Abstract. Peroxisome proliferator-activated receptors (PPARs) are ligand-activated transcription factors with essential functions in regulating lipid metabolism. Both the PPARβ (also referred to as PPARδ) and PPARγ subtype have been reported to either attenuate or potentiate tumorigenesis in a number of different models of intestinal and skin carcinogenesis. In the present study, we have addressed the role of PPARβ and PPARγ in lung tumorigenesis in a transgenic mouse model of RAF-induced lung adenoma using two different strategies: i) crossing with PPARβ null mice, and ii) chronic treatment with the PPARγ agonist rosiglitazone. Histological examination revealed a significant enhancement of tumor growth in mice lacking one or both alleles of Pparb, but no significant effect in response to rosiglitazone. These observations indicate i) that RAF-induced lung tumorigenesis is attenuated in mice with a disrupted Pparb gene, and ii) that chronic PPARγ activation does not affect lung adenoma growth. These results are relevant with respect to the clinical application of drugs modulating the activity of PPARβ or PPARγ.

Introduction

Peroxisome proliferator-activated receptors (PPARs) are transcription factors of the class of nuclear receptors that modulate target gene expression in response to endogenous and exogenous ligands (1-6). The PPAR family consists of three members, PPARα PPARβ/δ and PPARγ, whose major physiological functions are associated with the regulation of lipid metabolism. Intriguingly, all PPAR subtypes have also been associated with tumorigenesis. While PPARα has a species-specific role in rodent hepatocarcinogenesis, PPARβ and PPARγ have been implicated in a number of different types of cancer. Consistent with the physiological function of PPARβ in cell proliferation and differentiation in diverse cell types, including keratinocytes (7-9), trophoblastic cells (10) and intestinal epithelial cells (11), a role for PPARβ in chemically-induced skin carcinogenesis (12) and intestinal tumorigenesis (13-17) have also been described, although its precise function remains controversial. PPARγ is of major biomedical interest, since it is the target of the thiazolidinedione anti-diabetic drugs (such as rosiglitazone), and also because it is able to exert anti-oncogenic effects in diverse model systems. Activation of PPARγ inhibits tumor cell proliferation in vitro, suppresses tumor growth in mouse models and induces tumor cell apoptosis in vivo and in vitro (reviewed in refs. 18,19). However, similar to the function of PPARβ in carcinogenesis, there are also inconsistent reports suggesting that PPARγ may potentiate tumorigenesis as well. For example, administration of the PPARγ agonists BRL-49,653 and troglitazone to Apc Min mice paradoxically enhances the formation of intestinal polyps (20,21). Further, the targeted disruption of Pparg in mammary epithelium has no effect on tumor incidence (22). The role of PPARs using in vivo lung tumor models has not been analyzed to date. In the present study, we report the effects of modulating PPARβ or PPARγ activity in a transgenic mouse model of RAF-induced lung adenoma.

Materials and methods

Mouse experiments. Pparb−/− mice (12) and c-Raf-1-BxB (23) mice have been previously described. All experiments were performed with mice backcrossed with the C57BL/6N strain. Genotyping was performed by PCR. Rosiglitazone maleate (Avandia, Glaxo-Smith Kline-Beecham) was suspended in water and applied to mice per os at a dose of 30 mg/kg body.
weight once per day (24). Treatment was started at the age of 4 weeks and was continued for another 2 months until the end of the experiment.

**Histological evaluation.** Lungs were fixed in neutral buffered 10% formalin for 24 h and embedded in paraffin. Hematoxylin-eosin stained sections (3–4-μm thick) were viewed under a light microscope. Tumor nodules were identified as nodules of cuboid epithelial cells containing slightly enlarged and minimally atypical nuclei (23) some of which exhibited intranuclear inclusions. Tumors were classified according to their size into: i) small tumors (diameter corresponding to that of 1-2 average alveolar lumina), ii) intermediate-size tumors (diameter corresponding to 3-5 average alveolar luminal diameters), and iii) large tumors (diameter exceeding 5 average alveolar luminal diameters). For each animal, one section through one lung was used for morphological analysis and counting of the tumor nodules. For an assessment of the tumor load per lung, a tumor score was defined which was calculated according to the following formula: Score = tumor nodules per lung x F, where F=1 for small tumors, F=2 for intermediate-size tumors and F=3 for large tumors. The total area (including air spaces) of the lung tissue sections evaluated was measured morphometrically (Image-Pro Plus; Media Cybernetics, Silver Spring, MA, USA). Tumor numbers and tumor score values were related to the evaluated lung tissue area.

**RNA isolation.** RNA was isolated using the RNaseasy™ kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. Qia shredders (Qiagen) were used to break down tissue. cDNA was synthesized using 1 μg genomic DNA of lysed cells.

**Protocol.** Qia shredders (Qiagen, Hilden, Germany) following the manufacturer's protocol. RNA was isolated using the RNeasy™ kit for RNA isolation from the tissue area.

**Analysis and counting of the tumor nodules.** For an assessment of the tumor load per lung, a tumor score was defined which was calculated according to the following formula: Score = tumor nodules per lung x F, where F=1 for small tumors, F=2 for intermediate-size tumors and F=3 for large tumors. The total area (including air spaces) of the lung tissue sections evaluated was measured morphometrically. Tumor numbers and tumor score values were related to the evaluated lung tissue area.

**Real-time PCR (qPCR).** cDNA was synthesized using 1 μg of RNA, oligo(dT) primers and reverse transcriptase according to the manufacturer's protocol (Roche Diagnostics). qPCR reactions were carried out using the Absolute QPCR SYBR Green Mix (Abgene, Hamburg, Germany) at a primer concentration of 0.2 μM in a Mx3000P Real-Time PCR system (Stratagene, La Jolla, CA) for 45 cycles. Primers and annealing conditions were as described in Table I.

**Results**

**Effect of Pparb disruption of RAF-induced lung tumors.** c-Raf-1-BxB mice have an activated C-RAF transgene driven by the surfactant protein-C (SP-C) promoter (23). These mice develop multiple benign adenomas in type II pneumocytes within 3 months after birth (23). We used this strain to investigate whether PPARß plays a role in lung cancer. The c-Raf-1-BxB is of particular interest in this context, since previous studies have shown that PPARß expression can be induced by the Ras-RAF-Erk signaling pathway in different cell types in vitro (25,26).

To study the role of PPARß in RAF-induced lung cancer we crossed c-Raf-1-BxB onto a Pparb-/- background. The Pparb-/- mice used for this experiment harbor a phospho-riboseyltransferase II expression cassette in the last exon of the Pparb gene resulting in a lack of PPARß protein expression (12,14). c-Raf-1-BxB and Pparb-/- mice were crossed to obtain transgenic c-Raf-1-BxB positive mice on different PPARß genetic backgrounds. Pparb wild-type, Pparb+/- and Pparb-/- were analyzed for lung tumor development (n=28). Histological examination revealed a clearly increased incidence of lung adenomas in both Pparb+/- and Pparb-/- mice. While the average tumor incidence in Pparb wild-type mice was 1.37±0.45 nodules/mm², this was increased to 2.95±1.53 in Pparb+/- mice and 2.72±0.92 in Pparb-/- mice. These differences are statistically highly significant (wild-type versus heterozygous: P=0.010; wild-type versus null: P=0.002; Fig. 1A).

We also defined a tumor score taking the tumor size into account (for details see Materials and methods). Similarly, a significant increase was observed in both Pparb+/- and Pparb-/- mice: the average tumor score was 2.85±0.97 in Pparb wild-type mice, which was increased to 6.17±3.15 and 6.43±2.45 in Pparb+/- and Pparb-/- mice, respectively (wild-type versus heterozygous: P=0.009; wild-type versus null: P=0.002; Fig. 1B). We analyzed lungs both of 3 and 6 months old mice with essentially the same result (not shown). These data clearly indicate that PPARß attenuates the growth of C-RAF induced lung adenomas, similarly to the inhibitory effect of PPARß in intestinal adenoma (14,17).

**Pparb has been described as a target gene of the RAS pathway in cultured intestinal epithelial cells and fibroblasts (25,26). To determine whether Pparb expression was elevated in c-Raf-1-BxB lungs in vivo, we performed qPCR analysis to quantify mRNA transcripts for PPARß. No differences in Pparb mRNA were detectable in lungs from c-Raf-1-BxB mice compared to C-RAF transgene negative mice, indicating that RAF signaling does not regulate Pparb expression in alveolar cells in vivo (Fig. 2).
Effect of PPARγ activation on RAF-induced lung tumors. In most experimental systems used to date, PPARγ inhibits tumorigenesis, e.g. by inhibiting tumor cell proliferation and inducing apoptosis (reviewed in refs. 18,19). However, there are other findings that are inconsistent with this view including the observation that Apc Min mice treated with a PPARγ agonist exhibit increased intestinal adenoma growth (20,21). To evaluate whether a PPARγ agonist would influence the growth of lung adenomas in c-Raf-1-BxB mice, rosiglitazone maleate was administered orally to Raf-1-BxB mice for a period of 2 months (30 mg/kg/d) essentially as described (24). The experiment was initiated at the age of 4 weeks when lung tumors in Raf-1-BxB mice were just beginning to become microscopically detectable. At the end of the experiment, mice were euthanized, and tumor incidence and tumor score were determined as above. Neither tumor incidence (P=0.78) nor tumor score (P=0.87) showed any significant difference among treated and untreated mice (Fig. 3). We also measured expression of the PPARγ target gene CD36 and found a significant induction in rosiglitazone treated animals, demonstrating that the treatment regimen was effective at the molecular level (data not shown). These data indicate that in contrast to intestinal carcinogenesis, chronic PPARγ activation has no influence on lung adenoma growth.

Discussion

The present studies investigated the role of PPARγ and PPARβ in RAF-induced lung tumorigenesis using two different approaches: the genetic disruption of Pparb and the administration of the PPARγ agonist rosiglitazone. Previous reports suggested that PPARβ can either attenuate or potentiate tumorigenesis in a number of different models (reviewed in ref. 27). Results from the present study indicate that PPARβ is not required, but rather has an inhibitory effect on C-RAF-induced lung cancer. This is consistent with reports showing that PPARβ is dispensable for, or inhibitory to, intestinal tumorigenesis in both APCMin mice (13,14) and a mouse model based on a defect in mismatch DNA repair (28). Our observations are consistent with previous findings showing that PPARβ regulates terminal differentiation and has a negative regulatory role in the proliferation of certain cell
types, including keratinocytes (7-9), trophoblast giant cells (10) and intestinal epithelial cells (11). Collectively, these findings suggest that PPARβ is unlikely to possess pro-oncogenic properties, but rather seems to have a negative regulatory role in tumor growth. This is important in view of ongoing efforts to develop PPARβ agonists for the treatment of metabolism-related human diseases, in particular in view of disparate results regarding the effect of such drugs on intestinal adenoma growth in the mouse (16,17).

In contrast, administration of the PPARγ ligand rosiglitazone had no detectable influence on C-RAF-induced lung carcinogenesis. This is consistent with previous findings that the genetic status of Pparg has no effect on mammary tumor development in mice (22). On the other hand, other reports suggest that PPARγ can inhibit tumorigenesis-related processes in lung cancer model systems in cell culture (reviewed in ref. 19). Different explanations may account for this disparity, in particular differences in the model system used (in vivo versus in vitro) and differences in the oncogenic alterations mediating the carcinogenic effect. It thus remains possible that ligand activation of PPARγ could modulate lung carcinogenesis triggered by other oncogenic signaling pathways, as suggested by the paradoxical exacerbation of intestinal tumorigenesis by PPARγ agonists in ApcMin mice (20,21).

Alternatively, or additionally, the role of PPARγ in carcinogenesis is cell type-specific. Finally, PPARγ independent effects by PPARγ agonists on gene expression, cell proliferation and apoptosis have been described in several studies (29-35), including the inhibition of translation initiation through inactivation of eukaryotic initiation factor 2 (eIF2) (29) which might explain some of the anti-oncogenic effects of this class of PPARγ agonists and question the mechanistic interpretation of a number of published studies. Clearly, more experimental work on defined systems is required to clarify the role of PPARγ in tumor development and growth. This is particularly relevant with respect to the development and clinical application of drugs modulating the activity of PPARβ or PPARγ.

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References


Figure 3. Effect the PPARγ agonist rosiglitazone on the growth of RAF-induced lung adenomas. Effect of PPARγ activation on lung adenoma growth. Tumor incidence (A, expressed as nodules/mm²) and tumor score (B, for details see Materials and methods) were determined in untreated c-Raf-1-BxB mice (con) and in mice treated with the PPARγ agonist rosiglitazone (Rosi) for 2 months at a dose of 30 mg/kg/d.


