The Role of PPARγ in Min-Induced Tumorigenesis and Its Relation to Pla2g2a

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ABSTRACT

Peroxisome proliferator-activated receptor gamma (PPARγ) is a member of the PPAR family of nuclear receptor transcriptional activators. This family is important in body-plan specification, cell differentiation, and regulation of metabolism. PPARγ has undergone various experiments to determine whether it plays a role as a tumorigenesis suppressor. Some studies have found supporting evidence indicating that PPARγ is a tumor suppressor in B6 mouse strains. However, other studies found refuting evidence, labeling PPARγ as a tumor inducer in mouse strains mutant for the adenomatous polyposis coli (Apc) gene. Phospholipase A2 group IIA (Pla2g2a) gene is a component of the Modifier of Min-1 (Mom-1) locus found on distal mouse chromosome 4. B6 mouse strains of inbred mice carry a mutant non-functional version of Pla2g2a. In one study, a wildtype Pla2g2a gene demonstrated tumor resistance in the presence of a mutant Apc gene. Based on the results from the previous experiments, we designed a genetic experiment to determine if PPARγ is a tumor suppressor in the B6-ApcMin/+ mouse line and to determine if PPARγ action in the Min-mouse genetically interacts with a wildtype Pla2g2a gene.

Introduction

PPARγ is a member of the peroxisome proliferator-activated receptor (PPAR) family. This is a family of lipid and organic acid-activated molecules that are a subgroup in the nuclear hormone receptor super-family, which is a large group of ligand-dependent transcription factors that play important roles in body-plan specification, cell differentiation, and regulation of metabolism. The γ isoform of PPAR (PPARγ) affects pathways important in a variety of human diseases through actions mediating the differentiation of a number of unrelated cell types (i.e. adipocytes, hepatocytes, fibroblasts, myocytes, breast and colon epithelial cells, and macrophages/monocytes) (11). PPARγ-mediated regulation of cellular differentiation may also be important in carcinogenesis.
Recent studies by Girnun (3), Gupta (5), Kitamura (6), and Sarraf (9, 10) suggest that dysregulation of PPARγ may play a major role in colon cancer. Girnun (3) conducted a study on mice by treating them with azoxymethane, a chemical that induces colon tumors, and comparing the number of colon tumors between wildtype PPARγ mice and heterozygous PPARγ knockout (KO) mice. The heterozygous KO mice were more susceptible to chemical tumorigenesis, due to a decrease in PPARγ expression compared to that of the wildtype mice (3). Girnun (3) found PPARγ to function as a tumor suppressor that acted at the initiation stage of tumor formation. In their study, PPARγ was also found to regulate β-catenin levels, which were higher in the heterozygous KO mice compared to those of the wildtype mice (3). This result is significant because dysregulation of β-catenin is found in most human colorectal cancer.

Gupta (5) identified target genes of PPARγ, which were found to include genes linked to growth regulatory pathways, colon epithelial cell maturation, and immune modulation. Their results indicated that PPARγ decreases cell growth through a delay in the G1 phase of the cell cycle (5).

Kitamura (6) investigated the role of PPARγ expression in cell growth and differentiation in six human colon cancer cell lines. PPARγ mRNA was expressed in all six lines and the PPARγ protein present was found to be functional through treatment with troglitazone, a PPARγ agonist drug. Troglitazone also decreased the S and G2/M phases of the cell cycle due to an increase in the G0/G1 phase. This suggests that ligand activation of PPARγ inhibits cellular growth and may induce cell cycle arrest at G1 in colon cancer (6). Kitamura (6) and Gupta (5) both validated the findings of Girnun (3), that PPARγ functions in the initiation stage.

Sarraf (9) transplanted tumors from human colon cells into mice and treated them with troglitazone. They reported that PPARγ not only reduced tumor growth, but also was found to promote the reprogramming of colon cancer cells toward a more differentiated, less-malignant phenotype (9). Further, they also found that troglitazone-mediated activation of human PPARγ mRNA in the transplanted colon tumors is greater than or equal to the PPARγ mRNA expression in normal colon epithelium cells. This high PPARγ mRNA expression was associated with a reduction in mRNA of other proteins, which are typically over-expressed in colon tumors.

After it was shown that PPARγ reduced murine xenografted tumors, Sarraf (10) conducted another study of loss-of-function PPARγ gene mutations in human colorectal cancers. They examined 55 human primary sporadic colorectal carcinoma samples and observed four somatic coding region mutations: one nonsense and two missense mutations in exon
5 of PPARγ, and one frameshift mutation in exon 3 (10). They compared the transcriptional activity of wildtype PPARγ to the mutants after these mutants had been exposed to troglitazone. The activity in the wildtype PPARγ was increased and the activity in the mutants was either decreased or absent. These results signified that colon cancer in humans is associated with loss-of-function mutations in PPARγ (10).

Although it is apparent that PPARγ does reduce tumor growth, three studies have also shown that in the presence of a mutant adenomatous polyposis coli (Apc) gene, where mice are predisposed to develop intestinal polyps, the ability of PPARγ to regulate colon tumorigenesis is completely lost. Girnun (3) confirmed these results by crossing PPARγ wildtype and heterozygous knockout mice with B6-Apc<sup>1638N</sup> mice, who carry a mutant Apc gene. Their results showed no significant difference in tumor number between the wildtype and heterozygous PPARγ knockout mice (3).

Saez (6) gave troglitazone to wildtype mice and to C57BL/6J-Apc<sup>Min</sup> mice. Results indicated that troglitazone did not notably influence tumorigenesis in normal mice. However, colon polyps significantly increased in the C57BL/6J-Apc<sup>Min</sup> mice treated with PPARγ agonists (6).

Lefebvre (7) also confirmed these results by treating B6-Apc<sup>Min/+</sup> mice with PPARγ agonists. They found an increase in tumor number in mice treated with synthetic PPARγ activators compared to tumors in untreated mice. They stated that PPARγ activators seem to promote tumorigenesis only when acting on cells or tissues that have acquired the appropriate genetic predisposition (7).

Notably, almost all of these studies of PPARγ have not relied on genetic tools to dissect PPARγ’s function in colon cancer. For example, use of drugs, either as agonists or antagonists of PPARγ, can induce PPARγ-independent phenotypes because these drugs are often pleiotropic. In addition, drug dosage may be inappropriate to model normal physiological processes. Use of defined genetic models, however, can powerfully elucidate the specific effects that certain genes have on one another.

Cormier and colleagues (1) conducted a genetic study using Pla2g2a and B6-Apc<sup>Min/+</sup> mice. B6-Apc<sup>Min/+</sup> mice carry a germline mutation in the Apc gene that predisposes them to the development of hundreds of intestinal tumors. The phospholipase A2 group IIA (Pla2g2a) gene is a component of the Modifier of Min-1 (Mom-1) locus found on distal mouse chromosome 4 (2). Mom1 confers resistance to intestinal tumorigenesis. The B6 strains of inbred mice carry a mutant non-functional version of Pla2g2a. Cormier’s group made a transgenic B6 mouse that carried a wildtype functional copy of Pla2g2a. When this Pla2g2a transgene was expressed in the B6-Apc<sup>Min/+</sup> strain, a significant reduction in intestinal tumorigenesis was observed. Tumor resistance was found to be gene
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dosage-dependent and was greater in the large intestine than in the small intestine. It was also observed that Pla2g2a was expressed in the goblet cells of the intestines, a factor which is important in maintaining colonic homeostasis (2).

The results by Girnun (3), Saez (6), and Lefebvre (7), where PPAR\(_\gamma\) failed to suppress colon tumorigenesis in the presence of a mutant Apc gene, is completely opposite from the results found by Cormier (2), where Pla2g2a resists tumorigenesis in the presence of a mutant Apc gene. The purpose of my research project is to understand the role of PPAR\(_\gamma\) in Min-induced tumorigenesis and to determine whether PPAR\(_\gamma\) ’s tumor resistance is dependent on the presence of other genetic factors such as Pla2g2a.

In most of the studies mentioned, mouse models were used. Mouse models will also be used in this experiment because there are benefits to using mouse models when doing genetic research. One advantage is that mice breed quickly, thus significant results can be obtained quickly. Further, mouse models are valuable for studying colon cancer because similar germ line mutations, such as the Apc gene, exist in humans and mice (4).

The mouse models used for this study include B6-Apc\(^{\text{Min}+}\) mice, B6-Pla2g2a transgenic mice, B6-PPAR\(_\gamma^{\text{F/FI}}\) mice, and B6-Villin-Cre-Tg mice. The PPAR\(_\gamma^{\text{F/FI}}\) mice are a strain of mice in which the PPAR\(_\gamma\) gene is flanked by 42 base pair inverted repeat sequences called LoxP sites. The Villin-Cre-Tg mice are a strain of mice in which the villin promoter, which is only expressed in the intestine, is attached to the Cre recombinase gene. Cre is a site-specific recombinase that recognizes LoxP sites and excises intervening sequences. As a result, when the PPAR\(_\gamma^{\text{F/FI}}\) mice are crossed with Villin-Cre-Tg mice, the Cre recombinase will cut out the PPAR\(_\gamma\) gene in the mouse intestine. This Cre-Lox technology is being used because PPAR\(_\gamma^{+/+}\) mice are not viable. The Cre-Lox technology allows the PPAR\(_\gamma^{+-}\) genotype to occur in the intestine only, creating viable offspring of mice.

Since it was shown that PPAR\(_\gamma\), when activated by agonist drugs on the B6 strain (which is naturally mutant for Pla2g2a), was not effective in B6-Apc\(^{\text{Min}+}\) mice, our hypothesis is that PPAR\(_\gamma\) will reduce tumorigenesis in B6-Apc\(^{\text{Min}+}\) mice in the presence of a wildtype Pla2g2a transgene. The design of our experiment will permit us to examine the effect of intestine-specific nullizygosity of PPAR\(_\gamma\) in the presence or absence of Pla2g2a.
Materials and Methods

Breeding Scheme

Establishment of test mice
1. Villin-Cre-Tg (B6;D2) mice will be backcrossed to three generations of C57BL/6J mice; resulting in B6-Villin-Cre-Tg (N4) mice.
2. B6-PPARγ<sup>FL/FL</sup> mice will be crossed to B6-Apc<sup>Min/+</sup> mice; resulting in B6-Apc<sup>Min/+</sup> PPARγ<sup>FL/+</sup> mice.
3. B6-Apc<sup>Min/+</sup> PPARγ<sup>FL/+</sup> mice will be crossed with B6-Villin-Cre-Tg (N4) mice; resulting in B6-Apc<sup>Min/+</sup>PPARγ<sup>FL/+</sup>Villin-Cre-Tg (N5) mice.
4. B6-PPARγ<sup>FL/FL</sup> mice will be crossed with B6-Pla2g2a<sup>AKR-Tg</sup> mice; resulting in B6-PPARγ<sup>FL/+</sup>Pla2g2a<sup>AKR-Tg</sup> mice.

Crossing of test mice
The B6-PPARγ<sup>FL/Fl</sup>/Pla2g2a<sup>AKR-Tg</sup> mice (#4) will be crossed with the B6-Apc<sup>Min/+</sup>PPARγ<sup>FL/+</sup>Villin-Cre-Tg (N5) mice (#3); resulting in twelve classes of mice.

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**Testing for PPARγ expression**

**Western-blot analysis**
Five mice which test positive for the Cre-recombinase will be randomly selected from each PPARγ class (PPARγ<sup>+/+</sup>, PPARγ<sup>+/−</sup>, and PPARγ<sup>−/−</sup>), totaling 15 mice. The colon tissues will be removed and prepared as previously described (2). The intestine will be removed and opened. It will then be washed in phosphate-buffered saline (PBS) and cut with a razor blade. The tissues will be placed in 1M acetic acid and stored at 4°C overnight. The samples will be centrifuged for 30 minutes at 4000rpm in a swinging bucket rotor. The supernatant will then be transferred to a clean tube and stored at -20°C. Western blot analysis of protein lysates from colonic epithelium will be conducted as previously described (2), using a PPARγ antibody (Santa Cruz Biotechnology). Signals will be quantitated using a Molecular Dynamics densitometer and software.

**Immunohistochemistry**
The colons will be longitudinally cut and rinsed in 1X PBS. They then will be washed and flash frozen. Immunohistochemistry on the frozen sections will employ a PPARγ antibody (Santa Cruz Biotechnology).

**Genotyping of mice**
Qiagen DNeasy (a commercial kit) will be used to isolate DNA from tail snips. Each genotype will be determined by PCR using the specified primers for that gene.

**Phenotyping of mice**

**Number of test mice per class**
Each of the twelve classes will consist of 15-20 mice to show a significant difference between genotypes. The two-sided non-parametric Wilcoxon Rank Sum test will be used to determine the statistical significance between the classes.

**Tissue isolation and tumor scoring**
CO<sub>2</sub> asphyxiation will be used to sacrifice all mice. The entire intestinal tract will be removed, ready and fixed as previously described (1,2). Intestines will be stained with a 0.2% methylene blue solution to aid with tumor scoring. The entire intestine will be scored for tumor number and size at 10X magnification as previously described (2). Tumor size will be estimated by measuring the maximum diameter of a tumor using a calibrated eyepiece reticle mounted on a Zeiss dissecting microscope. One
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scorer will score each tumor. A coding method will be used so that the
scorer is blind to the genotypes of the samples. Afterwards, each sample
will be decoded.

Results

Currently, results have not been obtained. The study will be
completed by May 2004.

Discussion

The first hypothesis to be tested is that PPARγ is a tumor
suppressor in the B6-ApcMin/+ mouse line. This will be determined by
comparing the B6-ApcMin/+PPARγ KO mice, the B6-ApcMin/+PPARγ
heterozygous KO mice, and the B6-ApcMin/+PPARγ wildtype mice. If the
hypothesis is supported, a predicted loss of PPARγ will result in an increase
in Min tumors. This finding would be in agreement with the opinions of
Girnun (3), Gupta (5), Kitamura (6), and Sarraf (9,10) that PPARγ is indeed
a tumor suppressor.

Girnun (3) reported that PPARγ was a dosage-dependent tumor
suppressor in the mice given azoxythane, but the dosage of PPARγ did
not matter when a mutant Apc gene was present. However, in Girnun’s (3)
study, the B6-Apc1638N mice used in their experiment develop only 3-5
tumors. Strong deviations in the results of the heterozygous PPARγ mice
could have affected the mean tumor number causing the heterozygous and
wildtype mean statistics to be similar. Fortunately, the fact that B6-ApcMin/+ mice develop hundreds of tumors will be beneficial to our study in
determining statistical significance.

A key factor to this first hypothesis is the PPARγ floxxed mouse
strain. No other study used the Cre-Lox technology to abrogate PPARγ
function strain. Notably, Girnun’s group used only heterozygous PPARγ
KO mice. It may be that a significant effect will be observed in mice that
are completely null for PPARγ.

Three other outcomes may be possible. It may be determined that
PPARγ’s tumor resistance is not dosage-dependent and the tumor count in
the wildtype and the heterozygous KO mice are comparable. Another
possibility is that PPARγ may be found to increase tumorigenesis,
supporting the results by Saez (8) and Lefebvre (7). Last, PPARγ may not
have any effect on tumor resistance in B6-ApcMin/+ mice, coinciding with
the results by Girnun (3).

A second hypothesis is that PPARγ action in the Min-mouse
genetically interacts with a wildtype Pla2g2a gene. This will be
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determined through ingression of the B6-\(Apc^{Min/+}\) strain that are either positive or negative for a \(Pla2g2a^{AKR}\) transgene into the PPARγ floxxed strain, the heterozygous PPARγ KO strain, and the wildtype PPARγ strain.

Saez (8) and Lefebvre (7) concluded that PPARγ is a tumorigenesis enhancer when B6-\(Apc^{Min/+}\) mice are treated with PPARγ agonists. However, their results may have been affected by the use of drugs that can exert pleiotropic effects. Moreover, since B6 mice are naturally mutant for Pla2g2a, if PPARγ’s tumor suppression depends on Pla2g2a this phenotype would not be observed.

Since Cormier (1) proved that the \(Pla2g2a\) gene does cause tumor suppression in the B6-\(Apc^{Min/+}\) strain, it is proposed that Pla2g2a will affect mutual pathways in the B6-\(Apc^{Min/+}\) strain.

If the second hypothesis holds true, there should be a greater reduction in tumorigenesis in the presence of both Pla2g2a and PPARγ. A corollary hypothesis is that Pla2g2a’s robust tumor suppression would be weakened or completely abrogated in mice that are null for PPARγ.

An alternative outcome would be that PPARγ is not a tumor suppressor in the presence of Pla2g2a, resulting in comparable tumor counts to the strains containing only Pla2g2a. It is also possible that interaction between PPARγ and Pla2g2a could cause an increase in tumorigenesis due to the conflicting interaction between the two genes.

These two hypotheses will be supported or refuted when the results of the test classes are compared to the various controls in the study. The study will use comparison controls to determine whether a particular gene increases or decreases tumorigenesis. The B6-\(Apc^{Min/+}PPARγ^{+/+}\) strain will be compared to the B6-\(Apc^{Min/+}PPARγ^{+/+}Pla2g2a^{AKR}\)-Tg strain to verify that Pla2g2a decreases tumorigenesis. The B6-\(Apc^{Min/+}PPARγ^{+/+}\) will also be compared to the B6-\(Apc^{Min/+}PPARγ^{FIP1}Villin-Cre-Tg\) strain to test whether PPARγ is a factor in tumor resistance in Min mice. Comparing the B6-\(Apc^{Min/+}PPARγ^{+/+}Pla2g2a^{AKR}\)-Tg strain to the B6-\(Apc^{Min/+}PPARγ^{FIP1}Villin-CrePla2g2a^{AKR}\)-Tg strain will examine whether PPARγ does or does not interact with Pla2g2a in reducing tumorigenesis. The heterozygous classes in the study will not be used as comparative controls; however, they are important in determining if PPARγ is dosage-dependent.

Determination of statistical significance will be done using the two-sided non-parametric Wilcoxon Rank Sum test. This test is used when testing the equality of means of two continuous distributions that are non-normal, and samples are independent (12). The means and standard deviations will be calculated and the test classes will be compared to determine if there is a significant difference. The test will offer evidence in deciding if PPARγ and/or Pla2g2a increases or decreases tumor number.
Works Cited


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