A degenerative process which is extreme enough to cause local accumulation of low molecular weight catabolic products, which leads to increased tissue osmotic pressure that draws extra fluid, with release of heat large enough for significant increase of tissue temperature is defined as inflammation (Stankov, 2012). Five cardinal signs of inflammation include tumor - tissue swelling, calor – elevated temperature of the tissue, rubor – vascularized tissue has blood color-like redness at the inflammation site, dolor – intense sensation of a harmful stimulus, and functio laesa, i.e. impaired function of the affected organ; four of which have been proposed by Celsus as long as 2000 years ago (Rather, 1971; [No authors listed], 2005).

Inflammation is the normal response of living tissue to injury or infection. When a tissue is damaged, inflammation is expected to occur since it is a normal response. Also, there is need for an adequate blood supply to the tissues in order for an inflammatory response to be manifested, as inflammation occurs in living tissue. There are several factors that trigger the inflammatory response which include chemical toxins, mechanical injury, hypersensitivity reactions and invasion by microorganisms. Several events occur during the inflammatory response, the major ones include increased supply of blood to the affected area; increased permeability of the capillaries and the movement of leucocytes from the vessels of the capillaries into the surrounding interstitial spaces to the site of inflammation or injury (Rankin, 2004). The inflammatory response demonstrates a complex biochemical and biological process involving cells of the immune system and a plethora of biological mediators (Fig. 1.1).
Inflammation can be categorized as either acute or chronic. Acute inflammation is the initial response of the body to noxious stimuli and is attained by the increased migration of plasma and leukocytes (especially granulocytes) from the blood into the surrounding injured sites. Prolonged inflammation, also called as chronic inflammation, is demonstrated by the simultaneous destruction and healing of the tissue from the inflammatory process and leads to a progressive shift in the type of cells present at the site of inflammation (http://en.wikipedia.org/wiki/Inflammation).

**Inflammation and cancer:**

An undeniable link exists between inflammation and cancer. A well-regulated inflammatory response can be anti-tumorigenic and have a role in tumor suppression (Mantovani et al., 2008). Chronic inflammation, however, is detrimental and can frequently
predispose the cells for an oncogenic transformation since the accumulation of genetic lesions in cells is essential to the development of cancer. These genomic alterations like activation of proto-oncogenes and inactivation of tumor suppressor genes are required for the initiation and may also be involved in the promotion of tumor development (Rakoff-Nahoum, 2006).

Inflammation is one of the most important underlying etiology of carcinogenesis in the colon (Ballkwill et al., 2001). The incidence of colitis-associated colon cancer increases by 0.5-1.0% yearly, 8-10 years after diagnosis (Munkholm, 2003) and presently the mechanisms that link inflammation and cancer are being unraveled at a fast pace. Inflammatory microenvironment as well as active innate immune cells produce cytokines, which facilitates cell proliferation, migration and angiogenesis (Boland et al., 2005). However, inflammatory cells also produce cytokines that can limit tumor growth. (Lin and Karin, 2007). Also, the pro-inflammatory cytokines act as extracellular factors and activate receptor tyrosine kinases (RTKs) like Vegf receptor 2 (Vegfr2), thereby stimulating tumor growth and progression (Janeway et al., 2001). In this context, IL-2 elicits a unique pattern of signalling associated with JAK3-dependent activation of the PI3K/AKT pathway with little or no involvement of STAT5 and NF-κB (Cho et al, 2013).

A missing link between inflammation and cancer could be the activation of NF-κB, a hallmark of inflammatory response, and is frequently detected in malignant tumors (Pikarsky et al., 2004). Under normal circumstances NF-κB dimers are confined to the cytoplasm by the inhibitory κB (IκB). But stimulation from pro-inflammatory cytokines like TNF-α, apart from that in viral and microbial infections, leads to the activation of the canonical NF-κB pathway which activates the Inhibitory κB kinase (IKK) complex (Ghosh and Karin, 2002). IKK phosphorylates IκBs bound to the NF-κB, leading to the ubiquitination of the former and allowing the release of the p50-p65 subunits of NF-κB to enter the nucleus where they can initiate the transcription of various cell survival, proliferation and anti-apoptotic genes.
Chronic inflammation develops through the action of various inflammatory mediators, including TNF-α, IL-1β and IFN-γ, which remove anti-tumor immunity and facilitate tumor progression (Lin and Karin, 2007) as also through IL-6, as shown in Fig. 1.2. IL-2 is an anti-inflammatory cytokine which increases the natural killer cell activity as shown in animals as well as humans (Robinson and Morstyn, 1987). TNF-α has been found to be involved in all the stages of carcinogenesis such as cellular transformation, promotion,
survival, proliferation, angiogenesis and metastasis (Aggarwal et al., 2006). It acts primarily through the induction of genes encoding nuclear factor-κB (NF-κB)–dependent anti-apoptotic molecules (Luo et al., 2004).

Cancer development and tumor progression have also been hypothesized to be caused by chronic inflammation, which is an important epigenetic and environmental factor. Sustained cell proliferation in an environment rich in inflammatory cells, growth factors activated stroma, and DNA-damage-promoting agents, certainly potentiates and/or promotes neoplastic risk, however the proliferation of cells alone does not cause cancer (Coussens and Werb, 2002). Ulcerative colitis is one such inflammatory disease which may lead to colon cancer in the long run.

**Ulcerative colitis** is a long-term, chronic condition affecting the colon and the rectum. The colon becomes inflamed and, if this inflammation becomes severe, the lining of the colon is breached and ulcers may form. It is a form of the inflammatory bowel disease (IBD), the other form being the Crohn’s disease. Common symptoms include diarrhoea, with or without blood and mucus, abdominal pain, a frequent need to go to the toilet and weight loss. The cumulative probability of colorectal carcinoma in ulcerative colitis patients has been shown in meta-analysis to range from 2% after 10 years of disease, up to 18% after 30 years of disease (Eaden et al., 2001; Feagins et al., 2009). Presently the focus of intense research is the mechanisms through which inflammation results in carcinogenesis and the molecular insights need to be unravelled. Many pathways are supposed to be involved, including the production of reactive oxygen species, and immune cells produce cytokine and chemokine expression, which lead to the increased risk of mutagenesis, and interactions between cancer stem cells and the local microenvironment of the tumor, including immune cells and myofibroblasts (Shaker et al., 2010; Shaker and Rubin, 2011; Quante et al., 2011). DNA methylation patterns and histone modifications are also affected by inflammation. Also, inflammatory bowel disease (IBD) patients with a family history of colorectal carcinoma are at a higher risk of developing colitis associated colon carcinoma. In this regard, the role of cyclooxygenase-2 becomes critical as it affects apoptosis, angiogenesis and cell proliferation and exhibits increased expression in the inflamed tissues.
Cox enzyme and inflammation

During inflammation, the formation of prostaglandins such as PGE2 from arachidonic acid is catalyzed by the cyclooxygenase (Cox) enzyme (Krysan et al., 2006) which has been established as the most important proinflammatory mediator and implicated in the process of carcinogenesis. Cox has two isoforms: the housekeeping cox-1 and the inducible cox-2, the latter being highly upregulated in inflammation, lesions, carcinoma and other disorders (Rishikesh and Sadhana, 2003).

Inhibition of Cox enzyme by NSAIDs

Non-steroidal anti-inflammatory drugs (NSAIDs), which act primarily by the inhibition of the cox enzyme, have been found to be highly effective chemopreventive agents in animal cancer studies (Hofer et al., 2002). NSAIDs are categorized into traditional NSAIDs (inhibiting cox-1 and cox-2) and coxibs (selectively inhibiting cox-2 while sparing cox-1, thereby creating no hindrance to the homeostatic activities of cox-1). The present study is an attempt to understand the mechanism of action of coxibs, such as celecoxib in ulcerative colitis associated colon carcinogenesis and thereby its preventive role. The dose standardization of celecoxib, the NSAID under investigation has been done by carrageenan-induced hind paw edema test.

Di Rosa et al. (1971) used several NSAIDs and showed that the inflammatory response to carrageenan consisted of three phases. The primary phase consisted of histamine and 5-hydroxytryptamine release (Rouleau et al., 2000), followed by bradykinin mediated secondary phase and the final phase attributed to the production of prostaglandins (PGs) (Asano et al., 1997). Their precursor is derived from arachidonic acid and the reaction is catalyzed by cox enzymes. NSAIDs act basically by the inhibition of these enzymes during pain and inflammation (Vane and Botting, 1995), as stated earlier.

Cancer cells containing neither COX-1 nor COX-2 have been found to undergo proapoptotic effects by NSAIDs, indicating that other mechanisms (Cox-independent) are additionally involved such as reactive oxygen species (ROS) production and inhibition of nuclear factor κB-mediated signals (Seo et al., 2007).
Apoptosis as end effect in cancer cell killings

Whether an inflammatory immune response is pro- or anti-tumorigenic is a delicate balance between cell proliferation and apoptosis. A highly regulated form of cell death is called apoptosis and it is distinguished by the activation of a family of cysteine-aspartate proteases (caspases) which are known to cleave various proteins, resulting in biochemical and morphological changes, characteristic of apoptosis (Jana, 2008). Bad is a pro-apoptotic cytosolic protein, which translocates to mitochondria and antagonizes with anti-apoptotic Bcl-2, thereby activating pro-apoptotic Bax and causing mitochondrial membrane permeabilization for cytochrome c to escape (Castedo et al., 2002). Once mitochondrion has released cytochrome c, the latter orchestrates the assembly of an intracellular apoptosome complex that recruits the terminal caspase 3 leading to cell death (Keith et al., 2008). One of the necessary events in the development of the tumor cells is the dysregulated cell proliferation which is coupled with the obligate suppression of apoptosis (Evan and Vousden, 2001).

Oncogenic signal transduction in tumor promotion

As the cells proliferate, an intricate network of positive and negative signals tightly regulates the mitotic cycle progression, referred to as cell cycle control (Weaver and Cleveland, 2005). By phosphorylating their respective partner cyclins, cyclin dependent kinases (cdks) allow progression through the different phases of the cell cycle. For example, cyclin D associates with Cdk4 and Cdk6 during early G1, whereas cyclin E activates Cdk2 during G1 to S phase transition (Neganova and Lako, 2008). The latter acts by phosphorylation and thus inactivation of the tumor suppressor protein p53. p53 is the inducer of p21 gene family, which are the negative regulators of cell cycle (Gartel and Tyner, 2002). A critical feature of the tumor cells is the inappropriate cell cycle progression, which is also the hallmark of the transformed cells that they lack appropriate checkpoint control (Hall and Peters, 1996). Presently, we attempt to look further into the aspects of inflammation associated colon cancer by studying the RTKs activated MAPK pathway. The latter is well known to facilitate the upregulation of several oncogenic agents including the PI3K regulated downstream pathway (Shahbazian et al., 2006).
PI3K is a phospholipid kinase and generates PIP3 (Phosphoinositol-3 phosphate) while this reaction is reversed by Phosphatase and tensin homolog (PTEN), the negative regulator of this oncogenic PI3K pathway (Motoyama et al., 2009). PIP3 is a second messenger for the phosphorylation of Akt, when it is translocated to the plasma membrane where it is activated by PDK1 by phosphorylation. Fundamental cellular functions such as cell proliferation and survival are governed by the activation of Akt by phosphorylation of its downstream substrates like Glycogen synthase kinase 3β (GSK3β) and thereby its inactivation (Vasconsuelo et al., 2008). GSK3β in its active form doesn’t allow the activation of β-catenin (Chada et al., 2005). But when inactivated, β-catenin complex is degraded and β-catenin is phosphorylated, which moves to the nucleus and induces the transcription of several cell survival and proliferation related proteins (Hamad et al., 2013). Upon the inactivation of GSK3β, another oncogenic pathway led by Wnt activates β-catenin and promotes tumor growth (Larriba et al., 2013).

The PI3K/Akt signalling and the canonical Wnt/β-catenin signalling are well known to be mutated during neoplastic transformations (Saji and Ringel, 2010). PTEN, a tumor suppressor in both benign and malignant growth, is a potent antagonist of PI3K/Akt and Wnt signalling. Pancreatic cancer cells show that siRNA-mediated inhibition of PTEN gene expression leads to the increase in their Vegf secretion and upregulated the proliferation (Ma et al., 2009). The subsequent activation of PI3K and other signalling pathways and the phosphorylation of Vegfr2 occurs by the binding of Vegf to Vegfr-2. Recent reports demonstrated that normal vascular development and tumor angiogenesis is governed by the PTEN/PI3K pathway. Also, several studies have strongly indicated that PTEN was associated with tumor induced angiogenesis (Hamada et al., 2005). Therefore, the role of this oncogenic PI3K pathway in angiogenesis in the mice colon along with colitis is explored in the present thesis.

**Neo-vascularization or Angiogenesis**

Angiogenesis is the physiological/pathological process of the formation of new blood vessels from the pre-existing ones (Pratheeshkumar et al., 2012). Growing tumors release Vegf, which is received by Vegfr2, an RTK, leading to the upregulation of various inflammatory and angiogenic factors including Cox, MMPs, etc (Okumura et al., 2012). These make the
endothelial cells of the blood vessels more permeable thus increasing cell proliferation, migration and angiogenesis. Moreover, Vegf also activates the oncogenic PI3K/PTEN pathway (Jiang and Liu, 2009). In various kinds of human tumors such as leukemia, the mutations and amplification of PI3K/Akt and the functional loss of the PTEN are common (Shafee et al, 2009). Several downstream targets such as GSK-3β, mTOR and iNOS are involved in the regulation of angiogenesis by PI3K/Akt. HIF-1α and NF-κB activation by PI3K can also lead to the induction of Vegf expression.

**LACUNAE:**

Recent research in the field of cancer has shown the wide use of NSAIDs as chemopreventive agents against various types of cancers. But their role in ulcerative colitis and colitis associated colon cancer is still not clear. Besides, the molecular mechanisms on downstream signaling pathways in carcinogenesis, angiogenesis and anti-apoptotic pathways are largely not known. Therefore, the present study put emphasis on the interaction of NSAIDs with the PI3-K/Akt/Wnt signaling pathway along with the molecular agents of angiogenesis including MMPs, MIP, MCP and a variety of cytokines in experimentally induced colitis and colon cancer. The proteins of the apoptotic pathways, in particular the mitochondria mediated intrinsic pathway of apoptosis are also studied under the said experimental conditions.

**OBJECTIVES:**

**Inflammation (colitis) and Cancer:**

- To develop an animal model of colitis by the oral administration of DSS in drinking water, and also to develop i) early stage of colon carcinogenesis by subcutaneous administration of 1,2-dimethyl hydrazine dihydrochloride (DMH) and to develop ii) colitis-associated colon cancer model by co-administration of DMH and DSS for six and eighteen weeks in female Balb/c mice.

- To establish the role of celecoxib as a chemopreventive agent against colitis and subsequent colon cancer.

- Establishment of anti-inflammatory role of celecoxib by carrageenan-induced hind paw oedema in mice model.
➢ To determine disease activity index (DAI) by scoring the extent of body weight loss, stool consistency and rectal bleeding.

➢ To establish histopathologically, the prognostic biomarkers of colon carcinogenesis such as Multiple plaque lesions (MPLs), Aberrant crypt foci (ACFs), and dysplasia and hyperplasia in the different treatment groups in relation to cancer progression/regression in the presence and absence of celecoxib.

➢ To perform immunohistochemistry using Alcian blue stain to identify goblet cells, Periodic acid Schiff (PAS) stain and Mucicarmine to stain the epithelial mucins.

➢ To perform Myeloperoxidase (MPO) assay to determine the extent of neutrophil infiltration in inflammatory pathogenesis.

**Apoptosis and Cell Signalling:**

➢ To study apoptosis in the isolated colonocytes by fluorescent staining and TUNEL assay.

➢ Study of cell proliferation by the expression of cell proliferation marker Proliferating cell nuclear antigen (PCNA).

➢ To study the expression by western blots and immunofluorescence of the following proteins:
  
  - Two isoforms of cyclooxygenase enzyme: COX-1 and COX-2.
  - Matrix metalloproteinases and angiogenic factors: MMP2, MMP9, MIP-1, VEGF, MCP-1, iNOS.
  - Gelatin zymography for the matrix metalloproteinases activities.
  - PI3-kinase pathway and Wnt signalling: Wnt, β-catenin, GSK-3β, PI3-K, Akt, PTEN, NF-κB, IκBα, IKKα.
  - Cell cycle regulators: cyclin D1, cyclin E, CDK4, CDK2.
  - Intrinsic apoptotic pathway: Bcl2, Bad, Bax, Apaf-1, caspase-9, caspase-3.
• Tumor suppressors: p53, Rb.

• Cytokines: IL-4, IL-2, IL-1β.

**Membrane Dynamics and carcinogenesis:**

- To study membrane properties such as membrane fluidity using 1,6-diphenyl-1,3,5-hexatriene (DPH); membrane phase state determination by Laurdan (6-dodecanoyl-2-dimethylaminonaphalene); and lateral phase separation using NBD-PE fluorescence quenching studies.

- To estimate intracellular Ca+2 levels by fluorescent intensity using Fura-2.

- To study the generation of reactive oxygen species (ROS) and role of antioxidant scavengers in apoptosis by oxidation sensitive fluorescent probe 2’,7’-dichlorofluorescein diacetate.

- To study the role of nitric oxide in apoptotic signal transduction and the expression of inducible nitric oxide synthase.