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Lipoxygenase metabolism: roles in tumor progression and survival

Graham P. Pidgeon · Joanne Lysaght · Sriram Krishnamoorthy · John V. Reynolds · Ken O'Byrne · Daotai Nie · Kenneth V. Honn

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Abstract The metabolism of arachidonic acid through lipoxygenase pathways leads to the generation of various biologically active eicosanoids. The expression of these enzymes vary throughout the progression of various cancers, and thereby they have been shown to regulate aspects of tumor development. Substantial evidence supports a functional role for lipoxygenase-catalyzed arachidonic and linoleic acid metabolism in cancer development. Pharmacologic and natural inhibitors of lipoxygenases have been shown to suppress carcinogenesis and tumor growth in a number of experimental models. Signaling of hydro[peroxy] fatty acids following arachidonic or linoleic acid metabolism potentially effect diverse biological phenomenon regulating processes such as cell growth, cell survival, angiogenesis, cell invasion, metastatic potential and immunomodulation. However, the effects of distinct LOX isoforms differ considerably with respect to their effects on both the individual mechanisms described and the tumor being examined. 5-LOX and platelet type 12-LOX are generally considered pro-carcinogenic, with the role of 15-LOX-1 remaining controversial, while 15-LOX-2 suppresses carcinogenesis. In this review, we focus on the molecular mechanisms regulated by LOX metabolism in some of the major cancers. We discuss the effects of LOXs on tumor cell proliferation, their roles in cell cycle control and cell death induction, effects on angiogenesis, migration and the immune response, as well as the signal transduction pathways involved in these processes. Understanding the molecular mechanisms underlying the anti-tumor effect of specific, or general, LOX inhibitors may lead to the design of biologically and pharmacologically targeted therapeutic strategies inhibiting LOX isoforms and/or their biologically active metabolites, that may ultimately prove useful in the treatment of cancer, either alone or in combination with conventional therapies.

Keywords Lipoxygenase · Tumor survival · Apoptosis · Angiogenesis · Immune suppression

G. P. Pidgeon · J. V. Reynolds
Department of Clinical Surgery,
Trinity College Dublin, St. James Hospital,
Dublin 8, Ireland

J. Lysaght
Department of Haematology,
Trinity College Dublin, St. James Hospital,
Dublin 8, Ireland

S. Krishnamoorthy · K. V. Honn
Department of Pathology, Wayne State University School of Medicine & Karmanos Cancer Institute,
Detroit, MI 48202, USA

K. O'Byrne
Department of Clinical Medicine,
Trinity College Dublin, St. James Hospital,
Dublin 8, Ireland

D. Nie
Department of Medical Microbiology, Immunology, and Cell Biology, Southern Illinois University School of Medicine and Cancer Institute,
Springfield, IL 62794, USA

G. P. Pidgeon (✉)
Department of Clinical Surgery, Institute of Molecular Medicine,
Trinity Center for Health Sciences, TCD/St. James's Hospital,
Dublin 8, Ireland
e-mail: pidgeong@tcd.ie

1 Introduction

The functional relationship between polyunsaturated fatty acid metabolism, inflammation and carcinogenesis has been extensively examined in numerous molecular studies, re-

vealing potential novel targets for the treatment and chemoprevention of a number of different cancers [1, 2]. Three types of enzymes; cyclooxygenases, epoxygenases (cytochrome *P*450), and lipoxygenases can metabolize arachidonic acid to biologically active eicosanoids. The various lipid peroxides and bioactive lipids generated by this metabolism can regulate cellular proliferation, apoptosis, differentiation and senescence. The role of cyclooxygenase isoforms have been widely demonstrated in a number of studies and COX-2 in particular is regarded as a promising target for chemoprevention and treatment of several cancers [3, 4]. However, in the last decade there has been a significant rise in the number of reports examining the functional links between various LOX isoforms and mechanisms controlling cancer development and progression [5]. In this review we focus on the role of lipoxygenases in the most commonly diagnosed cancers, describing their involvement in aspects governing tumor development and progression, from tumor cell survival and immunosurveillance, to their involvement in the metastatic spread of tumors by influencing angiogenesis and migration. Throughout the review we also examine some of the mechanisms of action of particular agents targeting the LOX pathways of arachidonic acid metabolism. It is likely that in the future, treatments aimed at inhibiting LOX activity, either alone or in combination with other pathways, should improve the efficacy of conventional treatments aimed at inducing tumor cell apoptosis, such as chemotherapy and/or radiation therapy.

2 Lipoxygenase-catalyzed arachidonic acid metabolism

Lipoxygenases constitute a family of non-heme iron dioxygenases that insert molecular oxygen into free and/or esterified polyunsaturated fatty acids with regional specificity, and are designated 5-, 8-, 12- and 15-lipoxygenase (5-LOX, 8-LOX, 12-LOX, 15-LOX) accordingly [6]. The most studied LOX enzymes are 5-LOX, leukocyte and platelet-type 12-LOX and reticulocyte-type 15-LOX-1; however the mammalian family of LOX isozymes has recently grown to include isozymes preferentially expressed in both human and murine epidermis. These isozymes include epidermal 12-LOX and 12R-LOX (producing products with *R* chirality), mouse 8-LOX and its human ortholog 15-LOX-2, and epidermal LOX-3 exhibiting hydroperoxide isomerase activity [7, 8]. The LOX enzymes metabolize arachidonic acid (AA) to the biologically active metabolites hydroperoxy–eicosatetraenoic acids (HPETEs), which upon reduction result in the formation of corresponding HETEs (Fig. 1), while the metabolism of linoleic acid preferentially results in the formation of hydroxy–octadecadienoic acids (HODEs) [9]. Therefore, 5-, 8-, 12- and 15-HETE are the major AA metabolites formed by mammalian LOXs, and 9- and 13-HODE are the principle reaction products of linoleic acid oxygenation.

In the case of the 5-LOX enzyme, HPETE is further metabolized to form the unstable epoxide leukotriene A_4 (LTA_4) in the presence of 5-lipoxygenase-activating protein.

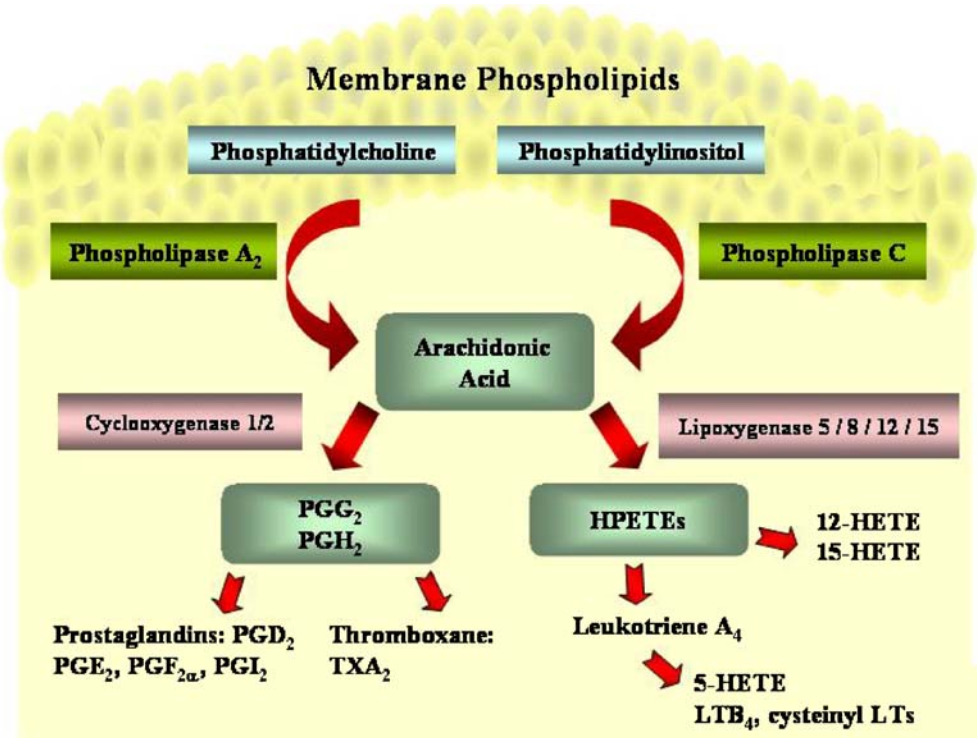

Fig. 1 The generation of bioactive lipid mediators. Membrane phospholipids are converted to arachidonic acid by phospholipase A_2 and phospholipase C. Arachidonic acid is then further metabolized by one of two distinct pathways. Cyclooxygenase pathways convert arachidonic acid to the unstable cyclic endoperoxides PGG_2 and PGH_2, which are converted to prostaglandins and thromboxanes by tissue specific synthases. The lipoxygenase pathway metabolizes arachidonic acid to cyclic hydroperoxides (HPETEs), which are then further converted to HETE and leukotriene A_4. Leukotriene A_4 is then further metabolized to LTB_4, LTC_4, LTD_4 and LTE_4

LTA_4 is subsequently converted to 5(*S*)-hydroxy-6-*trans*8, 11,14-*cis*-eicosatetranoic acid (5-HETE), or hydrolyzed into LTB_4 by leukotriene A4 hydrolase, or the cysteinyl leukotrienes, LTC_4, LTD_4 and LTE_4, following enzymatic conjugation with GSH [10].

Platelet-type 12-LOX exclusively uses AA released from glycerol-phospholipid pools to synthesize 12(*S*)-HPETE and 12(*S*)-HETE, whereas leukocyte-type 12-LOX can also synthesize 15(*S*)-HETE and 12(*S*)-HETE. In addition to leukocytes and platelets, the expression of 12-LOX isozymes has been detected in various types of cells, such as smooth muscle cells, keratinocytes, endothelial cells and tumor cells.

15-lipoxygenases (15-LOX) can be subdivided into two isoforms, named 15-LOX-1 and 15-LOX-2 and the intracellular activity of these enzymes is regulated at transcriptional, translational and post-translational levels [11]. 15-LOX-1 is mainly expressed in reticulocytes, eosinophils and airway epithelial cells, as well as in macrophages and in atherosclerotic lesions [12]. The enzyme plays a role in cell differentiation and maturation, inflammation, asthma, carcinogenesis and atherogenesis [11]. In terms of enzymatic characteristics, 15-LOX-1 preferentially metabolizes linoleic acid primarily to 13-(*S*)-HODE, but also metabolizes arachidonic acid to 15-(*S*)-HETE. 15-LOX-2, on the other hand, converts arachidonic acid to 15-(*S*)-HETE and metabolizes linoleic acid poorly [13]. While many lines of experimental evidence suggested that 5- and 12-LOX metabolites promote angiogenesis and carcinogenesis, in contrast 15-LOX may play an inhibitory role in tumor angiogenesis and thus, may slow carcinogenesis [14, 15]. Interestingly, Shureiqi and colleagues demonstrated that non steroidal anti-inflammatory drugs (NSAIDs) induce 15-LOX-1 expression in colorectal cancer cells and that this up-regulation is critical to NSAID-induced apoptosis [16].

The products of LOX metabolism represent either intermediary products such as HPETE, which are transformed enzymatically into secondary products including leukotrienes, hepoxilins, lipoxins and HETEs, which can act as signaling molecules in their own right, or give rise to the production of reactive oxygen species (ROS). Signaling of LOX-derived products can occur through either G protein-coupled cell surface receptors, in the case of lipoxins and leukotrienes [17], or through activation of nuclear receptors such as peroxisome proliferator activated receptors (PPAR) in the case of HETEs and HODEs [18].

3 Lipoxygenase expression in normal and malignant tissue

The role of LOX in the development and progression of cancer is complex due to the variety of LOX genes that have been identified in humans, in addition to different profiles of LOX observed between studies on human tumor biopsies and experimentally induced animal tumor models [14, 19]. Examining the expression and activity of LOX in matched normal and cancer epithelial tissues from both humans and mice, revealed that human 15-LOX-1 and -2 (or the corresponding mouse orthologs leukocyte 12-LOX and 8-LOX) are usually preferentially expressed in normal tissues and benign lesions, but not in carcinomas of the bladder, breast, colon, lung or prostate [20–24]. In contrast, 5-LOX and platelet type 12-LOX are generally absent in normal epithelia, can be induced by pro-inflammatory stimuli, and are often constitutively expressed in various epithelial cancers including colon, esophageal, lung prostate and breast cancer [25–29]. The literature emerging on the role of lipoxygenases in tumor growth, for the most part, suggests that distinct LOX isoforms, whose expression are lost during the progression of cancer, may exhibit anti-tumor activity, while other isoforms may exert pro-tumorigenic effects and are preferentially expressed during the development of various cancers (Fig. 2). This view is supported by experimental studies showing that forced expression of 8-LOX or induced expression of leukocyte type 12-LOX in transgenic mice reduced the formation of chemically induced skin tumors [30, 31]. There is also significant evidence from human studies supporting this idea, for example each of the four most prevalent cancers worldwide; breast, lung, colon and prostate cancer, have been shown to be differentially effected by LOX isoform expression.

In the case of breast cancer, both 15-LOX-1 and -2 were found to be poorly expressed compared to normal epithelium [32], while elevated levels of both 5-LOX and 12-LOX were identified and found to be associated with higher TNM staging [33]. In another study, elevated 12-LOX expression was shown to have prognostic value, when taken in combination with decreased levels of 15-LOX, in patients with breast cancer [26]. Similarly, in non small cell lung cancer (NSCLC), better differentiated tumors expressed greater 15-LOX-2 expression and that this expression was inversely correlated with tumor grade [22]. In a small study examining colorectal adenomas, 15-LOX-1 expression was down-regulated in 87% of tumors relative to normal epithelial mucosa, while no difference in either 5-LOX or 15-LOX-2 were observed [34].

The role of various LOX isoforms in prostate cancer has been extensively investigated. In a study involving over 130 prostate cancer patients, Gao et al. found that the level of 12-LOX mRNA expression is correlated with tumor stage [29]. In this study, the expression of 12-LOX and tumor stage, grade, positive surgical margins and lymph node positivity were evaluated. Overall, 38% of 122 patients demonstrated elevated levels of 12-LOX mRNA in prostate cancer tissue compared with their matching normal tissues. A statistically significantly greater number of cases were found to have an elevated level of 12-LOX among T3, high grade and sur-

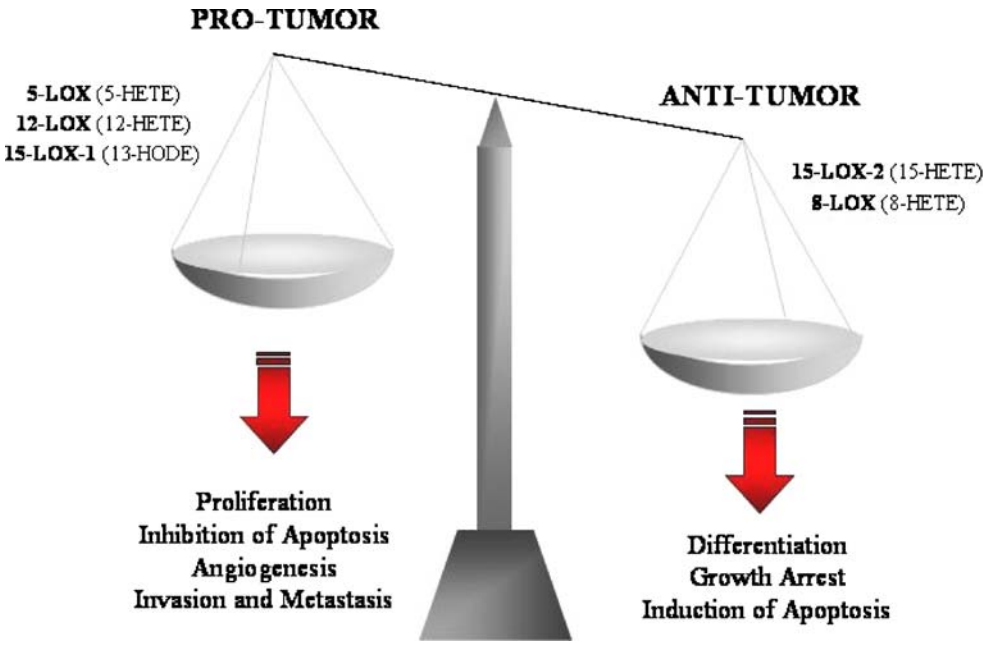

Fig. 2 Tumor development is modulated by both pro- and anti-tumor LOX isoforms. The expression pattern of individual LOX isozymes, and the biological activity of their corresponding products in malignant tissue, indicates a critical balance of pro- versus anti-tumor LOX enzymes may influence the course of tumor development in many cancer types

gical margin positive than T2, intermediate, and low grade and surgical margin negative prostatic adenocarcinomas. These data suggest that elevation of 12-LOX mRNA expression occurs more frequently in advanced-stage, high-grade prostate cancer [29]. In addition, urinary levels of 12-HETE, the metabolite produced by 12-LOX, have been reported to be significantly elevated in prostate cancer patients compared to normal individuals, and following radical prostatectomy these levels are significantly reduced, while the procedure has little effect on the levels of other LOX products [35].

Each of these studies implicate the involvement of lipoxygenases in tumor differentiation and progression, however the overall picture is controversial with regards to some isoforms. Like platelet-type 12-LOX, 15-LOX-1 over-expression has been reported in prostate tumors where its levels of expression correlate with the Gleason score of the cancer [36]. Higher Gleason scores correlated with expression of 15-LOX-1, while others have reported that 15-LOX-2 is expressed in normal prostate tissue, but poorly expressed in prostate tumors. The reduced 15-LOX-2 expression is inversely correlated with the Gleason score of the tumor.

This report would suggest that 15-LOX-1 is acting very differently in the prostate, compared to its tumor suppressive action in both colon and breast cancer [32, 34]. Therefore, it is likely that the role of LOX expression on cancer growth depends on both the isoforms expressed and the site of the particular tumor. To critically examine the role of each isoform in cancer development, it is important to investigate the effect of lipoxygenase metabolism on various aspects of cancer progression, from cell survival and growth, to invasion and angiogenesis.

4 Converging pathways of LOX and COX in cancer

It is evident that certain LOX isoforms and cyclooxygenase-2 display similarities in expression and function in human cancers. Firstly, 5-LOX, 12-LOX and COX-2 are co expressed and up-regulated in quite a large number of cancer cell lines and human tumors, including lung, colon, prostate and breast [37]. In addition, all three enzymes are pro-angiogenic with a convergent target on VEGF expression [38–40], and their inhibition induces apoptotic cell death in a number of tumor cell lines.

COX-2 selective inhibitors are being used *in vivo* to block cancer progression of colon cancer. COX-2 and 5-LOX have been shown to be over-expressed during the process of colonic adenoma formation promoted by cigarette smoke. Ye et al. investigated if a relationship exists between COX-2 and 5-LOX in this model, and whether dual inhibition of COX-2 and 5-LOX has an anti-carcinogenic effect in the colonic tumorigenesis promoted by cigarette smoke [39]. They found that pretreatment of colon cancer cells with cigarette smoke extract promoted colon cancer growth in the nude mouse xenograft model, and that inhibition of COX-2 and 5-LOX reduced tumor size.

NSAIDs can induce cancer cell apoptosis independent of their action on COX-2. For example in esophageal cancer cells the induction of apoptosis by NSAIDs is dependent on up-regulation of 15-LOX-1 [14]. On the other hand, free unmetabolized arachidonic acid has been reported to induce a concentration-dependent apoptotic response in cancer cells [41]. Therefore, when multiple metabolic pathways of arachidonic acid are present within the same cells, as in cancer

cells co-expressing COX-2, 5-LOX and 12-LOX, simultaneous blocking of each enzyme may be required to achieve substantial elevation in free arachidonic acid levels and prevent the shunting of metabolism to another active pathway. This concept is supported by studies showing that when either COX-2 or 5-LOX enzymes were inhibited alone in colon cancer cells, the result was activation of the other pathway [42]. In one study, Dr. Honn's laboratory found that, incubation of 12-LOX overexpressing PC-3 prostate cancer cells with 10 μM indomethacin, a COX inhibitor, resulted in elevated levels of 12(*S*)-HETE, which suggests channeling of the substrate to an alternate pathway, in this case to 12-LOX, when one pathway is inhibited (Fig. 3). Conversely, combined treatment with celecoxib and MK886, the 5-LOX inhibitor, had additive effects on inhibiting tumor cell proliferation and inducing apoptosis compared to either inhibitor alone. In a separate study, combined inhibition of COX-2 and 5-LOX significantly decreased incidence, number and size of chemically induced liver metastases in Syrian hamsters [43]. Interestingly, in the same study 5-LOX-inhibition decreased intra-metastatic PGE_2 concentration as well as $PGF_{1\alpha}$ and PGE_2 in non-metastatic liver, confirming a cross-talk between LOX and COX pathways in this cancer model.

The combination of COX and LOX inhibitors produced synergistic growth inhibition in Lewis lung cancer cell lines when used together and in combination with cisplatin [44]. In another study, both exisulind and sulindac sulfide produced synergistic cytotoxicity with doxorubicin and VP-16 against the lung adenocarcinoma cell line A549 [45]. These observations were furthered by Soriano et al. in a panel of human lung cancer cell lines where combination studies showed synergistic interactions for COX and LOX inhibitors with paclitaxel, cisplatin and 13-*cis*-retinoic acid regardless of drug-resistance phenotype [46].

The involvement of COX-2, 5-LOX and 12-LOX in human cancer progression is now supported by a growing body of literature. The co-localization of these enzymes and the similarities of their bioactions on cancer cell growth suggest that the simultaneous inhibition of these enzymes may represent novel and promising therapeutic approaches in selected cancer types. Therefore, when targeting the regulation of arachidonic acid metabolism, blocking COX-2, 5-LOX, 12-LOX and 15-LOX-1 without altering the expression of the anti-carcinogenic 15-LOX-2 may be the most effective, however at present no drug recapitulates these capabilities.

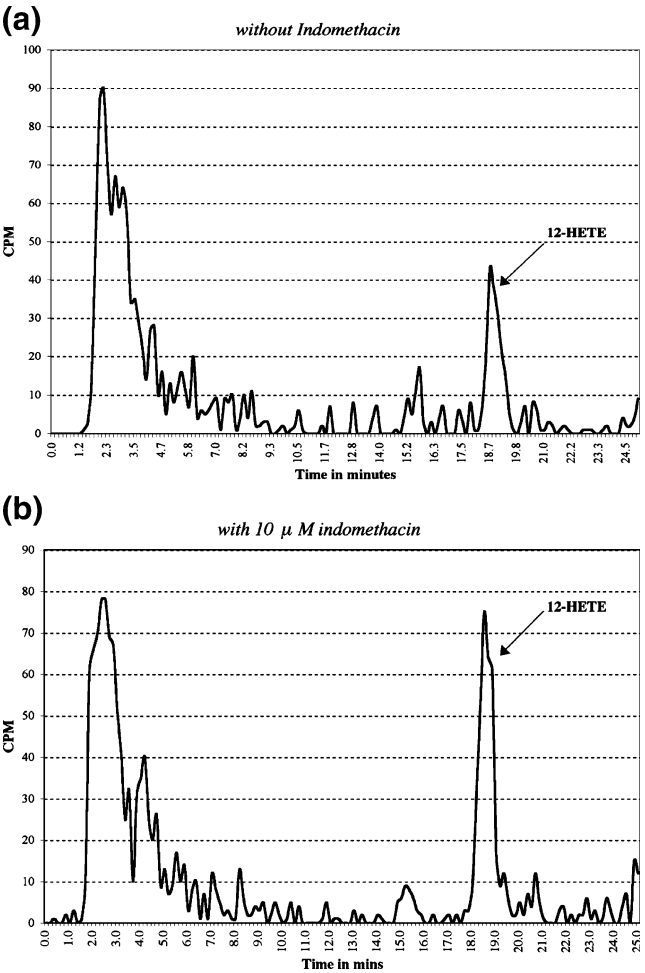

Fig. 3 *Shunting of substrate to alternate pathways triggered by specific inhibitors*. In this experiment exponentially growing 12-LOX stable transfected PC-3 cells were incubated either with vehicle or 10 μM of the COX inhibitor, indomethacin, in the presence of ^{3}H-arachidonic acid. Lipids were extracted using C-18 sep-pak columns and resolved using reverse phase HPLC. Note the increase in 12-HETE production from the 12-LOX stable transfected cells in the presence of indomethacin (**b**), compared to vehicle treated cells (**a**), suggesting the shunting of substrate to alternate enzymes when one arm of the metabolic pathway is inhibited

5 Effect of LOX on tumor cell signaling and proliferation

Deregulation of the fine balances controlling cellular proliferation and cell death is the hallmark of cancer. Many cell pathways are involved in the process by which cells choose between growth arrest, apoptosis or survival. The crosstalk of LOX derived products with different growth factor receptor-induced signaling cascades is involved in the stimulation of tumor cell growth.

The products of lipoxygenase metabolism may exert their biological effects in an intracrine manner, through the activation of transcription factors of the PPAR family, or they may interact with specific trans-membrane G protein-coupled cell surface receptors in an autocrine or paracrine manner [17, 47]. Leukotriene B_4, a downstream metabolite of the 5-LOX pathway, has been shown to regulate colon cancer growth through direct interaction with its receptor, BLT1. Targeting the receptor using either siRNA or a receptor antagonist significantly suppressed LTB_4 stimulated expression of ERK in

colon cancer cells, and high levels of BLT1 were observed in human colon cancer by immunohistochemistry [48]. Another pathway, potentially regulated by 5-LOX in colon cancer is the nuclear factor kappaB (NFκB) pathway, as reports have shown that inhibition of 5-LOX in colon cancer cells significantly inhibited the activation of NFκB and subsequent cell proliferation [49, 50]. The phosphoinositol 3 kinase (PI3 K)/Akt and NF-κB pathways control both proliferation and resistance to apoptosis of many cancer cells, and therefore signaling through these pathways critically regulate proliferation and survival mechanisms. 12(*S*)-HETE has also been shown to mediate the activation of NF-κB by 12-LOX in prostate cancer cells [51]. In this study, 12(*S*)-HETE treatment of PC-3 cells induced the degradation of IκB by the S6 proteasomal pathway and the activated NF-κB was shown to translocate to the nucleus, causing κB-induced transcription. Therefore, 12-LOX expression in prostate cancer may contribute to the observed constitutive activation of NF-κB in these tissues.

Other LOX products are thought to induce gene expression by transactivation of transcription factors of the PPAR family. The 15-LOX products, 15-HETE and 13-HODE, bind and activate PPARγ *in vitro*, and 15-HETE was shown to be an endogenous PPARγ ligand using a transactivation assay in various epithelial tissues expressing 15-LOX-2 [21]. Opposing effects of 13-HODE and 15-HETE on PPARγ transactivation have been reported in prostate cancer cells [52]. 13-HODE increased PPARγ phosphorylation while a subsequent decrease in PPARγ phosphorylation was observed with 15-HETE. Thus, 15-LOX metabolites have opposing effects on the regulation of the MAP kinase signaling pathway and on downstream targets of MAP kinase signaling like PPARγ.

The epidermal growth factor receptor (EGFR) and its downstream signaling pathways, Ras–Raf–MEK–ERK axis, play important roles in cancer development and are also regulated by LOX metabolism. In prostate cancer cells, EGF and neurotensin-induced phosphorylation of EGFR, ERK and AKT were inhibited by a general LOX inhibitor (NDGA), 5-LOX inhibitors (Rev 5901 and AA861) and a 12-LOX inhibitor (baicalein), while the COX-2 inhibitor, indomethacin, had no effect [53]. Therefore the proliferative effects of EGF may involve a feed-forward system requiring cooperative involvement between LOX isoforms and the ERK and AKT survival pathways.

13-HODE and 15-HETE up-regulate or down-regulate, both the MAPK and Akt pathways respectively following activation with IGF-1, demonstrating that pathways other than EGF and MAPK utilize LOX metabolism [52]. The results from this study provide a plausible mechanism explaining the apparent opposing effects of the pro-carcinogenic 15-LOX-1 and 13-HODE, compared to the anti-carcinogenic 15-LOX-2 and 15-HETE in prostate cancer. Supporting this data is the observation that PPARγ activation is associated with anti-carcinogenic effects in murine breast, colon and prostate cancer and PPARγ inactivating mutations are prevalent in human colon tumors [18]. Another study by Yoshinaga et al. demonstrated that over-expression of 15-LOX-1 in colon cancer cell lines resulted in the activation and phosphorylation of ERK, associated with decreased p21(Cip/WAF1) expression and increased proliferation of the cells [54]. Treatment of the cells with NDGA reversed these effects; while addition of the 15-LOX-1 product, 13(*S*)-HODE also decreased p21 and activated ERK in these cells. The ERK pathway has also been implicated in 12(*S*)-HETE induced proliferation of pancreatic cancer cells [55]. In this study, the authors found that intracellular protein tyrosine kinase activation is involved in the mitogenic effects of 12(*S*)-HETE and that both ERK and p38 MAPK, but not JNK/SAPK, were phosphorylated following treatment with 12(*S*)-HETE.

In addition to acting directly in cell survival pathways, there is evidence that products of LOX metabolism may act as intracellular second messengers facilitating the translocation of PKC_{α} to the nuclear and plasma membrane thereby regulating cell proliferation. This has been reported in human corneal epithelial cells, where pretreatment of the cells with 15(*S*)-HETE, followed by stimulation with either EGF or HGF, produced faster translocation of PKC_{α}, while 15(*S*)-HETE alone had no effect [56].

12(*S*)-HETE, the eicosanoid derived from 12-LOX action on arachidonic acid, has been proved to have a strong association with progression of various cancers [57]. Recently, the signaling pathways activated by 12-HETE have been delineated and the existence of putative cell surface receptors has been recognized (Fig. 4) [58, 59]. Studies are in progress to identify the specific receptor(s) for 12(*S*)-HETE, on cancer cells. We were able to identify the presence of 12(*S*)-HETE binding sites on a rat prostate cancer cell line, and competition studies with heteroligands showed that only 13 (*S*)-HODE competed for binding sites with 12(*S*)-HETE, compared to an array of eicosanoids (Fig. 5).

Therefore, extensive research within the last decade has shown that targeted inhibition of LOX isoforms can suppress the proliferation of a variety of tumor cells including prostate, breast, colon, lung and bladder. These effects appear to be accomplished through cell cycle arrest at the G_1/S-phase, induction of cyclin-dependent kinase inhibitors (such as p15, p21 and p27), down-regulation of anti-apoptotic machinery (e.g., Bcl-2 and Bcl-xL), and the inhibition of cell-survival kinases (AKT, PKC and MAPK) and inflammatory transcription factors (NFκB).

6 Regulation of tumor cell death by LOX

Tumor growth does not only depend on increased cell proliferation but also on prolonged cell survival through the

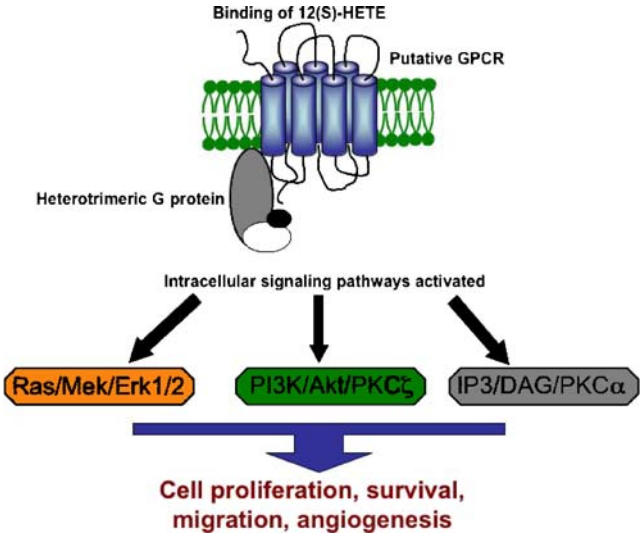

Fig. 4 *Signaling networks activated by 12(S)-HETE*. Putative 12(S)-HETE binding receptors have been identified as G-protein coupled receptors. Activation of these receptors by ligand interaction triggers G-protein linked intracellular signaling pathways ie, the Erk1/2, the PI3K-Akt, and the IP3/DAG pathways, which mediate the functional responses involved in tumor progression. The predominance of each of these pathways depends on the cell type involved. *Modified from* [58] *and* [59]

inhibition of cell death or apoptosis. 5-LOX and 12-LOX inhibition has been shown to induce apoptosis in human breast, colon, gastric, lung and prostate cancer cells [34, 60–63].

The induction of cell death is often preceded by an arrest in the cell cycle. There is substantial evidence that critical regulatory steps occur during the G_1 phase of the cell cycle, which can determine whether or not the cell will synthesize DNA and divide (Fig. 6). In a previous study in prostate cancer cell lines, treatment with the 12-LOX specific inhibitors, baicalein or BHPP (also known as BMD 122), resulted in a significant growth arrest in the G_0/G_1 phase of the cell cycle [63]. The mechanisms associated with this effect were studied in detail. This arrest was found to be associated with a time-dependent decrease in cyclin D1 and cyclin D3 levels, and a reduction of CDK-4 levels in the cells. Cyclin D1 functions upstream of the retinoblastoma (RB) protein, by binding to CDK-4 or -6 facilitating RB phosphorylation. The phosphorylation of RB in mid-to-late G_1 releases transcription factors bound by RB, and results in their subsequent binding to the promoter regions of various genes leading to DNA synthesis [64]. 12-LOX inhibition in prostate cancer cells, blocked the phosphorylation of RB, resulting in the RB protein remaining bound to transcriptional factors required for subsequent DNA synthesis [63]. Perhaps more interesting, is the fact that in this same study, 12-LOX inhibition also resulted in decreased levels of both p107 and p130 (RB family proteins) in RB mutant prostate cancer cells, indicating that 12-LOX inhibition may regulate other pathways that compensate for particular mutations in certain cells. Cell cycle arrest has also been reported following 12-LOX inhibition in lung cancer cells, however an S-phase arrest was observed with decreased levels of CDK-1 and cyclin B1, proteins shown to regulate S-phase transition to the G_2/M phase [62].

In colon cancer cells, induction of 15-lipoxygenase-1 decreased expression of p21 (Cip/WAF 1), an inhibitor of cyclin E/CDK2 complex formation [54]. It is therefore likely that inhibitors of 15-LOX metabolism, may induce a cell cycle arrest in G_1 in colon cancer cells. However, the role of 15-LOX-1 in the cell cycle is not as simple. In another report, conditional induction of 15-LOX-1 resulted in an inhibition of ribonucleotide reductase activity, cell cycle arrest in G_1 and accumulation of the pro-apoptotic protein Bax in a human embryonic kidney (HEK) cell line [65]. Therefore, like other aspects of tumor growth, the role of particular LOX isoforms need to be evaluated independently with regard to origin of the tumor and the particular products produced by LOX metabolism. 15-LOX-1 mediated reduction of p21 in colon cancer cells is mediated by the metabolite 13(*S*)-HODE, while the cell cycle arrest reported in HEK cells may have been as a result of 15(*S*)-HETE production which is more prevalent in normal cells.

In cancer cells, the triggering of apoptosis is due to a disturbance of the balance between pro- and anti-apoptotic proteins promoting a pro-apoptotic signaling pathways, which usually includes cytochrome c release from mitochondria and the activation of the caspase cascade.

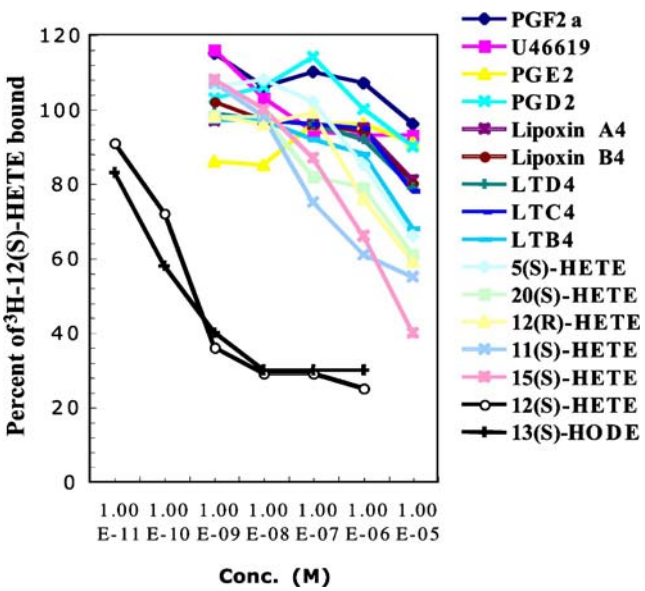

Fig. 5 *Competition binding studies—displacement of [3H]12(S)-HETE binding to AT2.1 cells*. Cell monolayers grown in 24-well plate were incubated for 120 min with 0.1 nM [^{3}H]12(S)-HETE in the absence and presence of either 1 fM—1 μM of unlabeled 12(S)-HETE or 1 nM—10 μM of the indicated eicosanoids. Competition for 12(S)-HETE binding (–o–) was seen only with 13(S)-HODE (–x–)

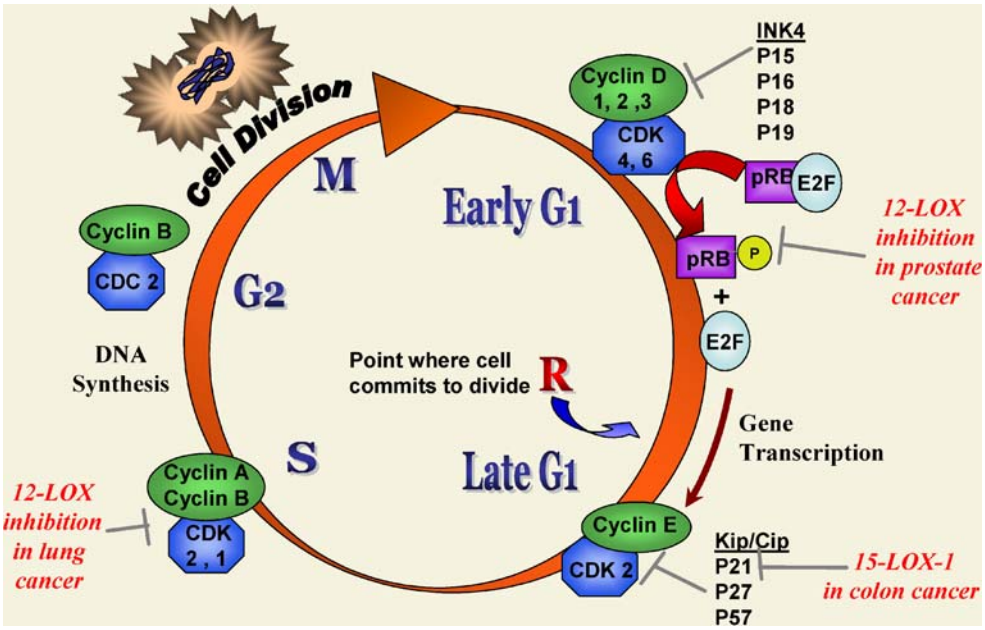

Fig. 6 LOX metabolism regulates many aspects of the cell cycle, from an early G1 progression past the restriction point (*R*) to later stages preceding DNA synthesis. The overwhelming evidence would suggest that LOX and their metabolites promote cell cycle progression and that their inhibition can induce specific cell cycle arrest in various cancer cells, followed usually by the induction of apoptosis

In human breast cancer cells, specific inhibition of both 5-LOX and 12-LOX resulted in significant apoptosis of cells, associated with induced cytochrome c release, activation of many caspase family members and resultant PARP cleavage [60]. This inhibition, observed in both ER negative and positive breast cancer cells, was also associated with a reduction in the levels of the anti-apoptotic protein Bcl-2 and Mcl-1 and elevation of the pro-apoptotic protein Bax. In another study in prostate cancer, 12-LOX inhibition resulted in down-regulation of Bcl-2 and Bcl-X_L, coupled to an up-regulation of Bax [63]. Therefore, it is clear that 12-LOX inhibitors alter the expression of Bcl-family protein members, resulting in a shift in their ratios favoring apoptosis, suggesting the broad applicability of these inhibitors to cancer treatment. We have recently examined the effect of the 12-LOX inhibitor, baicalein, on tumor cell apoptosis in a range of NSCLC lines (Fig. 7). Apoptosis was induced following treatment of the A549 adenocarcinoma cell line with 10 μM baicalein, indicated by approximately 40% of cells staining positive for Annexin V. The squamous lung cancer cells, SKMES-1, were even more sensitive to 12-LOX inhibition, with 28% apoptosis after 72 h with the lower concentration of 1 μM baicalein. In another NSCLC line, H460, that were also sensitive to baicalein-induced apoptosis, pretreatment of the cells with a specific caspase-3 inhibitor partially reduced cell death, suggesting that baicalein-induced apoptosis is partially dependent on caspase-3 [62]. These observations are supported by results in prostate cancer cells, where the induction of apoptosis associated with 12-LOX inhibition was found to be related to activation of activated caspase-3/7 [63]. In this study, it was also observed that levels of phosphorylated Akt-1 were decreased following 12-LOX inhibition. Akt activation has previously been shown to induce survival and suppress apoptosis through increased phosphorylation of Bad and subsequent liberation of anti-apoptotic proteins of the Bal family [66]. Interestingly, Akt has also been shown to enhance the translation of cyclin D1 [67], and previous reports have shown that treatment with antisepses cyclin D1 resulted in the strong induction of apoptosis in human squamous carcinoma [68], linking the Akt pathway to both the cell cycle arrest and apoptotic response observed, at least in prostate cancer cells. Also, cells exhibiting constitutively active 5-LOX show an impaired apoptotic response to geotaxis anticancer agents and ionizing radiation. In addition, geotaxis stress, induced by DNA damaging agents, was shown to increase 5-LOX expression and activity which suppressed p53-induced apoptotic pathways in human embryo fibroblasts [69]. Interestingly, we have also shown that combined treatment of a range of lung cancer cell lines with conventional chemotherapeutic agents and baicalein resulted in a synergistic inhibitory effect on tumor cell growth and induction of apoptosis [unpublished observations]. In addition to the reported observations of the effects of LOX inhibition on cell cycle arrest and apoptosis induction with associated ultra structural changes and DNA laddering, the inhibition of 5-LOX with the inhibitor MK886 induced cell death more consistent with an autophagic form of programmed cell death in lung cancer [70]. In either case, there is a very strong body of evidence supporting the induction of cell death by inhibitors of LOX metabolism, and it would highlight the potential of these inhibitors to be considered as novel therapies for the treatment of many cancers.

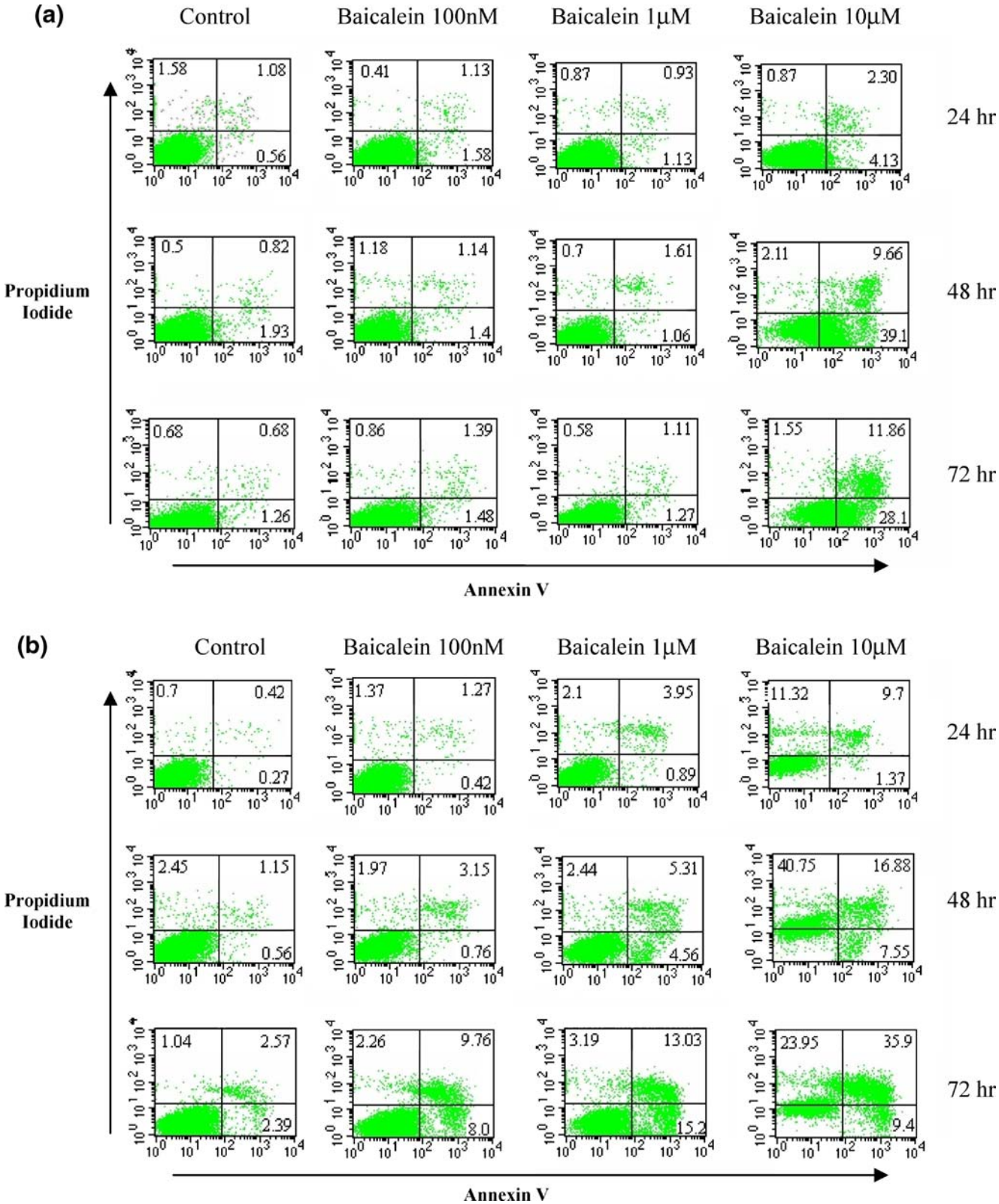

Fig. 7 The effect of selective inhibition of platelet type 12-LOX in NSCLC cell lines. (**a**) Treatment of A549 lung adenocarcinoma cells with baicalein at 10 μM resulted in 40% apoptosis after 48 h. (**b**) SKMES-1 squamous lung cancer cells were even more sensitive to 12-LOX inhibition, with 1 μM of baicalein inducing 28% apoptosis at 72 h, while 10 μM resulted in substantial cell death as early as 24 h post treatment

7 Effect of LOX on tumor angiogenesis

The induction of neovascularization and angiogenesis are prerequisites for tumor expansion beyond a limiting size of 2–3 mm^3 [71]. Many cells within the tumor microenvironment, including cancer cells, endothelial cells, stromal cells and inflammatory cells produce pro-angiogenic factors that induce endothelial cell recruitment, proliferation, migration and tubule formation. Angiogenesis is an intricate stepwise process, mediated by the interplay of a variety of factors which include tumor and endothelial cells, the balance between pro-angiogenic factors e.g., vascular endothelial growth factor (VEGF) and anti-angiogenic factors, proteolytic enzymes (MMPs), cell surface molecules (integrins) and many other factors [72]. Pro-angiogenic gene expression is activated by a multitude of factors, which include physiological stimuli such as hypoxia, oncogene activation, and tumor suppressor mutations [73]. VEGF is the most potent tumor angiogenic factor identified to date. Agents that target VEGF and its signaling pathways inhibit tumor growth and propagation in various experimental models [74]. The promoter region of the *VEGF* gene has binding sites for transcription factors like Sp1/Sp3, AP-2, Egr-1, STAT-3, and HIF-1 [75], with HIF-1 regulating transcriptional activation under hypoxic conditions.

High-fat, n-6 fatty acid-rich diets have been shown to be associated with a relatively poor prognosis in breast cancer patients; and in a nude mouse model the same diet enhanced breast cancer progression, while n-3 fatty acids exerted suppressive effects that were associated with impaired angiogenesis [76]. Products of n-6 fatty acid metabolism, by both the lipoxygenase and cyclooxygenase pathways, have been shown to be angiogenic in many systems. 12-LOX has been demonstrated to have a pronounced proangiogenic effect in prostate cancer. It was reported that prostate tumors in mice, derived from 12-LOX overexpressing PC-3 prostate cancer cells, were highly angiogenic. These tumors showed a remarkable increase in angiogenesis as evidenced by a high microvascular density [77]. Studies with matrigel implants in mice, generated by injection of matrigel plugs mixed with tumor cells clearly showed that there was a denser distribution of blood vessels in implants derived from PC-3 cells stably transfected with 12-LOX (Fig. 8) [77].

Studies indicate that 12(*S*)-HETE has direct stimulatory effects on multiple processes associated with angiogenesis. For example, 12(*S*)-HETE has been shown to be a mitogenic factor for microvascular endothelial cells [78] and stimulate endothelial cell migration [79]. It has been shown to up-regulate the surface expression of integrin $\alpha v\beta 3$, an integrin predominantly associated with angiogenic blood vessels in tumors and human wound granulation tissue, in both rat and murine endothelial cells [80]. In another study, 12(*S*)-HETE has also been shown to induce a reversible, nondestructive, time- and dose dependent retraction of endothelial cells by stimulating cytoskeletal rearrangement [81] and tumor cells can synthesize 12(*S*)-HETE in sufficient amounts to induce microvascular endothelial cell retraction [82]. Lewis lung carcinoma cell-induced endothelial cell retraction was blocked by a specific lipoxygenase inhibitor BHPP, but not by cyclooxygenase inhibitors.

The potential of 12(*S*)-HETE as a significant stimulator of pathological angiogenesis may lie in its ability to induce the expression of VEGF expression at both protein and promoter levels. In prostate cancer cells, this has been reported by two independent groups [38, 83]. Both NDGA and baicalein reduced VEGF expression, confirming that the enzymatic activity of 12-LOX plays a significant role in the regulation of *VEGF* gene expression. In the study by Nie et al., transient transfection with a 12-LOX expression construct enhanced VEGF promoter activity in the prostate cancer cell line, PC-3 [38]. Additionally the same study reported a potential mechanism for this phenomenon, with 12(*S*)-HETE capable of inducing signaling via the extracellular-signal related kinase1/2 (ERK1/2) mitogen-activated protein (MAP) kinase pathway in prostate cancer cell lines.

In another report by Ye et al., cigarette SMOKE extract was shown to enhance cell proliferation and the expression of 5-lipoxygenase (5-LOX), vascular endothelium growth factor (VEGF), matrix metalloproteinases (MMPs) 2 and 9 in colon cancer cells [39]. Notably, inhibition of 5-LOX decreased cell proliferation and expressions of VEGF, MMP-2 and MMP-9 induced by cigarette smoke extract. In addition, cigarette smoke extract indirectly stimulated HUVEC proliferation, an effect that was again blocked by the 5-LOX inhibitor. Furthering these observations, the same group also reported that inhibition of COX-2 or 5-LOX in the nude mouse xenograft model reduced colon tumor size induced by cigarette smoke. They further found that exposure to the mainstream smoke of unfiltered cigarettes enhanced the 5-LOX protein expression in the inflammation-associated colonic adenomas [50].

Using transgenic mice that over-express 15-LOX-1 in endothelial cells under the regulation of the murine preproendothelin-1 promoter, Harats et al. studied the effect on tumor and the growth of metastasis [84]. They reported that 15-LOX-1 inhibited tumor and metastasis growth in the transgenic mice in two different models of cancer (mammary gland and Lewis lung carcinoma). This inhibition was concomitant with a higher number of apoptotic cells in the metastases of the transgenic mice and with a complicated network of multiple small blood vessels, implicating 15-LOX-1 to have potential anti-angiogenic properties.

Treatment for cancer is now moving beyond traditional chemotherapy with the advent of specific targeted therapies, and much research effort is focused on developing treatments based on the inhibition of tumor angiogenesis. Neovascula-

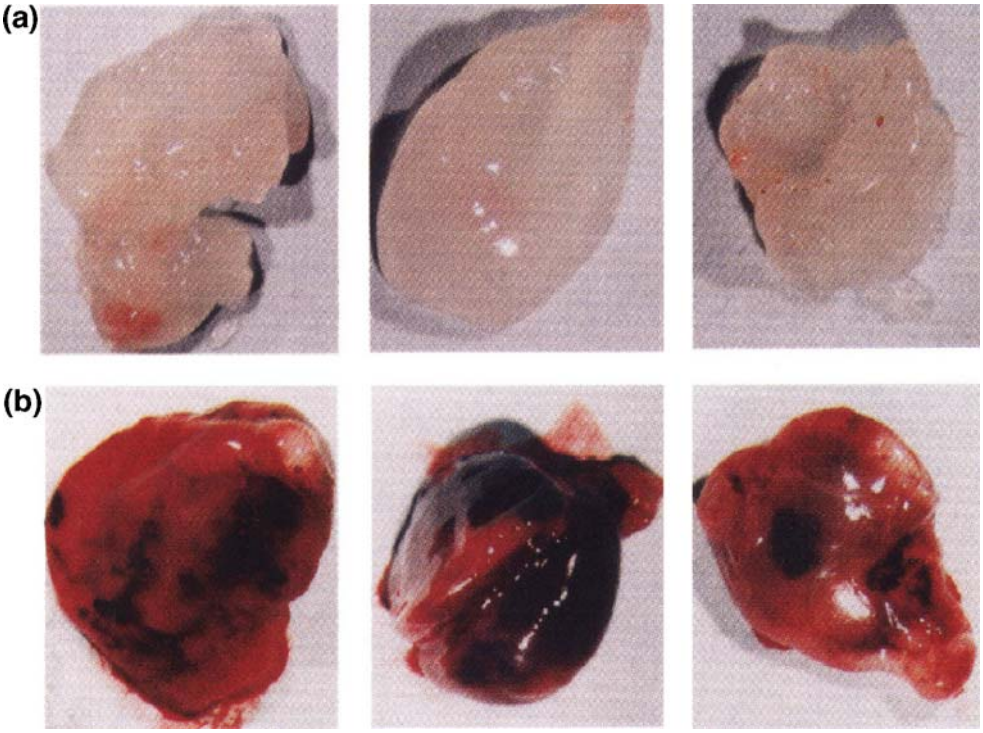

Fig. 8 *Matrigel assay demonstrating the ability of 12-LOX to stimulate angiogenesis*. In this assay, 12-LOX overexpressing or vector transfected PC-3 cells were mixed with matrigel and implanted SC in mice. Shown here are the matrigel implants harvested from these mice. Note the extent of angiogenesis in implants generated from 12-LOX transfected PC-3 cells (**b**) compared to vector transfected controls (**a**). Reproduced with permission from Cancer Research [77]

rization not only permits further growth of the primary tumor, but also provides a pathway for migrating tumor cells to enter the systemic circulation and establish distant metastases. Given its pivotal role in growth and survival, the tumor vasculature is an attractive target for anticancer therapy and targeting LOX metabolism may have potential as inhibitors of tumor angiogenesis.

8 Regulation of adhesion, migration and invasion by LOX

The ability of tumors to invade beyond hemostatic boundaries and form metastatic colonies requires the complex interplay of various cell surface-associated components regulating the proteolytic disruption of the ECM and the modification of cell adhesion properties. These cell-ECM interactions are mediated by a family of adhesive receptors, known as integrins, which mediate the attachment of cells to both structural and matrix-mobilized proteins to promote cell survival, proliferation and migration [85, 86]. Integrins perform a well-documented function in cellular invasion and metastasis [87, 88]. Non-ligated integrins are generally spread diffusely over the cell surface with no apparent linkage to the actin cytoskeleton. However, when ligated, integrins frequently cluster into specialized structures called focal adhesion complexes, thereby providing a convergence site for multiple signaling components [89, 90], while physically linking the receptors to actin microfilaments. Ligand binding to integrins triggers a number of signaling pathways, some of which are primarily related to cell adhesion, whereas others provide survival signals to cells. For example, prevention of cell adhesion to the ECM will trigger apoptosis in various cells in particular, epithelial cells [91–93], suggesting that integrin-mediated attachment relays important survival signals to the cells. Conversely, attachment or adhesion to the basement membrane or individual ECM components has been shown to promote cell differentiation and extend cell survival under various experimental conditions [94, 95].

The regulation of cell–cell and cell–matrix adhesion depends largely on the cellular profile of adhesion molecules and matrix metalloproteases and on the signaling mechanisms that regulate adhesive behavior and cellular responses to environmental stimuli. Thus, elucidating the effects of environmental components, such as fatty acids, on tumor cell adhesion is important for the understanding of metastasis. Previous studies have indicated a role for AA metabolism in cell–matrix interactions and integrin signaling. For example, inhibition of cyclooxgenase-2 by non-steroidal anti-inflammatory drugs blocks both platelet aggregation (by suppressing activation of integrin αIIbβ3) [96] and endothelial cell migration (by suppressing activation of αvβ3 integrin) [97]. Other studies have reported the role of LOXs, in particular, 12-LOX, in the regulation of surface integrin expression. For example, adhesion of B16 murine melanoma cells to microvascular endothelial cells was enhanced by pretreatment of the endothelial cells with the 12-LOX product, 12(*S*)-HETE, via up-regulation of αvβ3 integrin expression [80]. In the same cell line, ligation of αIIbβ3 integrin induced 12 (*S*)-HETE production [98], implying co regulation of in-

tegrin expression and LOX activity in these cells. Similarly, 12(*S*)-HETE treatment of human endothelial cells enhanced monocyte adhesion through increased very late-acting antigen-4 integrin expression [99]. In addition, the interaction of 12-LOX with a number of cellular proteins, including the integrin β_4 subunit, has previously been reported by yeast two-hybrid screening [100].

In a study by Pidgeon et al., over-expression of 12-LOX increased membrane expression of $\alpha v\beta 3$ and $\alpha v\beta 5$ in both prostate and epidermoid cancer cell lines, relative to wild-type or neo-transfected cells [101]. This increased expression at the cell surface was reported to mediate tighter cell–matrix adhesions and contribute to a more 'spread' morphology *in vitro*. The authors also reported, a definite cause and effect relationship between the increased surface integrin expression and survival as 12-LOX-transfected prostate cells demonstrating significantly reduced survival, when serum-starved in the presence of a monoclonal antibody to $\alpha v\beta 3$ or $\alpha v\beta 5$. The fact that over-expression of platelet-type 12-LOX in two tumor cell lines of different histological origin resulted in enhanced expression of the same integrin, implies a general phenomenon of 12-LOX regulating a specific subset of integrins. Integrins $\alpha v\beta 3$ and $\alpha v\beta 5$ have been shown to regulate cell migration; however, unlike $\alpha v\beta 3$, $\alpha v\beta 5$-mediated cell spreading and migration require activation of protein kinase C [102]. The stable end product of platelet-type 12-LOX metabolism, 12(*S*)-HETE, is a well-established protein kinase C activator [103] and more importantly, has been shown to increase the surface expression of $\alpha v\beta 3$ integrin on CD clone 3 endothelial cells [80].

The regulation of adhesion by LOX metabolism is not limited to prostate cancer. In breast cancer, exogenous arachidonic acid rapidly stimulates an increase in the adhesion of MDA-MB-435 cells to the prevalent basement membrane protein, type IV collagen, *in vitro* [104]. The same group reported that the inhibition of LOX activity blocks the ability of arachidonic acid to promote cell adhesion. They suggested that arachidonic acid or its metabolites could affect metastasis through alteration of the adhesive properties of tumor cells and that arachidonic acid activates both p38 MAPK and protein kinase C isozymes, mediating this enhanced cell adhesion [105, 106]. More recently, the intracellular signaling mechanisms mediating these effects in breast cancer cells have been elucidated. Overexpression of 15-LOX-2 sensitized breast cancer cells to AA addition, resulting in increased activation of p38 MAPK [107]. They reported that addition of exogenous 15(*S*)-HETE to MDA-MB-435 cells stimulated cell adhesion to type IV collagen and activated the p38 MAPK pathway, including the upstream kinases transforming growth factor-β_1-activated protein kinase-1 (TAK1) and MAP kinase 6. These reports are unusual, given the postulated 'anti-carcinogenic' role for 15-LOX-2 in cancer. However, the data nevertheless clearly demonstrates that 15(*S*)-LOX-2 generation of 15(*S*)-HETE activates specific growth factor receptor-related signaling pathways in breast cancer, thereby initiating signal transduction events leading to increased cell adhesion to the extracellular matrix.

There is less evidence for the role of 5-LOX in cell adhesion, although the generation of MMP2 is reported to be mediated by activation of phospholipase A_2 and 5-LOX activity in breast cancer cells [108]. The authors reported that breast cancer cells in culture release apparently full length soluble EMMPRIN that promoted the release of pro-MMP2 from fibroblasts. There is also evidence that 5-LOX and acetylated COX-2-derived eicosanoids regulate leukocyte–endothelial adherence in response to aspirin [109]. This mechanism itself could be potentially related to cancer biology as increasingly more aspects regulating normal physiological properties in cells are being adapted by cancer cells. On this note, stem cell mimicry of cancer cells has been reported, which is implicated in epithelial–mesenchymal transition and in the phenomenon of vascular mimicry. Certain cancer types acquire a geno–phenotype closely resembling platelets and express several megakaryocytic genes (adhesion receptors αIIbβ3, thrombin receptor and PECAM/CD31 and/or platelet-type 12-LOX) capable of activating the coagulation cascade or platelets themselves [110]. Platelet mimicry of cancer cells is typical of pancreatic, breast, prostate, colorectal and urogenital cancers and melanoma; and is reported important factor in their hematogenous dissemination and provides an attractive therapeutic target [110].

There is significant evidence for a role of LOXs, particularly 12-LOX in tumor invasiveness. In a study by Nie et al., 12-LOX transfected prostate cancer cells were reported to be more adhesive toward vitronectin and type I and IV collagen, and were more invasive through matrigel than cells transfected with the control vector [111]. In the same report, when the cells were subcutaneously grown in nude mice, invasion to the surrounding tissue was more frequently observed in the 12-LOX transfected cells. Similarly, when injected by tail vein into SCID mice with human bone fragment implants, increased metastasis to bone was observed in the cells over-expressing 12-LOX. Overall it would appear that the inhibition of LOX metabolism has wider implications than those targeting the primary tumor, and may be a potential therapeutic approach in the treatment of metastatic disease, particularly in the case of 12-LOX and prostate cancer.

9 5, 12, and 15-LOXs: a comparison on prostate cancer progression

Several researchers have worked on evaluating the role of each of the LOXs in prostate cancer progression. Previously

we have reported that inhibition of 12-LOX led to a drastic reduction in cell proliferation and promoted apoptosis in prostate cancer cells compared to inhibition of other LOXs [63]. Recent studies conducted in Dr. Kenneth Honn's laboratory have illustrated the contribution of the LOX isoforms in promoting prostate cancer progression (Figs. 9, 10 and 11). PC-3 and Du145 prostate cancer cells were stable transfected with each of the LOXs as fusion proteins with GFP. Tumors were established SC in 3–4 week old nude mice. Size and volume measurements revealed that tumors from Du145 cells overexpressing 5-LOX or15-LOX-1 (Figs. 9 and 10) were smaller compared to the vector control suggesting that these isoforms may inhibit tumor growth.

Similarly PC-3 cells overexpressing 5-LOX generated smaller tumors compared to vector control (Fig. 9). However 15-LOX-1 overexpressing PC-3 cells formed larger tumors [Fig. 10 (b)]. Conversely, 12-LOX was found to increase tumor growth and produced SC tumors larger than that of the control in both Du145 (Fig. 11) and PC-3 cells [77]. This suggests that among the three isoforms, 12-LOX may play a predominant role in driving prostate tumor progression. Clinical correlation to this observation was reported by Nithipatikom et al. who found that the levels of 12-HETE in urine samples from prostate cancer patients were higher than that of 5 and 15-HETEs. Radical prostatectomy in these patients led to a drastic drop in urinary levels of 12-HETE, while that of other HETEs remained unchanged [35].

10 Modulation of the immune system by LOX

Chronic inflammation results in the accumulation of inflammatory cells, with associated growth factors, cytokines and pro-inflammatory lipid mediators which may result in the activation of stromal compartments and the proliferation of epithelial cells, potentially resulting in tumor formation. One of the hallmarks of an inflammatory process is the production of inflammatory cytokines, including prostaglandins, leukotrienes and the HETEs. COX-2 and PGE_2 are by far the most studied components of the arachidonic acid to eicosanoid pathway in inflammation and immune modulation. COX-2 and its prostanoid product PGE_2 exert a wide range of pro- and anti-inflammatory effects on innate and adaptive immune

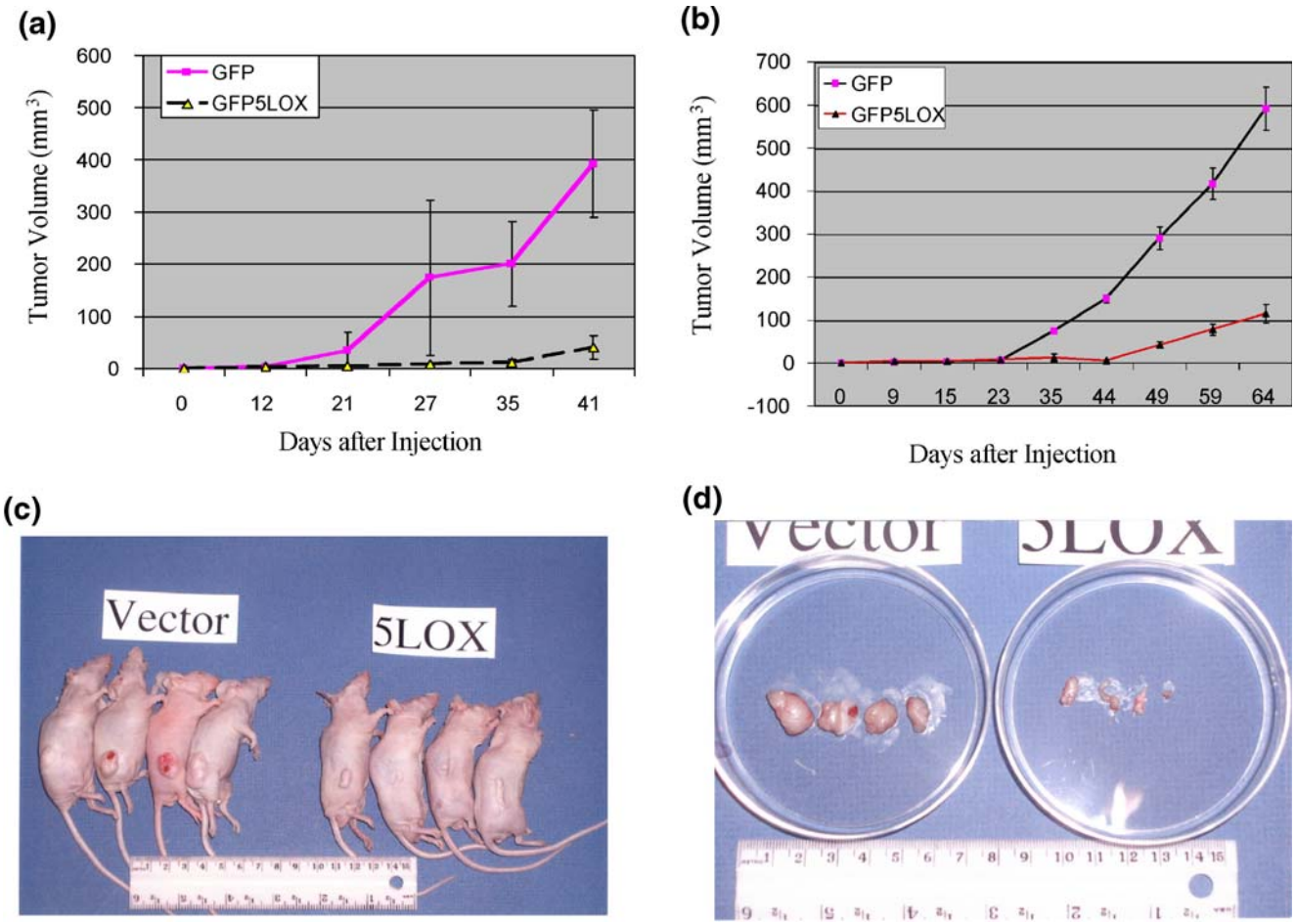

Fig. 9 Comparison of growth and volume for prostate tumors generated from SC injection of GFP tagged 5-LOX overexpressing Du145 cells (**a**, **c**, and **d**) and PC-3 cells (**b**)

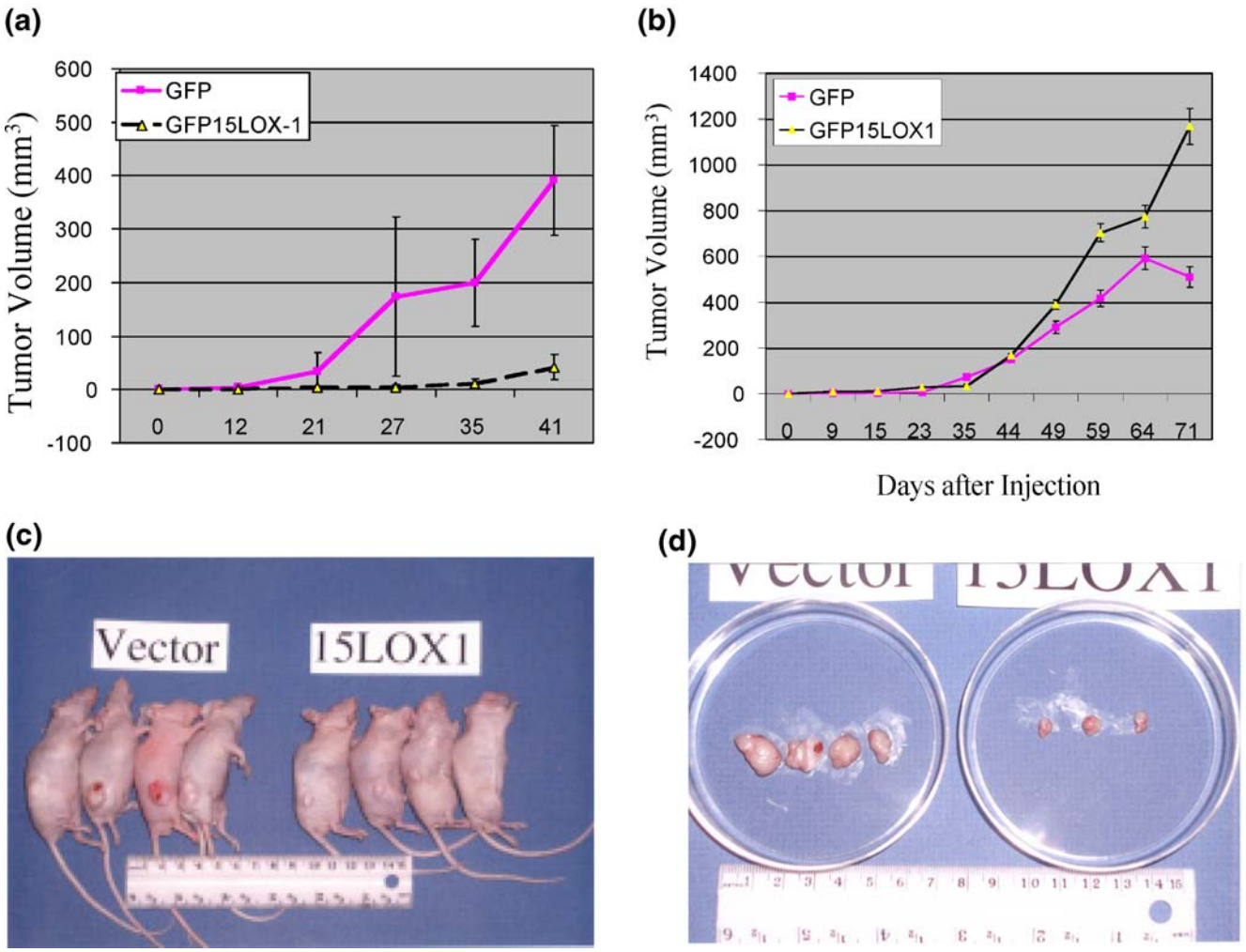

Fig. 10 Comparison of growth and volume for prostate tumors generated from SC injection of GFP tagged 15-LOX-1 overexpressing Du145 cells (**a**, **c**, and **d**) and PC-3 cells (**b**)

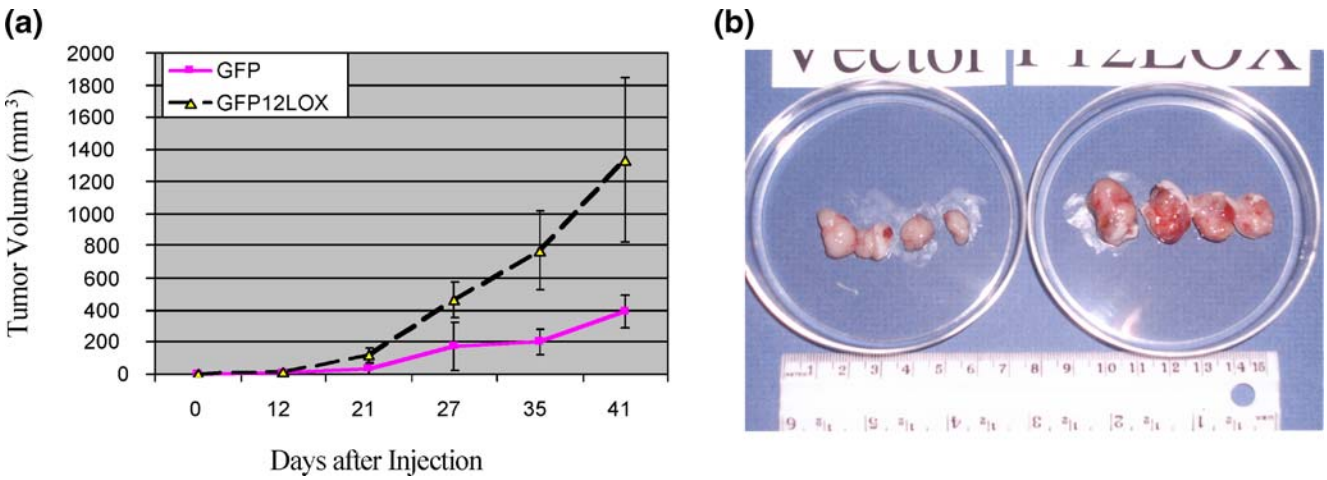

Fig. 11 Comparison of growth and volume for prostate tumors generated from SC injection of GFP tagged 12 LOX overexpressing Du145 cells (**a** and **b**)

cells. Dendritic cells (DC) are key regulators of immune responses and act as professional antigen presenting cells (APC) in linking the innate and adaptive immune responses to pathogens and tumors. The activation status of DC will direct the type of adaptive response and hence will determine how effective the immune system will be in clearing pathogens or eliminating tumor cells. A number of studies have examined the effect of PGE_2 on different subsets of DC, and found that treatment of bone marrow derived dendritic cells (BMDC) with exogenous PGE_2 induces the production of large amounts of the anti-inflammatory cytokine interleukin (IL)-10 and less IL-12p70, a potent bio-active inducer of inflammatory T helper (Th) 1 type immune responses. In addition, selective inhibition of COX-2, but not COX-1, was followed by significant decreases in PGE_2 and IL-10, restoration of IL-12p70 production and an enhancement of DC stimulatory ability [112, 113]. Paradoxically, while inhibiting IL-12p70 production by DC, PGE_2 and its receptors EP2 and EP4 have been found to be essential during the early stages of DC maturation and migration [114].

PGE_2 production also results in enhanced secretion of IL-10 by other immune cell subsets including macrophages and lymphocytes [115]. Further evidence that PGE_2 can enhance the immunosuppressive environment at a tumor site came from studies showing that PGE_2 can be over-expressed by tumors themselves or its production can be induced in cells in the tumor microenvironment, which can lead to the inhibition of endogenous IL-12p70 production at both the transcription and protein level. This inhibition is reversible by addition of a COX-2 inhibitor [116]. In addition to the effects PGE_2 has on IL-10 and IL-12 production, it has also been shown to inhibit production of the pro-inflammatory cytokine interferon-γ (IFN-γ) by T lymphocytes while enhancing the release of T helper type 2 cytokines, including IL-4 and IL-5 [117].

A novel mechanism of COX-2-induced immunosuppression is through the regulation of Indoleamine 2,3-di-oxygenase (IDO) expression. IDO catalyzes the conversion of L-tryptophan to L-kynurenine and has recently been established as a major player in the induction of immune tolerance to tumors and T cell suppression, as T lymphocytes are very sensitive to the loss of this amino acid [118, 119]. These studies revealed that COX-2 inhibitors attenuated IDO expression and this allowed for effective generation of tumor specific effecter $CD8^+$ cytotoxic T lymphocytes [120].

One of the naturally existing hurdles that cancer immunotherapies have to overcome is the existence and effecter functions of regulatory T cells. The main role of these T cells is to prevent autoimmune responses, which in its essence will interfere with an immune response to a self-originated tumor. While CD25 (IL-2Rα) has been used in the past to identify regulatory T cells, it is an activation marker and therefore not specific to regulatory T cells, although this population of cells are contained within the $CD25^+$ population. One specific marker for regulatory T cells is the transcription factor, forkhead box protein 3 (Foxp3) [121]. PGE_2 has been found to enhance the inhibitory capacity of *in vitro* purified human $CD4^+CD25^+$ regulatory T cells associated with a significant induction of Foxp3 expression. PGE_2 has also been shown to induce a suppressive regulatory phenotype in $CD4^+CD25^-$ T cells and the PGE_2-dependent acquisition of regulatory T cell function was correlated with induction of Foxp3 gene and protein expression [122].

Lesser studied than COX-2 and the prostaglandins are the lipoxygenases, and their role in the immune response to tumors and inflammation. However, the important role of 5-LOX in modulating inflammation is becoming more apparent and many studies have revealed many converging functions between 5-LOX and COX-2. These include roles in neoangiogenesis, cell proliferation and anti-apoptotic functions but importantly, also in inflammation [123]. Prostaglandins enhance leukotriene-mediated inflammation [124] and leukotrienes, in particular leukotriene B_4, are the major arachidonic acid derivatives produced when COX-2 expression is inhibited [125]. NF-κB, an important transcription factor involved in the generation of a wide range of inflammatory mediators, has also been implicated in the cross-talk between COX-2 and 5-LOX in the development of carcinogen induced colon cancer cell development [49]. As both COX-2 and 5-LOX are up regulated in a wide range of inflammatory and neoplastic diseases, inhibitors against these eicosanoids have proved beneficial in resolving inflammatory disorders and are now being examined for their anti-cancer properties [123].

Various inflammatory inducing immune cells including leukocytes, eosinophils, macrophages and mast cells produce leukotrienes from either endogenous or exogenous arachidonic acid in the presence of certain stimulants. Leukotriene B_4 (LTB_4) is derived by 5-lipoxygenation of arachidonic acid in activated innate immune cells at inflammatory sites [126]. LTB_4 is produced via enzymatically catalyzed reactions and can therefore be produced much more rapidly than other peptide chemokines and cytokines, which require a lengthier process of transcription and translation [127]. LTB_4 is a potent chemoattractant for myeloid and effecter T cells and potently induces their firm adhesion to endothelial cells [128]. LTB_4 is expressed on a minor population of $CD4^+$, $CD8^+$, Natural Killer T (NKT) cells and $\gamma\delta$ T cells in healthy individuals and this leukotriene mediates its activity via 2 G protein-coupled 7 transmembrane spanning receptors BLT1 and BLT2. BLT1 is a high affinity receptor and is preferentially expressed on peripheral leukocytes, antigen experienced pre-terminally differentiated lymphocytes and lymphocytes with a central memory phenotype [128]. BLT1 is also expressed on effecter $CD4^+$ T cells as they migrate out of the lymph nodes and are recruited into peripheral tissues

in response to chemoattractant proteins, including LTB_4. Other studies have demonstrated that BLT1 mediated the chemotaxis of T helper type 1 and 2 cells in response to LTB_4 and also induced the firm adhesion of these lymphocytes to endothelial cells under flow conditions [127]. BLT1 is also expressed by $CD8^+$ T cells in peripheral blood and these cells were found to secrete significantly more IFN-γ than BLT^-CD8^+ T cells, however they did not possess enhanced cytotoxic capabilities [128]. This is consistent with the ability of LTB_4 and other leukotrienes to enhance inflammatory responses but this inflammation may not accompany an effective anti-tumor immune response. In a model of pulmonary allergic inflammation, BLT1-LTB_4 mediated the early recruitment of $CD4^+$ and $CD8^+$ T cells, indicating an important role of this 5-LOX product in linking early immune system activation and effecter T cell recruitment to an inflammatory site [129, 130]. The LTB_4 receptor antagonist has been shown to inhibit T cell proliferation induced by Concavalin A, immobilized anti-CD3 monoclonal antibody, or IL-2. This inhibitory effect was abolished by addition of LTB_4 receptor agonist. The LTB_4 receptor antagonist inhibited IL-2, IFN-γ, and IL-4 production by anti-CD3-stimulated T cells and also inhibited IL-12-induced IFN-γ production [131]. LTB_4 has also been shown to enhance activation, proliferation and differentiation of human B cells [132] and also enhances human NK cell cytotoxicity by priming NK cells for increased target cell binding [133]. These data suggest that LTB_4 is capable of enhancing and recruiting a wide range of potential anti-tumor effecter immune cells to target tissues.

There is limited evidence to support the role of lipoxygenases in the development or function of regulatory T cells or myeloid derived suppressor cells but since a novel role for COX-2 in the differentiation, development and function of antigen specific regulatory T cells is emerging, it is feasible to think that maybe certain lipoxygenases products could also play a role in the development of these cells subsets. One such report has shown that $CD4^-CD8^-$ double negative thymocytes cultured with LTB_4 and IL-2 led to the generation of $CD8^+$ suppressor thymocytes and that these suppressive T cells are involved in tolerance to self antigens, which could be extended to include tumor antigens [134]. Another study examined the effect of LBT_4 treated thymocytes in a graft-versus-host disease (GVHD) model and reported that LTB_4 could influence the behavior of immature thymocytes and in the setting of allostimulation, as is in the case of GVHD, it induces a state of tolerance [135]. These studies support the theory that certain lipoxygenase products may play a role in preventing effecter anti-tumor immune responses while actively enhancing immune tolerance to tumors.

Other members of the leukotriene family appear to be important in the efficient functioning of certain immune cell subsets. These include the cysteinyl leukotrienes (cysLTs), LTC_4, LTD_4 and LTE_4, regulated by the action of 5-LOX [136]. One key function of LTC_4 is to render DC chemotactically responsive to the chemokine CCL19, an important chemokine involved in directing mature DC into secondary lymphoid organs where they can prime naïve T cells [136]. Leukotriene D_4 enhances IgE and total IgG production in CD40-activated human B lymphocytes [137] and it is also believed that LTD_4 is involved in the recruitment of activated γδ and αβ T cells to the site of inflammation by inducing a Ca^{2+} flux [138].

Another important class of lipoxygenases products are the HETEs and their role in inflammation have been well documented. A product of 12-LOX, 12(*S*)-HETE results in the induction of two potent pro-inflammatory cytokines, IL-6 and tumor necrosis factor (TNF)-α at both the mRNA and protein level, when cultured with peritoneal macrophages in a dose-dependent manner [139]. This study revealed an important role for protein kinase C, p38 MAPK, *c*-jun NH (2)-terminal kinase as well as nicotinamide adenine dinucleotide phosphate (NADPH) oxidase in the 12-(*S*)-HETE induced increase in IL-6 and TNF-α gene expression. Induction of 15-LOX was shown to induce the suppression of DC function through lipoxin A4, a product of 5-LOX and 15-LOX-1. Lipoxin A4 can reduce the ability of splenic DC to migrate out of the spleen and produce IL-12, an inflammatory cytokine essential for the priming of T helper type 1 cells [140]. HETEs can also modulate the activity of the anti-inflammatory peroxisomal proliferator-activating receptor-γ (PPAR-γ), however, both activation and inhibition can be observed depending on LOX product concentration [141]. PPARs are a family of ligand-dependent nuclear receptors that play a central role in dampening inflammation via their inhibitory activity on expression of the pro-inflammatory transcription factor, NF-κB [142]. Another 15-LOX product, monocyte-derived 13-HODE has the ability to suppress IL-2 production by human T cells in co-culture via PPAR-γ, this could have implications in the ability of these T cells to expand in response to antigen stimulation and to help mount an effective immune response against a tumor [143].

Overall, bioactive lipids play a multifaceted role in the immune response to tumors, the nature of this role depends largely on the eicosanoid itself, whether it has anti- or pro-inflammatory properties and whether they can effectively recruit and activate the cells of the innate and adaptive immune system in an appropriate manner to be effective in detecting and eliminating the tumor cells.

11 Targeting the lipoxygenase pathways using pharmacological and natural agents

Pharmacologic intervention studies with lipoxygenase inhibitors for the prevention or inhibition of cancers are difficult to

interpret, as there are very few isoform-selective LOX inhibitors available. Selective 5-LOX inhibition with zileuton significantly inhibited esophageal carcinogenesis in rats [25] and chemically induced lung cancer in mice [144].

The dual inhibition of 5-LOX and COX-2 has been shown to suppress colon cancer formation in xenograft mouse models induced by cigarette smoke [39, 50, 145]. In these studies, inhibition of COX-2 resulted in a shunt of arachidonic acid metabolism towards the leukotriene pathway during the tumorigenesis process, which did not occur following suppression of 5-LOX. The authors concluded that blocking both the COX-2 and 5-LOX pathways in cigarette smokers may present a superior anticancer strategy, at least in the context of colon carcinogenesis. Separately, the LTB_4 receptor antagonist, LY293111, in combination with gemcitabine significantly inhibited colon tumor growth in athymic mice, suggesting that targeting a single end product of 5-LOX metabolism has potential as a therapeutic strategy [146]. In a separate study, the most effective inhibition of colon cancer cell proliferation in response to treatment with the histone deacetylase inhibitor, butyrate, was achieved under combined treatment with indomethacin and nordihydroguaiaretic acid (NDGA), COX-2 and LOX inhibitors respectively [147]. The 5-LOX activating protein (FLAP) inhibitor, MK886, has been shown to reduce the proliferation and/or growth of lung, colon and prostate cancer in both cell lines or experimental models [42, 148, 149]. These effects were reported to involve up-regulation of $PPAR_{\alpha}$ and $PPAR\gamma$, and in the case of lung cancer cells, a greater apoptotic effect was observed when low-dose MK886 was given in combination with the PPAR agonist, ciglitazone [149]. A more in depth study, examining the pharmacologic mechanism of MK886 in lung cancer, was undertaken by Mayburd and colleagues using an affymetrix microarray study and ingenuity pathway analysis to validate results in the context of experimental proteomic networks [150]. The data analysis showed that genes altered were responsible for the regulation of actin B and focal adhesion complexes, confirming some of the targets discussed in relation to LOX inhibition previously.

In addition to the array of pharmacological agents under investigation for their effect on LOX metabolism, a number of naturally occurring agents that may interact with lipoxygenase activity are constantly being discovered, such as dietary carotenoids including lycophyll (present in tomatoes) that may modify the enzymatic function of 5-LOX through binding to the catalytic site with high affinity [151]. There are also numerous plant phenols including baicalein, kaempferol, quercetin, nordihydroguaretic acid and resveratrol which are known for their chemo preventive as well as LOX-inhibitory activity in different tumor models [152]. The 12-LOX inhibitor, baicalein, a flavonoid isolated from an important medicinal plant Scutellariae Radix (the root of Scutellaria baicalensis Georgi) has been shown to induce apoptosis in human colon, breast and prostate cancer cells [63, 153, 154]. Recently, work in our laboratory has shown that baicalein potently inhibits the proliferation of NSCLC cell lines, with an additive effect observed when the compound is used in combination with chemotherapy, both platinum based and topoisomerase inhibitor [Pidgeon et al, unpublished data]. Resveratrol, *trans*-3,5,4′-trihydroxystilbene, was first isolated in 1940 as a constituent of the roots of white hellebore (Veratrum grandiflorum O. Loes), but has since been found in various plants, including grapes, berries and peanuts. Resveratrol has been shown to suppress the expression of 5-LOX and to inhibit the proliferation of a wide variety of tumor cells, including lymphoid and myeloid cancers; multiple myeloma; cancers of the breast, prostate, stomach, colon, pancreas, and thyroid; melanoma; head and neck squamous cell carcinoma; ovarian carcinoma; and cervical carcinoma [155]. The growth-inhibitory effects of resveratrol are mediated through cell-cycle arrest; up-regulation of p21Cip1/WAF1, p53 and Bax; down-regulation of survivin, cyclin D1, cyclin E, Bcl-2, Bcl-xL and clAPs; and activation of caspases. It has been shown to suppress the activation of several transcription factors, including NF-κB, AP-1 and Egr-1; to inhibit protein kinases including IκBα kinase, JNK, MAPK, Akt, PKC, PKD and casein kinase II; and to down-regulate products of genes other than 5-LOX including COX-2, VEGF, IL-1, IL-6, IL-8, AR and PSA. Currently, structural analogues of resveratrol with improved bioavailability are being pursued as potential therapeutic agents for numerous forms of cancer. In another study, green tea was reported to inhibit aberrant crypt foci formation in mice fed a high corn oil diet, which was associated with a decrease cytosolic phospholipase A_2, 5-LOX and LTB_4 levels [156]. This report suggests a potential chemo preventive mechanism of green tea, and LOX inhibition, on colon carcinogenesis in populations consuming high amounts of fat, such as western countries.

In summary, there are a number of compounds that inhibit the metabolism of arachidonic acid by LOXs in general or specific LOX-isoforms. Results appear promising in numerous forms of cancer, however they need confirmation in the clinical setting as most of the published data thus far has been pre-clinical. The transition from a pre-clinical to clinical setting is not an easy one, particularly in the case of herbal products. Like any new target, the emphasis will be on the necessity for rigorous, randomized clinical trials.

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