

## Study on mechanism of multistep hepatotumorigenesis in rat: development of hepatotumorigenesis

Woo-song Ha<sup>1</sup>, Chi-kyeong Kim, Seung-hee Song and Chung-boo Kang\*

Department of Internal Medicine, College of Veterinary Medicine and Institute of Animal Medicine, Gyeongsang National University, Chinju 660-701, Korea

<sup>1</sup>Department of Surgery, College of Medicine, Gyeongsang National University, Chinju 660-701, Korea

With the aim of establishing bio-indices for the development of multistep hepatotumorigenesis, rats were fed water containing 0.01% diethylnitrosamine (DEN) *ad libitum* for 13 weeks. This treatment with DEN only made it possible to induce hepatic tumors in 100%. After the DEN administration, several clinical symptoms were observed including minor behavioral changes, brittleness of hair and a decrease in water and food intake. The concentration of total serum protein and albumin in all treated groups was significantly lower than in non-treated controls ( $P<0.05$ ). Increase of specific enzyme (AST, ALT and GGT) activity ( $P<0.05$ ), variable tumor size and hepatomegaly of the liver was observed in all rats treated with DEN for 10 weeks. Both hepatocellular carcinoma and cholangiocarcinoma were found in the same livers at the same time, and were prominently developed after 12 weeks. In case of carcinoma, some of the livers showed more or less advanced states over the 12-15 weeks period. In the present study, hepatocellular carcinoma was developed by treating DEN in only the drinking water, without any other carcinogens or without partial hepatectomy. These results indicate that DEN is a new carcinogen that acts directly on the liver, moreover, it might be very useful for investigating hepatotumorigenesis.

**Key words:** Diethylnitrosamine, tumorigenesis, hepatocellular carcinoma, enzyme activity, rat.

### Introduction

Hepatocellular carcinoma can be induced in the livers of laboratory animals by a variety of chemicals [1, 3, 10, 13, 14, 16, 17, 20, 23]. It has recently been reported that hepatoma can be induced by the administration of diethylnitrosamine (DEN) [9, 10, 15, 19].

Among laboratory animals, the rat has a wide variety of advantages, for example it has a short life span which allows observation of DNA transformation from its initial stage through to complete malignant cancer, in addition, variable factors may be fixed artificially during the course of experimentation. Several difficulties are encountered in the study of human hepatocarcinoma, since it occurs via several protracted processes, that obscure the detection of the early stages of the hepatocarcinogenic processes until its later development by which time it is influenced by a variety of factors.

Carcinogenotoxic substances are widely employed to develop cancers in specific organs of experimental animals, such as 1,2-dimethylhydrazine (DMH) [6] and azoxymethane [24] which have been frequently applied to evoke experimental cancers in the gastric intestinal organ, and DEN [19], a genotoxic carcinogen that exclusively resulted in liver cancer. Among these carcinogens, diethylnitrosamine (DEN) has been frequently used to study hepatocarcinogenic processes, treatments and drug effects, etc [18, 21]. Dunsford *et al.* [3] found the carcinogenic effects of DEN and used it opportunistically to improve cancer development in liver cells with enhanced multiplication caused by hepatocyte necrosis.

Chemical agents inducing hepatocarcinogenesis have been administered either as DEN alone or in combination with acetylaminofluorene (AAF), orthic acid, phenobarbital benzopyrene, N-amyl-N-methylnitrosamine and  $CCl_4$  [10, 14, 17, 23]. The chemical has advantages at inducing hepatocarcinogenesis, as it is able to induce hepatoma within a short time and can be administered by a variety of methods. The administration of carcinogenic substances may bring about changes in enzyme levels arising from clonic proliferation, so it is of some importance to analyze enzyme activity variation quantitatively in order to understand the processes involved [7, 8, 11, 12, 21].

In the present study, the development of a hepatocarcinogenesis model involved the administration of administering only water containing 0.01% DEN for 6-14 weeks to

\*Corresponding author

Phone: +82-55-751-5814; Fax: +82-55-751-5803

E-mail: cbkang@nongae.gsnu.ac.kr

an 8 weeks Sprague-Dawley rat strain, which involved neither partial hepatectomy nor the administration of carcinogenic promoters.

## Materials and Methods

### Animals and treatment

Eighty 6 weeks old male rats weighing 120-150 g of the Sprague-Dawley (SD) strain were supplied by the Asan Institute of Life Science, Korea. The animals were acclimated for 2 weeks and maintained under standardized environmental conditions, eg. lighting from 09.00 to 21.00 h, temperature 20°C, and relative humidity 45-60% and fed with commercial pellets. 0.01% DEN (Sigma Chemical Co., USA) was continuously administered to rats via drinking water for 14 to 16 weeks. No differences in the caring conditions of control rats and the DEN treated groups existed, except for the DEN dissolved in the drinking water. Each control group used 10 rats to compare with each DEN treated group. Rats were sacrificed to investigate their liver tissues on the same day after weeks 8, 9, 10, 11, 12, 13, and 14 of DEN administration. Serum samples were collected to investigate serum protein and albumin levels in both the DEN treated and the non-treated controls every week. The weight of each rat was taken before sacrifice, and the liver weight, number and diameter of all macroscopically visible liver tumours, and the weights of tumours over 1 cm in diameter were recorded. Liver tissues were taken from every lobe of the liver and fixed in 10% buffered formalin, for hematoxylin-eosin (H-E) sectioning as a routine procedure. The relative liver weight was calculated as the percentage ratio of liver to final body weight. Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activity were determined using a kit (Asan Pharm Co., Korea) by the modified Reitman-Frankel method, and gamma-glutamyl transferase (GGT) was measured according to a modification of the Orłowski method [7, 21].

### Statistical analysis

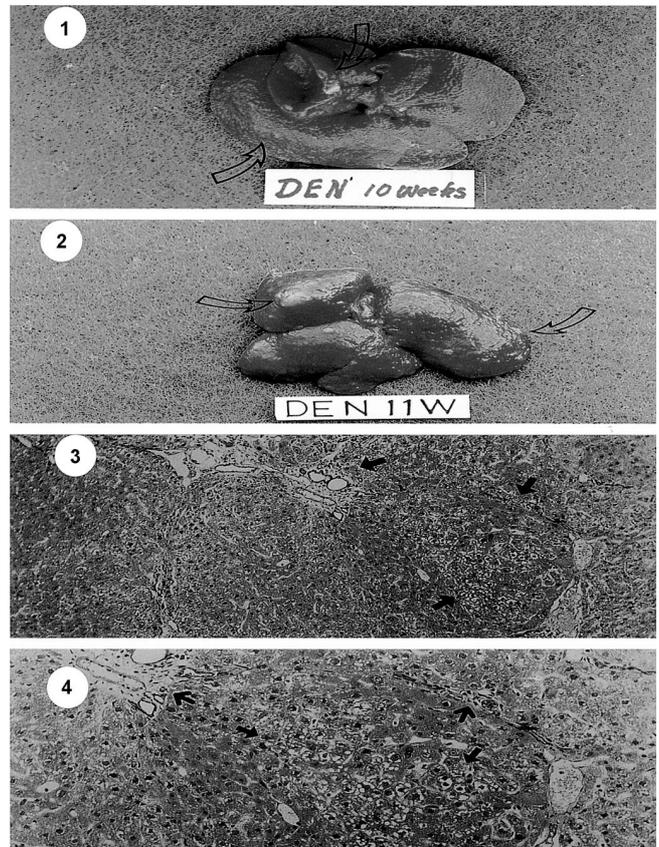
Data was evaluated using one-way analysis of variance (ANOVA: analysis of variance) and significance was tested using Duncan's new multiple range test.

Results were considered significant when  $p < 0.05$ .

## Results

### Clinical observations

A variety of minor clinical signs were observed, for example behavioural changes, brittleness of skin hair at 7 weeks and a decreased food intake from the 7-9th weeks after DEN administration. The amount of food intake was prominently lower, by one half, after 12 weeks of DEN administration. Even though the volume of urine excreted



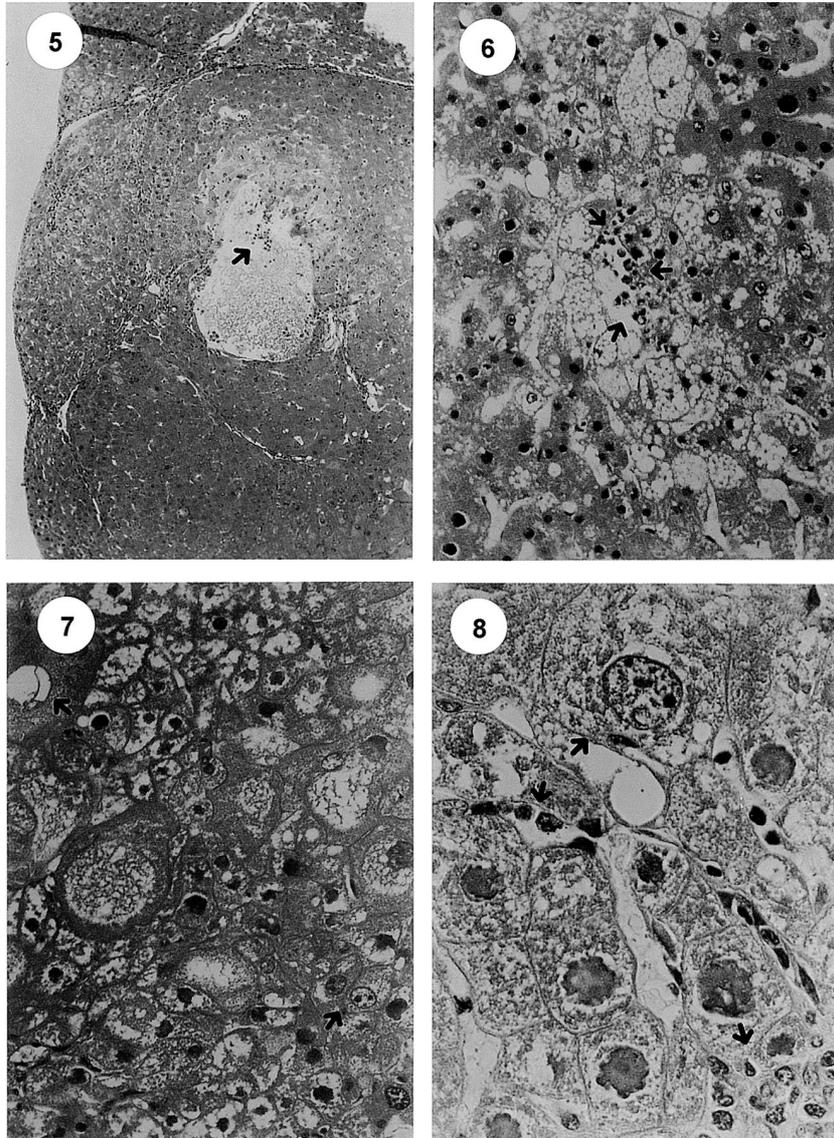
**Fig. 1.** Several foci and very small nodules on the peripheral areas in a rat treated with DEN at 10 weeks.

**Fig. 2.** Many persistent nodules in a rat at 11 weeks treated after with DEN.

**Fig. 3.** Numerous vacuoles are seen on the midzonal and peripheral areas of hepatic lobules and oval cells were seen on the vacuolated areas of hepatic lobules in a rat liver treated for 11 weeks with DEN. H-E stain,  $\times 50$ .

**Fig. 4.** Magnification of Figure 3. Numerous vacuoles and oval cells were seen in the midzonal and peripheral areas of hepatic lobules in a rat liver treated for 11 weeks with DEN. H-E stain,  $\times 100$ .

was slightly lower, no significant difference was found over the experimental period. No differences between the DEN-nontreated control group were found over the experiment. Liver weights of the DEN-treated group increased significantly over the experimental period compared with the negative controls. The increase of liver weight might have been caused by small tumours and well developed liver nodules. The ratio of liver weight/body weight was lower in the control group during the course of the experiment. The incidence of preneoplastic foci and enlarged liver were not significant at the 8th week in the DEN administrated group, while at the 10th week the incidence of nodules was almost 100% in the treated groups (Fig. 1). Thereafter, the numbers became greater and their sizes increased (Fig. 2). No such defects were observed in the control group.



**Fig. 5.** A large vesicle with proliferative oval cells on the periphery was seen in a rat liver treated for 11 weeks with DEN. H-E stain,  $\times 50$ .

**Fig. 6.** Several proliferative oval cells with dense nuclei on the vacuolated necrotic areas in a hepatic lobule of a rat liver treated with DEN for 13 weeks. H-E stain,  $\times 200$ .

**Fig. 7.** Vacuolated small or large eosinophilic hepatocytes in peripheral areas of hepatic lobules in a rat liver treated with DEN for 13 weeks. H-E stain,  $\times 200$ .

**Fig. 8.** Large or small vacuolated polymorphological hepatocytes with eosinophilic nuclei and prominent nucleoli or mitotic features in a rat liver treated with DEN for 13 weeks. H-E stain,  $\times 200$ .

### AST, ALT and GGT activities

The activity of serum AST gradually increased between the 11th and 13th weeks of DEN administration. AST activities were  $46 \pm 1.2$  IU/ml for the controls and prior to the 11th week in the DEN administered group. Although no significant weekly differences in AST activities were observed among the DEN administered rats after the 11th week (Table. 1), a rapid increase was observed to  $127 \pm 56.3$ ,  $106 \pm 19.7$ ,  $108 \pm 39.3$  IU/ml on the 11, 12, and 13th weeks, respectively.

ALT activities in the serum were also increased, and highest activity was observed on the 11th week, which then slightly decreased (Table. 2). GGT activities were  $37 \pm 19.2$  IU/ml in the control groups, but the GGT activities of the DEN treated groups were  $72 \pm 21.5$ ,  $68 \pm 28.5$ , and  $72 \pm 36.9$  IU/ml on 11th, 12th, and 13th weeks, respectively (Table. 3). Both the concentration of serum total protein and serum albumin were significantly lower than the respective controls.

**Table 1.** Changes of AST activities in DEN administrated rats

Duration of treatment (weeks)	No. of rats used	Activity (IU/ml)
0	10	46±1.2
11	12	127±56.3*
12	15	106±19.7*
13	12	108±39.3*

\*Significantly ( $p<0.05$ ) different from non-treated control group.**Table 2.** Changes of ALT activities in DEN administrated rats

Duration of treatment (weeks)	No. of rats used	Activity (IU/ml)
0	10	25±2.5
11	12	50±2.5*
12	15	34±3.2*
13	12	35±2.5*

\*Significantly ( $p<0.05$ ) different from non-treated control group.**Table 3.** Changes of GGT activities in DEN administrated rats

Duration (weeks)	No. of rats used	Activity(IU/ml)
0	10	3719.2
11	12	7221.5*
12	15	6828.5*
13	12	7236.9*

\*Significantly ( $p<0.05$ ) different from non-treated control group.

### Pathological findings

A small number of greyish-white foci or nodules of tumours developed on the surfaces of livers from 8 weeks, but were developed on all livers examined after 9 weeks. The numbers of developed tumors per rat liver were approximately 20 at 13 weeks and 50~60 at 15 weeks. The diameter of large tumours was 3~9 mm at 13 weeks and 7~15 mm at 15 weeks, higher numbers of tumours were developed on the visceral surfaces than on the diaphragmatic liver surfaces. The diameter of the largest tumour was 35.8 mm at 12 weeks. The parenchymae of livers were found to be fragile. The interlobular connective tissues of livers had proliferated by microscope examination by the early 8 week. Vacuolated or fatty degenerated liver cells were focally and widely distributed during the later stages.

Hepatocellular vacuolization was observed at the peripheral zone of some hepatolobules at 10 weeks, then these vacuoles appeared to agglomerate to form small vesicles at 11 weeks (Fig. 3-5). Finally hepatocellular vacuolization developed in almost all liver cells and progressed to necrosis at 13 weeks (Fig. 6, 7). Liver cells with large vacuoles tended to be crowded in focal areas later, and some lobules were transformed into round cells or eosinophilic polyhedral large cells. The nucleoli and eosinophilic karyoplasm of large cells were enlarged from 10 weeks after the administration of DEN, and nucleoli and eosinophilic karyosomes were distinct and concentrated, there was evidence of mitosis but hepatocellular degeneration, necrosis, and proliferation were distinct during the later weeks (Fig. 7, 8). These small round shaped oval cells and eosinophilic polyhedral large cells were believed to be precursors of hepatocarcinoma and hepatocarcinoma cells, respectively. The epithelial cells of bile ductules in the some hepatic lobules were transformed into vacuolated nucleic cells and were proliferated focally. These cells were evaluated to be cholangiocarcinoma cells. Hepatocellular carcinoma and cholangiocarcinoma developed simultaneously in the same liver and seemed to be markedly developed after 12 weeks, but the development of carcinoma in some livers on the same weeks was variable.

### Discussion

A variety of alterations in clinical symptoms were observed including, a loss of weight, decline of water and food intake, and rough hair with depilation. The results of autopsy made it possible to distinctly observe liver enlargement and the generation of tubercular tumour tissues from 10 weeks. Tumour tissues, of approximately 5mm in diameter, were distributed after 12 weeks, which indicated the progress of hepatoma since the liver tissues were tuberculated and became rigid.

In the present study, a number of new proliferative smaller circular masses, surrounding tubercles in necrotic portions, was first observed, and this was suspected as the initiation of malignant cancer.

DEN showed similar effects as aminoazo dyes by developing necrosis, hyperplasia, hyperbasophilia and tumor. It was bioactivated of by cytochrome P450, and necrosis and steatosis was induced near surrounding central vessels of

**Table 4.** Development of tumors in the livers of rats given DEN treatment

	Period after first DEN treatment (weeks)							
	8	9	10	11	12	13	14	15
Diameter(mm) of tumors or foci	0.51.5	24	23	34	35	39	25	59
No. of tumors > 1.5 mm in diameter		35	510	1020	47	18, 27, 12	50, 55	55

smooth endoplasmic reticulum [4, 5, 22]. The decrease of body weight observed in hepatoma is a symptom common in malignant tumours [9, 15]. In this experiment, body weight and liver weight also decreased in DEN administered rats. The weight of liver compared with the body weight was somewhat lower but not to the extent previously reported [9, 15].

The serum enzyme activities were highly increased in terms of all hepatoenzyme activities compared with the control, which is similar to results obtained with hepatotoxic and hepatocarcinogenesis substances.

Hepatospecific enzymes were activated when hepatocellular damage gave rise to abnormalities of liver function, and these enzymes were remarkably increased in hepatoma. In 1984, Simonsen *et al* [21]. reported that these enzymes exhibit high levels in the abnormally functioning liver, thus establishing them as an index of liver function recovery degree; these enzymes include, of AST, ALT, GGT and alkaline phosphatase (ALP) in liver transplant patients. In 1992 and 1994, Kim *et al* [11, 12]. reported greatly increased AST, ALT, and ALP enzyme levels in serum, after inducing hepatocellular tumours by administering  $\text{CCl}_4$  in rat.

It became clear that the DEN had not only carcinogenicity, as a potent alkylating agent, but also powerful toxicity. In our current study, the high levels of AST, ALT and GGT led to hepatocellular degeneration and the dissimilarity between the DEN administered group and the control were evident in liver disease and hepatitis.

AST and ALT activities in blood serum are generally accepted as an index of liver damage and this tendency is also known to be distinct in rodents. ALT is recognised to be a highly liver specific enzyme. On the other hand, AST might be a non-specific index because it was distributed not only in the liver but also in the heart, skeletal muscle, kidney and brain [9, 21]. The analysis of ALT and AST simultaneously, nevertheless, proved significant. ALT activity is known to increase in liver cells induced to necrosis by the agents other administration of than 2,2'-Azobis (2-amidinopropane) dihydrochloride (AAPH), such as chloroform and acetaminophen [8]. However, care must be taken when these enzymes are used as a diagnostic index. Even though it was believed advisable to evaluate the activities of AST and ALT 2 hours after administration, the ALT activity differed in pattern slightly by increasing gradually for 48 hours, and then over reached to the detection limit. That is why more exact verification was left to histopathological examination was necessary. The reported activity of GGT is known to vary. However, its activity is likely to have a high diagnostic value with respect to acute and chronic hepatitis [5, 7, 8, 21]. Its activity increased continuously during our study. The effect of carcinogenesis on the free radicals concentration needs further examination.

In the present study, DEN administration initially

induced hepatocellular degeneration around the central lobule in the liver tissue, and these degenerated liver cells then became the precursors of hepatoma by histopathological observation. These precursors were called neoplastic tubercles, degenerated lesions and proliferative tubercles by Farber *et al* [4]. In 1989, Dunsford *et al* [3], reporting on hepatocellular vacuolization in animals treated with DEN observed that vacuolar liver cells appeared from 5 weeks and increased in size and number up to 9 weeks. In addition, in the present study, hepatocellular vacuolization was observed as a small number of surrounding hepatolobules at 10 weeks, they then appeared to mass at the periphery and the centre and formed small vesicles at 11 weeks, lastly hepatocellular vacuolization developed in almost all liver cells and progressed to necrosis at 13 weeks.

With respect to nuclear variation in liver cells, the nuclei of the central lobular liver cells and cytosol appeared as large polymorphic cells in study by Tamano *et al.* [23] in 1994. However, in our present study, nuclei and basophilic karyoplasm were enlarged from 10 weeks after the administration of DEN, and nuclei and eosinophilic karyosomes were distinct and concentrated, with evidence of mitosis but hepatocellular degeneration, necrosis, and proliferation predominated in the following weeks, and tumour cells developed in the lesions.

In terms of the development stage of small tubercular lesions after DEN administration, Dunsford *et al.* [3] in 1989 reported having conducted a histological examination that a variety of small tumors were formed, and among them ovally shaped cells notably proliferated, these cells were presumed to be the precursors of hepatocarcinoma cells. In 1992, Lee *et al.* [15]. reported that trabecular tissues were formed and that the size of hepatocells were variable at 10 weeks, and tumor cells aggregated to form a spindle-shaped long nucleus insular. In this study, oval cells were viewed as precursors of carcinoma cells. Large eosinophilic polyhedral cells and the vacuolated nucleic cells of ductules were believed to be carcinoma cells. These three type cells were identified at 11 weeks and observed to develop in the liver tissues of all rats at 13 weeks. In conclusion, this study was able to develop tumors by treating DEN only through drinking water with other carcinogens and without performing hepatectomy. However, work remain to be done on the immunohistochemistry and the detection of glutathione S-transferase positive hepatocytes [2, 10], and on the development of a carcinogenic index an identification of carcinogenic index and a functional analysis for free radical assay.

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