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Expression of Oncogenes during Rat Chemical Hepatotumorigenesis Promoted by Estrogen

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To elucidate the role of oncogene expression in hepatocarcinogenesis, we examined the expression of 4 cellular oncogenes (c-*myc*, c-*fos*, Ha-*ras* and c-*erbA*) in liver tissues induced by chemical agents. Four groups of male Sprague-Dawley rats were examined in the present study. Rats of the first and second groups were given a single intraperitoneal injection of diethylnitrosamine (DEN), 200 mg/kg body weight. Two weeks later, these rats were divided into two groups; the DEN-C group received no further medication, whereas the DEN-DES group was given diethylstilbestrol (DES), 0.5 mg/day, for 12 months. The DEN group was given DEN, 100 ppm, in drinking water for five months as the hepatocellular carcinoma (HCC) group. The DES group was given DES, 0.5 mg/day, from the start for 8 months. Rats of the DEN-DES and DEN groups developed grossly visible hepatic tumors. Significantly higher levels of c-*myc* gene expression were observed in tissues of HCC of the DEN group and in neoplastic nodules of the DEN-DES groups than in the DES and DEN-C group. The increase of c-*myc* mRNA seemed to begin after 1 month of treatment and became significant at 4 months in the DEN-DES group. On the other hand, no significant differences in mRNA levels of c-*fos*, Ha-*ras* and c-*erbA* were observed among these four groups. Although the significance of increased c-*myc* gene expression in neoplastic liver is still not known, it is conceivable that the persistent elevation of c-*myc* gene expression in the DEN and DEN-DES groups might contribute to the development of rat chemical hepatotumorigenesis.

Key words: c-*myc* — Diethylstilbestrol — Rat — Hepatotumorigenesis

In recent years, the role of oncogenes in carcinogenesis has drawn increasing attention, because nucleotide sequences of cloned cDNAs have indicated that oncogene products may be involved in cell growth or cell proliferation as growth factors, their receptors and nuclear proteins. The activation of oncogene expression has been reported to be associated with a variety of human cancers.[1] Among several oncogenes, the expression of c-*myc* gene has been most extensively studied. In particular, the possible involvement of the c-*myc* gene in multi-step processes of hepatocarcinogenesis has been suggested from the strong association of an increased level of c-*myc* expression with cell proliferation in the regenerating liver.[2,3] Moreover, increased levels of c-*myc* transcripts have been found in both experimental liver tumors in animals[4–7] and in human liver tumors.[8]

Hepatic benign[9] and malignant[10] tumors occasionally occur in patients taking oral contraceptives. Exogenous estrogen promotes hepatocarcinogenesis in rats.[11–13] Therefore, estrogenemia associated with liver cirrhosis may accelerate the occurrence of hepatocellular carcinoma (HCC) in man. To further elucidate the significance of c-*myc* expression in hepatocarcinogenesis and its relationship with estrogen, we studied the expression of c-*myc* and some other oncogenes during estrogen-promoted hepatic tumorigenesis in rats. The expression of c-*erbA* gene was studied in the present experiments, since *erbA* might play a role in estrogen-stimulated hepatotumorigenesis because of its structural similarity with the estrogen receptor.[14] Histological examination was also performed to correlate oncogene expression with the development of tumor.

The abbreviations used are: DEN, diethylnitrosamine; DES, dietylstilbestrol; γ-GTP (GGT), γ-glutamyl transpeptidase; HCC, hepatocellular carcinoma; mRNA, messenger RNA.

MATERIALS AND METHODS

Animals and treatment Male Sprague-Dawley rats (Charles River Japan, Inc., Atsugi) were housed in an air-conditioned room at 24°C with a 12 h light-12 h dark cycle and were given Oriental M powdered basal diet (Oriental Yeast Co., Tokyo) and tap water *ad libitum*. Four groups of rats were examined in the present study. The rats of the first and second groups were given a single intraperitoneal injection of diethylnitrosamine (DEN) (200 mg/kg body weight) at six weeks of age. Two weeks later they were divided into two groups, of which one (the DEN-C group) was given only olive oil in diet, and the other (the DEN-DES group) was fed diethylstilbestrol (DES)(0.5 mg/day) in olive oil for 12 months. The third group of rats was given DEN (100 ppm) in water

for five months (the DEN group), and the fourth group of rats (the DES group) was fed DES in olive oil (0.5 mg/day) for 8 months. The administration of DES or DEN was stopped 48 h before the rats were killed and all rats were fasted for 24 h before death. The rats in the DEN-C group and DEN-DES group were killed one month, four months, eight months and one year after the start of DES administration to the DEN-DES group. The rats in the DEN group were killed five months after the start of DEN administration. The rats in the DES group were killed one month and eight months after the start of DES administration.

Histological study Under ether anesthesia, the liver was dissected out, and weighed. Each lobe was cut into 5 mm thick sections. Tissue blocks were fixed in cold acetone, stained with hematoxylin and eosin (H-E) by the routine method and stained for γ-glutamyl transpeptidase (γ-GTP) by the method of Rutenburg *et al.*[15] The most commonly used marker for the identification of preneoplastic lesions in rat is the appearance of γ-GTP activity in focal areas of hepatocytes, since most hyperplastic nodules and HCCs are known to have elevated levels of γ-GTP.[16]

RNA purification Details of RNA extraction have already been described.[17] Briefly, 1 g of liver tissue was homogenized with a Polytron homogenizer in a solution of 4 *M* guanidinium thiocyanate (Fluka), 25 m*M* sodium citrate, 0.5% Sarkosyl and 0.1 *M* β-mercaptoethanol (pH 7.0). Then, 1 g of CsCl/2.5 ml was added to the homogenate. The mixture was layered on a 1.2 ml cushion of 5.7 *M* CsCl in 0.1 *M* EDTA (pH 7.0), and centrifuged for 16–20 h in a Beckman SW50.1 swinging bucket rotor at 40,000 rpm at 20°C. The RNA pellet was resuspended in 10 m*M* Tris-HCl (pH 7.4), 5 m*M* EDTA (pH 7.5) solution and deproteinized with chloroform-2-butanol (4:1). RNA was collected by precipitation with ethanol.

Dot blot and Northern blot analysis of RNA The RNA preparation was immobilized on Genescreen membranes (New England Nuclear) as follows. Total RNA was redissolved in a small volume of sterile water and quantified for each sample at 260 Å with a spectrophotometer. For dot blot analysis, 2.5, 5, or 10 μg of total RNA was applied on the Genescreen membrane using a dot-blot manifold system (Schleicher & Schuell). For Northern blot analysis, 20 μg of total RNA was size-fractionated by 1% agarose gel electrophoresis in the presence of 6% formaldehyde. RNA was transferred to the Genescreen according to the instruction manual. After baking at 80°C for 4 h, the filters were prehybridized in 5×SSC, 5×Denhardt's solution (0.04% bovine serum albumin, 0.04% Ficoll 400, 0.04% polyvinylpyrrolidone), 1.0% sodium dodecyl sulfate (SDS), 100 μg/ml denatured salmon sperm DNA, and 50% formamide (deionized) for 16 h at 42°C. Hybridization was performed in the same solution with a nick-translated radioactive cDNA probe added for 24 h at 42°C. Filters were then washed twice in 2×SSC at room temperature for 5 min, followed by two washes each at 65°C for 30 min in 2×SSC and 1.0% SDS, followed by 0.5×SSC and 1.0% SDS. For dot blots, another 30 min wash in 0.1×SSC and 1.0% SDS at 65°C was added to decrease background radioactivity. Filters were exposed to Kodak XAR-5 X-ray film at −70°C with intensifying screens, and scanned by laser densitometry. The filters were stripped of the first probe (boiled for 8 min in water) and reprobed with an actin cDNA probe to confirm that all lanes and spots had equal amounts of RNA.

cDNA probes The probes were labeled with ^{32}P by nick translation of the following oncogenes; the *SalI-PstI* fragment of v-*myc* DNA of 0.9 kb (donated by Dr. R. C. Gallo, NIH)[18]; the *Hin*dIII fragment of v-*fos* DNA of 1.0 kb (purchased from Oncor Lab.)[19]; the *Hin*dIII fragment of Ha-*ras* DNA of 0.88 kb[20]; the *Pst*I fragment of v-*erbA* gene of 0.5 kb (provided by Dr. M. Yamamoto).[21]

RESULTS

More than four rats were studied at each stage in the DEN-DES group, three in the DEN group, five in the DES group and four in the DEN-C group. The body weight was decreased gradually in the DEN-DES group and most markedly in the DEN group. On the other hand, the liver weight was significantly larger in the DEN-DES group than in the DEN-C group and was the largest in the DEN group, as shown in Fig. 1. After four months of DES administration, all livers of the DEN-DES group were larger and had many nodules of various sizes, but livers of the DES group and the DEN-C group showed no macroscopic changes. In the DEN group, livers changed to large tumors with bleeding and necrosis. Microscopically, nodules of the DEN-DES group were defined as clusters of hepatocytes which were delineated from the surrounding hepatocytes. The major abnormality of cells within nodules was vacuolation of the cytoplasm. The foci and nodules were not accompanied with fibrosis or oval cell proliferation. Most of the nodules were histologically similar to those classified as "neoplastic nodules" in a workshop report.[22] In the DEN group, the tumors were histologically HCC. Figure 2 shows the liver and γ-GTP staining of liver tissue after 8 months of DES administration in the DEN-DES group. The number of γ-GTP-positive foci was larger in the DEN-DES group than in the DES group or the DEN-C group. Total area of the foci was already larger after 1 month of DES administration in the DEN-DES group than in the DES group or DEN-C group. Figure 3 shows

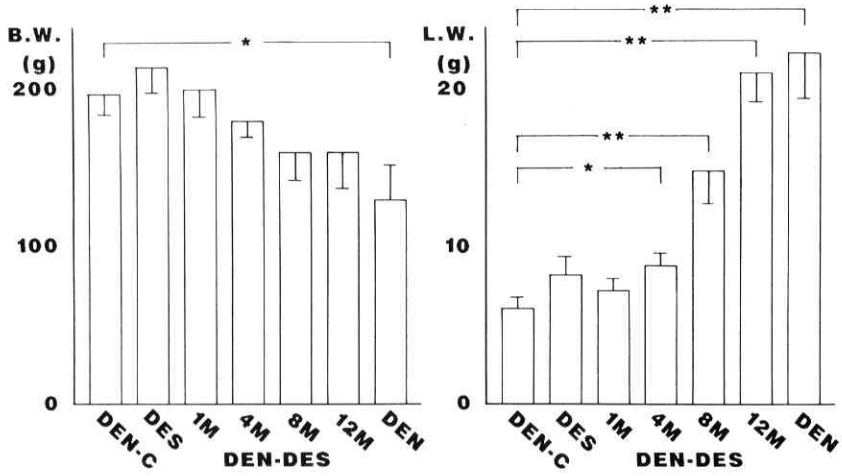
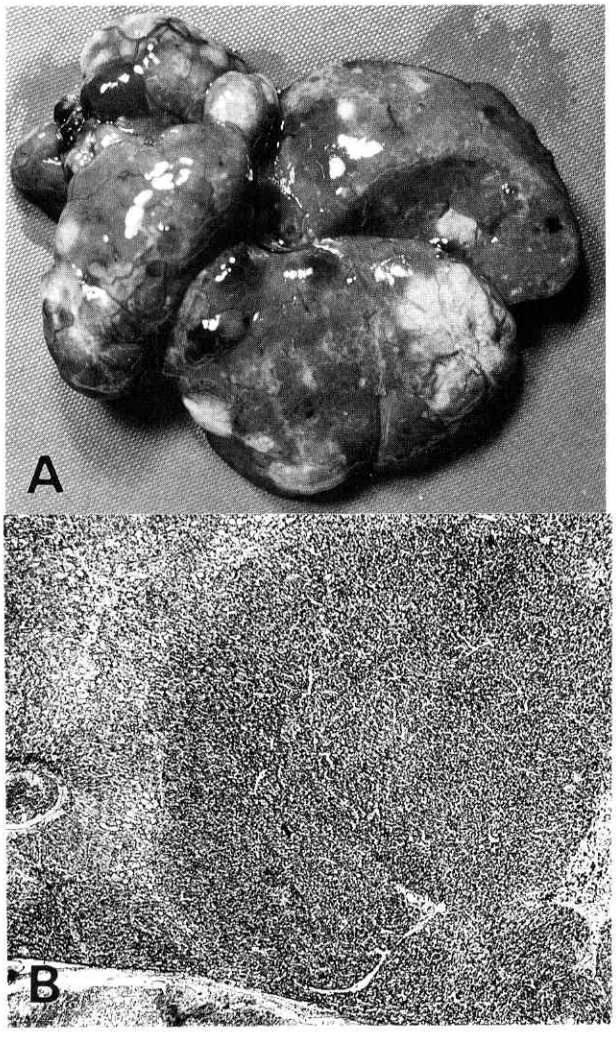


Fig. 1. Body weight (B.W.) and liver weight (L.W.) of rats in each experimental group (mean ± SE; *, $P<0.05$; **, $P<0.001$). Rats of the DEN-C and DES groups were killed after 1 month and rats of the DEN group were killed after 5 months.



the Northern blot analysis of c-*myc* mRNA in each group hybridized with the v-*myc* DNA probe. All mRNAs were of identical size of approximately 2.4 kb. Expression of c-*myc* gene, as demonstrated by the level of c-*myc* mRNA, was higher in the DEN-DES groups than in the DES group or DEN-C group, and was the highest in the DEN group. On the other hand, no significant difference was found in actin gene expression among the samples of different groups (data not shown). Autoradiograms of the Northern blots were densitometrically scanned to quantify the relative level of c-*myc* mRNA in the respective groups, as shown in Fig. 4. There were no significant differences in the c-*myc* mRNA level at 1 month in the DEN-DES, DES and DEN-C groups, although the level tended to be slightly higher in the DEN-DES group. No significant differences were found in the levels of Ha-*ras*, c-*fos* and c-*erbA* mRNAs among the DEN-DES, DEN, DES and DEN-C groups (data not shown).

DISCUSSION

Our previous studies showed that DES, a synthetic estrogen, promoted hepatic tumorigenesis initiated by DEN in male rats.[23] To elucidate the role of oncogenes in hepatotumorigenesis, we used this animal model and measured the expression of oncogenes in liver tissue. We showed that c-*myc* mRNA levels were increased in the DEN-DES group with developed γ-GTP-positive foci. The DEN group developed hepatocellular carcinoma and

Fig. 2. Liver (A) from a rat after 8 months of DES administration in the DEN-DES group and a photomicrograph showing γ-GTP-staining foci (B).

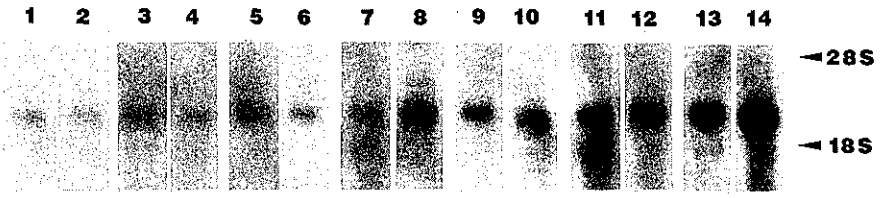


Fig. 3. Northern blot analysis of *myc*-hybridizing RNA of the DEN-C group (lanes 1 and 2 show samples at 1 month and 8 months from the start), the DES group (lanes 3 and 4 show samples at 1 month and 8 months from the start), the DEN-DES group (1 month, lanes 5, 6; 4 months, lanes 7, 8; 8 months, lanes 9, 10; 12 months, lanes 11, 12) and the DEN group after 5 months of DEN administration (lanes 13, 14). We used 20 μg of sample RNA in each well in this experiment. Total RNAs were size-fractionated by agarose gel electrophoresis and transferred to Genescreen filters as described in the text. The positions of 28S and 18S rRNA markers are indicated.

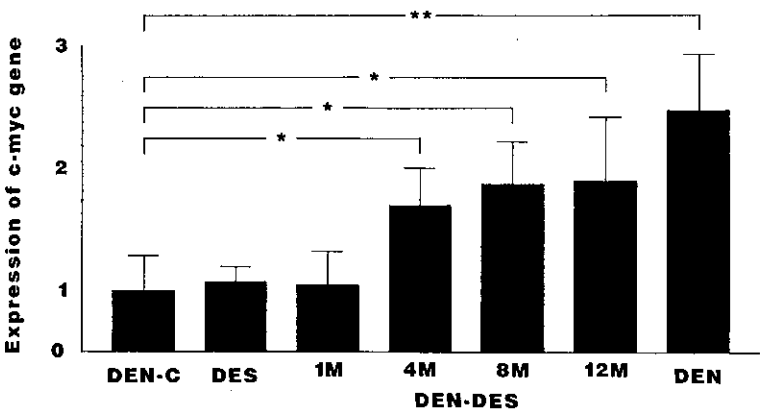


Fig. 4. Expression levels of c-*myc* RNA in tissues. The experimental period in each group was same as in Fig. 1. Histograms show the mean ($\pm$SE) levels of c-*myc* mRNA levels as measured by the densitometric scanning of autoradiograms resulting from Northern blot analysis (*, $P<0.05$; **, $P<0.01$).

showed high levels of c-*myc* mRNA. On the other hand, c-*myc* mRNA levels in the DES group were not significantly different from those in the DEN-C group. These results suggest that the increased expression of c-*myc* gene is associated with neoplastic changes and that estrogen, a promoter of hepatotumorigenesis, has little if any effect on the c-*myc* gene expression. The increased expression of c-*myc* gene seems to occur from an early stage of neoplastic change as shown in the present experiment. Other oncogenes we studied did not change significantly during the development of liver tumor. The *erbA* gene was previously considered to play a possible role in estrogen-dependent tumorigenesis because of its homology with estrogen receptor[14] but its mRNA levels were virtually unchanged throughout the development of hepatic tumors in the present experiment.

Studies on the expression of c-*myc* gene in experimental liver tumors have yielded conflicting results. Makino *et al.*[5] studied the expression of c-*myc* and c-Ha-*ras* genes in liver tumors induced by 3′-methyl-4-dimethylaminoazobenzene and observed that the c-Ha-*ras* expression was increased in tumor and non-tumor tissues, whereas expression of the c-*myc* gene was observed only in tumor tissues. They suggested, therefore, that the c-Ha-*ras* gene is related to proliferation of hepatocytes, while the c-*myc* gene is associated with carcinogenesis. Nagy *et al.*[6] observed an increase of the c-*myc* gene expression not only in hepatic tumors initiated by DEN from an early stage (preneoplastic foci) of the tumor development but also in liver tissues not initiated by DEN. Therefore, they related the c-*myc* gene expression to the undifferentiated stage of hepatocytes and not to carcinogenesis itself. Beer *et al.*[24] isolated γ-GTP-positive cells according to the protocol of Hanigan and Pitot[25] from liver tumors induced by partial hepatectomy followed by DEN and phenobarbital, and failed to detect an increase in the expression of the c-*myc* gene. Such discrepant results can be explained by differences in experimental protocols, chemical agents used and animal species used. Nevertheless, most of the previous studies in chemically induced hepatic tumors and in Morris tumors[4] suggest the possible involvement of c-*myc* gene in proliferation of hepatocytes.

The increased expression of c-*myc* gene can be observed in human hepatic tumors.[26] Our previous studies

showed that c-*myc* mRNA was increased in tissues of HCC, coinciding with previous reports and results of animal experiments.[8] Of more importance is that c-*myc* mRNA levels were almost consistently increased in cirrhotic tissue adjacent to HCC. This seems to be consistent with the increase in c-*myc* gene expression in preneoplastic models induced by chemical agents.[6,7] Therefore, the persistent elevation of c-*myc* gene expression in liver cirrhosis may contribute to the development of HCC. The c-*myc* gene encodes a nuclear chromatin-associated protein,[27] the production of which is closely correlated with cell proliferation[28] and is inversely correlated with cellular differentiation.[29] Goyette *et al.*[2] reported that the increased expression of c-*myc* gene was observed during liver regeneration in partially hepatectomized rats and we made a similar observation. However, the expression of cellular oncogenes increases in a regulated way in regenerating liver tissues,[3] different from the persistent elevation in neoplastic tissue. It is not yet clear whether c-*myc* mRNA is present in hepatocytes or other cell elements in the DEN-DES groups, and *in situ* hybridization is required to draw a conclusion about the location of c-*myc* mRNA. It seems probable, however, that hepatocytes in the neoplastic state have increased expression of c-*myc* gene, because Yaswen *et al.*[7] reported that the c-*myc* gene expression in isolated cell populations was elevated within 2 weeks after the start of a carcinogenic diet and remained elevated during the process of tumor formation in rats. The nodules we studied are considered to be a precancerous stage on the basis of our histological examination. It can be speculated, therefore, that the sustained activation of c-*myc* gene in hepatocytes contributes to the development of hepatocarcinogenesis.

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