Inhibition of adult liver progenitor (oval) cell growth and viability by an agonist of the peroxisome proliferator activated receptor (PPAR) family member γ, but not α or δ

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Multifaceted evidence links the development of liver tumours to the activation and proliferation of adult liver progenitor (oval) cells during the early stages of chronic liver injury. The aim of this study was to examine the role of the peroxisome proliferator activated receptors (PPARs): PPARα, δ and γ, in mediating the behaviour of liver progenitor cells during pre-neoplastic disease and to investigate their potential as therapeutic targets for the treatment of chronic liver injury. We observed increased liver expression of PPARα and γ in concert with expanding oval cell numbers during the first 21 days following commencement of the choline deficient, ethionine supplemented (CDE) dietary model of carcinogenic liver injury in mice. Both primary and immortalized liver progenitor cells were found to express PPARα, δ and γ, but not γ2, the alternate splice form of PPARγ. WY14643 (PPARα agonist), GW501516 (PPAR8 agonist) and ciglitazone (PPARγ agonist) were tested for their ability to modulate the behaviour of p53-immortalized liver (PIL) progenitor cell lines in vitro. Both PPAR8 and γ agonists induced dose-dependent growth inhibition and apoptosis of PIL cells. In contrast, the PPARα agonist had no effect on PIL cell growth. None of the drugs affected the maturation of PIL cells along either the hepatocytic or biliary lineages, as judged by their patterns of hepatic gene expression prior to and following treatment. Administration of the PPARγ agonist ciglitazone to mice fed with the CDE diet for 14 days resulted in a significantly diminished oval cell response and decreased fibrosis compared with those receiving placebo. In contrast, GW501516 did not affect oval cell numbers or liver fibrosis, but inhibited CDE-induced hepatic steatosis. In summary, PPARγ agonists reduce oval cell proliferation and fibrosis during chronic liver injury and may be useful in the prevention of hepatocellular carcinoma.

Introduction

The regenerative response of adult liver to injury is typically characterized by the repeated division of mature hepatocytes. However, under circumstances of prolonged or repeated liver injury, the damage sustained by hepatocytes often prevents their replication. In such cases, liver regeneration is accomplished through the coordinated proliferation and differentiation of adult liver progenitor (oval) cells. These cells have been identified in many different experimental injury models and also in human liver conditions such as chronic Hepatitis B and C, alcoholic liver disease and hereditary haemochromatosis (for reviews see (1,2)). In vitro and in vivo studies have conclusively shown the ability of these cells to undergo maturation into both biliary epithelial cells and hepatocytes (3). They also possess a high rate of proliferation, a feature typical of tissue-determined progenitor cells (4–6). Thus, oval cells represent a critical regenerative cell compartment in adult liver. In addition to their beneficial role, these cells have also been linked to the progression of chronic liver disease towards hepatocellular carcinoma (HCC). Most of the liver injury conditions in which liver progenitor cells are observed are associated with long-term risks for development of HCC, and a positive correlation exists between progenitor cell numbers and the stage of disease progression (7–9). They are easily transformed in culture, giving rise to solid tumours when injected into immune-challenged mice (10,11). They are phenotypically similar to hepatoma cells, and share the expression of several key oncodevelopmental marker genes (12–14). Finally, work in our laboratory has shown that inhibition of oval cell proliferation in mice, through gene knockout or drug treatment, results in a decreased risk for liver tumour formation (15).

These findings suggest that inhibition of the oval cell response during the early stages of chronic liver disease might delay or prevent the onset of liver tumorigenesis. Thus, understanding the pathways that govern the behaviour of the oval cell compartment is of particular interest, as these factors may represent novel therapeutic targets for the treatment of patients with chronic liver conditions that predispose them to development of HCC. At present, our knowledge of the mechanisms that control the oval cell response is limited. Several cytokine pathways have been implicated [for a review see (1)], however, in most cases definitive evidence for their involvement is lacking. Furthermore, although of scientific interest, cytokine systems have been generally found to make poor therapeutic targets, limiting the usefulness of these findings in a clinical setting.

The peroxisome proliferator activated receptors (PPARs) are nuclear receptors of the steroid receptor superfamily. They play a central role in the regulation of dietary lipid metabolism and fat storage in mammals. There are three members of the superfamily: α, δ (also sometimes referred to as β) and γ. They are structurally related and associate with the same nuclear co-receptor (retinoid x-receptor or liver x-receptor). The active

Abbreviations: CDE, choline deficient, ethionine supplemented; CK, cytokeratin; DMSO, dimethylsulphoxide; HCC, hepatocellular carcinoma; HSC, hepatic stellate cell; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide; PI, propidium iodide; PIL, p53-immortalized liver; PPAR, peroxisome proliferator activated receptor; SMA, smooth muscle actin.

Carcinogenesis vol.26 no.10 © Oxford University Press 2005; all rights reserved.
receptor complex binds to a peroxisome proliferator response element, which comprises two direct repeats of a hexad DNA motif, separated by a single nucleotide (16). This recognition sequence is common to all three PPAR isofoms; however, the relative affinity of each isoform to the peroxisome proliferator response element can be modulated through minor changes in the hexad DNA sequence or the presence of certain flanking 5’ sequences (16).

The PPAR isofoms have distinct patterns of expression across the different mammalian cell and tissue compartments (17). In accordance, each isoform differs in its function and cellular localization within the adult liver. PPARα is expressed by hepatocytes in both rodent (18) and human liver (19). In rodents, its activation is thought to promote hepatocyte proliferation both in vitro (20) and in vivo (21–23), although it should be noted that some conflicting data also exist (24). PPARα agonists are also known to promote hepatocarcinogenesis in rodent liver [for a review see (16,25)]. However, in human hepatocytes, no pro-proliferative or tumorigenic effects of PPARα activation have been observed. The mechanism underlying this discrepancy is not known at present; however, it appears to be related to the transcriptional activation of key downstream targets rather than the level of receptor expression (26).

In contrast to PPARα, PPARγ is expressed primarily by hepatic stellate cells (HSCs) and macrophages (Kupffer cells) in the adult liver (27,28). Its activation exerts anti-proliferative effects in hepatoma cells as well as in oval cell lines in culture (29–31). Administration of PPARγ agonists to mice has been reported to reduce liver injury, fibrosis and steatosis during experimentally induced non-alcoholic steatohepatitis (32) as well as to have beneficial effects on human patients with fatty liver disease (33). PPARδ has a relatively poorly characterized role in liver. Its pattern of expression has not been extensively studied, however like PPARγ, it is expressed by HSCs (34). Activation of PPARδ has been reported to augment hepatoma cell proliferation in vitro (35) and HSC proliferation during liver injury (34). In addition, a recent study found that targeted activation of PPARδ in adipose tissue dramatically improves fat accumulation in the liver of ob/ob mice, which are genetically predisposed to obesity (36).

These studies clearly support a role for all three PPAR isofoms in mediating the liver’s response to injury. However, to date, very little is known about the role of PPAR signalling in mediating the behaviour of the liver progenitor cell compartment. The aim of the current study was to examine the role of PPARα, γ and δ in mediating the oval cell response in vivo and in vitro, using experimental models developed in our laboratory.

Materials and methods

Animals

Male mice of the C57Bl/6 inbred strain were purchased from the Animal Resources Centre of Western Australia and housed at the Fremantle Hospital Animal Care Facility in accordance with the guidelines recommended by the National Health and Medical Research Council of Australia and the University of Western Australia Animal Ethics Committee. Five-week-old mice were fed a diet consisting of either normal chow and drinking water (control-diet) or a diet consisting of either normal chow and drinking water supplemented with 0.15% (w/v) ethionine (Sigma, USA) [choline deficient diet (CDE) diet, (37)]. Following 2, 5, 7, 14 or 21 days on the diet, animals were killed and saline-perfused liver portions collected for RNA or histological analysis.

For in vivo drug studies, ciglitazone (Biomol, Plymouth meeting, PA, USA) was dissolved in absolute ethanol at a final concentration of 20 mg/ml. GW501516 (CalBiochem, San Diego, CA, USA) was dissolved in dimethyl sulphoxide (DMSO) at a final concentration of 50 mg/ml. Aliquots (100 µl) of GW501516 or ciglitazone were stored at –20°C. Paired 100 µl aliquots of placebo (ethanol for the ciglitazone study, DMSO for the GW501516 study) were also prepared and stored at –20°C. Immediately prior to each injection, an aliquot of each (drug and placebo) was thawed and made up to 1.2 ml with sterile saline (with vortexing). Diluted drug (9 µg/ml body wt) was administered to mice via intraperitoneal injection every second day, giving a final dose of 20 or 50 mg/kg/48 h, for ciglitazone and GW501516, respectively.

Cell lines and primary oval cell isolation

Two murine p53-immortalized liver (PIL) progenitor cell lines were used in these studies. These cell lines were created in our laboratory from primary cultures of isolated oval cells, which spontaneously immortalized due to their deficiency in p53 (11). They have been extensively characterized and successfully employed in several recent publications to study the role of growth factor signalling on cultured oval cells (38,39). PIL-1 and PIL-2 cells were maintained at 37°C, 5% CO2 in William’s E medium supplemented with penicillin-streptomycin (Invitrogen, Carlsbad, CA, USA), Fungizone (Invitrogen) and 2–5% fetal bovine serum (Invitrogen). Medium was replaced every second day.

Primary oval cell cultures were prepared from the livers of mice fed with the CDE diet for 2 weeks using a Percel (Amersham-Phamacia, Buckinghamshire, UK) based method, as described previously (31). The cultures were determined to be ~80% pure by immunocytochemical analysis for the oval cell antigen, A6. The contaminating cells were primarily endothelial cells, with occasional Kupffer cells (positive for F4/80) and activated HSCs [positive for alpha smooth muscle actin (SMA)] also apparent. No hepatocyte contamination was observed. Following adherence to collagen-coated dishes, oval cell isolates were maintained in William’s E medium, supplemented as described previously, and RNA isolated following 48 h.

MTT assay for viable cells

Cells were seeded at an approximate density of 3000 cm−2 in 96-well plates and allowed to adhere overnight. The following morning, cells were re-fed with fresh medium containing either drug (0.5–100 µM dissolved in DMSO) or vehicle (DMSO alone). Treated cells were cultured for 72 h, with one change of medium (at 48 h). At the end of the 72 h incubation period, cell viability was assayed using a standard assay based on the conversion of 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) (5 mg/ml; Sigma) to a coloured formazan product (40).

Apoptosis assay

For analysis of programmed cell death, cells were seeded at a final density of 5000 cm−2 in chamber slides (BD Biosciences, USA) and allowed to adhere overnight. The following morning, cells were re-fed with fresh medium containing 15 µM ciglitazone or 50 µM GW501516 dissolved in DMSO) or vehicle (DMSO alone). Cells were incubated for 6–72 h and apoptosis analysed by staining for Annexin-V FITC (Molecular Probes, Eugene, OR, USA) and propidium iodide (PI, Sigma) according to the directions recommended in the Annexin-V product manual (Molecular Probes). Positive cells were visualized on a Nikon TE2000-U fluorescence microscope and cells positive for either Annexin-V only (green cytoplasmic stain), PI only (red nuclear stain) or both markers (green cytoplasm plus red nucleus) counted relative to total cell numbers (observed under phase-contrast). Ten fields of view were counted per sample, using a 40× objective. Final apoptotic cell counts were averaged and expressed as % of total cells. Only cells positive for Annexin-V were considered to be apoptotic.

Analysis of gene expression

RNA was isolated from cells or whole tissue using Ultraspec (Fisher Biotech, Australia), exactly as recommended by the manufacturer. Total RNA was DNase treated using the DNA-free kit (Ambion, Austin, TX, USA) and reverse transcribed to cDNA using the Thermoscript reverse transcription system (Invitrogen), according to the manufacturer’s instructions.

Levels of the PPAR isofoms: α, δ, γ and y2 in cDNA samples were determined by RT-PCR, either using conventional thermocycling or in real-time, incorporating SYBR1 green (Molecular Probes) at a final concentration of 3× to monitor production formation. Conventional PCR was performed using a DNA Engine (MJ Systems, Waltham, MA, USA). Real-time PCR was performed using a RotorGene (Corbett Biosystems, Sydney, NSW, Australia) and allowed to adhere overnight. The following morning, cells were re-fed with fresh medium containing 15 µM ciglitazone or 50 µM GW501516 dissolved in DMSO) or vehicle (DMSO alone). Cells were incubated for 6–72 h and apoptosis analysed by staining for Annexin-V FITC (Molecular Probes, Eugene, OR, USA) and propidium iodide (PI, Sigma) according to the directions recommended in the Annexin-V product manual (Molecular Probes). Positive cells were visualized on a Nikon TE2000-U fluorescence microscope and cells positive for either Annexin-V only (green cytoplasmic stain), PI only (red nuclear stain) or both markers (green cytoplasm plus red nucleus) counted relative to total cell numbers (observed under phase-contrast). Ten fields of view were counted per sample, using a 40× objective. Final apoptotic cell counts were averaged and expressed as % of total cells. Only cells positive for Annexin-V were considered to be apoptotic.
Histology
Specimens of liver tissue (3 mm × 3 mm × 3 mm) were immersed in formalin overnight, processed through graded ethanol and xylol and paraffin embedded. Liver specimens were also embedded in OCT compound and snap-frozen in liquid nitrogen. Sections (5 μm) were prepared from paraffin or frozen blocks and adhered to coated slides (Superfrost Plus; Menzel, Braunschweig, Germany). Paraffin sections were then dehydrated through xylene and graded alcohols, and rehydrated in tap water. Frozen sections were rehydrated in saline.

Hydrated sections were stained with haematoxylin and eosin (Sigma), Masson’s Trichrome (41) or immunohistochemically, as described below. Following staining, sections were mounted in Kaiser’s glycerol gelatin (Merck, Whitehouse Station, NJ, USA) and examined under a Nikon TE2000-U inverted phase microscope (Nikon, Japan).

Immunohistochemistry, oval cell counts and SMA scoring
Immunohistochemistry was performed to detect the presence of the oval cell antigen A6 or α-SMA, a marker of activated HSCs. Both stains were performed exactly as described previously (15,37,42). Oval cell numbers were quantified by counting the number of A6 positive cells in 10 sequential fields of view using a ×40 objective. Positive cell numbers were normalized to the number of hepatocytes present in each field. Semi-quantitative SMA scoring was performed according to our method published previously (42).

Data analysis
For comparison of two datasets, data were analysed using the Student’s t-test (for n > 5) or the non-parametric Mann–Whitney U test (for n < 5). For comparison of three or more datasets, analysis was performed using one-way ANOVA. A P-value of ≤0.05 was considered significant.

Results
Hepatic expression of PPARα and γ is increased during experimental chronic liver injury in mice fed a CDE diet
Hepatic levels of PPARα and γ mRNA remained at control-diet levels for the first week of CDE treatment. However, after day 7, levels of both isoforms rose rapidly. PPARα expression in CDE-fed liver increased to approximately five times that of control-diet liver by 14 days, and reached nearly eight times control-diet levels by day 21 (Figure 1A). PPARγ expression was upregulated ~3-fold in CDE-fed liver samples at both days 14 and 21 of the diet time course (Figure 1B).

Levels of PPARδ mRNA were below the threshold for detection in the majority (>80%) of samples (data not shown). As such, the pattern of PPARδ expression during the CDE time course could not be determined using the real-time RT–PCR assay developed for this project.

Oval cells express PPARα, δ and γ, but not γ2
To determine the pattern of PPAR isoform expression in oval cells, two immortalized murine oval cell lines (PIL-1 and PIL-2), as well as two primary isolates of oval cells, were subjected to RT–PCR for each PPAR subtype. PPARα, δ and γ were expressed in both the PIL cells and primary oval cell extracts (Figure 2). The alternate splice variant of PPARγ, PPARγ2, was not detected in any of the samples (Figure 2).

PPARδ and γ agonists induce dose-dependent growth inhibition and apoptosis in oval cell lines
Natural and synthetic ligands for each of the PPAR isoforms have been identified. These can be used to study the effects of PPAR activation in vitro and in vivo. To determine the effect of PPAR activation in cultured oval cells, PIL cells were exposed to various concentrations of WY14643 (PPARα agonist), GW501516 (PPARδ agonist) or ciglitazone (PPARγ agonist), and their growth and viability assayed following 3 days, compared with a vehicle control (Figure 3). WY14643 had no effect, even at concentrations approaching 100 μM (Figure 3A). Both GW501516 (Figure 3B) and ciglitazone (Figure 3C) invoked dose-dependent cell death in the PIL cell cultures. Based on these data, the ED50 for growth inhibition by these agents was estimated to be ~50 and 15 μM for GW501516 and ciglitazone, respectively.
To determine whether the growth inhibition observed in PIL cells treated with GW501516 and ciglitazone was due to apoptosis, cells treated for 6 or 72 h with agonist were stained with Annexin-V, a marker of early-stage apoptosis. PI co-staining was performed in conjunction, to exclude cells under-expressing Annexin-V, to verify the evenness of loading. All isoforms except PPARγ2 were detected in both PIL and primary oval cells. Successful amplification of PPARγ2 by RT–PCR was confirmed using a sample of RNA extracted from adipose tissue (+). Negative control samples (in which the reverse transcriptase enzyme was omitted from the cDNA synthesis reaction) were completely negative for all isoforms (no RT).

Fig. 2. Expression of the PPAR isoforms in oval cell lines and primary oval cell isolates. RNA was isolated from the PIL cell lines or primary oval cell isolates on two distinct occasions to ensure reproducibility. Expression of PPARα, δ, γ and γ2 were determined by RT–PCR. The housekeeping gene β-actin was also amplified, to verify the evenness of loading. All isoforms except PPARγ2 were detected in both PIL and primary oval cells. Successful amplification of PPARγ2 by RT–PCR was confirmed using a sample of RNA extracted from adipose tissue (+). Negative control samples (in which the reverse transcriptase enzyme was omitted from the cDNA synthesis reaction) were completely negative for all isoforms (no RT).

PPAR agonists do not modulate the maturation-status of an oval cell line

To determine whether treatment of the PIL cells with PPAR agonists modulated their maturation-status, we studied the effects of these agents on the expression of hepatocytic and biliary differentiation markers. The PIL-2 cell line expresses a variety of markers typical of oval cells observed in vivo, including the stem cell marker c-kit, the hepatic markers alpha-fetoprotein and albumin, and the biliary markers cytokeratin (CK)19 and CK7. We have recently demonstrated the ability of the PIL cells to undergo maturation down both the hepatic and biliary lineages, following stimulation (39). Culture of PIL-2 cells in either WY14643 or ciglitazone for 7 days did not significantly effect the expression of any genes examined (data not shown). Cells exposed to GW501516 showed significantly increased CK19 expression compared with vehicle-treated cultures (11.3-fold upregulated; one-way ANOVA, *P < 0.01); however, expression levels of all other markers remained similar to controls (data not shown).

Administration of ciglitazone, but not GW501516, inhibits the hepatic oval cell response and liver fibrosis in mice fed a CDE diet

Results of in vitro studies suggested that activation of either PPARδ or γ may effectively attenuate the hepatic oval cell response during chronic liver injury. To test this hypothesis, mice fed the CDE diet were treated with either placebo (vehicle), GW501516 (50 mg/kg) or ciglitazone (20 mg/kg) every second day for 14 days, and the livers examined for signs of injury and oval cell proliferation. These doses were selected based on previous rodent studies in which similar doses were shown to be efficacious (43–45). Animals receiving ciglitazone were generally in better health during the experimental...
Fig. 4. Induction of apoptosis by PPAR agonists. PIL-2 cells were cultured in the presence of vehicle (DMSO), GW501516 (50 μM) or ciglitazone (15 μM) for either 6 or 72 h and apoptosis assayed by Annexin-V/PI staining. Cell counts showed ~15 and 44% of cells to be undergoing apoptosis (Annexin-V⁻/PI⁻) following 6 and 72 h treatment with ciglitazone, respectively (A). GW501516 induced apoptosis in ~2% of cells at 6 h and 25% at 72 h (A). Data represent means ± SEM, n = 3. Statistical comparisons of the groups at 6 and 72 h revealed a significant difference between vehicle and drug treated groups at each time point (one-way ANOVA, P < 0.01). Representative photomicrographs are presented illustrating Annexin-V staining (B, D and E) and corresponding phase-contrast images (C, E and G) taken following 72 h treatment with vehicle (B and C), GW501516 (D and E) or ciglitazone (F and G).
period, and showed a trend towards having a higher body weight at the end of the CDE feeding period than corresponding placebo-treated mice (Table II). Body weights of GW501516-treated mice were comparable to placebo (Table II). Hepatic steatosis was evident in 5/5 of mice in both DMSO (Figure 5A) and ethanol placebo (Figure 5C) groups. Similarly, 5/5 ciglitazone-treated animals showed extensive liver fat deposition (Figure 5D). GW501516 improved the incidence and severity of steatosis induced by the CDE diet (Figure 5B). Only 2/5 GW501516-treated animals showed evidence of hepatic fat accumulation following the CDE feeding period.

Oval cells (identified by immunohistochemical staining for A6) were induced comparably in mice administered with DMSO placebo (Figure 6A), and those receiving GW501516 (Figure 6B). In contrast, ciglitazone-treated (Figure 6D) animals showed a reduced oval cell response compared with ethanol placebo (Figure 6C). Cell count data revealed that ciglitazone-treated animals had, on average, less than half the number of oval cells as placebo-treated mice (Figure 6E, \( P < 0.05 \)).

The CDE diet is known to induce HSC activation and liver fibrosis. Detection of activated HSCs in drug and placebo-treated livers showed that administration of ciglitazone (Figure 7D), but not GW501516 (Figure 7B), reduces the number of activated HSCs present following 14 days CDE feeding, compared with placebo (Figure 7A and C). Both placebo groups, as well as GW501516-treated animals, had a median SMA score of 2. In contrast, animals receiving ciglitazone had a median SMA score of 0. In accordance, Masson’s Trichrome revealed the presence of pericellular fibrosis in 3 out of 5 animals receiving placebo and 0 out of 5 receiving ciglitazone (Figure 7E and F).

**Discussion**

The nuclear hormone receptors of the PPAR family have been shown to modulate a variety of different biological effects, primarily related to lipid metabolism. In addition, several

| Table II. Body weights of mice before (pre) and after (post) a 14 day CDE feeding period with co-administration of either placebo or drug |
|---|---|---|---|---|---|---|---|
| Placebo (EtOH) | Placebo (DMSO) | Ciglitazone | GW501516 |
| Mean weight (g) | 18.9 | 17.7 | 18.4 | 18.0 | 15.8 |
| SD | 1.5 | 0.7 | 2.9 | 1.2 | 1.1 | 0.5 | 2.6 |

**Fig. 5.** Effect of GW501516 or ciglitazone treatment on liver histology following 14 days CDE feeding. Liver histology was examined in haematoxylin and eosin stained sections. The CDE diet induced fat accumulation in the livers of mice administered either DMSO (A) or ethanol (C) as a placebo. GW501516-treatment prevented hepatic steatosis induced by the CDE diet (B). Ciglitazone treatment did not effect hepatic fat accumulation (D).
reports have suggested they may be involved in regulating the hepatic response to injury. However, to date, the role of each PPAR isoform in mediating the behaviour of adult liver progenitor (oval) cells, was unclear.

We observed an increase in liver expression of both PPARα and γ during the progression of liver disease induced by a CDE diet in mice. The CDE diet has been well characterized as a rodent model of oval cell proliferation, and has been shown to...
promote tumorigenesis when administered over longer time periods (8,15). No difference in PPAR expression between control and CDE livers was detected for the first week of CDE feeding. However by day 14 of the CDE diet, levels of PPARα and γ were significantly increased over controls, by 3- and 5-fold, respectively. We have previously shown that oval cell numbers rapidly expand in the liver between days 7 and 14 of the CDE diet (6), suggesting a possible correlation between oval cell proliferation and PPARα/γ expression. PPARδ was not reliably detected using the method developed. This is somewhat surprising, as activated HSCs are known to express this isoform (27). It would be of interest to repeat the analysis on non-parenchymal cell isolates from CDE livers at each time point, to determine whether expression of PPARδ is modulated in these cells during the injury time course. Primary and immortalized oval cells were found to express all three PPAR isoforms. The alternate splice variant of PPARγ, PPARγ2, was not detected in oval cells; however, this is
not surprising as PPARγ2 is expressed primarily in adipose tissue (46). The increased levels of PPARα and γ mRNA detected following 14 and 21 days of CDE feeding may be, in part, due to their expression by oval cells. However, it is likely that PPAR expression by other cell types, such as hepatocytes and HSCs, also contributes to total liver mRNA levels.

We studied the effects of PPAR activation on cultured immortalized liver progenitor cells cloned from primary cultures of oval cells isolated from the livers of p53 knockout mice fed a CDE diet (PIL cells). These cells have been extensively characterized and found to express markers typical of oval cells in vivo (11). We have also demonstrated the ability of these cells to grow in response to increasing concentrations of serum, and to differentiate under certain culture conditions (38,39). PIL cells maintained in the presence of the PPARδ agonist GW501516 or the PPARγ agonist ciglitazone, underwent dose-dependent loss of viability. GW501516 was the less potent of the two reagents, with an approximate ED50 for growth inhibition of 50 µM in both cell lines, while the ED50 for ciglitazone ranged from 15 µM (in PIL-2) to 25 µM (in PIL-1). Annexin-V staining revealed that both agents were pro-apoptotic to the PIL cell lines, although to different degrees. Ciglitazone induced apoptosis rapidly and led to approximately half of all cells being in the early stages of programmed cell death following 72 h treatment. Additionally, a substantial number (~21%) of cells were necrotic in 72 h ciglitazone-treated cultures. In contrast, minimal apoptosis was detected following only 6 h treatment with GW501516 and a relatively small number (~26%) of cells were apoptotic following 72 h. Necrosis was minimal at both time points in GW501516-treated cultures. This suggests that the decreased numbers of viable PIL cells observed following 72 h treatment with GW501516 was probably due to a combination of pro-apoptotic and anti-proliferative effects. Ciglitazone analogues have been shown previously to induce apoptosis of both human hepatoma and rodent oval cell lines (30). However, no previous studies have reported induction of apoptosis following treatment with GW501516. In contrast, GW501516 has been shown to promote the growth of a variety of cell lines, including hepatoma cells (35,45,47). Treatment of cells with the PPARα agonist WY14643 did not affect PIL cell growth at any concentration tested. The finding that PPARα activation does not induce proliferation of cultured oval cells is of interest, as it has been shown previously to promote the growth of hepatocytes in vivo (21–23) and hepatoma cell lines in vitro (48–50). This suggests that the tumorigenic effects of WY14643 in rodent liver are not mediated by increased oval cell proliferation, as has been suggested previously (51).

Each of the PPAR isoforms has a recognized role in controlling the differentiation of one or more mammalian cell types (for a review, see (52)). As such, we investigated the ability of each PPAR agonist to modulate the maturation-status of PIL-2 cells following one week of treatment. A low dose (5–10 µM) was selected in order to avoid drug toxicity. Treatment of cells with the PPARδ agonist GW501516 resulted in upregulation of CK19 mRNA levels, suggesting a possible maturation of cells down the biliary lineage. However, as the expression of all other markers, including the mature bile duct marker CK7, were not affected, it is unlikely that this result represents a true differentiation effect. A previous report has suggested that PPARα activation induces the hepatic differentiation of oval cells in vivo (51). Our results do not support a role for either PPARα or PPARγ in modulating the differentiation of oval cells in culture.

Based on the finding that treatment with either ciglitazone or GW501516 caused growth inhibition of oval cells in vitro, we sought to determine whether either agent could abrogate the oval cell response to the CDE diet in vivo. The results show a significant reduction (of ~50%) in oval cell numbers in ciglitazone-treated mice compared with those receiving vehicle alone. In contrast, no appreciable effect on oval cell induction was observed in animals receiving GW501516. The overall health of the animals receiving ciglitazone was superior to the placebo group, reflected in the improved body weight of the animals at the termination of the experiment. Furthermore, our results show that ciglitazone treatment inhibited liver fibrosis and HSC activation in the CDE model of chronic liver injury, while again, no difference was apparent between placebo and GW501516-treated animals. The protective effects of PPARγ agonists against acute liver injury have been reported by several groups (53–55). PPARγ activation has also been shown previously to inhibit the activation of HSCs in vitro and in vivo, and therefore to prevent fibrosis following acute liver injury (56–58). Our results confirm and extend these findings, suggesting that the beneficial effects of PPARγ agonists may also extend to situations of chronic liver injury, such as that induced by the CDE diet in rodents or chronic hepatitis virus infection in humans. We did not observe a difference in HSC activation in mice receiving GW501516 versus those administered placebo. This was somewhat surprising, as PPARδ activation has been shown previously to induce stellate cell proliferation and increase SMA expression following acute liver injury induced by carbon tetrachloride treatment in rats (34). That no positive regulation of fibrosis was observed in GW501516-treated animals may reflect the difference between acute and chronic liver injury models with respect to the mechanisms underlying their pathogenesis.

Interestingly, administration of GW501516, but not ciglitazone, improved the incidence of CDE-induced fat accumulation in the liver. Agonists of both PPARγ and PPARδ have been reported previously to improve liver fat levels in rodent models of fatty liver disease (32,59,60). Thus, it is surprising that, in our study, ciglitazone did not reduce the extent of hepatic steatosis induced by the CDE diet. Although speculative, we suggest that the mechanisms mediating parenchymal fat accumulation in the CDE model may differ from that employed in other models of fatty liver disease. A detailed comparison of the different models and their susceptibility to treatment with PPARγ agonists might help to dissect where these differences lie.

Oval cell proliferation has been strongly linked to the progression of liver disease towards HCC, and we have shown previously that animals in which the oval cell response is diminished develop fewer liver tumours following sustained injury (15). As such, the attenuation in oval cell numbers seen following ciglitazone treatment in CDE-fed mice supports a potential role for PPARγ agonists as therapeutic agents for preventing or delaying the onset of tumorigenesis during chronic liver injury. The finding that ciglitazone treatment also reduced the severity of hepatic fibrosis strengthens this suggestion further. Our data suggest that PPARγ may represent a novel therapeutic target for the treatment of pre-neoplastic liver disease.
Effects of PPAR activation on hepatic oval cells

Acknowledgements

The authors thank Ms Carla Smith and Dr Alison Rose for excellent technical assistance and Dr Leon A. Adams for his advice in the grading of steatosis in the samples. Dr Belinda Knight is the recipient of a Richard Walter Gibbon Medical Research Fellowship from the University of Western Australia. Dr Bu Yeap was the recipient of a research grant from the Medical Research Fund of Western Australia (MEDWA). This work was supported by the Cancer Council of Western Australia.

Conflict of Interest Statement: None declared.

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Received March 1, 2005; revised May 13, 2005; accepted May 19, 2005