Pergamon

Life Sciences, Vol. 57, No. 26, pp. 2431-2437 1995

0024-3205/95 $9.50 + .00

0024-3205(95)02261-9

LOW DOSE EXPOSURE OF DIETHYLNITROSAMINE AFFECTS MICE LIVER THYMIDINE KINASE

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(Received in final form October 16, 1995)

Summary

Swiss albino mice exposed to 5 and 10 mg diethylnitrosamine kg^{-1} body weight by intravenous route up to four weeks showed cyto- and genotoxic effects. Distortion of cell and nucleus shapes and extensive necrosis were observed. Thymidine kinase activity in the liver declined in diethylnitrosamine dose and duration dependent manners. The adult-form of thymidine kinase isozyme declined continuously during this period. Simultaneously, two isozymic forms of thymidine kinase, with small anodic migrations in an electrophoretic field, were gradually induced. Significance of these changes in diethylnitrosamine induced precarcinogenic toxicity has been discussed.

Key Words: diethylnitrosamine, genotoxicity, hepatocarcinogenesis, thymidine kinase, liver

Diethylnitrosamine (DEN) is a genotoxic hepatocarcinogen. It induces liver cancer over a wide range of doses coupled with or without different promoters (1-6). One obvious requirement for the development of cancer is rapid cell proliferation. This has been shown for arecoline or betel-nut extract-induced carcinogenesis in vitro (7). To maintain rapid cell proliferation, DNA synthetic rate must also be high. This situation necessitates optimum availability of precursors of DNA. Availability of one of the precursors of DNA, thymidine nucleotide, is regulated by thymidine kinase (TK), an enzyme of the pyrimidine salvage pathway catalysing the transfer of

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phosphate from adenosine triphosphate to thymidine and to its mono- and diphosphate derivatives in the presence of Mg^{++} (8-10). Thus, the activity of TK may have profound influence on the genotoxicity of DEN and, eventually, on the process of carcinogenesis.

In mammalian cells TK is present in cytoplasm and mitochondria (11). Based on their electrophoretic mobilities, affinities for thymidine, phosphate donors and acceptors, two isozymic forms of TK have been reported, namely, adult form (TK-A) and fetal form (TK-F) (12). Rapidly proliferating cells show an increase in the activity of TK (8,9,13,14) and different tumours show even higher TK activities (15-20). The over all increase in TK activity in tumour cells is proposed to be due to TK-F, in contrast to that in the normal cells (14,15,17).

To look into the preneoplastic toxicity of DEN, five weeks old Swiss albino mice were exposed to very low concentrations of DEN (5 and 10 mg of DEN kg^{-1} body weight (b.w.) for 1 to 4 weeks in the absence of a promoter. Liver histology and activities of TK and its isozymes were monitored.

Methods

Chemicals: Diethylnitrosamine (DEN), tris(hydroxymethyl)-aminomethane, acrylamide, bis-acrylamide, N,N,N',N'-tetra-methyl-ethylenediamine, ammonium persulphate and ammonium formate were purchased from Sigma Chemical Co., USA whereas DEAE cellulose filter from Whatman, England and a toluene based scintillation cocktail, Ready-Solv.HP, and aquasol from Beckman Instruments Inc., USA. ^{14}C-thymidine (^{14}C-TdR; 5 mCi $mmol^{-1}$) was obtained from NEN, England. All other reagents were of analytical grade and were used without further purification.

Treatment of animals: Five weeks old Swiss albino mice (Assam Veterinary Biologicals, Guwahati), housed in polycarbonate cages with husk bedding at 25°C, were divided into 4 treatment groups each for the two doses of DEN. Stock solutions of DEN were prepared in glass double distiled water to deliver doses of 5 and 10 mg DEN kg^{-1} b.w. when 0.2 ml of DEN was intravenously (i.v.) injected. The 1-week-treatment group mice received 1 i.v. injection per mouse and were sacrificed by cervical dislocation on day 7. Similarly, the 2-week-, 3-week-, and 4-week-treatment group mice received 1 i.v. injection every week for 2, 3, and 4 weeks, respectively, and were sacrificed on day 7 after the last injection. Controls were age-matched mice. Standard mouse pellet and water were available ad libitum to all mice throughout.

Histology: Livers were removed, weighed, washed in 0.9% NaCl solution and either processed for histology studies or for TK assay. A slice of the liver, where gall-like nodules were observed in the treated animals, was placed on a glass slide and stained, in succession, with hematoxylin for 1 min and eosin for 2-5 min. The tissue was squashed and spread under a cover slip by gentle tapping and examined using a Zena microscope.

Assay of thymidine kinase: Liver was homogenized in cold 0.25 M sucrose (1 g of liver in 4.5 ml) in a glass-teflon homogenizer and centrifuged (15,000 xg, 4°C, 120 min). The supernatant (enzyme

preparation) was used for TK assay based on Cheng (21) with minor modifications. The enzyme preparation yielded approximately 90% of cellular TK activity. The assay mixture (0.25 ml) in a tube, containing 0.37 kBq of ^{14}C-TdR, 10 mM $MgCl_2$, 50 mM tris-HCl buffer (pH 8) and the enzyme preparation, was incubated at 37°C for 15 min and then plunged into a boiling water bath for 3 min. Aliquotes of 0.1 ml were applied on DEAE discs (1.5 cm diameter) held upright on needles for air drying. After 30 min, the discs were washed extensively in 1 mM ammonium formate solution (x 5) and in methanol (x 3) in succession. The residual ^{14}C-TdR counts on the discs were recorded as disintegration per min (DPM) by a Beckman liquid scintillation counter (L-1800) using Ready-Solv.HP scintillation cocktail. Protein concentrations were determined by Bradford's method (22) with BSA as a standard.

Detection of isozymes of TK by PAGE: The isozymes of TK were resolved by radio-label active staining of TK after their electrophoresis on a 8% native polyacrylamide gel at 4°C following the method of Gabriel (23). Enzyme preparations (0.25 ml) were loaded on pre-ran (150 V, 90 min) gels. After the main electrophoretic run (150 V, 120 min, 4°C), the gels were incubated in the TK assay mixture at 37°C for 90 min, washed in running buffer (x 5) and sliced (1 mm) by a gel slicer. The slices were placed in mini scintillation vials, crushed thoroughly, solubilized in 0.2 ml of aquasol and their DPM recorded in 3 ml of Ready-Solv.HP.

Results and discussion

Between 1 and 4 treated mice, constituting about 10-15%, did not survive DEN treatment regimes. The death, however, had no significant dependence on dose and duration of DEN-exposure, thus, representing undetermined factors (viz. infections etc) as the cause of death. There was no noticeable increase in the body or liver weight of the treated mice during the period of investigation. Except 5 mice out of 11 of 1-week-treatment group

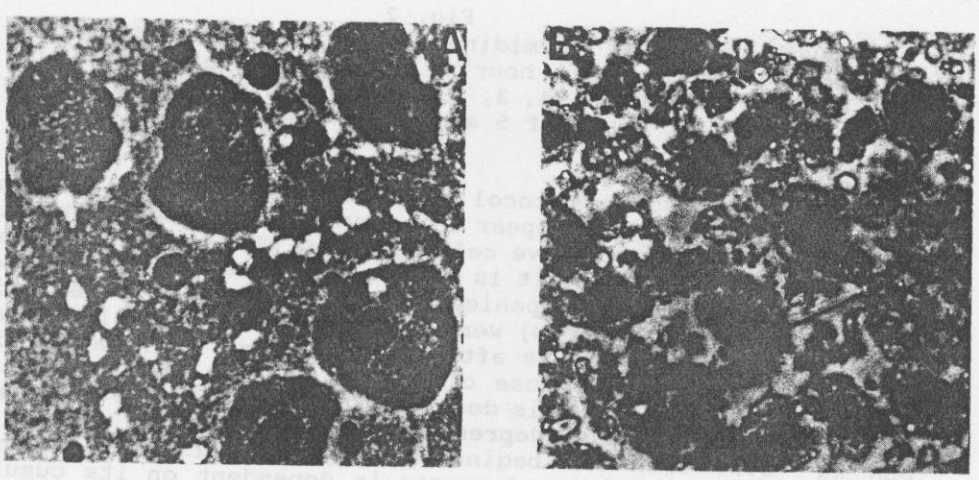

Fig. 1
Mouse liver microphotographs of normal (A) liver cells and that of DEN-exposed (B) cells (2 weeks of 10 mg DEN kg^{-1} body weight) (Magnification x 400).

(about 45%), all DEN-treated animals showed different sizes of gall-like nodules on their livers (24). The normal liver had well defined, symmetrical, mono- and bi-nucleate cells (Fig. 1A), while the DEN-exposed livers had relatively small and ill-defined cells without clear nucleus and at various stages of cell necrosis (Fig. 1B).

The general profile of TK activity was gradual decline following DEN-exposure, except at 2 weeks of the 5 mg DEN kg^{-1} b.w. (Fig. 2). The results are mean±SD of five independent experiments, each performed on a group of 2-3 mice. The changes in TK levels were statistically significant ($P \leq 0.01$; Student's t-test).

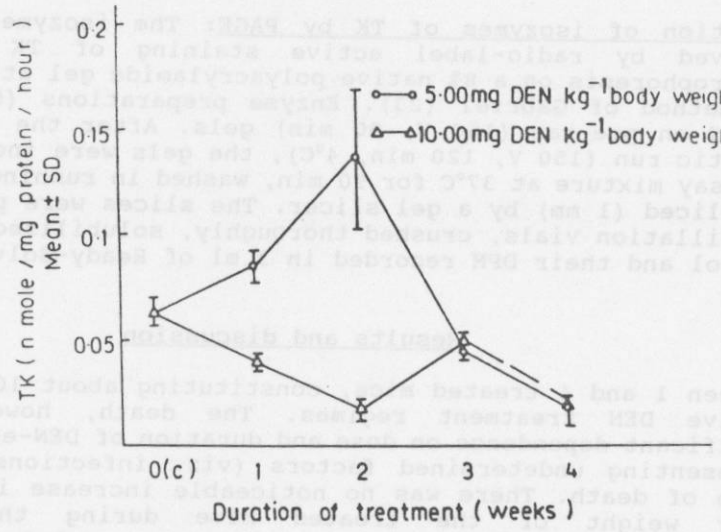

Fig. 2
Levels of total thymidine kinase (nmole of thymidine kinase mg protein^{-1} hour^{-1}) in mouse liver before (0; control) and after (1, 2, 3 & 4 weeks) diethylnitrosamine treatment at doses of 5 and 10 mg DEN kg^{-1} body weight.

In the DEN-exposure protocol used in this report, the initial effects of DEN on liver appear to be cyto- and genotoxicities. This is evident by (a) extensive cell necrosis (Fig. 1) and (b) decline in TK activity (Fig. 2). It is to be noted that DEN-exposure was at a low dose, was not accompanied or followed by a promoter and very early events (1 to 4 weeks) were monitored. The initial increase of TK activity up to 2 weeks after 5 mg DEN kg^{-1} b.w. may be due to sub-optimal cumulative dose of DEN to depress TK (Fig. 2). From third week onwards for this dose of DEN, the cumulative body burden was sufficient to cause depression of TK as was the case of 10 mg DEN kg^{-1} b.w. from the beginning. Lijinsky (25) has shown that development of DEN-induced cancer is dependent on its cumulative dose. The observed effects could be due to genotoxicity of DEN on TK gene expression as alterations of gene expression and lower abundance of some mRNA species in DEN-treated livers have been shown (2,3). The declining TK activity is in agreement with the

observations of Buchmann et al. (5) for several other enzymes from 3 strains of mice livers following one-time 20 mg kg^{-1} b.w. DEN-exposure.

Electrophoretically resolved TK isozymes fixed on gel in situ showed a single peak of TK with large anodic mobility in case of normal (control) liver (Fig. 3A; arrow) indicating that the normal adult cells had one homogenous TK-A isozyme (12). Two weekly i.v. administration of 5 mg DEN kg^{-1} b.w. caused TK-A to decline (Fig. 3B). By the third week of administration of the same dose of DEN, three isozymes were apparent with distinctly reduced activity of TK-A (Fig. 3C; arrow). In the fourth week of DEN administration (Fig. 3D), TK-A remained depressed while two isozymes on the cathodic end (marked 'a' and 'b' in Fig. 3D) showed significant

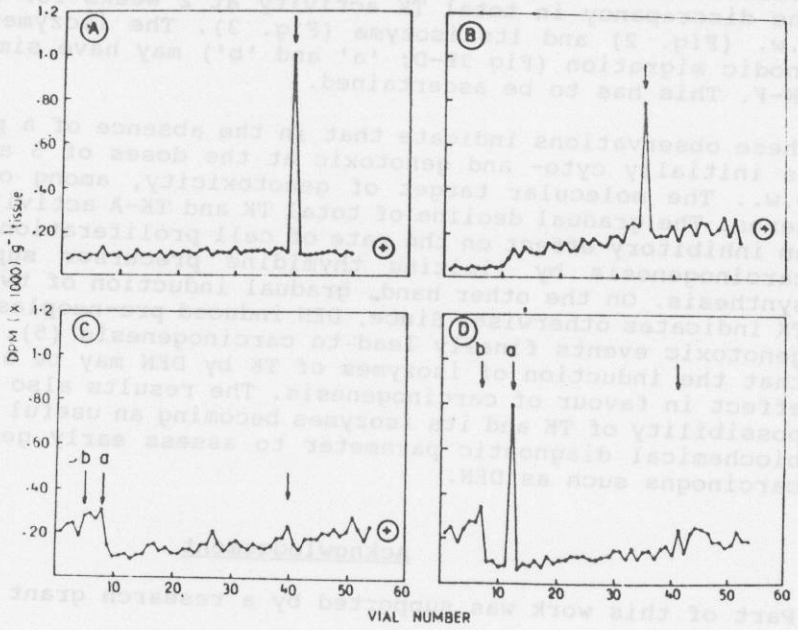

Fig. 3
Isozymes of thymidine kinase in mouse liver before (A; control) and after (B: 2 weeks; C: 3 weeks; D: 4 weeks) diethylnitrosamine (5 mg kg^{-1} body weight) treatment.

enhancement in their activities. Similar observations were made with 10 mg DEN kg^{-1} b.w. dose wherein the isozymic forms expressed approximately one week earlier than the 5 mg DEN kg^{-1} b.w. groups (results not shown) indicating that a relatively higher dose of DEN advanced the induction of these isozymes of TK.

Presence of two isozymic forms of TK, TK-A and TK-F, has been demonstrated (12). It is speculated that up to three additional isozymes, different from TK-A and TK-F, may be expressed in tumour cells (26,27). In this report, the TK-A isozyme declined with DEN dose and duration of treatments and two additional isozymes of TK appeared (Fig. 3A-D). This alteration of the pattern of TK isozymes following DEN-exposure is a pre-neoplastic character (26,27) as has

been suggested for ornithine decarboxylase and its genes (3). The declining TK-A activity (Fig. 3) could also be the cause of the over all decrease in TK activity (Fig. 2). However, an exact correlation between the total TK activity (Fig. 2) and that of its isozymes (Fig. 3) is not possible because of differences in their assay conditions. In the former, the enzyme preparation includes all TK isozymes and other cellular factors influencing their activities. In the later, on the contrary, the assay has been done after the isozymes have been resolved on a gel by PAGE. Electrophoresis might have removed other cellular factors normally present in the enzyme preparation. These could be the reasons for the discrepancy in total TK activity at 2 weeks for 5 mg DEN kg^{-1} b.w. (Fig. 2) and its isozyme (Fig. 3). The isozymes with small anodic migration (Fig 3B-D; 'a' and 'b') may have similarities to TK-F. This has to be ascertained.

These observations indicate that in the absence of a promoter, DEN is initially cyto- and genotoxic at the doses of 5 and 10 mg kg^{-1} b.w.. The molecular target of genotoxicity, among others, is TK genes. The gradual decline of total TK and TK-A activities indicate an inhibitory effect on the rate of cell proliferation and eventual carcinogenesis by limiting thymidine precursor supply for DNA synthesis. On the other hand, gradual induction of two isozymes of TK indicates otherwise. Since, DEN induced pre-neoplastic cyto- and genotoxic events finally lead to carcinogenesis (5), we speculate that the induction of isozymes of TK by DEN may be a compensatory effect in favour of carcinogenesis. The results also bring out the possibility of TK and its isozymes becoming an useful and sensitive biochemical diagnostic parameter to assess early genotoxicity of carcinogns such as DEN.

Acknowledgement

Part of this work was supported by a research grant from CSIR.

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