

# Vitamin A Deficiency Associated with Enhanced Proliferation of Bile Duct Epithelial Cells in the Rat

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**ABSTRACT:** **Background:** Vitamin A and its derivative retinoic acid regulate various aspects of cell behavior as growth, differentiation, and proliferation. Retinoic acid derivatives have been suggested to play a role in processes such as hepatic regeneration and fibrosis.

**Objectives:** To evaluate the influence of vitamin A on rat liver epithelial cell proliferation.

**Methods:** We performed common bile duct ligation in rats that had been subjected to differing vitamin A diets and compared their livers to control rats. Proliferation, apoptosis, and retinoic acid receptors were evaluated by histology and immunohistochemistry in bile duct cells and hepatocytes.

**Results:** Vitamin A deficiency was found to be associated with enhanced proliferation of bile duct epithelial cells following CBD ligation. The proliferation was manifested by increased numbers of ducts, by aberrant extended ductal morphology, and by elevated numbers of nuclei expressing the proliferation marker Ki67. The amount of vitamin A in the rat diet did not affect detectably ductal cell apoptosis. We observed up-regulated expression of the retinoid X receptor-alpha in the biliary epithelium of vitamin A-deficient rats that had undergone CBD ligation, but not in vitamin A-sufficient rats.

**Conclusions:** We speculate that the mechanism underlying the ductal proliferation response involves differential expression of RXR-alpha. Our observations suggest that deficiency of vitamin A may exacerbate cholestasis, due to excessive intrahepatic bile duct proliferation.

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**KEY WORDS:** vitamin A, deficiency, bile duct, proliferation

Vitamin A and its derivative retinoic acid regulate various aspects of cell behavior including growth, differentiation and proliferation [1]. In addition, they are modulators of embryogenesis [2], and important regulators of epithelial cell

terminal differentiation [3]. The effects of RA are mediated via its receptors, namely retinoid acid receptors and retinoid X receptors [4]. These receptors belong to a superfamily of nuclear steroid and thyroid hormone receptors. Members of this superfamily are ligand-dependent transcription factors that bind to specific regulatory sequences known as retinoic acid response elements and thus influence transcription of RARE-associated target genes [5]. Retinoic acid derivatives may also play a role in hepatic fibrosis via generation of transforming growth factor-beta [6,7].

Animals subjected to differing vitamin A diets are useful for investigating the involvement of vitamin A and/or RA in physiological processes. For example, hepatic oval cell (the progeny of the hepatic stem cell compartment) proliferation was observed to be increased in vitamin A-deficient rats in comparison with vitamin A-supplemented rats, indicating a role for vitamin A in oval cell proliferation [8]. Partial hepatectomy in VAD rats results in an inverse relationship of RAR-beta and RAR-alpha expression, and in elevated expression of TGF $\alpha$  [8]. A widely used rat experimental system for examining, specifically, proliferation of liver epithelial cells entails ligation of the common bile duct [9]. Using this system it was shown that proliferating bile-like cells are derived from existing biliary epithelium and have the same characteristics as the bile epithelium. Furthermore, it was found that the new lumen is usually continuous with the original one [10]. Two days after CBD ligation, bile duct cells exhibited a 17-fold increase in proliferation, accompanied by a three to fourfold increase in proliferation, accompanied by a three to fourfold up-regulation of hepatocyte renewal [11]. More generally, CBD ligation is a useful model for studying cholestasis.

The aim of the present study was to evaluate the influence of vitamin A on rat liver biliary epithelial cell and hepatocyte proliferation. Both types of epithelial cells arise from a common founder cell, which has bipotential differentiation capa-

RA = retinoic acid

RARE = retinoic acid response elements

VAD = vitamin A-deficient

RAR = retinoid acid receptors

TGF $\alpha$  = transforming growth factor-alpha

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CBD = common bile duct

RXR = retinoid X receptor

bilities during hepatic growth stimulation and regeneration. We examined the behavior of these cells after CBD ligation in rats subjected to a vitamin A-deficient diet, as compared to rats with a vitamin A-sufficient diet.

## MATERIALS AND METHODS

### ANIMALS

Male weanling Wistar, pathogen-free rats were obtained from the Harlan Laboratory, Weizmann Institute of Science, Rehovot, Israel. The rats were housed in metal cages in a room with controlled temperature ( $25 \pm 2^\circ\text{C}$ ), relative humidity ( $65 \pm 5\%$ ) and light (0800–2000 hr). Ethical approval was obtained for the study and all the procedures were conducted in full compliance with the strict guidelines of the Hebrew University Policy on Animal Care and Use.

### EXPERIMENTAL DESIGN AND DIETS

Male weanling rats (40–45 g) were randomly divided into two groups. Group 1 ( $n=8$ ) was fed a pelleted version of the VAD diet containing negligible amounts of vitamin A (cat. no. 960220, ICN Nutritional Biochemicals, Costa Mesa, CA, USA; see appendix); rats in group 1 are referred to as VAD. Group 2 ( $n=8$ ) was fed a vitamin A-sufficient diet containing retinyl palmitate (1200  $\mu\text{g}/\text{kg}$ ), which is the customary control diet for such studies (ICN Nutritional Biochemicals); rats in group 2 are referred to as VAS. All groups consumed food *ad libitum*. Food intake was monitored daily and the rats were weighed every other day.

### MEASUREMENT OF LIVER AND PLASMA VITAMIN A

After saponification with 50% ethanolic potassium hydroxide (KOH) vitamin A was extracted from plasma and liver samples using petroleum ether. Vitamin A concentration was determined using a high performance liquid chromatogram equipped with a fluorescence detector (MD-910, JASCO, Tokyo, Japan). HPLC was performed using a 5  $\mu\text{m}$  C18 reversible column and ethyl acetate-eluting solvent (20% in methanol). Retinyl acetate was used as an internal standard [12].

### COMMON BILE DUCT LIGATION

Eight VAD and eight VAS rats were prepared for operation. Five rats died during or soon after the procedure. A complete CBD ligation was induced in the rats by means of a double ligature as described previously [13]. Three VAD and four VAS rats survived the CBD procedure. Two VAD and two VAS rats had sham operations. Rats were sacrificed 48 hours after their operation; body and liver weights were measured, and livers were collected for histological evaluation.

### HISTOLOGY

The liver blocks were obtained from similar regions of the liver. All liver samples were blinded and viewed by one pathologist. Ten samples per liver were analyzed. Livers were embedded in paraffin and 4  $\mu\text{m}$  sections were cut. Sections were stained with hematoxylin and eosin.

### IMMUNOHISTOCHEMISTRY

To assess the number of bile ducts all bile duct-like structures that immunostained with cytokeratin-7 (MU255-UC, Biogenex, USA) were counted per portal area. Ten portal spaces were counted for each specimen. The tissue sections were dewaxed and microwave or pressure cooker antigen retrieval in buffer citrate pH 6.00 was performed. Sections were incubated for 1 hour at  $37^\circ\text{C}$  with one of the following antibodies: anti-TGF $\alpha$  (H-50) (SC-9043, Santa Cruz Biotechnology, USA) at a dilution of 1:50; anti-Ki67 (MM1) (NCL-Ki67-MM1, Novocastia, UK) at 1:100; anti-RXR $\alpha$  (D-20) (SC-553, Santa Cruz Biotechnology) at 1:20; anti-RAR $\alpha$  (C-20) (SC-551, Santa Cruz Biotechnology) at 1:10. Microwave antigen retrieval was done for anti-TGF $\alpha$ . Pressure cooker antigen retrieval was done for anti-Ki67. Detection was performed with a broad-spectrum detection kit (Histostain SP, 95-9943B, Zymed, USA). The chromogen used was AEC (aminoethylcarbazole) and sections were counterstained with hematoxylin.

To quantify the proliferation status of a specimen, 200 bile duct cells and 200 hepatocytes were surveyed and the number of nuclei immunostaining positive for the proliferation marker Ki67 was counted. The amount of apoptosis occurring in a specimen was assayed using the Tunnel In Situ Cell Death Detection Kit (AP Boehringer Mannheim, Germany) that monitors DNA double strand breaks. For each specimen 200 bile duct cells and 200 hepatocytes were viewed and the proportion of Tunnel-positive nuclei noted.

Vitamin A/retinoic acid receptors were detected using anti-RXR $\alpha$  (D-20) rabbit polyclonal antibody (SC-553, Santa Cruz) and anti-RAR $\alpha$  (C-20) rabbit polyclonal antibody (SC-551, Santa Cruz). Next, 200 bile duct cells and 200 hepatocytes per specimen were surveyed and the proportion with positive immunostaining noted.

### STATISTIC ANALYSIS

The data were analyzed using BMDP [14]. We calculated means and standard deviation for all relevant variables. Since the number of rats was small we compared the four groups using Mann-Whitney non-parametric analysis.  $P$  value  $< 0.05$  was considered significant.

## RESULTS

The animal characteristics including body and liver weight, and vitamin A levels of the VAD and VAS rat groups are summa-

VAS = vitamin A-sufficient

HPLC = high performance liquid chromatography

**Table 1.** Animal characteristics in the VAD and VAS rat groups

	CBD ligation		Sham		P
	VAS rats	VAD rats	VAS rats	VAD rats	
Body weight (g)	320.0 (310–330)*	246.0 (215–277)	332.5 (325–340)	285.5 (263–308)	0.007
Liver weight (g)	12.0 (11.35–12.7)	10.3 (9.0–11.5)	13.5 (12–15)	9.2 (8.7–9.8)	0.11
Liver/body weight (g)	0.037 (0.034–0.04)*	0.042 (0.04–0.046)	0.04 (0.035–0.046)	0.032 (0.028–0.037)	0.049
Vit A-liver (µg/L)	117.6 (104.3–131.2)*	0.92 (0.85–1.02)	121.3 (110.3–132.4)	1.05 (0.85–1.02)	0.034
Vit A-plasma (µg/L)	68.35 (60.1–74.3)*	14.9 (14.3–16.7)	72.9 (68.7–77.2)	16.8 (16.4–17.2)	0.034

Values are presented as median (range)

\*Significant difference between CBD ligated VAS and VAD rats

**Table 2.** Measures of bile duct proliferation in VAD versus VAS rats

	CBD ligation		Sham	
	VAS rats	VAD rats	VAS rats	VAD rats
No. bile duct profiles/portal area	3.4 ± 0.5*	14.3 ± 3.4*	1.3 ± 0.0	3.2 ± 0.9
No. Ki67 proliferation marker in bile ducts	28.4 ± 6.6	71.1 ± 6.2**	2.5 ± 0.7	10.5 ± 0.7**

\* Significant difference between these groups ( $P = 0.034$ )

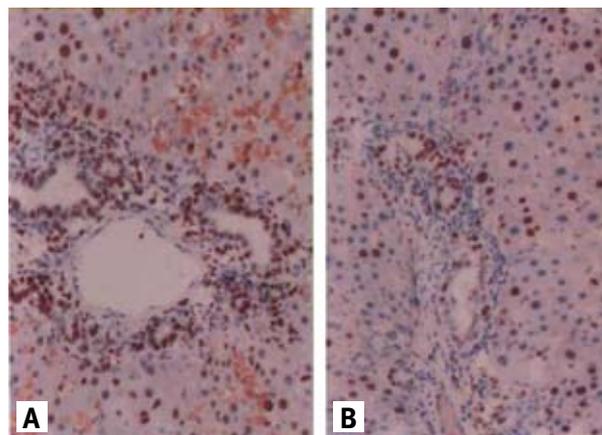
\*\* Significantly higher values for the VAD group both in the case of CBD ligated ( $P = 0.034$ ) and sham operated rats ( $P = 0.012$ ).

rized in Table 1. In the CBD-ligated rats the weight of the VAD rats was significantly lower than the VAS rats ( $P = 0.007$ ).

#### PROLIFERATION OF RAT BILE DUCT CELLS IN RESPONSE TO CBD LIGATION IS INFLUENCED BY DIETARY VITAMIN A

Histological inspection of specimens from CBD-ligated VAD and VAS rats revealed a more extensive bile duct proliferation in the livers of VAD than in the livers of VAS rats. It was notable that the extensive bile ductular proliferation observed in livers of VAD rats resulted in their extension beyond the limits of the portal tract and invasion of the parenchyme, most often in the direction of neighboring portal tracts. This periportal duct extension was associated with both acute and chronic inflammation. Areas of hepatocellular necrosis were observed in cholestatic regions. In sham operated rats the liver histology was normal.

In addition to the altered histology of the bile ducts observed in VAD rats, the absolute number of bile ducts was found to be significantly higher when compared to VAS rats. After CBD ligation, a mean of 14.3 duct-like profiles were counted in a portal field in VAD rats compared to 3.4 in VAS rats [Table 2]. Furthermore, the proliferation rate of bile duct cells, as measured by the proportion of bile duct cell Ki67 immunostaining, was significantly higher in the livers of the VAD than

**Figure 1.** Ki67 proliferation marker shows increased proliferation activity in bile ducts of VAD rats [A] in comparison to the sham group [B].

in the VAS rats, both for sham operated and CBD-ligated rats [Figure 1, Table 2]. However, it is noteworthy that CBD-ligated VAD and VAS rats exhibited overall increased proliferation rates compared to sham operated rats [Table 2]. Importantly, when the proliferation rate of hepatocytes was examined, no significant difference between VAD and VAS rats was found, indicating that the proliferation response to CBD ligation might be limited to ductal epithelial cells (data not shown).

#### THE LIVER APOPTOTIC RESPONSE TO CBD LIGATION IS NOT INFLUENCED BY DIETARY VITAMIN A

No significant differences were found between the VAD or VAS rats in the number of apoptotic hepatocytes or the number of apoptotic bile duct epithelial cells.

#### RXR $\alpha$ EXPRESSION IS DETECTABLE ONLY IN BILE DUCTS OF VAD RATS AFTER CBD LIGATION

There was no detectable immunostaining for RAR-alpha receptor in the bile ducts of VAD or VAS rats before or after operations. Immunostaining for RXR $\alpha$  was detectable in small amounts (5% of cells) after CBD ligation only in the bile ducts of VAD rats [Figure 2]. Both receptors were detected in the hepatocytes in sham and CBD-ligated VAD and VAD rats ( $P = NS$ ).

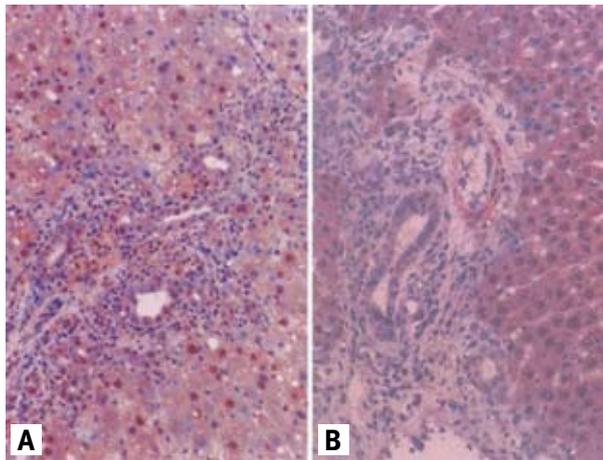
#### TGF $\alpha$ EXPRESSION IS NOT INFLUENCED BY DIETARY VITAMIN A

The number of inflammatory mononuclear cells stained for TGF $\alpha$  in the portal spaces was similar in all rat groups, independent of their diet.

## DISCUSSION

We found that vitamin A deficiency is associated with augmented proliferation of bile duct epithelial cells following CBD

**Figure 2.** RXR-alpha immunopositivity was detectable in 5% of bile duct cells **[A]** in comparison to lack of RXR alpha expression in bile ducts of the sham group **[B]**.



ligation. Although the number of rats in each group is small, the findings are novel, and the difference between the study and control groups is very prominent and statistically significant.

Previous related studies have not addressed this question. One earlier study looked at the effect of vitamin A deficiency on oval cells and found that oval cell proliferation is increased in response to bile duct ligation in VAD rats as compared with VAS rats [8]. Additionally, a study looking at the first 8 hours after partial hepatectomy described focal necrosis of the liver followed by rapid regeneration in VAD rats. The necrosis was associated with a fourfold increase in DNA strand breaks as assayed by Tunnel labeling of hepatocyte nuclei. However, in this hepatectomy study, no morphological or biochemical changes were found in the bile duct cells at this early time point [15]. Our study uniquely and specifically addresses the effect of vitamin A deficiency on bile duct cells and hepatocytes and revealed the extensive bile duct proliferation after CDB ligation in VAD rats.

The mechanism of the enhanced bile-duct proliferation associated with vitamin A deficiency is not well defined. In normal rat liver, cholangiocytes are mitotically dormant and can markedly proliferate in response to pathological triggers such as bile duct ligation [16]. Ductal hyperplasia after bile duct ligation is not associated with apoptosis, which makes this model ideal for evaluation of hyperplastic processes in the rat bile ducts. Proliferation of bile epithelial cells is a result of interactions between ductal and periductal cells, and is influenced by the secretion of cytokines such as interleukin-6 and hepatocyte growth factor [17]. Another investigation of the mechanisms controlling ductal proliferation was performed by examining oncogene expression after bile duct ligation. This study revealed absence or low levels of TGF $\alpha$  in combination with reduced expression of the gene encoding for hepatocyte-

specific prothrombin within 48 hours of ligation, and increased c-erb-B2 expression after 72–96 hours [11]. Expression of stem cell factor c-kit, hepatocyte growth factor and TGF $\alpha$  were found to increase in young rats after bile duct ligation, while no such increase was found in older rats [18]. In young rats Kit-signal transduction was found to play a crucial role in the proliferation of bile ducts [19]. In the current study we examined expression of TGF $\alpha$  in VAD compared to VAS rats after bile duct ligation, and no difference in the expression was found.

We found a mild expression of RXR $\alpha$  in the biliary epithelium of VAD but not VAS rats after CBD ligation. The role of RXR has not been completely clarified. This receptor serves as a partner for other nuclear receptors but may have a separate RXR-mediated signaling pathway [20].

The differential expression of RXR in the bile ducts of VAD rats after CBD ligation could be one of the mechanisms responsible for the enhanced proliferation of the biliary epithelium. The total abundance of RXR $\alpha$  was found to be reduced in the liver after CBD ligation [21], and mRNA level of RXR was reduced in hepatic stellate cells of rats with CBD ligation [22]. However, in both studies, RXR was not examined separately in the bile ducts as in the current study.

The findings of this study may be relevant to clinical practice. Patients exhibiting cholestasis in combination with fat malabsorption will experience vitamin A deficiency. Additionally, bile flow obstruction may lead to obstructive cholangiopathy and consequently vitamin A deficiency [23–25]. Our data suggest that the vitamin A deficiency in these patients is likely to result in increased proliferation of the intrahepatic bile ducts and thus exacerbate hepatic dysfunction.

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#### Appendix. Composition of a vitamin A-deficient diet

Ingredient	Quantity (g/kg)
Vitamin-free casein	180
DL-Methionine	3
Cornstarch	298.5
Sucrose	298.5
Alphacel, non-nutritive bulk	50
Cottonseed oil	50
Brewers yeast	80
Vioosterol	0.011
Minerals	6.534
Salt mixture No. 2, USP XIII	40