Hepatic Regeneration in Vitamin A-deficient Rats: Changes in the Expression of Transforming Growth Factor α/Epidermal Growth Factor Receptor and Retinoic Acid Receptors α and β

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Abstract
We have studied the effect of vitamin A deficiency on the expression of transforming growth factor α (TGF-α), hepatocyte growth factor, acidic fibroblast growth factor, and TGF-β1 after partial hepatectomy of vitamin A-supplemented and vitamin A-deficient rats. In addition, the expressions of epidermal growth factor receptor and retinoic acid receptors α (RARα) and β (RARβ) were studied.

Partial hepatectomy was performed on the animals from the vitamin A-supplemented and -deficient groups at the age of 10 weeks when the weights of the animals on the deficient diet had reached a plateau. Two animals from each group were sacrificed before the operation and also 12, 24, 48, and 72 h and 5 days after the operation. Partial hepatectomy of the vitamin A-deficient rats leads to a focal necrosis of liver followed by a rapid restoration of liver mass. Expression of the TGF-α and epidermal growth factor receptor was highly elevated in the livers of deficient animals after partial hepatectomy. In the vitamin A-supplemented animals, the level of epidermal growth factor receptor was down-regulated following partial hepatectomy. Proliferation of oval cells in vitamin A-deficient livers following partial hepatectomy and subsequent increase in 2.1-kilobase α-fetoprotein mRNA was observed, suggesting an activation of the stem cell compartment. Another unexpected result was an inverse relationship between RARβ and RARα expression, the latter becoming the major species after partial hepatectomy in animals on the vitamin A-deficient regimen. The level and time course of TGF-α expression following partial hepatectomy was different in vitamin A-deficient animals from those supplemented with vitamin A, whereas no significant difference in the expression patterns of hepatocyte growth factor, acidic fibroblast growth factor, or TGF-β1 was observed between vitamin A-supplemented and -deficient animals.

The present data indicate that the effect of vitamin A deficiency on hepatic proliferation may be similar to its effect on epithelial tissues in general, including occurrence of cellular necrosis and the activation of the undifferentiated cell (oval cell) population.

Introduction
The liver is a central organ for the storage and processing of retinoids and functions to maintain a constant plasma retinoid concentration in the animal body (1, 2). The exact mechanism by which hepatocytes take up retinoids from the plasma and subsequently transfer them to perisinusoidal stellate (Ito) cells is not entirely known, although retinol binding protein, produced by the hepatocytes, appears to participate in these processes (1, 2). Since the concentration of retinoid in the liver is quite high, a prolonged exposure to a diet devoid of retinoid is needed before any vitamin A deficiency is obvious. However, several carcinogens are known to rapidly deplete liver vitamin A reserves without significantly changing plasma retinoid concentrations (3–8).

Retinoids play a crucial role in embryonic development, cellular differentiation, and proliferation (9–16). In epithelial cells, vitamin A deficiency produces an excessive proliferation of primitive cells and leads to an aberrant differentiation (16). The involvement of retinoids on the control of various isoforms of TGF2-β in epithelial tissues has also been implicated (17).

Because the retinoids are known to exert their regulatory effects via nuclear RARs (Refs. 18 and 19, and references therein), the issue has been raised as to whether these receptors, either directly by binding to their response elements or indirectly by interacting with other nuclear regulatory proteins of the leucine zipper type, such as jun/AP-1, exhibit either stimulatory or repressive action on the expression of growth factors and/or their receptors (18). RARs are known to be developmentally regulated and exhibit tissue-specific distribution (20–22). In the adult liver, RARβ is the major form of the RAR subfamily. RARβ contains the retinoic acid responsive element and is inducible by RA (23–25). In contrast, RARα is not inducible and is present at low levels in the adult liver (25).

The partial hepatectomy model has been extensively used in studies dealing with the involvement of growth factors in the restoration of liver mass (26–29). Increase in the expression of TGF-α, TGF-β1, and HGF is observed as early as 4 h after partial hepatectomy, whereas the expression of aFGF is only evident 24 h after the operation (29). It is not known whether hepatocytes have the capacity to proliferate in vitamin A-deficient animals and whether the pattern of growth factors that are involved in hepatocyte proliferation is similar to that of vitamin A-supplemented animals. In the skin, bronchial and gastrointestinal epithel-

2 The abbreviations used are: TGF, transforming growth factor; RAR, retinoic acid receptors; RA, retinoic acid; HGF, hepatocyte growth factor; aFGF, acidic fibroblast growth factor; kb, kilobases; AFβ, α-fetoprotein; EGFR, epidermal growth factor receptor; cDNA, complementary DNA; bp, base pair(s); GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
Fig. 1. Histology of the livers 4 h after partial hepatectomy. Focal coagulative necrosis is present in the liver of vitamin A-deficient animals (A). × 200. The liver of vitamin A-supplemented animal is histologically normal (B). Hematoxylin and eosin staining. × 400.

Fig. 2. OV-6 staining of liver from the vitamin A-deficient animals 48 h after partial hepatectomy revealed infiltration of OV-6 positive cells from the periportal space into liver parenchyma (A). In contrast, no infiltration of OV-6-positive cells was observed in vitamin A-supplemented animals after partial hepatectomy (B). × 200.

Lium, vitamin A deficiency leads to atrophy and restorative proliferation of primitive cells (16). A reliable marker for activation of the liver stem cell compartment is the increase in the expression of the 2.1-kb species of AFP (30). In the normal liver, only a slight activation is observed after chemical (CCL4) or mechanical partial hepatectomy (30, 31).3 The known effect of TGF-α on hepatocyte proliferation in vitro and an early increase of TGF-α mRNA levels after partial hepatectomy (26) indicate an important role for TGF-α in the proliferation of hepatocytes in vivo.

In vitro studies have demonstrated participation of RA in the regulation of the TGF-α receptor (EGFR) level in the embryonic carcinoma cell lines (32). However transcriptional repression of the EGFR promoter region by RA has been reported in ligand-activated transcription assays (33). However, an induction has also been observed in a similar assay when high levels of RA were used (34).

The aim of the present study was to characterize the involvement of retinoids in the control of growth factors that are known to participate in hepatic proliferation and differ-

Fig. 3. Combination of in situ hybridization and immunocytochemistry. OV-6-positive oval cells express AFP in the liver and vitamin A-deficient animal 48 h after partial hepatectomy. Counterstained with hematoxylin. × 1000.

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1 H. Nakatsukasa, R. P. Evarts, and S. S. Thorgeirsson, unpublished observations.

2 The expression of these factors was studied during hepatic regeneration after partial hepatectomy of vitamin A-supple-

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mented and -deficient animals. In addition, the expression of RARs α and β as well as that of EGFR in the liver was included in the present study.

Results
Weights of the Animals and the Histology of the Liver. A slight decrease in the weights of the animals was first observed 7 weeks after birth, and this difference was further increased in 8 weeks. Between 9 and 10 weeks, the weights of the animals in the vitamin A-deficient diet did not change. This was regarded as a sign of severe vitamin A deficiency. In addition, several animals showed crusted eyelids and symptoms similar to xerophthalma. The weight difference between vitamin A-supplemented and -deficient animals at the time of partial hepatectomy was 36 g, 184 ± 12.4 g, and 158 ± 9.9 g, respectively. Histological examination of hematoxylin and eosin-stained liver sections did not reveal any obvious difference in livers of supplemented and deficient animals. However, 4 h after partial hepatectomy, a diffuse coagulative necrosis, which included all three zones of the liver acini, was present in deficient animals (Fig. 1). Similarly, after 12 h a widespread necrosis of the liver was observed in deficient animals, although the remaining hepatocytes between the necrotic areas looked very normal histologically. Livers from both group 48 and 72 h after partial hepatectomy appeared normal (data not shown). However, immunocytochemical staining using OV6 antibody, which recognizes oval cells and bile duct cells, revealed infiltration of OV6-positive cells into liver parenchyma 48 to 72 h after partial hepatectomy of the vitamin A-deficient animals (Fig. 2). In vitamin A-supplemented animals no, infiltration of OV-6 positive cells from the peripoortal space into liver parenchyma was observed (Fig. 2).

Expression of AFP and RARs α and β. The combination of immunocytochemistry using OV6 antibody, which recognizes oval cells, and in situ hybridization with 35S-labeled probe for AFP demonstrated that oval cells were heavily labeled with silver grains (Fig. 3). Similarly, Northern blot hybridization with the full-length AFP probe revealed a significant increase in the expression of 2.1-kb AFP mRNA species 24 to 72 h after partial hepatectomy in the deficient livers (Fig. 4). The expression of RARα in vitamin A-supplemented animals was low but increased 12 h after partial hepatectomy and remained elevated 72 h after the operation (Fig. 4). In vitamin A-deficient animals, the level of RARα was higher than in supplemented animals. This was especially evident 12–24 h after partial hepatectomy as demonstrated by the expression of transcripts of both 3.7- and 2.8-kb species of RARα expression in deficient animals (Fig. 4). In contrast, the constitutive expression of RA-inducible RARβ was relatively high in vitamin A-supplemented animals before partial hepatectomy and was not significantly affected following the operation. However, in vitamin A-deficient animals, RARβ expression was markedly decreased and remained so until 5 days, at which time only a weak expression of the 3.3- and 3.0-bands could be detected.

Expression of Growth Factors. Expression of HGF was significantly increased over control 12, 24, and 48 h after partial hepatectomy in supplemented animals and reached a peak by 12 h (Fig. 5). In the vitamin A-deficient animals, the peak of HGF expression was reached by 24 h and remained elevated at 72 h after the operation (Fig. 5).

Expression of TGF-β1 reached maximum between 12 and 24 h posthepatectomy, and no difference was observed between the two groups (Fig. 6). In contrast, the expression of TGF-α was significantly increased in vitamin A-deficient animals as compared to supplemented animals after partial hepatectomy (Fig. 7). In both groups, TGF-α expression increased 12 h after partial hepatectomy but was several-fold higher in deficient livers. Vitamin A-supplemented animals displayed increased expression of 6.0- and 10.0-kb
transcripts for EGFR, but the transcripts were down regulated after partial hepatectomy. In vitamin A-deficient animals, EGFR expression (with 6.0- and 10.0-kb transcripts) was up regulated following partial hepatectomy and stayed elevated for 5 days following the operation. Prior to partial hepatectomy, only a low level of the 6.0-kb transcript could be seen (Fig. 7).

The most significant change in the growth factor expression between the vitamin A-supplemented and -deficient animals was a prominent increase in the expression of TGF-α after partial hepatectomy and up-regulation of the EGFR in the deficient animals. No significant difference in the level of expression of HGF, αFGF, or TGF-β1 was observed between vitamin A-supplemented and -deficient animals.

Discussion

In retinoid deficiency, prominent histopathological changes are observed in the epithelium of the trachea, skin, and intestine (16, 35). In these tissues, mature epithelial cells are continuously generated from primitive cell types. In the adult liver, mature hepatocytes have a long life span but have retained their capacity to divide following partial hepatectomy. In our study, histological examination of the livers from vitamin A-supplemented and -deficient animals failed to reveal any conspicuous morphological differences. Only after partial hepatectomy did the effect of vitamin A deficiency become manifest as a widespread, coagulative focal necrosis. Electron microscopy studies have revealed multivesicular lysosomes in vitamin A-deficient livers (36). The recovery of the necrotic livers was somewhat surprising and may indicate that the damage caused by vitamin A deficiency may not have similarly affected all hepatocytes. A simultaneous activation of the stem cell compartment and the proliferation of the remaining hepatocytes were occurring in livers from vitamin A-deficient animals. Both atrophy and proliferation of primitive cells are known to occur in rapidly proliferating tissues such as skin, bronchial epithelium, and gastrointestinal epithelium of vitamin A-deficient animals (16). Therefore, a clear similarity exists in vitamin A deficiency between these extrahepatic epithelial tissues and the proliferating liver.

The most prominent effect of retinoids on the physiology of epithelial cells is promotion of cellular differentiation and inhibition of proliferation (Ref. 14 and references therein), whereas vitamin A deficiency has an opposite effect, delayed or inhibited differentiation and excessive proliferation of undifferentiated cells (16). Under normal conditions, the liver is capable of renewing itself without a constant supply of cells from the stem cell compartment. In addition, the liver stores most of the retinoids available to the animal (1) and, therefore, would be expected to be the last tissue subjected to vitamin A deficiency. However, as has been demonstrated by several studies, local vitamin A deficiency in liver is possible without systemic deficiency after injury caused by chemicals and carcinogens (3–8). In the present study, we have demonstrated a clear difference in the expression of TGF-α/EGFR system. A concomitant decrease in the mRNA level for EGFR and the increased expression of TGF-α after partial hepatectomy was observed in vitamin A-supplemented animals, which agrees with previous studies (26, 37). A clear understanding of the consequences of these changes for hepatocyte physiology is poorly understood, but the importance of TGF-α/EGFR system for early autocrine regulation of hepatocyte proliferation after partial hepatectomy is regarded to be of great importance (26). However, in vitamin A-deficient animals, a simultaneous increase in the expression of TGF-α and EGFR after partial hepatectomy was different than that observed in supplemented animals. Because a complete restoration of liver mass was observed in vitamin A-deficient animals, a long-term effect of TGF-α/ERGR system seems to be important when the vitamin A supply is low.

The level of RARβ transcripts was high in vitamin A-supplemented animals both before and after partial hepatectomy, whereas only a low level of RARα transcripts was present prior to partial hepatectomy, which was significantly increased when the hepatocytes proliferated after partial hepatectomy. In vitamin A-deficient animals, extremely high levels of RARα transcripts were present shortly after partial hepatectomy, which is in contrast to the level of RARβ transcripts that was scarcely visible 48 h after the operation. A prominent increase in the expression of RARα could be an indication of a compensatory increase in RARα mRNA when the expression of RARβ is low. Another possibility is that the RARα/RA system is important for the regulation of hepatocyte proliferation by repressing EGFR expression but is nonfunctional in the absence of the ligand in vitamin A deficiency, which then leads to a prominent increase in RARα. An in vitro study has demonstrated that at physiological levels RA exerts a repressive effect on the promoter region of the EGFR (33). Vitamin A deficiency led to a significant decrease in the level of RARβ expression, which agrees with the findings by others (23, 38, 39). It is possible that the RARβ controls events that lead to cellular differentiation, whereas RARα is involved in the control of proliferation.

An opposite effect between jun/AP-1 and RA operates in several biological systems (40). In adipose tissue, RA inhibits differentiation and promotes cellular proliferation, whereas jun/AP-1 promotes differentiation and inhibits growth (41). Also, in the regulation of collagenase, stromelysin, and osteocalcin gene expression, repression of jun/AP-1 transcription factors by RA has been demonstrated (42–44). Therefore, it is conceivable that in the liver the effect of RA and the jun/AP-1 system are counterbalancing each other. Following partial hepatectomy of vitamin A-supplemented (normal) liver, the level of EGFR transcripts is decreased due to the repressive effect of RARα/RA on the promoter region of EGFR, which leads to a slowdown of cell proliferation. Whereas in the vitamin A-deficient liver, an
up-regulation of the EGFR occurs after partial hepatectomy as a result of highly increased but nonfunctional RARα.

Materials and Methods
Ten pregnant female Fischer rats were divided into two groups. Animals in group I received a vitamin A-deficient diet (test diet TD 86143; Teklad, Madison, WI). Animals in group II received the same diet plus 50,000 units of all-trans retinol palmitate in polyethylene glycol (Sigma, St. Louis, MO) once a week. The offspring were weaned at the age of 4 weeks, and only male rats were included in the experiment. Starting at the age of 5 weeks, animals were weighed once a week, and the offspring of group II were administered retinol palmitate (20,000 units) by gavage once a week. Partial hepatectomy was performed on the animals from both groups at the age of 10 weeks, and two animals from each group were sacrificed immediately prior to and 12, 24, 48, and 72 h and 5 days after partial hepatectomy.

Probes. Mouse cDNA clones for RARs α and β were kindly provided by Dr. P. Chambon (Institut de Chimie Biologique, Strasbourg, France). A 620-bp EcoRI and PstI fragment from the 5′ end of RARs was subcloned to a pGEMz7 vector, whereas the 800-bp long fragment from the 3′ end of RARB cDNA was used. A 1.5-ko EcoRI/KpnI fragment from the 5′ end of murine EGFR in Bluescript M13 was kindly provided by Dr. Eileen Adamson (La Jolla Cancer Research Foundation, La Jolla, CA). A 520-bp fragment containing the 3′ and middle portion of rat AFP cDNA was subcloned into the pGEM2 vector. A 600-bp cDNA fragment encoding the 3′ end of rat HGF (kindly provided by Dr. Brian Carr, Department of Surgery, University of Pittsburgh, Pittsburgh, PA) was subcloned into the pBlueScript SK vector. A 335-bp rat TGF-α cDNA fragment between 1046 and 1381 bp of the cDNA (45) was amplified by polymerase chain reaction and cloned into a PCR II vector (Invitrogen, San Diego, CA). The sequence (45) was verified by the dyeodeoxy termination sequencing method. A 985-bp fragment of rat TGF-β1 in pBluescript II KS+ was kindly provided by Dr. Anita Roberts (National Cancer Institute). Antisense riboprobes for Northern blot hybridization were labeled with 32P. A cDNA probe coding for GAPDH (46) was labeled by the random priming method and used as an internal standard for Northern blot hybridization.

Northern Blot Analysis. Five μg of polyadenylated selected RNA from each sample was electrophoresed in 0.8% agarose gel and then transblotted to a nylon membrane. Blots were hybridized with 32P-labeled riboprobes at 60°C, and the cDNA probe was hybridized at 42°C overnight. The membranes were washed two times in 1× standard saline citrate (150 mM sodium chloride-15 mM trisodium citrate, pH 7.0) at room temperature, followed by two washes 15 min or longer in 0.1× standard saline citrate-0.1% sodium dodecyl sulfate at room temperature for the cDNA probe and 60°C for RNA probes. The membranes were then exposed to Kodak XAR-5 film with intensifying screen at 70°C. A monoclonal antibody that recognizes both ovary cells and bile duct cells was kindly provided by Dr. Harold Dunsford (University of Mississippi, Jackson, MS). This was used to identify ovary cells by the immunoperoxidase staining method using the Vectastain ABC Elite kit (Vector Laboratories, St. Louis, MO). In situ hybridization and combination with immunocytochemistry were performed as described earlier (47).

References


