Reversal of Liver Fibrosis in Aryl Hydrocarbon Receptor null Mice by Dietary Vitamin A Depletion

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Aryl hydrocarbon receptor (AHR)-null mice display a liver fibrosis phenotype that is associated with a concomitant increase in liver retinoid concentration, tissue transglutaminase type II (TGaseII) activity, transforming growth factorβ (TGFβ) overexpression, and accumulation of collagen. To test the hypothesis that this phenotype might be triggered by the observed increase in liver retinoid content, we induced the condition of retinoid depletion by feeding AHR-null mice a vitamin A-deficient diet with the purpose to reverse the phenotype. Liver retinoid content decreased sharply within the first few weeks on the retinoid-deficient diet. Analysis of TGFβ1, TGFβ2, and TGFβ3 expression revealed a reduction to control levels in the AHR-/- mice accompanied by parallel changes in TGaseII protein levels. In addition, we observed an increase in the TGFβ receptors, TGFβ RI and TGFβ RII, as well as in Smad4, and their reduction to wild-type mouse liver levels in AHR-/- mice fed the retinoid-deficient diet. Reduction of peroxisomal proliferator-activated receptor γ (PPARγ) messenger RNA (mRNA) and protein levels in AHR-/- mice was consistent with the presence of hepatic stellate cell (HSC) activation and liver fibrosis. Vitamin A deficiency normalized PPARγ expression in AHR-/- mice. In conclusion, livers from AHR-/- mice fed the vitamin A-deficient diet showed a decrease in collagen deposition, consistent with the absence of liver fibrosis. (HEPATOLOGY 2004;39:157–166.)

Liver fibrosis is characterized by an abnormal hepatic accumulation of extracellular matrix that results from both increased deposition and reduced degradation of collagen fibers. The key cellular event associated with this phenomenon is the injury-triggered activation of hepatic stellate cells (HSCs) to proliferating fibrogenic myofibroblastic cells.1–3 Activation of HSCs is also associated with a reduction in their lipid granules, the body store of vitamin A. Even though a wide spectrum of autocrine and paracrine factors (mainly cytokines and growth factors) can activate the HSCs, transforming growth factor β (TGFβ) plays a major role in fibrogenesis.4 Active TGFβ not only up-regulates the synthesis of extracellular matrix components, but also prevents their degradation by inhibiting the synthesis of matrix-degrading proteases (e.g., collagenase, stromelysin, plasmin) and by simultaneously inducing the synthesis of their inhibitors (e.g., tissue inhibitor of metalloprotease-1 and plasminogen activator inhibitor-1).5 More recently, the peroxisome proliferator-activated receptor, isoform gamma (PPARγ), also has been shown to be involved in the molecular regulation of HSC activation. Loss of PPARγ expression and reduction of its signaling are associated with activation of HSC,6–9 Furthermore, PPARγ ligands have been shown to affect collagen deposition in both animal models of liver fibrosis and in vitro. PPARγ ligands inhibit collagen gene expression through transcriptional inhibition of the collagen gene promoter, thus retarding fibrosis.8,9

Abbreviations: HSC, hepatic stellate cells; TGFβ, transforming growth factor-β; PPARγ, peroxisomal proliferator-activated receptor γ; RA, retinoic acid; AHR, aryl hydrocarbon receptor; TGaseII, tissue transglutaminase type II; RT-PCR, reverse transcription polymerase chain reaction; mRNA, messenger RNA; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HPLC, high-performance liquid chromatography.

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Even though liver retinoid status was shown to be an important factor in fibrogenesis, the exact mechanism by which retinoids play their role is still quite controversial. Loss of cytoplasmic retinyl palmitate and other retinyl esters is often associated with HSC activation in advanced fibrosis. In vitro treatment of quiescent HSCs with retinol, the hydrolysis product of retinyl esters, leads to inhibition of their activation, which results in down-regulation of cell proliferation and collagen synthesis. These data suggest that loss of retinoids from HSCs promotes their activation. However, other studies showed that hypervitaminosis A is also associated with liver fibrosis. In an in vitro and in vivo rat model, exposure of HSCs to supraphysiologic concentration of retinoids is profibrogenic and exacerbates liver fibrosis, possibly by inducing production and activation of latent TGFβ in HSCs. This would suggest an interplay between the retinoid and TGFβ pathways in the development of fibrotic liver injury. Other studies supported the hypothesis that the liver fibrotic phenotype observed in aryl hydrocarbon receptor (AHR)-null mice is associated with an impaired retinoid homeostasis. The observed increase in the levels of retinoic acid (RA) and tissue transglutaminase type II (TGaseII) activity could account for activation of the latent TGFβ complex, resulting in altered deposition of collagen, abnormal cell cycle control, and increased apoptosis in the livers of AHR-/- mice. We hypothesized that, if retinoid excess is a causative agent for overexpression of TGFβ in AHR-null mice, dietary depletion of vitamin A should reduce TGFβ levels and signaling through downstream Smad proteins and eventually reverse the fibrotic phenotype of AHR-null mice.

Materials and Methods

Experimental Design. AHR-null mice (C57BL6/CNrlX129/Sv) were generated as described previously. Control (AHR +/+ ) and AHR-null mice were housed in a pathogen-free facility using air-filtered controlled environment racks and autoclaved water, cages, and bedding. All manipulations of mice were performed in sterile conditions and in accordance with the National Institutes of Health guidelines recommended and enforced by the National Cancer Institute Animal Care and Use Committee. After copulation, female mice were divided into two groups. One group was fed a control autoclaved Purina rodent chow and the other group received a vitamin A-deficient rodent diet (TD 69523; Harlan Teklad, Indianapolis, IN). The vitamin A-deficient diet is a highly purified research diet containing no sources or precursors of vitamin A in a protocol that has consistently generated vitamin A deficiency within 15 to 20 weeks for other strains. At weaning, the male pups, 40 per dietary group, from both genotypes were culled and maintained on their respective diets for up to 18 weeks. At each time point (i.e., 3, 6, 9, and 12-18 weeks of age), two mice per dietary group and genotype were weighed and killed by CO2 narcosis. The liver specimens were quickly removed and weighed. For retinoid determination by high-performance liquid chromatography (HPLC), liver sections were snap frozen in liquid N2 and stored at −70°C or fixed in formalin (10% formaldehyde in phosphate-buffered saline) and used for immunohistochemical and biochemical analysis.

Measurement of Liver Retinyl Palmitate. Retinoids were extracted as described previously. Briefly, liver tissue sections were homogenized in saline and extracted with chloroform/methanol (2:1). The organic extracts were evaporated to dryness under nitrogen. The residues were dissolved in ethanol, filtered, and kept at −20°C until aliquots were analyzed. Retinyl palmitate was detected by HPLC. A Beckman model 110A pump (Beckman Instruments, Palo Alto, CA) was connected to an ultraviolet detector (Knauer variable-wavelength; Sonntek, Woodcliff Lake, NJ) set at 325 nm. Analyses were performed on a C-130 guard column (Upchurch Scientific, Oak Harbor, WA) in series with a Waters C-18 (5 mm) “Resolve” column (3.9 mm inside diameter × 30 cm). The mobile phase consisted of acetonitrile/dichloromethane/methanol/1-octanol (90:15:10:0.1) plus 0.1% butylated hydroxytoluene at a flow rate of 1.2 mL/min.

Immunohistochemistry. For the immunocytochemical localization of TGFβ and their receptors in 10% formalin-fixed, paraffin-embedded tissue sections, the avidin-biotin complex technique was employed (Vector Laboratories, Burlingame, CA). Five liver sections per mouse were analyzed for each antibody. Affinity-purified polyclonal antibodies to TGFβ1 (V) for TGFβ1, TGFβ2 (V) for TGFβ2, TGFβ3 (III) for TGFβ3, TGFβ RI (V-22) for TGFβ RI, and TGFβ RII (L-21) for TGFβRII were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). After deparaffinization and blocking of endogenous peroxidase in hydrogen peroxide/methanol, the tissue sections were blocked with 1.5% goat serum/0.5% bovine serum albumin, incubated overnight at 4°C with affinity-purified antisera at 0.5 mg/mL, washed extensively, and incubated with biotinylated goat anti-rabbit immunoglobulin G and the avidin-enzyme complex. Tissue sections were stained with 3,3’-diaminobenzidine (Sigma, St. Louis, MO) and hydrogen peroxide and counterstained with Mayer’s hematoxylin. Controls included the use of primary antisera preincubated with a 20-fold...
excess of the appropriate peptide and the replacement of primary antisera with normal rabbit immunoglobulin G for 2 hours at room temperature before this mixture was applied to the tissue section. For Smad4 and PPARγ immunolabeling, tissue sections were microwaved in 10 mmol/L citrate buffer (pH 6) for 12 minutes for antigen retrieval. The tissue sections were then blocked for 1 hour in 1.5% horse serum and incubated overnight with the mouse monoclonal anti-Smad4 and polyclonal PPARγ (E8) antibody (Santa Cruz Biotechnology), applied after a 1:200 dilution. Antibody binding was visualized.

Collagen Deposition in Liver. The collagen content was evaluated using Sirius red/Fast green staining as previously described.16

Real-time Reverse-Transcription Polymerase Chain Reaction. The expression of PPARγ messenger RNA (mRNA) was quantified by real-time reverse-transcription polymerase chain reaction (RT-PCR). Total RNA samples were extracted from crushed, frozen mouse liver tissue sections using TRIzol reagent (Invitrogen, Life Technologies, Carlsbad, CA) according to the manufacturer’s protocol. To avoid possible contamination of genomic DNA, RNA samples were treated with DNaseI and purified using the MegaClear kit (Ambion, Austin, TX) according to the manufacturer’s protocol. In the complementary DNA synthesis, purified total RNA samples (1 µg) underwent RT using the SuperScript First-Strand Synthesis System for RT-PCR kit (Invitrogen), according to the manufacturer’s protocol. Reactions were primed with Oligo(dT)12-18 primers in a total volume/reaction of 20 µL. Quantitative PCR was performed in a total volume of 50 µL using the SYBR Green PCR core reagent kit (Perkin-Elmer Applied Biosystems, Foster City, CA) and analyzed on an ABI PRISM 7900 HT sequence detector system (Perkin-Elmer Applied Biosystems). One microliter of complementary DNA per sample was used as the template. Both glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and PPARγ amplifications were performed in triplicate for each sample. Primer sequences for PPARγ (GenBank accession number NM_0111146) were as follows: forward primer 5’-ATTCTGGCCCCACCACTTGGG1703’; reverse primer 5’-488TGGAAAGCTTGTGCTTTTATGCCCA4633’. The primer pair set for GAPDH was purchased from Applied Biosystems. The thermal cycling conditions included 2 minutes at 50°C and 10 minutes at 95°C, followed by 40 cycles at 95°C for 15 seconds and 60°C for 1 minute. At the end of the PCR cycles, the PCR products were analyzed using the dissociation curve protocol to make sure that only one single PCR product was detected by SYBR Green dye. Quantitative values for PPARγ expression were obtained from identification of the cycling point when the PCR product is detectable (threshold cycle value). Results are expressed as relative levels of PPARγ mRNA, referred to samples from AHR+/+ mice on the normal diet (the calibrator), chosen to represent 1 times the expression of this gene. Samples from AHR-null mice on both the normal and vitamin A-deficient diets and from AHR+/+ samples on vitamin A-deficient diet express n-fold PPARγ mRNA relative to the calibrator. The amount of target, normalized to an endogenous reference (GAPDH) and relative to the calibrator, is defined by the ΔΔ threshold cycle method as described by Livak (Sequence Detector User Bulletin 2, Applied Biosystems). Data are expressed as means. The Student’s t-test was used to determine the significance of differences between groups (P < .05 was significant).

Western Blotting. Frozen liver tissue specimens were homogenized in lysis buffer (30 mmol/L Tris, pH 7.5; 150 mmol/L NaCl; 1% NP-40; 0.5% Na+ deoxycholate; 0.1% sodium dodecyl sulphate; 10% glycerol; 1 mmol/L ethylenediaminetetraacetic acid) to which the protease inhibitor cocktail (Complete Mini, Roche, Indianapolis, IN) was added just before use. After incubation for 30 minute at 4°C, tissue samples were centrifuged at 14,000g for 10 minutes. Pellets were discarded and supernatants used for protein quantification by the dye binding method (MicroBCA kit, Pierce, Rockford, IL) using bovine serum albumin as a standard. Thirty micrograms was separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis on a 4% to 12% Bis-Tris NuPAGE gel (Invitrogen) and transferred to nitrocellulose membrane. Membranes were probed with antibodies against TgaseII (Ab-4, NeoMarkers, Lab Vision, Fremont, CA). Binding of antibody was detected using horseradish peroxidase-conjugated secondary antibodies and the SuperSignal Chemiluminescence Substrate for detection of horseradish peroxidase (Pierce). Densitometric analysis was performed using Kodak 1D image analysis software (Kodak, Rochester, NY).

Results

Establishment of Vitamin A Deficiency in AHR+/+ and AHR-/– Mice. No major difference (P = .75) between the body weights of AHR+/+ and AHR-/– mice was evident in mice maintained on a normal diet. However, a highly significant difference was found for liver weights (P = 1.8 × 10⁻⁶) between the two genotypes. The AHR-/– mice weighed approximately 30% less than the wild-type mice (Fig. 1A). Vitamin A deficiency caused a small (10%) difference in body weight (P = .045) and a highly significant difference in liver weight (P = 4.9 × 10⁻⁵) between the two genotypes (Fig. 1B). The AHR-/– mice weighed approxi-
mately 30% less than the wild-type mice. This experimental design induced complete retinoid depletion in different mouse strains (refs. 18, 19 and this manuscript). To confirm the vitamin A-deficient status of the mice, liver retinyl ester levels were determined by HPLC. Figure 2A shows that liver retinyl palmitate levels decreased sharply in both AHR+/+ and AHR-/- mice 3 weeks after they received the deficient diet and reached near zero levels at 9 weeks (Fig. 2B). The retinyl palmitate levels remained undetectable thereafter (Fig. 2C). This finding reproduced previous results in a different strain of mice.19 It is noteworthy that consumption of the vitamin A-deficient diet in our protocol abolished the 2.5-fold difference in retinyl palmitate content between the two genotypes.

Expression of TGFβ (TGFβ1, TGFβ2, and TGFβ3) in AHR+/+ and AHR-/- Mice Fed a Normal Diet Versus Mice Fed a Vitamin A-Deficient Diet. The liver fibrosis phenotype in the AHR-/- mice correlated with an increased level of TGFβ expression as well as with higher levels of retinyl esters, retinol, and RA.12,17 To test whether it is possible to reverse liver fibrosis by dietary vitamin A deficiency, we determined the expression of TGFβ (TGFβ1, TGFβ2, and TGFβ3) in the liver of AHR+/+ and -/- mice fed vitamin A-deficient versus control diets. Figure 3 shows the results of the
Fig. 3. Immunohistochemical analysis of TGFβ1, TGFβ2, and TGFβ3 in AHR +/+ and AHR -/- mice fed normal and vitamin A-deficient diets. Liver tissue sections of AHR -/- and AHR +/+ mice were stained for TGFβ1, TGFβ2, and TGFβ3 isoforms. AHR -/- mice fed the normal diet (left panels) had higher levels of these cytokines (in particular TGFβ1) compared with their AHR +/+ counterparts (inserts). When AHR -/- mice were fed a vitamin A-deficient diet (right panels) expression levels of TGFβ1, TGFβ2, and TGFβ3 decreased to normal levels, that is, levels were similar to those of AHR +/+ controls (inserts). (Original magnification, ×400.)
immunohistochemistry studies. Hepatocytes, HSC, and Kupffer cells displayed immunoreactivity for TGFβ1, TGFβ2, and TGFβ3 in both AHR+/+ and AHR−/− mice. However, AHR−/− mice maintained on a normal diet exhibited higher levels of TGFβ1 compared with AHR+/+ mice, in agreement with previous reports.12,17 Furthermore, AHR−/− mice fed a normal diet showed higher expression of both TGFβ2 and TGFβ3 compared with AHR+/+ liver tissue sections (Fig.3, panels showing normal diet; inserts show AHR+/+ counterpart controls). Figure 3 also demonstrates that vitamin A deficiency reduces the expression of TGFβ to the same basal level observed in AHR+/+ mice maintained on a normal diet (Fig.3, panels showing vitamin A-deficient diet). The levels of TGFβ in AHR+/+ mice are similar for the two diets.

Expression of TGFβ RI and RII in AHR+/+ and AHR−/− Mice Fed a Normal Diet Versus Mice Fed a Vitamin A-Deficient Diet. In animals fed a normal diet, immunodetectable levels of TGFβ RI and RII were higher in hepatocytes and HSC from liver tissue sections of AHR−/− mice compared with wild-type control mice (Fig. 4). Basal levels of expression of TGFβ RI and RII were observed in both wild-type and null genotypes maintained on a vitamin A-deficient diet. These results indicate that overexpression of TGFβ and TGFβ RI and TGFβ RII in liver tissue sections from AHR−/− mice can be normalized by equalizing liver retinoid content through a vitamin A-deficient diet.

Smad4 Expression. Smad4 expression was studied to assess whether the observed higher expression of the TGFβs results in an increased TGFβ signal transduction
in AHR -/- mice. Figure 5 shows increased expression of Smad4 in AHR -/- mice maintained on a normal diet. The majority of AHR -/- hepatocytes displayed nuclear localization of Smad4 (Fig. 5B, arrows). In contrast, AHR -/- mice fed a vitamin A-deficient diet showed lower Smad4 expression levels (Fig. 5C) similar to the expression levels found in AHR +/+ mice maintained on either a normal or vitamin A-deficient diet (Fig. 5A).

**AHR-Null Livers Show a Reduction in PPARγ and Dietary Vitamin A Deficiency Normalizes PPARγ Expression to Endogenous Levels.** Because HSC activation is accompanied by a reduction in PPARγ expression,6–9 we studied by immunohistochemistry PPARγ expression in the livers of AHR-null mice. Consistent with HSC activation,6–9 PPARγ levels in HSC (red arrows) from AHR -/- (Fig. 6B) mice decreased compared with their wild-type counterparts (Fig. 6A). Next, we tested whether a lower HSC activation is observed in AHR-null mice fed a vitamin A-deficient diet. PPARγ expression was normalized in these mice (Fig. 6C). We further validated these immunohistochemistry data by checking PPARγ mRNA expression levels by real-time RT-PCR. Figure 6D shows a 50% reduction of PPARγ mRNA levels in the AHR-null mice and normalization to endogenous levels in mice fed the vitamin A-deficient diet (P < .05).

**AHR-Null Livers Show an Increase in TGaseII and a Decrease to Normal Levels After Receiving a Vitamin A-Deficient Diet.** Figure 7 shows the results of a Western blot analysis of liver tissue sections. As shown in a previous study,12 the densitometric analysis in the current study showed that TGaseII levels were twofold higher in the AHR-null mouse liver tissue sections compared with the liver tissue sections from wild-type mice (Fig. 7B). The results in the current study are consistent with the increase in RA concentration observed in this genotype. The vitamin A-deficient diet caused a marked reduction in TGaseII levels in both genotypes. As a result of this reduction, TGaseII levels in AHR -/- mice fed a vitamin A-deficient diet were normalized to levels observed in AHR +/+ mice maintained on a normal diet.

**Dietary Vitamin A Deficiency Reverses Excess Deposition of Collagen Fibers in the Liver of AHR -/- Mice.** Collagen and noncollagen fiber expression was determined by Sirius red/Fast green double staining.16 Increased collagen deposition (stained in red) versus noncollagenous fibers (stained green) was observed in the liver tissue sections of AHR -/- mice fed a normal diet (Fig. 8B) compared with wild-type control mice on the same diet (Fig. 8A). Inserts represent smaller fields at higher magnification to appreciate that the staining for collagen fibers, when present, is consistent with fibrosis.
The vitamin A-deficient diet reduced the collagen fibers to levels similar to expression in wild-type mice (Fig. 8C).

Discussion

The deficiency in AHR expression is associated with increased collagen deposition and liver fibrosis.13–17 This phenotype was characterized by a marked accumulation (twofold to threefold) of liver retinyl esters, retinol, and RA, as well as by an increase in TGaseII activity12 and an accumulation of TGFβ1 and TGFβ3.17 In the current study, we tested the hypotheses that the retinoid excess status of AHR-null mice would contribute to the observed phenotype of liver fibrosis and that vitamin A-deficient diet would deplete liver retinoids, reduce TGFβs and their downstream signaling molecules, and reverse the phenotype of liver fibrosis. The deficient diet reduced liver retinyl ester levels by 90% within the first 3 weeks and retinyl esters could not be detected after 9 weeks on the diet. This retinyl ester depletion rate is similar to that reported previously for SENCAR mice,19 suggesting that our ability to induce vitamin A deficiency in mice is dependent on the diet and the methodology used.18,19

We confirmed the increase in TGFβ1 and TGFβ3 reported previously17 and showed increased TGFβ2 expression in the hepatic portal areas of the AHR-/- mice compared with their wild-type counterparts. Higher expression levels of TGFβ were detected in both parenchymal (hepatocytes) and nonparenchymal (HSC, Kupffer cells) cells from AHR-/- mouse liver tissue sections. This increase was more pronounced for the expression of TGFβ1 compared with the TGFβ2 and TGFβ3 isoforms. In addition to the TGFβs, TGFβRI and TGFβRII and their downstream signaling molecule, Smad4, were overexpressed in AHR-/- mouse liver tissue sections. Remarkably, the expression of the TGFβ isoforms and of the RI and RII receptors was normalized in mice fed a retinoid-deficient diet. This was also associated with decreased TGFβ signaling, as demonstrated by the normalized Smad4 expression in AHR-/- mice fed the deficient diet.

Because HSC activation is accompanied by down-regulation of PPARγ6–9 our observed normalization of PPARγ levels in the vitamin A-deficient AHR-null mouse liver tissue sections (Fig. 6) supports the hypothesis that vitamin A deficiency reduces the level of HSC activation
in AHR-null mice with a consequent reversal of liver fibrosis. Our data with TGaseII are consistent with the role of this enzyme in the activation of latent TGFβs. An increase in TGaseII protein levels was found and its down-regulation resulted from a vitamin A-deficient diet. Finally, staining with Sirius red/Fast green confirmed14 an increase in collagen fiber deposition in the liver tissue sections of AHR-/- mice, consistent with liver fibrosis. In mice fed the vitamin A-deficient diet for 18 weeks, collagen levels were normal, demonstrating a reversal of liver fibrosis. Real-time RT-PCR experiments for α1(I) collagen gene expression (data not shown) confirmed the results obtained by Sirius red/Fast green staining.

Our results support the importance of a direct link between retinoids and TGFβ in fibrogenesis, as also emphasized by Friedman1 and Blomhoff.3 Retinoids stimulated the activation of latent TGFβ1 through TGaseII17 and we demonstrated an increase in TGaseII activity in the AHR -/- mouse liver.12 Similarly, Okuno et al.11 showed that RA exacerbates liver fibrosis obtained in response to subcutaneous injection of heterologous serum. However, Leo et al.20 showed that ethanol-induced fibrosis in rat liver tissue sections is coincident with a retinoid deficiency rather than an excess. Senoo and Wake21 suggested that excess retinyl esters protect from liver fibrosis by inducing a quiescent status in HSC, which are believed to be the main cell type responsible for fibrogenesis. Therefore, liver fibrogenesis can be induced by different mechanisms.

The current study shows that nutritional intervention with a diet designed to specifically lower the concentration of an essential nutrient (i.e., vitamin A) can counteract and reverse the phenotype induced by a genetic mutation. Therefore, vitamin A status, although not necessarily the only factor responsible for fibrogenesis and its prevention, may be an important contributor especially in the presence of a genetic imbalance.

References


