8

Cyclooxygenase-2 Overexpression in Non-Melanoma Skin Cancer: Molecular Pathways Involved as Targets for Prevention and Treatment

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1. Introduction

Non melanoma skin cancers (NMSCs) are the most frequent malignancies in humans (Geller & Annas, 2003). It comprises many different types of tumors, such as Squamous Cell Carcinoma (SCC) and Basal Cell Carcinoma (BCC), which are the main ones among the NMSCs. There are 100.000 new cases of these neoplasms per year in the UK (Aitken *et al.*, 2007) and about a millon in the US (Kuchide, 2003). NMSC incidence is estimated to increase in a 10% by 2060 -taking into account the Copenhagen Amendments scenario for protection of ozone layer- (Slaper *et al.*, 1996).

The development of both SCC and BCC implies a malignant transformation of keratinocytes, the most abundant cells in the epidermis. In addition to genetic factors, there are a variety of stimulus that can induce this transformation, like viral or bacterial infections and chemical agents (such as 7,12-dimethyl benzanthracene -DMBA- or Benzo[a]pyrene-7,8-diol-9,10-epoxide -B[a]PDE-), (Aubin *et al.*, 2003; Bouwes Bavinck *et al.*, 2001; Madan *et al.*, 2006). However, the most important carcinogen of the skin is the solar ultraviolet radiation (UVr), mainly the UVB component (280-310 nm), which is able to reach the earth surface (Armstrong & Kricker, 2001).

All of those carcinogens promote molecular and cellular changes, modifying different functions like cell cycle control or apoptotic cell death. In this way, the affected cells lose their capacity to regulate their proliferation properly and have the potential to generate an uncontrolled linage of cells, which may ultimately lead to tumor formation and growth.

There are many gene products that have been found to be involved in skin cancer development. Some of them are related to cell cycle -like cyclin D1- (Liang *et al.*, 2000), apoptosis -like Bax, Bcl-2, and others- or in both processes -like p53 and p21- (Coultas & Strasser, 2003; Murphy *et al.*, 2002). Additionally, skin UVB irradiation has been proven to induce an increment in the levels of a significant number of immune system mediators, such as tumor necrosis factor-α (TNF-α), interleukins 6 (IL-6), 10 (IL-10), 8 (IL-8), 4 (IL-4), prostaglandins (PGs), nitric oxide (NO) and vascular endothelial growth factor (VEGF),

among others (Paz *et al.*, 2008; Trompezinski *et al.*, 2001). Some of these molecules are known to mediate an important outcome of UVB irradiation: immunosuppression. It has been vastly proved since Margaret Kripke's first description (Fisher & Kripke, 1977) that UVr-induced DNA damage is not sufficient to lead to skin cancer development; there must be a concomitant decrease in normal immunosurvillance (Aubin, 2003; Ullrich & Kripke, 1984). This immunosuppression is mainly mediated by IL-4, IL-10 and prostaglandin-E_2 (PGE_2) (Shreedhar *et al.*, 1998).

PGs -including PGE_2- and thromboxanes (TXs), are subproducts of key enzymes in the inflammatory response: cyclooxygenases (COXs). There are two isoforms described for COX: COX-1, which is constitutively expressed in many tissues and the inducible isoform COX-2, which is up-regulated under physiological and pathophysiological conditions.

The relationship between COX-2 over-expression and non-melanoma skin cancer development and progression has been very well documented and described. Along this chapter we will discuss different aspects concerning this relationship, including molecular pathways involved in COX-2 expression, cellular effects of COX-2 products and experimental treatments for non-melanoma skin cancer, which comprises the modulation of the COX-2 pathway.

2. Molecular pathways involved in COX-2 expression

COX-2 is a single copy gene, whose location was identified in chromosome 1 by a fluorescence in situ hybridization technique (locus 1q25.2-25.3) (Tay *et al.*, 1994). Its gene size is about 8 kb and it includes 10 exons with 9 introns, resulting in a primary mRNA of 4.5 kb. The expression of this gene, as it was mentioned before, is inducible. There are many factors which can contribute to the induction of COX-2 expression, including cytokines, growth factors, hormones and bacterial components.

2.1 COX-2 gene structure

COX-2 was first described in 1991 by Kujubu et al. (Kujubu *et al.*, 1991) as a primary response gene, which encodes an amino acid sequence sharing 61% of identity with the COX-1 gene, described in 1988 by Merlie et al. (Merlie *et al.*, 1988).

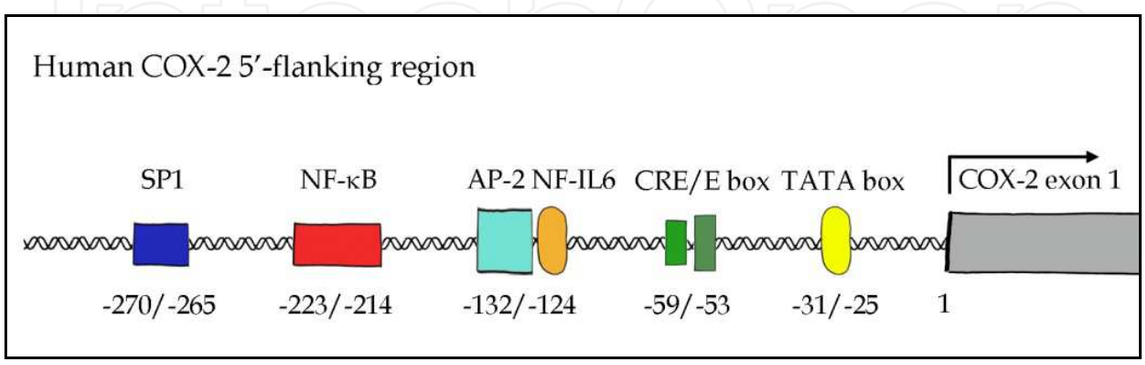

Fig. 1. Estructure of COX-2 gene regulatory region. Transcription regulatory elements are shown, their names (above) and upstream position (below with numbers) are indicated.

In 1995 Inoue et al. analyzed the sequence of the human COX-2 5'-flanking region, describing several transcription regulatory elements (Figure 1), which include a TATA box, a CRE motif, an E-box, a NF-IL6 motif, three Sp1 sites, two AP-2 sites and two NF-κB sites (Inoue *et al.*, 1995). Many animal COX-2 5'-flanking sequences have been characterized, exhibiting similar structures in mouse, horse, cow and rat.

All of these sequence elements have a potential role in the induction of the expression of COX-2. Some inducers and the molecular pathways involved in the activation of transcription factors, which finally lead to COX-2 expression, are described in the next section.

2.2 Stimulus and mechanisms involved in COX-2 expression

Prostaglandins and thromboxanes production are intimately related to inflammation, besides the fact that they produce a regulation of the overall inflammatory response (Ricciotti & FitzGerald, 2011). In this way, it is reasonable that many cytokines and molecules involved in the inflammatory process may play some role in the production of PGs and TXs. Certainly, TNF-α, IL-1 and Toll-like receptors agonists (like bacterial lipopolysaccharide -LPS-) can highly induce the expression of COX-2 gene in many cell types.

Besides the three mentioned molecules, there are other stimuli which are capable of inducing COX-2 expression. Other cytokines and different growth factors –like IL-6, EGF, FGF and PDGF- may lead to its expression (Tanabe & Tohnai, 2002). Keratinocytes, the most abundant cells in the skin, are a cell type that is particularly exposed to natural UVr. This stimulus is sufficient to induce the production of many inflammation-related molecules by the keratinocytes -such as TNF-α, IL-6 and COX-2 itself- among others (Paz *et al.*, 2008). Finally, there are many chemical agents used as tumor promoters (Lim *et al.*, 2011; Rundhaug *et al.*, 2007), which are able to induce the expression of COX-2.

There are two main molecular pathways involved in COX-2 expression (Figures 2 and 3). The first one includes the activation of a transcription factor (NF-κB, nuclear factor kappa B) and the second one involves the mitogen-activated protein kinases (MAPKs) cascade -with three subgroups: p38 MAPK, ERKs and JNK-.

NF-κB is normally inhibited by IκB binding; this inhibitor can be phosphorylated and degraded in the proteasome, releasing the nuclear factor to translocate to the nucleus and to start its transcriptional effects. In this pathway the trigger stimuli is related to the interaction of membrane receptors with their corresponding agonists, like TNF-α receptor (TNFR), IL-1 receptor (IL-1R) and Toll-like receptor 4 (TLR-4). As it is shown in Figure 2 there are several molecules involved in the pathway, relating the membrane receptors with the phosphorylation of IκB. TNFR recruits TRADD (TNFR-associated death domain protein), which recruits TRAF-2 (TNFR-associated factor 2), leading to the final common path: phosphorylation of NIK (NF-κB-inducing kinase), IKK (IκB kinase complex) and the final phosphorylation (and consequent inactivation) of IκB. The other side of this pathway comprises IL-1R and TLR-4. Both molecules use MyD88 (Myeloid differentiation factor 88) as a linker for the recruitment of IRAK (IL-1R-activated kinase) and the consequent phosphorylation of TRAF-6 (TNFR-associated factor 6), which leads to the same final common path above mentioned. Once released, NF-κB translocates to the nucleus activating the transcription of target genes, like COX-2.

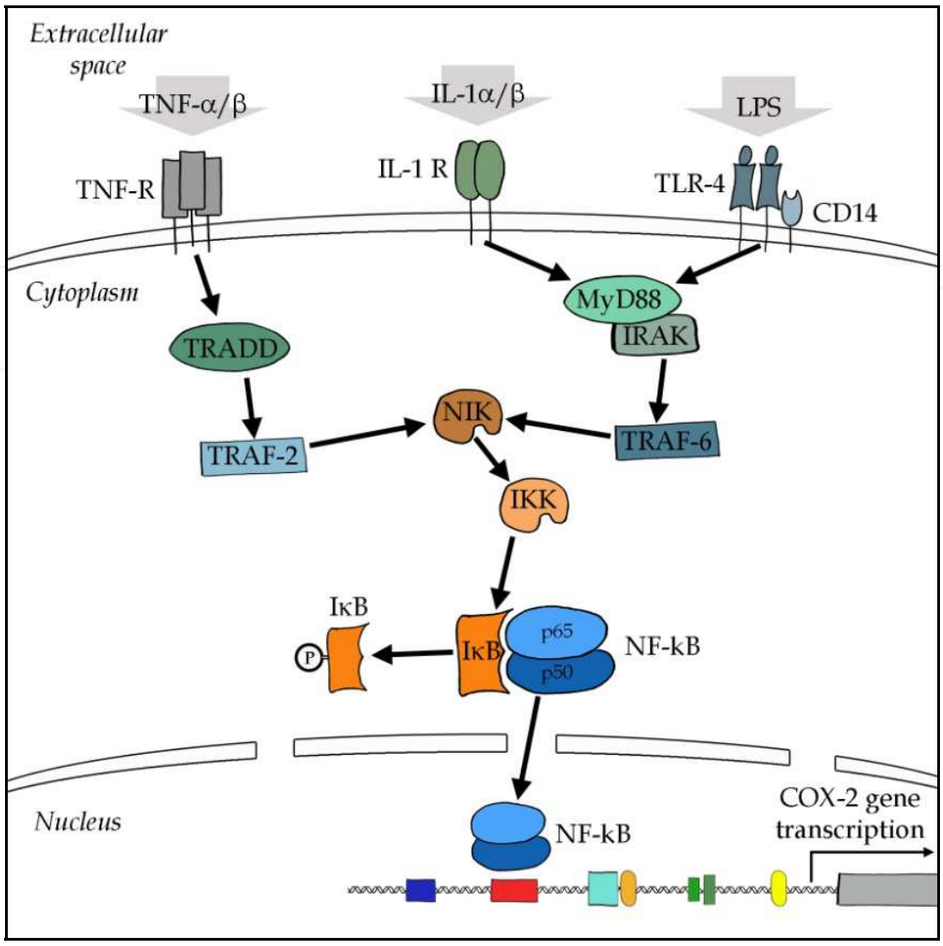

Fig. 2. Nuclear factor kappa B -NF-κB- molecular pathway. Membrane receptors with their corresponding agonists as trigger stimuli (grey arrows) are shown, also the different downstream molecules involved. The common final path, that leads to NF-κB activation and subsequent COX-2 expression, can also be seen. P: phosphorylated molecule.

MAPKs pathway includes a cascade of kinases with specific activity, each of them phosphorylates (and activates) the next kinase in the path, ultimately leading to the activation of transcription factors which promote the transcription of target genes. This pathway is one of the most significant in the activation of COX-2 expression and prostaglandins biosynthesis. JNK (c-Jun N-terminal kinase) and p38 MAPK share some of the activation stimuli -like proinflammatory cytokines and bacterial endotoxins- also environmental stressors, such as heat shock, oxidative stress and ultraviolet light (highly relevant in the development of skin cancer). On the other hand, ERKs (extracellular-regulated kinases) are mainly activated by growth factors and oncogenes, like Ras. As it is shown in Figure 3, the three MAPKs have individual upstream kinases involved in their activation (MAPK kinase). MKK-4/7 activate JNK, while MEK-1/2 activate ERKs and MKK-3/6 activate p38. MKKs and MEKs are known as MAPK kinases, and all of them are under the control of an upstream kinase (generally named as MAPKK kinase). MAPKK kinases are not specific, and in this step the pathways begin to crosslink. We describe only a few examples of these MAPKK kinases: the phosphorylation of MKK-4/7 by MEKK 1,

MEK-1/2 by Raf-1 (which in turn is activated by Ras oncogene) and MKK-3/6 by RIP. Besides these kinases, a family of proteins named SFKs -Src family kinases, including Fyn kinase- can directly activate MAPK kinases. Considering the beginning of the pathway, MAPKK kinases are activated by STE20 kinases or by GTP-binding proteins (Chang & Karin, 2001). Finally, at the end of the pathway MAPK activates transcription factors such as AP-1 (homodimers or heterodimers conformed by Jun, Fos, JDP and ATF families) leading to an increment in COX-2 expression. Another activated transcription factors can be C/EBPs, which play different roles in COX-2 expression, according to the cell type involved (Chun & Surh, 2004).

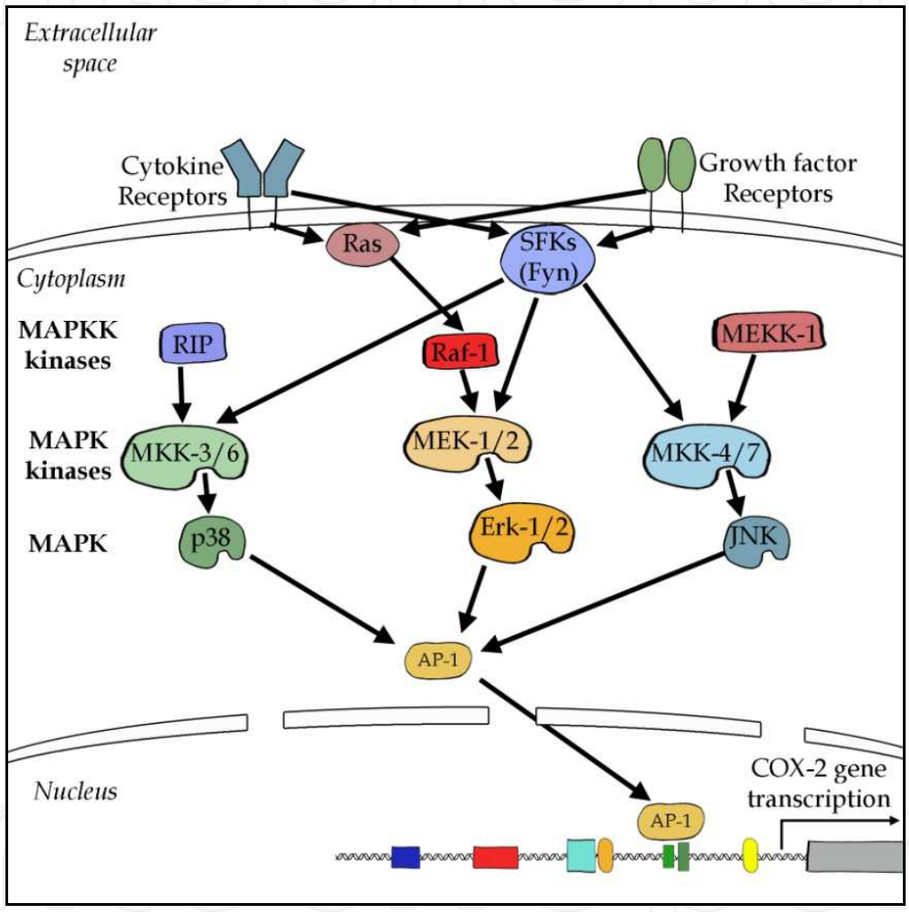

Fig. 3. Mitogen-activated protein kinases –MAPKs- molecular pathway. Membrane receptors which receive the trigger stimuli are shown, also the different downstream molecules involved and their crosslinks. This cascade includes kinases which phosphorylate and activate the next kinase. The common final path through the activation of the transcription factor AP-1, that finally leads to COX-2 expression, can also be seen.

In addition to the described mechanisms, there is an intense crosstalk between NF-κB and MAPKs pathways (Figure 4). For example, MEKK 1 and RIP are activated by TRAF-2 (Wu & Zhou, 2010) and MEKK 1 and JNK can be activated in LPS-treated macrophages through and adapter protein, named ECSIT (Kopp *et al.*, 1999). Besides, MEKK 1 is capable of phosphorylating IκB, releasing in this way an active NF-κB (Chun & Surh, 2004).

Finally, it is worth to notice that there are other pathways involved in the activation of NF-κB or MAPKs (Figure 4). For example, Phosphoinositol triphosphate (PIP_3, produced by active PI3K) activates Akt protein, which is able to phosphorilate IκB, therefore releasing active NF-κB (Ouyang *et al.*, 2006).

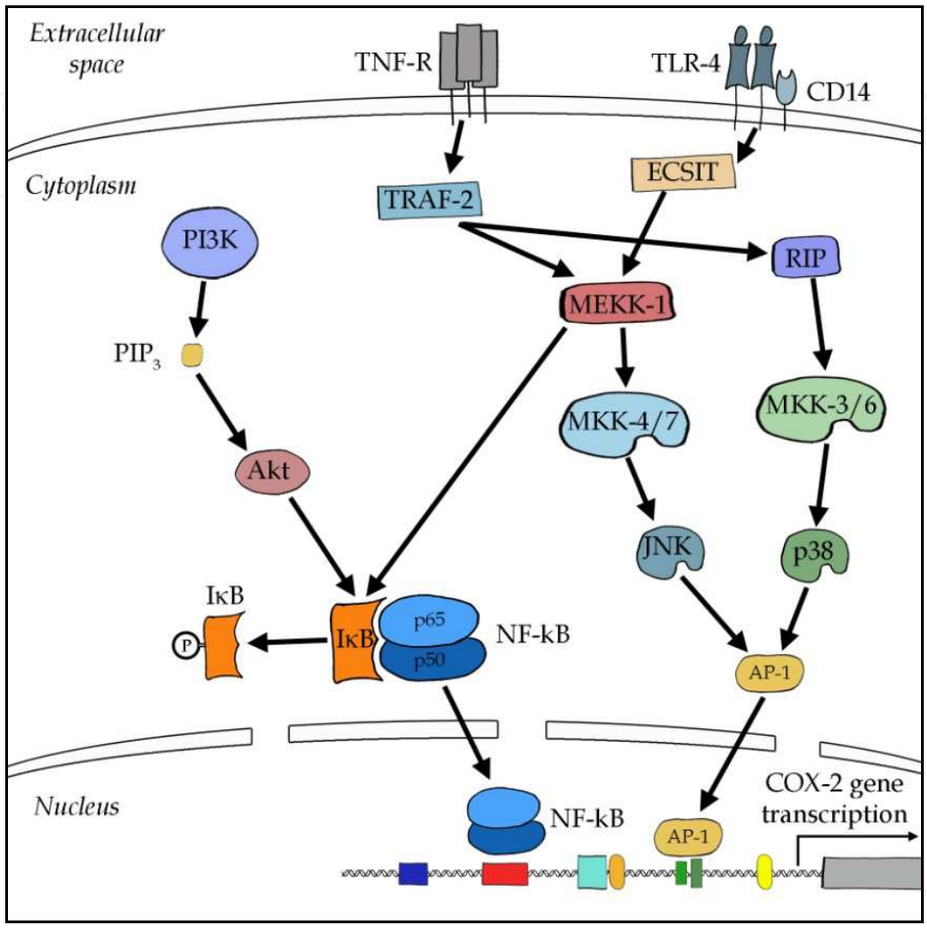

Fig. 4. Crosstalk between MAPKs, PIP_3 and NF-κB pathways. Membrane receptors, the different downstream molecules involved and their crosslinks are shown. The final path, which leads to COX-2 transcription and expression, can also be seen. P: phosphorylated molecule.

3. Molecular and cellular effects of COX-2 products

COXs -also known as PGH synthases- are the enzymes responsible for the conversion of arachidonic acid (AA) into PGH_2. AA is released from the cell membrane phospholipids by Phospholipase-A_2. AA conversion chemical reaction is catalyzed by the two enzymatic activities of COXs: cyclooxigenase (or bis-dioxygenase) activity generates a cyclopentane hydroperoxy endoperoxide (PGG_2) from AA. Peroxidase activity reduces the hydroperoxy group yielding the final product PGH_2 (Figure 5.a). PGH_2 by turns can be transform to different products (PGI_2 or prostacyclin, PGD_2, PGF_{2a}, PGE_2 and TXA_2) by specific synthases (Figure 5.b).

All of these AA-derived molecules, which play different roles in many physiological and pathophysiological mechanisms, have a short half-life *in vivo* (from seconds to minutes), performing its actions in an autocrine and paracrine fashion, rather than in an endocrine way (Ricciotti & FitzGerald, 2011).

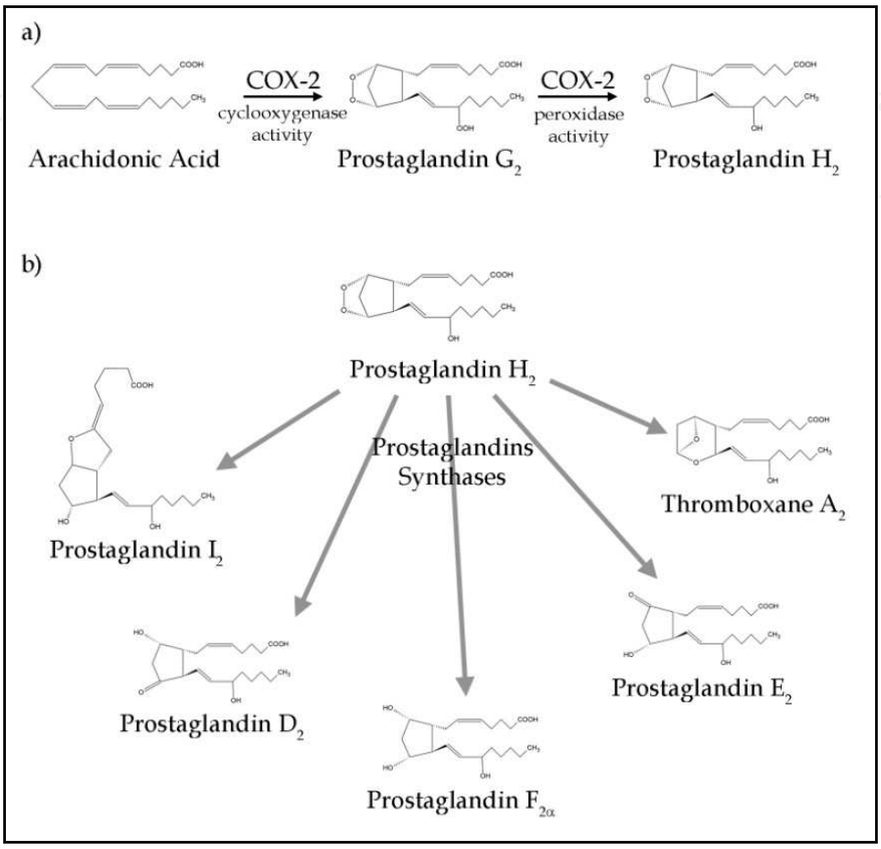

Fig. 5. Prostaglandins synthesis. a) Conversion of arachidonic acid –AA- into prostaglandin H_2 -PGH_2-, by the two enzymatic activities of COX-2. b) Conversion of PGH_2 into different final products, by specific synthases. The chemical structures and names of each product are shown.

3.1 Effects of PGE_2 on cell cycle and proliferation

Prostaglandins, particularly PGE_2, have been related to cell growth in some cell types. The role of PGE_2 has been widely described in keratinocytes, as a cell proliferation inducer. Indeed, it was described at the end of the 1970 decade, that there was an increase in the production of PGE_2 in a mouse model of epidermal hyperproliferation -induced by 12-O-tetradecanoylphorbol-13-acetate (TPA)- (Ashendel & Boutwell, 1979). In this TPA-induced proliferation model, keratinocyte growth was effectively blocked using the Non-Steroidal Anti-Inflammatory Drug indomethacin and successfully restored with an external supplementation of PGE_2 (Furstenberger *et al.*, 1979; Furstenberger & Marks, 1978). A few years later, Pentland described not only the capacity of keratinocytes to produce their own PGs from an external source of AA, but also the autocrine effects of these PGs on the cellular growth rate (Pentland & Needleman, 1986).

Interestingly, the final effect of PGE_2 in a specific cell type depends on the kind of receptor expressed in the target cell. There are four types of PGE_2 receptors, identified as E Prostanoid receptors 1 to 4 (EP_1-EP_4) (Narumiya *et al.*, 1999; Negishi *et al.*, 1993). These receptors are G-protein-coupled receptors which can activate different molecular pathways. EP_1 acts through the activation of Phospholipase C-β leading to an increase of intracellular calcium, while the other three receptors regulate adenylate cyclase activity. Meanwhile EP3 is able to down-regulate its activity, EP_2 and EP_4 can up-regulate it (Negishi *et al.*, 1995). Other PGs also exert their effects through G-protein-coupled receptor, according to Table 1.

Prostaglandin	**Prostanoid Receptor Subtype**	**G-protein coupled**	**Molecular Effects**
PGE_2	EP_1	G_q	$\uparrow IP_3$
	EP_2	G_s	$\uparrow$ cAMP
	EP_3	G_i, G_{12}, G_{Rho}	$\downarrow$ cAMP, $\uparrow Ca^{2+}$
	EP_4	G_s	$\uparrow$ cAMP
PGD_2	DP	G_s	$\uparrow$ cAMP
	CRTH2	G_i	$\downarrow$ cAMP, $\uparrow Ca^{2+}$
$PGF_{2\alpha}$	FP_A, FP_B	G_q, G_{Rho}	$\uparrow IP_3$
PGI_2	IP-IP	G_s, G_q, G_i	$\uparrow \downarrow$ cAMP, $\uparrow IP_3$
	IP-TP_α	G_s	$\uparrow$ cAMP
TxA_2	TP_α, TP_β	G_q, G_s, G_i, G_h, $G_{12/13}$	$\uparrow Ca^{2+}$, $\uparrow \downarrow$ cAMP, $\uparrow IP_3$

Table 1. Molecular effects of prostaglandins –PGs-. The type of PG, subtype of prostanoid receptor involved, G-protein-coupled receptor activated and final molecular effect are shown. ↑: increase, ↓: decrease, ↑ ↓: both effects are possible.

Recently, in a study performed using primary mouse keratinocytes, Ansari et al. demonstrated that PGE_2 is capable of inducing an indirect phosphorilation of epidermal growth factor receptor (EGFR), leading to the activation of Ras/MEK/ERK/AP-1 and PI3-K/Akt/NF-κB pathways; besides the direct activation of Adenylate cyclase/PKA/CREB through EP receptor. Transcription factors CREB, AP-1 and NF-κB lead to its proliferative effects through the activation of the expression of cyclin D1 and VEGF (Ansari *et al.*, 2008).

3.2 Prostaglandins effects on inflammation and immune system

The involvement of PGs in the inflammatory response has been largely known. The typical signs of inflammation -heat, redness, swelling and pain- result from PGs direct effects. Indeed, redness and edema are a consequence of an increased vasodilatation and vascular endothelium permeability, mediated by the interaction of PGI_2 and PGE_2 with vascular smooth muscle cells, respectively (Funk, 2001; Yamamoto *et al.*, 1999). Besides vascular modifications, inflammation-related pain depends on PGE_2 stimulation of peripheral sensory neurons (Ricciotti & FitzGerald, 2011). Additionally, fever caused in many inflammatory processes is also due to the interaction of PGE_2 with the nervous system, in this case with neurons of the organum vasculosa lamina terminalis at the midline of the preoptic area (Funk, 2001). In order to relieve one or all of these effects, inhibitors of PGs synthesis have been used as anti-inflammatory drugs for more than a century (acetylsalicylic acid was released in 1899 by Bayer), even when their molecular target was

unknown. Nowadays, all of the Non-Steroidal Anti-Inflammatory Drugs (NSAIDs) are well-known inhibitors of COXs enzymes. There are different groups of NSAIDs each of them with diverse mechanisms of action and also selectivity for the two isoforms of the enzyme.
As it was stated before, PGs and COXs implication in the inflammatory response have been described many decades ago. However, the positive feedback loop that involves these molecules (Figure 6) should be highlighted, since PGE_2 (a sub-product of the enzyme) is able to regulate both COX isoforms expression, as it has been proved in keratinocytes. Amazingly, not only COX-2 expression was increased but also was COX-1 (Maldve *et al.*, 2000). The mechanism underlying this feedback involves EP_2 receptor and the activation of cAMP/CREB pathway (Ansari *et al.*, 2007).

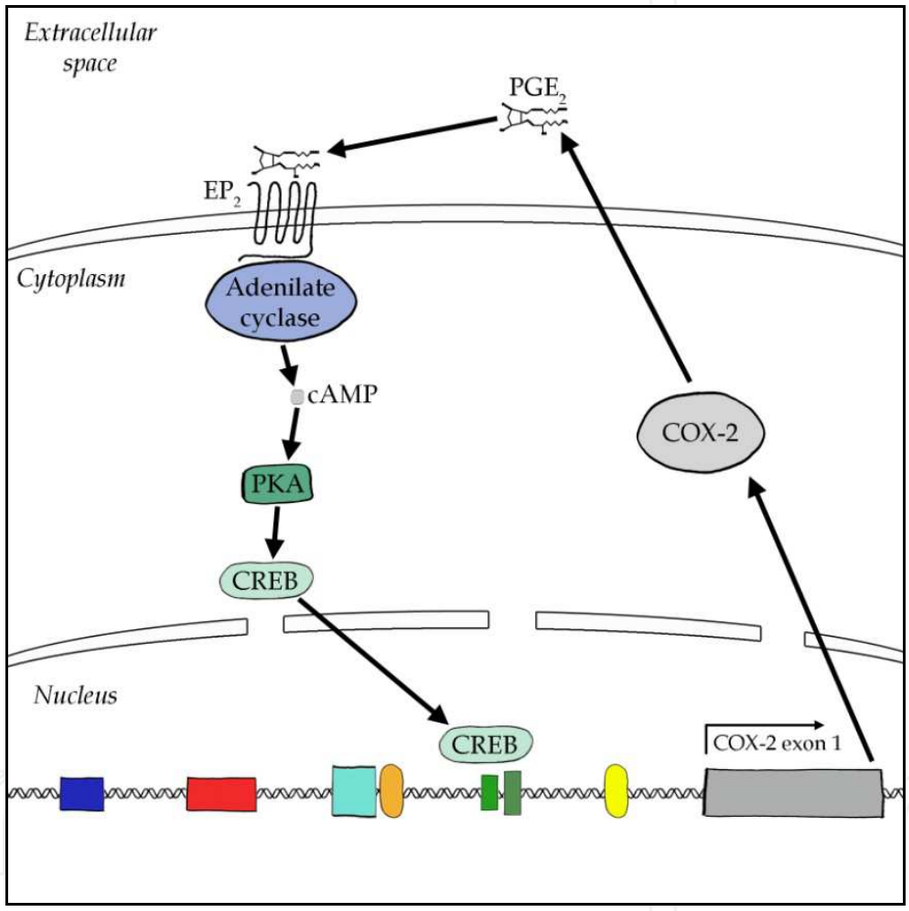

Fig. 6. COX-2 expression positive feedback loop. PGE_2, through its binding to EP_2 receptor, activates the cAMP/CREB pathway that finally leads to COX-2 expression, which synthesizes more PGE_2.

Besides their direct effects on inflammation, PGs play key roles in the development of the immune response, being capable of modifying dendritic cells and T helper (T_h) cells responses. During the activation of naive T_h cells by dendritic cells, or other antigen presenting cells, they can differentiate into different phenotypes: T_h1 cells (characterized by IFN-γ production), T_h2 cells (characterized by IL-4 production), T_h17 cells (characterized by IL-17 production) or T_{reg} cells (characterized by IL-10 production). PGE_2

plays an important role during this phenotypic differentiation of T_h cells. As it has been recently reported, PGE_2 -through its interaction with both EP_2 and EP_4- facilitates T_h1 cells differentiation, by the activation of the PI3K signalling pathway. Moreover, even when it was observed that T_h17 differentiation was not affected directly by PGE_2, an important role in IL-23-mediated T_h17 cells expansion was proved for PGE_2. IL-23 is the cytokine involved in the differentiation of naive T_h cells into T_h17 subset and is produced by dendritic cells, which generate endogenous PGE_2 that acts in an autocrine way through EP_4, activating the cAMP-Epac pathway. PGE_2 did not substantially affect T_h2, while it did inhibit T_{reg} differentiation (Yao *et al.*, 2009). Finally, Langerhans cells (the skin-homing dendritic cells) require the PGE_2-EP_4 signalling to mature and migrate from the epidermis to the draining lymph nodes, in order to perform an appropriate T cells activation (Kabashima *et al.*, 2003).

4. Skin cancer development and COX-2 over-expression

Under physiological conditions a balance exists between cell growth and death, resulting in a "homeostatic regulation". In this way, cells are normally under the control of their environment. Basically, cancer consists of a group of cells -probably originated from only one cell- that presents an "out of control" growth and functional characteristics. Cancer cells exhibit six basic acquired capabilities : i) self-sufficiency in growth signals, ii) insensitivity to anti-growth signals, iii) evading apoptosis, iv) sustained angiogenesis, v) tissue invasion and metastasis and vi) limitless replicative potential (Hanahan & Weinberg, 2000). Different signalling pathways can be affected by environmental stimuli, affecting normal cell growth. UVB radiation, as it was mentioned before in this chapter, is the main environmental carcinogen, affecting in a severe way cell growth control (de Gruijl *et al.*, 2001). As it has been vastly proved, specific mutations in the p53 gene and over-expression of the p53 protein are present in different types of skin cancer (Bolshakov *et al.*, 2003; Brash *et al.*, 1991). However, it is not the only affected gene, as it was demonstrated by Kim et al., among others, who evaluated the expression of p53 and COX-2 in human skin cancers: SCC, BCC, Bowen's disease and actinic keratosis. In this study it was found that both proteins are over-expressed in skin cancer, but there is not a direct correlation between them (Kim *et al.*, 2006). COX-2 over-expression could be a consequence of UVB radiation exposure and the subsequent activation of different signaling pathways, like the ones which include MAPK, PI3K and NF-κB.

4.1 Epidemiological data

It was described that up-regulation of COX-2 expression, which is normally undetected in most human tissues, is related to cancer promotion and development. Indeed, COX-2 has been evaluated in different human and animal malignancies, and it was stated as a probable target for cancer prevention (Hull, 2005; Subbaramaiah & Dannenberg, 2003).

COX-2 over-expression in different types of non-melanoma skin cancers have been assessed by different research groups, mainly by immunohistochemical techniques on human biopsy samples. In this way, human samples of SCC, BCC, Bowen's disease (BD, essentially SCC *in situ*), actinic keratosis (AK, pretumoral lesions that may evolve to SCC) and benign papillomas were studied. Sample positive reaction for COX-2 expression (in many cases with a highly positive result) ranged between 50 and 91% in SCC, 56 and 80% in BCC, 40

and 88% in BD, 50 and 83% in AK and it was of 25% in benign papillomas (An *et al.*, 2002; Kagoura *et al.*, 2001; Kim *et al.*, 2006). COX-2 over-expression has also been determined in mice models of skin abnormalities, such as hyperplasia of the skin, benign papillomas and SCC –resulting from UV chronic irradiation-. In these animal models COX-2 expression was also found increased in all cases, in accordance with the results obtained in human biopsy samples abovementioned (An *et al.*, 2002; Athar *et al.*, 2001).

4.2 Relationships between COX-2 expression, UV radiation and skin cancer development

UV radiation, as the main inducer of skin cancer, activates many molecular pathways in skin cells. There is a vast bibliography about all of these processes, which we described briefly, and it has also been reviewed elsewhere by other authors (Rundhaug & Fischer, 2008).

COX-2 expression can be induced in humans, proved both in cultured skin cells and in whole tissues, after a single or multiple exposures to UVr (Buckman *et al.*, 1998; Tripp *et al.*, 2003). This molecular response to UVr plays a pivotal role in the development of skin malignancies. UVr is able to increase reactive oxygen species (ROS) production, due to an excitation of molecules which act as chromophores and that can finally transfer the absorbed-energy to molecular oxygen, generating ROS. These molecules produce the oxidation of a phosphatase (receptor-type protein tyrosin phosphatase-κ), whose function is to keep the epidermal growth factor receptor (EGFR) unphosphorilated and inactive. Once the phosphatase is inactivated, EGFR becomes active and can transduce the signal through the downstream pathway, which includes Ras/Rac1/p38, leading ultimately to COX-2 transcription (Xu *et al.*, 2006a; Xu *et al.*, 2006b). Besides this mechanism, UVr may transfer its energy to generate a photoproduct of tryptophan, named 6-formylindolo[2,3-b]carbazole (FICZ). This molecule is a ligand for arylhydrocarbon receptor (AhR), which activates Src kinase and finally leads to the over-expression of COX-2 (Fritsche *et al.*, 2007). Another described mechanism underlying UVr-induced COX-2 over-expression is mediated by genotoxic damage. UVr directly affects cells by the generation of thymine dimers in the DNA; this genotoxic damage activates p53 protein, which arrests cell cycle and regulates the activation of apoptotic cell death. p53 also leads to the induction of heparin-binding EGF, which activates Ras/Raf/ERK pathway finally mediating the over-expression of COX-2 (Han *et al.*, 2002).

COX-2 over-expression leads to the many molecular events described before in this chapter. There are some more evidences of the involvement of those molecules and pathways in the development of skin cancer. Transgenic mice that express COX-2 constitutively in the skin-under the regulation of keratin 5 promoter- were studied in terms of alterations of growth or differentiation of the keratinocytes. It was found that constitutive expression of COX-2 in keratinocytes generates epidermal hyperplasia, mainly due to a lack of differentiation instead of an increased growth rate, and other pathological signs–like epidermal invaginations, loss of cell polarity and formation of horn pearls- (Neufang *et al.*, 2001). Moreover, studies performed in knock-out mice for both COX isoforms demonstrated that the absence of any of these enzymes reduces the incidence of skin cancer development, in DMBA-TPA model (Tiano *et al.*, 2002a). Finally, the carcinogenic effect of COX-2-derived PGE_2 is: i) dependent on the expression of EP_2, but not of the expression of EP_3; since knock-out mice lacking these two receptor type respond in different ways to the carcinogenic stimuli of DMBA/TPA treatment (Sung *et al.*, 2005) and ii) dependent on EP_1;

since its specific blockade reduced the number of tumor per mice in a UVB-induced skin cancer model (Tober *et al.*, 2006). However, the four prostanoid receptors EP_1-EP_4 are expressed differentially throughout the skin, and developed different staining patterns after UVB irradiation, suggesting that each receptor might play dissimilar roles in carcinogenesis (Tober *et al.*, 2007).

5. Chemoprovention and treatment of skin cancer

Classical treatment for NMSC consists mainly in surgical excision. Particularly, for the case of BCC, there is no need to perform a follow-up of the patients, since this type of cancer does not have the capacity to metastasize (MacKie, 2006). However, there are enormous efforts being made in order to evaluate alternative non-surgical therapies for NMSC. Many of them are being studied in animal models, and many others in human patients. We make here a brief summary of treatments, highlighting the molecular pathways involved, both for some pharmacological and natural compounds.

5.1 Pharmacological compounds in the skin cancer treatment

As it was mentioned before, most of the knowledge about the role of COX in the development of skin cancer was achieved by studies performed with NSAIDs. In this way, the oldest report on the topic was first published, as far as we know, in 1995 (Reeve *et al.*, 1995). In this study indomethacin (a non-specific NSAID) was administrated in a mouse model of UV-induced skin cancer. Many studies were performed since then, facing the issue in different ways. Non-specific NSAIDs orally administered have the great disadvantage of gastric side-effects, in prolonged treatments. Considering this aspect, the pioneering work of Reeve et al. was discussed by Pentland et al., whose work was performed using celecoxib –a specific COX-2 inhibitor- in the same model: SKH:1 mice chronically exposed to UV radiation (Pentland *et al.*, 1999), results which were further confirmed (Orengo *et al.*, 2002). Pentland demonstrated that, beyond the absence of side-effects, there were much better anti-tumor results using celecoxib instead of indomethacin. Moreover, these conclusions were also achieved by other research group, which directly compared the anti-tumor effects of both celecoxib and indomethacin in parallel experiments (Fischer *et al.*, 1999). Oral celecoxib treatment was recently evaluated in a randomized, double blind, placebo-controlled trial, showing that it would be helpful in the prevention of SCC and BCC in patients who have an extensive actinic damage (Elmets *et al.*, 2010).

In addition to these studies, the idea of skin cancer oral treatments was challenged by another way for drugs application: topical treatment. The present book includes a full chapter related to the topical administration of drugs and skin cancer, written by Taveira & Lopez, which could be of interest. Celecoxib topically applied demonstrated to be useful in the suppression of UVB-induced cutaneous inflammation (Wilgus *et al.*, 2000). Besides, topically applied naproxen –a non-selective NSAID- was useful in the reduction of skin tumors after chronic irradiation of SKH:1 mice, with a marked decrease in the levels of PGE_2 and a surprising increment in the epidermal production of TNF-α (González Maglio *et al.*, 2010). Accordingly, long ago it has been reported that TNF-α production increases after the inhibition of COX (Hart *et al.*, 1989). PGE_2, as it was stated before in this chapter, may act both increasing and decreasing the levels of cAMP (a well-known repressor of TNF-α expression). The NSAID-mediated decrease in PGE_2

production might avoid the inhibition of TNF-α transcription, increasing in this way the level of this cytokine in the treated cells (Grandjean-Laquerriere, 2003). Increases in TNF-α production may explain, at least in part, another important effect of NSAIDs treatment: the induction of apoptosis in the target cells (Fecker *et al.*, 2007). This induction could be mediated by TNF-receptor triggered cell death pathway. Moreover, it is worth to notice that topical treatment could dramatically reduce the gastric side-effects of non-specific NSAIDs prolonged treatment. If this kind of drug application was performed, there would be better results in the anti-tumor effects, since both isoforms of COX mediates skin cancer development (Tiano *et al.*, 2002b). However, in neither of the commented studies the incidence of tumors in treated mice was null, showing that there are cellular alterations remaining that cannot be faced with this kind of drugs.

Aspirin or acetylsalicylic acid –the oldest NSAIDs in use- may capture some special attention, since it has been demonstrated that this drug is not only capable of inhibiting COX enzymatic activity, but also of inhibiting the activity of IκB kinase-β, inhibiting in this way the NF-κB pathway (Yin *et al.*, 1998).

It is of great interest to analyze another drug, imiquimod, recently approved by the US Food and Drug Administration (FDA) to be used topically in the treatment of human SCC and BCC (Love *et al.*, 2009). This drug activates directly TLR-7 and TLR-8 and leads to the activation of NF-κB and AP-1 nuclear factors, resulting in the expression of pro-inflammatory cytokines, and probably –but not proven up to date- COX-2. Another mechanism of action includes a decrease in the cellular levels of cAMP which, as it was explained before, inhibits the transcription of inflammatory cytokines, like TNF-α. Finally, imiquimod can induce directly apoptotic cell death of tumoral cells through the activation of death receptors (Schon & Schon, 2007; Schon *et al.*, 2006).

Drug treatment of skin cancer might affect the COX-2 pathway, but a reduction in this enzyme expression or activity does not necessarily ensure an anti-tumor effect. Besides, by only blocking COX activity, the incidence of cancer can be reduced, but not entirely abolished.

5.2 Natural compounds in the skin cancer treatment

Historically, different human malignancies and pathologies have been treated with medicinal plants and different natural extracts. Nowadays, a great number of natural compounds with anti-tumor effects have been identified and isolated. Many of them have anti-oxidant effects, which are responsible for their protective effects against malignancies. A whole chapter of the present book, written by Filip and Clichici, is completely dedicated to analyze the use of natural compounds in the treatment of skin cancer. Hereafter, we comment the use of some compounds in this field, with some mention to the corresponding molecular pathway involved.

Many natural compounds have the ability of down-regulate *in vitro* or *in vivo* the UV-induced over-expression of COX-2 or the production of PGE_2. Some very well documented examples are curcumin (Cho *et al.*, 2005), apigenin (Tong *et al.*, 2007), nobiletin (Tanaka *et al.*, 2004), cyanidin (Kim *et al.*, 2010), cyaniding-3-glucoside (Lim *et al.*, 2011) and α-linoleic acid (Takemura *et al.*, 2002). This capacity of inhibiting COX-2 expression or function is promising for the use of these compounds in skin cancer treatment. However, not all of the above-mentioned compounds have been properly assayed in animal tumor models.

As an example, cyanidin-3-glucoside (C3G) has been proven to effectively reduce the number of papillomas in TPA-treated mice (Ding *et al.*, 2006). C3G was also demonstrated to attenuate activation of transcription factors AP-1 and NF-κB, and the kinases MEK, MKK4, Akt and MAPKs. However, it does not affect the phosphorylation of the upstream kinase Fyn. Molecular docking analysis has demonstrated the capacity of C3G to directly fit in the pocket responsible for enzymatic activity of Fyn (Lim *et al.*, 2011).

Resveratrol was shown to be useful in the treatment of UV-induced skin cancer in mice (Aziz *et al.*, 2005). Moreover, it has exhibited an interesting decrease in the activation of NF-κB pathway in UVB-exposed cultured keratinocytes (Adhami *et al.*, 2003).

Pathways which are activated by peroxisome proliferator-activated receptors (PPARs) are also of interest. These pathways may be activated by a great number of ligands, including pharmacological compounds, fatty acids and fatty acid-derived molecules. PPAR-α can modulate the expression of COX-2 and VEGF by antagonizing NF-κB and AP-1, inducing the expression of IκB and interfering with the MAPKs pathway (Grau *et al.*, 2008). Indeed, PPAR-α expression is down-regulated in human SCC and AK biopsies (Nijsten *et al.*, 2005) and its activation has shown a partial inhibition in the development of skin tumors in a mice model (Thuillier *et al.*, 2000). Finally, α-linoleic acid (ligand of PPAR-α) can mediate a decrease in the UV-induced PGE_2 production in SKH:1 mice (Takemura *et al.*, 2002).

It is worth to notice that all of these compounds, although promising, are not being used in humans yet. Many of them remain to be thoroughly tested in animal models, before they can be approved for human treatment. Additionally, for natural compounds it is also vacant the analysis of the topical administration, since the majority of the studies have been performed using the classical oral administration. Topical administration can offer the advantages of direct treatment of the lesion and reduction of unwanted side-effects, which have not been well-described and reported yet, for the doses of the natural compounds needed to exhibit anti-tumor effects.

6. Conclusion

COX-2 over-expression is highly related to human NMSC development and progression. Many very well known carcinogens can activate different molecular pathways leading to COX-2 gene transcription. Over-expression of COX-2 enzyme was determined in human NMSC biopsies as well as in animal tissues by different techniques. *In vitro* studies have demonstrated its expression in keratinocytes after carcinogenic stimuli.

The sub-products of COX-2 enzyme activity, mainly PGE_2, lead to many cellular and molecular events related with carcinogenesis. These events include apoptosis resistance, growth signalling, angiogenesis and selective immunosuppression. Many of these events are regulated by the expression of specific receptors expressed in the target cell.

A great number of studies have been performed in order to obtain alternative or adjuvant treatments for NMSC, different from surgical excision. Treatments which regulate COX-2 expression pathways or its own enzymatic activity, altered the whole inflammatory response, as well as the cellular and molecular events mentioned before. All anti-tumor therapies must be analyzed taking into account that malignant-transformed cells can be directly eliminated –by avoiding growth stimuli or inducing cytotoxicity- or indirectly –by the proper activation of the immune response-. In this way, some of the above-mentioned treatments induce preferentially cytotoxic effects, while others increase the immune response. For some others, even when the overall anti-tumor effects were effectively proven,

the exact mechanism was not. Finally, it is important to highlight that opposite effects on the same pathway may result in an equally effective anti-tumor response, i.e. imiquimod activates NF-κB pathway while PPAR-α ligands inhibit it, and both are useful treatments for NMSC.

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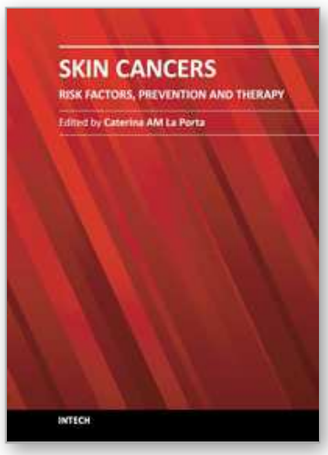

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Skin cancers are the fastest growing type of cancer in the United States and represent the most commonly diagnosed malignancy, surpassing lung, breast, colorectal and prostate cancer. In Europe, the British Isles have been the highest rates of skin cancer in children and adolescents. The overall idea of this book is to provide the reader with up to date information on the possible tools to use for prevention, diagnosis and treatment of skin cancer. Three main issues are discussed: risk factors, new diagnostic tools for prevention and strategies for prevention and treatment of skin cancer using natural compounds or nano-particle drug delivery and photodynamic therapy.

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