Abstract: Hepatocellular carcinoma represents one of the most common malignancies worldwide with a rising incidence in western countries. Chronic inflammation is recognised as a threat factor for cancer progression. Cyclooxygenase-2 is the major mediator of inflammation. Various studies on Cox-2 suggest its possible association with HCC differentiation. Sufficient genetic and pharmacologic evidences implicate its crucial role in neoplasia and it is also now clear that Cox-2 plays a crucial role in tumor progression. Cox-2 overexpression is associated with maintaining tumor microenvironment and has crucial implication for angiogenesis. Cox-2 operates in multifactorial fashion. Cox-2 selective inhibition has been reported as a successful tool in suppressing angiogenesis and metastasis. The pharmacological suppression of Cox-2 represents a bright future as a therapeutic tool for treatment of various malignancies. This review is an attempt to discuss the critical issue of overexpression of Cox-2 and its role in the development of HCC in particular and cancer in general.

Keywords: Cox-2; PGE2; HCC; Tumorigenesis.

1. Introduction
Hepatocellular carcinoma represents one of the most common malignancies worldwide with a rising incidence in western countries. With advancement in science and medical facilities, early clinical diagnosis of HCC and its management is possible. Yet the complicated mechanisms involving infectious, genetic and epigenetic factors contributing to its development have not been completely understood and remain a serious medical issue (Cervello and Montalto, 2006). Causative agents like Hepatitis C Virus and Hepatitis B Virus infection, excessive alcohol consumption and aflatoxin ingestion lead to HCC progression (Abou-Shady et al., 1999; Trujillo-Murillo et al., 2007; Xue, 2005). Primary liver cancer caused by HCV has affected more than 170 million people world-wide (Xu et al., 2001). In about eighty percent of infected people, HCV is responsible for acute and chronic liver diseases resulting in fibrosis, cirrhosis, and most importantly HCC (Choi et al., 2004; Xu, 2002). HCC is a highly malignant tumor characterized by active angiogenesis and extracellular matrix formation (Abou-Shady et al., 1999). Common clinical manifestations include severe abdominal pain and deteriorated hepatic function (Cervello and Montalto, 2006). Earlier incidences of HCC were reported to be more prevalent in east Asian countries, but in recent years the incidence is rising fast in the western world too and it has become the leading cause of liver transplant in USA and Europe (Chi-Man Tang et al., 2005; Tang and Grise, 2009; Xu, 2002). This review is an attempt to look at the critical issue of overexpression of Cox-2 and its role in the development of HCC.

2. HCC and Inflammation
HCC development is a multi-pathway process involving activation of proto-oncogenes, inactivation of tumor suppressor genes, dysregulation of RB1, p53 and Wnt pathways (Xue, 2005). Chronic liver inflammation coupled with active neovascularisation are characteristic...
pathological changes associated with HCC (Chi-Man Tang et al., 2005; Tang and Grise, 2009). Chronic inflammation is recognised as a threat factor for cancer progression. There is sufficient evidence to support its crucial role in pathogenesis related to several types of cancer including pancreatic, breast cancer, colorectal cancer, squamous cell carcinoma in head and neck, ovarian cancer, gastric adenocarcinoma, lung cancer and HCC (Cianchi et al., 2001; Costa et al., 2002; Fujiwaki et al., 2002; Gallo et al., 2002; Li et al., 2003; Tang et al., 2005; Wolff et al., 1998; Yip-Schneider et al., 2000). Inflammation is an immediate response of natural immunity and plays a key role in physiological and pathological conditions like pathogenic invasion or wound healing. It can be activated by various compounds such as lipopolysaccharides during bacterial confrontation, presence of toll like receptors that are produced to detect viruses, physical injury such as by UV radiation and chemical compounds like reactive oxygen species. When afflicted with pathogens, specific receptors are activated which trigger a multi-factorial network of signal cascade mediated by NFkB, p38 or MAPKs, which in turn regulate production and crosstalk between pro-inflammatory cytokines, chemokines and cell adhesion molecules. All these incidences dictate recruitment and activation of immune cells. Constant persistence of cytokines, chemokines, various immune cells like lymphocytes, macrophages, and dendritic cells potentiate production of tumor microenvironment. Tumor microenvironment orchestrated by inflammatory cells foster neoplastic growth, proliferation and metastasis (Coussens and Werb, 2002; Kulinsky, 2007; Sobolewski et al.).

3. Mediator of inflammation: Cyclooxygenase

Cyclooxygenase exist as two isoforms: Cox-1 and Cox-2

Production of various prostaglandins is directed by coordinated activity of eicosanoid forming enzymes named Cyclooxygenase. In humans, Cox is present as two isoforms designated as Cox-1 and Cox-2. Cox-1 has been reported to function as a housekeeping isoform of cyclooxygenase and is constitutively expressed to serve functions such as regulation of renal blood flow, imparting protection to stomach against ulcers, production of prostacyclin and PGE$_2$ to maintain coherence and structure of gastric mucosal surface, and production of prostanoid thromboxane in platelets (Leng et al., 2003; Li et al., 2002; Williams et al., 1999). Cox isoforms differ a lot in their genomic structure, expression and regulation in spite of similar structure and enzymatic activity. In 1976, Cox-1 was purified and characterised for the first time from bovine vascular glands and was isolated in 1988. The molecular weight of unmodified and unglycosylated enzyme lacking signal peptide is 65 kDa, but after post translation modifications it increases to 70 kDa in fully functional enzyme (DeWitt and Smith, 1988; Merlie et al., 1988). The cox-2 gene was cloned and characterised in 1993 (Jones et al., 1993; Smith et al., 1990). The genes encoding Cox-1 and Cox-2 proteins are located on separate chromosomes. Gene coding for Cox-1 is located on chromosome nine, contains 11 exons and generates mRNA of 2.8 kb (Cervello et al., 2005; Funk et al., 1991). It lacks TATA or CAAT box at transcription sites (Kraemer et al., 1992). On the other hand, cox-2 is 7.5-9 kb in size and present on chromosome one. It consists of 10 exons. Its mRNA transcript is 4 kb long. Cox-1 consists of 602 residues while Cox-2 comprises 604 residues. The two isoforms share significant homology in sequence and enzymatic mode of action. They exhibit high similarity both structurally and mechanistically, and have almost identical size. The central part of both proteins consists of catalytic and substrate binding site but functional differences in their activity confers that Cox-2 has larger catalytic site. The analysis of amino-acid sequences of Cox proteins suggests noteworthy difference at N terminal. The signal peptide is comprised of 17 less amino acids in Cox-2 than in Cox-1. On the contrary, C terminal of Cox-2 has 18 more residues than Cox-1 (Bakhle and Botting, 1996; Gierse et al., 1996).

Cyclooxygenase-2 is the major mediator of inflammation

A number of reports by different groups have pointed to the involvement of Cox-2 prostanoid pathway in inflammation leading to hepatocellular carcinoma. Cox-2 differs markedly in expression and functioning from Cox-1. It is an inducible early response gene and is activated
in response to various extracellular or intracellular physiological stimuli. These factors comprise of lipopolysaccharide (LPS), interleukin-1 (IL-1), tumour necrosis factor (TNF), epidermal growth factor (EGF), platelet activating factor (PAF), serum, endothelin, and arachidonic acid. It is not expressed constitutively in all the tissues at all the time except in placenta, brain and kidney (Williams et al., 1999). The up-regulation and over-expression of Cox-2 is mainly associated with inflammation, loss of apoptosis, uncontrolled cell proliferation, growth, metastasis, neovascularisation, and angiogenesis finally leading to cancer. Cox-2 generated prostaglandins have also been reported to function as immuno-suppressors. It has been shown that macrophage mediated and natural killer cell mediated cytotoxicity is suppressed by PGE2 (Leng et al., 2003; Williams et al., 1999).

Transcriptional regulations of Cox-2

Transcriptional activation of Cox-2 occurs rapidly in response to broad spectrum of stimuli such as pathogen, cytokine, nitrous oxide (NO), growth factors, and extracellular ligands. Cross talks between various signalling pathways induced by pro-inflammatory and growth promoting stimuli converge to the activation of MAPK cascade which govern Cox-2 expression at transcription and post transcription level (Tsatsanis et al., 2006). Highly specialised machinery is involved in the generation of these signalling molecules as they are highly specific for the stimulus and cell type (Kang et al., 2007). Sequence analysis have shown numerous potential regulatory transcription factor sites including TATA box, C/EBP motif, AP-2, NF-KB sites present at 5’flanking region of cox-2 gene promoter region (Appleby et al., 1994; Tsatsanis et al., 2006). Consequences of chromatin remoulding, like differential acetylation status of histone and non-histone proteins also pose impact on transcriptional control of Cox-2. Literature has revealed decisive impact of p300, a histone acetyltransferase on Cox-2 translation through diverse mechanisms including acetylation of NF-kB. Elevated incidences of p300 binding to NK-kB play prominent role in cox-2 promoter activation (Deng et al., 2003). Hypermethylation of CpG islands (cytosine-guanine rich dinucleotides) located on cox-2 promoter repress its efficiency of promoting transcriptional silencing (Song et al., 2001). It has been documented that the 3’UTR of cox-2 gene encodes multiple copies of AU rich elements (AREs) motif which when targeted by various trans acting ARE binding factors influence Cox-2 mRNA stability and increase or decrease Cox-2 mRNA expression and enzyme activity level (Appleby et al., 1994; Song et al., 2001). Transcription of Cox-2 is controlled by numerous pathways, which also regulate its expression. It has been hypothesized that Cox-2 is down regulated by APC. Mutations in APC activate wnt signal pathways leading to increased accumulation of β-catenin that binds to Tcf-4. Tcf-4 binding element has been identified in cox-2 promoter (Araki et al., 2003). Combined dysregulation of Wnt and Ras pathways increase Cox-2 mRNA production thus implicating their role in Hepatocellular carcinoma (Abou-Shady et al., 1999; Araki et al., 2003).

Expression of Cox-2 is considered to be associated with de-differentiation of adult hepatocytes (Abou-Shady et al., 1999). Cox-2 is rapidly induced in foetal hepatocytes when challenged with pro-inflammatory stimuli like LPS but there is a rapid decline in hepatocytes after birth. It has been suggested that high levels of C/EBP (CCAAT/ enhancer binding proteins) in adult hepatocytes impairs Cox-2 expression when exposed to pro-inflammatory stimuli (Callejas et al., 2000). PPAR” activation in human hepatocellular carcinoma cells results in induction of Cox-2 expression and increased cellular proliferation. The proposed mechanism behind induction is enhanced activity of proximal promoter of cox-2 gene (Glinghammar et al., 2003). HBV infection is a major etiological cause of HCC. Up-regulation of Cox-2 has been reported in HBV mediated hepatocellular carcinoma. A close association has been investigated between HBx and Cox-2 (Cheng et al., 2004a). HBx intensifies metastasis in hepatic tumor cells and is the only HBV protein expressed in hepatocytes during HCC. It is suggested that Cox-2 is major cellular effector of HBx in HBV associated hepatic tumorogenesis and HBx activates cox-2 gene promoter activity mediated by NF-AT transcription factors thus magnifying tumor cell invasion (Lara-Pezzi et al., 2002). It has been reported that ER stress induced due to expression
of mutant HBV viral surface proteins stimulate Cox-2 expression by activating NF-kB and p38 MAPK pathways (Hung et al., 2004). Also, studies have indicated modification of NF-kB activity through HBx protein of HBV and HCV suggesting its potential role in triggering up activities of many cellular anti-apoptotic genes, perpetuation of inflammation and inhibition of differentiation of antigen presenting cell (APC) leading to chronic hepatitis (Mozer-Lisewska et al., 2006; Waris et al., 2002). Another study showed that there was marked repression in rate of cox-2 gene transcription by wild type p53. Any mutation in p53 or loss of its expression increased cox-2 gene transcription many fold (Subbaramaiah et al., 1999).

3.4. Post-transcriptional Regulation of Cox-2

Post transcriptional regulation plays a significant role in maintaining molecular regulation of cox-2 gene expression. Dysregulation at this level may elevate considerably incidences of tumorigenesis (Dixon, 2004). It has been found that RNA binding proteins HUR and TTP bind to AU rich elements located in 3' UTR of cox-2 gene and regulate its expression (Young et al., 2009). HuR has been reported to be highly crucial for its post transcriptional mRNA stability and its overexpression has shown to augment cox-2 expression (Sengupta et al., 2003; Young et al., 2009). Recently, elevated HuR level has been found in HCC cell lines, emphasizing its probable role in cox-2 associated inflammation and tumor invasion (Sheflin et al., 2001). A key study on CUG triplet repeat RNA- binding protein 2 (CUGBP2) showed that it binds to specific AREs present in first 60 nucleotides of the 3' UTR of cox-2 and plays role in opposing function of imparting stability to cox-2 mRNA coupled with inhibition of its translation (Mukhopadhyay et al., 2003). Genetic make of the tumor cells also regulates Cox-2 expression. Tumor suppressor genes like p53 tend to restrain Cox-2 expression while oncogenes like ras and src stimulate it (Koga et al., 1999).

4. Biological Function of the Cox-2 Pathway and Role in Cancer Prostanoid Synthesis Pathway

Cox-2 regulates key step in prosatnoid (i.e. prostaglandin and thromboxanes) pathway (Fig. 1) (Wang and Dubois, 2006). Various inflammatory mediators implicated in pathological process associated with cancer include prostaglandin, thromboxanes and leukotrienes. They belong to the family of hormonally active, oxygenated C18, C20, C22 fatty acids called eicosanoids derived from polyunsaturated fatty acids (Meric et al., 2006). The precursor molecule for prostanoids is arachidonic acid, which is a 20 carbon unsaturated ω-6 fatty acid, usually esterified at sn-2 position of phospholipids and dispersed throughout the lipid bilayer of the cell membrane.

![Figure 1: Phospholipase A2 (cytosolic or secreted) hydrolyse plasma membrane lipids or lipids derived from diet to generate Arachidonic acid (AA). AA is a 20 carbon unsaturated fatty acid. Cox-1/ Cox-2 catalyse AA to various prostaglandins having several physiological effects. AA is first converted to PGH2. This conversion is a two-step process. The first step includes introduction of 2 oxygen molecules to AA, to form unstable PGG2, and second step consists of peroxidation reaction in which PGG2 is reduced to stable PGH2 which is a substrate to a number of specific prostaglandin and thromboxane synthases. Prostaglandins are critical molecules, as they regulate various physiological functions. Cox-1 is a constitutive enzyme and is expressed in almost all the tissues of the body. Whereas, the other isoform Cox-2 is induced by several external and internal stimuli and its overexpression mediates abnormal PGE2 production. Excessive production of PGE2 leads to chronic inflammation and carcinogenesis]
(Wang et al., 2007). In response to various stimuli like growth factors, hormones and cytokines, arachidonic acid is liberated from membrane and metabolised to various bioactive lipids. This conversion involves three major steps. First is the liberation of arachidonic acid from phospholipids by phospholipase A2 enzyme (secretory or cytoplasmic). Second is addition of two molecules of oxygen to arachidonic acid forming bicyclic peroxide PGG2, which is an unstable intermediate. The catalytic site for the next step is located on a different site of the enzyme. Lastly, PGG2 diffuses to the requisite site and here its peroxidation leads to reduction of unstable PGG2 to stable PGH2 (Smith, 1992).

**Cox-2 and Tumor Progression**

Over expression of Cox-2 cause accumulation of pro-inflammatory molecule PGE2. It acts as a weapon in maintaining tumor survival (Fig. 2). It potentially increases tumor aggressiveness and inhibits apoptosis by various mechanisms. Most evident effect of PGE2 seen on tumor cells is mediated by synthesis of metastasis promoting matrix metallo-proteinases (MMPs). It has also been documented that production of Cox-2 and PGE2 modulates replication in hepatitis B virus, cytomegalovirus and gammaherpes virus (Nie and Honn, 2002; Symensma et al., 2003; Tsujii et al., 1998; Zhu et al., 2002). Recent studies emphasise that Cox-2 is stimulated in cancer and this accelerates and intensifies tumor growth, tumor vascularization, angiogenesis, invasion and metastasis (Cheng et al., 2004b; Cianchi et al., 2001; Gallo et al., 2002; Li et al., 2003). In another study, Cox-2 levels were measured and quantified in tumor cytosol. It was found that Cox-2 expression in tumor cytosol potentially influenced tumor division rate, venous invasion, advanced tumor stage and progression. In contrast there was complete lack of association between cytosolic Cox-2 and tumor size (Chi-Man Tang et al., 2005). It is probable that Cox-2 favours phenotypic changes that reduce apoptosis, thereby favouring tumor progression. Cells expressing increased Cox-2 levels elucidated increased adhesion properties to extracellular matrix proteins (ECM) and also mediate in part resistance to apoptosis. Direct relation between Cox-2 and Bcl-2 protein has yet not been established and is still under speculation. Yet it has been found that Cox-2 mediates increased expression of Bcl-2. Interestingly, Cox-2 inhibitors have shown to down regulate Bcl-2 protein expression suppressing tumorigenesis (Tsujii and DuBois, 1995). The correlation between Cox-2 and serine threonine kinase Akt signalling cascade is under investigation. However, their interaction has been hypothesized to have significant implication in angiogenesis (Gately, 2000). In a recent report it has been mentioned, that increased phosphorylation events of Akt and its downstream substrate glycogen synthase kinase-3 beta and pro-apoptotic Bad are observed in Hepatitis C virus expressing cells. These phosphorylation events were sensitive to selective Cox-2 inhibitors (Waris and Siddiqui, 2005). Genetic studies using mice model also strongly support the correlation between Cox-2
over-expression and cancer progression. Inhibition of intestinal polyposis has been reported in APCΔ716 mice that were Cox-2 knockouts. Moreover treating APCΔ716 mice with Cox-2 inhibitor further restrained the number and size of polyps (Oshima et al., 1996). In another study on multistage mouse skin model, engineered homozygous deficient for isoforms Cox-1 and Cox-2 showed reduction in skin tumor development by 75% (Tiano et al., 2002). Significant data in literature strongly support that overexpression of Cox-2 accelerates incidences of tumorigenesis in transgenic mice model; and use of chemo-preventive approach to restrain Cox-2 may be a logical therapeutic method to combat Cox-2 mediated cancer (Liu et al., 2001).

Interestingly, in some reports the pattern of Cox-2 expression does not suggest its principle role in tumorigenesis. A recent report on colon cancer has reported that Cox-2 expression is absent in early premalignant lesion human aberrant crypt foci and Cox-2 level seems to increase at adenoma stage, when minimum 45% adenoma are positive (Eberhart et al., 1994). Another fascinating fact advanced by Takeda et al. on polyp of APCΔ716 suggest that Cox-1 expression in stromal cells was basal, present in polyps of any size. The Cox-2 was induced only when the polyp grew bigger than 1mm in diameter (Takeda et al., 2003).

Immunosuppressive Tumor Microenvironment

Cooperative interaction between pro-inflammatory eicosanoids, cytokines, chemokines and carcinoma cells contribute to formation of immunosuppressive tumor microenvironment. PGE₂ functions as immune modulator and plays a crucial role in maintaining microenvironment that favours tumor cell growth and invasion. It has been reported that PGE₂ switches anti-tumor TH₁ microenvironment to TH₂ immunosuppressive microenvironment. It induces down-regulation of TH₁ cytokines like TNFα, IFNγ, IL-2 and IL-12, and upregulates TH₂ cytokines such as IL-4 and IL-10, which have immunosuppressive effect (Huang et al., 1998; Kambayashi et al., 1995; Snijdewint et al., 1993; Stolina et al., 2000). Another interesting finding demonstrated that PGE₂ directly inhibits cytotoxic T cell activity. It has been found that PGE₂ up-regulates CD94/NKG2A heterodimer complex, which is a natural killer receptor. Cross-linking reaction between CD94 and T cells expressing this heterodimer prevents cytotoxic T cell activity (Zeddou et al., 2005). In another study, it has been reported that PGE₂ indirectly eliminates anti-tumor effects of cytotoxic T cells. It inhibits dendritic cell maturation, down-regulates antigen presenting cells and causes abortive activation of naive CD8(+)T cells (Ahmadi et al., 2008).

5. Hepatocellular Carcinoma

Chronic liver infection, when associated with dreaded complications like liver cirrhosis and hepatocellular carcinoma, is followed by liver damage. Multiple factors including infection with HCV, HBV, chemical mutagens like aflatoxin and other environmental or host factor contribute to the development of HCC. However, HCV infection remains one of the major reasons for this disease. It has been documented that HCC consists of many different histological grades of HCC tumor. Mostly, two or more different grades have been observed inside a single tumor. As the size of tumor grows, the foci of less differentiated tumor tissue arises and starts growing inside the pre-existing well differentiated tumor tissue till they replace the original well differentiated HCC tumor (Kenmochi et al., 1987; Sugihara et al., 1992). HCC emerge as well differentiated tumors in early stages and progressively become less differentiated in advance stages (Araki et al., 2003). Recently, HCV mediated HCC has been found to be closely associated with activation and overexpression of Cox-2 gene. Various studies of Cox-2 suggest its possible association with HCC differentiation. It is possible that it plays a pivotal role in early stages of HCC. Its overexpression has been well documented in well-differentiated HCC; however in advanced HCC and in less differentiated HCC, its expression is negligible (Bae et al., 2001; Koga et al., 1999). A recent study reported that Cox-2 expression is significantly influenced by iNOS presence (Rahman et al., 2001). Combined iNOS and Cox-2 expression may modulate prognosis of HCV positive HCC and may be responsible for increased tumor growth and larger tumor size. Together they may also be accountable for modulating angiogenesis in HCC (Rahman et al., 2001).
Cox-2 and De-differentiation of HCC

HCC emerge as well differentiated tumors, and as they advance they progressively become less differentiated (Kenmochi et al., 1987). Tumor de-differentiation has been observed during HCC tumor growth. Liver is supplied with blood through two channels, one is arterial and other is portal (Koga et al., 1999). During pathological conditions, proinflammatory cytokines, notorious reactive chemical species and lipopolysaccharide are present particularly in the portal blood and they victimise hepatocytes. Most of them contribute to induce Cox-2 thus exaggerating the amplification of prostanoids (Belvisi et al., 1997; Feng et al., 1995). Studies have shown that hepatocarcinogenesis comprises of series of sequential changes, which alter haemodynamic status. All these changes are associated with pathological conditions and have been studied using imaging techniques (Winter et al., 1994). It has been speculated that early HCC is mainly characterised by well-differentiated HCC tumor tissue and are bestowed with abundant arterial supply and relatively less pre-existing portal tracts (Sakamoto et al., 1993; Ueda et al., 1992). It has been suggested that Cox-2 inducers attack by portal tracts leading to overexpression of cox-2 gene in early well-differentiated HCC. But on the other side, due to sequential heamodynamic changes, advance HCC are moderately or poorly differentiated and devoid of pre-existing portal tracts. Hence, they are not influenced by Cox-2 inducers (Koga et al., 1999).

Cox-2, Virus Infection and HCC

Both HCV and HBV accelerate Cox-2 expression. HBV mediated HCC involves expression of HBx viral protein. It facilitates Cox-2 overexpression by transactivation of NF-AT transcription factor. A recent study showed that mutation in HBV surface protein caused due to endoplasmic stress activates NF-kB and p38MAPK cascade resulting in up-regulation of Cox-2 (Hung et al., 2004). Furthermore, another report suggested that significant association exist between Cox-2 expression and acute inflammation in adjacent non-tumor liver tissue. Cox-2 mediated inflammation is actively involved in HCC relapse after surgery, imparting shorter disease free survival to patients afflicted with HCC (Kondo et al., 1999). These studies provide new approach and broaden horizon to speculate the mechanism by which Hepatitis viral infection operates through upregulation of Cox-2 and accelerate PG production. They also provide substantial evidence that Cox-2 may prove to be a logical therapeutic target to combat HCC.

Cox-2 and Tumor Angiogenesis

A close link between Cox-2 and angiogenesis has been observed in several human malignancies including HCC. Angiogenesis can be described as budding of new capillaries from the pre-existing vasculature and is the prerequisite for successful establishment of tumor and its growth (Joo et al., 2003). Using pharmacological and genetic methodologies, it has been established that Cox-2 produced prostanoids are responsible for facilitating angiogenesis function in autocrine or paracrine manner (Williams et al., 2000). Potential role played by Cox-2 in angiogenesis and tumor growth becomes more evident when Cox-2 expressing tumor cells were detected to grow larger and showed greater degree of angiogenesis in contrast to tumor cells deficient in Cox-2 expression. In tumor cells expressing Cox-2, it has been elucidated that its growth, aggressiveness and angiogenesis could be repressed by selective Cox-2 inhibitors, but inhibitors failed to inhibit the production of angiogenic proteins in Cox-2 negative tumor cells (Tsujii et al., 1998). Together, these observations support that Cox-2 mediates tumor progression through angiogenesis and neovascularisation. The inhibition of tumor progression in Cox-2 expressing cells by selective Cox-2 inhibitors may pave for a new path in fight against cancer (Sawaoka et al., 1999). Recently, Cox-2 expression in HCC has also been closely related to increased vascularity and sprouting of new capillaries from pre-existing vessels (Rahman et al., 2001). A recent study was designed to explore the association between Cox-2 and angiogenesis and also the significance of the role played by other inflammatory cells such as macrophages, kupffer cells etc. in the progression of primary HCC, using anti-CD-34 antibody. CD34 is commonly used as a marker for the identification and isolation of hematopoietic stem cells (Nielsen and McNagny, 2008). A positive correlation was found between
Cox-2 expression and CD-34. Comparative analysis between early stage HCC and advanced HCC showed decreased level of CD-34 positive cells with dedifferentiation. Cox-2 was reported to be the only independent variable, positively associated with CD-34 in multivariate analysis (Cervello et al., 2005). All these findings support the hypothesis, that inhibiting Cox-2 selectively, by treating with selective Cox-2 inhibitors may be a good strategy in combating HCC associated angiogenesis, thus providing an additional rational approach for treating the deadly disease. However, several safety and efficacy related issues are associated with prolonged usage of Cox-2 selective inhibitors.

Modulation of VEGF
Angiogenic phenotype that can support tumorigenesis results from sequential upregulation of products of angiogenic inducers. It involves both activation of oncogene, production of VEGF, loss of wild type p53 and suppression of thrombospondin, an inhibitor of angiogenesis, which can counteract VEGF. All the above events in step wise manner result in acquisition of pro-angiogenic phenotype in which Cox-2 over-expression has a key role to play (Volpert et al., 1997). Significant correlation exists between Cox-2 expression and VEGF in various human cancers such as endometrium cancer, head and neck cancer (Fujiwaki et al., 2002; Gallo et al., 2001). However, sufficient data is not yet available to prove direct correlation between Cox-2 and VEGF protein expression in HCC. In a recent study it has been shown that VEGF may also function as a biomarker for tumor invasion in HCC as its elevated level was found to mediate venous invasion and metastasis in HCC (Poon et al., 2001). Another study revealed that prostaglandins derived from Cox-2 enhance VEGF protein expression and the use of Cox-2 specific inhibitors down regulates VEGF level (Gallo et al., 2001; Kirkpatrick et al., 2002; Leahy et al., 2000). Studies have also indicated that VEGF expression is considerably very high in differentiated HCC, followed by moderately differentiated HCC and then poorly differentiated HCC. Thus it establishes relation between VEGF expression pattern and histological grade of HCC. It has also been hypothesized that Cox-2 and VEGF genes are down-regulated during de-differentiation, thus expressing low levels of Cox-2 and VEGF in moderately and poorly differentiated HCC. It may be concluded that Cox-2 and VEGF operate in sequential manner and combined together promote HCC in humans (Koga et al., 1999).

Also Cox-2 association with iNOS and VEGF at molecular level has been reported. In mice model for cox-2(-/-) mouse fibroblasts, 94% reduction was found in production of VEGF as against wild type (Williams et al., 2000). Other reports suggest that Cox-2 produced prostaglandins can potentially induce VEGF (Cheng et al., 1998; Majima et al., 2000). Cox-2 and associated prostaglandin are bioactive factors that act in paracrine fashion effecting neighbouring tumor cells (Rak et al., 1996). Prostaglandins exaggerate expression of IL-6, which increases expression of VEGF leading to increase in consequences of angiogenesis (Gruber et al., 2000).

Growth Factors
Well differentiated HCC show high expression level of epidermal growth factor receptor and Transforming growth factor alpha as compared to less differentiated HCC which reveal reduced level of TGFα and EGFR, suggesting high expression level of TGFα and EGFR during early stages of HCC progression (Morimitsu et al., 1995). Recently, it has been found that Cox-2 expression is linked to TGFα (DuBois et al., 1994) and EGFR activation and their expression (Asano et al., 1997). Also elevated TGFα levels are expressed in 82% of human HCC, and it is closely associated with and augments cascade of events during HBV mediated HCC (Hsia et al., 1992). Another study reported that dysregulation of adenomatous polyposis coli (APC) and k-ras genes encourage progression of human HCC. Mutation and consequent loss of function of APC protein upregulates wnt cascade allowing building up of β-catenin, which has strong affinity for T-cell factor (tcf-4). This interaction affects downstream processes. It has been hypothesised that this activation augments Cox-2 expression. In HCC pathogenesis, Cox-2 expression is suppressed by APC and intensified by nuclear accumulation of β-catenin (Araki et al., 2003).
Cox-2 and HCC

Inflammatory Cells
In addition, tumor tissue also manifests prominent correlation between Cox-2 expression and presence of inflammatory cells like macrophages, kupffer cells, mast cells. As HCC progresses towards advance stages there is a sudden drop in the number of Cox-2 expressing cells and inflammatory cells, suggesting their possible role in early HCC (Cervello et al., 2005). Interestingly, Cox-2 down-regulation with tumor progression seems to be an extraordinarily odd event. A possible answer to this peculiar behaviour, as proposed by Trifan is that Cox-2 overexpression during early HCC possibly causes growth disadvantage. He has proposed that Cox-2 overexpression may bring about cell cycle arrest in various cell types (Trifan et al., 1999).

Cox-2 and tumor Metastasis
A major event observed during solid tumor progression is their ability to invade locally and metastasize to distant organs. We have previously shown that Cox-2 over-expression is also mediated by interaction between human metastasis suppressor protein Nm23-H1 protein and Epstein Barr Virus latent nuclear antigen EBNA3C (Kaul et al., 2006). This suggests the key role of Cox-2 in regulation of metastasis potential of cancer cells. Studies have demonstrated Cox-2 role in modulating the invasive aggressiveness of transformed cancer cells. Tsuji et al. (1997) proved experimentally that cells programmed to express Cox-2 constitutively exhibited increased metastatic potential as compared to control cells. Biochemical changes involved in the process include activation of MMP-2 and overexpression of membrane type matrix metalloproteinase 1 (MT-MMP-1). Both these effects were reversed by Cox-2 inhibitor sulindac sulphide. It may be possible that Cox-2 controls MMP activity (Tsuji et al., 1997). MMPs and Cox-2 play critical role in colorectal cancer. Their activation and overexpression has been reported in more than 85% of human colorectal tumor samples. Sufficient data in literature support the proposal that Cox-2 actively participates in MMP-2 production and secretion (Hong et al., 2000; Shattuck-Brandt et al., 1999). The basic underlying mechanism by which Cox-2 modulates MMPs expression has not been fully understood and is under investigation (Dempke et al., 2001). It has also been proposed that combination therapy using selective Cox-2 and MMPs inhibitors may offer therapeutic benefits (Shattuck-Brandt et al., 1999).

Several human malignancies exhibit a fundamental link between Cox-2 expression and tumor metastasis (Murata et al., 1999). It has been reported that colon cancer cells expressing Cox-2 constitutively, acquired increased lymphatic invasion and metastatic potential. These phenotypic changes comprise of activation and overexpression of membrane type metalloprotein-2 (MMP-2). Also it was shown that regular treatment with Cox-2 inhibitors like NSAIDs (non-steroidal anti-inflammatory drugs) potentially altered the metastatic potential of cancer cells (Tsuji et al., 1997). On these grounds, it was explored that there could be strong possibility of close link between prostanoid activation and secretion of MMPs, which were reported to mediate migratory capacity and adhesion properties in human hepatoma cell lines (Mayoral et al., 2005). There is compelling evidence that showed Cox-2 produced PGE_2 can induce expression and activation of MMP-2 in HCC tumor cells (Mayoral et al., 2005). Human hepatoma cell lines also exhibited link with integrins in mediating cell invasion (Mayoral et al., 2005). Integrins belong to the family of heterodimeric cell adhesion receptor. Their function is to mediate cell movement on matrix molecules and regulate expression of matrix degrading enzymes called as MMPs, thereby playing a key role in cell invasion. Dysregulation of integrin’s is closely linked to increased metastatic potential (Ivaska and Heino, 2000). Recent studies have demonstrated that integrin α5 is repressed (Yao et al., 1997) and α8, β1, β7 and β8 are upregulated in aggressive HCC phenotype (Liu et al., 2002). Another recent study on integrins demonstrated that αV integrin have affinity for vitronectin which is produced by liver. The interaction between them mediates early stage liver metastasis (Kikkawa et al., 2002). The significant role played by Cox-2 and PGE_2 pathway in tumor metastasis is further strengthened by the fact that treatment with aspirin and Cox-2 selective inhibitor NS398 inhibits Hepatocyte Growth Factor (HGF)
induced invasiveness of human hepatoma cells (Abiru et al., 2002).

6. Cox-2 inhibition as a Potential Therapeutic Strategy

6.1. Non-steroidal anti-inflammatory Drugs (NSAIDs)

Cox-2 is an inducible enzyme and is not expressed constitutively. Rather it is overexpressed in neoplasm and malignant tissue. Studies have shown substantial increase in Cox-2 expression in human HCC cell lines including HuH7 (Kern et al., 2002), but the mechanism involved remains a mystery (Kern et al., 2004). Clinical trials using specific Cox-2 inhibitors suggest that it may prove to be a viable molecular target in cancer treatment (Dang et al., 2002). The data suggest that Cox-2 inhibitors might serve as an effective therapeutic tool to combat HCC (Cheng et al., 2002). It has been found that NSAIDs and selective Cox-2 inhibitors block Cox-2 prostanoid pathway followed by reduction in production of inflammatory mediators, thus reducing inflammation (Jachak, 2007). NSAIDs act in three favourable ways: analgesic, anti-pyretic and anti-inflammatory. They have a wide spectrum with varying degree of inhibitory capability against Cox-1 and Cox-2. NSAIDs having moderate selectivity for Cox-1 include ketorolac, fluriprofen, ketoprofen etc. Inhibitors having dual specificity i.e. against both Cox-1 and Cox-2 include indomethacin, aspirin and ibuprofen. But recently, it has been found that selective inhibition of Cox-2 is more promising strategy in fighting tumor progression. Drugs that fall under this category include celecoxib, rofecoxib, valdecoxib, etoricoxib, and lumiracoxib. Recently limitations associated with extensive use of Cox-2 inhibitors have been found. They are highly toxic and can potentially cause gastrointestinal bleeding and myocardial infarction (Jachak, 2007). Strong evidences in literature suggest that aspirin and other NSAIDs inhibit tumor progression by inducing apoptosis (Qiao et al., 1998). Aspirin blocks Cox-2 enzymatic activity by acetylation of Ser-530 in Cox-1 and Ser-516 in Cox-2. Most of the other NSAIDs block Cox cascade by competing with arachidonic acid for active site (Williams et al., 1999).

6.2. Other Inhibitors

Separate study on hepatoma cell lines show that selective Cox-2 inhibitor NS-398 and Sulindac (NSAID analogue) potentially suppress growth of tumor by reducing metastasis and proliferation and by inducing apoptosis (Bae et al., 2001; Hu, 2002). Another report suggests that in HCC tumor cells, NS398 and U0126 (MEK inhibitor) act synergistically to accelerate apoptosis, thus exhibiting anti tumor activity (Schmidt et al., 2003). Also Cox-2 is regarded as determinant of differentiation level in HCC during tumor growth, therefore inhibition of Cox-2 by NS-398 may suppress HCC (Bae et al., 2001). In a recent study on hepatoma cell lines it was found that Cox-2 inhibition using specific inhibitor meloxicam and Sulindac decreased solid tumor formation. Thus Cox-2 inhibitors may have substantial preventive and therapeutic potential in treating HCV (Kern et al., 2002). Another report suggest that Cox-2 selective inhibitor NS-398 and indomethacin, which inhibits both Cox isoforms repress invasion and metastatic potential of tumor cells by down-regulating expression of VEGF and MMP-2 (Li et al., 2002). Recent study on Celecoxib which is a selective Cox-2 inhibitor, and SC560, which is a selective Cox-1 inhibitor, showed that both induced G0/G1 cell cycle arrest by reducing expression of cyclinA, cyclinB and cyclin dependent kinase I. It was further assessed that Celecoxib functions in Cox-2 dependent and independent pathway and are not limited to tumors expressing Cox-2 (Grosch et al., 2001). In HCC cell lines, Celecoxib has been shown to arrest cell cycle by down regulating cell cycle protein cyclin D1 (Chi-Man Tang et al., 2005). For long term treatment of HCC, it is thought that use of selective Cox-2 inhibitors reduce the consequences of undesirable side effects as they specifically down regulate pro-inflammatory prostaglandins (PGE2) (Kern et al., 2004).

6.3. Adverse Effects of Cox-2 Inhibition

Benefits of using NSAIDs as therapeutic tool in reducing incidences of tumorigenesis have been extensively shown in preclinical and clinical studies. Yet the adverse gastrointestinal and cardiovascular side effects remain. Although precise data showing the incidences of side effects
of prolonged use of NSAIDs is unavailable, yet it is estimated that up to 4% of the patients per year suffer catastrophic complications (Bjorkman, 1999). Safety and efficacy are the two prohibitive limitations that NSAIDs used today cannot overcome (Rigas and Kashfi, 2005). The discovery of potential role played by Cox-2 selective inhibitors such as NSAIDs in fighting cancer opens new horizon in treating cancer. However, the inevitable side effects associated with their prolonged use is a matter of concern (Rigas and Kashfi, 2005). Therefore more work need to be done to study the downstream cascade of PGs, as PGE₂ is found in huge quantities in tumor microenvironment (Cha and DuBois, 2007).

6.4. Cox-2 and Multidrug Resistance
Multidrug resistance in cancer cells is a big hurdle in successful chemotherapy. Substantial body of evidences suggest that overexpression of Cox-2 may also lead to enhanced level of MDR1 gene expression and its downstream product, the P-glycoprotein. In tumor tissue P-gp has been implicated as major cause of MDR and it acts as multidrug efflux pump. A causal link between Cox-2 and P-gp has been established and it is also predicted that use of Cox-2 inhibitors NS398 may check Cox-2 mediated MDR-1 over-expression (Patel et al., 2002; Sorokin, 2004). Thus the idea of selective Cox-2 inhibition might reinforce tumor suppressive action of conventional chemotherapy by opposing P-gp expression. A recent study on human liver cancer cell lines indicated that MDR-1 is linked with overexpression of angiogenic phenotype including cox-2 gene (Fantappie et al., 2002).

7. Problems with Cox-2 inhibition: Activation of Alternative Pathway and Generating Cancer?
A little shift from focus of Cox-2 as central mediator is required as there is accumulated data conferring role of eicosanoids in cancer progression. A well designed system of talented enzymes referred to as ‘terminal enzymes’ include phospholipases, Coxs and Loxs as they metabolise poly unsaturated fatty acids to end products forming biologically active eiconsanoids (Rigas and Kashfi, 2005). Lox products have also drawn attention as some have pro-tumorigenic properties while others have anti-tumorigenic activities (Shureiqi and Lippman, 2001). The terminal enzymes act in peculiar manner and play individual role in cancer. A recent report on murine model for pulmonary cancer suggested that over-expression of prostacyclin synthase results in exaggerated pulmonary PGI₂ production and prevents murine lung cancer. These finding broaden our horizon for new therapeutical approach that may include manipulation of PG metabolism downstream from Cox in lung cancer prevention rather than focussing on Cox-2 inhibition alone (Keith et al., 2002). Literature reveals that inhibition of Cox-2 using selective inhibitors further complicates the situation by channelizing its substrate fatty acid to non-cox cascade resulting in production of pro-tumorigenic end product. Also, Cox-2 inhibition could switch arachidonic acid to Lox pathway, thus repressing apoptosis and thinning chances of cancer prevention (Rigas and Kashfi, 2005). Another interesting finding on human lung cancer showed that oral administration of celecoxib accelerated leukotriene B4 level in lung microenvironment under physiological conditions, though there is no functional significance of this effect (Mao et al., 2004).

8. Summary
Cox-2 metabolise production of eicosanoids. Their downstream products contribute to various physiological processes including inflammation, immune and development function. Extensive literature evidences have shown that Cox-1 is housekeeping in function while Cox-2 is inducible. It is activated by various inflammatory, chemical, mutagenic and physiological stimuli and acts in pro-inflammatory manner. Sufficient genetic and pharmacologic evidences implicate its crucial role in neoplasia and it is also now clear that Cox-2 plays a crucial role in tumor progression. Cox-2 overexpression is associated with maintaining tumor microenvironment and has crucial implication for angiogenesis. Cox-2 operates in multifactorial fashion. It not only promotes production of pro-angiogenic proteins but also results in production of PGE₂, PGI₂ and TXA₂ that are directly linked to cancer development; not to forget its key role in tumor
enhancement, survival and metastatic aggression by upregulating several anti-apoptotic proteins and several signalling cascades. Cox-2 contribution at several points in angiogenic and inflammatory cascade makes it an ideal target to fight cancer. Cox-2 selective inhibition has been reported as a successful tool in suppressing angiogenesis and metastasis. Thus, pharmacological suppression of Cox-2 represents a bright future as therapeutic tool for treatment of various malignancies. Yet the extent to which conventional NSAIDs are involved in prevention of various malignancies is confined. Although selective pharmacological repression of Cox-2 may present a bright future as therapeutic tool for prevention of various malignancies, yet multiple safety and efficacy issues associated with regular use of NSAIDs limits their role. There is a compelling need to shift the focus from selective Cox-2 inhibition alone, and device strategy to identify agents or combinations of molecular targets offering high efficacy and minimal toxic side effects. A new era in cancer therapy has already begun, involving not only generation of mechanistic insight but also taking them to clinical evaluation and trials.

9. Future Prospects

Discovery of Cox-2 upregulation and overexpression in various cancers has provided a major turning point and strong stimulus in last few years to unfold several mechanisms related to cancer pathogenesis thus narrowing the focus on combating cancer. The conventional concept of dominant role of Cox-2 in preventing cancer has critical complications. These subtle issues necessitate reappraisal in devising strategies that may include multi-pathway suppression as chemo-preventive measure. Further development of dual inhibitors such as of Lox and Cox pathway like Licofelone may pave next milestone in the fight against cancer (Rigas and Kashfi, 2005).

Acknowledgement

RK is supported by grants from University of Delhi (Seed Money grant, R&D grant) and DST (DU-DST PURSE grant), Government of India. JG is supported by Junior Research Fellowship from UGC, Government of India.

Abbreviations

HCC, hepatocellular carcinoma; Cox-2, cyclooxygenase-2; HCV, Hepatitis C Virus; HBV, Hepatitis B Virus; EGFR, epidermal growth factor receptor; TGFá, transforming growth factor alpha; NSAIDs, non-steroidal anti-inflammatory drugs; PGE₉, prostaglandins; MDR1, multidrug resistance; P-gp, P-glycoprotein.

References

Cox-2 and HCC


