of the tumor was measured with a digital calliper, and the volume was calculated by the formula $width^2$ x length x 0.44. A tumor was considered to be established when it had grown to a volume of 0.2 mL and the mice were followed for tumor development for up to 72 days after the tumor cell injection. The mice were euthanized in carbon dioxide and the tumor was excised, weighted, divided, and snap frozen in liquid nitrogen for storage at -80°C, or fixed in 4% PFA and stored at 4°C. All animal experiments were approved by the regional ethics committee and in accordance with the Swedish national regulations, including the Animal Protection law (SFS1988:534), the Animal Protection regulation (SFS1988:539), and the Regulations for the Swedish National Board for Laboratory Animals (SFS1988:541).

3.4.2 The TH-MYCN transgenic model

Transgenic TH-MYCN mice spontaneously develop neuroblastoma due to a targeted expression of the MYCN oncogene to migrating cells of the neural crest (Weiss *et al.*, 1997). The animals develop predominantly palpable abdominal tumors, but thoracic tumors may also occur. In our hands, 100% of the homozygous develop tumors within seven weeks of age and 50% of the hemizygous mice develop tumors within 6-19 weeks (Rasmuson *et al.*, unpublished). The animals were obtained from the Mouse Model of Human Cancer Consortium Repository as an N16 backcross to the 129X1/SvJ background, and have since been kept as a continuous inbreeding. Breeders were kept in harems, and pups were earmarked and biopsied at two weeks of age. The animals were housed at a maximum of six per cage in an enriched environment with access to food and water *ad libitum*. At 4.5 weeks of age, homozygous mice were randomized to receive either no treatment or a daily treatment with 10 mg/kg ASA (Sigma Aldrich) by oral gavage for 10 consecutive days. All animals underwent abdominal palpations two to three times weekly to monitor tumor development. Upon palpable tumor, the animals were monitored closely and euthanized upon signs of discomfort, tumor-related problems, or heavy tumor burden. At sacrifice, the animals were euthanized using carbon dioxide and cardiac blood was drawn. The blood was put into EDTA tubes and centrifuged at 3000 rpm for 10 min, and the cell component was collected used for FACS analysis. The tumor was excised and put in PBS on ice, weighted and divided for: FACS analysis, fixation in 4% PFA or storage at -80°C for further analysis. All animal experiments were approved by the regional ethics committee and in accordance with the Swedish national regulations.

3.5 STATISTICAL ANALYSIS

For the *in vitro* studies, parametric tests were used to determine statistically significant differences. Comparisons between two independent groups were analyzed by t-test, the data was log transformed when indicated, and several independent groups were analyzed by two-way ANOVA. One sample t-test was performed to test if the mean of a single sample was significantly different from the control. For the *in vivo* studies, non-parametric tests were used. The Mann-Whitney U-test was performed for the comparison between two independent groups, and the Kruskall-Wallis test, followed by Dunn's multiple comparison test, was used for the comparison between several independent groups. The Spearman test was performed to determine if a correlation existed between two paired observations. All tests were two-sided, and $p<0.05$ was considered significant.

4 RESULTS AND DISCUSSION

4.1 PGE_2 SIGNALING IN CANCER

4.1.1 PGE_2 signaling in neuroblastoma (Paper I)

Our group has previously shown that COX-2 is overexpressed in neuroblastoma (Johnsen *et al.*, 2004) and that therapeutic treatment of neuroblastoma xenografts with diclofenac, a dual COX-1 and COX-2 inhibitor, or celecoxib, a specific COX-2 inhibitor, induces apoptosis and reduces tumor growth (Johnsen *et al.*, 2004). To further investigate why COX-2 is important for neuroblastoma cell survival, we studied the role of PGE_2 and its receptors.

4.1.1.1 Production of PGE_2 and expression of PGE_2 receptors

To determine if neuroblastoma cells produce PGE_2, SK-N-SH, and SK-N-BE(2), cells were incubated with arachidonic acid, the substrate for PG biosynthesis, alone or in combination with the inflammatory mediator IL-1β, and PGE_2 production was measured using LC-MS/MS. The MYCN-amplified, drug-resistant cell line SK-N-BE(2) produced PGE_2 under normal culture conditions, whereas SK-N-SH cells did not produce detectable levels of PGE_2. When the cells were cultured with arachidonic acid, both cell lines produced PGE_2, and stimulation with IL-1β further increased the production. Hence, these results are consistent with earlier findings about the inducible nature of COX-2 and mPGES-1 (Samuelsson *et al.*, 2007). We also investigated if PGE_2 could rescue neuroblastoma cells from apoptosis induced by COX-2 inhibition. Treatment of SK-N-BE(2) cells with 35 μM celecoxib resulted in a 70% reduction in cell viability. A concomitant addition of PGE_2 rescued the cells from celecoxib-induced cytotoxicity, and the cell viability after 48 hours of incubation was 86% of the control. To further determine whether neuroblastoma cells expressed any of the PGE_2 receptors, EP1-EP4, 28 neuroblastoma primary tumors were evaluated using immunohistochemistry, and a panel of seven neuroblastoma cell lines was analyzed with RT-PCR, western blotting and immunofluorescence. Expression of all four receptors was detected in all primary tumors and cell lines investigated, independent of biological characteristics and clinical stage. In addition, some expression was also detected in the adjacent stroma. The immunofluorescence staining revealed that all four receptors are expressed at the cell membrane, and that EP1 and EP4 are also expressed in the nuclear compartment.

The existence and differential expression of EP1-EP4 enable a wide range of PGE_2 actions. Our results here agree with our previous study on the childhood brain tumor medulloblastoma, which also shows expression of all

four receptor subtypes (Baryawno *et al.*, 2008), and with the many studies on adult epithelial cancers that show a complex EP receptor profile that often has several receptors expressed, but at a different level compared to normal tissue (Lee *et al.*, 2005; Miyata *et al.*, 2006; Gustafsson *et al.*, 2007a). Very little is known about the precise role of each EP receptor in malignant behavior. Studies using mice deficient in each of the EP1-EP4 receptors have implicated all four receptors in different stages of carcinogenesis. However, the results are difficult to interpret because they vary depending on the model used (Fulton *et al.*, 2006).

The production of PGE_2 takes place at the endoplasmic reticulum and at the nuclear envelope (Morita *et al.*, 1995). The presence of nuclear EP receptors indicates that PGE_2 can act intracellularly, and can potentially mediate distinct functions from receptors expressed at the cell membrane, thereby possibly affecting gene transcription (Zhu *et al.*, 2006; Reader *et al.*, 2011). Support for the idea of a different function for nuclear EP receptors comes from a breast cancer study that showed that a nuclear expression of EP1, though not cytoplasmic, is associated with a significantly better prognosis (Ma *et al.*, 2010). The study proposes that EP1 may act to suppress metastatic spread.

4.1.1.2 PGE_2 and neuroblastoma cell survival and proliferation

Because our results demonstrated that neuroblastoma cells express all four receptor subtypes and produce PGE_2, we decided to focus this study on the response of neuroblastoma cells to PGE_2. SK-N-SH and SK-N-BE(2) cells were serum-starved overnight before being incubated with PGE_2 at different concentrations and durations. A significant dose-, time-, and cell line-dependent increase in cell viability and proliferation was observed. To elucidate the mechanisms behind the observed effect on cell viability, and to test the functionality of the receptors, we investigated the activation of second messengers and effectors by PGE_2. We performed confocal laser scanning microscopy, using the calcium fluorescent dye Fluo-4/AM to detect changes in the intracellular calcium concentration, since activation of EP1 results in an increased calcium level. PGE_2 induced a rapid increase in the concentration of cytoplasmic calcium. EGTA, a calcium chelator, did not alter the response, which shows that calcium is released from intracellular stores. Because EP2, EP3, and EP4 act through adenylate cyclase and cAMP, we also investigated the effect of PGE_2 on cAMP. A net response of increased cAMP was observed in neuroblastoma cells after 20 min of PGE_2 incubation. NF-449, a Gαs inhibitor, prevented cAMP accumulation. We also performed western blotting to investigate the effect of PGE_2 on the phosphorylation status of Akt, which is a key protein involved in cell proliferation and the survival of cancer cells and neuroblastoma. Incubation with PGE_2 increased the phosphorylation, thus activating Akt signaling in both neuroblastoma cell lines investigated.

In agreement with our results, one other study has shown that PGE_2 increases the intracellular calcium concentration in cholangiocarcinoma cells (Zhang *et al.*, 2007). Several studies using epithelial cancer cell lines have confirmed functional EP2 and EP4 receptors (Castellone *et al.*, 2005) and a subsequent generation of cAMP in response to PGE_2 (Chang *et al.*, 2005; Subbaramaiah *et al.*, 2008). cAMP has been shown to increase proliferation in some cancer cells, whereas in other cancer types cAMP induces differentiation (Borland *et al.*, 2009; Naviglio *et al.*, 2009). Neuroblastoma cells have been shown to contain very low levels of cAMP, and incubation with dibutyryl cAMP, a cAMP analog, induces differentiation *in vitro* (Prasad *et al.*, 2003). In addition, retinoic acid-differentiated neuroblastoma cells respond to PGE_2 by an elevated production of cAMP compared to control cells (Prasad *et al.*, 2003). To my knowledge, only one other study has investigated the role of PGE_2 receptors in neuroblastoma, and it revealed that incubation with butaprost, an EP2 receptor agonist, or overexpression of the EP2 receptor with a concomitant increase in cAMP, reduced cell growth and induced apoptosis (Sugino *et al.*, 2007). Taken together, our results show that neuroblastoma cells respond to PGE_2 by the activation of several EP receptors. However, PGE_2 receptor signaling is more complex than the effect on calcium and cAMP. For instance, the receptors often couple to more than one type of G-protein, the βγ subunits can activate downstream effectors and G-protein independent signaling has been reported (Hirata and Narumiya, 2011).

Numerous studies have shown that PGE_2 increases the proliferation of cancer cells of epithelial origin (Wang and Dubois, 2010). Both this current study and our previous study on medulloblastoma (Baryawno *et al.*, 2008) show that PGE_2 also increases the proliferation of cancer cells derived from pediatric tumors of neural origin. PGE_2 has been shown to activate many different pathways in epithelial cancers that promote proliferation and survival and inhibit apoptosis, such as Akt, Erk, Wnt, EGFR, and the downregulation of Bcl-2 (Wang and Dubois, 2010). In neuroblastoma, the activation of Akt has been demonstrated to correlate with a poor prognosis (Opel *et al.*, 2007), and the inhibition of Akt signaling represents a promising target for neuroblastoma therapy (Sartelet *et al.*, 2008). Akt is an important regulator of various cellular processes and stimulates protein synthesis, cell growth, and proliferation while inhibiting apoptosis (Sartelet *et al.*, 2008). Here, we found that PGE_2 activates Akt in neuroblastoma.

PGE_2 released from neuroblastoma cells could also influence the behavior of immune cells and other stromal cells within the tumor to help subvert an anti-tumor response and promote the establishment of an immunosuppressive tumor microenvironment. Experimental data indicates that metastatic neuroblastoma cells present in the bone marrow produce PGE_2 (Ara *et al.*, 2009) and galectin-3 (Song *et al.*, 2009). This stimulates bone

marrow stromal cells to produce IL-6, which in turn activates ostoclasts and thereby facilitates bone invasion (Sohara *et al.*, 2005). In addition, IL-6 released by the bone marrow stromal cells stimulates neuroblastoma cell proliferation and survival (Ara *et al.*, 2009; Song *et al.*, 2009).

An alternative approach for targeting COX-2 activity to inhibit PGE_2 signaling is to block the binding of PGE_2 to its receptors. To determine if any of the receptors are crucial for neuroblastoma cell survival, we treated neuroblastoma cells with a panel of EP receptor antagonists at different concentrations for 48 hours. The most effective drugs in reducing cell viability were antagonists that inhibited signaling through the EP1, EP3, and EP4 receptors. Because of a lack of specific antagonists, the EP2 receptor could not be specifically inhibited. These results indicate that more than one receptor is important for neuroblastoma cell viability, though the results are also dependent on differences in affinity and the specificity of the antagonist for the respective receptor.

Together, these data show that PGE_2 acts as an autocrine and/or paracrine proliferation and survival factor for neuroblastoma, and suggests that the specific inhibition of PGE_2 signaling may be effective in neuroblastoma treatment.

4.1.2 mPGES-1 in prostate and lung cancer (Paper II)

Unlike with the other PGE synthases and COX-1, the expression of both mPGES-1 and COX-2 can be induced by inflammatory stimuli, and both are inhibited by treatment with glucocorticoids. mPGES-1 has been suggested to predominantly couple with COX-2 (Jakobsson *et al.*, 1999; Thoren and Jakobsson, 2000); therefore, inhibition of this enzyme may represent a more specific target for preventing both PGE_2 signaling in cancer and the side effects of COX-2 inhibitors.

4.1.2.1 Expression of mPGES-1

To investigate if mPGES-1 is expressed in prostate cancer, tumor tissues were analyzed with western blotting, and three cell lines were analyzed using western blotting and immunofluorescence. We observed a differential expression of mPGES-1 in prostate cancer, with a tendency towards a higher expression in malignant tumors. Two out of three prostate cancer cell lines, DU145 and PC3, constitutively expressed detectable levels of mPGES-1 in a noninducible manner, and the expression level correlated well with the production of PGE_2. However, the expression of mPGES-1 can also be inducible in cancer cells, as exemplified by the non-small lung cancer cell line, A549.

An intermediate expression of mPGES-1 has been detected in normal prostate tissue (Jakobsson *et al.*, 1999). In fact, PGs are named after the prostate gland because they were first discovered in seminal fluid and were therefore thought to be produced in the prostate gland, though, PGs were later found to be produced in seminal vesicles. The expression of mPGES-1 has also been detected in various other epithelial cancers, such as those of the colon, pancreas, cervix, and stomach; and in non-small cell lung cancer; but also in brain tumors such as glioma (Nakanishi *et al.*, 2010) and medulloblastoma (Baryawno *et al.*, 2008).

The expression of mPGES-1 and COX-2 can be induced by inflammatory stimuli such as IL-1β, TNFα, and lipopolysaccharides, with a tandem induction of mPGES-1 and COX-2 being most often observed (Samuelsson *et al.*, 2007). However, cancer tissues frequently express mPGES-1 and COX-2 differentially, even in individual tumors (Samuelsson *et al.*, 2007). Here, we show that the prostate cancer cell line, DU145, constitutively expressed high levels of mPGES-1. However, immunofluorescence stainings revealed a translocation of mPGES-1 to the nucleus upon IL-1β stimulation, which could promote PGE_2 production. In contrast, COX-2 is expressed at a low level in these cells, and the expression can be induced by IL-1β stimulation. Similarly, another study has shown the differential constitutive expression of mPGES-1 and an inducible expression of COX-2 in gastric cancer cell lines (van Rees *et al.*, 2003). On the other hand, we show here, and others have shown previously, that stimulation of the A549 lung cancer cell line, with IL-1β (Jakobsson *et al.*, 1999), TNFα (Yoshimatsu *et al.*, 2001), or PMA (van Rees *et al.*, 2003) increases the expression of both mPGES-1 and COX-2. However, two of the previous studies documented that the time course and magnitude of induction of mPGES-1 and COX-2 after incubation with TNFα and PMA were markedly different (Yoshimatsu *et al.*, 2001; van Rees *et al.*, 2003). In contrast, TNFα did not have any effect on mPGES-1 and COX-2 in non-transformed control cells, but in these cells an overexpression of the oncogene *Ras* could enhance the transcription of mPGES-1 (Yoshimatsu *et al.*, 2001). In summary, these results show that the expression of mPGES-1 and COX-2 is controlled by different pathways in cancer cells, and indicate that the regulation is not even similar in all cancer cells.

PGE_2 can also be produced by transcellular metabolism, and A549 cells can take up PGH_2 that is released from human umbilical vein endothelial cells and further convert it to PGE_2 (Salvado *et al.*, 2009). Consequently, a concomitant expression of mPGES-1 and COX-2 may not be necessary for cancer cells to produce PGE_2.

4.1.2.2 Phenotypic changes due to mPGES-1 knockdown

No clinical trials with mPGES-1 inhibitors have yet been reported. The development of specific mPGES-1 inhibitors has been greatly hampered by

interspecies differences that complicate animal testing and preclinical studies. Potent inhibitors of human mPGES-1 either partially or totally lose their inhibitory effect against the rodent form. Three amino acid residues in human mPGES-1, thr-131, leu-135 and ala-138, correspond to the rat mPGES-1 residues val-131, phe-135 and phe-138, which line the entrance of the active site. These amino acid residues account for most of this effect (Smith *et al.*, 2011).

To study the role of mPGES-1 in tumorigenesis, we established stable DU145 and A549 mPGES-1 knockdown clones by using shRNA. The downregulation of mPGES-1 expression was confirmed by real-time PCR and western blotting, and the production of PGE_2 was reduced by 85-90%. mPGES-1 knockdown clones had a significantly smaller clonogenic capacity than control cells that were transfected with non-targeting shRNA. To investigate the tumorigenic potential of these cells *in vivo*, athymic mice were injected with either DU145 or A549 wild type cells, non-targeting shRNA control cells, or mPGES-1 knockdown cells, respectively. The time to tumor take (0.2 mL) was monitored for all six groups after tumor cell injection. A significant delay in tumor development was observed for mPGES-1 knockdown xenografts, and only nine of 20 injections of DU145 mPGES-1 knockdown cells and five out of 20 injections of A549 mPGES-1 knockdown cells developed into tumors. Tumors from mPGES-1 knockdown xenografts also appeared less proliferative and more necrotic compared to control. An *ex vivo* analysis of mPGES-1 revealed an expression of mPGES-1 in all tumors independent of what cells had been injected, which could be explained by the expression of mPGES-1 in other tumor stromal cells (Kamei *et al.*, 2009; Sasaki *et al.*, 2011).

Thus, the production of PGE_2 by the cancer cells is important for tumor development and growth, although the effect seems to be independent of the expression pattern of mPGES-1. Other studies have shown that chemical carcinogen-induced colon and intestinal tumorigenesis is suppressed in mPGES-1 knockout mice (Nakanishi *et al.*, 2008; Nakanishi *et al.*, 2011; Sasaki *et al.*, 2011) and that these mice show a reduced metastatic bone cancer growth (Isono *et al.*, 2011). However, one study displays the opposite effect, an accelerated intestinal tumorigenesis when mPGES-1 is genetically deleted (Elander *et al.*, 2008). The authors propose that this could be explained by a metabolic shunt towards the production of other pro-tumorigenic PGs and TXA_2 (Elander *et al.*, 2008). In addition, two studies have shown that host-associated mPGES-1 expression also significantly contributes to tumor growth, angiogenesis and metastatic spread (Kamei *et al.*, 2009; Sasaki *et al.*, 2011).

Adriamycin, or doxorubicin, is a chemotherapeutic drug that intercalates DNA and inhibits macromolecular biosynthesis. DU145 mPGES-1

knockdown cells were significantly more sensitive to adriamycin-induced apoptosis than control cells, and the addition of PGE_2 to the cell cultures could rescue the cells from cell death. However, the same effect was not observed for A549 knockdown cells. One possible explanation for this observed difference is the high anti-oxidative capacity of A549 cells, which protects them from genotoxic stress (Radmark and Samuelsson, 2010). No other study has yet investigated the effect of mPGES-1 inhibition on the cytotoxic effect of chemotherapeutic drugs, but studies have shown that celecoxib and NSAIDs can act in synergy with adriamycin (Duffy *et al.*, 1998; Ponthan *et al.*, 2007; Roy *et al.*, 2010).

Together, these results confirm that the inhibition of mPGES-1 may represent an alternative, more specific therapeutic approach for NSAIDs and COX-2 inhibitors in cancer therapy.

4.2 5-LO AND THE LEUKOTRIENES IN NEUROBLASTOMA (PAPER III)

As with the COX enzymes, the main substrate for 5-LO and LT biosynthesis is arachidonic acid. Since neuroblastoma cells are enriched in arachidonic acid (Reynolds *et al.*, 2001), and because LTs are pro-inflammatory mediators, we decided to investigate if the 5-LO pathway is present in neuroblastoma, and if so, clarify the role of this pathway in neuroblastoma cell survival and proliferation.

4.2.1 Expression of the 5-LO pathway and production of leukotrienes

We used immunohistochemistry to analyze 27 neuroblastoma tissue samples, three ganglioneuromas, and three nonmalignant adrenals from children for the expression of enzymes and receptors in the 5-LO pathway. Some neuroblastomas spontaneously differentiate or mature into a benign tumor called a ganglioneuroma. The characteristic of these tumors is that they are stroma rich, with differentiating neuroblasts and/or mature or maturing ganglion cells (Brodeur, 2000). The staining revealed that neuroblastoma tumor cells and tumor-derived ganglion cells of ganglioneuromas express 5-LO, FLAP, LTA_4H, and LTC_4S, as well as the LTB_4 receptor BLT1 and the CysLT receptors CysLT1 and CysLT2. Nonmalignant adrenals demonstrated an expression of 5-LO and FLAP in the cortex but not in the medulla, where neuroblastomas arise. Moreover, nonmalignant adrenals expressed LTA_4H, LTC_4S, and the LT receptors both in the cortex and medulla. Using RT-PCR, western blotting and flow cytometry, we could detect mRNA and protein that corresponded to each of the enzymes and receptors in a panel of seven

neuroblastoma cell lines. Hence, neuroblastoma cells express all enzymes required for the synthesis of both LTB_4 and LTC_4. To determine if neuroblastoma cells could produce LTs, we measured *de novo* production from primary neuroblastoma tumors and cell lines. Indeed, both LTB_4 and CysLTs could be detected, which shows that neuroblastoma cells endogenously produce LTs. In tumor tissue, however, infiltrating leukocytes could also contribute to the total production.

The role of the 5-LO pathway and LTs in the adrenal gland is not fully clear, though it has been suggested that they are involved in hormone production by adrenal cortical cells (Hirai *et al.*, 1985; Schroder *et al.*, 2005). The expression of 5-LO has previously been shown in cells of neuronal origin, and 5-LO has been detected in neural stem cells (Wada *et al.*, 2006) and immature cortical neurons, although to a lesser extent in mature cultures (Uz *et al.*, 2001). Functional studies suggest that 5-LO and LTB_4 are important for the proliferation and differentiation of these cells (Uz *et al.*, 2001; Wada *et al.*, 2006). 5-LO is also expressed in various brain tumors (Boado *et al.*, 1992; Ishii *et al.*, 2009; Lim *et al.*, 2010), and an increased production of LTs is correlated with a more advanced disease (Simmet *et al.*, 1990). Furthermore, 5-LO is also expressed in a variety of epithelial cancers (Steinhilber *et al.*, 2010).

Neuroblastoma expressed LTA_4H and produced LTB_4. Only one other study has investigated and demonstrated an expression of LTA_4H in cancer cells and infiltrating inflammatory cells of esophageal adenocarcinomas, and the study showed that treatment with bestatin, a LTA_4H inhibitor, reduced LTB_4 production and tumor incidence *in vivo* (Chen *et al.*, 2003). Increased levels of LTB_4 have also been detected in oral (el-Hakim *et al.*, 1990) and prostate (Larre *et al.*, 2008) cancer tissues. Here, we show that neuroblastoma cells endogenously produce LTB_4 and express the BLT1 receptor, thereby suggesting that LTs could affect the behavior of neuroblastoma cells. An upregulated expression of BLT1 has been shown in pancreatic (Hennig *et al.*, 2002), colon (Ihara *et al.*, 2007), and ovarian cancer (Rocconi *et al.*, 2008), while no increased expression was detected in bladder cancer (Kim *et al.*, 2010). At the time of this study, no commercial antibodies were available to study the expression of BLT2, although it has now been shown that BLT2 is overexpressed not only in pancreatic (Hennig *et al.*, 2008) and breast cancer (Choi *et al.*, 2010), but also in ovarian (Rocconi *et al.*, 2008) and bladder cancer (Kim *et al.*, 2010), in which the expression of BLT2 significantly correlated with an advanced disease stage and poor prognosis. Signaling through BLT2 has been shown to promote tumor cell proliferation (Hennig *et al.*, 2008), survival (Choi *et al.*, 2010), invasion, and metastasis (Kim *et al.*, 2010).

In addition, neuroblastoma cells express LTC_4S, produce CysLTs and express the receptors CysLT1 and CysLT2. According to the literature, no other study has investigated the expression of LTC_4S in solid tumors; however, an elevated concentration of CysLTs has previously been detected in brain tumors (Simmet *et al.*, 1990). An increased expression of the CysLT1 receptor has also been found in tissues from urological (Matsuyama *et al.*, 2009), colon (Ohd *et al.*, 2003), breast (Magnusson *et al.*, 2011), and brain (Zhang *et al.*, 2004) cancers. In colon cancer, CysLT1 has been shown to be expressed both in the cellular and nuclear membrane (Nielsen *et al.*, 2005). Interestingly, the activation of nuclear CysLT1 receptors triggered a calcium response, induced the phosphorylation of Erk1/2, and stimulated cell proliferation (Nielsen *et al.*, 2005), which indicates a distinct nuclear CysLT1 signaling. By contrast, signaling through the CysLT2 receptor may be involved in differentiation (Magnusson *et al.*, 2007) and decreased cell growth and migration (Magnusson *et al.*, 2011). A high expression of CysLT1, together with a low expression of CysLT2, correlates with a worse prognosis for colon (Magnusson *et al.*, 2010) and breast (Magnusson *et al.*, 2011) cancer patients. Moreover, no expression of CysLT2 was detected in brain tumors, although a high expression was detected in neuronal and glial cells in the surrounding stroma (Hu *et al.*, 2005).

4.2.2 5-LO and the leukotrienes in neuroblastoma cell survival and proliferation

To investigate the effect of LTs on neuroblastoma cell viability, we serum-starved cells overnight and added LTB_4 or LTD_4 at different concentrations and durations. A significant time- and cell line-dependent increase in cell viability was observed for both SK-N-AS and SK-N-BE(2) cells. A panel of neuroblastoma cell lines was also treated with 13 different LO pathway inhibitors and LT receptor antagonists at different concentrations for 48 hours. The most effective compounds to decrease neuroblastoma cell survival were AA-861, a 5-LO inhibitor; MK-886, a FLAP inhibitor; and montelukast, a CysLT1 receptor antagonist. The drug concentration that was needed to inhibit 50% of the cell viability ranged from 2-14 μM. These results suggest that LTs are important for neuroblastoma cell survival and proliferation. To investigate the mechanisms behind the decreased cell survival, we performed cell cycle distribution analysis using DAPI staining, and we observed a sub-G1 peak, an indicator of apoptosis, in SK-N-SH cells treated with AA-861, MK-886, or montelukast. For SK-N-BE(2) cells, we observed an increase in G1, which indicates cell cycle arrest after treatment with AA-861 or MK-886 and a sub-G1 peak after treatment with montelukast. To further determine the effect of AA-861 and MK-886, we stained cells with annexin V, which binds to phosphatidylserine on the cell surface of early apoptotic cells. Treatment with AA-861 or MK-886 resulted

in a positive annexin V staining. We also investigated the mitochondrial membrane potential using TMRE, since depolarization of the mitochondrial membrane is one of the first steps of apoptosis. All three compounds induced depolarization of the mitochondrial membrane in the two neuroblastoma cell lines that were investigated. The release of cytochrome c from the mitochondria activates caspase-9, which subsequently cleaves and activates the effector caspase-3. Treatment with AA-861, MK-886, or montelukast resulted in caspase-9 and -3 cleavage, and a co-incubation with zVAD-fmk, a pan caspase inhibitor, attenuated the cytotoxic effect. Together, these results show that treatment with AA-861, MK-886, or montelukast induces apoptosis of neuroblastoma cells through the intrinsic apoptotic pathway.

This shows that neuroblastoma cells produce LTs that can promote their own survival. Other studies suggest that the mechanism underlying the increased proliferation and survival induced by LTB_4 and LTD_4 involves the activation of MEK/Erk and PI3K/Akt pathways (Tong *et al.*, 2005; Massoumi and Sjolander, 2007) and the upregulation of anti-apoptotic Bcl-2 (Massoumi and Sjolander, 2007). One research group has shown that BLT2 activates Nox1/4, followed by the generation of ROS and then activation of NF-κβ signaling, which increased cell proliferation, invasion, and metastasis (Choi *et al.*, 2010; Kim *et al.*, 2010).

Numerous studies on epithelial cancer cells have revealed cytotoxic and anti-proliferative effects by treatment with 5-LO pathway inhibitors (Steinhilber *et al.*, 2010). Similar to our findings, other studies have shown that 5-LO pathway inhibitors primarily induce apoptosis by activation of the intrinsic apoptotic pathway (Steinhilber *et al.*, 2010). Remarkably, the treatment of neuroblastoma cells with montelukast, a CysLT1 receptor antagonist, was as potent as treatment with AA-861 or MK-886 in reducing cell survival and inducing apoptosis. Montelukast, which is sold under the trade name Singulair, is used in clinics for the treatment of asthma in children and adults. In addition to binding to the CysLT1 receptor, montelukast has been reported to directly inhibit 5-LO activity (Ramires *et al.*, 2004).

It has been shown that 5-LO inhibitors can induce apoptosis by mechanisms independent of 5-LO inhibition (Fischer *et al.*, 2010). To investigate this, we added LTs to cells treated with the 5-LO inhibitor AA-861. Addition of LTB_4 and LTD_4 alone or in combination with the 5-LO product 5-HETE could abrogate the cytotoxic effect of AA-861. We also specifically suppressed 5-LO protein expression, and the subsequent LT production by siRNA transfection, which resulted in a 35% reduced neuroblastoma cell growth. The same anti-proliferative effect was observed when CysLT1 expression was reduced. These data show that LTs are important for neuroblastoma cell

growth, but nevertheless we cannot exclude the possibility that off-target effects of the compounds may contribute to the observed cytotoxicity.

One LTB_4 receptor antagonist, LY293111, has shown a promising efficacy in the treatment of cancer, both *in vitro* and *in vivo*. Three phase I studies proved that LY293111 in combination with cytostatic drugs was safe and well tolerated in patients. However, the results from two phase II studies in patients with non-small cell lung or pancreatic cancer failed to demonstrate any improvement in a progression-free survival (Adrian *et al.*, 2008).

4.3 TARGETING PROSTAGLANDIN SIGNALING *IN VIVO* (PAPER IV)

A lot of research has focused on the role of inflammation and inflammatory cells and mediators in adult cancers, while much less is known about the importance of inflammation in childhood cancer and in neuroblastoma (Seeger, 2011). To investigate the composition of immune cells in neuroblastoma and the effect of COX-inhibition, we used the TH-MYCN mouse model that spontaneously develops neuroblastoma-like tumors originating from the neural crest (Weiss *et al.*, 1997; Hansford *et al.*, 2004). In our hands, 100% of the homozygous mice developed palpable tumors at four to seven weeks of age, and 50% of the hemizygous mice developed tumors between six and 19 weeks (Rasmuson *et al.*, unpublished). To be able to analyze small, unpalpable tumor lesions, we used homozygous mice that were four and a half to six weeks old. At the age of five weeks, we could detect small poorly vascularized tumor lesions in all dissected homozygous animals. One week later, when the mice were six weeks old, the tumors had grown much larger and comprised a significant portion of the abdominal cavity, often growing onto and displacing the kidneys.

4.3.1 Characterization of the TH-MYCN tumor microenvironment during tumor progression

4.3.1.1 T cells

To investigate whether T cells were present in TH-MYCN tumors, the tumors were stained with the T cell marker CD3. The staining revealed that tumor-infiltrating $CD3^+$ T cells were present in the tumors as well as in blood vessels. FACS analyses of whole tumor single-cell suspensions showed that in small tumor lesions, 0-0.2 g, 63% of the total number of $CD45^+$ hematopoietic cells consisted of T cells. In larger tumors, 0.5-2.2 g, the proportion of T cells was significantly smaller, and comprised only 27% of the $CD45^+$ cells. T cells can be subdivided into T-helper cells, expressing the CD4 co-receptor, and CTLs, expressing the CD8 co-receptor. We further

characterized the T cells by anti-CD4 and CD8 staining. FACS analysis showed that both T-helper cells and CTLs are present in TH-MYCN tumors, with a higher number of T-helper cells. Calculating the CD4/CD8 ratio in relation to the tumor weight revealed a significant increase in T-helper cells during the tumor progression. Moreover, we investigated the presence of Treg, an immune suppressive subpopulation of T-helper cells that expresses the transcription factor FOXP3. Treg constituted 8-20% of the T-helper cell population, and this proportion was consistent throughout the tumor progression.

Tumor infiltration of $CD4^+$ T cells correlates with a good prognosis for colon and lung cancer patients, whereas the opposite has been documented for breast and renal cancer patients (DeNardo *et al.*, 2010). One important factor contributing to these paradoxical findings is that the $CD4^+$ T-cell population can be subdivided into several functionally different subtypes, including Th1, Th2, and Treg. Activation of Th1 type responses activates anti-tumor programs, while the activation of Th2 and Treg responses promotes tumor progression by stimulating immune suppression, angiogenesis, and tissue remodeling. The Th1 and Th2 cells exert their effect by the release of different cytokines. Th1 cells express interferon γ (IFNγ), tumor necrosis factor α (TNFα), IL-2, and IL-12, whereas the Th2 cells typically secrete IL4, IL5, IL-6, IL-10, and IL-13 (DeNardo *et al.*, 2010).

Th1 cells can initiate an effective anti-tumor response by activating CTLs, one of the cell types that can directly kill tumor cells, and by releasing high levels of IFNγ, TNFα and cytotoxic granules, all of which are directly toxic to cancer cells (DeNardo *et al.*, 2010). We could detect CTLs in TH-MYCN tumors, although with a gradual decrease during tumor growth. To elucidate whether the tumor-associated T cells had the capacity to mount an effective Th1 effector response, we stimulated the cells *ex vivo* with PMA and ionomycin for five hours, and analyzed the intracellular concentration of IFNγ by flow cytometry. The analysis revealed that both $CD4^+$ and $CD8^+$ cells could produce IFNγ, thus indicating that these cells have the capacity to initiate a Th1 response. The ability of CTLs to kill tumor cells is also dependent on the expression of major histocompatibility complex (MHC) class 1 on tumor cells. Many tumor cells have lost or downregulated their expression of MHC class 1, and thus cannot be recognized and killed by the CTLs. Therefore, we evaluated the expression of MHC class I on TH-MYCN tumor cells. We detected a low expression of the $H\text{-}2D^b$ allele, but no expression of the $H\text{-}2K^b$ allele.

4.3.1.2 Myeloid cells

We also stained whole tumor single-cell suspensions with F4/80 in combination with CD45 for the detection of macrophages. FACS analysis revealed TAMs in all tumors of all stages. In smaller tumors, TAMs

constituted less than 10% of the $CD45^+$ cells, while larger tumors had a significantly higher proportion at 25%. Macrophages can exert both tumor-promoting and anti-tumor activities depending on their phenotype. Generally, in established tumors, TAMs have an M2-like phenotype and promotes tumor growth and progression, which is the opposite effect of the M1 phenotype (Mantovani *et al.*, 2008). To characterize the phenotype of TAMs that infiltrate TH-MYCN tumors, we evaluated the expression of MHC class II and the macrophage mannose receptor (MMR). In small tumors, TAMs displayed a high expression of MHC class II and a low expression of MMR, thus indicating an M1 polarization, while the opposite expression pattern was found for TAMs in larger tumors. Larger tumors expressed significantly lower levels of MHC class II and showed a tendency towards a higher expression of MMR, both of which indicate an M2 polarization.

Furthermore, we investigated the presence of dendritic cells, defined as $CD45^+CD11c^+$, in TH-MYCN tumors. Similar to the TAM, the dendritic cells accumulated during the tumor progression. In small tumors, only 6% of the $CD45^+$ cells were constituted of dendritic cells, whereas larger tumors had a median of 22%. The dendritic cells present in large tumors displayed a reduced expression of MHC class II, indicating an immature phenotype (Fainaru *et al.*, 2010). Dendritic cells within tumors often display an immature phenotype, with a subsequent impaired ability to initiate adaptive anti-tumor immune responses (Gabrilovich, 2004). Immature dendritic cells also directly promote tumor growth by stimulating angiogenesis (Fainaru *et al.*, 2010). Studies have shown that TAMs, MDSCs and various other tumor-derived factors can restrict the maturation and function of the dendritic cells within tumors (Gabrilovich, 2004).

Similar to the TAMs and dendritic cells, we detected an accumulation of MDSCs in tumors during progression. MDSCs are a diverse group of immature myeloid cells with immunosuppressive functions that are characterized by the expression of GR1 and CD11b in mice (Ostrand-Rosenberg and Sinha, 2009). The immunosuppressive actions of MDSCs include the suppression of T cell activation, the induction of Treg, and the production of cytokines, which helps skew the tumor microenvironment towards a tumor-promoting Th2 type (Ostrand-Rosenberg and Sinha, 2009). The MDSCs are activated by pro-inflammatory mediators such as PGE_2, IL-1β, and IL-6 (Ostrand-Rosenberg and Sinha, 2009).

4.3.1.3 Cytokines

By quantitative real-time RT-PCR we characterized the expression level of the cytokines IL-10, IL-6, IL-2, tumor growth factor β (TGF-β) and IFNγ relative to the control gene HPRT in TH-MYCN tumors of various sizes. The highest expression was detected for the Th2 cytokines IL-10 and TGF-β,

while the expression of IL-6 and the Th1 cytokines IL-2 and IFNγ were close to or under the detection limit. We did not detect any correlation between the level of expression and tumor weight. A significant difference, however, was detected when comparing the expression level of IL-10 and TGF-β in tumors of homozygous versus hemizygous mice, which could reflect a slower disease progression with a subsequently prolonged time to develop a state of chronic inflammation. Surprisingly, we detected very low levels of the Th2 cytokine IL-6. One possible explanation for this is that the overexpression of MYCN downregulates the expression of IL-6 (Hatzi *et al.*, 2002).

Together these results show the presence of an early tumor-associated inflammation in tumors from five-week-old mice. These tumors shift toward a more immunosuppressive microenvironment with an accumulation of M2 TAM, immature dendritic cells, and MDSC at the expense of T cells and CTLs.

4.3.2 Expression of COX and therapeutic treatment with a low-dose ASA

As previously mentioned, our group has shown that human neuroblastoma cells overexpress COX-2 and produce PGE_2 (**paper I**), and that treatments with COX inhibitors reduce the growth of neuroblastoma xenografts (Johnsen *et al.*, 2004). To investigate the effect of COX inhibition on tumor-associated inflammation, we first evaluated the expression of COX-1 and COX-2 in TH-MYCN tumors, and we detected the expression of both enzymes by use of immunohistochemistry. FACS analysis confirmed the expression of COX-1 and COX-2 and revealed that 63% of the neuroblastoma cells expressed COX-1, whereas only 31% of cells expressed COX-2. A similar pattern was observed for $CD45^+$ hematopoietic cells, in which 95% expressed COX-1, though only 11% expressed COX-2. Because of the high expression of COX-1 in the tumor cells, we decided to treat animals with a low-dose ASA. Eight homozygous mice were treated with 10 mg/kg of ASA, which corresponds to 60 mg for an adult human, calculated according to body surface area (Reagan-Shaw *et al.*, 2008). The treatment was given by oral gavage, and treatment started when the animals reached 4.5 weeks of age and continued for 10 consecutive days. Strikingly, the low-dose ASA treatment significantly reduced tumor growth, as the median tumor weight for treated animals was 0.25 g compared to 1.15 g for the control tumors. FACS analysis of whole tumor single-cell suspensions showed that ASA-treated tumors had a composition of inflammatory cells that resembled those found in small tumors. Because all ASA-treated tumors had a small tumor weight, it is difficult to conclude whether the altered pattern of inflammatory cells is due to the treatment or to the bystander effect of reduced tumor growth.

In general, a high dose of ASA (1 g) is anti-inflammatory by its inhibition of the COX enzymes, whereas a low dose (75-150 mg) is cardioprotective through the inhibition of platelet-derived TXA_2. However, a recent study documented that even low doses of ASA could act in an anti-inflammatory manner on acute inflammatory responses in humans (Morris *et al.*, 2009).

During the time of this study, several articles have been published that describe a prominent effect with low doses of ASA (75-300 mg) in cancer prevention (Rothwell *et al.*, 2010; Salinas *et al.*, 2010; Rothwell *et al.*, 2011). Additionally, one randomized control trial showed that 600 mg of ASA per day significantly reduced the risk of developing cancer in patients with Lynch syndrome, a hereditary form of colorectal cancer (Burn *et al.*, 2011). The underlying mechanism behind this observed effect is not yet clear, although many speculations have been reviewed by Langley *et al* (Langley *et al.*, 2011). These include COX-specific effects with an inhibition of COX-1 in platelets with a subsequent inhibition of TXA_2 biosynthesis, the inhibition of COX-2 and tissue PGs, and COX-independent effects such as the inhibition of NF-κβ, Wnt, and TGF-β signaling. Another proposed mechanism by which ASA may inhibit tumor growth is the production of ATLs (Greene *et al.*, 2011). In this study, we have shown that even a therapeutic treatment with a low dose of ASA results in a significantly reduced neuroblastoma tumor growth in the aggressive TH-MYCN model.

5 SUMMARY AND CONCLUSIONS

In this thesis, the importance of PGE_2 and LTs in cancer has been investigated. COX-1, COX-2, and mPGES-1 are key enzymes in the metabolism of arachidonic acid to PGE_2, whereas the 5-LO pathway catalyzes the production of LTs. We have investigated the expression of key proteins in these two pathways and their role in tumor cell growth and survival. The result may be summarized as follows:

- Neuroblastoma cells express the four PGE_2 receptors EP1-EP4.
- PGE_2 acts as an autocrine and/or paracrine proliferation and survival factor for neuroblastoma.
- mPGES-1 is differentially expressed in prostate cancer.
- Downregulation of mPGES-1 reduces the clonogenic capacity and delays tumor growth of prostate and lung cancer xenografts.
- Enzymes and receptors of the 5-LO pathway are expressed in neuroblastoma.
- LTs act as autocrine/paracrine proliferation and survival factors in neuroblastoma.
- Inhibition of enzymes within the 5-LO pathway or blocking the LT receptors leads to neuroblastoma cell death by induction of apoptosis.
- A progressive inflammatory tumor microenvironment is present in tumors from the transgenic TH-MYCN neuroblastoma model.
- COX-1 and COX-2 are expressed in TH-MYCN tumors.
- Therapeutic treatment with a low-dose ASA reduces neuroblastoma TH-MYCN tumor growth.

6 FUTURE PERSPECTIVES

A compelling piece of evidence demonstrates that COX-2 is involved in tumor growth and progression in multiple cancers, but the exact mechanism behind its tumor-promoting effects is still not fully clear. Metabolism through COX-2 involves several different substrates and downstream synthases, with the subsequent formation of a whole group of bioactive compounds that signals through multiple receptors. In general, a given cell type forms only one or two of these products in abundance. We and others have shown that PGE_2 signaling in cancer cells is complex and involves several EP receptors. In addition, stromal cells within the tumor may also express one or several of these receptors (Reader *et al.*, 2011). Accordingly, targeting only one EP receptor to inhibit tumor progression and improve patient outcome may prove insufficient. Inhibition of mPGES-1 may represent a more promising target for cancer therapy, because this stops most, if not all, PGE_2 production and subsequently all PGE_2 signaling. Future studies will reveal if PGE_2 accounts for all the tumor-promoting properties of COX-2, and if mPGES-1 inhibitors and COX-2 inhibitors have a similar potential in reducing tumor growth and metastatic spread.

Inhibition of COX-2 in cells expressing both the COX-2 and 5-LO pathways could, theoretically, result in a metabolic shunt of free arachidonic acid over to the 5-LO pathway, with a subsequent increased production of pro-inflammatory LTs. This has indeed been reported in a study in which non-smokers and smokers were given celecoxib for one week, which resulted in lower urinary levels of PGE_2 but significantly increased urinary LTE_4 (Duffield-Lillico *et al.*, 2009). Since neuroblastoma cells express both the COX-2 and 5-LO pathways, and both PGE_2 and LTs promote cell survival and growth, the best therapeutic treatment strategy would be to inhibit both pathways through combination therapy.

The high expression of COX-1 in TH-MYCN tumors also raises the question of the importance of COX-1 in human neuroblastoma. To date, no studies have investigated this. Furthermore, one previous study reported that the TH-MYCN tumors were non-immunogenic and lacked infiltrating immune cells (Moore *et al.*, 2008). In contrast, our data clearly demonstrates a progressive inflammatory tumor microenvironment in TH-MYCN tumors, making this a useful model for studying the role of cancer-related inflammation in neuroblastoma *in vivo*.

Whether a low-dose ASA should be included in the treatment for neuroblastoma remains to be further investigated, and the exact mechanism behind its anti-tumoral effect remains to be further clarified. Before incorporating a low-dose ASA in the treatment protocol, the most effective dosing and time scheduling must also be established. Today ASA is not

recommended for children under the age of 16 years due to an increased risk of developing Reyes syndrome when using ASA for viral illnesses. Therefore, a careful evaluation of the potential pros and cons is essential.

7 POPULÄRVETENSKAPLIG SAMMANFATTING

I Sverige drabbas varje år över 50.000 vuxna och cirka 300 barn av cancer. Trots stora framsteg så är cancer den näst vanligaste dödsorsaken för både barn och vuxna i Sverige. Behovet av att hitta nya behandlingar som kan förbättra överlevnaden och minska de sena komplikationerna är stort. Genom en ökad biologisk förståelse kan man utveckla mer målstyrda behandlingar som förhoppningsvis kan förbättra behandlingsresultaten.

Neuroblastom är en cancersjukdom som drabbar små barn, de flesta insjuknar redan innan två års ålder. Neuroblastom uppkommer i omogna celler av det sympatiska nervsystemet, som är en del av det icke-viljestyrda nervsystemet som har till uppgift att anpassa kroppen till fara och stress. Det sympatiska nervsystemet stäcker sig längs med båda sidorna av ryggraden och förgrenar sig i bålen till bl.a. binjuren. Neuroblastom kan utvecklas var som helst längs det sympatiska nervsystemet men uppkommer oftast i binjuren. Neuroblastom är en av de vanligaste cancerformerna hos barn men det är också den barncancer som har sämst prognos. Trots en intensiv behandling med kirurgi, cytostatika och strålning så är överlevnaden för barn i högriskgruppen endast 55% och den totala överlevnaden för alla barn med neuroblastom är 74%.

Inflammatoriska celler och signalsubstanser är en viktig del av tumörmiljön där de bidrar till en ökad tumörtillväxt. Antiinflammatoriska behandlingar har visat sig kunna minska canceruppkomst och tumörtillväxten. Neuroblastomceller har en förändrad fettsyrabalans jämfört med normala nervceller. De har en ökad nivå av den inflammatoriska omega-6 fettsyran arakidonsyra men en lägre nivå av antiinflammatoriska omega-3 fettsyror. Arakidonsyra som finns lagrat i cellens membraner omvandlas via tre enzymvägar, cyklooxygenaser (COX), lipoxygenaser (LO) och cytokrom p450 till olika signalsubstanser, eikosanoider. Vi har i en tidigare studie visat höga nivåer av COX-2 i neuroblastom, som omvandlar arakidonsyra till inflammatoriska prostaglandiner. Vi har också visat att om man behandlar neuroblastom med COX hämmare så minskar tumörtillväxten. I min avhandling har jag undersökt betydelsen av eikosanoider i cancer, med fokus på neuroblastom.

För att utreda varför COX-2 är viktigt i neuroblastom undersökte vi betydelsen av dess produkt prostaglandin E_2 (PGE_2). Våra resultat visar att alla fyra PGE_2 receptorer finns i neuroblastom. Neuroblastomceller kan också själva producera PGE_2 som stimulerar cellöverlevnad och celldelning. Om signaleringen via PGE_2 receptorerna förhindras, hämmas celltillväxten. Resultaten pekar på att flera av receptorerna är viktiga för neuroblastomcellernas överlevnad. Vi undersökte också effekten av att specifikt blockera bildandet av PGE_2 genom att hämma det enzym som ligger

nedströms om COX-2 som specifikt ansvarar för omvandlingen till PGE_2, mikrosomalt PGE syntas 1 (mPGES-1). Resultaten visar att om man tar bort mPGES-1 så reduceras prostatacancercellers och lungcancercellers förmåga att klona sig och tumöruppkomsten hindras.

En annan väg för arakidonsyra är via 5-Lipoxygenas (5-LO) och en produktion av inflammatoriska leukotriener. Denna avhandling visar att alla enzymer i 5-LO kaskaden samt leukotrienreceptorerna finns i neuroblastom. Neuroblastomceller kan också producera leukotriener vilka verkar som cellens egentillverkade tillväxtfaktorer. Blockerar man produktionen av leukotriener eller leukotriensignaleringen, induceras en programmerad celldöd hos neuroblastomceller.

I barncancer är den inflammatoriska komponenten av tumörmikromiljön inte så väl studerad. Vi visar här på en tidig närvaro av inflammatoriska celler, i tumörer från en transgen musmodell som spontant utvecklar neuroblastom. Vi visar också att kompositionen och fenotypen av dessa celler förändras när tumören växer. Sammansättningen och fenotypen av de inflammatoriska celler som finns i små tumörer är mer gynnsam än i stora tumörer. De stora tumörerna domineras av celler som bidrar till tumörtillväxt och förhindrar ett aktivt angrepp mot tumörcellerna. Behandlar man dessa djur med en låg dos av anti-inflammatorisk acetylsalicylsyra (aspirin) bibehålls den gynnsamma kompositionen av inflammatoriska celler och tumörerna växer långsammare.

Sammanfattningsvis visar studierna i den här avhandlingen att både PGE_2 och leukotriener verkar som neuroblastomcellers egentillverkade tillväxtfaktorer. Resultaten visar på att en specifik blockering av PGE_2 och leukotriensignaleringen skulle kunna utgöra nya behandlingsmål får barn drabbade av neuroblastom.

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