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Effect of Vitamin A Deficiency on the Integrity of Hepatocytes after Partial Hepatectomy

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The effect of vitamin A deficiency on hepatic regeneration in male and female rats was studied after partial hepatectomy. A fourfold increase in the number of positive dUTP end-labeled nuclei was observed in the deficient animals as early as 30 minutes after partial hepatectomy and their number reached a peak by 8 hours after the operation. The bile duct cells were both morphologically and biochemically intact at all time points. Administration of retinyl palmitate 1 hour before partial hepatectomy significantly reduced the number of positive nuclei, and treatment with retinyl palmitate 24 or 48 hours before the operation reduced the number of positive cells to the level observed in control vitamin A-supplemented rats. The level of transcripts for c-jun, c-fos, c-myc, and transforming growth factor-β1 were increased for an extended period of time in livers of deficient animals, whereas the expression of both p53 and max were unchanged. Immunocytochemistry demonstrated the presence of latent transforming growth factor-β1 in cells showing evident apoptotic or necrotic changes in their nuclei. This study demonstrates the importance of vitamin A for the survival of hepatocytes both in intact vitamin A-deficient liver and after partial hepatectomy, whereas the ductal cells appear to be less sensitive to vitamin A deficiency. (Am J Pathol, 147:699–706)

Cell death can occur either by means of necrosis or apoptosis, both of which have distinct morphological differences.[1,2] In the course of embryonic development and metamorphosis, apoptosis eliminates the unwanted cells of the organism. In the rapidly proliferating adult tissues apoptosis maintains cellular homeostasis, whereas in the lymphoid tissue it is involved in the negative selection of cells.[3,4] Although many of the factors that participate in apoptotic cell death are known, the ultimate mechanism is still unknown. It is evident, however, that several different pathways can lead to cell death via apoptosis (reviewed in Ref. 5). One of the hallmarks of apoptosis is the breakdown of DNA into nucleosomal oligomers probably as a result of the accessibility of the internucleosomal linker region to endonuclease. DNAse I, the activation of which is frequently implicated in the apoptotic process, is a Ca^{2+}- and Mg^{2+}-dependent endonuclease. DNAse I-like endonuclease is present in several tissues in the inactive state complexed with G-actin.[6] Therefore calcium is regarded as an important regulator of apoptosis. Several proto-oncogenes and growth factors that control proliferation are also involved in the control of apoptosis. Immediate early genes, c-*fos*, c-*jun*, and c-*myc*, play an important role in the regulatory network that controls proliferation and differentiation.[7,8] The *myc* and *max* heterodimers are the biological active form of *myc*[9] and proteins encoded by c-*jun* and c-*fos* form the AP-I complex, which regulates the expression of genes with the AP-I consensus sequence in their promoter region. The participation of the immediate early genes is also implicated when cells enter the death pathway.[10–13] The inhibition of cell cycle progression with high c-*myc* expression is frequently regarded as a prerequisite for apoptosis.[10] The tumor suppressor gene p53 produces growth arrest at the G1/S boundary of the cell cycle and its expression increases dramatically in response to various DNA-damaging agents.[14–16] p53 has a dose-dependent effect on apoptosis induced by DNA damage and thus protects the organism from the proliferation of unwanted and damaged cells. Several cytokines are known to protect the

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cells from apoptosis, but expression of transforming growth factor β1 (TGF-β1) has been linked to cell death both *in vitro* and *in vivo*.[17–19] Other physiological mediators of apoptosis, especially in the lymphoid tissue, include steroid hormones,[3] which via their cognate receptors, function as transcriptional regulators. Retinoid receptors belong to the same nuclear family of receptors as the steroid hormones,[20,21] and retinoic acid has also been shown to inhibit apoptosis.[22,23]

Our earlier study demonstrated a prominent focal necrosis 4 hours after two-thirds partial hepatectomy of livers from vitamin A-deficient (VAD) male rats.[24] This observation suggested that in VAD animals the response of hepatocytes to growth signals might be abnormal. This prompted us to study more closely the events that preceded hepatocyte death. In the present study we determine the types of cell death that occur after partial hepatectomy of VAD animals as well as the expression of genes that are involved in both the normal cell cycle and that under certain conditions also participate in apoptotic cell death. Recently a cytochemical method was described that is based on terminal deoxynucleotidyl transferase-mediated incorporation of biotinylated dUTP into DNA.[25,26] The method is based on the specific binding of TdT to 3′-OH ends of DNA produced by activated endonucleases. We demonstrate that vitamin A deficiency leads to a widespread activation of nuclease activity when hepatocyte proliferation is induced by partial hepatectomy and in addition that the administration of vitamin A to these animals enhanced the capacity of the liver to regenerate by preventing apoptosis and necrotic cell death.

Materials and Methods

Test diet (TD 86143, Teklad, Madison, WI), which is deficient in vitamin A, was given to 25 pregnant Fischer rats until the progeny were 5 weeks old. The animals (171 rats) were then weaned, sexed, and divided into four groups: females on VAD diet, males on VAD diet, females on vitamin A-supplemented (VAS) diet (test diet TD 8614, Teklad), and males on VAS diet. Animals were weighed once a week and when the weights had leveled off (10 weeks of age for males and 13 to 14 weeks for females), partial hepatectomy was performed by removing the right lateral and median lobes. Animals from each group were sacrificed at 0 time (no partial hepatectomy) and 10 or 30 minutes or 1, 2, 4, 8, 12, 24, or 48 hours after partial hepatectomy. Some of the VAD animals received 40,000 U of retinyl palmitate in corn oil by gavage 1 hour or 2 or 3 days before partial hepatectomy. These animals were sacrificed 8 hours after the operation.

Cytochemical Methods

Liver samples were fixed in 10% formalin in phosphate-buffered saline and stained with hematoxylin and eosin (H&E). DNA breaks in nuclei were detected by DNA end-labeling as described by Gavrieli et al.[25] At least 1000 hepatocytes of 20 randomly selected microscopic fields were counted. Antibodies for TGF-β1 were kindly provided by Dr. Michael Sporn (National Cancer Institute, Bethesda, MD). LC antibody, raised in rabbit to amino acids 1 to 30, recognizes the mature TGF-β1, and another antibody raised to amino acids 266 to 278 of the TGF-β1 precursor (anti-Pre) recognizes the latent form of TGF-β1. Immunocytochemistry was performed with formalin-fixed slides according to the method described by Heine et al[27] with the avidin-biotin-peroxidase Elite kit (Vector Laboratories, Burlingame, CA). For controls, the primary antibody was replaced with normal rabbit serum IgG at 3 μg/ml. The slides were counterstained with hematoxylin.

Probes

The following probes were used for the Northern hybridization: 2600-bp *Eco*RI fragment of mouse c-*jun* in pGEM2, 2116-bp *Eco*RI fragment of rat c-*fos* in pSP65, 1410-bp fragment of mouse c-*myc* in pGEM4z, 550-bp *Eco*RI fragment of human *max* in pVZ1 (kindly provided by R. Eisenman, Hutchinson Cancer Research Center, Seattle, WA), a 985-bp fragment of rat TGF-β1 in Bluescript II KS+ (kindly provided by Dr. Anita Roberts, National Cancer Institute, Bethesda, MD), and 1400-bp p53 cDNA fragment. Antisense riboprobes for Northern hybridization were labeled with ^{32}P. The cDNA probe was labeled by the random priming method.

Northern Blot Analysis

Five micrograms of poly(A) mRNA from each sample was electrophoresed in 0.8% agarose gel and transblotted to a nylon membrane. Blots were hybridized overnight with ^{32}P-labeled riboprobes at 60°C or with the cDNA probe at 42°C. The membranes hybridized with riboprobes were washed two times in 1× standard saline citrate (SSC; 150 mmol/L sodium chloride, 15 mmol/L trisodium citrate, pH 7.0) at room temperature, followed by two washes of 15 minutes or longer in 0.1× SSC/0.1% sodium dodecyl sulfate

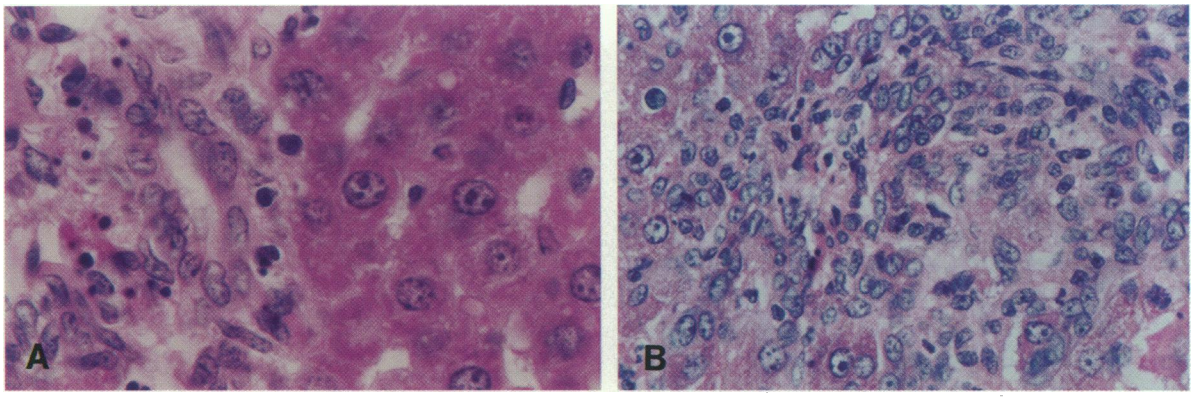

Figure 1. A: *H&E staining of liver from VAD female rat 8 hours after partial hepatectomy. Several apoptotic cells are present in the periportal area but the ductal cells are unaffected. Magnification, ×1000.* B: *H&E staining of the liver from VAD male rat 8 hours after partial hepatectomy. Oval cells are present in the periportal area and between hepatocytes. ×900.*

at 60°C. The membranes hybridized with cDNA probes were washed for 15 minutes with 1× SSC followed by a 10-minute washing with 0.5× SSC. The membranes were then exposed to Kodak XAR-5 film with intensifying screen at −70°C.

Results

Histology of the Liver

As has been reported earlier,[28] female rats retained their vitamin A storage capacity for a longer time period than did male rats. However, both male and female rats on VAD diet showed similar histology of the liver once their body weights leveled off and ocular lesions were evident (10 weeks for male rats and 13 to 14 weeks for female rats). Livers from the deficient animals looked normal except for some hepatocytes with condensed nuclei and increased cellularity in the periportal areas. The number of cells with nuclear changes, including chromatin condensation and chromatin lining on the nuclear membrane, clearly increased 30 minutes past partial hepatectomy and continued to increase with time after the operation, reaching a maximum at 8 hours after partial hepatectomy. Necrotic hepatocytes were present either as single cells or as groups of cells composed of three to four hepatocytes already 1 to 2 hours after partial hepatectomy. Frequently in the vicinity of necrotic cells a few polymorphonuclear leukocytes were also present. At all time points after partial hepatectomy cells were present that were undergoing typical apoptotic changes with shrinkage of cytoplasm and fragmentation of nuclei. In rare cases apoptotic cells of unknown origin were seen in the periportal areas (Figure 1A), whereas no apoptotic or necrotic cells were found among the bile duct cells. Sometimes an excessive proliferation of oval cells was seen (Figure 1B). At 2 days after partial hepatectomy, empty spaces left behind by necrotic hepatocytes could be seen. When vitamin A was administered 1 hour before partial hepatectomy to the VAD animals that were sacrificed 8 hours after the operation, necrotic and apoptotic cells were still present although the nuclear changes were less frequent. However, when vitamin A was administered 24 to 48 hours before the operation the liver histology was not significantly different from that observed in control VAS animals.

In Situ *End-Labeling of Tissue Sections*

Incorporation of terminal transferase-mediated biotinylated dUTP into nuclear DNA was observed in some hepatocytes of VAD animals, whereas in control animals the presence of positive cells was rare. This difference, however, was not statistically significant. A four- to fivefold increase occurred 30 minutes to 1 hour after the operation (Figure 2), whereas no labeling was observed when terminal deoxynucleotidyl transferase was excluded from the incubation mixture. The positive cells were located either in the periportal or midzonal areas of the liver acini at the early time points, but 4 to 8 hours after the operation they were present mainly in the midzonal areas (Figure 3). By 24 to 48 hours a clear shift in the location of positive cells toward pericentral areas was evident. When vitamin A was administered 1 hour before partial hepatectomy to the VAD animals, and the animals were sacrificed 8 hours after the operation, the number of cells with activated endonuclease dropped from 118 ± 16 to 28 ± 20 per thousand cells (mean ± SE), which is statistically significant ($P < 0.01$). When vitamin A was administered 24 and 48 hours before partial hepatectomy, only a few positive cells were present.

Northern Hybridization

There was an increased expression of both c-*jun* and c-*myc* in livers of unoperated VAD animals as compared with the supplemented animals (Figure 4, A and B, lane 1). Supplemented animals showed normal expression of immediate early genes; c-*jun* and c-*fos* reached their peak in 30 minutes, in 1 hour their expression was significantly reduced, and by 12 and 24 hours a slight second peak was observed. No difference between male and female rats was observed (female data not shown). In livers of VAD animals the expression of both c-*jun* and c-*fos* remained at high levels for an extended period of time after partial hepatectomy (Figure 4A). The same was also true for the c-*myc*. Its expression remained high for 4 hours and then decreased to the level seen before the operation in VAD animals (Figure 4B). The expression of *max*, which encodes a dimerization partner for *myc*, did not change after partial hepatectomy and no difference was found between VAD and VAS animals (Figure 4B). p53 was expressed in both groups, and the vitamin A status did not cause any change in its expression (Figure 5). In contrast, the expression of TGF-β1 increased in the deficient animals at the time when activation of endonucleases was prominent. Because the number of cells with activated endonucleases was highest 8 hours after partial hepatectomy, and a significant reduction in the number of the positive cells was observed after vitamin A administration 1 hour before partial hepatectomy, we studied the expression of TGF-β1, c-*myc*, and p53 in livers of VAD animals to whom 40,000 U of vitamin A as retinyl palmitate was administered 24 hours and 48 hours before partial hepatectomy. The animals were sacrificed 8 hours after the operation. Figure 6 shows that the expression of TGF-β1 was less than that in deficient animals but still above the level seen in livers of VAS animals. The expression of c-*myc* was similarly decreased especially when retinyl palmitate was administered 48 hours before the operation. No consistent difference in the expression of p53 was observed.

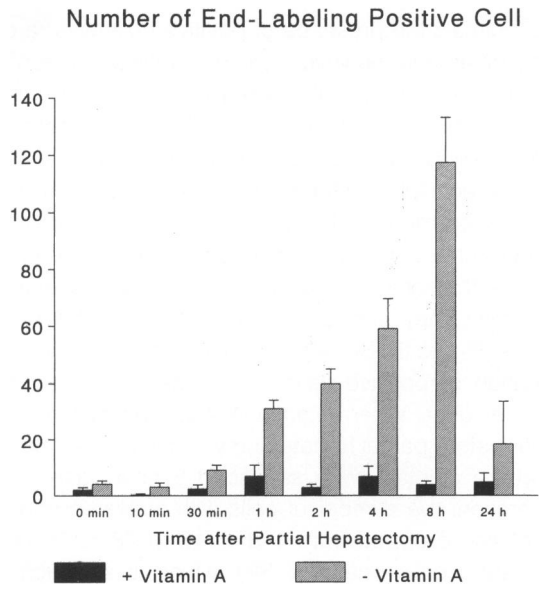

Figure 2. *Number of cells with nuclei (mean ± SE) that gave a positive reaction for activated nucleases when measured by* in situ *DNA end-labeling. The number of samples at each time point ranged from 3 to 16.*

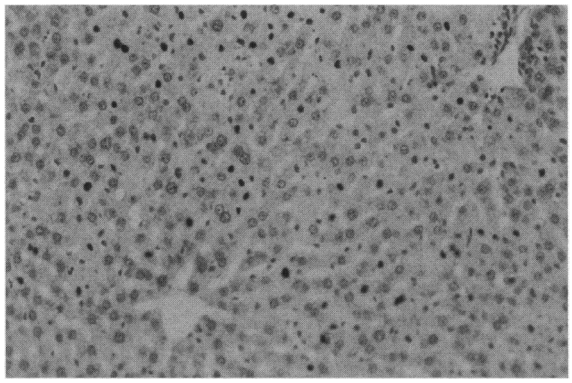

Figure 3. *Presence of several cells with nuclei positive for DNA end-labeling 8 hours after partial hepatectomy. The highest concentration of positive cells was located in the midzonal areas. Magnification, ×200.*

Immunocytochemistry for TGF-β1

As the participation of TGF-β1 has been implicated in the process of apoptotic cell death, we studied the presence of both the mature and latent form of TGF-β1 in livers from VAS and VAD animals. Mature TGF-β1 was found in bile duct cells, bile ductular cells (data not shown), and in hepatocytes around the pericentral areas in both VAS and VAD animals (Figure 7A). A few dispersed hepatocytes in parenchyma were also TGF-β1 positive. We did not find any significant correlation between the presence of mature TGF-β1 in the hepatocytes and morphological changes in their nuclei (Figure 7A). A weak signal for pre-TGF-β1 was found in bile duct cells or bile ductular cells and also in the connective tissue around the blood vessels. The staining in the hepatocytes in general was somewhat above the background especially in the periportal and pericentral areas (data not shown). Some individual hepatocytes in VAD animals had a clear signal for pre-TGF-β1, and several of them also showed nuclear fragmentation (Figure 7B).

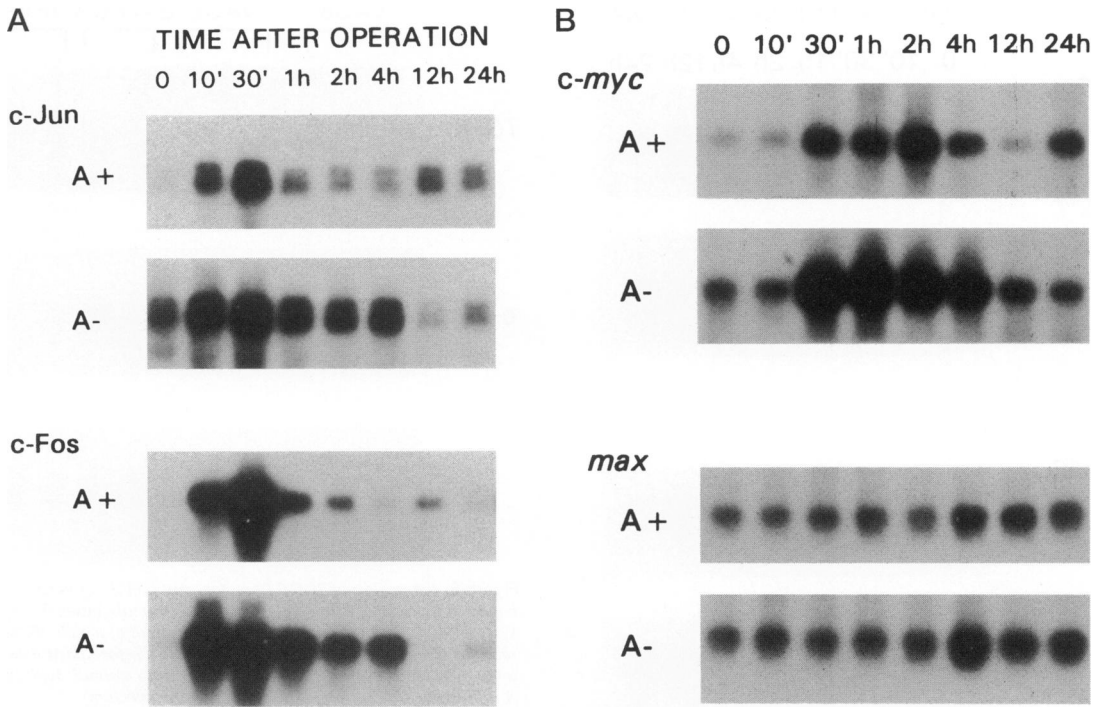

Figure 4. A: *Northern hybridization for* c-jun *and* c-fos *of livers from male rats on VAS (+A) or VAD (−A) diet.* Lane 1, *non-operated animals;* lane 2, *10 minutes;* lane 3, *30 minutes;* lane 4, *1 hour;* lane 5, *2 hours;* lane 6, *4 hours;* lane 7, *12 hours; and* lane 8, *24 hours after partial hepatectomy.* B: *Northern hybridization for* c-myc *and* max *of livers from VAS (+A) and VAD (−A) male rats.* Lane 1, *non-operated animals;* lane 2, *10 minutes;* lane 3, *30 minutes;* lane 4, *1 hour;* lane 5, *2 hours;* lane 6, *4 hours;* lane 7, *12 hours; and* lane 8, *24 hours after partial hepatectomy.*

Discussion

Our study demonstrates the importance of vitamin A for the survival of the hepatocytes during hepatic regeneration. Without the proliferative stimulus the liver looked morphologically normal except for occasional apoptotic or necrotic hepatocytes and increased cellularity in the periportal space. Mild hypertrophy of the tissue of the portal tracts and moderate degenerative changes of the parenchymal tissue have been described in VAD animals,[29] but in general the liver is regarded to be unaffected by vitamin A deficiency. This is in contrast to the continuously proliferating epithelial tissues such as skin and gastrointestinal and urogenital tracts, which undergo apoptotic, necrotic, and metaplastic changes when exposed to severe vitamin A deficiency.[29] It is known that, after partial hepatectomy, first the cells in the periportal area enter the cell cycle and later are followed by cells in the midzonal and pericentral areas. The distribution of cells with activated endonuclease in the liver acini seems to precede the entering of cells into the S phase of the cell cycle. On the basis of histological examination, both necrotic and apoptotic cell deaths appeared simultaneously after partial hepatectomy. DNA end-labeling could not separate these two forms of cell death; at the very beginning both prenecrotic and preapoptotic cells were positive. These data agree with recent findings by Gold et al,[26] although the authors demonstrated that DNA end-labeling was somewhat more sensitive for the detection of apoptotic cells than *in situ* nick translation.

Oxidative stress is one of the inducers of apoptotic cell death and living cells have various means to counteract oxidative damage,[30] which include *Bcl-2*, which is thought to protect the cells against cell death by functioning as an antioxidant[31] and carotenoids that provide protection against free radicals in addition to functioning as provitamin A. We demonstrated a protective effect against nuclear changes by administering retinyl palmitate, which is ineffective as a free radical scavenger,[32] but its metabolites function as ligands for transcriptional regulators and are shown to be potent negative effectors of activation-induced T cell apoptosis.[23]

We have previously demonstrated the presence of transcripts and protein (unpublished observation) for stemcell factor (SCF) and its receptor c-*kit* in liver ductal cells.[33] The presence of the SCF/c-*kit* system in the ductal cells may explain the lack of endonuclease activation in these cells, similar to the protective effect of SCF that has been demonstrated for the

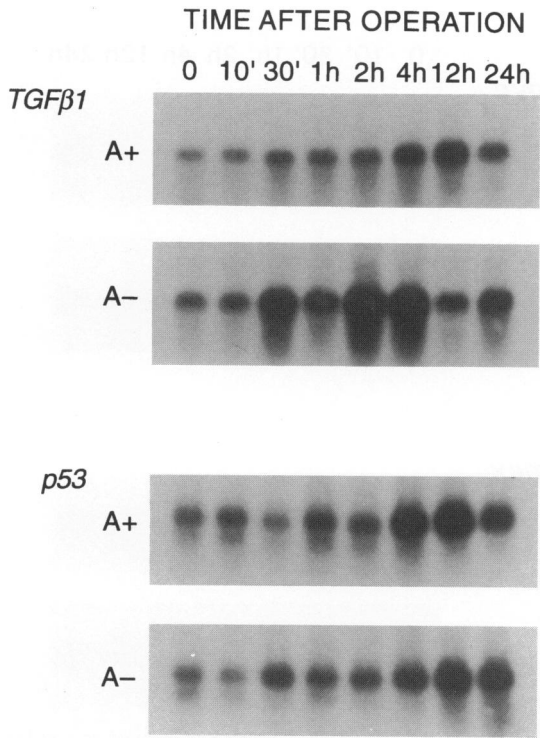

Figure 5. *Northern hybridization for p53 and TGF-β1 of livers of male rats on VAS (+A) or VAD (−A) diet.* Lane 1, *non-operated animals;* lane 2, *10 minutes;* lane 3, *30 minutes;* lane 4, *1 hour;* lane 5, *2 hours;* lane 6, *4 hours;* lane 7, *12 hours; and* lane 8, *24 hours after partial hepatectomy.*

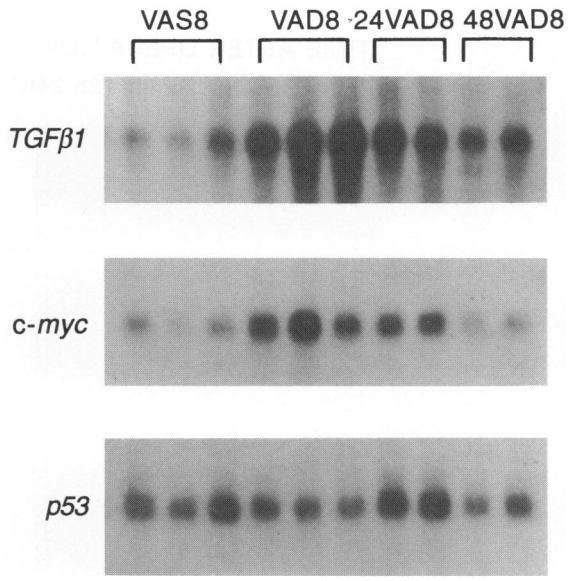

Figure 6. *Expression of TGF-β1,* c-myc, *and p53 8 hours after partial hepatectomy.* Lanes 1 *to* 3, *liver from VAS animals;* lanes 4 *to* 6, *from VAD animals;* lanes 7 *and* 8, *from VAD animal to which 40,000 U of retinyl palmitate was administered 24 hours before partial hepatectomy; and* lanes 9 *and* 10, *from corresponding animals with vitamin A administration 48 hours before partial hepatectomy.*

survival of hemopoietic cells.[34–37] It seems that bile duct cells possess a protective mechanism against cell death that is apparently not dependent on vitamin A status, whereas the hepatocytes in VAD animals frequently enter the death pathway, either by apoptosis or by undergoing necrosis.

As has been shown in several studies, c-*myc* plays a central role in the death process.[10] Extended expression of c-*myc* was also evident in our study. It was preceded by an increased and extended expression of c-*jun* and c-*fos*. The hepatocytes seem to be especially sensitive to cell death when they have entered the cell cycle and a high expression of immediate early genes is present. The maximal number of nuclei with activated endonucleases was observed 8 hours after partial hepatectomy. c-*myc* controls cellular proliferation and a decrease in c-*myc* expression is observed after addition of inducers of differentiation. c-*myc* can also sensitize cells towards apoptosis, especially when combined with a block in cell proliferation[10] and/or in cooperation with p53, which blocks the S phase progression.[38]

In the abrogation of the cell cycle of damaged cells, both p53 and TGF-β1 are thought to be involved. p53 transcripts were present at all time points after partial hepatectomy at equal levels in livers from both VAS and VAD animals. In contrast, a clear difference in the expression of TGF-β1 was evident between livers from VAS and VAD animals at the time when nuclear changes were present. We tried to further elucidate the possible role of TGF-β1 in the death of the hepatocytes by immunocytochemical means. The expression of the mature TGF-β1 was restricted to the periportal and pericentral hepatocytes and to a few dispersed hepatocytes throughout the liver acini in VAD animals. No evidence for the increase in the concentration of the mature form of TGF-β1 among apoptotic or necrotic hepatocytes was found, but this form of TGF-β1 was always present in the bile duct cells, which did not enter the death pathway. In contrast, the expression of pre-TGF-β1 was observed frequently in both necrotic and apoptotic cells as well as in hepatocytes having normal morphology. This observation agrees with the earlier report by Bursch et al.[19] However, simultaneous DNA end-labeling and immunocytochemistry for pre-TGF-β1 are needed to further clarify the relationship between pre-TGF-β1 and apoptosis. It is possible that TGF-β1 in cooperation with c-*myc* and p53 may promote the elimination of cells having activated endonucleases. Depending on the severity of the damage caused by the vitamin A deficiency, the participation of proto-oncogenes concomitantly with TGF-β1 may result in either apoptotic or necrotic cell death.

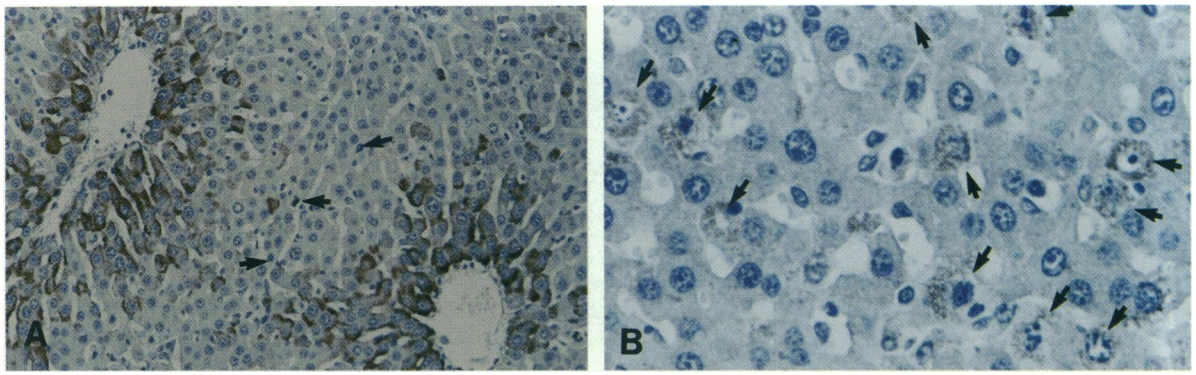

Figure 7. A: *Immunocytochemistry of mature TGF-β1 in livers from VAD animals 4 hours after partial hepatectomy. The positive cells are located mainly in the pericentral areas with only a few dispersed positive cells in the midzonal area, which is the location for cells with nuclear changes (arrows). Magnification, ×200.* B: *Immunocytochemistry for pre-TGF-β1 in liver from VAD liver 1 hour after partial hepatectomy. Several of the apoptotic or necrotic hepatocytes are positive for pre-TGF-β1.*

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