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Involvement of eicosanoid signalling in epithelial cell migration

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Faculty of Medicine

Academic dissertation

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Involvement of eicosanoid signalling in epithelial cell migration

Abstract

The development of inflammatory bowel diseases (IBD) and colon cancer (CC) has been shown to involve the up-regulation of inflammatory mediators and the machinery producing them such as the eicosanoids. Changes in the expression of extracellular matrix proteins and their related integrin receptors have also been shown to be important in the advancement IBD and CC. In light of this, we have investigated the role of eicosanoids in cell migration a key process in IBD and CC. This was through direct stimulation, with the eicosanoid leukotriene D4 (LTD4) or indirectly through inducing cyclo-oxygenase-2 (COX-2; an enzyme involved in the production of various eicosanoids) expression, by activating integrin collagen receptors. We observed that direct stimulation with LTD4, induced intestinal epithelial cell migration, through activation of the CysLT1 receptor, phosphotidylinositol-3 kinase, Vav2 and Rac localisation to membrane ruffles. Indirect stimulation, by activating COX-2 in an alpha 2 beta 1 integrin dependent manner was able to elicit a migratory response. The integrin dependent COX-2 expression was shown to be mediated through the activation of CD47 and its associated G alpha i3 protein, which in turn lead to the protein kinase C alpha, Ras GTPase and NF kappa B activation. COX-2 expression is synonymous with activation of the protein. Inhibition of COX-2 expression or specifically inhibiting COX-2 activity, resulted in cell adhesion being favoured over cell migration in a CD47 dependent manner. Thus understanding cellular mechanisms leading to cell migration may reveal novel targets which lead to alternative therapeutic strategies in treating IBD and CC.

Key words: Leukotriene D4, COX-2, cell migration, integrin, CD47, inflammation, colon cancer

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Character cannot be developed in ease and quiet. Only through experience of trial and suffering can the soul be strengthened, vision cleared, ambition inspired, and success achieved.

Helen Keller

Table of contents

List of papers

This thesis is based on the following papers, referred to in the text as papers I-III:

I The Pro-inflammatory Mediator Leukotriene D_4 Induces Phosphatidylinositol 3-Kinase and Rac-dependent Migration of Intestinal Epithelial Cells. Sailaja Paruchuri, Oliver Broom, Karim Dib and Anita Sjölander (2005). J Biol. Chem 280, 13538-13544

II $\alpha 2\beta 1$ Integrin Signalling Enhances Cyclooxygenase-2 Expression in Intestinal Epithelial Cells. Oliver Jay Broom, Ramin Massoumi and Anita Sjölander (2006). J Cell Physiol 209, 950-958

III CD47 dependent COX-2 expression and migration of intestinal epithelial cells. Oliver Jay Broom, Ramin Massoumi, Per-Arne Oldenborg and Anita Sjölander. Manuscript

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Abbreviations

5-LO	5-lipoxygenase
AA	Arachidonic acid
BM	Basement membrane
cAMP	Cyclic adenosine monophosphate
CD	Crohns disease
Col I	Collagen I
Col IV	Collagen IV
COX	Cyclo-oxygenase
cysLTs	Cysteinyl leukotrienes
DAG	Diacylglycerol
ECM	Extracellular matrix
EGF	Epidermal growth factor
Erk1/2	Extracellular signal regulated kinase 1 and 2
FLAP	Five lipoxygenase associated protein
FAK	Focal adhesion kinase
GALT	Gastrointestinal associated lymphoid tissue
GAP	GTPase activating protein
GDI	GDP dissociation inhibitor
GDP	Guanosine Dinucleotide phosphate
GEF	Guanosine exchange factor
GPCR	G-protein coupled receptor
GTP	Guanosine triphosphate
H_2O_2	Hydrogen Peroxide
IBD	Inflammatory bowel disease
IκB	Inhibitor of κ B
ILK	Integrin linked kinase
Jnk	c-Jun NH2 terminal kinase
LTA_4	Leukotriene A_4
LTB_4	Leukotriene B_4
LTC_4	Leukotriene C_4
LTD_4	Leukotriene D_4
LTE_4	Leukotriene E_4
MAP	Mitogen activated protein
mDia	Mammalian homologue of the *Drosophila* gene *Diaphanous*
MMP	Matrix metalloproteinase
MMS	Multiply membrane spanning
NFκB	Nuclear factor κ B
NSAIDS	Non-steroidal anti-inflammatory drugs
$O_2^{\cdot-}$	Superoxide anion

OH^-	Hydroxyl radical
$p90^{RSK}$	p90 kDa ribosomal S6 kinase
PGE_2	Prostaglandin E_2
PGF_2	Prostaglandin F_2
PGG_2	Prostaglandin G_2
PGH_2	Prostaglandin H_2
PGI_2	Prostaglandin I_2
PGJ_2	Prostaglandin J_2
PI	Phosphatidylinositols
PI-3K	Phosphatidylinositol-3 kinase
PIP	Phospahtidylinositol-4-phosphate
PIP_2	Phospahtidylinositol-3,4-bisphosphate
PIP_3	Phospahtidylinositol-3,4,5-trisphosphate
PLIC	Protein linking IAP to cytoskeleton
PTX	*Bordetella pertussis* toxin
PKC	Protein kinase C
RasSFM	Ras super family member
ROCK	Rho associated coiled coil forming protein kinase
ROS	Reactive oxygen species
SIRPα	Signal regulatory protein α
TXA_2	Thromboxane A_2
UC	Ulcerative colitis
VVM	Valine-Valine-Methionine
WASP	Wiskott-Aldrich syndrome protein

Introduction

The intestines of a healthy human are constantly being challenged by foreign environmental factors. In defence to these factors the body mounts an immune response, and as such, a low level of inflammation is constantly present in the intestines. Unfortunately this, like any other process in the body, can become defective and this is thought to lead to the development of chronic inflammatory bowel disease (IBD) which is an umbrella term for conditions such as Crohns' disease, ulcerative colitis and collagen colitis. The precise aetiology of these diseases is as yet unclear, however, what is clear, is that IBD is characterised by the over production of inflammatory mediators, which leads in part, to remodelling of the extracellular matrix (ECM).

The ECM acts as a structural support for the intestinal epithelial cells, and for the cells in the underlying sub-epithelial tissue. ECM remodelling during IBD involves the dysregulated increase in production of ECM proteins such as collagen I, III, IV and V. Integrins are proteins used by cells to attach and migrate through, the ECM. They are also capable of transducing signals from the ECM, which in turn stimulate various cellular processes such as cell migration.

The eicosanoids are a family of lipid derived inflammatory mediators that have been identified as playing a key role in IBD and additionally in the development and progression of colon cancer. Indeed our laboratory has also shown that leukotriene D_4 (LTD_4; a cysteinyl leukotriene and member of the eicosanoid family), is able to induce cell proliferation and survival, whilst one of the receptors binding LTD_4, $CysLT_1$, is up-regulated in colon cancer patients, and correlates with a poorer disease prognosis. Additionally we have also published data, indicating that, cyclooxygenase-2 (COX-2; an important enzyme in the synthesis of prostanoids) and prostaglandin E_2 (a downstream metabolite produced from the action of COX-2), are able to regulate the surface expression of the $\alpha 2\beta 1$ integrin, which affects colon cancer cell migration.

Taking into account the previous data from our laboratory and others, the aim of this work was to investigate eicosanoid mediated cell migration either by, direct stimulation with LTD_4 or indirectly by, stimulating COX-2 expression and production of prostanoids, with collagen.

Background

1. The physiology of the normal intestine

The intestines are part of the gastrointestinal tract and are the main area for nutrient and water absorption. The structure could be considered as being a highly convoluted tube, lined by a layer of epithelial cells [1]. These cells are the barrier between the outside and the internal environment of the body, similar in effect to the function of the epithelial layer of the skin and respiratory system. The intestines can be roughly divided into two parts; the small and large intestines. The small intestine is composed of the ileum, duodenum, and caecum, whilst the large intestine composes the colon and rectum. The fundamental difference between the small and large intestines is the structure; the small intestine's structure is made up of depressions in the epithelium called crypts and raised areas called villi (See Figure 1.). This is in stark contrast to the large intestine which only has crypt structures [1]. There also exists functional differences between the two; the small intestine is mainly responsible for nutrient absorption and is relatively devoid of microflora, whilst the large intestine is highly populated by commensal and probiotic bacterial microflora. The colorectal region is mainly responsible for fatty acid (produced by the inhabiting microflora) absorption, water absorption and collection of indigestible waste products to be excreted [2, 3].

The normal physiological role of the intestines includes the production of a low level of inflammation in response to the constant environmental challenges. Specialised areas of the epithelium known as Payer's patches or the Gastrointestinal Associated Lymphoid Tissue (GALT) are responsible for mediating the level of the immune/inflammatory response in a process known as immunosurveillance [4, 5]. This tissue is designed to allow sampling of the microflora in the intestinal lumen by the specialised antigen presenting cells. The system has evolved to be able to distinguish potential pathogens from the microflora normally present in the gut, through the Toll-like and NOD-like pattern recognition receptors [6]. This in effect means that a full immune response is not mounted against the normal microflora. A possible defect in this system has been proposed to be one of the underlying causes of chronic inflammatory bowel diseases (IBD).

2. Inflammatory Bowel Disease

Inflammatory Bowel Disease (IBD) is an umbrella term for a group of related diseases. Most notable amongst these are, Crohn's disease (CD) and ulcerative colitis (UC), which make up the vast majority of patients [7]. The precise aetiology of IBD is still unknown. Two main theories have been championed in the literature as to the cause of IBD, however it is recognised that a combination of factors is more likely to lead to the onset of the condition. Firstly it has been proposed that defects in the "sensing" and related inhibition of an immune response to commensal microflora

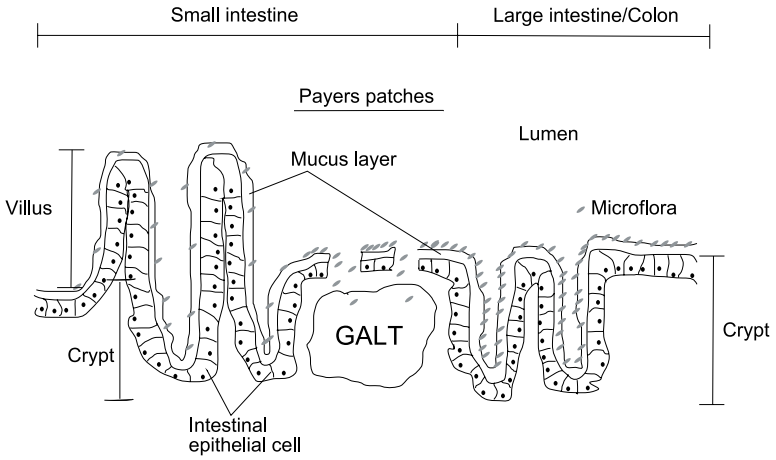

Figure 1. The structure of the intestines, including the Payers patches, the sites for immunosurveillance.

is responsible for a heightened inflammatory response. Evidence supporting this recently came with the discovery of the first gene found to be mutated in CD. Mutations in the *CARD15* gene, which codes for the NOD2 protein, have been shown to significantly increase the risk for CD [6, 8]. Usually NOD2 dampens the inflammatory response against the commensal microflora. Mutations to the NOD2 protein remove the inhibitory ability of NOD2 and an enhanced inflammatory response is seen. Secondly, changes to the microfloral population and epithelial barrier have been proposed to induce an inflammatory response from a normal immune system. This has been speculated to be through a mechanism which allows a greater number of commensal microflora to come into contact with the GALT, thus mimicking the situation when the intestinal mucosal layers are being invaded by a pathogen, and therefore invoking a full immune response. In addition, mutations to the *N-cadherin* gene and subsequent protein (which is involved in the preservation of the impervious epithelial barrier, thus mutations could lead to greater epithelial permeability), have been shown to induce IBD in mice [6, 9]. Several similar secondary effects are observed to be in common between the various inflammatory conditions, such as inflammatory cell infiltration, ulceration and systemic effects upon other tissues within the body (for example uveitis, in the eye) [10, 11]. Two such effects have been addressed in greater detail in this thesis, focussing specifically on their role in cell migration, these are; the observed marked up-regulation of the eicosanoid inflammatory mediators including the cellular machinery responsible for producing them [12], and the remodelling of the extracellular matrix [13].

3. The eicosanoid family of bioactive lipids

The eicosanoids are a family of bioactive lipids derived from the pre-cursor lipid arachidonic acid (AA)[14]. AA is found esterified usually at the carbon 2 position in membrane phospholipid bilayers, as phosphatidyl choline/inositol/ethanolamine.

Upon activation of a phospholipase A_2 or to a much lesser extent phospholipase C enzyme, AA is liberated from the phospholipid bilayer whereupon it is further metabolised into a plethora of lipid mediators each responsible for many individual and overlapping signalling events [15]. This catalysis is performed by various processes, however the eicosanoids are produced by the action of the lipoxygenases and the cyclo-oxygenases (See Figure 2.).

3.1 Lipoxygenases and leukotriene production

There are three lipoxygenses responsible for producing several families of eicosaoids (5-lipoxygenase, 12-lipoxygenase and 15-lipoxygenase), these are named after the position at which they insert a molecular oxygen into the carbon backbone, i.e. either at position 5, 12 or 15 [16, 17]. The leukotrienes are produced by the action of 5-lipoxygenase (5-LO; Figure 2.). In-conjunction with the Five Lipoxygenase

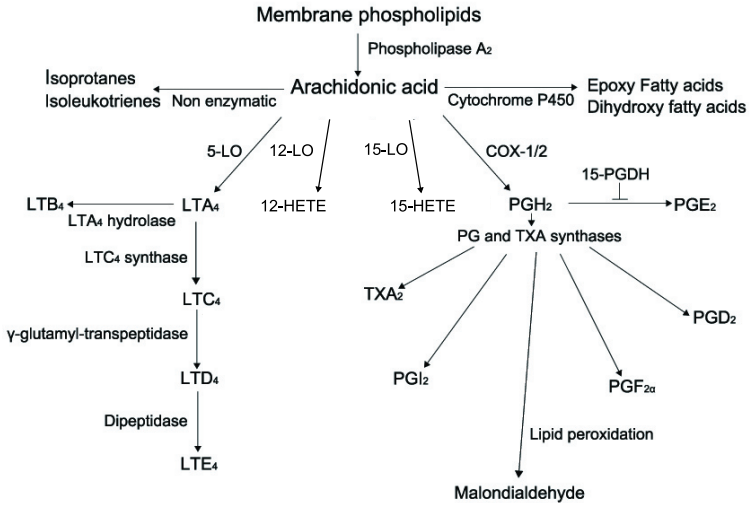

Figure 2. The metabolism of AA and synthesis of eicosanoids.

Activating Protein (FLAP), at the outer nuclear membrane, 5-LO converts AA into 5-hydroperoxyeicosatetreanoic acid, and then LTA_4 [18]. This compound is then either hydrolysed to form leukotriene B_4 (LTB_4), or a glutathione residue is conjugated by LTC_4 synthase, to form leukotriene C_4 (LTC_4). Pumps located in the cell membrane, pump out LTC_4 of the cell into the external milieu, where membrane bound γ-glutamyl transpeptidase and dipeptidases act to produce, leukotriene D_4 (LTD_4) and

leukotriene E_4 (LTE_4), respectively [19-21]. These alternative derivatives to LTB_4, are known as the cysteinyl leukotrienes (cysLTs) due to the presence of a cysteine residue in their structure.

3.2 Leukotrienes

Although firstly discovered in the 1930's (then termed slow-reacting substance of anaphylaxis, due to their effect on bronchioconstriction) their structure was not solved until 1979 by Samuelsson and co-workers, and they were named after the cells in which they were found, namely the leukocytes and their carbon-carbon double bond structure (-triene) [22].

Leukotrienes are primarily synthesised in response to an inflammatory stimulus, usually acute inflammation [17], although their presence has been shown to be important in chronic inflammatory conditions such as pulmonary fibrosis, asthma and IBD [23-25]. The vast majority of leukotrienes are produced by inflammatory cells such as macrophages, neutrophils and eosinophils, although other cells have been shown to be able to produce them, if in smaller quantities [26]. Although structurally different, LTB_4, like LTD_4, is able to function as a powerful chemotractant for leukocytes, increasing tissue cellularity, a characteristic of inflammation. The cysLTs are primarily known as potent inducers of smooth muscle contraction that is involved in bronchioconstriction [21]. Indeed they have been shown to be 10,000 times more potent than histamine [27], thus this has lead to the development of several asthma medicines directed against the effects of the cysLTs, through antagonising the receptors they bind to (see below for further details; [28, 29]).

Apart from their effects on smooth muscle cells, cysLTs have been known to induce other cellular responses, such as in pulmonary fibrosis, which is associated with an overproduction of extracellular matrix proteins and mucus [24]. Our group has also described several roles for cysLTs signalling, especially LTD_4 (which is the most potent of the cysLTs) in both non-transformed intestinal epithelial cells and colon cancer. Our research has indicated that LTD_4 is involved in cell proliferation and survival through activating alternative pathways from the $CysLT_1$ receptor, which in turn stimulate a panoply of signalling intermeadiates [30-32]. In relation to the current work, we have previously shown that LTD_4 is capable of inducing greater surface expression of the $\alpha 2\beta 1$ integrin and cell migration in a colon cancer cell line, mediated in part by the action of cyclo-oxygenase-2 [33].

3.3 The Cyclo-oxygenases

Apart from the lipoxygenases, an additional family of eicosanoids, namely the prostanoids, are produced by the action of the cyclo-oxygense (COX) enzymes. The COXs are dualistic haem containing enzymes performing a cyclo-oxygenase

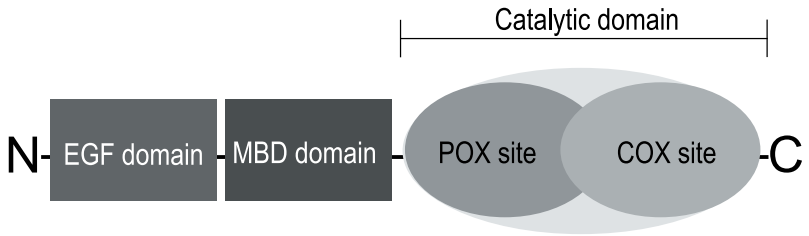

Figure 3. The structure of COX-2. MBD: Membrane Binding Domain; POX: Peroxide catalytic site; COX: Cyclo-oxygenase catalytic site.

and peroxidase function [34]. Two COX isoforms exist; COX-1 and COX-2, although a derivative of COX-1 (COX-3) has been found in cerebral tissue [35]. Human COX-1 and COX-2 proteins share a 60% homology [36].

COX-1 is on the whole constitutively expressed in most tissues, and is required for homeostasis, such as maintenance of the epithelial barrier [37]. Conversely COX-2 is an inducible enzyme, its expression being activated by a plethora of factors from inflammatory mediators to changes in oxygen levels, [38] and although it is mainly considered to be pro-inflammatory, recent data has shown COX-2 to be involved in the resolution of inflammation, thus anti-inflammatory [39, 40]. Both enzymes however are located to the luminal side of the endoplasmic reticulum, the inner and outer nuclear membranes or the mitochondria [34, 41], and are found at these sites as homo- or heterodimers [42]. Each COX monomer is composed of three domains; an epidermal growth factor domain, a membrane binding domain and a catalytic (the site for both the cyclo-oxygenase and peroxidase function) domain (See Figure 3.). AA is metabolised by the COXs to form the prostaglandin precursor PGH_2, (hence their alternative name, the prostaglandin H_2 synthases). Initally the cyclo-oxygenase function converts AA into prostaglandin G_2, which is then quickly transformed into prostaglandin H_2 (PGH_2) by the peroxidase action [39].

Although the majority of AA is converted into PGH_2 a smaller but significant amount of alternative metabolites are also produced: 11*R*-HPETE, 15*R*-HPETE, 15*S*-HPETE [43]. The COXs can also metabolise other molecules for example dietary polyunsaturated fatty acids such as linoleic acid and potential carcinogens can be activated for example polycyclic hydrocarbons and halogenated pesticides [36]. PGH_2 can also be degraded to form malondialdehyde, a free radical molecule and potent mutagen [44].

COX-2 has been intensively studied since it was first identified to be over-expressed in colon cancer tissue in 1994 by DuBois and co-workers. Since then COX-2s expression has been shown to be elevated in IBD, colorectal cancers and many other cancers, including pancreatic and breast cancers [45-47]. Indeed COX-2 is over-expressed in 90% of colorectal adenocarcinomas, and occurs relatively early in the carcinogenic process. Specific COX-2 inhibitors have been shown to be able to

reduce colonic polyp number and development. This correlates well with the wealth of data showing that inhibition of COX-2 is able to reduce tumour size, and significantly reduce the development of colorectal cancer [48]. Several COX-2 specific, non-steroidal anti-inflammatory drugs (NSAIDS) have been used clinically to treat adenomatous polyp formation, however their unexpected cardiovascular side effects have forced several to be withdrawn from the clinic and those remaining such as celecoxib, come with a strong warning for the possible development of cardiovascular side effects [49]. Although COX-2 is involved in several physiological roles such as ovulation, its prominent role in cancer has portrayed it as the "bad" COX and conversely COX-1 has been thought of as the "good" COX [50]. However this has proved to be an oversimplification as emerging data has presented a role for COX-1 in various cancers for example in ovarian cancer [51]. Thus current strategies, are reconsidering using NSAIDS which are not specific for either COX, or alternatively targeting the downstream enzymes such as the PGE_2 synthases, which acts to inhibit the production of the potent prostaglandin, PGE_2 [49].

3.4 Prostaglandins and thromboxane A_2

Firstly discovered in 1935, after being isolated from seminal fluid, prostaglandins have since been recognised as potent lipid mediators, controlling a panoply of cellular events [52]. These inflammatory mediators are produced firstly by the action of the COXs to produce PGH_2, which in turn can be selectively metabolised to PGI_2, PGE_2, PGD_2, $PGF_{2\alpha}$ or Thromboxane A_2 by specific prostaglandin and thromboxane synthases, which can also vary between cells. The evolution of PGE_2 is also controlled by the 15-hydroxyprostaglandin dehydrogenase enzyme, which degrades PGE_2 to an inactive 15-keto PGE_2 thus protecting against PGE_2 overproduction [48].

Prostaglandin signalling is required for many physiological processes, for example vascular smooth muscle constriction and maintenance of the intestinal epithelial barrier [53]. However these COX metabolites have a more sinister side, with PGE_2 debatably being the chief protagonist. Like COX-2, PGE_2 overproduction has been heavily implicated in the progression of IBD and colorectal cancer [48]. Several different studies both *in vitro* and *in vivo* have implicated PGE_2 in cancer cell proliferation, survival and migration by directly stimulating cell migration and inducing angiogenesis to allow dissemination from the tumour site [54-56]. For example Hansen-Petrik and co-workers recently showed that PGE_2 supplements can protect intestinal adenomas against regression induced by NSAID treatment, in APC^{min} mice [57]. Due to the increased risk with using NSAIDS against COX-2, the enzymes specifically producing the individual prostaglandins have come into focus, likewise the prostaglandin receptors, as potential alternative therapeutic targets, to hopefully negate the side effects seen with the COX-2 specific NSAIDS [48].

3.5 The leukotriene and prostaglandin receptors

The receptors responsible for transducing the signals induced by the leukotrienes or prostaglandins are members of the G-protein coupled receptor (GPCR) family [58]. These receptors are the largest class of surface membrane receptors and are found in most tissue and cell types, integrated not only into the cell membrane, but also the nuclear membranes [59, 60].

GPCRs are a single protein that threads through the lipid bilayer seven times. The N-terminal extracellular domain and loops are crucial for ligand recognition, whilst the cytoplasmic tail and loops are important for regulation of the signalling, by controlling the binding of the different heterotrimeric G-proteins [61].

Signalling from GPCRs is propagated by the heterotrimeric G-protein composed of the α, β and γ subunits. In the inactive state, the α subunit is bound to guanine dinucleotide phosphate (GDP). Upon activation the GDP is exchanged for guanine trinucleotide phosphate (GTP), which results in the dissociation of the α subunit from the $\beta\gamma$ subunits [62]. The dissociated subunits can then stimulate different pathways, usually inducing a calcium ion influx [63]. Inactivation of the α subunit is performed by its intrinsic ability to hydrolyse the GTP to GDP, which causes inactivation and re-association with the $\beta\gamma$ subunits.

The signalling pathways emanating from these receptors are involved in a huge range of physiological and pathological conditions, thus are the targets of many drugs in use [64, 65].

Two GPCRs have been found to specifically bind LTB_4, BLT1 and BLT2. Two GPCRs have also been found and characterised for the cysLTs, the $CysLT_1$ and $CysLT_2$ receptors. However recent data has revealed that the GPCR orphan receptor, GPR17, is able to bind LTC_4 and D_4, as well as uracil neucleotides [17]. The $CysLT_1$ receptor has the highest affinity for the cysLTs, binding LTD_4 the highest ($IC_{50} \approx 350nM$), followed by a 100 fold lower affinity for LTC_4 then LTE_4. The $CysLT_2$ receptor on the other hand, has an equally low affinity for LTD_4 and LTC_4 ($IC_{50} \approx 3\text{-}7nM$), and binds LTE_4 even less [66]. Several antagonists targeted against the $CysLT_1$ receptor are used clinically to counteract bronchioconstriction encountered by asthma patients [29].

Research from our group, has supported the role for LTD_4 and the $CysLT_1$ receptor in being pro-inflammatory and capable of promoting the production of pro-inflammatory/carcinogenic factors, such as COX-2 [67, 68]. The metabolites produced by COX-2 also bind to receptors which are members of the GPCR family.

There are nine receptors in total which bind the different metabolites, thus some metabolites can bind to several receptors. For example there are four receptors for PGE_2, called EP1-4 [69]. These are quite probably the most studied of the prostaglandin

receptors, unsurprisingly since PGE_2 has been shown to be the major prostaglandin synthesised in colon cancer [54].

Although all four receptors bind PGE_2, they are the products of different genes, with splice variants existing for the individual receptors and structurally only share 20-30% homology to one another. Naturally the EP receptors are differentially expressed in various tissues (although mRNA has been found for all of them in the intestinal epithelium) and as such play out their differing roles through inducing quite separate intracellular pathways [70].

In relation to IBD and colorectal cancer, EP2 and EP4 have been defined as playing major roles in the aetiology of these diseases. EP2 can either directly induce cancer cell proliferation and survival, or as has recently been shown, transactivate the epidermal growth factor receptor (itself a key mediator of carcinogenesis) to propagate its signal. In contrast to this, specific inhibition of the EP4 receptor leads to a decrease in cancer cell migration. Thus through the differential signalling of the different receptors, PGE_2 can control a wide range of cell responses [49].

Leukotrienes and prostaglandins through binding to their respective receptors mediate inflammatory processes such as wound healing which requires remodelling of the extracellualar matrix (ECM). Dysregulation of this process, a characteristic of chronic inflammatory conditions, results in the development of fibrosis and a change in the composition of the ECM [7]

4. The extracellular matrix

The formation of cell-cell junctions requires many proteins, E-cadherin being the main protein involved. These junctions are relatively stable and tight, however these alone are unable to maintain the structure of tissues and organs which is vital for the functionality of physiological processes [71]. Therefore in addition to the cell-cell junctions there exists cell-extracellular matrix bonds.

The ECM is a composite of many different proteins, glycoproteins and proteoglycans and is produced mainly by fibroblasts, or myofibroblasts, although other cell types such as epithelial cells can produce ECM proteins to a lesser extent [72]. The ECM provides not only a vital structural and mechanical function to the surrounding cells but is now known to also provide various cellular cues such as proliferation and survival. In addition to this the ECM is able to bind growth factors and cytokines, in effect acting as a store, to provide a rapid response (for example to insult) without the need for de novo synthesis [73].

The composition of the ECM varies between organs and different tissues, however one or several of the collagen types are usually present. The intestinal epithelium sits upon and is bound to, the ECM known as the basement membrane (BM). The BM facilitates the maintainance of the epithelial barrier function whilst

separating it from the underlying stromal and connective tissue ECM, which contains the interstial cells (such as fibroblast and myofibroblast cells) [13, 74]. In contrast to the stromal tissue, the BM contains mainly the network forming collagen type IV, which is interlinked by differentially expressed (depending upon the position in the villus/crypts) proteins, like laminins, fibronectin and elastin. The underlying connective tissue however contains little collagen IV, which is substituted by other fibrillar collagen types, namely collagen I, III and V, which again are cross linked by elastic fibres, and supported by proteoglycans such as hyaluronan [7, 73].

Changes to the ECM occur, for example during inflammatory conditions, where an insult results in the prompt release of inflammatory mediators, enzymes which can degrade the matrix and highly reactive molecules such free radicals. All of which results in the remodelling and/or degradation of both the BM and stromal tissue [75]. If the antigen is dealt with, the inflammation is resolved and things return to the *status quo.* However, if the inflammation persists, then it can become chronic. A key characteristic of chronic inflammation is the establishment of fibrosis [73]. Fibrotic tissue is associated with an over production of ECM proteins, in particular collagens I, III, IV and V, and therefore the balance between synthesis and degradation, is shifted towards synthesis [7]. As a result, the composition of the ECM changes, as do the mechanical properties and associated architecture, of the epithelium and underlying tissue [13, 72]. This has profound consequences for the cell population within the area.

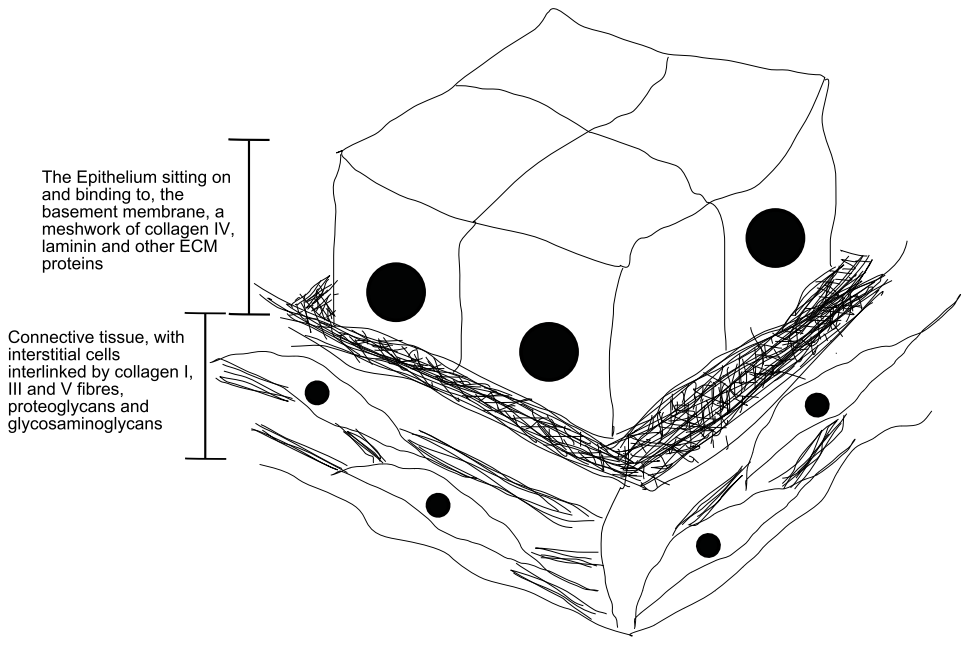

Figure 4. A simplified diagram of the basal lamina and connective tissue.

Alterations to the ECM, like fibrosis seen in IBD, are also apparent in the tumour microenvironment, and has therefore lead to speculation that such modifications can lead to or aid, the progression of carcinogenesis [76]. This has been demonstrated, by investigating the intracellular signals which are generated by the cell-ECM interactions. A cell can respond to environmental changes (for example changes in ion and oxygen levels.) through many different ways. One way, which has gained evermore significance, is through ECM-cell interactions mediated by a group of proteins known as the integrins.

4.1 The integrin family of ECM receptors and cell adhesion molecules

The integrins are heterodimeric transmembrane proteins, which are chiefly responsible for cell anchorage (to their surrounding ECM and other cells), but are now also recognised as being important signalling molecules transducing signals from the ECM. Integrins have been likened to velcro™, as individually they bind relatively weakly to the ECM, however together they tightly bind the cell to the ECM. This also has the advantage of allowing a cells ECM interactions to be highly dynamic yet still strong [77].

Composed of an α and β subunit, there are currently 18 different α and 8 different β subunits known, although splice variants have been found for some integrins [78]. Not all α and β subunits can bind to each other, as only 24 combinations have been found so far (See Figure 5.) and the particular combination of α and β determines the ligand specificity [79]. Integrins are ubiquitously expressed in all tissues, although differences between their expression patterns exist and the post-translational modifications for the same integrin, can result in functional differences [80, 81].

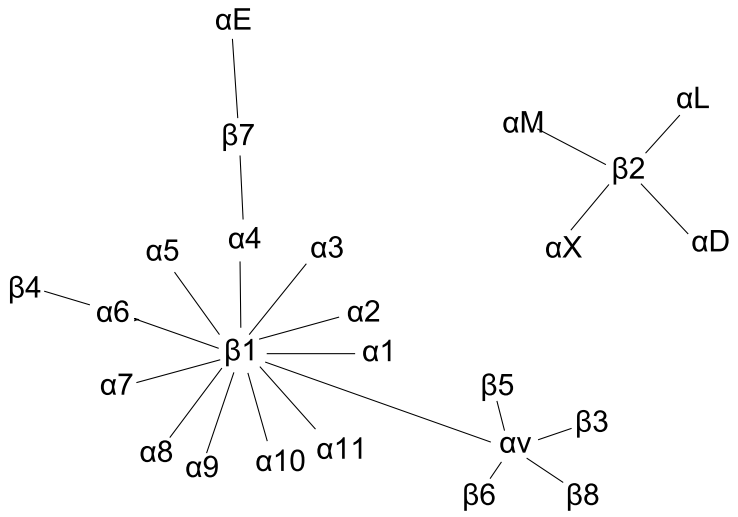

Figure 5. The integrin heterodimer combinations.

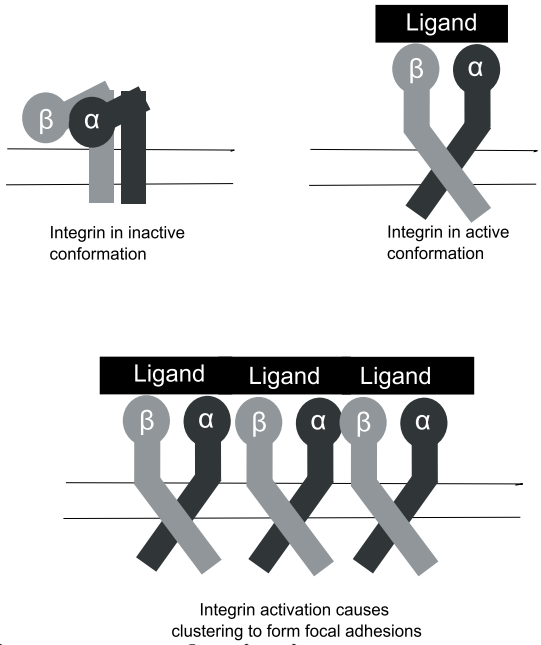

Figure 6. Integrin structure and activation.

Differential integrin expression and post-translational modifications are also seen in cancer, and play a key role in many aspects of carcinogenesis, including increasing proliferation, migration and survival of cancer cells [82, 83]. Binding of the different ligands results in differing signalling pathways being activated which is complicated by the effects of the post-translational modifications.

Integrin signalling is now recognised as playing an important role in normal physiology and also in pathological circumstances. Epithelial cells are required to be in constant contact with the surrounding ECM, which is "detected" by the integrins and reported to the cell via discrete intracellular pathways. Loss of this contact and the integrin signalling can lead to anoikis; controlled cell death which is similar to apoptosis [84]. Cancer cells are often thought of as being anchorage independent thus are insensitive to anoikis, although, as mentioned previously, this is not to say that signalling from the tumour microenvironment becomes redundant [76].

Integrin signalling is affected by the state of the integrin i.e. if the integrin is in an open active or a folded inactive state (See Figure 6.) [85]. Binding to an ECM ligand activates the integrin and causes clustering of many activated integrins. This in turn causes intracellular signalling, which is known as outside-in signalling.

The alternative to this is inside-out signalling; an intracellular signalling path--way activates the R-Ras and Rap1 GTPases and talin proteins (See Figure 7.), to induce the extracellular conformational change of the integrin, which is required for activation of the integrin [86, 87]. Both types of integrin signalling are hijacked by

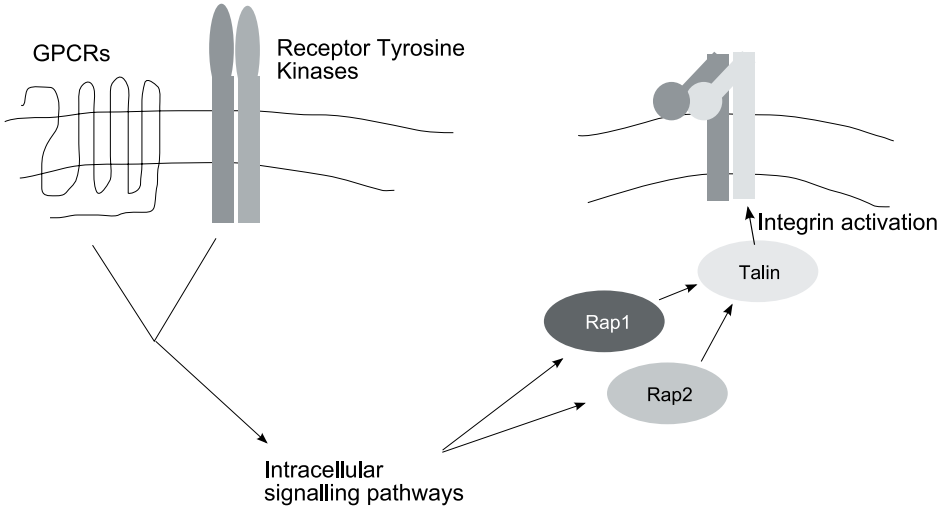

Figure 7. Inside-out signal pathway leading to integrin activation

cancer cells to further their progression. It is well established that many growth factors like epidermal growth factor (EGF) are over expressed in cancer and through their respective transmembrane receptors, have been shown to control integrin activation via inside-out signalling [88].

The outside-in signalling can take several forms. As mentioned above, post-translational changes to the integrin structure can mediate integrin related cell fates, indeed hyper-sialylation of the β1 integrin was shown to increase cell adhesion to collagen I and cell migration [83]. Differential regulation of integrin expression is another form of controlling outside-in signalling, as fewer or enhanced expression of "anti-" or "pro-carcinogenic" integrins respectively, would be favourable for cancer cells [89]. Perhaps the most well known example of this is the αvβ3 integrin, which is highly up-regulated in many tumours, resulting in enhanced cell migration, proliferation and angiogenesis and this has led to the development of anti-cancer drugs specifically targeting this integrin [90]. Other integrins are also known to be im -portant for tumour progression, indeed the α2β1 integrin collagen receptor was shown to mediate colon cancer cell migration and cell cycle progression [91, 92].

Augmenting the modulated integrin functions are the changes to the ECM. In both the tumour microenvironment and IBD, fibrosis is prevalent, and as a result integrin signalling is enhanced. Fibrotic tissue contains a higher content of collagens I, III IV and V, which results in the rigidity of the ECM to be significantly increased. This in turn leads to the development of larger and prolonged signalling complexes (see below), which naturally has the knock-on effect of prolonging stimulation of, for example, cell proliferation or producing other pro-carcinogenic molecules such as reactive oxygen species (ROS) [7, 76, 93].

Integrin heterodimers contain no intrinsic catalytic ability, therefore the conformational change induced by ligand (from the outside) or talin binding (from the inside), allows them to act as a scaffold for other proteins to dock into them and form signalling complexes known as focal adhesions (FA) [85]. For example, other scaffolding proteins such as paxillin and vinculin or enzymatic proteins such as focal adhesion kinase (FAK) and integrin linked kinase (ILK) [86], can bind and interact with other docked proteins, which can become, in the case of interaction with FAK and ILK, phosphorylated. A common example is the phosphorylation of c-Src by FAK, which leads to the propagation of the integrin signal, through in turn activating Extra cellular regulated kinase 1/2 (Erk 1/2), which is a key MAP (Mitogen activated protein) kinase regulating many signalling pathways [92, 94]. Thus by spatially and temporally regulating the scaffolding proteins binding to the integrins, this dictates the particular enzymatic proteins (such as kinases, phophatases, lipases and proteinases), present at any one time, thereby shifting the emphasis towards, proliferation, survival, differentiation or migration [79].

It is logical therefore to think of the FA as a dynamic structure, which is able to react rapidly to stimulation, however FAs are crucial for stable binding to the cytoskeleton. This is achieved through linker proteins, such as α-actinin, which serve to stabilise the cellular structure and morphology and facilitates the formation of tissues and organs [95]. Being linked to the cytoskeleton is also crucial in cell migration, as the cytoskeleton is the driving force behind cellular protrusions like lamellipodia and filopodia [96]. Again the FAs need to be dynamic not just assembling quickly, but disassembling as well (this the role of proteinases like calpain, which cleaves the actin filaments attached to the FAs), which releases the trailing edge of the cell from the ECM, and allows forward movement [97].

A vast number of FA associated proteins have been discovered especially for the β integrin subunit however the list is now growing for the proteins which interact with the α subunit. Additional layers of complexity of FA formation come in the form of the membrane location that they are formed and the other transmembrane proteins that are involved. Membrane lipid rafts are areas within the lipid bilayer that are rich in cholesterol and often are associated with caveolin proteins. Lipid rafts form descrete complexes and integrin signalling from the lipids rafts is distinct from non-raft signalling [98-100]. An expanding group of transmembrane spanning proteins are known to regulate integrin signalling, examples include the syndecans, tetraspanins and CD47. Differential association of integrins with these proteins, many of which act as scaffolding proteins, allows for the coupling to different pathways. For example the syndecans are coupled to protein kinase Cα, a kinase known to be involved in regulating cell proliferation and migration, whilst the integrin associated protein, CD47 has been shown to associate with integrins in membrane lipid rafts and couple to cyclic adenosine monophosphate (cAMP) signalling through a heterotrimeric G-protein [79, 101].

4.2 The integrin associated protein, CD47

The integrin associated protein, CD47, was firstly isolated from leukocytes and placenta in a complex with the αvβ3 integrin. Since then its cDNA has been cloned and found to be the same protein as the OV-3 antigen known to be up-regulated in ovarian cancer [102].

Although originally named IAP (Integrin Associated Protein), because it could be co-purified with a select number of integrins (αvβ3, α2β1 and αIIbβ3) and antibodies against it could regulate the integrins signalling capacity, it is now known to be able to bind ligands and function independently of integrins [103]. Proteins containing the VVM motif are able to bind and activate signalling from the protein and typical ligands are the ECM protein thrombospondin and the transmembrane protein SIRPα [102, 104]. CD47 is a pentameric transmembrane protein. The extracellular domain contains an IgV like domain, a multiply membrane spanning (MMS) domain and a differentially spliced cytoplasmic domain (See Figure 8.). The IgV like domain is responsible for ligand recognition and together with the MMS domain, for binding to the various integrins [102]. Four splice forms of the cytoplasmic domain have been found, with the fourth variant having the longest cytoplasmic domain and being the predominant form present in the intestines [105].

However all forms bind a class of heterotrimeric G-protein (same as GPCRs) which is sensitive to the modulation by the toxin produced from the *bordetella pertussis* (PTX) bacterium [101]. Additionally two new intracellular proteins have been discovered called PLICs (Proteins Linking IAP to Cytoskeleton, PLIC1 and PLIC2), which bind to the 2 and 4 splice form of CD47. These have been proposed to be involved in regulating the cytoskeleton, as their over-expression resulted in enhanced cell spreading and altered intermediate filament distribution [106].

CD47 signalling via the PTX sensitive G-protein mediates various cellular pr-

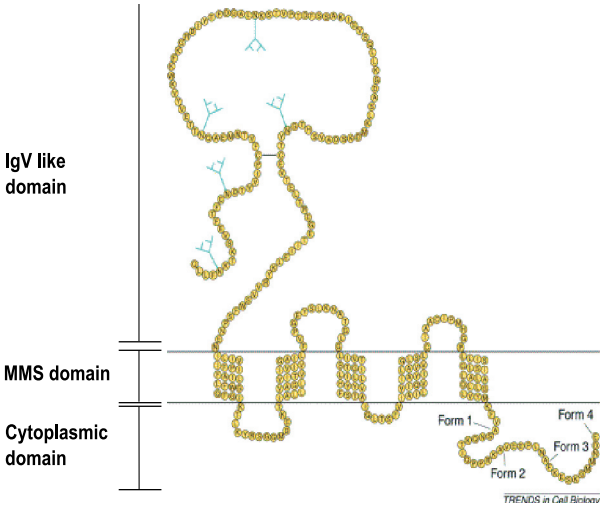

Figure 8. The structure of the integrin associated protein, CD47, indicating the domains and the four splice forms of the protein (Adapted from Brown et al. 2001)

-ocesses including apoptosis, leukocyte activation, cell adhesion and migration, all of which are dependent upon the cell type [103, 107, 108].

Cell migration in response to CD47 stimulation has been investigated in several cell lines including neurons and smooth muscle cells, where chemotaxis was through association with the α2β1 integrin and required MAP kinase activation [109]. It could also be speculated that the association of the α2β1 integrin with CD47, requires the localisation to low density lipid rafts (which contain a high cholesterol content and are enriched with detergent insoluble glycolipids), as was previously shown to be necessary for the functionality of the αvβ3-CD47 signalling complex [99], however integrin-CD47 complexes have not been detected in FA complexes but in focal contacts.

5. Intracellular signalling proteins

Activation of integrins which leads to the formation of FAs or focal contacts, proceeds to stimulate an overwhelming number of proteins and signalling pathways, many of which overlap with those activated by the GPCRs. It has been estimated that the proteome contains approximately 10,000 proteins [110], many of which are involved in intracellular signalling pathways, thus a few notably examples pertaining to the current project, will be described in greater detail in the following section.

5.1 Ras superfamily of small GTPases

The progression of many signalling pathways involves the activation of a key group of proteins namely the Ras superfamily of GTPases [111]. Intense research into the activity and role of these proteins has revealed their function as molecular switches, at the crux of many pathways. Indeed as the cell relies so heavily upon them, aberrations in their structure or dysregulation of their activation can have disastrous consequences. This was demonstrated firstly for the Ras protein, mutations to which have been found in approximately 20% of all cancers [112]. Studies in cell lines corroborated the *in vivo* data detailing certain Ras mutations alone, as being capable of causing cellular transformation; thus the *ras* gene has been designated as an oncogene [111].

The superfamily contains five subfamilies of proteins: Ras, Rho, Rab, Ran and Arf, altogether composing approximately 150 proteins [113]. These proteins like the α subunit of the heterotrimeric protein complex coupling GPCR signalling to the cell, bind GDP when inactive, GTP when active and possess an intrinsic ability to hydrolyse GDP to GTP. Cycling between the activation states (GDP versus GTP bound forms) is additionally controlled by the action of GEFs (Guanine Exchange Factors), which shift the equilibrium towards the active, GTP bound form. Counter-

-acting the GEFs are the; GAPs (GTPase Activating Proteins), which increase the hydrolysis of the GTP to the GDP form and in effect cause deactivation, and the GDIs (Guanine Dissociation Inhibitors), which inhibit dissociation of the nucleotide from the GTPase active site [114] (See Figure 9.).

Each of the five subfamilies have their own shared and distinct GEFs, GAPs and GDIs. Localisation with in the cell is determined in part by the post-translational lipid modification attached to the protein. Ras and Rho subfamily members can be farnesyl or geranylgeranyl isoprenoid modified, which allows their insertion into different membranes such as the endoplasmic reticulum or the outer lipid bilayer [113].

5.2 The Ras GTPase

The Ras sarcoma protein was the first to be found of all the Ras superfamily members. Within the cell, three genes code for four Ras proteins (HRAS, NRAS and the alternatively spliced KRAS4A and KRAS4B) whilst sharing a high homology, they however differ at the C terminal hyper variable domain, their subcellular localisation and the functions they perform [112].

Ras mutations are commonly found in tumours, with usual amino acid substitutions at positions 12, 13 and 61, which results in the ablation of the intrinsic GTPase function and inhibition association with the GAPs, thus the protein becomes constitutively active [115]. This has dire consequences for the cell as the Ras protein is a key mediator in epidermal growth factor (EGF) signalling, which promotes cell proliferation [114]. Therefore the presence of a constant proliferative signal leads to uncontrolled growth and also aberrations in DNA replication, which in themselves can induce cell transformation.

5.3 The Rac GTPase

The Rac GTPase, whilst being a member of the Ras superfamily of small GTPases, is part of the Rho subfamily. This subfamily is composed of five proteins, the most famous of which are Rho, Rac and Cdc42 [113]. Typically the Rho GTPases are thought to be primarily involved in cell migration and cytoskeletal rearrangements [116]. Activation of Rho leads to the formation of actin stress fibres and non-apoptotic membrane blebs seen in amoeboid type migration, whilst Cdc42 is implicated in filopodia formation, small spike like protrusions of the cell membrane [117]. Rac 1 and 2 again induce cytoskeletal rearrangements however, lamellipodia or membrane ruffles are typically formed by their activation [118].

Recent data has also shown that the Rho family proteins are able to influence other pathways which can influence cell proliferation and survival. Accordingly, overexpression or mutation to form constitutively active forms of the proteins induces cell transformation and can lead to enhanced cell migration[117].

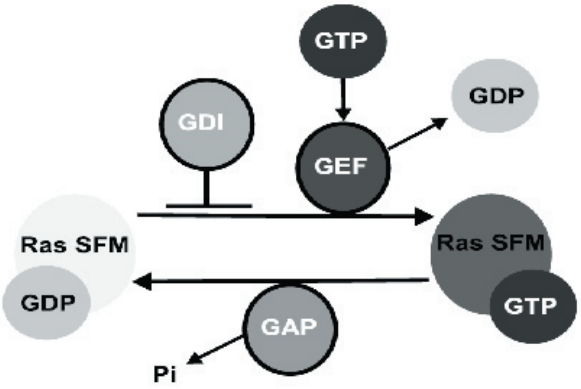

Figure 9. GTPase cycling. Activation of the Ras superfamily (Ras SFM) members is regulated, by the GEFs, GDIs and GAPs. Pi: Phosphate released by GTPase activity. RasSFM-GDP represents the inactive form whilst RasSFM-GTP represents the active form.

5.4 The Vav2 GEF

Three Vav proteins have thus far been discovered, imaginatively titled Vav1, 2 and 3. Vav1 is primarily found in haemopoetic cells, (although other cell types have been reported to express it), whilst Vav2 and 3 are widely expressed. Vav GEFs are responsible for controlling the activity of the Rho family proteins [119].

The three homologues share a similar structure, that contains several different functional domains. The *dbl* homology domain, is responsible for the GEF activity towards the Rho proteins, whilst the plekstrin homology domain binds to the lipid products of the phosphatidylinositol-3 kinase (PI-3K) enzyme. Binding of polyphosphates serves to activate the particular Vav GEF, which involves phosphorylation of specific tyrosine residues. The Src homology 2, Src homology 3 domains along with a proline and acidic rich region mediate Vav's protein-protein interactions [119].

Mutations of the Vav proteins, which result in a constitutively active protein, mimic the cell phenotype seen when the Rho family proteins are mutated, namely that the cells become transformed and increase their migratory potential, key features of tumour spreading to distant sites [118].

5.5 The Phospahtidylinositol-3 kinase

In the late 1980's a new signalling mechanism was proposed whereby intracellular lipid metabolites could act as signalling intermediates controlling a whole range of cellular effects from proliferation to migration. The phosphatidylinositol-3 kinase was thus found to be a key enzyme producing some of these lipid molecules.

There are three classes of PI-3K. Class I can use phosphoinositols (PI), phosphoinositol-4-phosphate (PIP) and phosphoinositol-3,4-bisphosphate (PIP_2) as substrates whilst class II can use PI and PIP, and class III can only use PI as a substrate. However only class I will be addressed here [121].

Two functional domains make up PI-3K, the catalytically active p110 subunit and the regulatory p85 subunit, which also contains SH2, SH3 and proline rich domains (which function similarly to the Vav domains in mediating protein-protein interactions such as binding to Ras, and cellular localisation) [122, 123]. Together they form the active enzyme which catalyses the addition of a phosphate to PI, with the preferred substrate of PI-3K seemingly being PIP_2, which results in the formation of phosphatidylinositol-3,4,5-trisphosphate (PIP_3).

The products of PI-3K are important factors mediating tumorigenesis as they are able to activate many different pathways, not only GEFs which lead to increased migratory potential, but also Akt is a major downstream target [124]. Akt/PKB is important regulator of proliferative signalling pathways, many of which are dysregulated in cancer a hallmark of carcinogenesis [125]. Thus unsurprisingly, PI-3K and its metabolites are known to be up-regulated in various cancers. This is exacerbated by the fact that PI-3K is a downstream target of many membrane surface receptors mutations to which increase their signalling in cancerous cells [121].

5.6 Protein Kinase C

There are many ways of transmitting signals, one fundamental way is through the phosphorylation of particular amino acids within a protein. This will invariably change the properties of the protein, for example activating it or allowing association with another protein. Three amino acids are known to be phosphorylated; tyrosines, serines and threonines.

The protein kinase C (PKC) enzyme is a family of kinases which are able to phosphorylate serine and threonine residues. In total, 10 mamalian isoforms have been classified and divided into three subfamilies; the classical, novel and atypical PKCs [126]. Whilst all PKCs share a similar general structure of having an amino terminal regulatory domain coupled to a carboxyl-terminal via a variable region [127], descrete structural differences between the three subfamilies exist, which are accompanied by differing modes of activation.

The classical PKCs require the presence of calcium ions and diacyl glycerol (DAG) for full activation, while the novel PKCs need only DAG, whereas the atypical isoforms require neither calcium ions or DAG [128]. The different PKC isoforms have varying expression patterns, however PKCα and β are ubiquitously expressed [129].

Since their discovery, the PKCs have been extensively studied and are found

to have a role in most cellular processes. PKCα is a member of the classical PKCs and once activated, unfolds to associate with the cell membrane, although a direct interaction with the β1 integrin cytoplasmic domain through the PKCα V3 domain, has been observed [130]. This was shown to be crucial for cancer cell migration, which corresponds well with data we have published, indicating a role for PKCα in up-regulating active β1 integrin at the cell surface after stimulation with LTD_4 [131].

5.7 Reactive oxygen species

Reactive oxygen species (ROS) are a group of compounds which are produced via reduction-oxidation reactions within the cell. These are highly reactive molecules because they contain oxygen in a reduced form and examples include hydrogen peroxide (H_2O_2), the superoxide anion ($O_2^{\cdot-}$) and the hydroxyl ion (OH^-).

The major source for endogenous ROS production comes from the mitochondrial electron transport chain, responsible for the cells energy production, where it has been predicted that 1-2% of electrons leak out and become available for ROS generation (although only H_2O_2 can pass through the mitochondrial membranes, and $O_2^{\cdot-}$ is dismutated to H_2O_2, by a superoxide dismutase) [132]. Another major source of ROS comes from the oxidation of unsaturated fatty acids such as AA. As previously mentioned lipoxygenases, COXs, the cytochrome P450 complex and γ-glutamyl-transpeptidase are involved in the metabolism of for example AA. All of these enzymes have been identified as sources of ROS production, metabolising not only endogenous unsaturated fatty acids but also exogenous sources such as thalidomide into ROS [133].

In phagocytes, which are known to produce large quantities of ROS, a specialised enzymatic complex, namely the NADPH oxidase is responsible for a considerable part of the ROS produced. Five NOX isoforms have been discovered, which are not restricted to phagocytic cells, with NOX1 being the main isoform found in the colon and intestinal barrier tissues. This enzymatic complex which at least in phagocytes, requires the binding of the Rac2 GTPase for full activation (but has not been shown in non-phagocytic NOX complexes) can also produce significant quantities of ROS in non-phagocytic cells [134]. Hepatocytes contain a specialised enzyme called xanthine oxidase, which is also capable of producing ROS [135].

The cellular production of ROS is balanced by antioxidant enzymes and molecules such a superoxide dismutase and glutathione, respectively. These serve to react with the ROS and neutralise their cellular effects by converting them to more stable and thus less reactive compounds [132, 133].

ROS are often cast in a “bad” light because of the large body of data which points to their detrimental effects on the cell. These include binding to DNA to ca-

-ause mutations to the strands which results in mutant proteins, but also through functioning as signalling intermediates.

Under both physiological and pathogenic conditions ROS are able to activate various key proteins. Studies with antioxidants reduced cancer cell proliferation, which indicates a role for ROS in the cell cycle [134]. This was further strengthened when ROS were shown to not only be able to activate the MAP kinase Erk1/2, but also $p90^{RSK}$ (both potent mitogenic inducers).

Oxidative stress i.e. ROS production is also known to activate two stress related proteins namely c-Jun NH_2-terminal kinase (Jnk) and Nuclear Factor κ B transcription factor (NFκB), both of which are involved in pro-carcinogenic pathways, with NFκB being intimately linked to IBD and the development of colorectal cancer [136].

5.8 Nuclear Factor κ B

The elucidation of DNA and the genes encoded therein, has in turn led to the discovery of a whole magnitude of transcription factors responsible for transcribing the genetic information into proteins. Many of these transcription factors are implicated in the pathogenesis of several diseases, and this is especially applicable to the NFκB transcription factor.

Firstly discovered in the late eighties in inflammatory cells, NFκB has since been shown to be present in the majority of cell types [137]. Although involved in many signalling pathways, its primary function is the transcription of proteins mediating the inflammatory response, such COX-2, and as such is highly activated during inflammation and tightly regulated otherwise [138].

This family of proteins is comprised of five members (p50, p52, p65, RelB and c-Rel), which can homo- or heterodimerise [139]. Typically the complex formation leads to gene transcription, however p50 or p52 homodimers alone, act as transcriptional repressors [140].

To perform its function NFκB must translocate from the cytoplasm to the nucleus to transcribe its target genes. In the cytoplasm it is bound to special proteins called inhibitors of κ B (IκB), which mask the nuclear localisation signal which NFκB requires to translocate. Upon cell stimulation, IκB kinases phosphorylate the IκB, which targets them for proteosomal degradation and releases NFκB to translocate into the nucleus [141, 142].

A considerable amount of effort has been put in to understanding the signalling pathways and mechanisms of NFκB, as it is implicitly entwined with the development and progression of several chronic inflammatory diseases such as IBD and many cancers [143]. Often dysregulated, NFκB signalling occurs due to dysfunctional regulation of the protein as opposed to mutations to the protein *per se* [144].

6. Cell migration

The activation of signalling pathways and the proteins transducing them, results in the stimulation of different cellular responses such as proliferation, survival and migration. The ability of cells to move is vitally important for mammalian development and homeostasis. Under physiological conditions cell migration is required in many processes, such as wound healing, where initially leukocytes (white blood cells) migrate into the area to induce inflammation and attack any potential pathogens. These are later followed by both epithelial and fibroblastic cells migrating in to rebuild and repopulate the wound. Indeed severe wounds may require angiogenesis, inducing the migration of endothelial and smooth muscle cells to renew the blood supply [145].

Wound healing will be required in any damaged cell layers including the epithelium in the intestines [7]. However cell migration plays an additional role in the intestines in facilitating the constant renewal of the epithelial barrier [1]. Intestinal stem cells at the bottom of the crypts provide new enterocytes (through their division) which differentiate and migrate along the crypt/villus axis (depending on the location in the intestines i.e. small intestine versus the large intestine). This is until they slough off at the top of the crypt or villus tip, into the intestinal lumen. The constant renewal has been hypothesised to be necessary to remove potentially damaged cells, which has a significant chance of occurring due to the epithelium being constantly exposed to potentially harmful factors [1, 80]. Thus without cell migration it is plausible that intestinal diseases would be more common.

The movement of a cell(s) requires the mobilisation of the cytoskeleton, in-conjuction with directional coordination. Directional stimulus comes in several forms: chemotaxis (movement along a chemical gradient); haptotaxis (movement along an ECM gradient); durotaxis (movement towards a more rigid ECM) and mechanotaxis (movement in response to shear stress forces) [145]. The dynamics and remodelling of the cytoskeleton are controlled by the Rho GTPases (and their associated GEFs, GAPs and GDIs), which in turn activate the Arp2/3 complex through proteins in the Wiskott-Aldrich syndrome protein (WASP) family, in the case of Rac and Cdc42; whereas Rho mediates its effects on the cytoskeleton through the formin protein, mDia1 (and possibly mDia2) [117]. These effector proteins dictate the structure and dynamics of not only the actin cytoskeleton (which is the main player in cell migration), but also microtubule and intermediate filaments.

Activating the different Rho GTPases results in different cellular protrusions [146] such as, lamellipodia, membrane blebs (migratory protrusions) and filopodia (exploratory protrusions). Rac and Cdc42 are commonly found at the leading edge of cells (the edge moving forward) in distinct morphological membrane protrusions called lamellipodia (See Figure 11.) [146]. In cells using lamellipodial migration, Rho is usually employed to stimulate retraction of the cell through activating actinomyos-

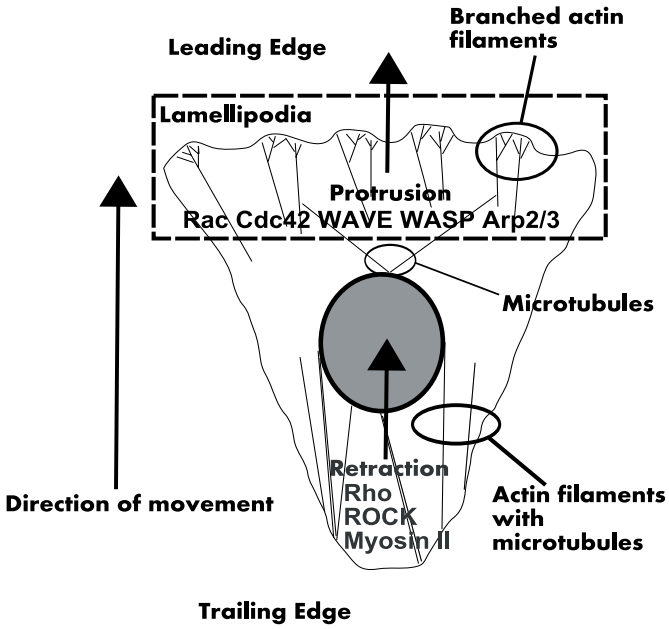

Figure 11. A simplified view of cell migration and the main players involved.

-in contraction. This is via Rho kinase (ROCK) phosphorylation of myosin light chain which induces myosin II crosslinking of actin filaments [117].

However, this Rho function has been proposed as the mechanism behind amoeboid type migration, which does not require lamellipodia formation, but instead non-apoptotic membrane blebbing is used as the morphological structure to facilitate forward migration [156].

Lamellipodial migration (the most studied type of migration) results in the formation of the lamellipodial cell protrusion, which is driven primarily by the extension of the cytoskeleton (mainly the polymerisation and branching of actin to form filaments supported by microtubules) from the underside of the cell membrane [98]. This has been hypothesised to be augmented by a fluid pressure force from the cytoplasm flowing into the lamellipodia, which is attracted by the hydrogel effect of the actin polymers and the physical outward movement of the membrane [71]. When the protrusion comes into contact with the ECM, this induces the formation of integrin mediated FA complexes and provides the traction force required to retract the rear of the cell. Thus as new FAs are formed at the leading edge of the cell, FA disassembly takes place primarily at the trailing edge which is mediated by, for example, the calpain protein which is a proteolytic enzyme responsible for cleaving FA associated proteins and attachments to the cytoskeleton [71, 121, 144].

It is possible to crudely divide cell migration into two broad catagories; metalloproteinase (MMP) dependent (requiring lamellipodial formation) and MMP

independent. MMPs are proteinases which cleave the extracellular matrix, that allows the cells to migrate through the degraded material, a mechanism commonly used by mesenchymal cells [43, 156]. These proteinases require the presence of a metal ion at their active site to function and can be membrane bound or released into the surrounding ECM.

MMP independent migration is a conserved form of migration, as it is used by the primitive single celled *dictostylium* and cells of the immune system in higher organisms. In contrast to MMP dependent migration, no degradation of the ECM takes place, instead cells, purely use the cytoskeletal pressure, which results in the cells physically pushing their way through the ECM (via actinomyosin contraction, see above) [151, 152].

Like many physiological processes such as proliferation and survival, cell migration is hijacked by cancer cells to further their propagation by allowing them to spread to new sites and exploit the available resources. Cancer cell metastasis (i.e. migration) accounts for a significant proportion of the aetiology and morbidity of cancer. Both types of migratory phenotypes are used by cancer cells to migrate, which has been proposed to be the reason for the relative failure of inhibitors of MMPs to block metastasis in cancer patients as was expected from the pre-clinical trials [151, 153, 154].

The present investigations

7. Aim

The aim of the investigations presented in this thesis was to further delineate the connection between eicosanoids and intestinal epithelial cell migration.

Specifically the following questions were addressed:

1. **Can leukotriene D_4 induce intestinal epithelial cell migration, and if so through which signalling intermediates?**
2. **Are the collagen binding integrins able to induce the expression of cyclo-oxygenase-2, and does this play a role in cell migration?**
3. **Is CD47 involved in the integrin mediated cyclo-oxygenase-2 expression and cell migration?**

Results and Discussion

8. LTD_4 induces intestinal epithelial cell migration through a PI-3 K and Rac dependent pathway (Paper I)

During the inflammatory response an overwhelming amount of cytokines, chemokines and bioactive lipids are produced by the different cell types present or infiltrating the inflamed area [155]. The eicosanoids are a family of bioactive lipids that have wide ranging effects upon various cells [18].

We have previously shown that the potent eicosanoid, LTD_4, is capable of inducing proliferation and protection from apoptosis, non-transformed intestinal epithelial cells [26, 67]. Although clearly necessary under physiological conditions, such as wound healing or indeed homeostasis, it is well documented that dysregulation of either of these processes leads to, or exacerbates, IBD or even colorectal carcinomas [156].

Cell migration is another mechanism which cancer cells have usurped for their own benefit. In light of the previous results with LTD_4 [33] and cancer cell migration, we decided to investigate effects of LTD_4 on non-transformed intestinal epithelial cell migration. Stimulation of the non-transformed intestinal epithelial cell line, Int 407, with LTD_4 induced a concentration dependent increase in migration of these cells. The Rho family proteins are known players in cell migration [117]. We have previously shown that LTD_4 can induce RhoA activation [157] and in the present study we found that another family member namely Rac, was also able to be activated by LTD_4 signalling. This was achieved by immunoprecipitation with a PAKcrib construct which binds the active forms of Rac and Cdc42. Using this construct we observed that Rac but not Cdc42 was in the GTP bound form after LTD_4 stimulation. This differential activation was accompanied by the phosphorylation of the Rho family specific GEF, Vav2.

Using a dominant negative protein to compete with the endogenous Vav2, we were able to deduce that the LTD_4 induction of Vav2 and Rac activation were related and not purely coincidental. The structure of Vav2 includes a domain which can bind to the products produced by the action of the PI-3K enzyme, PIP_2 and PIP_3 which correlates well to previous reports which have linked PI-3K and Vav2 activation to one another. Immunoprecipitation with an antibody against phosphorylated tyrosines or the p85 subunit of PI-3K indicated that at the same time as Rac activation is seen, an increase in phosphorylation of the p85 subunit occurs, which might indicate PI-3K activation. To further substantiate the potential link between PI-3K and Rac, the ability of LTD_4 to activate Rac was assessed in the presence of synthetic inhibitors against PI-3K and a dominant negative form of the p85 subunit. Immunoprecipiptation of the GTP form of Rac was reduced in the presence of the various inhibitors, wortmannin and LY294002.

Additionally the LTD_4 signal was shown to be mediated by a $G\alpha_i$ –protein coupling to the $CysLT_1$ receptor, as PTX could block Rac activation.

For a cell to move it needs to reorganise its cytoskeleton which, in turn induces morphological changes to the cell [146]. Staining of the actin cytoskeleton, allows detection of any morphological changes. Membranes ruffles were observed upon stimulation with LTD_4 which are typical migratory morphological changes associated with Rac activation [117]. Pre-incubation of the cells with the synthetic inhibitors of PI-3K were able to significantly block the LTD_4 mediated membrane ruffles.

Co-staining cells with an antibody against Rac, revealed that both Rac and actin co-localised in the membrane ruffles when stimulated with LTD_4, which were sensitive to the $CysLT_1$ receptor antagonist, ZM198,615.

Finally a Boyden chamber assay was used to test the hypothesis that the PI-3K dependent Rac activation was mediating LTD_4 cell migration. Int 407 cells were pre-incubated with PTX, or the two synthetic PI-3K inhibitors and allowed to migrate towards a LTD_4 concentration gradient. Cellular migration was enhanced in the presence of LTD_4 which could be significantly attenuated by PTX or the PI-3K inhibitors, supporting the hypothesis.

In summary we have shown that intestinal epithelial cell migration in response to LTD_4 is mediated by a signalling pathway requiring the activation of a $G\alpha_i$-protein, PI-3K and the GTPase Rac.

8.1 Collagen mediated cyclo-oxygenase-2 expression and cell migration is regulated by the integrin associated protein, CD47 (Papers II and III)

Changes to the ECM are gaining ever more importance in relation to their capability to influence pathological conditions [7]. Indeed it is well established that the proteins binding to, and transducing the signals from the ECM, mainly the integrins, have important roles in various pathological scenarios including cancer [158].

We have previously shown that the potent eicosanoids PGE_2 and LTD_4 are capable of increasing colon cancer cell migration in a COX-2 dependent manner. In addition these two eicosanoids could also increase colon cancer adhesion to collagen through up-regulating the surface expression of the $\alpha 2\beta 1$ collagen binding integrin [33]. In light of these findings we decided to further investigate any possible link between the ECM and COX-2 expression with special interest in the effect on cell migration.

Changes to the ECM which occur in IBD are before any neoplastic transformation, thus non-transformed intestinal epithelial cells were used. In an attempt to model the situation in IBD where, theoretically, the epithelium may come into

contact with the underlying stromal ECM, two collagens were used. Collagen IV (Col IV) found mainly in the basement membrane, was compared to collagen I (Col I), a protein found in stromal tissue and up-regulated in fibrotic tissue. Plating of the non-transformed intestinal epithelial cell line, Int 407, onto Col I or Col IV induced a significant increase in the surface expression of the α2β1 integrin. The increased integrin expression was accompanied by a significant increase in COX-2 expression and translocation to the peri-nuclear membrane, which was significantly different from any basal COX-2 expression (plausibly derived as a response to the cellular stress of being re-plated onto a new ECM) seen when the cells were plated onto the control surface. To date, two collagen binding integrins have been described the α1β1 and the α2β1 integrins [80]. However only the α2β1 in our system is important for the collagen mediated COX-2 expression, as pre-incubation with a specific α2β1 blocking antibody could dramatically reduce the COX-2 expression.

Many proteins are known to bind and transduce integrin signals, one of which is the integrin associated protein, CD47 [79]. Investigation of the integrin partners of this protein have revealed that it is relatively selective, binding only four integrins one of which is the α2β1 integrin. CD47 is known to signal through coupling to a PTX sensitive $G\alpha_i$-protein (similar to GPCRs) [102]. Int 407 cells plated onto collagen were unable to induce significant COX-2 expression in the presence of PTX, or a small blocking peptide targeting the $G\alpha_{i3}$ protein. Furthermore a functional blocking antibody (which inhibits the formation of the CD47-α2β1 complex seen upon activation with collagen), and a dominant negative form of CD47, were similarly able to inhibit collagen dependent COX-2 expression. Conversely, direct activation of CD47 with thrombospondin-1 (a known activator of CD47), resulted in COX-2 expression and augmented the collagen stimulation. Therefore there is evidence to suggest that the CD47-α2β1 complex formation is required for downstream signalling leading to COX-2 expression.

Previous reports in the literature have indicated that Erk1/2 and a Src family kinase are important signalling intermediates regulating integrin mediated COX-2 expression [159]. However we could find no evidence of their involvement in the collagen mediated COX-2 expression in the Int 407 cell line or in the Rat non-transformed intestinal epithelial cell line, IEC-6. Although inhibitors against these proteins had no effect on the collagen mediated COX-2 expression, inhibitors targeting PKCα, Ras and NFκB did show significant inhibition. The role of PKCα and Ras was further established when PKCα was down regulated or Ras was out competed using as dominant negative form of the protein. Both treatments resulted in the significant decrease of the collagen induced COX-2 expression.

COX-2 as mentioned before, is an inducible enzyme [47], therefore the mRNA and protein are quickly degraded upon the cessation of inflammation [160]. Thus it was of interest to assess if PKCα, Ras and NFκB were involved in the activation of the

COX-2 gene promoter, which was measured using a luciferase assay. Correspondingly the promoter activity resembled the protein expression, in that it was controlled by the activation of PKCα, Ras and NFκB.

The collagen binding integrins have been implicated not only in cancer cell migration but also in the progression of the cell cycle and ROS production [91, 92]. A recent article by Edderkaoui et al. defined a role for the ECM proteins fibronectin and laminin, in 5-LO dependent ROS production in a pancreatic adenocarcinoma cell line [161]. Consequently it was of interest to investigate the possibility that collagen could induce ROS production in a COX-2 dependent manner. Plating the Int 407 cells onto collagen resulted in a production of ROS in a time dependent manner. This stimulation could be significantly reduced when a specific inhibitor against COX-2 was used, thus providing similar evidence to the pancreatic cancer model, of a link between fibrotic ECM proteins and ROS manifestation.

Integrin signalling often leads to an increase in cell adhesion and migration [96]. Thus far, experiments had demonstrated that a signalling pathway existed from the formation of the CD47-α2β1 integrin complex to the production of ROS via COX-2 induction. A further question was if the CD47-α2β1complex formation could lead to reduced cell adhesion and lead to an increase in cell migration, and was this via COX-2?

Using the functional blocking antibody against CD47, cell migration across a collagen coated membrane was significantly reduced, in contrast to the non-specific IgG control antibody. Complementing this finding was the observation that wounding an Int 407 monolayer plated onto collagen, healed i.e. migrated, in a COX-2 dependent manner. Thus it appears from these data that the signalling emanating from the α2β1-CD47 complex controls multiply processes through regulating the expression of COX-2.

The effect seen on cell migration could plausibly be explained by a change in the cell morphology, which was observed specifically when the cells were plated onto collagen as opposed to fibronectin. As compared to the cell spreading seen when the cells were plated onto fibronectin, cellular membrane blebs, in-conjunction with a more rounded cell morphology, were apparent after plating onto the collagen matrix. In addition, it appeared the development of the blebs, were under the control of signalling from the α2β1-CD47 complex, as the functional blocking antibody or PTX could reduce bleb occurrence.

Membrane blebbing has previously been described as a mechanism used by leukocytes and some cancer cells to facilitate their migration and is associated with a reduced affinity for the ECM thus enabling cell movement, as their adherence has been lowered [94, 150]. In order to define a potential role for the bleb development in relation to CD47s signalling role, CD47 was inhibited with the functional blocking

antibody or stimulated with TSP-1, and the cell adhesion to collagen measured. In the presence of the blocking antibody as opposed to the IgG control, cell adhesion was greater than that of the untreated cells. Conversely enhanced stimulation of CD47 with TSP-1 and plating onto collagen significantly reduced cell adhesion.

In conclusion, it is plausible to hypothesise, that the membrane blebs identified here, play a similar role to those seen in leukocytes in facilitating cell migration, and inhibition of their formation reduces migration through increasing cell affinity and adhesion for the collagen matrix.

In summary, the work present here provides further evidence for the importance of the eicosanoids in regulating cell migration, either directly or indirectly, by using their synthesis to facilitate the conversion to the migratory phenotype.

Summary

In summary we have shown the following that:

1. LTD_4 can induce cell migration through the activation of PI-3K, Vav2 and Rac.
2. Collagen I and IV are capable of inducing COX-2 dependent cell migration and ROS production via PKCα, Ras and NFκB.
3. CD47 plays a central role in mediating the collagen I induction of COX-2 and cell migration.

A general summary

The intestines, constituting the small and large intestines are under constant duress, as they are continually exposed to potentially harmful substances and pathogens. In order to manage and respond to these foreign substances a constant low level of inflammation is present, which is able to distinguish between the foreign substances which are normally present and contrive little or no threat, and those which are harmful.

Inflammation is a physiological process which is a crucial first line of defence against pathogens and is intricately controlled by a multitude of substances/mediators. The balance between pro-inflammatory and anti-inflammatory mediators is shifted towards one side or the other, depending on the stage of the inflammatory response. Inflammatory mediators perform their functions by interacting with proteins, known as receptors which span the cell surface membrane and transmit signals into the cell interior to produce different cellular responses. Pathological conditions arise from inflammation, when the inflammatory response is prolonged and/or constant due to the regulation of the balance being lost, thus it is no longer acute (short lived), and has become chronic.

Chronic inflammation is the underlying cause of several common diseases such as arthritis, bronchiectasis and inflammatory bowel diseases (IBD) and in some cases such as IBD, patients are known to have an increased risk of developing cancer. Chronic inflammation has several characteristics, such as the continued presence of pro-inflammatory mediators, and the development of fibrosis. Elements of these two characteristics have been addressed in this thesis. The eicosanoid family of bioactive lipids are a group of potent inflammatory mediators that are up-regulated in IBD. There are two subfamilies namely the leukotrienes and the prostanoids. Various members of both subfamilies have even been heavily linked to the development and progression of colorectal cancer (CC). This is also true for the enzymes which are responsible for producing the eicosanoids, indeed cyclo-oxygenase-2 (COX-2), which is involved in prostanoid production, has been demonstrated to have a central role in driving cancer progression.

Cells are surrounded and held in place by a molecular scaffold known as the extracellular matrix (ECM). The ECM ensures the correct architecture and organisation of tissues and cells within the tissues. This matrix is composed of many proteins, glycoproteins and proteoglycans. As well as fulfilling a structural role the ECM is able to transmit signals to the cells through for example the physical interactions with the cells.

Integrins are proteins which span the cell membrane and anchor cells to the ECM. However they are also able to transmit signals into the cell interior and facilitate movement through the ECM, both of which are processes hijacked by cancer cells to aid their propagation.

Fibrosis is when the balance between production and degradation of the ECM, is shifted towards production, and is associated with a change to the composition and structure of the ECM.

The aim of the thesis was to further understand the link between eicosanoid signalling and cell migration of intestinal epithelial cells.

In the first article we use the potent eicosanoid, leukotriene D_4 (LTD_4), to stimulate intestinal epithelial cell migration. We found that the LTD_4 receptor, $CysLT_1$ was important for transmitting the LTD_4 signal into the cell via activating a heterotrimeric $G\alpha_i$-protein. This in turn could stimulate cytoskeletal (the internal scaffold of a cell) remodelling and cell migration through activating the Rac GTPase, which required the activation of the phosphatidylinositol-3 kinase and Vav2.

In the next two papers we investigated the effect of the ECM protein, collagen, on intestinal epithelial cell migration through inducing COX-2. We found that cell adhesion to the collagen types I (Col I) and IV (Col IV), could increase production of COX-2, which in turn could stimulate cell migration and reactive oxygen species (ROS) production. The signalling pathway leading to COX-2 expression was observed to require the activation of the $\alpha 2\beta 1$ integrin in-conjunction with another transmembrane protein, CD47. Similar to the $CysLT_1$receptor, the signal is passed through a $G\alpha_i$-protein and subsequently on to protein kinase $C\alpha$, the Ras GTPase and the NFκB transcription factor. Blockage of the action of COX-2 could reduce cell migration and ROS production. Cell morphological changes induced specifically on a Col I matrix are proposed to be involved in facilitating the intestinal epithelial cell migration.

In summary these results suggest that the eicosanoids play an important role in cell migration, in collaboration with signals from the extracellular matrix.

So it's finally here...the bit you've all been waiting for (assuming that you've *actually* waded through the first bit)

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