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An Arachidonic Acid Metabolizing Enzyme, 8*S*-Lipoxygenase, in Mouse Skin Carcinogenesis

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The involvement of arachidonic acid (AA) metabolizing enzyme, lipoxygenase (LOX), in the development of particular tumors in humans has gradually been acknowledged and LOX has emerged as a novel target to prevent or treat human cancers. In the mouse skin carcinogenesis model, which provides an excellent model to study multistage nature of human cancer development, many studies have shown that some of the LOXs are constitutively upregulated in their expression. Moreover, application of LOX inhibitors effectively reduced tumor burdens, which implicates the involvement of LOX in mouse skin tumor development as well.

8*S*-LOX is a recently cloned LOX, which is specifically expressed in mouse skin after 12-*O*-tetradecanoylphorbol-13-acetate (TPA) treatment but not in normal skin. Unlike other members of the LOX "family" expressed in mouse skin, this TPA-induced expression of 8*S*-LOX is prominent only in the skin of the TPA tumor promotion-sensitive strains of mice (SENCAR, CD-1, and NMRI) but not in the promotion-resistant C57BL/6J mice. This is a very unique phenomenon among strains of mice. Constitutive upregulation of 8*S*-LOX was also found in early stage papillomas and the expression was gradually reduced as the tumors became malignant. Based on these observations, it has been thought that 8*S*-LOX is involved in TPA-induced tumor promotion as well as in tumor conversion from papillomas to carcinomas. In accordance with this hypothesis, several studies have suggested possible roles of 8*S*-hydroxyeicosatetraenoic acid (HETE), an AA metabolite of 8*S*-LOX, in mouse skin tumor development. A clastogenic activity of 8*S*-HETE was demonstrated in primary keratinocytes and a close correlation between the levels of etheno-DNA adducts and 8*S*-HETE during skin carcinogenesis was also reported. On the other hand, it has been reported that 8*S*-LOX protein expression is restricted to a differentiated keratinocyte compartment. Moreover, reported findings on the ability of 8*S*-HETE to cause keratinocyte differentiation appear to be contrary to the procarcinogenic features of the 8*S*-LOX expression, presenting a question as to the role of 8*S*-LOX during mouse skin carcinogenesis. In this review, molecular and biological features of 8*S*-LOX as well as current views on the functional role of 8*S*-LOX/8*S*-HETE during mouse skin carcinogenesis are presented.

Key words: Mouse skin carcinogenesis, Arachidonic acid metabolism, Lipoxygenenase, 8*S*-Lipoxygenase

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INTRODUCTION

Arachidonic acid (AA) (5,8,11,14-eicosatetraenoic acid, 20:4, n-6) is a polyunsaturated fatty acid which can be obtained from either an endogenous biosynthetic pathway of desaturation and elongation of linoleic acid (LA) (18:2, n-6)[1] or dietary sources such as fats of animals and egg lipids. Synthesis of AA is, however, limited due to the absence of both Δ6-desaturase and Δ5-desaturase in certain tissues including human and mouse epidermis.[2] Therefore, AA in these tissues must come from dietary intake or from transportation to the tissues from other endogenous sources such as liver, which is capable of synthesizing AA. Arachidonic acid exists in an esterified form in the *sn*-2 position of membrane phospholipids, primarily in phosphatidylcholine or phosphatidylinositol, and rarely exists in a free form. Upon a variety of stimuli (e.g., wounding, growth factors, hormones, 12-*O*-tetradecanoylphorbol-13-acetate (TPA) treatment etc.), however, AA can be promptly released by phospholipase A_2 (PLA_2) and phospholipase C (PLC) and is metabolized to bioactive lipid mediators by cyclooxygenases (COXs), lipoxygenases (LOXs) and cytochrome P450 monooxygenase (Fig. 1). These metabolites, including prostaglandins (PGs), thromboxanes (from COXs), leukotrienes, and hydroxyeicosatetraenoic acids (HETEs) (from LOXs) are known to execute a num-

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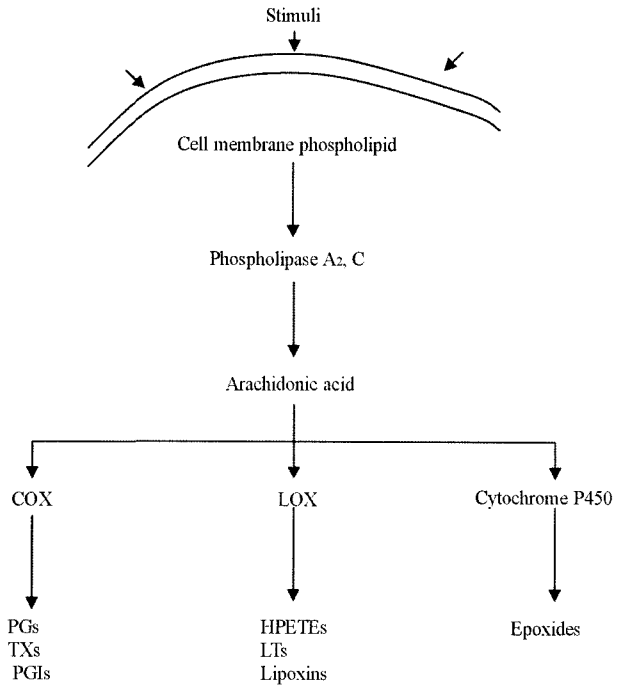

Fig. 1. Arachidonic acid metabolism

Upon variety of stimuli, arachidonic acid is released from cell membrane phospholipid by the action of phospholipase A_2 or phospholipase C. Free arachidonic acid undergoes enzymatic oxygenation by the COX, LOX, or cytochrome P450 monooxygenase pathway, and is converted to biologically active metabolites, called eicosanoids. PGs: Prostaglandins; TXs: Thromboxanes; PGIs: Prostacyclins; HPETEs: Hydro peroxyeicosatetraenoic acid; LTs: Leukotrienes

ber of physiologically important functions[3] and aberrant metabolism has been implicated in the pathogenesis of many human diseases such as cancer.[4]

A large body of evidence has shown an excessively activated COX pathway in a variety of human cancers.[5] Protumorigenic roles of PGs have been documented[5] and moreover, some of the COX-specific inhibitors are already in clinical use for the prevention of colon cancer.[6] On the other hand, various reports suggesting that LOX metabolites also exert a substantial impact on the development of animal as well as human cancers.[7-9] However, research on the role of LOX metabolites in tumorigenesis has lagged behind that of the COX metabolites. This is partly because LOXs are a more complex system in terms of number of isozymes, tissue specificity as well as substrate usage than the COXs. For example; 1) there are two forms of COX. One is COX-1 which is a constitutively expressed form, and the other is COX-2 which is transiently and highly induced by tissue stimulation (e.g., hormonal or growth factor stimulation, tissue damage, irritation, and TPA treatment).[10,11] Whereas there are at least 5 different LOXs exist in mouse skin. 2) COXs metabolize exclusively AA and LOXs can metabolize both AA and LA. 3) COX metabolites, especially PGs, have mainly procarcinogenic role, whereas, LOX metabolites appear to play a role as either pro- or anticarcinogenic lipid mediators in a metabolite- and tumor type-specific manner.[8] Therefore, clear understanding of the roles of each LOX/LOX metabolite is indispensable to further understand their contribution to carcinogenesis as well as their potential as preventive or therapeutic targets.

Recently, a new AA metabolizing enzyme, 8*S*-lipoxygenase (8*S*-LOX), was cloned from mouse skin and showed a unique feature of expression compared to other LOXs present in mouse skin. That is, it is not expressed at a detectable level in normal skin, however, it is highly induced by treatment of the skin with tumor promoter, TPA and is constitutively expressed in early stages of papilloma (benign skin tumors) development.[12-15] In this review, molecular and biological features of 8*S*-LOX will be covered and the functional role of 8*S*-LOX/8*S*-HETE during mouse skin carcinogenesis will be discussed. For better understanding of 8*S*-LOX, however, the review will start with background information on mammalian skin structure, mouse skin carcinogenesis, and lipoxygenase.

1. Structure and Differentiation of the Skin Epidermis

The skin is composed to two major compartments (Fig. 2). One is the dermis, a dense fibroelastic connective tissue layer, and the other is the thin stratified epithelial layer lying above the dermis, called epidermis. These distinct layers are physically separated by the basement membrane located between them. Probably because of the innate function of the epidermis in protecting important body organs from the environment, the epidermal cells are primarily composed of keratinocytes, which produce the most rigid intermediate filament protein, keratin, as the major cytoskeletal protein. Moreover, the epidermis consists of multiple layers of keratinocytes and continuously repeats the renewing process in which old, fully differentiated cells are sloughed off and are replaced by young daughters of progenitor cells. The self-renewing process of the epidermis is a result of an elaborate orchestration of epidermal differentiation (Fig. 2). The process of keratinocyte differentiation is accomplished by migration of epidermal cells from the basement membrane to the skin surface and is marked by specific marker proteins that are characteristic of each stage of differentiation.[16] The very inner layer of the epidermis lying on top of the basement membrane is composed of a proliferative epidermal cell population referred to as basal cells. These undifferentiated basal cells express

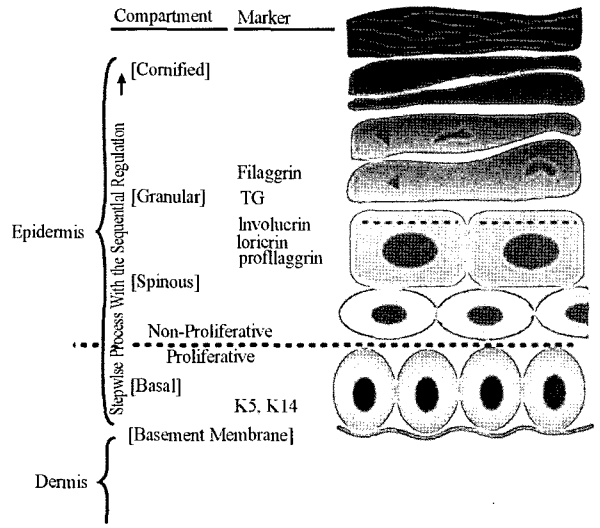

Fig. 2. Mammalian skin structure

Simplified schematic illustrating skin layers with representative differentiation-specific markers and epidermal compartments. Mammalian skin is composed of two different layers, dermis and epidermis, which are physically separated by a basement membrane (illustrated as a waved line). When the proliferative basal cells cease their mitotic activity, they start to migrate from the basement membrane to the suprabasal cell layers and to differentiate. The epidermal cells at each stage of differentiation express specific marker proteins and compose specific compartments of epidermis

keratin (K) 5 and K14 as the cytoskeletal proteins.[17,18] When the basal cell ceases its mitotic activity and migrates to the suprabasal layer, it starts to differentiate, which is marked by an alteration of the major cell products from K5 and K14 to K1 and K10.[19] The basal cells moving outward assume a polyhedral and spinous shape. Thus, the first cell type seen in the suprabasal cell layer is called the stratum spinosum.[20] The shape of the spinous cells, however, becomes progressively more flattened as it moves toward the skin surface. As the spinous cell further matures and differentiates, the cytoplasm of the cell is increasingly filled with filament proteins including involucrin[21] and loricrin,[22] which results in aggregation of those proteins. Since the aggregation is seen as lamellar granules under the electron microscope, this layer of cells is called the stratum granulosum.[20] A subsequent differentiation of the granular cell is associated with several remarkable events in which mitochondria and ribosomes are degraded, the nuclear envelope disappears, and ultimately the nucleus is degraded.[20] Additionally, the interstices of the cell are filled with a filament aggregating protein, filaggrin,[23] which results in a dense marginal band adjacent to the inner face of the plasma membrane.[20] Later on, the marginal band is attached to the plasma membrane by formation of ε-(γ-glutamyl) lysine bonds by transglutaminase.[24] Since this dense marginal band makes up the thickened cell envelop, this terminally differentiated cell layer is called the stratum corneum and provides resistance of the epidermis to mechanical and chemical destruction. Subsequently, filaggrin in the cornified cell is degraded into free amino acids, which raises osmolarity in the cell and contributes to the retention of moisture and the maintenance of flexibility of the cornified cell.[25]

2. Two-stage Mouse Skin Carcinogenesis

The development of a malignant neoplasm is a multistage process that usually starts from a single mutated cell that begins to proliferate abnormally in a limitless manner. In terms of multistage carcinogenesis, the mouse skin model provides an excellent model since it shows distinct physiological and molecular events in each process.[26-28] The multistage carcinogenesis process in skin has been subdivided into three distinct stages termed initiation, promotion, and progression (Fig. 3).[29]

Initiation of carcinogenesis is generally accomplished by a single, topical application of subcarcinogenic dose of chemical (e.g., 7,12-dimethylbenz[*a*]anthracene (DMBA), tobacco tar, etc.) or physical (e.g., X-rays, ultraviolet (UV) radiation) initiating agents to the dorsal mouse skin.[30] These initiating agents have the property of binding to DNA, introducing methylation, or causing damage to chromosomal DNA, which leads to genetic changes such as mutations, deletions, translocations, or loss of regions of chromosomes. In any case, these genetic changes may produce constitutive growth signals in the initiated cells. Since every cell has a DNA damage repair system, however, application of an initiating agent itself does not always generate an "initiated" cell.[31] It needs several rounds of DNA synthesis and cell division in order to "fix" the mutation permanently.[32] Therefore, proliferating cell populations including basal cells and epidermal stem cells residing in hair follicles are prime targets for initiation. Once the mutation is fixed, it is not reversible. The most frequently mutated gene in the initiation stage is the *ras* oncogene.[33-36] Initiation alone at the subcarcinogenic dose is, however, not sufficient for the development of visible tumors during the life span of the animal. The selective clonal expansion of the initiated cells follows in the promotion stage. This is called two-stage skin carcinogenesis in which tumor development is accomplished through the stages of both initiation and promotion.[37] On the other hand, in the complete carcinogenesis protocol, application of the initiating agent at a carcinogenic dose alone is sufficient to cause tumors.[37]

Unlike the initiation stage, tumor promotion is accomplished by repetitive application of tumor promoting agents to the initiated skin.[36] Upon repeated treatment,

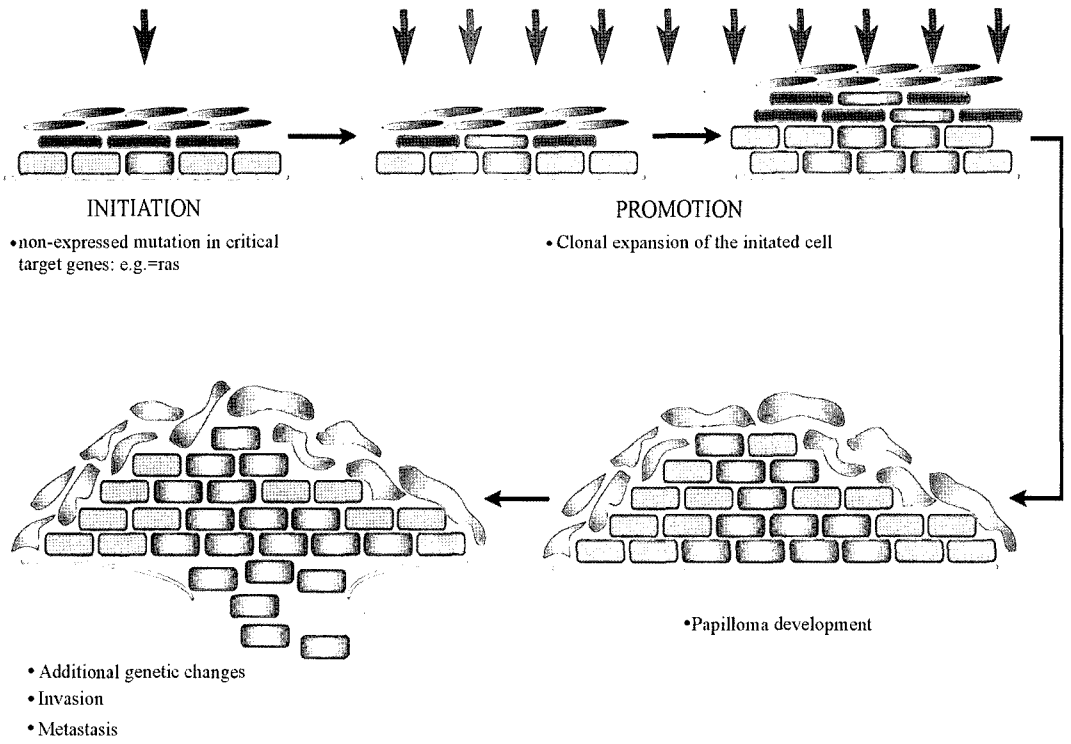

Fig. 3. Mouse skin multistage carcinogenesis

A multistage process of mouse skin tumor development is demonstrated. In the initiation stage, a critical target gene in epidermal cells is mutated by a single application of subcarcinogenic dose of carcinogen to mouse skin. In the promotion stage, clonal expansion of initiated cells occurs by repetitive treatment of chemical or physical tumor promoters, such as 12-*O*-tetradecanoylphorbol-13-acetate (TPA) to the initiated skin. During this process, the initiated cells may develop into papillomas. In the progression stage, additional genetic mutations occur within rapidly proliferating cells, which convert benign papillomas to malignant carcinomas. Further progression allows them to invade and metastasize to other tissues

mouse skin develops benign tumors, papillomas, which are characterized as a contained, differentiated lesion of uncoordinated growth, and lacking vascularization. It is generally accepted that tumor promoters do not bind to DNA and are not mutagenic like a carcinogen. Therefore, normal mouse skin usually does not develop tumors with application of the promoter alone.[37] However, the promoting agents bring about several important epigenetic changes (e.g., alteration of gene expression, signal transduction, differentiation, and intercellular communication) to the cell, which is thought to help in the selective growth of the initiated cell.[38] These epigenetic changes are reversible in the early stages of promotion and are not inheritable like a carcinogen-induced DNA mutation in the initiation stage. Therefore, continued and repetitive promoter treatment is required to maintain the promoting activity of the agents and a cessation of promoter treatment causes regression or disappearance of benign tumors at least up to some point. The epigenetic changes induced by the promoter elicit many diverse genetic and physiological changes in the skin, including induction of epidermal hyperplasia, inflammation and ornithine decarboxylase expression.[38] Although the mechanism of tumor promotion has not been fully elucidated, induction of sustained hyperplasia of the skin has been the best correlated with the tumor promoting activity of the promoter.[37] TPA, the most potent and frequently used tumor promoter in mouse skin carcinogenesis, elicits those events mainly through activation of protein kinase C (PKC).[39,40] An endogenous ligand for PKC is diacylglycerol (DAG). However, TPA binds to the same cysteine-rich zinc fingers in PKC with much higher potency since it has a longer half-life then DAG.[41] Activated PKC exerts its major effect in skin via various mitogen-activated protein kinase (MAPK) cascades and stimulates expression of genes involved in hyperproliferation, tissue remodeling, and inflammation such as transforming growth factor α and COX-2.[42] In the multistage model, many skin tumors remain as papillomas but some go further into irreversible progression stage, depending on strain and protocol.

In the tumor progression stage, benign papillomas convert into malignant carcinomas which are characterized by rapidly growing, invasive, and vascularized phenotypes. The conversion process occurs spontaneously in the absence of further tumor promoter treatment. Although how this process progresses is not understood very well, a number of gene and chromosome alterations are associated with tumor progression in mouse skin, which are thought to be responsible and/or permissive for this stage of carcinogenesis. Those alterations include trisomy of chromosome7[7] loss of the normal Ha-*ras* allele, loss of E-cadherin, loss of K1 and K10, and stable expression of certain proteases.[36] In particular, increased expression of proteases is thought to be responsible for the acquisition of an invasive phenotype of highly aggressive tumor cells.[43,44] Metastasis from skin squamous cell carcinomas, however, is uncommon.

3. Lipoxygenase

Overall Structure and the Catalytic Action of Lipoxygenase

Lipoxygenase proteins are long, single polypeptide chains with a molecular mass of ~75-80 kDa in animals and ~94-104 kDa in plants. From the three dimensional structure of soybean LOX 1 which is the most studied enzyme in both mechanistic and spectroscopic aspects, it was revealed that the polypeptide chain is organized into two three-dimensional domains.[45,46] Those are β -barrel (domain Ⅰ) and α-helical (domain Ⅱ) domain in N-terminal and C-terminal of the peptide, respectively. Domain I is composed of a much smaller portion of the protein compared to domain Ⅱ (e.g., in the case of soybean LOX 1; 146 and 693 amino acid residues for N-terminal and C-terminal domains, respectively, out of a total 839 residues). It also stands quite separate from and only loosely connects with the rest of the molecule. Domain II, which is the major domain of the protein, contains a single atom of non-heme iron in the center of the domain and executes the catalytic function of the enzyme.[46]

Although the mechanism for the catalytic reaction of the enzyme is not completely understood, it appears that the iron atom in domain Ⅱ composes a central part of the catalytic activity of a lipoxygenase. It has been observed that when the metal exists in Fe^{2+} (ferrous) form, the enzyme is inactive, whereas, as the ferrous ion is oxidized to Fe^{3+} (ferric), the enzyme becomes active and can oxidize substrates such as arachidonic acid or linoleic acid.[46] From this observation, many studies have proposed a model for the iron-mediated catalytic reaction of LOX. One of the commonly proposed models is as follows[46]:1) Molecular oxygen reaches the iron center from outside the enzyme and oxidizes iron from Fe^{2+} to Fe^{3+}; 2) The Fe^{3+} ion abstracts one electron from the 1,4 diene system of the unsaturated fatty acid substrate, which produces a free radical form of the 1,4 diene system, and is reduced to Fe^{2+} 3) Now, O_2 reacts with this substrate radical and forms a peroxyl radical. The peroxyl radical subsequently abstracts an electron from the metal, which regenerates Fe^{3+}, and produces a peroxidate anion; 4)The peroxidate anion receives the proton from the base, which finally results in hydroperoxide products. Notably, all these reactions occur in a regio-and stereo-selective manner. Most known LOXs form hydroperoxide products with '*S*' stereochemistry.[47] Recently, however, several LOXs forming the mirror image '*R*' configuration have also found in coral,[48] mouse,[49] and human.[50]

Occurrence

Wide distribution of lipoxygenases has been found in plants, fungi, and animals.[51-55] However, it has been reported that most bacteria, yeast, and other prokaryotes do not contain LOX genes,[56] partly because these organisms do not have proper substrates for the LOXs.[57] In the case of yeast, lack of desaturases, which are necessary for the synthesis of polyunsaturated fatty acids, was reported.[56] On the other hand, higher plants as well as animals have multiple LOXs in various tissues. At least eight different LOXs were identified in soybean and seven and five different LOXs are found in mouse and in humans, respectively.[56]

Nomenclature

Typically, lipoxygenases are classified according to the specific carbon position of oxygen insertion in the substrate and if it is necessary, the stereoconfiguration is specified. For example, 8*S*-LOX incorporates an oxygen molecule onto the carbon-8 from the carboxyl end of arachidonic acid and produces the '*S*' configuraion of hydroperoxide products, 8*S*-HETE. However, since this is based on a single property of LOXs and does not take into account other enzymatic, protein-chemical, and molecular biological characteristics of the enzyme, this type of nomenclature has several inherent problems. Those problems typically occur in the following cases: 1) When the carbon length of substrate is changed; 8*S*-LOX produces 8*S*-HETE from AA (C20:4), but produces 9*S*-hydroxyoctadecadienoic acid (9*S*-HODE) from LA (C18:2). 2) When more than one isozyme is present in the same species; mouse has three

different 12-LOXs that differ in tissue distribution, sequence homology, substrate affinity, and biological function.[54] To solve this problem, currently prototypical tissues of their predominant occurrence are specified for the mammalian LOXs such as platelet, leukocyte, or epidermal type of 12-LOX. 3) When LOX has dual activity on a specific substrate; leukocyte type 12*S*-LOX can oxidize both C-12 and C-15 of AA although C-12 is the preferred site.[54] Despite these problems, classical nomenclature is still popularly used because it conveys the useful message in a most simple way.

The Importance of Lipoxygenase Pathway in Mouse Skin Tumor Development

In mouse skin, at least 5 different LOXs , 5*S*-,[58] platelet-type (*p*) 12-,[59] leukocyte-type (*l*) 12-[59] epidermal type (*e*) 12-,[60] and 8*S*-LOX,[12-14] are expressed. In the course of mouse skin tumor development, it has been found that the expression of some of these LOXs is constitutively upregulated.[15,59] Moreover, inhibition of LOX enzyme activity was at least as effective as that of COX in the inhibition of tumorigenesis,[7,61-65] which suggests a critical role for LOX in mouse skin tumor development as well. The function of individual LOXs and their metabolites in mouse skin carcinogenesis is, however, only slowly being elucidated. Some of the reported functions for the LOX are described below.

Of all the LOX family members, 5*S*-LOX is the only enzyme involved in leukotriene (LT) synthesis.[66] It oxidizes AA to 5*S*-HPETE, which is either reduced to 5*S*-HETE or further metabolized to LTA_4 by the LTA_4 synthase activity of 5*S*-LOX. The LTA_4, an unstable C-5 epoxide of AA, can be converted to the glutathione-conjugated LTC_4 and its metabolites, LTD_4 and LTE4. These leukotrienes are well known participants in host defense reactions and in pathophysiological conditions such as immediate hypersensitivity and inflammation.[67] Since the immediate response of mouse skin to external stimuli, such as wounding or TPA treatment, is an inflammatory reaction, which is an important component of tumor promotion, the implication of LTs in the reaction as well as tumor promotion was expected. In fact, the presence of LTB_4, LTC_4, LTD_4, and LTE_4 was identified in mouse skin[68] and in particular the level of the latter three LTs were strongly increased by TPA.[68] Many studies also reported the effective inhibition of two-stage as well as complete skin carcinogenesis by application of specific 5*S*-LOX inhibitors.[69,70] The production of 5*S*-HETE by epidermal keratinocytes has been controversial. Fischer et al. reported that the levels are nearly undetectable.[71]

12-LOX is the most abundant LOX expressed in mouse skin.[72] It occurs in 3 isoforms, platelet (*p*)-, leukocyte (*l*)-, and epidermis (*e*)-types, which are encoded by different genes and converts AA primarily to 12-HETE.[54] The function of *p*12*S*-LOX in normal epidermis appears to be involved in normal permeability barrier function of the skin.[73] However, constitutive overexpression of *p*12*S*-LOX in papillomas, squamous cell carcinomas,[59] and several epithelial tumor cell lines[74] suggest its involvement in mouse skin tumorigenesis as well. When the *p*12*S*-LOX gene was disrupted by gene targeting, the papilloma and carcinoma incidence was in fact significantly reduced in two-stage carcinogenesis, although there was variability depending on the genetic background of the mice.[75] Many reported studies on the procarcinogenic functions of 12*S*-HETE further support a critical role of *p*12*S*-LOX in mouse skin tumor development. Those functions include stimulation of epidermal proliferation,[76] infiltration of neutrophils and monocytes to the skin,[77] angiogenesis,[78] metastasis,[79] or repression of K1 expression.[80]

The presence of *l*12*S*-LOX in keratinocytes has been controversial. The transcripts of *l*12*S*-LOX were not detectable in normal epidermis and were detectable only in a few tumors.[59] Since the increased level of *l*12*S*-LOX mRNA coincided with the infiltration of granulocytes to the epidermis in the course of TPA-induced inflammation, it has been suggested that *l*12*S*-LOX specific mRNA found in tumor samples may originate from non-epithelial cells such as infiltrated inflammatory cells or Langerhans'cells residing in the epithelium.[59]

Epidermis type 12-LOX was recently found in mouse skin and has not been found in humans yet. So far, three isoforms of *e*12-LOX have been cloned, *e*-LOX-1,[81] *e*-LOX-2 (12*R*-LOX),[49] and *e*-LOX-3.[82] The biological functions of each individual *e*12-LOX are not known yet; however, observations on the constitutive expression of *e*12-LOX in differentiated stratified epithelia[49,81] suggest its potent role in keratinocyte differentiation. It has been reported that the *e*12-LOX expression is not further induced by TPA[49,81] and is transcriptionally downregulated in the course of skin tumorigenesis,[83] which suggest this enzyme may not produce 12-HETE and may have an anticarcinogenic effect.

Besides *e*12-LOX, mouse skin expresses another unique epidermal type LOX, 8*S*-LOX, which is described in detail in the following section. This enzyme is distinguished from other LOXs expressed in mouse skin because it is not expressed in normal mouse skin, however, is highly

induced by a single topical treatment of TPA.[12-14] Since constitutively overexpressed 8*S*-LOX was found in papillomas,[15] it has been hypothesized that 8*S*-LOX also plays an important role in skin tumor development.

Another important feature of the LOX pathway in skin tumorigenesis is the generation of reactive oxygen species (ROS) as byproducts of LOX metabolism. Since LOX reactions are carried out by hydrogen abstraction, radical rearrangement and oxygen insertion processes,[46] besides the hydroperoxide products of the substrate, many ROS can be generated during these processes.[84] In fact, it has been shown that either LOX inhibitors (nordihydroguaiaretic acid and benoxaprofen) or inhibitors for both COX and LOX (eicosatetraynoic acid and phenidone) were effective in inhibiting ROS production, whereas, COX inhibitors (indomethacin and flurbiprofen) were not good inhibitors.[85] The excess level of oxygen radicals has been shown to be involved in lipid peroxidation, enzyme activation or inactivation, and DNA strand breaks, which can contribute to tumorigenesis. Interestingly, many tumor promoters and progressors are free-radical generating compounds such as benzoyl peroxide. Considering that antioxidants in general are effective inhibitors of mouse skin tumor development[86-88] and that many LOX inhibitors are in fact antioxidants, it is very likely that LOX contributes to the skin tumor development through ROS by-production as well.

4. Murine 8*S*-Lipoxygenase

The possible involvement of 8*S*-LOX in mouse skin carcinogenesis originated in a report that the most highly elevated LOX metabolite in TPA treated mouse skin was 8-HETE.[12] Since this 8-HETE was subsequently shown to be the 8*S* enantiomer,[89] which is an indication of an enzyme-mediated reaction product, the presence of 8-HETE synthesizing enzyme in mouse skin was expected. Later on, 8*S*-LOX was identified as the enzyme responsible for the synthesis of 8*S*-HETE.[13] Of note is that 8*R*-HETE occurs only as an autoxidation product of AA.

Molecular Biology of Murine 8S-LOX

8*S*-LOX cDNA was cloned by two independent groups while searching for a murine homolog of human 15*S*-LOX-2 in mouse skin.[14,49] RT-PCR strategy was applied in which cDNA template was prepared from phorbol ester-treated mouse skin and degenerate primers corresponding to well conserved sequences in mammalian lipoxygenases were used. Full-length cDNA sequence was also isolated by subsequent screening of mouse skin cDNA library. The full-length cloned cDNA is 3.2 kb in length and encodes a protein of 677 amino acids with a calculated molecular weight of 76 kDa and a pI of 6.72[14,49] The amino acid sequence has 78% identity with human 15*S*-LOX-2 and approximately 40% identity with other mammalian LOXs (54% identity with 12*R*-LOX, 50% with *e*-LOX-3, 43% with 5*S*-LOX, 38% with 12*S*-, and 12/15*S*-LOX).[14,49] A phylogenetic tree of mammalian LOXs showed that 8*S*-LOX and its human and bovine orthologue 15*S*-LOX-2 are placed close to other epidermal- type LOXs, including 12*R*-LOX (*e*-LOX-2) and *e*-LOX-3.[90]

Despite high sequence identity between 8*S*-LOX and 15*S*-LOX-2, 8*S*-LOX exclusively metabolizes AA to 8*S*-HETE, whereas, 15*S*-LOX-2 metabolizes AA to 15*S*-HETE. Jisaka et al. sought out to determine the positional specificity of 8*S*-LOX and 15*S*-LOX-2.[91] They generated a variety of chimeras starting from a large portion of protein domain and narrowed down to a single amino acid sequence by swapping between 8*S*-LOX and 15*S*-LOX-2. The resulting chimeras were expressed in HeLa cells and tested for enzyme activity. From this experiment, it turned out His^{604} of 8*S*-LOX appears to be a key amino acid determinant of positional specificity, and the neighboring Tyr^{603} seems to support its function. Change at both positions is required to switch the 8*S*-LOX to an enzyme with predominantly 15*S*-LOX-2 activity.

There is another interesting feature shared by 8*S*-LOX and 15*S*-LOX-2. Based on reported crystal structures of plant and mammalian LOX, the non-heme iron in the catalytic domain is ligated by three histidines, the C-terminal isoleucine, and in certain structures also by a fifth amino acid ligand, which is represented by either an asparagine or a histidine.[92] However, 8*S*-LOX and 15*S*-LOX-2 have a serine residue (Ser^{558} for 8*S*-LOX, Ser^{557} for 15*S*-LOX-2) in the putative fifth iron ligand position. To test the significance of this serine residue in the structure-function of 8*S*-LOX, Ser^{558} was replaced by asparagines, histidine, or alanine by site-directed mutagenesis.[92] The wild-type and the mutant cDNA were expressed in HeLa cells and tested for enzymatic activity. Remarkably, all three mutants still exhibited significant 8-LOX acitivity, indicating that the putative fifth iron ligand binding site represented by Ser^{558} is not essential for the 8*S*-LOX enzyme activity.

Gene structure as well as chromosomal localization of the 8*S*-LOX gene was only recently reported.[90] Approximately 14.5 kb-long, the 8*S*-LOX gene (designated *Alox15b* referring to the previously annotated human 15*S*-LOX-2 gene, *ALOX15B*) is composed of 14 exons and is located in the central region of mouse chromosome

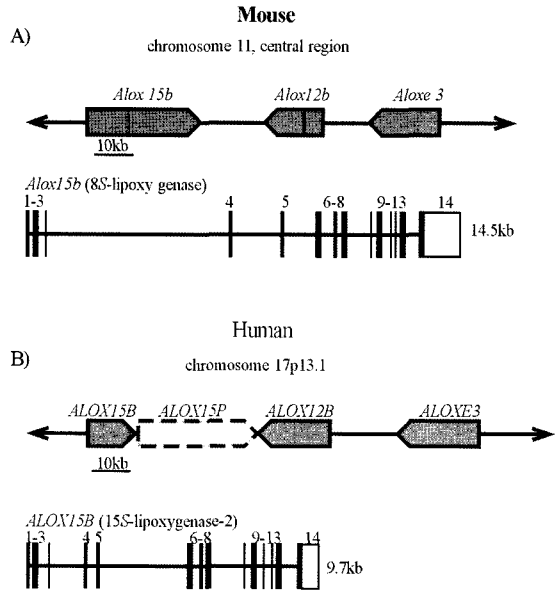

Fig. 4. Gene structure and chromosomal localization of murine 8*S*-LOX and human 15*S*-LOX-2 (*Taken from F?stenberger et al. Prostaglandins Other Lipid Mediat.68-69, 235-243, 2002*)

(A) A mouse gene cluster encoding 8*S*-LOX (*Alox15b*), 12*R*-LOX (*Alox12b*), and *e*-LOX-3 (*Aloxe3*) is shown in the linear arrangement. (B) A human gene cluster encoding 15*S*-LOX-2 (*ALOX15B*), 12*R*-LOX (*ALOX12B*) and *e*-LOX-3 (*ALOXE3*) and the 15*S*-LOX pseudogene (*ALOX15P*) is depicted. The orientation of the genes is illustrated as arrows. The exon/intron organization of the 8*S*-LOX and 15*S*-LOX-2 genes is drawn to scaleand numbered. The closed boxes indicate exons and the open boxes indicate 5'- and 3'-untranslated regions.

11 (Fig. 4). It was also shown that the *Alox15b* gene is adjacent to the genes encoding 12*R*-LOX (*Alox12b*) and *e*-LOX-3 (*Aloxe3*). Interestingly, these genetic findings of 8*S*-LOX, such as gene organization and LOX gene cluster in the chromosome, is very similar to those of human 15*S*-LOX-2 gene (*ALOX15B*) except that the latter gene is located on human chromosome 17.[93] Along with high sequence identity between 8*S*-LOX and 15*S*-LOX-2, this genetic similarity further supports an idea that 15*S*-LOX-2 represents the human orthologue of mouse 8*S*-LOX. Recently, approximately 2 kb of 8*S*-LOX gene promoter was cloned.[94] The promoter does not have a TATA box or a CCAAT box and a transcription initiation site was mapped to -27 bp from the ATG translation start site. The promoter was highly responsive to TPA in TPA promotion-sensitive SSIN but not in promotion-resistant C57BL/6J primary keratinocytes. A Sp1 binding site, located -77 to -68 from the ATG was identified as a TPA responsive element (TRE) of the promoter, and Sp1, Sp2, and Sp3 proteins were identified as the proteins that bind to the TRE. Since the binding of these proteins to the TRE was significantly increased by TPA treatment and inhibition of the binding decreased TPA-induced promoter activity as well as 8*S*-LOX mRNA expression,

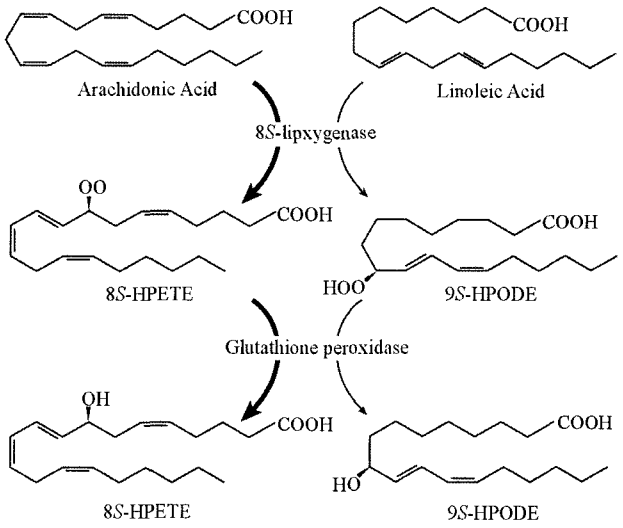

Fig. 5. Metabolic action of murine 8*S*-LOX

8*S*-LOX metabolizes arachidonic acid and linoleic acid to 8*S*-HPETE and 9*S*-HPODE, respectively. The 8*S*-HPETE and 9*S*-HPODE are subsequently reduced to 8*S*-HETE and 9*S*-HODE, respectively, by glutathione peroxidase. Arachidonic acid metabolism occurs more efficiently than linoleic acid metabolism by 8*S*-LOX, which is illustrated by a thicker line. 8*S*-HPETE: 8*S*-Hydroperoxyeicosatetraenoic acid; 8*S*-HETE: Hydroxyeicosatetraenoic acid; 9*S*-HPODE: Hydroperoxyoctadecadienoic acid; 9*S*-HODE: Hydroxyoctadecadienoic acid

it has been suggested that increased binding of Sp1, Sp2, and Sp3 to the TRE of the 8*S*-LOX promoter is a mechanism through which 8*S*-LOX expression is regulated by TPA in keratinocytes.

Enzymatic Properties of 8S-LOX

8*S*-LOX can metabolize both AA and LA to 8*S*-HPETE and to 9*S*-hydroperoxyoctadecadienoic acid (9*S*-HPODE), respectively, however, it uses AA as a preferred substrate (Fig. 5).[14,15] The enzyme has an optimum pH of 8.0 and does not require Ca^{2+} or ATP for the activity.[13] Most enzyme activity of 8*S*-LOX is detected in the cytosolic fraction of suprabasal keratinocytes.[13] Of particular interest is that the enzymatic activity is very low in normal mouse skin, however, it is strongly induced by a single, topical treatment of TPA to mouse skin.[12,13] This increased enzyme activity was found to depend on 8*S*-LOX protein biosynthesis.[13] In NMRI mice, the activity became apparent 3 h after TPA treatment, reached a maximum between 18 and 36 h and disappeared 3-5 days after treatment.[13] However, the enzyme sensitivity to TPA is largely mouse age-dependent. Newborn pups could not be induced to generate 8*S*-HETE in response to TPA, whereas 6-7-day-old mice showed the most prominent response.[13] TPA, however, could not induce 8*S*-HETE production in a cell free system prepared from either the rat tongue epithelial cell line (RTE2) or the murine

epidermal cell line (HEL30), although both systems were active in metabolizing AA to 12- and 15-HETE.[12] Other than the TPA, 12-*O*-retinoylphorbol-13-acetate was also effective 8-HETE production in mouse skin.[12] However, non-promoting phorbol esters including 12-*O*-ethacrynyl-phorbol-13-acetate and 4-*O*-methyl TPA and Ca^{2+} -ionophore were inefficient in causing 8-HETE production.[12]

Tissue and Cellular Localization of 8S-LOX

The presence of 8-HETE was first identified in human neutrophils,[95] mouse peritoneal macrophages,[96] mouse and rat liver,[97] rat kidney glomeruli,[98] human psoriatic skin,[99] human tracheal cells,[100] and human primary squamous cell carcinomas of head and neck.[101] However, 8*S*-LOX enzyme expression has not yet been detected in those tissues or cell types.

So far, only limited tissues of mice have been reported to express 8*S*-LOX transcripts and/or protein. In black Swiss mice, strong constitutive 8*S*-LOX mRNA expression was detected in brain but not in heart, spleen, lung, liver, skeletal muscle, kidney, or testis.[14] Immunohistochemical analyses showed constitutive 8*S*-LOX protein expression in the hair follicles of this mouse as well.[14] In NMRI mice, low levels of constitutive 8*S*-LOX mRNA expression were detected in the footsole.[49] Recently, the possibility of the presence of 8*S*-LOX protein in rat cornea has been suggested.[102]

In normal mouse skin, including dorsal and tail skin, 8*S*-LOX is not expressed at a detectable level. However, the expression is highly induced by a single, topical treatment of TPA.[14] This TPA-induced expression is, however, highly mouse strain-dependent.[65,71] That is, using 6-10-day-old mice, low constitutive 8*S*-LOX expression and strong TPA-induced expression was observed in black Swiss and NMRI mice.[12,14] In the SENCAR strain, high constitutive 8*S*-LOX activity with little extra induction by TPA was observed.[14] In a TPA tumor promotion-resistant strain, C57BL/6J mice, very low constitutive expression was observed and little induction was seen with TPA treatment.[71] Given the fact that many researchers have extensively studied C57BL/6J mice from genetic, molecular, biologic, and biochemical points of view to find critical genetic or cellular components that are responsible for their resistance to TPA tumor promotion,[71,103-106] this is one of the most interesting phenomena observed between promotion-sensitive and -resistant strains of mice. Notably, 8*S*-LOX protein expression in skin was limited to the differentiated keratinocyte compartment, the stratum granulosum.[14] Since this compartment is highly expanded after TPA treatment, it has been suggested that TPA-induced 8*S*-LOX expression is correlated with an increase in the number of cells that produce 8*S*-LOX in skin.

Biological Functions of 8S-LOX in Mouse Skin Carcinogenesis

Implication in Skin Tumor Development

Despite several reports showing that LOX metabolites are critically involved in skin inflammation as well as tumor promotion, and that LOX inhibitors effectively inhibit mouse skin carcinogenesis, 8*S*-HETE is so far the only highly induced LOX metabolite in mouse skin after TPA treatment. Although 12*S*-HETE is the most abundant LOX metabolite in the skin, the level of 12*S*-HETE is not significantly increased by TPA.[71] This observation led to the hypothesis that 8*S*-HETE/8*S*-LOX may contribute to mouse skin tumor development. Supporting this idea, constitutively upregulated 8*S*-LOX expression as well as activity was also observed in early stage of papilloma development (reversible papillomas).[15] Interestingly, however, in the course of malignant progression of papillomas to carcinomas, it was observed that the level of 8*S*-LOX expression and activity decreased.[107] That is, the level was reduced in irreversible papillomas, which are precursor lesions of carcinomas, and in carcinomas the level returned completely back to that of normal epidermis. This profile of 8*S*-LOX expression is quite different from that of COX-2 and *p*12*S*-LOX, which were found to be constitutively upregulated in carcinomas.[59,108] Since conversion of papillomas to carcinomas occurs spontaneously without any other treatment, it has been thought that endogenous genotoxic agents in papillomas are responsible for further genetic mutations or instability, which contribute to the malignant progression. In this context, strongly increased 8*S*-HETE in papillomas was suspected to be one of the genotoxic agents contributing to tumor progression.[90] In fact, an ability of 8*S*-HETE to induce chromosomal damage, predominantly gaps and breaks, in primary keratinocytes was reported.[15] Moreover, an observation that the level of 8*S*-HETE was closely correlated with promutagenic etheno-DNA adduct formation in skin tumors[107] appears to further support a critical role of 8*S*-HETE in mouse skin tumor development as well as in malignant tumor progression.

Induction of Terminal Differentiation in Keratinocytes

On the other hand, histochemical analyses showed that 8*S*-LOX protein in mouse skin was localized in a terminally differentiated epidermal cell compartment, the stratum

granulosum.[14] Since TPA-induced 8*S*-LOX expression as well as enzyme activity was closely related with expansion of this compartment after TPA treatment, a potential role of 8*S*-LOX in keratinocyte differentiation has also been suggested. In fact, targeted FVB transgenic mice overexpressing 8*S*-LOX gene under control of the loricrin promoter exhibited a highly differentiated and keratinized epidermal phenotype.[109] Moreover, K1 staining in the transgenic epidermis was not only more intense, but also occured in many of the basal cells as well as in the suprabasal layers, whereas the staining in wild type epidermis was primarily in suprabasal layers. Interestingly, the transgenic mice also showed increased rates of proliferation in inter- and intra-follicular keratinocytes without exhibiting significant hyperplasia. This phenomenon was explained in the context of a compensatory mechanism in which loss of suprabasal cells resulting from rapid migration of epidermal keratinocytes from the basement membrane and terminal differentiation was compensated for by increased proliferation of basal cells. More direct evidence for the ability of 8*S*-HETE to induce K1 expression was also demonstrated in primary keratinocytes and the induction of K1 was a peroxisome proliferator-activated receptor (PPAR)-α mediated process.[109] Recently, we also investigated the functional role of 8*S*-LOX/8*S*-HETE in mouse skin carcinogenesis using a series of gain-of-function studies.[110] Targeted C57BL/6J transgenic mice overexpressing the 8*S*-LOX gene under control of the loricrin promoter showed a more differentiated epidermal phenotype as well as reduced tumor development compared to wild type mice in a two-stage skincarcinogenesis protocol. Forced expression of the 8*S*-LOX gene or exogenous addition of 5 μM 8*S*-HETE in skin tumor cell lines either caused a more differentiated cell phenotype or inhibited cell proliferation. From immuno-histochemical analyses, we also found 8*S*-LOX expression was strictly limited to the differentiated compartment of skin epidermis even in the process of skin tumor development. These observations strongly suggest that 8*S*-LOX is not only closely related to, but also actively participates in the process of keratinocyte differentiation and inhibit mouse skin tumorigenesis as well.

Murine 8S-LOX vs. Human 15S-LOX-2

Although 8*S*-LOX is not encoded in the human genome, it shares 78% identity in amino acid composition with human 15*S*-LOX-2.[14] Moreover, both of them use arachidonic acid as a preferred substrate and produce 8*S*-HPETE and 15*S*-HPETE, respectively. Despite these structural and catalytic similarities, however, their tissue and cellular localization are quite distinct from each other. That is, the expression of 15*S*-LOX-2 was identified in human prostate, lung, cornea, skin, and brain, whereas the expression of 8*S*-LOX has been reported only in mouse skin and brain.[14,111] Moreover, in skin, 8*S*-LOX was detected in differentiated keratinocytes and in hair follicles, whereas 15*S*-LOX-2 was detected in the basal layer of the epidermis, sebaceous glands, and other adnexa.[112] Based on these observations, it had been thought that they are not functional homologs but just structural homologs of each other. However, recently reported studies suggest that they may share functional roles as well. Although 15*S*-LOX-2 is not generally expressed in differentiated keratinocytes of epidermis, strong immunostaining for 15*S*-LOX-2 was observed in differentiated secretory sebocytes.[112] Considering that 15*S*-HETE is a ligand of PPAR, which is involved in adipocyte differentiation,[113,114] both the 8*S*-LOX and 15*S*-LOX-2 gene products appear to be involved in tissue differentiation through PPARs. That is, 8*S*-HETE causes keratinocyte differentiation through PPARα and 15*S*-HETE contributes to sebocyte differentiation through PPARγ. In prostate, 15*S*-LOX-2 was specifically expressed in the glandular prostate epithelial cells *in vivo* but not in basal cells or other cell types including stromal cells.[115] More interestingly, the protein level and enzymatic activity of 15*S*-LOX-2 have also been shown to be downregulated in prostate cancers compared with normal and benign prostate tumors.[116,117] Moreover, exogenous 15*S*-HETE treatment or restoration of 15*S*- LOX-2 expression in prostate cancer cells inhibits cell proliferation *in vitro* and *in vivo*.[115,118] These observations are very similar to what has been observed with 8*S*-LOX in mouse skin and skin cancer cells in a reported study.[110] Again, although the tissues in which of 8*S*-LOX and 15*S*-LOX-2 are expressed are different, both genes appear to function as a suppressor of specific tumor development. Considering this suggested functional similarity, further investigation on the functional role of 8*S*-LOX in keratinocyte differentiation as well as in skin tumor inhibition may increase our understanding on the functional role of human 15*S*-LOX-2.

CONCLUDING REMARKS

AA is one of the most actively metabolized fatty acid in animals and the metabolites of AA exhibit many important biological functions in non-pathological and pathological conditions including cancer. Murine 8*S*-LOX

is a recently cloned AA metabolizing enzyme and appears to have many interesting biological features during mouse skin carcinogenesis. Considering its promising contributions to understanding other LOX family members, the mechanisms of tumorigenesis, as well as differentiation, we expect that a more thorough understanding of the regulation and function of 8S-LOX in the future could lead to novel approaches to the prevention or treatment of many human diseases including cancers.

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