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Inhibition of PPARα/RXRα-Mediated Direct Hyperplasia Pathways During Griseofulvin-Induced Hepatocarcinogenesis

Yasuyuki Nagao, Barbara A. French, Yan Cai, Samuel W. French, and Yu-Jui Y. Wan*

Department of Pathology, Harbor-UCLA Medical Center, Torrance, California 90509

Abstract Chronic griseofulvin (GF) feeding induces preneoplastic foci followed by hepatocellular carcinoma in the mouse liver. Our previous study suggested that GF-induced hepatocellular proliferation had a different mechanism from that of peroxisome proliferator (PP)–induced direct hyperplasia. The GF-induced hepatocellular proliferation was mediated through activation of immediate early genes such as Fos, Jun, Myc, and NFκB. In contrast, PP-induced direct hyperplasia does not involve activation of any of these immediate early genes. It has been shown that nuclear hormone receptors including peroxisome proliferator activated receptors (PPARs) and retinoid x receptors (RXRs) play important roles in mediating the pleiotropic effects of PPs. To examine the possible roles of PPARs and RXRs during non-PP-induced hepatocellular proliferation and the interaction between PP and non-PP-induced proliferation, we have studied the expression of the PPAR and RXR genes in the GF model using northern blot hybridizations and gel retardation assays. The data showed that the expression of PPARα and RXRα genes was down-regulated in the livers containing preneoplastic nodules and in the liver tumors induced by GF. The mRNA down-regulation was accompanied by a decrease in the amount of nuclear protein–bound to peroxisome proliferator and retinoic acid responsive elements. Down-regulation was also associated with the suppressed expression of the PPARα/RXRα target genes (i.e., acyl-Co oxidase and cytochrome P450 4A1) and the catalase gene. The RXRγ gene was also down-regulated, but the RARα, β, and γ and PPARβ and γ genes were up-regulated. These results indicated that the hepatocarcinogenesis induced by GF is accompanied by suppression of the PPARα/RXRα-mediated direct hyperplasia pathway. The differential expression of these nuclear hormone receptors reveals a new aspect for understanding the individual roles and intercommunication of PPAR, RXR, and RAR isoforms in the liver. J. Cell. Biochem. 69:189–200, 1998.

Key words: peroxisome proliferator activated receptor; retinoid x receptor; retinoic acid receptor; liver hyperplasia; hepatocarcinoma

Chronic griseofulvin (GF) feeding induces neoplastic changes as well as cytokeratin alterations in the mouse liver [Denk et al., 1976; Tazawa et al., 1983; Cadrin et al., 1995; Yuan et al., 1996]. Five months of GF feeding induces preneoplastic foci, and longer feeding (12 months) causes tumors in the liver [Tazawa et al., 1983]. Using γ-glutamyl transferase (GGT) as a marker for hepatoma induction, we have shown that the number of GGT-positive foci per square millimeter of area was 3.55 + 0.56 and 6.09 + 0.75 after 4 and 10 months of GF feeding, respectively. The morphological appearance of the tumor shows a characteristic trabecular pattern which is similar to hepatocellular carcinoma observed in human livers [Tazawa et al., 1983]. In recent studies, it was found that the expression of the immediate early genes (c-myc and c-jun) was induced in the GF-induced liver hyperplasia [Nagao et al., unpublished data] and the staining score (mitotic index) of proliferating cell nuclear antigen was increased during GF feeding [Yuan et al., 1996]. These data suggested that GF-stimulated hepatocelluar proliferation in the mouse might be mediated through activation of the immediate early genes.

Hepatocellular proliferation is thought to play an important role in the carcinogenic process, such as initiation, promotion, and progression [Farber and Sarma, 1987]. There are two dis-

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*Correspondence to: Yu-Jui Yvonne Wan, Department of Pathology, Harbor-UCLA Medical Center, 1000 West Carson Street, Torrance, CA 90509. E-mail: agarose@ucla.edu

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tinct patterns in hepatocellular proliferation: compensatory hyperplasia and direct hyperplasia [Columbano and Shinozuka, 1996]. Compensatory hyperplasia follows the loss of liver cells such as a partial hepatectomy [Higgins and Anderson, 1931; Columbano and Shinozuka, 1996]. GF-induced liver hyperplasia involves cell necrosis and is also classified as compensatory hyperplasia. Direct hyperplasia has two different intracellular pathways. One pathway can be stimulated by peroxisome proliferator (PP) and 9-cis retinoic acid, while another can be stimulated by certain mitogens such as lead nitrate and ethylene dibromide [Coni et al., 1993; Ohmura et al., 1996]. Heterodimers of peroxisome proliferator activated receptors (PPARs) and retinoid x receptors (RXRs) play a role in PP-induced direct hyperplasia [Bardot et al., 1993]. The activation of the immediate early genes is not involved in PP-induced hyperplasia [Coni et al., 1993]. Tumor necrosis factor is involved in mitogen- but not PP-induced hyperplasia [Shinozuka et al., 1994; Ledda-Columbano et al., 1994].

PPARs are members of the steroid hormone receptor superfamily [Isseman and Green, 1990]. PPARs/RXRs heterodimers can regulate the transcription of genes such as acyl-CoA oxidase (AOX) in peroxisomal β-oxidation and cytochrome P450 4A1 (CYP4A1) in microsomal ω-hydroxylation through the peroxisome proliferator responsive element (PPRE) [Dreyer et al., 1992; Kliewer et al., 1992; Tugwood et al., 1992; Aldridge et al., 1995]. To date, three subtypes of PPARs have been described in amphibians, rodents, and humans: PPARα, β (also called δ or Nuc-1), and γ [Isseman and Green, 1990; Schmidt et al., 1992; Gottlicher et al., 1992; Sher et al., 1993; Zhu et al., 1993; Kliewer et al., 1994]. These receptors are differentially expressed and regulate a distinct set of genes. Little is known about how these receptors are expressed and regulated during carcinogenesis. It has been reported that PPs play a role in rodent hepatocarcinogenesis by stimulating proliferation of peroxisome and the β-oxidation pathway followed by overproduction of hydrogen peroxide leading to DNA damage [Reddy and Lalwani, 1983; Elliot et al., 1986]. Therefore, the activation of the PPARα-related transcriptional pathways has been thought to be important for PP-induced hepatocarcinogenesis in rodents.

Retinol (vitamin A) and its derivative, retinoic acid, have pleiotropic effects in regulating cell growth, differentiation, proliferation, and embryogenesis [Lotan, 1980]. The liver plays a central role in the uptake, storage, and metabolism of retinol. Two types of nuclear hormone receptors, retinoic acid receptors (RARs) and retinoid x receptors (RXRs), are involved in mediating the biological functions of retinoic acid [Mangelsdorf et al., 1992, 1995; Mangelsdorf and Evans, 1995; Chambon, 1996]. It has been postulated that RAR genes are involved in carcinogenesis as shown by the downregulation of the RARβ gene in hepatoma cell lines [Wan et al., 1992; Li and Wan, 1997].

The objective of this study is to understand the possible roles of PPARs, RXRs, and RARs in non-PP (GF)-induced hepatocellular proliferation and hepatocarcinogenesis and to elucidate the intercommunication between non-PP- and PP-induced proliferation pathways. We demonstrated that the PPARα/RXRα-mediated pathways were downregulated in the livers containing preneoplastic foci and the hepatocellular carcinomas induced by GF. In addition, the expression of the PPAR, RXR, and RAR genes are differentially regulated. These results suggest the presence of a negative feedback mechanism in inhibiting the proliferation of hepatocytes in the non-PP-induced liver hyperplasia. The differential roles of PPAR, RXR, and RAR in GF-induced hepatocarcinogenesis are discussed.

MATERIALS AND METHODS

Animals

Male C3H mice were fed 2.5% (w/w) GF in a semisynthetic complete diet for 5 months and 16 months. As controls, mice were fed the same diet without GF for the same period of time. Three to eight mice were used for each treatment. The liver was removed under anesthesia (pentobarbital) and cut into small pieces. The pieces were snap-frozen in isopentane precooled by liquid nitrogen at -155°C and kept in a freezer maintained at 80°C. For studying tumor tissues, parts of macroscopic tumors of which sizes were larger than 5 mm^3 were selectively used, and their histologies were confirmed by hematoxylin and eosin staining as hepatocellular carcinomas. These same mouse livers had been used in previous studies [Nagao et al., submitted]. All mice were handled in

compliance with the Animal Committee Guidelines of Harbor-UCLA Medical Center.

Northern Blot Hybridization

Total RNA was isolated from frozen tissues described above using modification of the single-step acid guanidinium thiocyanate-phenol-chloroform extraction method [Chomczynski and Sacchi, 1987]. The probes used in the hybridization include the 1.9 kb BamHI-XbaI fragment of pSG5-mouse PPARα [Isseman and Green, 1990], HindIII-BamHI fragment of pCMX-mouse PPARδ [Kliewer et al., 1994], PvuII fragment of pCMX-mouse PPARγ1 [Zhu et al., 1993], full-length cDNA insert of pTZ18R for rat AOX [Miyazawa et al., 1987], EcoRI fragment of pUC9-rat CYP4A1 [Hardwick et al., 1987], 1.5 kb SstI-BamHI fragment of pGEM-human RARα [Giguere et al., 1987], full length cDNA insert of BI-human RARβ [Benbrook et al., 1988], full-length cDNA insert of pBS-RH for human RARγ [Ishikawa et al., 1990], KpnI fragment (~1.4 kb) of mouse RXRα, 5′ end to AccI site (~1.5 kb) of mouse RXRβ, 5′ end to HindIII site of mouse RXRγ [Mangeldorf et al., 1992], 1.9 kb EcoRI-KpnI fragment of pBR322-human 18S ribosomal RNA [Gonzalez and Schmickel, 1986], ~1.2 kb fragment of pBR322-pcfos-1 [Curran et al., 1983; Miller et al., 1984], and 2.4 kb EcoRI fragment of pSP65 for human catalase [Quan et al., 1986].

Twenty micrograms (for all probes except PPARγ1) or 35 μg (for PPAR γ1 probe) of RNA were fractionated by electrophoresis on 1.2% agarose gels containing 0.22 M formaldehyde and then transferred to nylon membranes (Micron Separation Inc., Westboro, MA). Then, RNA was immobilized to the membrane with a UV linker (UV stratalinker 1800; Stratagene, La Jolla, CA). The membranes were hybridized to ^{32}P-labeled probes (1×10^6 cpm/ml) prepared by random priming in 7% (w/w) SDS, 0.5 M sodium phosphate, pH 6.5, 1 mM EDTA, and 1 mg/ml bovine serum albumin at 68°C for 18 h. The membranes were washed with 1% SDS, 50 mM NaCl, 1 mM EDTA at 68°C for 30 min and then autoradiographed at -70°C using Kodak XAR-2 films with intensifying screens. Parts of the images were quantified using a densitometric analysis (GS-670; Bio-Rad, Richmond, CA). Correlation between two groups (control vs. GF treatment, including both 5 months and 16 months treatments) were determined using linear regression analysis.

Gel Shift Assay

Hepatocyte nuclei were isolated according to the method of FitzGerald et al. [1995]. Nuclear protein was prepared by the method of Dignam et al. [1983]. Binding reactions were performed by the method of FitzGerald et al. [1995] with slight modifications. Briefly, 10 μg of nuclear protein were incubated with binding buffer containing 10 mM HEPES, pH 7.9, 50 mM KCl, 5 mM $MgCl_2$, 8% glycerol, 1 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, 0.2 mM EDTA, and 2 μg of poly (dI-dC) for 10 min at room temperature and then incubated with radiolabeled oligonucleotide probes (2×10^5 cpm) for 30 min at room temperature. For supershift experiments, 1 μl of antibodies was added after probe incubation and further incubated for 10 min on ice. After the incubation, reactions were loaded on 5% nondenatured polyacrylamide gels and electrophoresed in 0.5× Tris–boric acid–EDTA buffer. Antibodies against RXRα and β were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), and the antibody against PPARα was purchased from Affinity Bioreagents, Inc. (Golden, CO). For oligonucleotide probe, a consensus oligonucleotide including PPRE, composed of GGGGACCAGGACAAAGGTCA, which is located in the 5′ upstream of the AOX gene, was used [Tugwood et al., 1992]. The RAREs located on the RARβ2 (DR-5) (gatactGTAGGGTTCACCGAAAGTTCACTCa) [De The et al., 1990; Hoffmann et al., 1990; Leid et al., 1992] and cellular retinoic acid binding protein type II (DR1) (gatactGCTGTCACAGGTCACAGGTCACAGTTCAa) [Mangelsdorf et al., 1991] genes were also used as probes. Probes also included the consensus oligonucleotides of AP-1 and nuclear factor κB (NFκB) binding sites composed of CGCTTGATGAGTCAGCCGGAA and AGTTGAGGGGACTTTCCCAGGC, respectively (Promega Co., Madison, WI).

RESULTS

Changes in the Expression of the c-fos and Activation of AP-1 and NFκB in the GF model

c-Jun and c-fos play important roles in transcription regulation, and their products, AP-1, are closely related to hepatocellular proliferation and cytokeratin expression [Pankov et al., 1994; Shiota et al., 1994; Taube, 1995]. Our previous study using the same livers demonstrated that the expression of c-jun mRNA was upregulated during GF-induced hepatocarcino-

genesis and Mallory body formation [Nagao et al., unpublished data]. To further analyze the roles of AP-1 in GF-induced hepatocarcinogenesis, we examined the expression of c-fos mRNA in the GF model. The expression of c-fos mRNA was induced in the livers containing preneoplastic nodules (5PN) and in the tumors (16T) compared with the controls (5C and 16C) after 5 and 16 months of GF treatment, respectively (Fig. 1). In addition, the amounts of AP-1 bound to its response element were also increased indicating the activation of AP-1 in the GF model (Fig. 2a). These results suggested that AP-1–mediated transcriptional pathways were activated through the up-regulation of c-jun and c-fos mRNAs in the GF model.

NFκB is considered to be important as a transcriptional factor for hepatocellular proliferation since it is immediately activated after partial hepatectomy [FitzGerald et al., 1995; Columbano and Shinozuka, 1996; Ohmura et al., 1996] and intravenous injection of lead nitrate [Ohmura et al., 1996]. However, NFκB was not activated in PP-induced direct hyperplasia [Ohmura et al., 1996]. Our previous results indicated that the hepatocellular proliferation induced by GF operated by a different pathway from PP-induced direct hyperplasia. To further examine the mechanism of hepatocellular proliferation in the GF model, we performed gel retardation assays using the NFκB DNA bindng site as the probe. The results showed that the amount of nuclear proteins bound to the NFκB probe was increased in the livers after 5 months of GF feeding (Fig. 2b), which indicated the activation of NFκB-mediated transcriptional pathways in the GF model. These results indicated that, similar to non-PP-induced liver hyperplasia, GF-induced hepatocyte proliferation involves activation of early signal transduction genes.

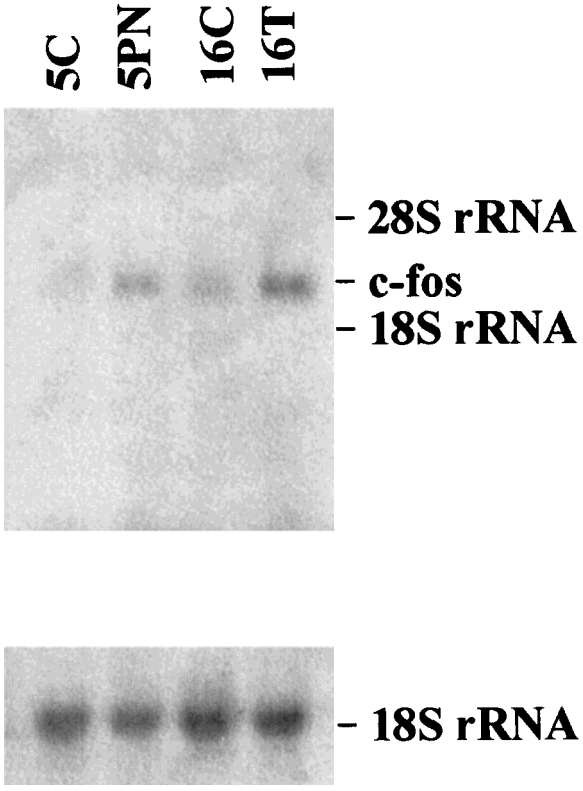

Fig. 1. Expression of the c-fos gene in the GF model. RNA was extracted from normal livers of mice fed with control diet for 5 months (5C) or 16 months (16C). RNA was also extracted from the livers containing preneoplastic nodules (5PN) and the tumors (16T) after 5 and 16 months of GF feeding, respectively. Twenty micrograms of total RNA were fractionated by formaldehyde agarose gel, electroblotted onto nylon membrane, and then hybridized with ^{32}P-labeled c-fos cDNA probes. The membrane was rehybridized with ^{32}P-labeled 18S rRNA probe.

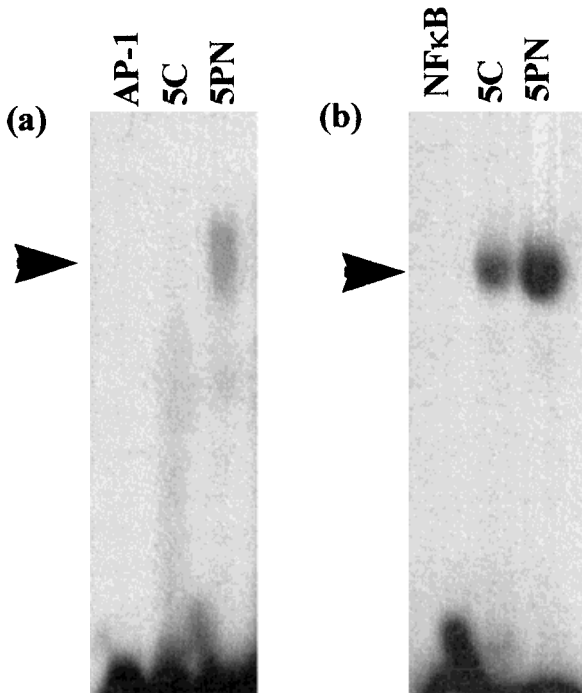

Fig. 2. Activation of AP-1 and NFκB in the GF model. AP-1 and NFκB were employed as probes for gel shift assays. 5C and 5PN are the nuclear proteins derived from the livers of mice fed with control diet and GF diet for 5 months, respectively. Arrowheads indicate the positions of the DNA-protein complexes.

Expression of the PPAR (α, β, γ1) Genes in the GF Model

In 5 month and 16 month control mouse livers (5M controls and 16M controls, respectively), PPARα mRNA was highly expressed. The livers which contained preneoplastic nod-

ules (5PN) showed a four- to five-fold down-regulated expression of PPARα mRNA. The downregulation was also observed but to a lesser extent (four-fold) in the tumors after 16 months of GF feeding (16T) (Fig. 3). The PPARβ gene was weakly expressed in 5M and 16M control livers. Contrary to the PPARα gene, the expression of the PPARβ gene was threefold up-regulated in 5PN and 16T (Fig. 3). Although the expression of the PPARγ1 gene could not be detected in 5M and 16M control livers, the PPARγ1 gene was induced more than fifteen-fold in 5PN and 16T (Fig. 3).

It has been reported that c-jun inhibited the expression of the PPARα gene in rat liver tumor [Sakai et al., 1995]. As indicated before, our previous study showed up-regulation of the c-jun gene in 5PN and 16T using the same mouse livers [Nagao et al., unpublished data]. It is possible that the up-regulated c-jun gene expression may have an interaction with the PPARα expression. Thus, we performed linear regression analysis between the amounts of c-jun and PPARα mRNA in the GF model. The down-regulation of PPARα mRNA was negatively correlated with the up-regulation of c-jun mRNA observed in both 5PN and 16T ($P < 0.001$, $r = 0.837$).

Expression of the RAR (α, β, γ) and RXR (α, β, γ) Genes in the GF Model

PPAR binds PPRE as a heterodimer with RXR [Kliewer et al., 1992; Bardot et al., 1993]. As the expression of PPARα mRNA was suppressed in GF treatment, we studied the expression of RXR mRNAs in the GF model. In GF-treated mouse livers, the expression of RXRα mRNA was three- to four-fold down-regulated in 5PN and 16T, which was correlated with the down-regulation of the PPARα gene ($P = 0.018$, $r = 0.582$) (Fig. 4). The expression of RXRβ mRNA was not significantly changed, and the expression of RXRγ mRNA was markedly down-regulated (more than tenfold) in 5PN and 16T (Fig. 4).

RARα, β, and γ mRNAs were induced in 5PN (three-, ten-, and six-fold, respectively). However, RARα and RARγ mRNAs were only weakly induced (two- to three-fold), and RARβ mRNA showed no remarkable change in 16T compared with the control livers (16C) (Fig. 5). Thus, the inductions of the RAR genes in 16T were weaker than 5PN. The level of RARβ mRNA was slightly increased in the 16 month control livers compared with that in 5 month control livers. This

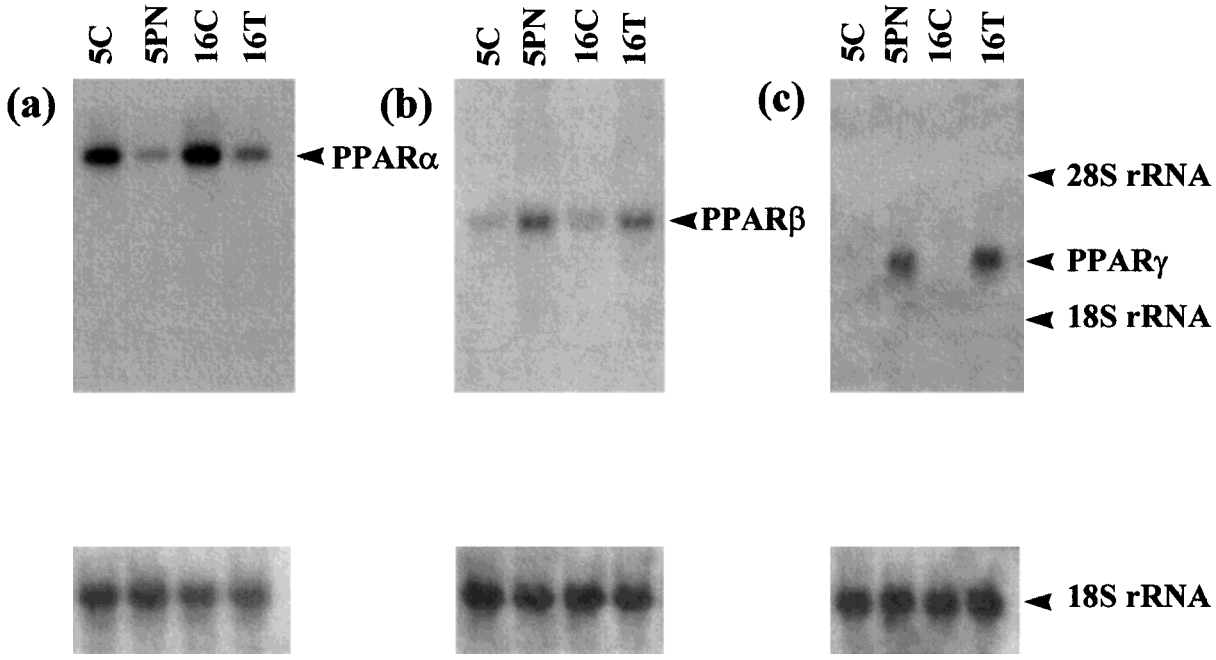

Fig. 3. Expression of the PPARα **(a)**, β**(b)**, γ1**(c)** genes in the GF model. RNA was extracted from normal livers of mice fed with control diet for 5 months (5C) or 16 months (16C), livers containing preneoplastic nodules (5PN), and liver tumors (16T) induced by GF after 5 and 16 months of GF feeding, respectively. Twenty micrograms of total RNA (for PPARα and β probes) or 35 μg of total RNA (for PPARγ1 probe) were fractionated by formaldehyde agarose gel, electroblotted onto nylon membrane, and then hybridized with ^{32}P-labeled PPAR (α, β, γ1) cDNA probes. The membranes were rehybridized with ^{32}P-labeled 18S rRNA cDNA probe.

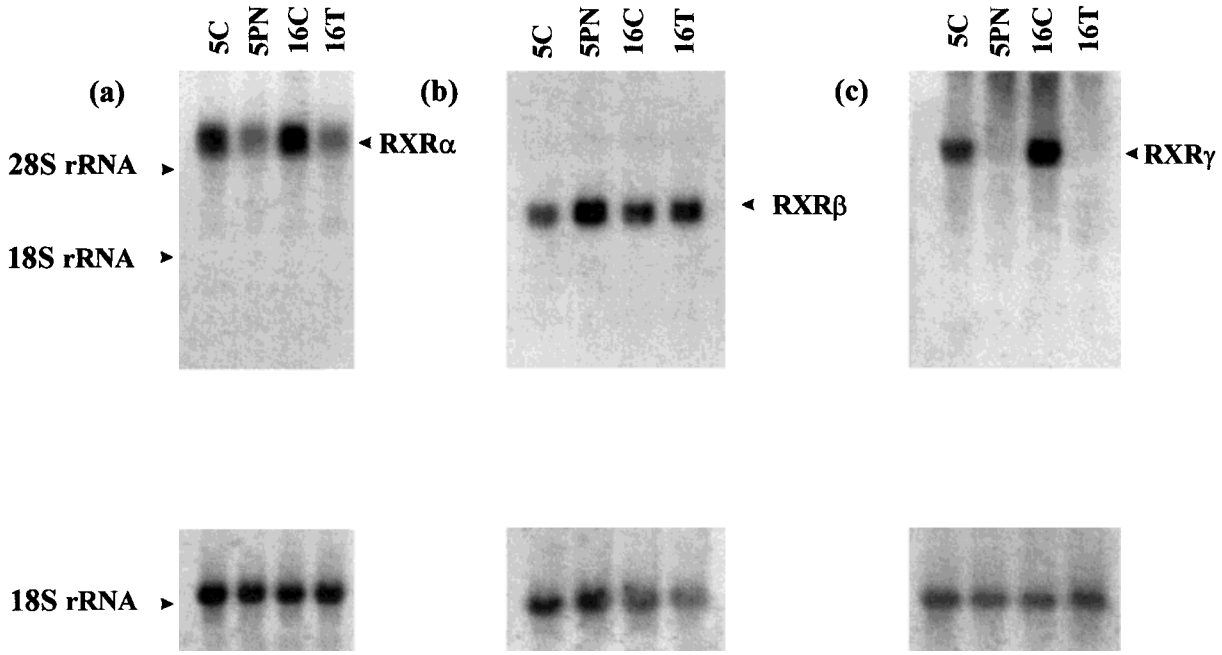

Fig. 4. Expression of the RXRα**(a)**, β**(b)**, γ**(c)** genes in the GF model. Northern blot hybridizations were performed as described in the legend for Figure 1. The blots were hybridized with ^{32}P-labeled RXR (α, β, γ) cDNA probes. The blots were rehybridized with ^{32}P-labeled 18S rRNA cDNA probe.

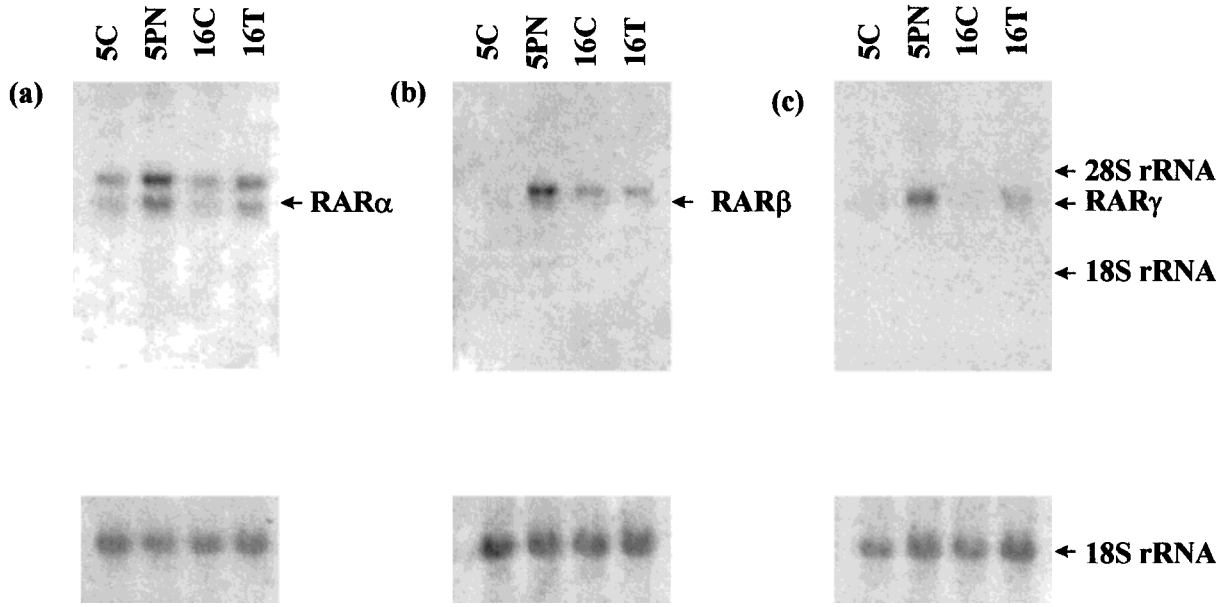

Fig. 5. Expression of the RARα**(a)**, β**(b)**, γ**(c)** genes in the GF model. Northern blot hybridizations were performed as described in the legend for Figure 1. The blots were hybridized with ^{32}P-labeled RAR (α, β, γ) cDNA probes. The blots were rehybridized with ^{32}P-labeled 18S rRNA cDNA probe.

change probably was related to aging since all the mice were fed with the basic control diet.

Expression of the AOX, CYP4A1, and Catalase Genes in the GF Model

PPAR is involved in the activation of genes for both peroxisomal and microsomal fatty acid–oxidizing enzymes (i.e., AOX and CYP4A subfamily, respectively) [Tugwood et al., 1992; Aldridge et al., 1995]. We studied the expression of AOX and CYP4A1 mRNAs to elucidate the significance of changes in the PPARα/RXRα gene expression. The expression of AOX mRNA was fourfold down-regulated in 5PN and 16T,

which was correlated with the down-regulation of PPARα mRNA ($P = 0.039$, $r = 0.521$) (Fig. 6). The expression of CYP4A1 mRNA was also down-regulated (more than tenfold in 5PN and four-fold in 16T) (Fig. 7). These results suggested that the expression of AOX and CYP4A1 mRNAs might be suppressed through the inhibited transcriptional mechanism mediated by PPARα/RXRα heterodimers.

Catalase is a peroxisomal enzyme which catalyzes the decomposition of hydrogen peroxide produced by fatty acid β-oxidation to oxygen and water. We studied the expression of the catalase gene in the liver. The expression of catalase mRNA was two- to three-fold down-regulated in 5PN and 16T compared to the control livers (Fig. 8). These results indicated that GF inhibited the activity of peroxisome.

Changes in the Binding of Nuclear Hormone Receptors in the GF Model

Northern blotting analysis showed the expression of the PPAR, RXR, and RAR genes is differentially regulated, and the PPARα/RXRα target genes including AOX and CYP4A1 are down-regulated in the GF model. To study the effects of GF on the binding of nuclear proteins to the hormone response elements in the GF model, we performed gel retardation assays using PPRE, DR-1, and DR-5 as probes. PPRE is composed of two direct repeats with a spacer of one nucleotide and is preferentially bound by PPAR/RXR heterodimers. DR-1 has five direct repeats and can be bound by RXR homodimers and polymers. DR-5 is preferentially bound by RXR/RAR heterodimers [Pfahl, 1993]. We compared 5PN with 5 month control livers because these two stages showed more prominent changes in the expression of PPARs, RARs, and RXRs mRNAs. Gel retardation assays showed that the amounts of nuclear proteins bound to PPRE, DR-1, and DR-5 response elements were markedly decreased in 5PN (Fig. 9). Competition experiments indicated that the bindings were specific. Since both RXRα and β mRNA were still detected in 5PN by Northern blot hybridization, supershift experiments were performed to confirm that the binding of RXRα to the response elements was decreased. As shown

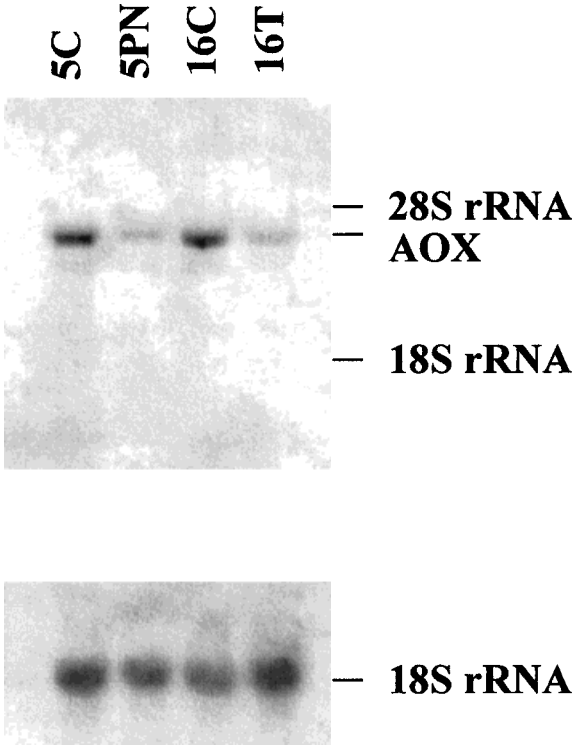

Fig. 6. Expression of the AOX gene in the GF model. Northern blot hybridizations were performed as described in the legend for Figure 1. The blot was hybridized with ^{32}P-labeled AOX cDNA probe and then rehybridized with ^{32}P-labeled 18S rRNA cDNA probe.

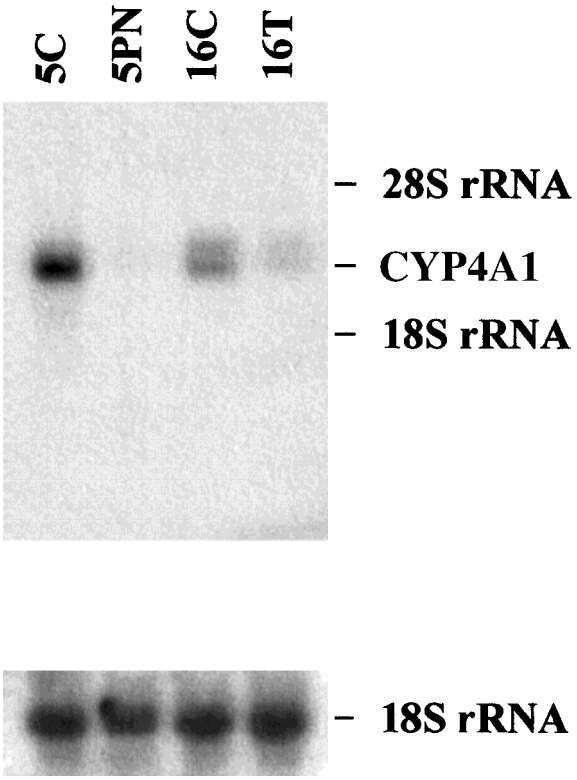

Fig. 7. Expression of the CYP4A1 gene in the GF model. Northern blot hybridizations were performed as described in the legend for Figure 1. The blot was hybridized with ^{32}P-labeled CYP4A1 cDNA probe and then rehybridized with ^{32}P-labeled 18S rRNA cDNA probe.

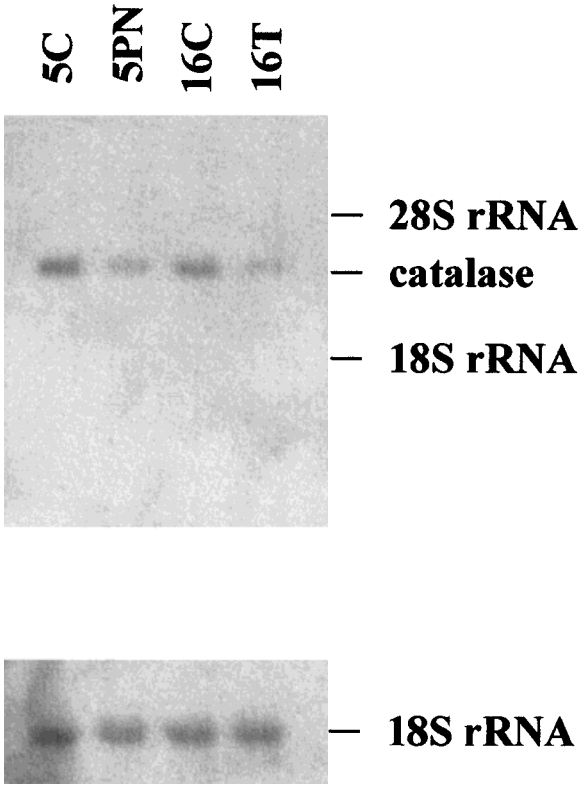

Fig. 8. Expression of the catalase gene in the GF model. Northern blot hybridizations were performed as described in the legend for Figure 1. The blot was hybridized with ^{32}P-labeled catalase cDNA probe and then rehybridized with ^{32}P-labeled 18S rRNA cDNA probe.

in Figure 9, the binding of RXRα and PPARα to the response elements was decreased in 5PN, which was correlated with the Northern blot hybridization results. The amounts of RXRβ bound to the RA response elements were also decreased in 5PN. It is possible that RXRβ was post-transcriptionally regulated; thus, the actual amount of RXRβ expressed in 5PN was low. However, the majority of the protein found in the 5C livers that decreases in the 5PN livers was unknown since the antibodies only shifted small amounts of protein. The extracts used in the experiment demonstrated in Figure 9 were also used for the experiment shown in Figure 2, which indicates that the decreased binding of nuclear protein to the RA and PP response elements was not due to degradation of protein. RXRα but not RXRβ antibody supershifted purified RXRα, indicating the specificity of the antibodies. These results suggest that PPAR/RXR-, RXR/RXR-, and RXR/RAR-mediated transcriptional pathways were inhibited during GF treatment.

DISCUSSION

Our previous results showed that GF treatment–induced hepatocellular proliferation was accompanied by induction of the immediate early genes and the increase in proliferating cell nuclear antigen [Yuan et al., 1996]. In this paper, we have demonstrated the activation of AP-1 and NFκB in the GF model, indicating the involvement of early signal transduction genes in GF-induced liver hyperplasia which is distinct from PP-induced liver hyperplasia [Columbano and Shinozuka, 1996]. Furthermore, in GF-induced liver hyperplasia and hepatoma, the expression of the PPARα and RXRα genes and their target genes was suppressed, which was in contrast to PP-induced hepatocyte proliferation. Induction of rodent hepatic tumors by PP would increase the expression of the PPARα and its target genes [Matsushima-Nishiwaki et al., 1996; Miller et al., 1996; Sterchele et al., 1996]. Considering that PPARα and RXRα play roles in peroxisomal β-oxidation and direct hyperplasia as heterodimers [Bardot et al., 1993], this correlated down-regulation and the decreased amount of nuclear protein bound to response elements suggested that the transcriptional pathway regulated by heterodimers of PPARα and RXRα was inhibited during hepatocarcinogenesis induced by GF.

It is possible that the suppression of the PPARα/RXRα transcriptional pathway was down-regulated by the gene expression of c-jun. It has been reported that jun serves as a negative regulator and controls the expression of the PPARα gene [Sakai et al., 1995]. Our results showed the induction of c-jun mRNA and the activation of AP-1 transcriptional factors in GF-treated mice livers. Therefore, we postulate that the activation of a c-jun–related transcriptional pathway may inhibit the PPARα/RXRα pathway directly or indirectly.

It has been demonstrated that retinoic acid induced the expression of the RAR genes, but the levels of RXRs might be the key factors for control of the action of retinoic acid, since RARs tend to form heterodimers with RXRs [Pfahl, 1993]. This notion is further supported by the data presented in this study. We have shown that all three RAR genes are up-regulated in the GF model. However, the RXRα and RXRγ genes were down-regulated, and the amounts of nuclear protein bound to response elements were decreased. These results indicate that all

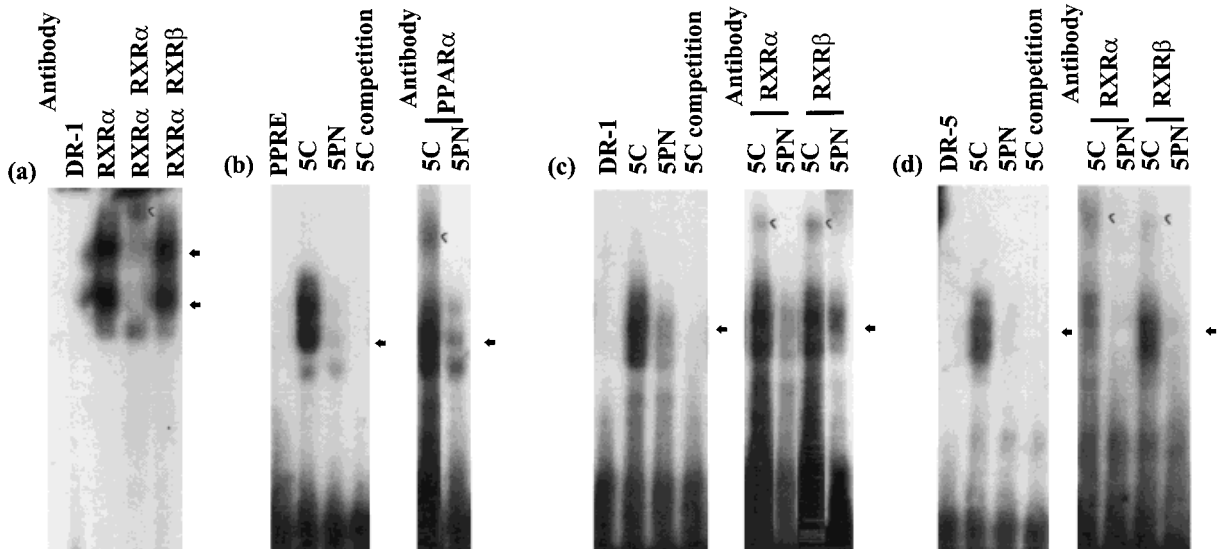

Fig. 9. Changes in the amounts of nuclear protein bound to (**b**) PPRE, (**c**) DR-1, and (**d**) DR-5 in the GF model. DR-1 (a,c), PPRE (b), and DR-5 (d) oligonucleotides were labeled with ^{32}P and served as probes for gel shift analysis. 5C and 5PN are the nuclear proteins derived from the livers of mice fed with the control diet and the GF diet for 5 months, respectively. Arrows indicate the positions of the specific DNA-protein complexes. RXRα protein (**a**) was purified from expression plasmid pGEX.2T RXRα [Mangelsdorf et al., 1991]. Competition experiments (b,c,d) were done by addition of 25× more of the unlabeled probes. Supershift experiments (a–d) were performed using anti-RXRα and β and -PPARα antibodies. Arrowheads indicate the specific spershifts.

RXR-related transcriptional pathways are suppressed in spite of the up-regulated the RAR genes.

The significance of the up-regulation of the RAR genes is not clear. It is possible that the RAR genes do not interact with the transcriptional factors which are involved in suppression of the expression of the PPARα, RXRα, and RXRγ genes. The RAR genes might be induced by a negative feedback mechanism in order to maintain the hepatocellular differentiation and compensate for the down-regulation of RXR-mediated pathways. The evidence that 5PN showed greater induction of the RARs genes than tumors may indicate that this negative feedback regulation pathway was impaired in tumors.

During tumorigenesis induced by GF, the PPAR genes demonstrated differential expression patterns. Although the expression of the PPARα gene was down-regulated, the PPARβ and PPARγ1 genes were induced in the livers containing preneoplastic foci and in the liver tumors. There is little known about the intercommunication among PPARs. These PPARs may respond differently to various physiological activators [Kliewer et al., 1994; Wahli et al., 1995]. It was suggested that PPARα is the major mediator of peroxisome proliferator action in the liver, and other PPARs can repress its activity [Green, 1995; Jow and Mukherjee, 1995]. It is possible that the PPARβ and PPARγ genes were induced to take over the down-regulated PPARα activity for lipid metabolism. However, the decreased expression of the AOX and CYP4A1 genes suggested that the activity of lipid metabolism and other pathways related to PPARs may still be suppressed, as shown in the decreased amounts of nuclear protein bound to PPRE in spite of the increased PPARβ and PPARγ1 mRNAs. This suppression might be due to decreased expression of RXRα and RXRγ. Another possible role for the induction of the PPARβ gene is that PPARβ may be important for hepatocellular development or proliferation because PPARβ was highly expressed in the mouse embryo [Kliewer et al., 1994].

It has been reported that the expression of catalase mRNA was down-regulated in the liver of tumor-bearing nude mice and immortalized and transformed mouse hepatocytes [Yamaguchi et al., 1992; Sun et al., 1993]. In the GF model, the expression of catalase mRNA was also down-regulated in both 5PN and 16T. Given that the expression of catalase mRNA was induced by PP [Nemali et al., 1988], it is possible that the down-regulation of the peroxisomal β-oxidation pathway, which may produce less hy-

drogen peroxide, was involved in the down-regulation of catalase mRNA in GF-treated mouse liver.

Down-regulation of the PPARα and RXRα genes also has been found in diethyl 1,4,6-triethyl-3,5-pyridine-dicarboxylate–induced hepatocellular proliferation [Nagao et al., unpublished data]. Inhibition expression of the PPARα gene also has been documented in liver tumors of rats with spontaneous hereditary hepatitis and hyperplastic nodules induced by the Solt-Farber model (diethylnitrosamine-2-acetylaminofluorene partial hepatectomy) [Sakai et al., 1995]. Thus, it is possible that the PPARα/RXRα pathway is inhibited in all of the non-PP-induced hepatocellular proliferations. Transforming growth factor β1 and activin are the potential inhibitors of liver regeneration and guardians of quiescence [Braun et al., 1988; Yasuda et al., 1993; Fausto et al., 1995]. In the GF model, the PPARα and RXRα genes are specifically inhibited, which in turn would decrease the basal transcription rate of all the pleiotropic effects mediated through PPARα/RXRα, and this could be another negative regulatory mechanism in controlling the non-PP-induced hepatocellular proliferation. Our data suggest the presence of intercommunication between PP- and non-PP-mediated proliferation. It also demonstrated the interaction between liver differentiation and proliferation.

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