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PPAR δ is pro-tumorigenic in a mouse model of COX-2-induced mammary cancer

Mallika Ghosh^a, Youxi Ai^a, Kirsi Narko^a, Zhenglong Wang^b, Jeffrey M. Peters^c, and Timothy Hla^{a,*}

^a Center for Vascular Biology, University of Connecticut Health Center, Farmington, CT 06030-3501, United States

^b Vascular Biology Center, Yale University School of Medicine, New Haven, CT 06510, United States

^c Department of Veterinary and Biomedical Sciences, Pennsylvania State University, University Park, PA 16802, United States

Abstract

Cyclooxygenase-2 (COX-2), overexpressed in inflammatory conditions and cancer, regulates angiogenesis and tumorigenesis via the production of biologically active prostanoids. Previously, we showed that COX-2 over-expression in the mammary gland of transgenic mice induces an angiogenic switch and transforms the mammary epithelium into invasive mammary carcinoma. Since COX-2-derived prostanoids can activate the nuclear receptor PPAR δ , we crossed *Ppar δ ^{-/-}* mice with COX-2 transgenic mice in the FVB/N background. PPAR δ was expressed constitutively in the mammary gland of virgin, pregnant and lactating mice. Mammary hyperplasia and tumorigenesis in the COX-2 transgenic mice was markedly reduced in the *Ppar δ ^{-/-}* mice compared to their wild type counterparts. Analysis of the mammary tissues indicated that immunoreactive Ki-67, cyclin D1 and phosphorylated histone 3 (Phospho H3) were reduced in *Ppar δ ^{-/-}* mice, suggesting that PPAR δ activation regulates cell proliferation in the mammary gland. We postulate that activation of the nuclear receptor PPAR δ by COX-2-derived prostanoids may be involved in the proliferation of mammary epithelial cells and therefore contribute to mammary cancer development.

Keywords

COX-2; PPAR; Mammary cancer; Peroxisomal proliferator-activated; receptor-delta

1. Introduction

Cyclooxygenase (COX)-2 is an inducible enzyme overexpressed in inflammation and cancer. Several lines of evidence support the concept that COX-2 plays a critical role in cancer development and progression [1,2]. Inhibitors of COX-2 and non-steroidal anti-inflammatory drugs which inhibit both COX isoenzymes, suppress cancer development in many animal models [3,4]. Epidemiological studies and clinical trials also indicate that inhibitors of COX-2 reduce cancer incidence and adenoma progression [5,10]. In addition, COX-2 is overexpressed in numerous cancer tissues including breast, colon, prostate, gastric, pancreatic and urinary bladder [2,6–8]. More importantly, exaggerated expression of COX-2 is a negative prognostic factor in breast cancer [9–11]. Genetic studies in which the *COX-2* (*Ptgs2*) gene was

*Corresponding author. Tel.: +1 860 679 4128; fax: +1 860 679 1201. E-mail addresses: hla@nso2.uchc.edu, hninyee@msn.com (T. Hla).

overexpressed or deleted in mice resulted in concomitant induction or suppression of neoplasia, respectively [12–14]. These studies strongly suggest that COX-2 expression in the tumor microenvironment is important for the progression of tumors.

We have shown that over-expression of human *COX-2* gene in the mammary gland of transgenic mice resulted in the formation of mammary tumors in multiparous mice [12]. Using this mouse model, we have also determined that COX-2 enzyme induces the angiogenic switch and epithelial cell hyperplasia [7]. In previous studies we documented the important role played by the major prostanoid metabolite of COX-2, namely PGE₂ in the induction of angiogenic response, and epithelial hyperplasia. Signaling by the cell surface receptor EP2 was shown to be critical for these responses [6].

The COX-2 enzyme is also known to activate nuclear receptors of the PPAR family. In particular, the PPAR δ receptor was shown to be activated prostacyclin and related products. We and others have shown that COX-2 pathway couples to the PPAR δ *in vitro* and *in vivo* [15–19]. The role of PPAR δ in mammary hyperplasia and cancer induced by COX-2 has not been examined. In this report, we assessed the role of the PPAR δ in the development of mammary cancer in MMTV-COX-2 transgenic mice. We show that PPAR δ activation by COX-2 is an important player in mammary tumorigenesis.

2. Materials and methods

2.1. Animals

COX-2 transgenic mice was generated as described previously [12]. *Ppar* $\delta^{-/-}$ mice in the 129/C57/BL/6 background developed by Peters et al. [20], was first back-crossed >5 times into the FVB/N background (Taconic Farms, NJ). *Ppar* $\delta^{-/-}$ mice obtained in $\geq 97\%$ FVB/N background were then crossed with the MMTV-COX-2 transgenic mice in FVB/N background to obtain *Ppar* $\delta^{-/-}$ COX-2-TG mice.

2.2. Whole mount staining and histological analysis

Mammary glands were dissected and processed for whole mount analysis as described. For histological analysis mammary glands embedded in paraffin were performed as described before [12]. Immunohistochemical staining was conducted with the following antibodies, PPAR δ (1:400, Santa Cruz), cyclooxygenase 2 (COX-2) (1:300, Cayman Chemical), phospho H3 (1:1000, Upstate) Ki-67 (1:200, Calbiochem), CyclinD1 (1:1000, Santa Cruz) with a Vectastain ABC kit (Vector) according to the manufacturer's instructions. The peroxidase staining was visualized with 3,3'-diaminobenzidine (Vector), and the sections were counterstained with methyl green.

2.3. Immunoprecipitation

In order to observe the expression of PPAR δ in *Ppar* $\delta^{-/-}$ mice and wild type mice, immunoprecipitated mammary gland extracts were detected with the PPAR δ antibody in an immunoblot assay. PPAR δ K-20 antibody (30 μ g; Santa Cruz) was cross-linked with 100 μ l of protein A beads. Protein extracts from 12-week-old virgin (5 mg), pregnant and lactating (10 mg) of *Ppar* $\delta^{-/-}$ and wild type mice were loaded onto the beads. The PPAR δ protein was eluted with 2 $\times$ sample buffer. Proteins were then loaded into 10% gel and transferred onto nitrocellulose membrane. The membrane was then incubated in 1:1000 PPAR δ K-20 and 1:10,000 rat anti-goat sequentially and visualized by the ECL reagent (Amersham).

2.4. Statistical analysis

Results are shown as mean $\pm$ S.E.M. Statistical significance was calculated by using Student's *t*-test.

3. Results

3.1. Expression of PPAR δ in the murine mammary gland

To establish the role of PPAR δ in COX-2 induced mammary tumorigenesis, we obtained *Ppar δ ^{-/-}* mice developed by Peters et al. [20] in the 129/C57BL/6 background and back-crossed to >5 times into the in FVB/N background. The resulting mice were crossed with the MMTV-COX-2 transgenic mice, which is in the FVB/N background. The *Ppar δ ^{-/-}* mice in the FVB/N background had small litters, consistent with the role of PPAR δ in uterine implantation [19,20].

To verify the expression of PPAR δ in different stages of mammary gland development, total protein was extracted from mammary gland of virgin, pregnant and lactating COX-2-TG or COX-2-TG/*Ppar δ ^{-/-}* mice and immunoprecipitated using the PPAR δ -specific K-20 antibody. Cytosolic extracts of HEK-293T cells transfected with either PPAR δ construct was used as positive control and PPAR γ construct as negative control. Fig. 1 shows that PPAR δ is expressed in virgin (V, 16 weeks), pregnant (P, 18 days pregnant) and lactating (L, 7 days postpartum) COX-2-TG mice but not in the *Ppar δ ^{-/-}* mice. The expression was constitutive and did not change during the development and involution of the mammary gland.

3.2. Decreased tumor incidence and hyperplasia in PPAR δ ^{-/-}COX-2-TG mice

We previously showed that multiparous COX-2-TG mice develop mammary hyperplasia and invasive mammary carcinoma [12]. Mammary glands from female COX-2-TG/*Ppar δ ^{-/-}* mice or COX-2-TG mice were isolated after three pregnancy/lactation cycles and whole mount preparations were stained and analyzed under a dissecting microscope. Tumor incidence was further confirmed by histological sectioning and H&E staining. As shown in Fig. 2A, marked reduction in tumor incidence was observed in COX-2-TG/*Ppar δ ^{-/-}* mice in comparison to the COX-2-TG mice. High tumor incidence was noticed in COX-2-TG mice (10 out of 11, 90%) while it was substantially lower in COX-2-TG/*Ppar δ ^{-/-}* mice (1 out of 7, 14%). Estimates of total tumor size per mammary gland was also markedly reduced in COX-2-TG/*Ppar δ ^{-/-}* mice, 0.0023 cm³ compared to COX-2-TG mice, 0.28 cm³.

Mammary glands were isolated from multiparous COX-2-TG/*Ppar δ ^{-/-}* mice in the weaned stage that have undergone multiple cycles of pregnancy and lactation. Whole mount analysis indicated alveolar development in the COX-2-TG mice whereas COX-2-TG/*Ppar δ ^{-/-}* mammary glands showed attenuated lobuloalveolar development. Histological sections indicated that adeno squamous-type, well differentiated mammary tumors were observed in COX-2-TG mice while the occurrence of epithelial hyperplasia and adenocarcinoma were markedly reduced in COX-2-TG/*Ppar δ ^{-/-}* mice. These observations suggest a critical role of the PPAR δ in mammary tumorigenesis.

3.3. Cell proliferation is attenuated in the mammary glands of COX-2-TG/*Ppar δ ^{-/-}* mice

The expression of PPAR δ , COX-2 and various markers of cell proliferation were examined in mammary tissues isolated from COX-2-TG or COX-2-TG/*Ppar δ ^{-/-}* mice. As shown in Fig. 3, PPAR δ expression was absent in COX-2-TG/*Ppar δ ^{-/-}* mice. As expected, COX-2 was expressed in an equivalent level in both genotypes. Ki-67, a nuclear protein expressed in proliferating cells was used as a standard index of overall cellular proliferation. Because histone 3 is phosphorylated rapidly cycling cells, phospho H3 staining provided a direct indication of mitotic activity. We observed a drastic reduction of Ki-67 and Phospho H3 immunoreactivity in the COX-2-TG/*Ppar δ ^{-/-}* mammary tissue sections compared to COX-2-TG mice. Furthermore, expression of CyclinD1, a mammary oncogene was also substantially reduced in the COX-2-TG/*Ppar δ ^{-/-}* mammary tissues. These observations suggest that the absence of

PPAR δ in COX-2-TG mice resulted in reduced cell proliferation of mammary tissues in COX-2-TG mice.

4. Discussion

In this report, we show that the PPAR δ receptor is critical for mammary tumorigenesis in the COX-2-TG mice. Our data indicate that mammary epithelium in COX-2-TG mice that lack the PPAR δ receptor contained less lobuloalveolar differentiation, hyperplasia and tumorigenesis. Markers of mammary epithelial cell differentiation were also down-regulated. These data suggest that PPAR δ is a critical mediator in mammary cancer development and is likely to regulate cell proliferation. The expression of PPAR δ was observed in mammary epithelial cells as well as stromal cells in the mammary gland. Thus, direct effect on epithelial cells and/or stromal activation, which could indirectly influence epithelial cell proliferation could be involved.

Our data suggest that activation of the PPAR δ receptor by prostanoid produced from the COX-2 pathway induces proliferation of mammary epithelial cells and thereby promote hyperplasia and progression of mammary cancer. Previous studies have shown that COX-2 over-expression induces PPAR δ activation in colon cancer cells [16]. We recently showed that endocannabinoid metabolism by the vascular endothelial cell COX-2 enzyme results in the activation of the PPAR δ receptor selectively [15]. Such a mechanism may be important in mammary tumorigenesis.

Our previous work has shown that COX-2 induced mammary tumor development is also dependent on the presence of the EP2 receptor [6,7]. This receptor is potently activated by PGE₂ which is one of the major prostanoids in the mammary gland. EP2 signaling regulated the cAMP-dependent activation of amphiregulin and vascular endothelial cell growth factor, which are potent growth factors for epithelial cells and vascular endothelial cells, respectively. Our results on the PPAR δ receptor raise the question about relative importance of EP2 and this nuclear receptor. It is likely that unique as well as overlapping pathways are regulated by both cell surface as well as nuclear receptors for prostanoids and thus contribute to the tumorigenic phenotype.

The role of the PPAR δ receptor in tumorigenesis remains unclear [21–24] and somewhat controversial particularly in colon carcinogenesis [22]. Although some studies using the PPAR δ knock-out mice and the use of PPAR δ agonists have suggested the pro-tumorigenic role, some studies have found that PPAR δ receptor knock-out mice have increased tumor development, especially in the intestinal system [16–18,25–30]. The reason for these apparent discrepancies is not well understood [21,31]. It is possible that factors such as genetic background and differences in tumorigenic models contribute to these apparent differences. However, further studies are required to examine the precise role of this pathway in tumor biology. Our results suggest that in the mammary gland, PPAR δ receptor has a pro-tumorigenic role.

In summary, we show that PPAR δ receptor knock-out mice exhibits an attenuated tumorigenic phenotype induced by COX-2 overexpression. Our data support the pro-tumorigenic role for PPAR δ receptor in mammary cancer.

Abbreviations

PPAR

peroxisome proliferator-activated receptor

COX

cyclooxygenase

MMTV

murine mammary tumor virus

MTT

3(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide

TG

transgenic

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Fig. 1.

PPAR δ is expressed at different stages of mammary gland development. Total protein extracted from 16-week-old virgin (V), 18-days pregnant (P), 7-day postpartum (L) from *Ppar* $\delta^{-/-}$ or wild type mice was immunoprecipitated and immunoblotted with the PPAR δ antibody as described. Cytosolic extracts of HEK293T cells transfected with PPAR δ or PPAR γ constructs served as positive (+) or negative (-) controls respectively.

Fig. 2.

Diminished tumor incidence in $Ppar\delta^{-/-}$ COX-2-TG mice. Panel A shows tumor incidence from COX-2TG ($N = 11$) and $Ppar\delta^{-/-}$ COX-2-TG ($N = 7$) mice observed after three cycles of pregnancy and lactation ($p < .01$). Panel B shows whole mounts of mammary glands as described in Section 2. Note the suppression of lobuloalveolar development in $Ppar\delta^{-/-}$ COX-2-TG mice. (Panel C–F) Hematoxylin and eosin sections of $Ppar\delta^{-/-}$ COX-2-TG mice and COX-2-TG mice (C and D—10 $\times$ magnification; E and F—40 $\times$ magnification).

Fig. 3. Reduced cell proliferation in the mammary gland of *Ppar δ ^{-/-}* COX-2-TG compared to COX-2-TG mice. Mammary tissues from *Ppar δ ^{-/-}* COX-2-TG (A, C, E, G and I) and COX-2-TG (B, D, H, and J) mice were analyzed for the expression of PPAR δ (A and B), COX-2 (C and D), Ki-67 (E and F), and CyclinD1 (G and H) Phospho H3 (I and J) by immunohistochemical staining. Sections were viewed with a 40 $\times$ objective lens.