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CORRELATION OF REPRESSED TRANSCRIPTION OF α -TOCOPHEROL TRANSFER PROTEIN WITH SERUM α -TOCOPHEROL DURING HEPATOCARCINOGENESIS

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Using a subtraction-enhanced display technique, we identified a rodent α -tocopherol transfer protein (α -TTP) cDNA which exhibited markedly lower messenger RNA (mRNA) amounts in rat hepatocellular carcinoma (HCC) than in healthy controls. Several lines of evidence have substantiated that abnormal α -TTP results in isolated vitamin E deficiency. In this study, we investigated the hepatic mRNA amounts of α -TTP during rat hepatic carcinogenesis and liver regeneration on Northern blot, localization of α -TTP mRNA in HCC of rats and humans by *in situ* hybridization, and we analyzed the correlation between α -TTP mRNA and α -tocopherol. α -TTP mRNA concentrations of the rats were decreased at the early stage of hepatic carcinogenesis, and remained 3–5-fold reduced as the tumor progressed. In parallel, serum α -tocopherol concentrations were significantly decreased to 40% of those in the controls at the early stages of rat hepatic carcinogenesis ($p < 0.01$). The 2 data sets were strongly correlated ($r = 0.834$, $p < 0.001$). *In situ* hybridization revealed that a decrease of α -TTP mRNA was preferentially localized in the tumor nodules of rats and humans with HCC. Our data suggest that repressed transcription of α -TTP is associated with a decrease of serum α -tocopherol and with hepatic carcinogenesis. *Int. J. Cancer* 71:686–690 1997.

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A major effort in cancer studies is aimed at identifying new parameters that enable better understanding of carcinogenesis, earlier establishment of diagnosis and more precise assessment of prognosis in patients with cancer. Several methods, including subtractive hybridization (Wang and Brown, 1991), differential display (Liang and Pardee, 1992) and serial analysis of gene expression (Velculescu *et al.*, 1995), have been developed for identifying and analyzing the genes that are over- or under-expressed in one cell population relative to another.

Hepatocellular carcinoma (HCC) is a frequent malignancy of humans, mainly in areas of Asia and Africa (Lotze *et al.*, 1993). It results from a stepwise process involving different pre-neoplastic lesions that reflect multiple genetic events like activation of proto-oncogenes, inactivation of tumor-suppressor genes and over- or re-expression of growth factors (Vogelstein and Kinzler, 1993; Wands and Blum, 1991). The majority of HCC cases is associated with interactive effects of hepatitis virus and chemical carcinogens. However, the latter have been widely used for analyzing multistep carcinogenesis and for generating animal models (Farber and Sarma, 1987; Beer *et al.*, 1986).

Since altered gene expression is a common feature of neoplastic cells, the steady-state concentration of particular transcripts may provide information on the differentiation status of hepatocytes, both during carcinogenesis and in fully developed tumors (Lasserre *et al.*, 1992). We previously reported that by utilizing the subtraction-enhanced display technique, several up-regulated gene products, including c-myc, α -prothymosin, glutathione-S-transferase Yb1 (GST), and ferritin-H from rat HCC, were identified (Wu *et al.*, 1996). Ferritin-H was further shown to be overexpressed at the early stage of rat hepatic carcinogenesis, and shown to be a specific and sensitive marker for rat HCC (Wu *et al.*, 1997). Moreover, one cDNA clone was found to be 100% similar to rat α -tocopherol

transfer protein (α -TTP), and its mRNA concentrations in HCC were lower than those in control rats.

The α -TTP is a cytosolic liver protein with high binding affinity for α -tocopherol (Sato *et al.*, 1991; Yoshida *et al.*, 1992). Although its complete physiological function is unclear, α -TTP purified from rat liver preferentially transfers α -tocopherol between liposomes and mitochondrial membranes *in vitro* (Sato *et al.*, 1991), and incorporates α -tocopherol into the very low density lipoprotein (Traber, 1990). Frame-shift mutations have been found in the α -TTP gene in families of North African and European ancestry suffering of ataxia with isolated vitamin E deficiency (Ouahchi *et al.*, 1995). In agreement with these findings, a missense mutation in the α -TTP gene that results in the production of a partially defective α -TTP molecule of a Japanese patient with delayed expression of spinocerebellar dysfunction was reported as a determinant of low-serum vitamin E concentrations (Gotoda *et al.*, 1995). However, no study on the association of α -TTP and α -tocopherol in subjects with cancer has ever been reported.

The most biologically active form of vitamin E is α -tocopherol and is believed to be a lipid-soluble antioxidant that prevents lipid oxidation in membranes (Sato *et al.*, 1993). As one of the major free radical scavengers, vitamin E may be important in inhibiting the cancer process *in vivo* (Wattenberg, 1992). We have now investigated the temporal and spatial gene expression pattern of α -TTP and its correlation with serum α -tocopherol during rat hepatic carcinogenesis.

MATERIAL AND METHODS

Material and histology

HCC was induced in male Wistar rats, weighing 200–250 g, by supplying diethylnitrosamine (DENa) (Sigma, St. Louis, MO) at a concentration of 1:10,000 via drinking water (Wu *et al.*, 1997). The rats were kept in a temperature- and humidity-controlled environment and had free access to a laboratory diet and water. At 0, 3, 6, 9, 12 and 16 weeks of treatment with DENa, 4 rats were sacrificed after overnight fast. For a 70% partial hepatectomy, the medial and left hepatic lobe were excised according to the method of Higgins and Anderson (1931). All procedures involving animals were performed according to the Guidelines for Animal Use and Care of the University of Amsterdam.

Liver tissues from both rats and 8 European patients (7 males and 1 female, mean age 65 ± 9 years) with HCC (6 with co-existing cirrhosis and 2 without cirrhosis) were examined by standard histopathologic techniques employing hematoxylin-eosin (H-E) and reticulin staining on paraffin-embedded liver sections.

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cDNA cloning and sequencing

An α -TTP cDNA fragment (approximately 0.6 kb), isolated by the subtraction-enhanced display technique (Wu *et al.*, 1996), was cloned in the pCR II vector (Invitrogen, Leek, The Netherlands) and sequenced from both the 3' and 5' end using a PCR cycle sequencing kit (Perkin-Elmer, Nieuwerkerk a/d IJssel, The Netherlands) with T7 and SP6 primers. The sequence was compared with those reported in the GenBank database via the Blast search program.

RNA preparation and Northern blot analysis

Total RNA was extracted from frozen liver tissue of controls, of rats treated with DENA and of 70% hepatectomized rats at the indicated time points using Trizol, according to the vendor's protocol (GIBCO BRL, Breda, The Netherlands). The amount of RNA was determined by measuring absorbance at 260 nm, and RNA quality was confirmed by electrophoresis on an agarose gel stained with ethidium bromide. Total liver RNA (20 μ g) was separated on a 1% formaldehyde-agarose gel and transferred to a Hybond-N nylon membrane (Amersham, Aylesbury, UK). After fixation at 80°C for 2 hr, the Northern blots were prehybridized for 2 hr at 65°C in 6 $\times$ SSC, 5 $\times$ Denhardt's solution, 0.5% SDS and 100 μ g/ml of herring sperm DNA. Rat α -TTP and ferritin-H cDNA (0.83 kb), which we previously identified (Wu *et al.*, 1997), were isolated from low melting point agarose and labelled according to the hexamer-random primed method following the manufacturer's instructions (Promega, Leiden, The Netherlands). Membranes were hybridized overnight under the same conditions as stated for prehybridization and afterwards were washed four times for 15 min with 1 $\times$ SSC/0.1% SDS and once with 0.2 $\times$ SSC/0.1% SDS at 65°C. The membranes were exposed and scanned with a Phosphorimager radio-analytic scanning system (Molecular Dynamics, Sunnyvale, CA) to quantify the amount of radioactivity of individual bands, which were standardized by the intensity of ethidium bromide-stained 28S rRNA as scanned with the EAGLE EYE II (Stratagene, La Jolla, CA).

In situ hybridization

In situ hybridization was performed on serial paraffin-embedded liver sections of treated and control rats and of patients with HCC as previously described (Wu *et al.*, 1997). The probes used in this study were made from a 0.6-kb rat α -TTP cDNA fragment cloned in the pCR II vector and a full-length (0.83-kb) rat ferritin-H cDNA cloned in the pCDNA3 vector (Invitrogen). A sense probe was used as negative

control. Labelling was carried out by the T7 or SP6 RNA polymerase method using [α -³⁵S] UTP (Amersham), to a specific activity of 10⁸ cpm/ μ g. After pretreatment of the tissue sections as described (Wu *et al.*, 1997), 5 $\times$ 10⁴ cpm/ μ l of the labelled probe resuspended in hybridization mixture were applied to each section. Hybridization was performed overnight at 52°C. Sections were washed by gently shaking in 2 successive baths of 50% formamide in 1 $\times$ SSC at 52°C for 15 min. Sections were then rinsed twice in 1 $\times$ SSC for 10 min and once in 0.1 $\times$ SSC for 10 min at room temperature. After dehydration in graded ethanol containing 0.3 M ammonium acetate, the sections were dipped in Ilford Nuclear Research Emulsion K-5 (Ilford, Leiden, The Netherlands). After 5–14 days of exposure, the sections were developed in amidol-developer (4-hydroxy 1,3-phenylenediammoniumdichloride) (Merck, Amsterdam, The Netherlands), fixed in 30% Na₂S₂O₃·H₂O in distilled water and stained with 0.1% nuclear-fast-red. All sections were examined by dark-field microscopy.

Biochemical analysis of serum α -tocopherol, vitamin A and cholesterol

Serum samples were obtained from the overnight-fasted rats which had been exposed to DENA treatment for up to 16 weeks, and from the control rats. Serum α -tocopherol and vitamin A concentrations were determined by a reverse-phase HPLC method, essentially as described by Catignani and Bieri (1983). Detection was done by fluorimetry. Tocopheryl-acetate and retinylacetate were used as internal standards to correct for losses during extraction and analysis of the respective vitamins. Cholesterol was determined on a Cobas Bio analyzer (Roche, Basel, Switzerland) by an enzymatic method (Boehringer Mannheim, Germany). The low ratios of serum α -tocopherol to total cholesterol in the rats with DENA indicate that the low-serum α -tocopherol concentrations were not due to abnormal lipoprotein profiles (Gotoda *et al.*, 1995).

FIGURE 1 – Northern blot analysis of α -TTP mRNA levels during rat hepatic carcinogenesis as compared with ferritin-H (fer-H). Lanes 0–16 represent RNA derived from liver of rats exposed to DENA for 0, 3, 6, 9, 12 and 16 weeks, respectively. Intensity of the bands was quantified with the Phosphorimager and standardized by comparison to 28S rRNA.

FIGURE 2 – α -TTP mRNA expression during liver regeneration after a 70% partial hepatectomy detected by Northern blot (top); 20 μ g of total RNA from liver of rats before (0) and 3, 6, 12, 18, 24 and 30 hr after 70% hepatectomy were loaded on the membrane and hybridized with the α -TTP probe. 28S rRNA was used as reference. The same quantitative method as in Figure 1 was conducted for α -TTP mRNA levels during liver regeneration (bottom).

FIGURE 3 – Gene expression and localization of α -TTP and ferritin-H mRNA in the liver from a rat exposed to DENA for 9 weeks and from a patient with HCC as histopathologically (H-E staining) detected (a,d). Overexpressed ferritin-H mRNA (b,e) is indicated by arrows in the tumor nodules of both of rat and human (exposed for 5 days), in contrast to decreased α -TTP mRNA (exposed for 2 weeks) (c,f). Scale bar: 0.5 mm.

Statistical analysis

Results are expressed as means $\pm$ SD. The differences between means were analyzed with Student's *t*-test. Correlation between α -TTP mRNA levels and serum α -tocopherol concentrations was examined by Pearson's correlation coefficient, and the corresponding *p* values were calculated. Significance was defined as *p* < 0.05 (double-sided test).

RESULTS

α -TTP mRNA levels during rat hepatic carcinogenesis and liver regeneration

In order to detect the temporal alterations of α -TTP mRNA levels during hepatic carcinogenesis, equal amounts of total RNA, isolated from liver of rats before, and 3, 6, 9, 12, and 16 weeks after treatment with DENA, were blotted and hybridized with the

ferritin-H probe and with the α -TTP probe. As shown in Figure 1, mRNA levels of α -TTP were decreased as early as 3–6 weeks of treatment with DENA and remained low after 9 weeks. This contrasts with the inverse expression pattern of ferritin-H mRNA that reaches its highest levels after 9 weeks (Fig. 1). The mRNA amounts of α -TTP from rat livers after 9 weeks were 3–5-fold lower than those seen at early stages or in control rat livers ($p < 0.01$). In comparison, α -TTP mRNA levels remained unchanged during liver regeneration after 70% partial hepatectomy (Fig. 2).

Localization of α -TTP mRNA in the liver of rats treated with DENA and patient with HCC

To study the tissue distribution of decreased α -TTP mRNA, a radio-labelled antisense, as well as a corresponding sense probe RNA probe, was hybridized to serial liver sections. These were examined by standard histopathologic H-E and reticulin staining, thereby showing the tumor nodules and the surrounding non-tumor tissue as indicated with arrows from a rat after 9 weeks of DENA treatment (Fig. 3a) and from a HCC patient with co-existing cirrhosis (Fig. 3d). In agreement with the findings on Northern blot, overexpressed ferritin-H mRNA was seen in the tumor nodules as compared to the surrounding non-tumor tissue in rats (Fig. 3b). By contrast, decrease of α -TTP mRNA was preferentially localized in the tumor cell islands rather than in the surrounding non-neoplastic tissue (Fig. 3c). Similarly, a low α -TTP mRNA level was also found in the tumor nodule of 3 of 8 patients with HCC (Fig. 3f), whereas ferritin-H was overexpressed in all 8 HCC patients (Fig. 3e). All sections which were hybridized with sense probes did not show any specific signal (data not shown).

Serum α -tocopherol and vitamin A concentrations in rats treated with DENA

To investigate whether the decrease of α -TTP mRNA would correlate with serum α -tocopherol concentrations during hepatic carcinogenesis, the ratios of serum α -tocopherol to total cholesterol concentrations were determined. As shown in Figure 4, these ratios were 72% ($p < 0.05$) in rats treated with DENA for 3 and 6 weeks compared with those of control rats. The ratios of rats at 9 weeks were 40% ($p < 0.01$) of controls. The ratios remained low as the tumor progressed, up to 16 weeks. In comparison, serum vitamin A concentrations were unchanged and seemed to be slightly de-

creased only after 9 weeks of DENA initiation, but not significant ($p > 0.05$).

Correlation between mRNA levels and serum α -tocopherol

As shown in the Figure 5, hepatic α -TTP mRNA levels from 24 rats treated with DENA and from the controls quantified on Northern blots standardized with 28S rRNA were shown to be significantly correlated with the ratios of serum α -tocopherol to cholesterol concentrations. The correlation coefficient of these 2 data sets is 0.834 ($p < 0.001$).

DISCUSSION

Hepatic carcinogenesis models in rodents have provided an important and useful framework for studying the multistep process by which malignant tumors develop (Lotze *et al.*, 1993; Richardson *et al.*, 1988). In this study, we investigated the α -TTP mRNA expression pattern in rat hepatic carcinogenesis induced by DENA and in 8 patients with HCC, and its possible association with hepatic carcinogenesis. We previously showed that an increase in ferritin-H mRNA started after 6 weeks of DENA treatment, and its overexpression was restricted to the preneoplastic foci, well-defined tumor nodules and the cancer cells invading blood vessels (Wu *et al.*, 1997), paralleling the alteration of GST expression, a well-established early tumor marker (Liu *et al.*, 1995). In contrast to the overexpression of these 2 genes, a marked decrease in α -TTP mRNA levels was observed in rats at early stages (3–6 weeks) of hepatic carcinogenesis. In comparison, unchanged α -TTP mRNA levels during liver regeneration after 70% partial hepatectomy indicated that repression of α -TTP during hepatic carcinogenesis was not due to liver regeneration, which usually accompanies hepatic carcinogenesis. Subsequently, we studied the spatial expression pattern in the rats with HCC, and found that the decreased α -TTP mRNA was preferentially localized to the tumor nodules in the rat livers. Since human and rat α -TTP mRNA are highly conserved (Arita *et al.*, 1995), we were able to detect a similarly decreased α -TTP expression pattern in 3 of 8 European patients with HCC associated with hepatic cirrhosis by *in situ* hybridization using rat α -TTP RNA probe. These data strongly suggest that the decrease of α -TTP is associated with hepatic carcinogenesis.

From recently accumulating evidence, it has been substantiated that mutations in the α -TTP gene are responsible for a decrease of serum vitamin E concentrations in patients with isolated vitamin E deficiency (Gotoda *et al.*, 1995; Ouahchi *et al.*, 1995). The mutated α -TTP protein had impaired ability to incorporate α -tocopherol into very low-density lipoprotein secreted by the liver (Traber, 1990), a function that accounts in normal individuals for efficient

FIGURE 4 – Relative amounts of α -TTP mRNA (solid bars), ratios of serum α -tocopherol to total cholesterol concentrations (α -T, fine dashed bars) and serum vitamin A concentrations (Vit. A, rough dashed bars, values shown as 3-fold to the real determined) are shown as the means $\pm$ SD ($n = 4$) from the fasted rats treated with DENA for various weeks. Asterisk indicates significant difference ($p < 0.05$) compared with rats before DENA administration.

FIGURE 5 – Correlation between hepatic α -TTP mRNA levels and serum α -tocopherol concentrations of 24 rats treated with DENA and control rats.

recycling of plasma vitamin E that would otherwise be eliminated (Traber *et al.*, 1994). However, no study on the association between α -TTP, α -tocopherol and the incidence of cancer in those patients has been reported. Therefore, we studied whether repression of α -TTP would affect serum α -tocopherol concentrations during rat hepatic carcinogenesis. To avoid misinterpretation due to variations in lipoprotein levels or profiles, serum α -tocopherol concentrations were expressed as the ratios of α -tocopherol to total cholesterol concentrations. We found that the serum α -tocopherol concentration profile closely paralleled hepatic α -TTP mRNA levels throughout the whole carcinogenesis process, except for the last point (at 16 weeks) at which the α -T/cholesterol ratio was raised, but remained lower as compared to that of controls. Furthermore, concentrations of serum vitamin A, another lipid-soluble vitamin, were determined during rat hepatic carcinogenesis. The insignificant changes of vitamin A showed that alteration of α -tocopherol during hepatic carcinogenesis was not likely due to malabsorption. Hence, the findings obtained from the rat hepatic carcinogenesis model are in agreement with previous observations that α -TTP is a determinant of serum vitamin E concentrations (Gotoda *et al.*, 1995).

A wealth of data has documented that vitamin E, one of the major antioxidants, acts as a scavenger of free radicals. Epidemiological and biological studies have implicated that vitamin E, together with other factors, is associated with carcinogenesis

(Wattenberg, 1992; Diplock *et al.*, 1994; Feig *et al.*, 1994). Our results demonstrate that repression of α -TTP gene expression paralleled a decrease of serum α -tocopherol during hepatic carcinogenesis. Although this does not provide direct proof, our findings support the hypothesis that α -TTP affects serum α -tocopherol concentration, probably due to reduced recycling of α -tocopherol (Traber *et al.*, 1994), which may be associated with a decrease of α -tocopherol in patients with cancer (Greenberg and Sporn, 1996). However, it remains unclear by which mechanism the transcription of the α -TTP gene is repressed in HCC. Frame-shift mutations (Ouahchi *et al.*, 1995) and a missense mutation (Gotoda *et al.*, 1995) in α -TTP gene have been characterized in patients with isolated vitamin E deficiency. In this context, results showing loss of heterozygosity of chromosome 8q, where the α -TTP gene is located (Ben Hamida *et al.*, 1993), and detection of mutations of α -TTP in patients with HCC, are intriguing.

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