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Apoptosis, mitosis and cyclophilin-40 expression in regressing peroxisome proliferator-induced adenomas

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Chronic exposure to peroxisome proliferators (PP), including certain industrial and pharmaceutical chemicals, causes liver cancer in rodents. Continuous exposure to PP is needed for tumor development since the frequency of hepatocellular neoplasms is decreased in animals returned to control diet. To determine cellular and molecular events responsible for enhanced growth in PP-induced liver tumors, we evaluated the relationships of WY-14,643 levels, apoptosis, mitosis and cyclophilin-40 (Cyp-40) expression in regressing tumors induced by WY-14,643, a potent PP. Male F344 rats were fed WY-14,643 (0.1%) in the diet for 43 weeks and then switched to control diet for 2, 3, 5 or 36 days. Mean serum and hepatic concentrations of WY-14,643 were decreased as early as 2 days following removal of WY-14,643 as compared with rats continuously fed WY-14,643. Adenomas from rats maintained on WY-14,643 markedly compressed surrounding parenchyma. Evidence of adenoma regression was observed by 3 days of WY-14,643 withdrawal and was characterized by loss of compression. Decreased compression corresponded to increases in the apoptotic index and decreases in the mitotic index in regressing adenomas at 2, 3, and 5 days following the switch to control diet. Cyclophilins are multifunctional receptor proteins involved in numerous signal transduction pathways, including those mediated by cyclosporin, a liver tumor promoter in rats. Cyp-40 expression was markedly increased in adenomas from continuously exposed rats, but expression returned to levels similar to surrounding parenchyma in adenomas after 5 days of WY-14,643 withdrawal. Taken together, these results indicate that WY-14,643-induced adenomas regress rapidly following withdrawal of the PP in association with declining liver WY-14,643 levels, suggesting that peroxisome proliferator-activated receptor α may mediate PP-induced alterations in mitogenic and/or apoptotic regulation in growing tumors, in conjunction with alterations in Cyp-40 signal transduction.

Introduction

Peroxisome proliferators (PP) are mitogens and carcinogens in rodent liver (1,2). Proliferative lesions induced by PP, including hepatocellular adenomas, regress following cessation of exposure, indicating that development of the lesion is dependent upon continuous PP-induced alterations in the growth regulatory pathway (3–5).

The peroxisome proliferator-activated receptor α (PPARα) is a transcription factor responsible for mediating the pleiotropic effects of PP, including hepatomegaly, acyl CoA oxidase activity, hepatocellular DNA synthesis and hepatocarcinogenesis (6,7). While the genes involved in fatty acid metabolism have been identified and contain peroxisome proliferator response elements (PPRE), the downstream effectors of PP-induced tumor development, are yet to be determined (8–11).

Evaluation of PPARα-associated effectors of growth control or other effectors of tumor induction in rodents is necessary since the health significance of differing levels and activation of PPARα in human liver tissue relative to rodents is not clear (12–16).

Liver growth is controlled by tight regulation of the balance between hepatocyte replication (mitosis) and apoptosis. PP cause disruption of this balance within the liver, particularly within subsets of preneoplastic hepatocytes. This disruption eventually leads to overt neoplasia (4,10,17–19). Preneoplastic and neoplastic lesions induced by PP have increased frequencies of mitosis and apoptosis. Rather than resulting solely from stable mutations in oncogenes and tumor suppressor genes, the changes in mitosis and apoptosis might also result from continued activation of PPARα by the PP ligand. If so, then the regression would presumably result from decreased PPARα activity and restoration of more normal rates of mitosis and apoptosis.

Cyclophilins are multifunctional receptor proteins involved in numerous signal transduction pathways, including those mediated by cyclosporin, recently identified as a liver tumor promoter and as enhancing hepatocyte mitogenesis in rats (20,21). Expression of cyclophilin A, a receptor for cyclosporin, has recently been shown to be altered upon exposure to PP (22). A similarity between PP- and cyclosporin-mediated liver tumor promotion has been noted, including regression of neoplastic lesions upon cessation of exposure (20). The mechanism of cyclosporin-induced hepatocarcinogenesis is not known, but it is possible that cyclophilin-mediated regulation of apoptosis is disrupted (23,24). One member of the cyclophilin family, cyclophilin-40 (Cyp-40), is involved in heat shock protein and c-Myb growth regulatory pathways and has homology to the p59 component of the steroid receptor complex (25–27). Although rapid regression of PP-induced adenomas might involve a cyclophilin-mediated pathway(s), expression of Cyp-40 in PP-induced adenomas has not been characterized.

To determine cellular and molecular events responsible for enhanced growth in PP-induced liver tumors, we evaluated the relationships of WY-14,643 levels, apoptosis, mitosis and Cyp-40 expression in regressing tumors induced by WY-14,643, a potent PP. Our results indicate that levels of

Abbreviations: Cyp-40, cyclophilin-40; H&E, hematoxylin and eosin; PP, peroxisome proliferators; PPARα, peroxisome proliferator-activated receptor α; PPRE, peroxisome proliferator response elements.

the PPARα ligand WY-14,643, lesion growth and Cyp-40 expression are closely related.

Materials and methods

Chemicals

WY-14,643 [4-chloro-6-(2,3-xylidino)-2-pyrimidinylthio]acetic acid, >98% pure] was obtained from ChemSyn Science Laboratories (Lenexa, KS).

Animals and dosing

A total of 65 male F-344 rats ~10 weeks of age were obtained from Charles River Breeding Laboratories (Raleigh, NC). Care and use of these animals was in accordance with the recommendations listed in the *Guide for Care and Use of Laboratory Animals* (DHEW Publication no. NIH 86-033). Rats were housed two to a cage and exposed to a 12 h light/dark cycle with room temperature and humidity maintained at 22 ± 2°C and 50 ± 5%, respectively. Deionized, filtered water and the standard base diet (NIH-07) were available *ad libitum*.

WY-14,643 was blended into the NIH-07 chow to a concentration of 1000 p.p.m. Beginning at about 12 weeks of age, rats were fed chow containing WY-14,643 *ad libitum* for 43 weeks. Following which, groups of rats (withdrawal groups) were returned to the control (NIH-07) diet for 2, 3, 5 or 36 days.

Necropsy

Rats were anesthetized by inhalation of isoflurane (~2% in air). When rats were under deep anesthesia, a ventral midline incision was made and ~3 ml of blood were collected from the right ventricle using a vacutainer. Blood samples were kept on ice until serum was separated from the cells by centrifugation. The serum was then stored at –20°C until further analysis. The rats were exsanguinated by transecting the caudal vena cava and abdominal aorta. The liver was immediately harvested. Slices of liver and liver tumors were fixed in 10% neutral buffered formalin, routinely processed and stained with hematoxylin and eosin (H&E). H&E stained liver sections were examined using light microscopy and tumors were diagnosed as adenomas or carcinomas according to standard criteria. The two key features used to distinguish adenomas from carcinomas were cord thickness (three or more cells thick in carcinomas) and amount of invasion of surrounding parenchyma (more invasion and infiltration in carcinomas). In addition, carcinomas tended to have greater pleomorphism. Adenomas were selected for further analysis. Compression was assessed in H&E stained sections by subjectively evaluating the amount of distortion of surrounding non-neoplastic parenchyma and the ease of delineation of the tumor margins. Neoplastic cells in tumors induced by WY-14,643 are more basophilic than the surrounding tissue and this feature was used to aid in defining the margin of the tumor, particularly in those from the withdrawal groups. The remaining portions of unfixed liver tumors (>5 mm) were dissected away from non-lesion liver prior to freezing and were frozen in liquid nitrogen and stored at –70°C.

Liver (~1 g) that did not contain tumors was also collected from the median lobe and homogenized in a buffer solution of 154 mM KCl, 50 mM Tris, pH 7.4 (final volume 5 ml) and stored at –20°C until further analysis.

WY-14,643 liver and serum concentrations

Liver and serum concentrations of WY-14,643 were measured by HPLC using a Fisher 250×4.6 mm i.d. CN column, containing 5 µm particles with 80 Å pores (gradient mode). The mobile phases used acetonitrile and 30 mM $NH_4H_2PO_4$ in H_2O, pH 3.70 (acetonitrile concentrations were 25, 50 and 75%). The internal standard solution contained azoxybenzene in 20:80 isopropanol:cyclohexane (final concentration 6 µg/ml). Standard curves were prepared using blank serum, blank homogenate and WY-14,643 in concentrations of 0, 0.1, 1.0, 10 and 100 µg/ml.

Acyl CoA oxidase enzyme detection

Acyl CoA oxidase activity was measured in samples of liver homogenates using a modification of a photometric assay previously described (28,29).

Apoptotic and mitotic indices

Apoptotic activity was determined by counting all apoptotic bodies in seven contiguous 200× fields on H&E stained sections of adenomas. All apoptotic bodies in the fields were counted (i.e. those with and without chromatin) at 200× magnification. The total number of hepatocytes was determined in the same fields by counting hepatocyte nuclei in 25% of the grid and multiplying by 4. Adenoma areas were measured on H&E stained sections on an Image-1 image analyzer (Universal Imaging Corp., West Chester, PA). All hepatocyte mitotic figures within each adenoma were counted and are presented as number of mitotic figures per unit area (cm^2).

Western blot analysis

Liver protein extracts from whole cell homogenates (50–120 µg total protein) made according to Wilcke and Alexson (30) were denatured and size separated by 12% SDS–PAGE. A test gel was stained with Coomassie blue to evaluate loading prior to blotting. Proteins were transferred to nitrocellulose membranes and visualized with Ponceau Red to confirm transfer and equal loading by visual inspection. The blotted proteins were probed with polyclonal anti-Cyp-40 antibody (ABR Inc., Golden, CO) followed by anti-rabbit IgG coupled to horseradish peroxidase and visualized by enhanced chemiluminescence (ECL™; Amersham).

Densitometric analysis was carried out using a Scanjet 4c flatbed scanner (Hewlett-Packard, Palo Alto, CA) with PhotoShop software (Adobe Systems, Seattle, WA).

Statistics

Mean apoptotic and mitotic indices in hepatocellular adenomas from each withdrawal group were compared with mean apoptotic and mitotic indices in hepatocellular adenomas from continuously exposed animals using a Kruskal–Wallis rank test followed by a Bonferroni analysis with the significance level set at $P \leq 0.05$.

Acyl CoA oxidase levels and densitometric data from withdrawal and continuously exposed animals were compared using Tukey–Kramer analysis with the significance level set at $P \leq 0.05$. Withdrawal group liver weights were compared using Tukey–Kramer analysis with the significance level set at $P \leq 0.05$.

Results

Declining levels of liver WY-14,643 paralleled the rapid decreases in WY-14,643 measured in the serum of rats in the withdrawal group and was observed as early as 2 days after these rats were returned to the control diet. Acyl CoA oxidase levels, in turn, declined following 3 and 5 days of withdrawal and were similar to 43 week control values after 36 days of PP withdrawal (Table I).

Livers from rats continuously exposed to WY-14,643 were uniformly enlarged, consistent with hepatomegalic effects reported previously for these compounds (1). As expected, PP-induced hepatomegaly diminished soon after PP withdrawal (Table I).

Adenoma multiplicity decreased in livers from rats that were returned to control diet for 36 days (Table II). Livers from these animals contained hemorrhagic depressions on the natural surfaces of the liver, likely representing sites of previously existing adenomas. Microscopically, these depressions contained few hepatocytes and consisted of loose aggregates of lipofuscin- and hemosiderin-laden macrophages, often near collections of extravasated red blood cells (Figure 1). Hepatocytes adjacent to the depressions were typically disorganized, most likely due to previous compression. Some of the remaining tumors from the day 36 withdrawal group had a mixture of morphological features, including portions that lacked compression and contained abundant hemosiderin and liposfuscin, contiguous with regions more typical of non-regressing PP-induced neoplasms, i.e. extensive compression and numerous mitoses. These lesions probably represent mixtures of PP-dependent and PP-independent subclones of hepatocytes.

Morphological evidence of tumor regression was seen as early as 2 days after withdrawal but was more pronounced on withdrawal days 3 and 5. Microscopically, regression was manifested by decreased compression of surrounding parenchyma by the adenomas, lesions that are noteworthy for displacing nearby hepatocytes (Figures 2A and 3). Decreased compression in regressing lesions was supported by alterations in mitotic and apoptotic activity. Numerous apoptotic bodies and mitotic figures were observed in adenomas from continuously exposed rats, indicating intense cell cycle activity (Figures 4A and 5A). In contrast, adenomas from the withdrawal groups were characterized by increased numbers of

Table I.

Group	Time point	Liver weight (g)	Serum WY (μg/ml)	Liver WY (μg/ml)	Acyl CoA ox. (g/dl)
Control		13.8 ± 0.3	n.d.	n.d.	12.3 ± 1.3
Withdrawal	Day 2	28.9 ± 1.2	0.3 ± 0.0	n.d.	–
	Day 3	23.2 ± 1.0	0.2 ± 0.0	n.d.	69.6 ± 4.3
	Day 5	20.1 ± 0.6**	0.2 ± 0.0	n.d.	36.8 ± 2.6
	Day 36	14.4 ± 0.4	n.d.	n.d.	11.0 ± 1.1
Continuous	Day 2	25.3 ± 0.5	26.7 ± 0.9	2.1 ± 0.2	–
	Day 3	24.4 ± 0.6	22.3 ± 2.5	2.0 ± 0.2	120.5 ± 11.4*
	Day 5	22.8 ± 0.6	19.9 ± 2.6	1.7 ± 0.1	151.1 ± 11.8*

WY, WY-14,643. Values represent the mean of at least five animals +/– the SEM. Acyl CoA oxidase mean for control animals based on $n = 6$. Control animals were not exposed to WY-14,643. Limit of detection for serum levels, 0.2 μg/ml; and for liver levels, 0.4 μg/ml. n.d., none detectable. *Significant difference from withdrawal group. **Significant difference from withdrawal day 2.

Table II. Liver tumor multiplicity in rats exposed to WY for 43 weeks

Treatment	Total adenomas	Adenomas per liver	Total carcinomas	Carcinomas per liver
Exposure to WY for 43 weeks ($n = 14$)	9	0.64	1	0.05
Exposure to WY for 43 weeks + 36 day withdrawal ($n = 10$)	3	0.30	2	0.20

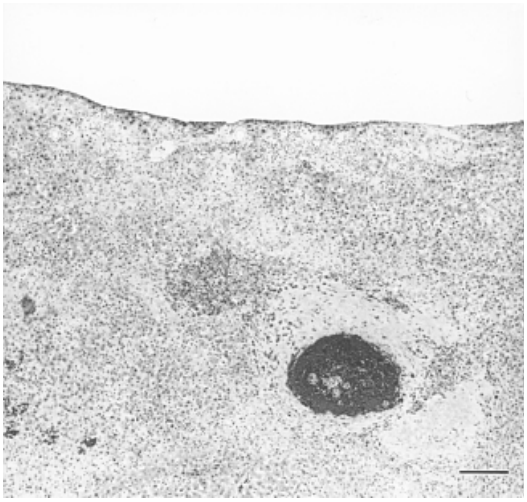

Fig. 1. Section of liver corresponding to depressed area seen grossly from a rat withdrawn from WY-14,643 exposure for 36 days. Note the large area of hemorrhage, disorganized hepatic parenchyma, abundant hemosiderin and lipofuscin. H&E stain. Bar, 200 μm.

apoptotic bodies with fewer mitoses on days 2 and 3 (Figures 4B and 5B). Larger amounts of mitotic activity in the day 5 withdrawal group may be indicative of adenomas containing PP-independent growth pathways, likely representing lesions that are at a more advanced stage of tumor progression.

Cyp-40, a mediator of numerous signal transduction pathways, was measured by western analysis using protein extracted from tumors and surrounding parenchyma of continuously exposed rats and from the day 5 withdrawal group. Cyp-40 was expressed at low levels in non-neoplastic surrounding parenchyma but expression was significantly increased in adenomas from the continuously exposed rats. Following 5 days of WY-14,643 withdrawal, Cyp-40 expression levels in the adenomas decreased to levels approximately equivalent to surrounding parenchyma (Figure 6). There was no significant difference in expression of Cyp-40 in liver from unexposed animals as compared with non-neoplastic hepatic parenchyma from continuously exposed rats or from rats returned to control diet for 5 days.

Discussion

Evidence of rapid regression following chemical withdrawal in PP-induced adenomas is consistent with transcriptionally mediated tumor induction and indicates that a significant part of the mechanism is not the result of stable mutations.

Liver tumor growth is ultimately determined by the proportion of cells entering and leaving the cell cycle. The results of these studies suggest that disruption of both apoptosis and mitosis by PP contributes to growth of the neoplasm. The relative importance of apoptotic versus mitotic pathways in lesion growth is difficult to determine since direct quantitative comparison is complicated by end-point differences in duration and stoichiometry. For some time, PP have been well known as potent hepatic mitogens. A mitogenic component of PP-induced tumor growth is certainly supported by the studies reported herein. However, the role of apoptosis cannot be discounted. *In vitro* and *in vivo* evidence for PP-altered regulation of apoptotic control within liver has been documented (18,31) and the early increase observed at 2 days of withdrawal in these studies suggests that apoptosis is an important component of expansion of neoplastic cell populations. The importance of both apoptosis and mitosis is inferred from the rapid increases in apoptotic and decreases in mitotic activity seen as early as 2 days post-withdrawal of PP exposure. The slight rebound of mitotic activity in the 5 day withdrawal group may be due to replacement of PPARα-dependent hepatocytes. The alterations in apoptosis and mitosis in WY-14,643-induced tumors are consistent with alterations observed in nafenopin-induced tumors and suggest that dysregulation of both are a general mechanism of PP-induced liver tumor development (4), specifically that tumor growth is a function of decreased apoptosis and increased mitosis in developing neoplasms.

Regarding the mechanism of growth and regression of PP-induced neoplasms, clearly PPARα is at least partly responsible for the development of neoplasia, even until late

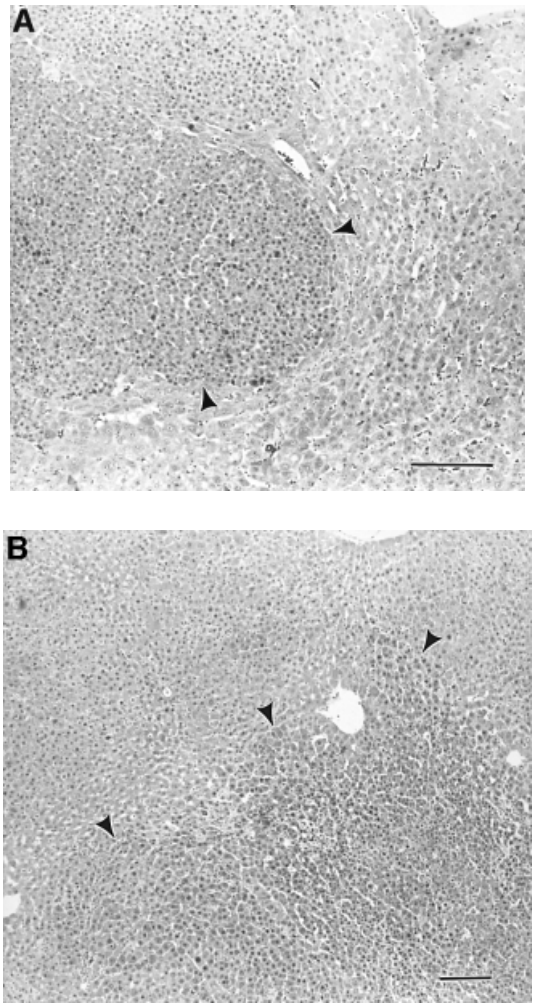

Fig. 2. (**A**) Section of hepatocellular adenoma from a rat continuously exposed to WY-14,643 for 43 weeks. Note the compression of the adjacent parenchyma along the margin of the neoplasm (arrowheads). H&E stain. Bar, 200 μm. (**B**) Section of hepatocellular adenoma from a rat continuously exposed to WY-14,643 for 43 weeks and returned to control diet for 5 days. Note the poor delineation of the neoplasm due to minimal compression of adjacent parenchyma (arrowheads). H&E stain. Bar, 200 μm.

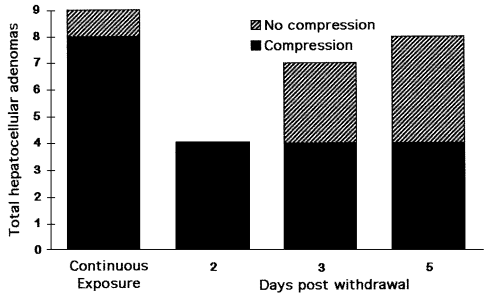

Fig. 3. Proportion of hepatocellular adenomas significantly compressing surrounding parenchyma from rats continuously exposed to WY-14,643 for 43 weeks and from rats switched to control diet for 2, 3 or 5 days. For continuous exposure, $n = 9$ adenomas from eight rats; 2 days withdrawal, $n = 4$ adenomas from three rats; 3 days withdrawal, $n = 7$ adenomas from four rats; 5 days withdrawal, $n = 8$ adenomas from five rats.

in the neoplastic process (7). However, the specific genes responsible for the enhanced mitogenesis and apoptosis in these lesions are unknown since significant alterations of established liver growth control genes with or without PPRE remain to be identified (9–11). The requirement for WY-

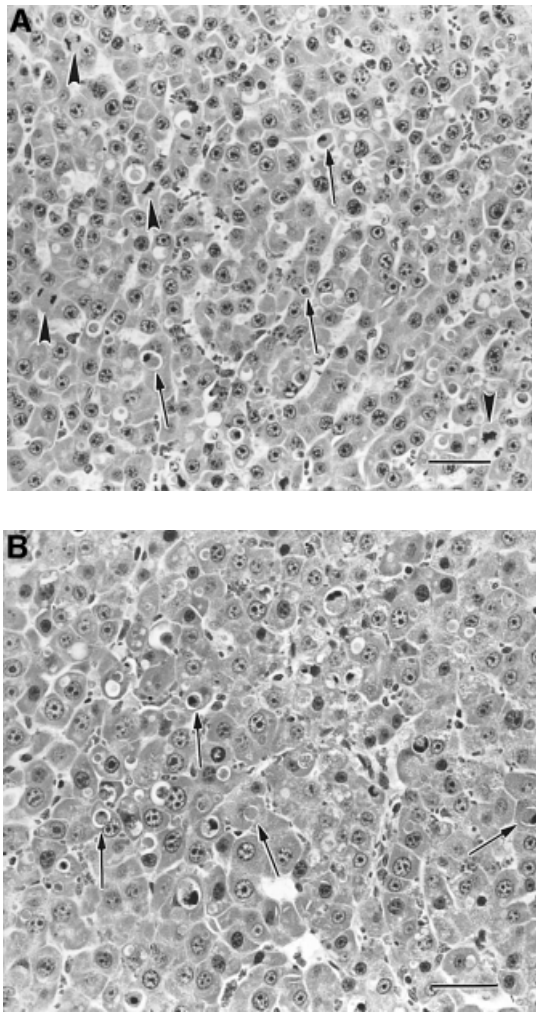

Fig. 4. (**A**) Section of hepatocellular adenoma from a rat continuously exposed to WY-14,643. Numerous mitotic figures (arrowheads) and apoptotic bodies (arrows) are present throughout the neoplasm. H&E stain. Bar, 50 μm. (**B**) Section of hepatocellular adenoma from a rat continuously exposed to WY-14,643 for 43 weeks and returned to control diet for 3 days. Numerous apoptotic bodies (arrows) are observed, but few mitotic figures are present. H&E stain. Bar, 50 μm.

14,643-induced PPARα activation late in tumor development is interesting in light of lower levels of the PPARα-dependent gene acyl CoA oxidase seen in preneoplastic lesions (32). This dislinkage has also been observed for the hepatic lobular distribution of PP-induced cell replication (periportal) and enzyme induction (centrilobular). Recent data suggest a role for Kupffer cells and their production of tumor necrosis factor α and possibly other immunomodulators, raising the intriguing possibility of an indirect pathway for mitogenesis and possibly tumor development in response to PP exposure (33).

The extent to which the immunophilin Cyp-40 may be necessary for PP-induced lesion growth is not clear since a direct regulatory link between PP and Cyp-40 is not established, thus the WY-14,643 withdrawal-associated decrease in Cyp-40 protein expression in adenomas requires further study. Alterations in Cyp-40 expression may be important given the broad ranging effects observed for members of this receptor class, especially in light of the fact that this is one of the few growth regulatory proteins shown to be up-regulated in PP-induced tumors.

Cyclophilins mediate apoptosis and interact with heat shock

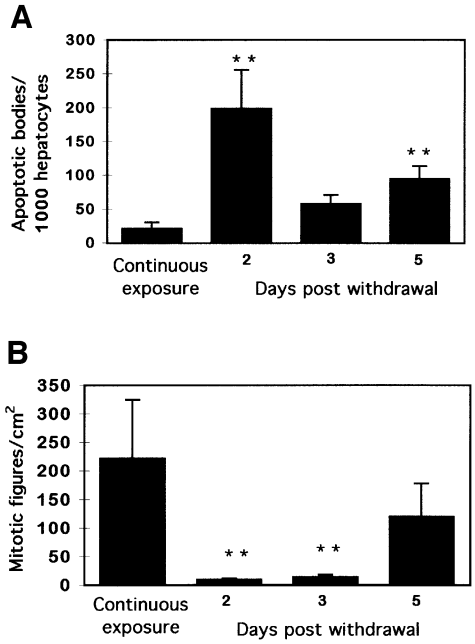

Fig. 5. (**A**) Apoptotic activity in hepatocellular adenomas from rats continuously exposed to WY-14,643 and from those withdrawn from WY-14,643 exposure. Apoptotic activity is increased in hepatocellular adenomas from rats as early as 2 days of withdrawal. (**B**) Mitotic activity in hepatocellular adenomas from rats continuously exposed to WY-14,643 and from those withdrawn from WY-14,643 exposure. Mitotic activity is greatly diminished in hepatocellular adenomas from rats as early as 2 days of withdrawal. For both graphs for continuous exposure, $n = 9$ adenomas from eight rats; 2 days withdrawal, $n = 4$ adenomas from three rats; 3 days withdrawal, $n = 7$ adenomas from four rats; 5 days withdrawal, $n = 8$ adenomas from five rats. **Significant difference from continuously exposed animals, $P < 0.05$.

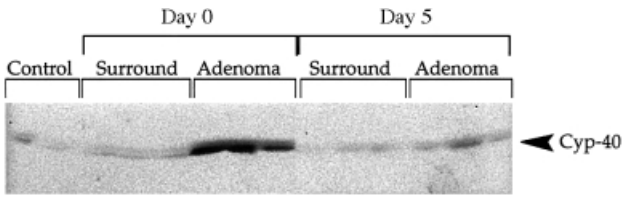

Fig. 6. Cyp-40 expression in hepatocellular adenomas from rats continuously exposed to WY-14,643 (Day 0) and from those withdrawn from WY-14,643 exposure for 5 days (Day 5). Increased Cyp-40 expression observed in hepatocellular adenomas from the continuously exposed group is nearly absent following 5 days of WY-14,643 withdrawal [$n = 2$ for control and $n = 3$ for surrounding tissue and adenomas; each band represents an individual and unique (non-pooled) sample]. Mean optical density values ± SEM for: control, 2.65 ± 1.05; Day 0 surround, 2.37 ± 0.90; Day 0 adenomas, 53.03 ± 12.19; Day 5 surround, 1.73 ± 0.32; Day 5 adenomas, 4.50 ± 1.67. Cyp-40 expression in Day 0 adenomas was significantly different from Day 0 surrounding tissue whereas there was no significant difference between Cyp-40 expression in Day 5 adenomas as compared with Day 5 surrounding tissue ($P < 0.05$).

proteins and other immunomodulatory pathways; these effects led us to consider the possibility of a role for this protein in Kupffer cell interactions with the target cell for neoplastic development (23,24,33–35). The apoptotic effects mediated by cyclophilins require clarification in the liver since cyclophilin-related apoptotic data have, to date, only been reported in non-hepatic tissues. Although a mitogenic role for Cyp-40 has not been previously reported, Cyp-40 over-expression in this study was observed in the group of adenomas that had high mitotic activity (from continuously exposed animals). However, a causal relationship has not been established in these studies. A possible interaction between PPARα and Cyp-40 requires further study. Few genes have been shown to be strongly up-regulated in PP-induced tumors, Cyp-40 expression can be used in future studies to characterize earlier appearing lesions and non-PP rodent hepatocarcinogens.

In conclusion, these results suggest that modulation of apoptosis and mitosis, possibly through cyclophilins, is an important mediator of PP-dependent growth in hepatocellular neoplasms.

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