INTRODUCTION

Cancer may not be at all the dreadful disease as commonly perceived but could be an welcome defense response in maintaining cellular homeostasis to the chronic low grade inflammation and the consequent pathogenesis. Inflammation is identified to be the outcome of local synthesis and release of the prostaglandin (PG) molecule (PGE$_2$) which is derived from a long chain polyunsaturated fatty acid, arachidonic acid (C$_{20}$:4). PG is formed by prostaglandin synthase which carries out the dehydrogenation and cyclooxygenation reaction of the linear chain and the subscript 2 refers to the two double bonds that occur outside the ring structure.

Today there is a paradigm shift in our understanding and treatment modalities of cancer, where we focus more on the prevention strategies and intend to block the process of tumorigenesis rather than using extremely cytotoxic chemotherapeutic agents in a full blown cancer. The disappointing results with the currently available therapeutic modalities for advanced colon cancer have stimulated various cancer prevention efforts in recent times which are classified as primary, secondary and tertiary (Krishnan et al., 2000) effort, respectively. Primary prevention is concerned with the preventable etiological factors of cancer. Cessation of smoking is an excellent example of an effective intervention for the primary prevention of lung cancer. In secondary prevention premalignant conditions are identified and treated in the subjects at risk. Screening colonoscopy with removal of benign colonic polyps (the premalignant stage in colon carcinogenesis) represents such an intervention. In tertiary prevention or chemoprevention a naturally occurring or pharmacological agent is administered to individuals at risk to prevent the development or the recurrence of cancer (Rigas, 2007).

Chemoprevention, which means the use of drugs to inhibit carcinogenesis, is an important and rapidly evolving subject of cancer research. Among the treatment modalities actively pursued at present, includes the chemoprevention by non-steroidal anti-inflammatory drugs (NSAIDs) which block the prostanoid production
from the arachidonates through the inhibition of the enzymes, cyclooxygenase (COX) (Smith et al, 1998).

COX enzymes are a part of the PG synthase complex which is responsible for cyclic modification of the fatty acid and had been identified to exist in three isoforms (Smith and Langenbach, 2001). COX-1 is constitutively expressed whereas COX-2 is a highly inducible early response gene product that is activated by cytokines, growth factors, phorbol esters, oncogenes and chemical carcinogens, and therefore, has a putative role in inflammation and carcinogenesis (Levy, 1997; Smalley and DuBois, 1997). COX-3 which is a COX-1 variant has been tentatively found in the neuronal tissues (Chandrasekharan et al, 2002). COX-1 is involved in the maintenance of tissue homeostasis, with near constant levels and activity in many tissues, including the mucosa of the gastrointestinal tract where it is believed to protect against gastric damage. COX-2 is minimally expressed in most tissues, but capable of induction at sites of inflammation as evidenced by an increased mRNA, protein and enzymatic activity as high as 10-fold or more, which then promptly returns to the basal level once the stimulation subsides. Exceptions to this phenomenon include components of the central nervous system, the kidney and the seminal vesicles, which contain constitutively high levels of COX-2.

Increased COX-2 expression has been demonstrated in several cancers, including esophageal, gastric, colorectal, pancreatic, prostatic and cervical tumors (Eberhart et al, 1994; Sano et al, 1995; Wolff et al, 1998; Zimmemmann et al, 1999; Yip-Schneider 2000; Gupta et al, 2000: Kulkarni et al, 2001). COX-2 is also overexpressed in breast, lung and stomach cancers (Ristimaki et al, 2002; Soslow et al, 2000; Tucker et al, 1999; Van Rees et al, 2003; Yamagata et al, 2002).

Genetic and pharmacological studies suggest that COX-2 induction is an early step in colorectal tumorigenesis, as seen by COX-2 expression in the aberrant crypt foci, one of the earliest detectable premalignant lesions in colorectal tumorigenesis (Subbaramaiah and Dannenberg, 2003). COX-2 has also been shown to be overexpressed in 40-50% of premalignant adenomas and 80-90% of human colorectal carcinomas (Eberhart et al, 1994). Perhaps the most striking evidence implicating COX-2 in colon carcinogenesis is the finding that a null mutation for COX-2 markedly reduced the number and size of intestinal tumors in the APCΔ716 knock out mice, a murine model of familial adenomatous polyposis (FAP) (Oshima et al, 2001). Also, in two different APC knockout mouse models, APCΔ716 and min mice, adenomas at early stages showed loss of heterozygosity of the normal
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APC allele concurrent with the COX-2 overexpression (Williams et al, 1996; Oshima et al, 2001) and of Wnt signaling (Araki et al, 2003; Uematsu et al, 2003). These results suggest that COX-2 is a rate-limiting step in the physiological manifestation and formation of intestinal polyps.

In colon cancer, COX-2 catalyzes the oxidation of arachidonic acid, which produces prostaglandins and highly reactive by-products that may accelerate the carcinogenic process while a high concentration of free arachidonic acid can promote apoptosis (Cao et al, 2000). It is possible that the increased level of COX-2 may serve to lower the intracellular level of free arachidonic acid and thereby prevent apoptosis. Thus COX-2 catalysis may simply be a process that depletes an apoptotic signal. Alternatively, products formed by the enzymatic action of COX-2, presumably one of the prostaglandins (PGE$_2$), alter cell growth, apoptosis, angiogenesis or other steps involved in tumorigenesis (Dempke et al, 2001; Sheng et al, 1998). COX-1 and COX-2 have also been shown to cooperate to induce angiogenesis and as a result PGE$_2$ inhibits programmed cell death by inducing expression of the Bcl-2 protooncogene (Sheng et al, 1998). PGE$_2$ and other prostaglandins often elevate intracellular cyclic AMP concentrations which can also suppress apoptosis.

The increased levels of PGs in the tumors provided the rationale for the use of non-steroidal anti-inflammatory drugs (NSAIDs), which inhibit the COX enzymes, as potential chemopreventive agents. The fact that COX-2 gets induced, and aspirin and other NSAIDs taken on regular basis decrease the relative risk of colorectal cancers in epidemiological studies (DuBois et al, 1996), suggests a possible role for COX-2 and PGs in the induction of colorectal cancers. Thus, in addition to the general use as inhibitors of inflammation, pain and fever, the NSAIDs have a potential utilization as chemotherapeutics for the prevention of human cancer (Marnett, 1992; Duperron and Castongnay, 1997).

NSAIDs of older generation like Aspirin, Ibuprofen and Indomethacin nonspecifically inhibit the COX-enzymes, thereby blocking not only the inflammatory PGE$_2$ but also other PGs which are cytoprotective, particularly in the gastrointestinal mucosa. Therefore, although these drugs are found to cause regression of colon cancer, these may also produce the undesirable side effects like intestinal bleeding and ulcers. Newer generation NSAIDs on the other hand, such as those specifically inhibit the COX-2 but spare the beneficial effects of COX-1 are now the centre of attraction as chemotherapeutic models in cancer of colon and other tissue. Newly developed NSAIDs such as the coxibs (eg, Celecoxib, Rofecoxib, Lumiracoxib, Etoricoxib, etc.) held tremendous potential in cancer
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Chemoprevention as demonstrated in epidemiological studies, animal experimentation, cell culture systems and also some of them are approved for clinical studies (Shi and Klotz, 2008).

For many years the cytotoxic actions of the chemotherapeutic drugs were ascribed solely to the ability to induce genotoxic damage. During the past decade or two however, the evidence is gradually accumulated that many agents induce cell death by a process known as programmed cell death or apoptosis. The accumulating evidences suggest that defects in the process of apoptosis may be closely associated with carcinogenesis and that many cancer cells have defective machinery for self destruction (Yano et al., 1994). The susceptibility to apoptosis-inducing effects of the chemotherapeutic drugs may depend upon the intrinsic ability and or resistance of the tumor cells to respond to the apoptotic signals. (Yano et al., 1994). Evidence strongly suggests a protective effect of the NSAIDs in colon cancer by promoting apoptosis (Chung-Faye and Kerr, 2007) while gene knock out studies in mice suggests that inhibition of the COX-2 pathway by NSAIDs may be important in the mechanism of their action (Di Pace et al., 2006).

Prior studies have utilized various chemical carcinogens to induce colonic neoplasia in experimental animals as the means of investigating some of the biological characteristics of these tumors (Weiseburger, 1971; LaMont and O’Gorman, 1978). One such carcinogen, 1, 2- dimethylhydrazine (DMH) has been extensively studied. DMH is a colonic procancerogen that requires metabolic activation within the host to become active (LaMont and O’Gorman, 1978). DMH induced tumor in the rodents closely parallel human colonic cancer with respect to clinical and pathological features (Lingeman and Garner, 1972). Using this model of experimental colon cancer, several investigators have reported various toxicological, enzymatic and biochemical changes in rat colonic tissue prior to the development of overt tumors (Lingeman and Garner, 1972; Ball et al., 1976). Few other studies, utilizing the DMH model of colonic adenocarcinoma, have shown the alterations in the composition and dynamicity of colonic plasma membrane (Brasitus et al., 1986;1988). Also, the alterations in the membrane lipid composition and fluidity in the cancer cells had been reported, thus indicating the relationship of membrane fluidity to the malignant transformation process. It has also been reported that the intracellular pH (pHi) changes to alkalinity and associated Na’/H’ antipporter activity may cause the condition favorable for carcinogenesis in the colonocytes (Brasitus et al., 1988).
Lacuna

Although extensive research is done on the molecular pathogenesis of colon cancer, not much information is available on the drug membrane interaction in the preneoplastic and neoplastic tissue. Till date, various studies have explained the chemopreventive capabilities of NSAIDs and their actions through the enzymatic changes and metabolic pathways, however, studies have not been performed in rat colonocytes to explain the role of NSAIDs in membrane events during carcinogenesis. In view of these lacunae in the knowledge, the present study was designed to examine the various plasma membrane associated changes in the rat colon under the influence of DMH alone or in combination with the NSAID administration.

Clearly, the ability of the NSAIDs to induce cell cycle arrest and apoptosis has received attention in recent years and also a number of studies strongly demonstrated a positive correlation between COX-2 expression and down regulation of apoptosis in cancer cells (Reddy et al, 1999; Pardhasaradhi et al, 2003; Subhashini et al, 2004, 2005; Shi and Klotz, 2008). However, the underlying molecular mechanisms of such resistance to cellular death in cancer are still not fully understood. Therefore, the present study is also a detailed account of the evidence for the regression in early carcinogenesis studies with aspirin which is a benchmark NSAID and two recently developed coxibs, celecoxib and etoricoxib, in an animal model where colon cancer is induced with DMH.

Hypothesis

Membrane dynamicity is known to be changed in many pathophysiological conditions which could therefore be the initial biophysical event that alters the cellular pH and calcium homeostasis leading to the carcinogenesis as well. This may be influenced by the NSAIDs to trigger the signal of apoptosis as a dominant end-effect of cancer cell killings.

Objectives

- Establishment of an animal model of experimental colon carcinoma by subjecting the rats to a colon specific carcinogen 1-2, dimethylhydrazine (DMH)
- Histopathological evaluation of the various prognostic biomarkers of colon cancer in the different treatment groups in relation to cancer progression/regression.
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- Study of the role of membrane lipids in carcinogenesis, by analyzing the phospholipids, cholesterol and following their biosynthesis in the colonic tissues

- Elucidating the membrane dynamics of colonic epithelia such as membrane fluidity, membrane phase state and membrane phase separation as initiating biophysical events potentially responsible for colon carcinogenesis.

- Study of the alterations in chemical environment of colonocyte membranes by studying the functional groups in the biomolecules using FTIR spectroscopy.

- Elucidating the pathway underlining NSAIDs induced apoptosis:
  - Role of Na⁺/H⁺ exchange in colonocyte membranes, intracellular pH changes during the malignant transformation and chemoprevention as studied by fluorescence spectroscopy.
  - Study of intracellular Ca²⁺ reserve, Ca²⁺ATPase and Ca²⁺ uptake in the colonocytes by fluorescent and radioactivity analysis.
  - Study of the role of reactive nitrogen species like nitric oxide as the stable product along with generation of reactive oxygen species and the antioxidant defense system during colon carcinogenesis.

- Elucidating the molecular events underlining NSAIDs induced apoptosis as the dominant end effect:
  - Study of the expression of the important interacting proteins like COX-1 and COX-2, PARP cleavage, iNOS, cytochrome-c, Bcl-2 and caspase-3 expression by Western immunoblot assay.
  - Immunohistochemical localization of COX-2 in colonic sections
  - Determining the status of apoptosis by fluorescent microscopy, DNA fragmentation analysis, Terminal de-oxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay and caspase activity measurement.