Review

Cox and mesothelioma: an overview

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Summary. Cyclooxygenases catalyze the rate limiting step in the production of prostanoids. Accumulating data demonstrate that overexpression of these enzymes, and in particular of cyclooxygenases-2, promotes multiple events involved in tumorigenesis; in addition, numerous studies show that inhibition of cyclooxygenases-2 can delay or prevent certain forms of cancer.

Malignant mesothelioma is a lethal pleural, peritoneal and pericardial neoplasia that actually lacks valid therapies and in which cyclooxygenases-2 is recognized as an important adverse prognostic factor. Hence, there is an increasing interest in the development of new treatments based on cyclooxygenases-2 inhibitors, to prolong survival and even potentially cure this neoplasia.

Key words: COX-2, Mesothelioma, Chemotherapy

The COX enzymes: structure and function

Prostaglandin-endoperoxide synthases, also known as cyclooxygenases (COXs), are key regulatory enzymes in the biosynthesis of prostanoids, a class of hormones including prostaglandins, prostacyclins, and thromboxanes responsible for multiple inflammatory, mitogenic, and angiogenic activities in various tissue and organ systems.

Increasing interest on COXs is due to much evidence showing the involvement of these enzymes not only in physiologic but even in pathophysiologic processes such as development and progression of cancer (Prescott and Fitzpatrick, 2000), Alzheimer (Minghetti, 2004) and Parkinson disease (Sánchez-Pernaute et al., 2004).

There are two related but distinct genes codifying for enzymes that posses COX activity named cox-1 and cox-2, respectively cloned in 1988 (DeWitt and Smith, 1988; Merlie et al., 1988; Yokoyama and Tanabe, 1989) and

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1991 (Kujubu et al.,1991; Xie et al., 1991) and localized to 9q32-q33.3 (COX-1) (Funk et al., 1991) and to 1q25.2-q25.3 (COX-2) (Tay et al., 1994).

Although encoded by different genes, COX-1 and COX-2 share a relatively conserved primary structure (~60% of amino acid identity) and a similar structural topology (Picot et al., 1994; Kurumbail et al., 1996; Luong et al., 1996; Garavito et al., 2002; Kulmacz et al., 2003). Moreover, the two COXs catalyze the same reaction, the conversion of arachidonic acid in prostaglandin-H2 (PGH2), through two different activities, a cyclooxygenase (bis-oxygenase) one which converts arachidonic acid into prostaglandin-G2 (PGG2) and a peroxidase one which reduces PGG2 to form PGH2 (Miyamoto et al., 1976; van der Ouderaa et al., 1977).

Both enzymes are integral membrane glycoproteins (~70 kDa) that have been localized on the lumenal surface of the endoplasmic reticulum and on the outer membrane of the nuclear envelope (Fletcher et al., 1992; Kraemer et al., 1992; Otto and Smith, 1994), although they have also been detected in other structures (e.g., lipid bodies and mitochondria) (Bozza et al., 1997; Coffey et al., 1997; Liou et al., 2000, 2001). Histochemical staining shows that COX-1 is primarily localized in the cytoplasm whereas COX-2 is localized both in the cytoplasm and on the surface of the nucleus (Morita et al., 2005). The lumen of the endoplasmic reticulum is important for the correct function of the enzymes, because its oxidative potential allows formation of the disulfide bonds and because N-glycosylations, necessary for proper protein folding, occur here (Otto et al., 1993).

COXs are dimers of identical subunits, so there are two cyclooxygenase sites and two peroxidase sites for each complex. Each subunit (~600 amino acids) comprises three domains: an epidermal growth factor domain, thought to be responsible for initiating or maintaining protein-protein interaction (Picot et al., 1994); a membrane-binding domain, containing four helices that interdigitate into only one leaflet of the lipid bilayer (Picot et al., 1994; Otto and Smith, 1996;

Chandrasekharan and Simmons, 2004) and a catalytic domain that contains the clooxygenase and the peroxidase sites separated by the heme prostetic group (Marnett et al., 1999).

The clooxygenase site is a deep pocket within the protein (Fig. 1); the access to this channel (8Å wide and 25Å long) (Picot et al., 1994; Thuresson et al., 2001) is gained from the interior of the bilayer through the mouth formed by the four amphipathic helices of the membrane-binding domain. On the opposite side, on the surface of the enzyme, is the peroxidase site, which is needed to activate the heme group that partecipate in the clooxygenase reaction (Picot et al., 1994; Otto and Smith, 1996). The clooxygenase site is easily reachable from an hydrophobic molecule like arachidonic acid that is liberated from the membrane by cellular phospholipases (e.g. PLA2). After its formation, PGG2 diffuses to the peroxidase site where it is reduced to PGH2. Finally, PGH2, which is sufficiently non polar to diffuse through the membrane, is captured by synthases, responsible for the production of other prostanoids, that are located on the cytosolic surface of the endoplasmic reticulum or in the cytosol (Chandrasekharan and Simmons, 2004).

More recently, a research group (Chandrasekharan et al., 2002) described a third enzyme with COX activity, named COX-3, that have been found in canine and human cerebral cortex. This isozyme is a splice variant of COX-1 from which it differs in the retention of intron 1 (Chandrasekharan et al., 2002). COX-3 contributes about 5% of total COX-1 and has a cyclooxygenase activity that is about 80% lower than that of COX-1, probably because intron 1 retention modifies the conformation of the active site (Berenbaum., 2004). The majority of studies promoting the existence of COX-3

are rooted in an effort to explain the pharmacology of acetaminophen (paracetamol). This molecule is a common analgesic and antipyretic drug with a little antiinflammatory activity that shows only a weak inhibitor effect on isolated COX-1 and COX-2 (Ouellet and Percival, 2001; Graham and Scott, 2003). Since the distinctive characteristic of COX-3 is its greater sensitivity to acetaminophen, the hypothesis is that COX-3 could represent the primary target through which paracetamol reduces pain and fever. However the physiological function of this isozyme has yet to be fully elucidated.

Since the discovery of a second gene encoding for a cyclooxygenase, there has been a considerable interest about the reason for the existence of two isoforms.

The answer to this question seems to be that COX-1 is a constitutive enzyme expressed in most mammalian tissues (even if some evidence shows that its levels change during development) (Brannon et al., 1994) that produces prostaglandins involved in cellular "house-keeping" functions like cytoprotection, tissue homeostasis and cell-to-cell signaling (Smith, 1989); on the contrary, the rapidly (2-6h) inducible COX-2 (Hla and Neilson, 1992; Niiro et al., 1997) that produces larger amounts of prostaglandins in response to a variety of stimulants such as bacterial lipopolysaccharides (LPS), growth factors, cytokines, and phorbol esters (Han et al., 1990; O'Sullivan et al., 1992; Sirois et al., 1992; DeWitt and Meade, 1993; Evett et al., 1993; Jones et al., 1993; Kujubu et al., 1993; Smith et al., 1996), is generally undetectable in most tissues in the absence of stimulation and is present in cells only during early stages of cell differentiation or replication (Smith et al., 2000).

For many years, COX-1 was considered uniquely responsible for platelets aggregation (Patrignani et al., 1994) and maintenance of gastrointestinal integrity (Whittle et al., 1980; Smith, 1989) and COX-2 the primary source of prostanoids that contribute to both the initiation and the resolution of the inflammatory response (Smith and Langenbach, 2001). Currently we know that the situation is more complicated.

Through studies on isoform-specific inhibitors and knock-out mice it is emerged that there are processes in which each isozyme is uniquely involved and others in which both function coordinately or in which one can compensate the lack of the other (reviewed in Smith and Langenbach, 2001).

For example, studies on COX-1-null mice show that COX-2 cannot compensate COX-1 in promoting platelet aggregation (since the second isozyme is not expressed in platelets). On the contrary, COX-1 deficient mice have a 99% reduction in gastric prostaglandin-E2 (PGE2) levels but don't spontaneously develop ulcers (Langenbach et al., 1995) suggesting the presence of compensatory mechanisms: the use of selective inhibitors show that just COX-2 compensates the lack of COX-1. In fact, COX-1 selective inhibitor (SC-560) is not sufficient to induce gastric damage, which instead
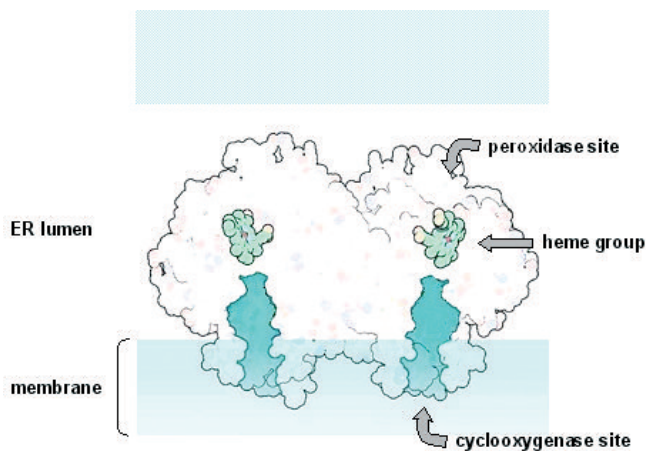
Fig. 1. Localization and structure of COX dimer in the endoplasmic reticulum (ER). (Modified from Protein Data Bank, http://www.rcsb.org/pdb)

occurs when SC-560 is administered together with celecoxib (a COX-2 selective inhibitor) (Wallace et al., 2000). Finally, both isoforms contribute to reproductive success: COX-2 being required for ovulation and blastocyst implantation (Lim et al., 1997; Davis et al., 1999) and COX-1 for parturition (Langenbach et al., 1995; Gross et al., 1998).

The role of COX-2 in tumorigenesis: a close look at mesothelioma

Amongst all the actions mediated by COXs, the one currently most investigated is the involvement in tumorigenesis, since several reports indicate that NSAIDs (non steroidal anti-inflammatory drugs), which are COXs inhibitors, can prevent the development of various human tumors including colon (Suh et al., 1993; Thun et al., 1993; Muscat et al., 1994), breast (Harris et al., 1996, 1999), lung (Schreinemachers and Everson, 1994) and gastric (Thun, 1996; Farrow et al., 1998) neoplasias. Indeed, to date, most studies have implicated COX-2, rather than COX-1, in multiple events throughout the tumorigenic process (Smith and Langenbach, 2001).

The mechanism through which COX-2 exerts its tumorigenic action can be directly mediated by the enzyme or due to the effects of its products. COX-2 is an oxygenase and its intermediates are highly reactive. It is possible that this compound may cause free radical damage, for example, against DNA molecules. Moreover, prostaglandins synthesis may contribute to oncogenesis by directly stimulating mitogenesis in fibroblasts (Nolan et al., 1988), osteoblasts (Goin et al., 1993; Quarles et al., 1993), and mammary epithelial cells (Bandyopadhyay et al., 1987). Furthermore, PGE2 can inhibit programmed cell death by inducing the expression of the Bcl-2 protooncogene (Sheng et al., 1998) as well as elevating cyclicAMP concentration (which can suppress apoptosis) (Orlov et al., 1999). PGE2 has also been shown to suppress cell mediated immunity, which may otherwise block tumor growth (Huang et al., 1996; O'Byrne et al., 2004), and to promote angiogenesis (Leahy et al., 2000, 2002; Masferrer et al., 2000) that is essential for tumor growth beyond 1-2 mm in diameter. In addition, PGH2 can isomerize to form the potent mutagen malondialdehyde, which can induce frame shifts and base pairs substitutions (Marnett, 1992).

All these mechanisms should not occur in normal tissues since COX-2 is expressed at very low levels but several studies show that this isozyme is consistently overexpressed in premalignant lesions and in a variety of cancers (Koki and Masferrer, 2002) (while COX-1 does not increase during trasformation (Prescott, 2000). Moreover, COX-2 is also detected in noncancerous cells immediately adjacent to tumor cells and in the vasculature within and in the proximity of tumors (while it is absent in the vasculature of normal tissue) (Buckman et al., 1998; Koki et al., 1999). The amount of

COX-2 seems to be important, since there is a correlation between its level of expression and the size and invasiveness of tumors and survival of patients with colon (Tsujii et al., 1997; Fujita et al., 1998; Tomozawa et al., 2000; Chen et al., 2001), gastric (Murata et al., 1999; Chen et al., 2001), and lung (Achiwa et al., 1999) cancers.

The question is why COX-2 is induced. Some studies suggest that COX-2 overexpression is correlated with HER-2/neu (also known as c-erbB2) (Subbaramaiah et al., 2002), a receptor protein kinase whose expression is widely accepted as an adverse prognostic factor (Slamon et al., 1987), as well as with loss of heterozygosity of APC gene (Kinzler and Vogelstein, 1996), a tumor suppressor. The induction of expression appears to occur mainly through increased gene transcription but also via translational regulation (Prescott and Fitzpatrick, 2000).

For several types of cancer the real risk factor seems to be chronic inflammation (reviewed in Prescott and Fitzpatrick, 2000) that mantains a high level of COX-2 and increases events that promote tumor formation. A tragic example of this mechanism is malignant mesothelioma (MM).

MM is a rare tumor of the mesothelial surface of the pleural and peritoneal cavities that is increasing in incidence in most countries. This is an highly aggressive tumor and despite advances in surgery, radiotherapy and chemotherapy of the last decades, it is commonly known that the prognosis is poor for patients, with a median survival of 4-9 months in either treated or untreated patients (Curran et al., 1998; Sugarbaker et al., 1999). Several clinical prognostic factors (TNM, extension of surgery, histopathologic subtype, cell proliferation, mitotic count) have been tentatively correlated to survival, but their significance still remains to be defined (Johansson and Linden, 1996; Beer et al., 1998; Rusch and Venkatraman, 1999; Sugarbaker et al., 1999). The disease is mainly associated with exposure to asbestos fibers, a substance that has been widely used in the past, but several studies report the presence in some MM of DNA encoding SV40 T antigen or SV40 T antigen protein expression, suggesting that the presence of this viral gene may also be associated with the pathogenesis of this neoplasm (Carbone et al., 1999). Although molecular mechanisms of asbestos tumorigenicity have not been elucidated, research has shown that deposition of insoluble amphibole fibers results in a chronic inflammatory state (Mossman and Churg, 1998) and that this state generates reactive oxygen and nitrogen species, as well as cytokines and growth factors, through the activation of macrophages and other cell types (Kamp and Weitzman, 1999).

As expected, the prolonged inflammation causes the increase of COX-2 level, which is actually recognized as an important MM prognostic factor (Marrogi et al., 2000; Edwards et al., 2002). It has been shown that cultured human mesothelial cells contain cyclooxygenase activity (Baer and Green, 1993) and that

Table 1. Studies on the role of COX-2 in mesothelioma.

REFERENCES	DATA
Prognotic significance of COX-2 in MM	
Edwards et al., 2002	↑ COX-2 = poor prognostic factor
Baldi et al., 2004	↑ COX-2 and ↓ p21 and p27 = poor survival
O'Byrne et al., 2004	↑ COX-2 = poor survival
Anti-proliferative effect of COX-2 inhibitors (in vitro)	
Marroni et al., 2000	NS-398 shows dose- and time- dependent antiproliferative activity on MM cell line
Catalano et al., 2004	Antiproliferative effect of celecoxib, acetylsalicylic acid, indometacin and NS-398
Anti-proliferative effect of COX-2 inhibitors (in vivo)	
DeLong et al., 2003	Rofecoxib ↓ growth of small MM tumors
Catalano et al., 2004	Celecoxib ≠ survival in >37% of nude mice bearing intraperitoneal MM

COX-2 expressing mesothelioma cell lines are associated with increased proliferative and invasive potential (Marrogi et al., 2000). A recent study clearly demonstrated that COX-2 expression is a strong prognostic factor in human mesothelioma, which contributes, independently to the other clinical and histopathologic factors, in determining a short survival (Edwards et al., 2002). Finally, COX-2 has been proposed to exert its influence on mesangial cell proliferation *in vitro* by a novel mechanism involving the tumor suppressor p53 and the cell cycle inhibitors p21 and p27 (Zahner et al., 2002). Interestingly, several recent studies have investigated the potential prognostic value of p53, p21 and p27 in malignant mesotheliomas, thus reinforcing the evidence of a primary role of COX-2 in the pathogenesis and progression of MM (De Luca et al., 1997; Esposito et al., 1997; Bongiovanni et al., 2001; Beer et al., 2001; Isik et al., 2001; Baldi et al., 2002, 2004). Due to the lack of reliable treatment capable of achieving long-term control in mesothelioma patients, these enzymes are becoming more and more appealing as potential therapeutic targets.

Indeed, most chemotherapeutic agents are not very effective against MM, with typical single-agent response rates of ≤ 20% (Ryan et al., 1998), which is why recent studies are investigating the clinical usefulness of COX-2 selective inhibitors in the treatment of MM (Table 1). A selective COX-2 inhibition could minimize side effects due to COX-1 inhibition but maintain anti-inflammatory activity. Unfortunately, the majority of these studies are still in an *in vitro* phase, so there is need of further studies in animal models to prove the real efficacy of these inhibitors. Preliminary data from our laboratory have shown, in an *in vivo* model of peritoneal mesothelioma in nude mice, that the antitumor effect of cisplatin is augmented by the simultaneous treatment with piroxicam, a COX-2 inhibitor (Spugnini E.P., Cardillo I., and Baldi A. unpublished). These results further support the possibility that multimodal therapies, involving the use of COX-2 inhibitors together with more traditional chemiotherapic agents, will achieve better results for the treatment of this malignant tumor.

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