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Impaired Testosterone Biosynthesis in Rat Testis Treated with DiethylNitrosamine and/or Nodularin
Impaired Testosterone Biosynthesis in Rat Testis Treated with Diethylnitrosamine and/or Nodularin

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ABSTRACT

Gynecomastia and hypogonadism such as testicular atrophy, loss of libido and impotence are frequent complaints of cirrhotic patients, and chronic hepatitis and cirrhosis are commonly associated with hepatocellular carcinoma. Aims of this study are to investigate changes of testosterone level and its metabolism in Sprague-Dawley male rats treated with carcinogens such as N-nitrosodiethylamine (DEN) and nodularin. Serum testosterone level dramatically decreased after DEN as well as nodularin injections. Reduction of serum testosterone was due to combination of reduced activities of cholesterol side chain cleavage enzyme and 17α-hydroxylase, and also decreased expression of steroidogenic acute regulatory protein (StAR) gene, all of which constitute rate limiting steps for testosterone biosynthesis in testes. Light and electron microscopic examinations revealed apoptotic and vacuolar changes of glandular epithelium in the ventral and dorsal prostates, and separation of spermatogonia and disarray of the spermatogenesis with vacuolar change in testes. In conclusion, reduction of serum testosterone during carcinogen-treatment was due to
decreased biosynthesis of testosterone in testes, resulting from reduction of two rate
limiting enzyme activities and inhibition of StAR gene expression

Key words: Testosterone, Diethylnitrosamine, Nodularin, Hepatocarcinogenesis,
Hypogonadism, 17-α-hydroxylase, Side chain cleavage enzyme(SCC), Steroidogenic
acute regulatory protein(StAR).
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Impaired testosterone biosynthesis in rat testis treated with diethylnitrosamine (DEN) and/or nodularin

I. INTRODUCTION

Feminization such as gynecomastia and hypogonadism such as testicular atrophy, loss of libido, and impotence are frequent complaints of cirrhotic subjects. The degrees of feminization and hypogonadism are not related with etiology of liver cirrhosis but correlated well with the severity of liver failure in human. Various endocrine disturbances are often observed in liver damage. Chief abnormalities of sex hormones are decrease in serum testosterone and increase in serum estrogen levels accompanied by an increase in the ratio of estrogen to testosterone in the liver cirrhosis patients with severe hepatic dysfunction. Serum free testosterone and the ratio of testosterone/sex hormone binding globulins decreased, while serum free and total estrogen levels increased in liver cirrhosis, resulting in estrogen predominance and feminization of male patients. Peripheral aromatase activity and serum estrogen
level were increased in cirrhotic patients\textsuperscript{5,6}. Nevertheless, molecular mechanism of the change of serum testosterone level was not fully understood. Together with earlier cited observations\textsuperscript{1-7}, the fact that severe hepatic dysfunction with change of sex hormone could induce feminization and hypogonadism, led us to reevaluate change of testosterone level induced by diethylnitrosamine (DEN) and/or nodularin treatment, which are well known hepatocarcinogens in the animal model. DEN and/or nodularin treatment induced liver damage, apoptosis, alteration of hepatocyte and hepatocyte proliferation. Severe liver damage including necrosis and apoptosis, regeneration of hepatocyte and liver fibrosis were also observed during hepatocarcinogenesis. Therefore, in animal model, the pathological change of liver induced by DEN/nodularin treatment appears to mimic pathological finding observed in human liver. In the present study, we investigated changes of serum testosterone level after DEN and/or nodularin treatment and biochemical mechanism involved in the reduction of serum testosterone.

A. Molecular events during hepatocarcinogenesis.
Many carcinogens such as hepatitis B and C viruses, aflatoxin B1 and alkylating chemical agents can cause hepatocellular carcinoma in human.\textsuperscript{8-10} Primary liver tumors, which include hepatocellular carcinoma occurring in human can be produced by chemical carcinogens in laboratory animals\textsuperscript{11}, and the sequential cellular and molecular changes preceding these neoplasms have been well documented with experimental hepatocellular carcinogenesis models induced by chemicals\textsuperscript{12}: Initial inflammatory processing in the liver proceeded, followed by formation of preneoplastic foci of altered hepatocytes. This is then followed by the appearance of hepatocellular adenomas and hepatocellular carcinoma in experimental hepatocarcinogenesis\textsuperscript{13}. Inflammatory reactions are very similar regardless of the type of initial injury; that is, whether viral infection (hepatitis B or C virus), toxic materials including alkylating agents, immune or metabolites\textsuperscript{14}. During the inflammation, damaged hepatocytes can undergo two pathways. First, the most heavily damaged hepatocytes go apoptosis pathway, whereas survived hepatocytes can undergo carcinogenesis pathway. Thus, survived altered hepatocytes can proliferate during the promotion stage and thereafter go through the stages of altered
foci, adenoma and eventually hepatocellular carcinoma. Their early appearance and progression to hepatocellular adenoma and carcinoma in animal models is extremely beneficial to the study of human hepatocarcinogenesis.

In the Solt-Farber\textsuperscript{15} model of multistage hepatocarcinogenesis, N-nitrosodiethylamine (DEN) as an initiator\textsuperscript{16} and 2-acetylaminofluorene (2-AAF) as a promoting agent were administered in combination with partial hepatectomy in the middle of the promotion period. It should be noted here that DEN is a well studied liver carcinogen which, when administered continuously to rats, produces a well characterized dose-response relationship between concentration in the drinking water and tumor incidence\textsuperscript{17}. DEN is converted to monoethylnitrosamine in vivo, which is capable of ethylating cell component (Fig. 1). Metabolic conversion of the compound endows itself with electrophilic properties and can covalently bind to cellular macromolecules, including protein and DNA. Thus, the tissue specific enzymatic conversion of DEN to an electrophilic intermediate, and ethylation of DNA by the intermediate is thought to be responsible for mutagenic and carcinogenic properties of this chemical. Therefore, DEN administration in animal results in the formation of
O^4\text{-ethyldeoxythymine} and O^6\text{-ethyldeoxyguanine} adducts of DNA, a promutagenic adducts\textsuperscript{18}, and replication of such damaged DNA may lead to somatic mutation and can develop cancer.

In our early studies, we induced hepatocellular adenoma and carcinomas, by employing DEN as an initiator and nodularin as a liver-specific promotor without partial hepatectomy\textsuperscript{19}. Here, nodularin (Fig. 2), isolated from the toxic brackish water cyanobacterium *Nodularia spumigena*, is hepatotoxic and cyclic pentapeptide, and is closely related to the cyclic heptapeptide microcystin-LR. It has previously been reported that the incidence of human primary liver cancer in Quidong and neighboring cities in China, where the people drink pond and ditch water, is about eight times higher than that in the other provinces of China\textsuperscript{20}. These waters are highly contaminated with the cyanobacteria, which produce nodularin\textsuperscript{21}. Microcystin-LR and nodularin belong to the okadaic acid-type tumor promotor\textsuperscript{22}, which inhibit protein phosphatase types 1 and 2A (PP1, 2A)\textsuperscript{23,24}, and protein phosphatases are known to play significant roles in signal transduction pathways pertaining to cell proliferation and gene expression. One \(\mu\text{M}\) nodularin induced
expression of TNF-α as well as of proto-oncogenes of the *fos* and *jun* family in primary hepatocyte cultures isolated from Fischer 344 rats. Nodularin treatment (25μg/kg) after DEN initiation proliferates hepatocytes and eventually induces hepatocellular carcinoma in Fischer 344 rat livers; nodularin injection persistently induces proliferating cell nuclear antigen (PCNA) in the glutathione-S-transferase placental form (GST-P) positive dense nodule, whereas it is expressed transiently in the surrounding hepatocytes. This indicates that nodularin affects the PCNA index differentially in the altered and the unaltered hepatocytes. On the other hand, nodularin injection without DEN initiation rarely induced hepatocellular carcinoma, but induced rather shrinkage of left and caudate lobes with accompanying necrosis and regeneration. Eventually left and caudate lobes became atrophy, as opposed to enlargement of right and median lobes. Heterogeneous effect of nodularin on left and right lobes was accompanied with a significant inhibition of protein phosphatase 1/2A (PP1/2A) in the left lobe as compared with that of right lobe.

B. Testosterone metabolism.
Feminization and hypogonadism are frequent complaints of cirrhotic subjects\textsuperscript{1-3}. It has been well established that over 95% of testosterone is synthesized in Leydig cells of testis, 2-3% in adrenal gland and 1-2% in peripheral conversion. Precursor of testosterone biosynthesis is supplied as plasma LDL-cholesterol to testicular mitochondria where all the biosynthetic pathways are localized. Cholesterol transported as plasma LDL complex enters mitochondria through mediation of a regulatory protein called steroidogenic acute regulatory protein (StAR)\textsuperscript{27}. The intramitochondrial transport of cholesterol is rate-limiting step in the steroidogenesis and the main site for regulation by physical stimuli during acute stimulation of steroid production. The entered cholesterol activates cholesterol side chain cleavage enzyme (CSCC) to produce pregnenolone in testis mitochondria\textsuperscript{28}. Pregnenolone is released to the cytoplasm and converted to progesterone by the action of 17α-hydroxylase, and the latter is eventually to testosterone through a few more enzymatic steps\textsuperscript{29} (Fig. 3). Testosterone is C-19 steroids that provide major regulatory influences on male reproductive function. Target organs of testosterone include prostate, seminal vesicle, liver and skin that have androgen receptor, and the
effect of testosterone is produced by the receptor-hormone complex. Testosterone and its 5 alpha reduced derivative, 5 alpha-dihydrotestosterone (DHT), are physiological ligands for the androgen receptor. Ligand-activated receptor acts as a nuclear transcription factor and mediates androgen action\textsuperscript{30}. Also testosterone increases DNA synthesis by about 50% in HepG2 human hepatoma cells\textsuperscript{31}.

Testosterone biosynthesis is regulated by hormone and various cytokine. Hypothalamus-pituitary-testis axis is a main regulation system in testis for testosterone secretion. Gonadotropin releasing hormone (GnRH) is secreted in hypothalamus and it activates pituitary gland. Activated pituitary gland secretes luteinizing hormone (LH) and LH induces testosterone biosynthesis in testis. Serum testosterone level is negatively regulated GnRH and LH secretion by feedback mechanism. Also various cytokines including transforming growth factor beta 1 (TGF β1)\textsuperscript{32} and tumor necrosis factor α (TNF α) can regulate testosterone biosynthesis in testis. TGF β1 and TNF α inhibit testosterone biosynthesis in testis. TGF β1 decreases steroidogenic acute regulatory protein expression in the transcription level, and decrease the cholesterol supply to P450scc\textsuperscript{32}. TGF β1 is
recognized as a physiological mediator of growth and differentiation of various cell
type\textsuperscript{33}. Its major effects are on the cell cycle in epithelial cells on the synthesis of extracellular matrix proteins in most cell types. During hepatocarcinogenesis initiated by DEN and promoted by nodularin, the TGF β1 expression was induced in the liver\textsuperscript{40}. In normal liver, TGF β1 is produced in the endothelial cells, but hepatocyte and stellate cell secret TGF β1 during hepatocarcinogenesis. Interestingly, TGF β1 level in the serum and urine is increased in the hepatocellular carcinoma patients and used as a tumor marker\textsuperscript{34,35}.

Approximately 50-70\% of the testosterone is degraded in liver\textsuperscript{36} and converted to androsterone, etiocholanolone, or dihydrotestosterone, which is more active than testosterone. These testosterone metabolites are converted to glucuronide derivatives form and excreted in urine. Therefore, liver is the major target organ in testosterone metabolism (Fig. 4).

In the present study, we initiated with DEN and promoted with nodularin in male rats, and found that serum testosterone level was significantly reduced during the initial stage. We also found that the reduction of the hormone was due to
decreased testosterone biosynthesis in testes rather than increased testosterone catabolism. Decrease of testosterone biosynthesis resulted from the reduced activities of both cholesterol side chain cleavage (CSCC) enzyme and 17α-hydroxylase both of which are rate-limiting steps in biosynthetic pathway of the hormone in testes. Moreover, expression of StAR gene, whose protein is involved in transport of LDL-cholesterol through mitochondrial membrane, was also reduced in rats treated with nodularin alone or with DEN-nodularin as compared with its control. Morphologically, DEN and/or nodularin injection induced degeneration of ventral and dorsal prostate with vacuolar change in the glandular epithelium as well as apoptosis, disarray of spermatogenesis, and vacuolar change of spermatids with formation of secondary lysosomes in testes.
Diethylnitrosamine

\[
\begin{align*}
\text{C}_2\text{H}_5 & \quad \text{N} - \text{N} = \text{O} \\
\text{C}_2\text{H}_5 & \quad \text{N} - \text{N} = \text{O} \\
\text{C}_2\text{H}_4\text{OH} & \quad \text{N} - \text{N} = \text{O} \\
\text{C}_2\text{H}_5 - & \quad \text{N} - \text{N} = \text{O} \\
\text{H} & \\
\text{Monoethyl nitrosamine} \\
\text{Spontaneous} \\
\text{C}_2\text{H}_5^+ & \quad \text{N} - \text{N} + \text{OH}^- \\
\text{Ethylation of cell components}
\end{align*}
\]

Fig. 1. Bioactivation of diethylnitrosamine
Fig. 2. Structure of microcystin LR and nodularin
Fig. 3. Testosterone biosynthesis in testis
Plasma Testosterone (5mg/day)

- Aromatase
  - 0.3% plasma E2
  - (0.02mg/day)

- 5α-reductase
  - 6-8% DHT
  - (0.3mg/day)

- 17β-OH steroid dehydrogenase
  - 40% 17-ketosteroids
  - (2.0mg/day)

- Hydroxylase conjugating enzyme
  - 50% polar metabolites
  - (2.5mg/day)

Fig. 4. Peripheral metabolism of plasma testosterone
II. MATERIALS AND METHODS

A. Materials.

1. Animals

Sprague-Dawley (SD) male rats purchased from Dae-Han experimental animal center (Choong Chung Buk Do, Korea) were used. Animals were provided with an irradiated and microbial controlled diet (PicoLab Rodent Diet 20, 5053, PMI Feeds, Inc., Richmond, IN.), and water *ad libitum*. The environmental condition of the animal housing was controlled at a constant temperature (22±1°C) and humidity (55±5%). The room was ventilated 17 times/hour and illuminated for 12 hour/day. The other cares were followed by the "Guide for the Care and Use of Laboratory Animals" prepared by the Korea Research Institute of Bioscience and Biotechnology (Daejun, Choong Chung Nam Do, Korea).

2. Materials

N-Nitrosodiethylamine (DEN) was obtained from Sigma Chemical Co (St.
Louis, MO) and nodularin was purified by Dr. Carmichael (Wayne state university, Michigan). [\textsuperscript{125}I] Testosterone (4\muCi/100 tests tube), [\textsuperscript{4-\textsuperscript{14}}C]cholesterol (51.0mCi/mmol), [\textsuperscript{4-\textsuperscript{14}}C]progesterone (55.4mCi/mmol) and [\textsuperscript{4-\textsuperscript{14}}C]testosterone (57.0mCi/mmol) were purchased from New England Nuclear (Boston, MA), and [\textsuperscript{\alpha-\textsuperscript{32}}P]dCTP (3000Ci/mmol) from Amersham life science products (Buckinghamshire, U.K.). Progesterone, testosterone, dihydrotestosterone, 17\alpha-hydroxyprogesterone and NADPH were obtained from Sigma Chemical Co. (St. Louis, MO). Whatman plate coated with silica gel 60 (F254) was purchased from Merck Chemical Co. (Darmstadt, Germany). The rest of chemicals were obtained from various commercial sources and were of the highest grade available.

B. Methods

1. Tissue preparation

Seven week-old SD male rats (weighing approximately 180~200g) were i.p. injected with DEN (200mg/kg body weight, diluted in corn oil) and the animals were sacrificed 24 hrs later (D24) or nodularin (25\mug/kg body weight) was injected twice
per week for 2 weeks after DEN initiation (DN2). When needed, 5 weeks old SD male rats were orchietomized (Ox), then DEN was injected 2 weeks later (7 weeks of age), and twice/week nodularin injections were performed for 2 weeks (OxDN2). For control, saline was injected into intact (C) and orchietomized rats (Ox) (Fig. 5). The animals were sacrificed at 10:00 AM after ether anesthesia in order to avoid diurnal variation, and liver, prostate and testes were removed and stored at -70°C until use. Tissues for morphologic examination were fixed immediately after resection in 10% formalin for 24 hr.


Level of testosterone in serum was quantified by a solid-phase radioimmunoassay with $^{125}$I-testosterone assay kit (Coat-A-Count), according to the manufacturer’s instruction (Diagnostic Products Corporation, Los Angeles, CA). Five ml of whole blood from the control and the test rats were collected by cardiac puncture. Serum was prepared after centrifugation of the blood at 12,000 rpm, and 50μl of the serum was used to quantify testosterone level. In the assay, testosterone
assay kit tube was coated with testosterone antibody, and 50µl of the serum and 3ml of $^{125}$I labeled testosterone would bind competitively to the coated testosterone antibody. After 3 hr of incubation at 37°C, the reaction tubes were washed 3 times with 5ml of distilled water and dried at room temperature for 30min. Radioactivity was then counted by γ-counter (Wallac, Turku, Finland). Known concentration (0, 0.2, 1, 2, 4, 8ng/ml) of testosterone was used as a control.


Ten % testicular homogenates prepared in 0.01M Tris-HCl (pH 7.4) from the control and the test rats were extracted with 8 volumes of ether and the extract was dried over N$_2$ gas under a fume hood. The dried sample was resuspended in testosterone zero serum in the kit. Testosterone concentration was measured by the method described above. Testosterone concentration in testis was represented as nanogram per gram testis.

4. Measurement of lutenizing hormone and cholesterol levels
Serum lutenizing hormone and cholesterol levels were measured by an automated chemiluminescence system (Chiron Diagnostic ACS:180). Two-site sandwich immunoassay method was used for the measurement of lutenizing hormone using 50μl of serum. Enzymatic method was used for measurement of total cholesterol, HDL-cholesterol and triglyceride (TG) levels. LDL-cholesterol level was calculated from the above data on lipid:

\[
\text{LDL-cholesterol} = \text{Total cholesterol} - \text{HDL cholesterol} - \frac{\text{TG}}{5}
\]

5. Analysis of testosterone metabolites in liver.

Liver was homogenized in 5 volumes of ice-cold 0.01M Tris-HCl (pH 7.4) containing 0.25M sucrose, and the homogenate was centrifuged at 800xg for 10 min. The supernatant spun at 9,000xg for 20 min (the precipitate represents mitochondrial fraction) was further centrifuged at 105,000xg for 1 hour and the final precipitate was used as microsomal fraction. Testosterone metabolites were analyzed by the method described\textsuperscript{32} with 10 nmoles of [4-\textsuperscript{14}C] testosterone dissolved in toluene.
(1.25x10^5 dpm), 10-40μg of microsomal fraction and 0.1mM EDTA in 100mM HEPES (pH 7.4) at 37°C for 10min. The reaction was started by the addition of 1mM NADPH. Analysis was carried out by thin layer chromatography (TLC) on Whatman plate coated with silica gel 60 (F254, Merck Chemical Co. Darmstadt, Germany). The plate was developed in the following solvent systems; initial separation in dichloromethane:acetone (4:1 by v/v) for 30 min, followed by air-dried for 10 min, and finally in chloroform:ethylacetate:absolute ethanol (4:1:0.7 by v/v/v) for 30 min. Steroid spots were identified by autoradiography with control spots of standard testosterone and dihydrotestosterone.


In order to measure the urinary testosterone metabolites in the control and DEN treated rat, 500μg of testosterone (mixture of 10 nmoles of [4-¹⁴C] testosterone (1.25x10^5 dpm) and cold form of testosterone) was i.p. injected to the control and the D24 rats. Urine was collected at 1,2,3,5,7,14,16,20 and 24 hr after injection, and radioactivity in urine was counted by liquid scintillation counter (Wallac, Turku,
7. Assay for cholesterol side chain cleavage enzyme (CSCC)

CSCC in testis was measured by conversion of [4-\textsuperscript{14}C] cholesterol to [4-\textsuperscript{14}C] pregnenolone according to the published method\textsuperscript{28}. After incubation of 500\mu l reaction mixture containing 100\mu M [4-\textsuperscript{14}C] cholesterol, 500\mu g testicular mitochondrial fraction, 20mM KCl, 5mM MgCl\textsubscript{2}, 2mM NADP\textsuperscript{+}, 10mM isocitrate and 250mM sucrose in 10mM potassium phosphate buffer (pH7.4) at 37\degree C for 10 min, the products were resolved by TLC and sprayed with 3% cupric acetate in 5.5% perchloric acid. The plate was autofluorographed, and the fluorescent spots were scraped off into scintillation vials. One-half ml of methanol was added to extract steroids from the silica gel and the mixture was incubated at 37\degree C for 1 hr. Five ml of scintillation cocktail solution was added and the radioactivity was counted by liquid scintillation counter (Wallac, Turku, Finland). Each radiolabeled steroid metabolite analyzed by TLC was expressed by either percentage of the total radioactivity recovered or by specific activity. The specific activity represents \textit{pmoles}
of cholesterol converted to pregnenolone per minute per mg protein.

8. Assay for 17α-hydroxylase activity

17α-Hydroxylase activities were measured by the conversion of [4-14C] progesterone to 17α-[4-14C] hydroxyprogesterone29. [4-14C] Progesterone at 100μM was suspended in 50mM phosphate buffer (pH 7.4) containing 0.5mM NADPH. Five hundred μg of testicular microsomal fraction were then incubated with the above radiolabeled progesterone in a final volume of 400μl for 30 min at 32°C. The reaction was terminated by adding 100μl 1M NaOH, the 17α-[4-14C] hydroxyprogesterone formed was extracted with 3ml of chloroform:ether mixture (1:3 by v/v), and TLC analysis was carried out in freshly prepared solvent system of chloroform:cyclohexane (1:1 by v/v) for 30 min and sprayed with 3% cupric acetate in 5.5% perchloric acid. The plate was autofluorographed and fluorescent spot was scraped off into scintillation vials. The rest of the procedures including extraction of radiolabeled steroids from the silica gel and expression of radioactivity in the metabolites are the same as described above for CSCC activity.
9. Northern blot analysis

Total cellular RNAs were isolated from testis by the method routinely used in our laboratory. Ten volume of guanidine-thiocyanate-phenol solution was added to the testis and homogenized, and chroloform was added to denature protein. After precipitation of total RNA by ethanol, the RNA concentration was measured. Thus, samples containing 20μg RNA were separated in 1.2% agarose gels containing 2.2M formaldehyde in 1xMOPS buffer and transferred onto nylon membranes (QIAGEN, Hilden, Germany) by capillary action in 10x sodium citrate saline solution. Probe cDNAs were prepared by digesting rat cDNA plasmid encoding StAR gene [cloned and donated by Dr. Jaemog Soh 38] with EcoR I and Sal I, and labeled with [α^32P]dCTP for hybridization and autoradiography. The amount of RNAs loaded onto each lane was shown by the level of CHOβ expression in the same blot.

10. Morphologic analysis of testes and prostate

To examine morphologic changes of testis and prostate treated with DEN
and/or nodularin in relation to reduced testosterone level, fresh testis from the treated
animal was fixed overnight in Bouin solution, whereas prostate was fixed in 10% formalin solution overnight, subsequently, paraffin embedding and serial sections (5\textmu m) were carried out by routine procedures, and were stained with H&E\textsuperscript{12}. For transmission electron microscopic examination, small pieces of testis and prostate were prefixed in Karnovsky’s solution and then post-fixed as described previously\textsuperscript{39}.

11. Statistical analysis

Student \textit{t}-test was used to compare characteristics between control and test groups. Values of \( p < 0.05 \) were considered as statistically significant.
Fig. 5. Experimental scheme of DEN-nodularin-induced hepatocarcinogenesis.

Seven week old- Sprague-Dawley male rats were used. Group C rats were intraperitoneally (i.p.) injected with saline and used as control. Group D24 was initiated with a single i.p. injection of DEN (200 mg/kg B.W.) and sacrificed 24 hours later. Group DN2 was initiated with DEN, followed in 24 hours by nodularin i.p. injections (25 μg/kg B.W, twice/week) for 2 weeks. Group Ox was orchiectomized at the age of 5 weeks, initiated with saline i.p. injection and promoted with saline injections at the age of 7 weeks, and used for the orchiectomy control. Group OxDN2 was orchiectomized at the age of 5 weeks, and initiated with DEN i.p., followed in 24 hours by nodularin injections for 2 weeks. Arrowheads indicate the time to sacrifice rats. Identical experiments were performed three times.
III. RESULTS

A. Reduction of serum testosterone level by DEN initiation as well as DEN/nodularin treatment.

As shown in Fig. 6, serum testosterone concentration decreased dramatically within 24 hours of DEN initiation (D24) to the levels of the orchiectomized rats, (Ox and OxDN2). In rats with nodularin injections for 2 weeks after DEN initiation (DN2), the level recovered somewhat, however, it was still significantly depressed as compared with the control.

B. Change of serum LH level by DEN and/or nodularin injections into intact and orchiectomized rats

As a first step to elucidate mechanism of the decreased level of serum testosterone after DEN initiation, serum lutenizing hormone (LH) level was measured. LH is released in pituitary gland and stimulates the testis for secretion of testosterone. During DEN alone (D24) or DEN/nodularin injected intact male rats
(DN2), the serum LH level did not change significantly as compared with the control (C). On the other hand, the serum LH levels in the Ox and OxDN2 were significantly elevated (Fig. 7). These results indicate that DEN-nodularin treatment did not suppress pituitary LH secretion, demonstrating an intactness of pituitary-testis axis during DEN-nodularin treatment.

C. Increased LDL-cholesterol level in serum by DEN and/or nodularin treatment.

Precursor of testosterone biosynthesis is supplied as plasma LDL-cholesterol to testis and adrenal gland. Serum LDL-cholesterol level was much higher in DEN and/or nodularin treated rats than the control (Fig. 8). This data indicate that the reduction of serum testosterone in the hepatocarcinogen treated rats is not due to the limitation of the source of testosterone.

D. Decreased testosterone catabolism in the liver of DEN-treated rats

Since more than 50-70% of testosterone is degraded in liver microsomes, a possibility of whether the reduction of serum testosterone resulted from increased
degradation of testosterone in liver was examined. When [4-\textsuperscript{14}C] labeled metabolites derived from [4-\textsuperscript{14}C] testosterone were analyzed by TLC after incubation of the radio-labeled testosterone with microsomal fraction for 15 min (Fig. 9), conversion of testosterone to its metabolites was found to be significantly diminished by DEN treatment (D24) as compared with the control (C). Validity of the result in Fig. 9 was also confirmed by the finding that formation of the metabolites in the control group (C), shown in the lower spots, was dependent on the amount of microsomes added. The result demonstrates that the marked reduction of serum testosterone in DEN-treated rats was not due to its increased catabolism of testosterone in liver.

E. No difference in change of testosterone metabolite between DEN treated and control rat in urine

Testosterone metabolites are converted to the soluble form by glucuronization and excreted in the urine. Therefore, \textsuperscript{14}C labeled testosterone was i.p. injected to the DEN treated and control rat, and urine was collected at indicated time from the control and DEN treated rats and the radioactivity was counted by liquid
scintillation counter. The two groups (control and DEN treated rats) did not show any significant change in the amount of radioactivity excreted in urine (Fig. 10). The above data indicate that the reduction of testosterone in carcinogen treated rats is not due to the increased catabolism of testosterone.

F. Decrease of testosterone level in testis after treatment with DEN and/or nodularin

There is a strong possibility that the decrease of serum testosterone level after DEN-treatment might be due to decreased biosynthesis of testosterone in testis. Indeed, as shown in Fig. 11, the amount of testosterone in testis was significantly reduced after treatment with DEN (either D24 or DN2) as compared with the control.

G. Decreased 17α-hydroxylase activities

Testosterone biosynthesis proceeds through multi-steps, and 17α-hydroxylase is one of the rate-limiting steps for testosterone biosynthesis in testis; it converts pregnenolone and progesterone to 17α-hydroxy pregnenolone and 17α-hydroxyprogesterone, respectively\textsuperscript{29}. When measured with microsomal fractions
isolated from rat testes treated with either saline, DEN or DEN/nodularin (Fig. 12),
the activities of 17α-hydroxylase were found to be inhibited by approximately 40%
in the D24 (210.0 ± 44.5 pmoles/mg protein/min) and 15% in the DN2 (285.9 ± 22.0
pmoles/mg protein/min), compared with the control (336.4 ± 66.2 pmoles/mg
protein/min).

H. Decreased CSCC enzyme activity in testis.

The cholesterol side-chain cleavage enzyme (CSCC) system is an important
rate-limiting, hormone-regulated step to convert cholesterol to pregnenolone. Therefore, we examined the CSCC by analyzing the formation of pregnenolone from
[4-14C] cholesterol. As shown in Fig. 13, incubation of [4-14C] cholesterol with
testicular mitochondria for 10 min showed significantly reduced pregnenolone
formation in the DN2 and D24 rats as compared with the control.

I. Marked reduction of StAR gene expression in testis by either DEN/nodularin or
nodularin alone treatment
Biological function of StAR is to deliver cholesterol through the inner mitochondrial membrane to CSCC located in mitochondrial matrix when the cells are stimulated by LH and FSH\textsuperscript{27,33}. Therefore, the mRNA expression of StAR gene was measured in rats injected twice with nodularin at 2 weeks after a DEN (D2NN) or saline (S2NN) initiation. Both treatments significantly reduced the mRNA expression of StAR gene as compared with the control (Fig. 14), showing more reduction in the D2NN than S2NN. Moreover, DEN itself (D24) completely abolished StAR expression in testis. Densitometric analysis revealed decreased of about 80\%, 60\%, and 20\% of StAR mRNA expression in D24, D2NN, and S2NN, respectively. It should be noted that each lane shows individual variations among the animals.

J. Morphological changes of prostate and testes during DEN/nodularin induced hepatocarcinogenesis

Light and electron microscopic examination revealed that DEN and/or nodularin on the morphological effects of prostate and testes were basically similar,
although combined treatment of DEN and nodularin induced more atrophic and degenerative changes in the glandular epithelium than nodularin or DEN alone treatment (data not shown). Normal glandular epithelium of prostate showed tall columnar with a few mitotic figures with luminal infoldings (Fig. 15A, x200), however, DEN-nodularin treatment revealed cuboidal change of epithelium with apoptosis in the ventral prostate (Fig. 15B, x200). In dorsal part of the prostate, vacuolar degeneration was also seen with glandular atrophic changes by nodularin alone treatment (Fig. 15C, x100). However, no significant change was found in testes by DEN-nodularin treatment with rare tubules of dissociation (Fig. 15D, x100). Leydig cell proliferation was not noted in DEN-nodularin treated testes.

Although the cells appeared normal in ultrastructural examination of the testes, (Fig. 16A, x3000), DEN alone treatment induced separation of spermatogonia from each other, suggesting mild dissociation, and DEN-nodularin (D2NN) treatment induced disarray of the spermatogenesis with vacuolar changes of spermatids with secondary lysosomes (Fig. 16B, x3000). Even though not shown, treatment of animals with nodularin alone (S2NN) also showed clearing of cytoplasm and
hydropic changes of spermatogonia.
Fig. 6. Changes of serum testosterone levels by DEN and/or nodularin injections to the intact and orchietomized rats.

Serum testosterone level was measured by a solid-phase radioimmunoassay kit with $[^{125}]$I-testosterone (Coat-A-Count). Whole blood was collected by heart puncture right before sacrifice of the rats of D24, DN2, Ox and OxDN2. Serum was prepared by centrifugation at 10,000g for 10 min after 4 hours in refrigerator.
Fig. 7. Change of LH level during DEN and nodularin treatment

Serum level of LH was measured by an automated chemiluminescence system (Chiron Diagnostic ACS:180) using sandwich immunoassay.
Fig. 8. Change of serum LDL-cholesterol level by DEN and/or nodularin treatment.

Precursor of testosterone biosynthesis is supplied as plasma LDL-cholesterol to testis and adrenal gland. Serum LDL-cholesterol level was much higher in DEN and/or nodularin treated rats than control.
Fig. 9. Thin layer chromatographic separation of [4-\textsuperscript{14}C]testosterone metabolites, visualized by autoradiography.

[4-\textsuperscript{14}C]Testosterone (1.25\times10\textsuperscript{5}dpm) was mixed with unlabeled T\textsubscript{4}, and the mixture was air dried under hood. Subsequently, 150\mu l of HEPES buffer containing EDTA and different amounts of microsomal fraction were added to the tube. The mixture was preincubated, and the reaction was then started by addition of NADPH in a final total volume of 200 \mu l and terminated by ethyl acetate after 15 min of incubation. After centrifugation of the reaction mixture at 12,000xg for 10 min, clear organic phase was dried and developed on Whatman thin layer silica gel plate. Steroid spots were visualized by autoradiography. DHT and T indicated by arrows represent migration distance of the corresponding standards, and the other spots indicate unidentified metabolites.
Fig. 10. No difference of testosterone metabolite between DEN treated and control rats in urine

$[^{14}\text{C}]$ labeled testosterone was i.p. injected to the DEN treated and control rats. Urine was collected from the rat at the indicated times and the radioactivity was counted by liquid scintillation counter.
Fig. 11. Change of testicular testosterone level by DEN and/or nodularin treatment.

Ten % testicular homogenates prepared in 0.01M Tris-HCl (pH 7.4) from the control and the test rats were extracted with 8 volumes of ether and the extract was dried over N2 gas under a fume hood. The dried sample was resuspended in testosterone zero serum in the kit. Testosterone concentration was measured by the method described above. Testosterone concentration in testis was represented as nanogram per gram testis.
Fig. 12. Activities of 17α-hydroxylase.

17α-Hydroxylase activity was measured by the conversion of [4-14C]progesterone to 17α-[4-14C]hydroxyprogesterone. [4-14C]Progesterone was incubated with testicular microsomes for 30 min and the 17α-[4-14C]hydroxyprogesterone was extracted with chloroform:ether mixture (1:3 by v/v). After centrifugation of the mixture at 12,000xg, the clear supernatant was removed and dried over N2 gas. TLC analysis was carried out on Whatman plate in solvent system of chloroform:cyclohexane. After development and visualization of the compounds, the plate was autofluorographed, and the compounds were scraped off into scintillation vials.
Fig. 13. Cholesterol side chain cleavage (CSCC) enzyme activity.

CSCC in testis was measured by the conversion of [4-\textsuperscript{14}C] cholesterol to [4-\textsuperscript{14}C] pregnenolone. After incubation of reaction mixture containing [4-\textsuperscript{14}C] cholesterol and 500\mu g testicular mitochondrial fraction at 37°C for 15 min, the products were resolved by TLC and autofluorographed, and the fluorescent spots were scraped off into scintillation vials. One-half ml of methanol was added to extract steroids from the silica gel and the mixture was incubated at 37°C for 1 hour. Five ml of scintillation cocktail solution was added and the radioactivity was counted by liquid scintillation counter (Wallac, Turku, Finland). Each radiolabeled steroid metabolite analyzed by TLC was expressed by either percentage of the total radioactivity recovered or by specific activity. The specific activity represents pmoles of cholesterol converted to pregnenolone per minute per mg protein.
Fig. 14. mRNA expression of steroidogenic regulatory protein gene

Total cellular RNAs obtained from testes were separated in 1.2% agarose gel and transferred onto nylon membranes in 10x sodium citrate saline solution. StAR gene cDNAs were digested with the EcoRI and Sall, and labeled with [$\alpha^{32}$P]dCTP for hybridization and autoradiography (A), and quantitated by densitometer (B).
Fig. 15. Light microscopic changes of prostate and testes during DEN-nodularin treatment

Light microscopic findings (H&E): (A) Normal glandular epithelium of prostate showing tall columnar and a few mitotic figures (arrows, x200). (B) Arrows indicate a cuboidal change of epithelium with apoptosis by DEN-nodularin treatment (D2NN, x200). (C) In dorsal part of the prostate, degenerative changes are seen with vacuolation of glandular epithelium (arrows) by treatment with nodularin alone (S2NN, x100). (D) Change in testes was minimal and revealed generally well preserved seminiferous tubules with rare dissociation by DEN-nodularin treatment (x100).

Fig. 16. Electron microscopic changes of testes during DEN-nodularin treatment

Electron microscopic findings: (A) Treatment of rats with DEN induced separation of spermatogonia from each other (arrows), even though the germ cell itself appeared normal. White bar indicates 2.5mm, x3000. (B) Treatment with DEN-nodularin induced disarray of spermatogenesis with vacuolar changes of the spermatids (thin arrows) and formation of secondary lysosomes (thick arrow). Black bar indicates 2.5mm, x3000.
IV. DISCUSSION

It has been well established that over 95% of testosterone is synthesized in Leydig cells of testis and approximately 50-70% of the hormone is degraded in liver\textsuperscript{36}. Precursor of testosterone biosynthesis is supplied as plasma LDL-cholesterol to testicular mitochondria where all the biosynthetic pathways are localized. Cholesterol transported as plasma LDL complex enters mitochondria through mediation of a regulatory protein called StAR\textsuperscript{27} and the entered cholesterol activates cholesterol side chain cleavage enzyme (CSCC) to produce pregnenolone\textsuperscript{28}. Pregnenolone is in turn converted to progesterone by the action of 17α-hydroxylase, and the latter is eventually to testosterone through a few more enzymatic steps\textsuperscript{29}.

The above three enzymatic reactions mentioned are known to be rate-limiting steps in the biosynthesis of testosterone in testis\textsuperscript{29}. We, therefore, studied these enzymatic reactions in rats during DEN-nodularin treatment in order to elucidate the molecular mechanism behind the decrease of serum testosterone level; the observed reduction of serum testosterone might be either due to decreased
biosynthesis or increased degradation of testosterone. Indeed, we found that these enzymatic activities were immediately reduced in 24 hours after DEN and/or nodularin treatment, with a marked increase of LDL-cholesterol in serum. On the other hand, the liver where over 50-70% of testosterone is degraded did not indicate any increased capacity of testosterone catabolism, but rather diminished catabolism of this hormone after DEN injection (Fig. 9). The above results clearly indicated that marked reduction of serum testosterone might be due to decreased biosynthesis in testes rather than increased catabolism in liver during the DEN and/or nodularin treatment. In this study, we found that injection of DEN and/or nodularin to SD-male rats inhibited StAR expression in testis as well as 17α-hydroxylase and CSCC activities (Fig. 12, 13, 14). We also found in the present study that nodularin alone (S2NN) or sequential treatment of DEN and nodularin (D2NN) significantly damaged seminiferous tubules in testes, and induced vacuolation of glandular epithelium and dilatation of endoplasmic reticulum in prostate. However, it is also possible that the DEN-damaged liver released a higher-to-unidentified factor, which subsequently acted on testis. In our DEN-nodularin model, hepatocarcinogenesis was
frequently found to be associated with rapid induction of TGF-β1 mRNAs after DEN initiation and its protein expression only in the GST-P(+) hepatic nodules\textsuperscript{19}. They also manifested severe cirrhotic changes after nodularin injection\textsuperscript{19,40}. Hepatic expression of TGF-β1 in transgenic mice containing albumin enhancer sequences at 4 weeks of age induced multiple tissue lesions including atrophy of seminiferous tubules in testes among them\textsuperscript{41}. It is, therefore, highly tempting to suggest that expression of TGF-β1 was first induced by DEN or nodularin treatment, and subsequently induced TGF-β1 caused degenerative changes in testes, thereby resulting in the reduction of serum testosterone level. On the other hand, however, an alternative possibility that testis might be directly targeted by DEN or nodularin also exists. Related to this, sterility has been known to be one of the long-term effects of cisplatin\textsuperscript{42} and cisplatin-mediated reduction of serum testosterone is caused by reduced responsiveness to gonadotropin of the testicular Leydig cells and accompanying depression of cytochrome P450 dependent CSCC activity\textsuperscript{43}.

At this point, some discussion on the biological significance of the present finding is in order. It has been known that HCC in human occurs more frequently in
male than in female⁴⁴-⁴⁷, and the presence of physiologic level of testosterone has been shown to promote tumor development in animal liver⁴⁸-⁵⁰. It is, therefore, an enigma to observe such reduction of testosterone during hepatocarcinogenesis. However, such a mystery could also be found in the seemingly contradictory epidemiological observation that human HCC occurs more commonly in man than female, however, gynecomastia and hypogonadism are frequently associated with hepatic cirrhosis¹⁴.

Finally, it is quite possible that the reduction of testosterone at the very early stage of carcinogenesis might be due to toxic effect of DEN-nodularin. Nevertheless, orchiectomy itself induces high levels of proapoptotic signals such as degradation of Bcl-xL and induction of Bax in response to DEN-nodularin treatment⁵¹. Also, treatment of FaO rat HCC cells with DHT and TGF-β1 together significantly inhibited TGF-β1 induced apoptosis in cell culture system⁵⁷. Since GST-P positive hepatocytes lost TGF-β1 responsiveness and proliferated under the pressure of TGF-β1 during primary culture of hepatocytes⁵², and DEN-nodularin induced carcinogenesis in Fisher 344 male rats was due to the absence of TGF-β receptor
expressions, our present results indicate that the reduction of testosterone helped the altered hepatocytes, which did not express TGF-β receptors, to escape from apoptosis, whereas the surrounding hepatocytes with strongly expressed TGF-β receptors underwent apoptosis under the highly reduced testosterone level in serum. In conclusion, the marked reduction of testosterone biosynthesis in testes might be one of the various maneuvers during carcinogenesis, which helped the mutated hepatocytes to be selected for proliferation, while the surrounding hepatocytes undergoing TGF-β1 induced apoptotic death.
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국문 초록

남성에게서 여성화 유방, 고환의 위축, 성욕의 감소등과 같은 여성화증상은 만성 간 경화 환자나 간 경화가 동반된 간암 환자에게서 종종 발견되는 임상 증상이다. 이 실험의 목적은 백서에게서 간 발암 개시 물질인 Diethylnitrosamine(DEN)과 간 발암 촉진 물질인 Nodularin을 병행 또는 단독 투여하였을 때 혈액내의 남성호르몬의 변화를 측정하고 그 원인을 규명하는 것이다. 그 결과 간 발암물질을 투여하였을 때 혈액내의 남성호르몬은 감소되었다. 그 원인을 규명하기 위하여 남성호르몬의 대사 과정과 생성 과정을 측정한 결과 남성 호르몬 감소의 원인은 간세포 내에서 남성 호르몬의 대사가 증가에 의한 것이 아닌, 고환 내에서 남성 호르몬의 생 합성이 저하 된 것으로서, Leydig 세포 내에서 Cholesterol side chain cleavage(CSCC) enzyme과 17-α-hydroxylase의 활성도 저하, 그리고 Steroidogenic acute regulatory protein(StAR)의 발현저하에 의한 것임을 알 수 있었다. 또한 광학 현미경과 전자 현미경 촬영으로 고환 내에서 spermatogonia의 분리, 세포 내에 vacuolar의 생성 등이 관찰되었다. 결론적으로 혈청내의 남성 호르몬의 감소는 고환내의 생합성이 저하에 의한 것이며, 이는 3개의 rate-limiting단계인 Cholesterol side chain cleavage(CSCC), 17-α-hydroxylase의 Steroidogenic acute regulatory protein(StAR)의 저하에 의한 것이다.
ENZYMATIC METHYLATION OF RECOMBINANT TIS21 PROTEIN-ARGININE RESIDUES

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Summary: Recombinant TIS21 protein was overexpressed in Escherichia coli harboring the expression vector plasmid pQE-30 carrying the TIS21 cDNA coding sequence containing an extra 120 nucleotides upstream. Employing this protein consisting of 158 amino acid residues of the main chain plus 40 residues of the fusion peptide, it was found that one of the protein methylase I group [S-adenosylmethionine:nuclear protein/histone-arginine N-methyltransferase; EC 2.1.1.23; J. Biol. Chem., 269, 1075 (1994)] methylated this protein. The methylation products were identified as guanidino-N-methylated arginines. Some of the kinetics of the reaction are described.

Key Words: recombinant TIS21 protein, protein methylase I, enzymatic methylation, Nγ-methylarginines

Introduction

The TIS21 gene is one of the early response genes transiently induced by a potent tumor promoter tetradecanoylphorbol-13-acetate (TPA) in Swiss 3T3 fibroblast (1). A sequence of 158 amino acid residues has been deduced from the cloned cDNA and was found to have 59% sequence homology with the human BTG gene which was isolated from human B-cell chronic lymphocytic leukemia (2, 3). The biological significance of the TIS21 gene/protein has yet to be elucidated. However, increasing evidence indicates that several homologues of the TIS21 gene, such as PC3, BTG2 and TOB are involved in growth inhibition (4-7).

Abbreviations used are: MBP, myelin basic protein; hnRNP, heterogenous nuclear ribonucleoprotein particle; GST, glutathione S-transferase; PMSF, phenylmethylsulfonyl fluoride; MOPS, 3-N-(morpholino)propanesulfonic acid.
TIS21 expressions in thymus, lung, stomach and spleen of the BALB/c mouse are constitutive from the newborn until 6 months of age, whereas its expression in liver is transient only in the embryo and the newborn liver (8). However, expression was diminished significantly in thymic carcinoma tissues obtained from transgenic mice containing the SV40 T antigen and NCIH69 human lung cancer cell lines (9).

TIS21 protein with additional 40 amino acid linker sequence in the upstream was overexpressed in *Escherichia coli* harboring the expression vector plasmid pQE-30 carrying the Pst I fragment of TIS21 cDNA (10). The possibility was explored of posttranslational modification of the recombinant TIS21 protein, and it was found that one of the protein methylase I group of enzymes (11) methylated this protein *in vitro*.

**Materials and Methods**

**Materials**: S-Adenosyl-L-[methyl-14C] methionine (sp. act., 56 mCi/mmol) was purchased from Amersham International, England. Calf thymus histone type II-AS (arginine-rich), N-lauroyl sarcosine and γ-globulin were obtained from Sigma Chemical Co. (St. Louis, MO, USA) and isopropyl-β-D-thiogalactopyranoside (IPTG) from Promega (Madison, WI, USA). The rest of the chemicals were purchased from various commercial sources and were of the highest grade available.

**Preparation of recombinant TIS21 protein**: TIS21 protein was overexpressed in *E. coli* M15 [pREP4] harboring the expression vector plasmid pQE-30 (QIagen Inc. USA) carrying the Pst I fragment of TIS21 cDNA (10). One litre of bacterial culture was grown at 37°C until the cell density reached 0.7–0.9 at A600 nm and was then induced with 2mM IPTG for 3 hrs. Subsequently, the cells were harvested and sonicated for 2 min in homogenization buffer containing 20mM Tris-HCl buffer (pH 8.0) and 100mM NaCl. N-Lauroyl sarcosine was then added to the mixture at a final concentration of 0.3%. This process extracts the TIS21 protein. The supernatant fluid obtained by centrifuging the above mixture at 2,000 g for 15 min was passed through a Ni-NTA agarose column (1.0 l.d.) x 2 cm; Qiagen), the column was washed twice with 5 bed-volumes of washing buffer containing 20mM Tris-HCl (pH 8.0), 100mM NaCl, 10mM imidazole and 0.1% N-lauroyl sarcosine, and was eluted twice with elution buffer containing 20mM Tris-HCl (pH 8.0), 100mM NaCl, 150mM imidazole and 0.1% N-lauroyl sarcosine. The eluted sample was dialyzed against a solution containing 20mM Tris-HCl (pH 8.0) and 50mM NaCl. The yield of TIS21 protein preparation was approximately 0.5 mg from one litre of the culture fluid. In order to check the purity of the prepared TIS21 protein, SDS-PAGE (sodium dodecylsulfate-polyacrylamide gel electrophoresis) was carried out in 2% SDS according to the method (12).

**Purification of S-adenosylmethionine:histone-arginine N-methyltransferase (Protein methylase I)**: Rat liver protein methylase I was purified by the published method (13) with a slight modification. Ten g of fresh rat liver was processed up to the stage of DEAE-cellulose column chromatography by the method described. The eluate from the column was concentrated to an approximate 2 ml volume by Centriprep (Amicon) and the
concentrate was applied onto a Sephadex G-200 column [1.5 (i.d.) x 110 cm; Pharmacia] which has been equilibrated with buffer A [containing 10 mM MOPS (pH 7.4), 2 mM EDTA, 0.5 mM PMSF and 1 mM 2-mercaptoethanol] and the column was eluted with buffer A containing 2% glycerol. The flow rate was 2 ml/hr. Two ml of each fractions were collected and the fractions containing the protein methylase I were pooled. After concentration of the active fractions, the sample was reapplied onto the Sephadex G-200 column as above, and the active fractions were pooled and concentrated. The specific activity of the final preparation was 10 pmol methyl groups transferred/min/mg enzyme protein under the assay condition, using histone as the methyl acceptor. Protein concentration was estimated by the method of Bradford (14), using bovine serum albumin as a standard.

Assay of protein methylase I: Protein methylase I activity was assayed according to the published method (15). One hundred twenty five μl total incubation mixture contained 12.5 μl of 1 M K-phosphate buffer (pH 7.6), 30 μl of partially purified protein methylase I (15 μg), 10 μl of S-adenosyl-L-[methyl-14C]methionine (40 μM; 123 dpm/pmol) and 60 μg of recombinant TIS21 protein (this amount used for routine assay). For a control, boiled enzyme replaced the active enzyme. The mixture was incubated at 37°C for 1 hr, and the reaction was terminated by the addition of 3 ml 15% trichloroacetic acid (TCA) into the mixture followed by 40 μl of γ-globulin (4 mg). γ-Globulin was added as a carrier to facilitate the precipitation of protein. The reaction mixture was mixed thoroughly by vortex and was centrifuged with 12,000 g for 3 min. The precipitate was washed three more times with 3 ml each of 15% TCA, and the final precipitate was transferred to scintillation counting solution for measurement of radioactivity.

Amino acid analysis: The TIS21 protein labelled with [methyl-14C] by the method described above was hydrolyzed in 6N HCl at 110°C for 24 hrs in vacuo and the hydrolysate was dried using drying reagent (a solution of ethanol:triethylamine:water = 2:2:1) by lyophilization. After repeating the drying two more times, the sample was dissolved in 50 μl of derivatizing reagent (a solution of ethanol: triethylamine: water: phenylisothiocyanate = 7:1:1:1), and the sample was dried by lyophilization. The lyophilized derivatized sample was dissolved in 20 μl of solvent A [2.5% (v/v) acetonitrile in 70 mM Na-acetate buffer (pH 6.5)] and was analyzed for [methyl-14C]-labelled amino acid on HPLC by the following program:

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<tr>
<th>Time (min)</th>
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* Solvent B consisted of 15%(v/v) methanol in 45% (v/v) acetonitrile, and the column temperature was 43°C.
The HPLC system consisted of Waters solvent delivery system (Waters 501 pumps and Waters 680 gradient controller), a Shodex C18 5B column (4.6 x 250 mm, 5 mm particle size), Waters 480 absorbance detector and Gilson autoinjector system. The flow rate was 1.2 ml/min/fraction, absorbance was measured at 254 nm, and the total fraction was counted for radioactivity.

Results

Purity of recombinant TIS21 protein: As shown in Fig. 1, the recombinantly prepared TIS21 protein is electrophoretically pure, evidenced by a single band on SDS-PAGE. The molecular weight of the protein is approximately 22 kDa.

Relationship between the concentration of TIS21 protein and protein methylase I: Fig. 2 illustrates the effect of recombinant TIS21 protein concentration on the nuclear protein/histone-specific protein methylase I activity. There exists a linear relationship between the amount of TIS21 protein and the protein methylase I activity up to 40 μg of TIS21 protein (15 mM), providing an approximate Km value of 0.5 x 10^{-7} M which is close to that of hnRNP protein A1 [1.9 x 10^{-7} M (11)].

The protein methylase I activity in increasing concentrations with a fixed amount of TIS21 protein (60 μg) is illustrated in Fig. 3. There is a linear increase of protein methylase I activity up to 40 μg of the protein.

Effect of incubation time on protein methylase I activity: The relationship between the incubation period and activity of protein methylase I is shown in Fig. 4. There is a direct relationship between the incubation time and protein methylase I activity during the 120 min of incubation.

Product analysis: The [methyl-14C]TIS21 protein prepared by incubating protein methylase I and S-adenosyl-L-[methyl-14C]methionine was hydrolyzed in 6 N HCl, and the hydrolysate was analyzed on HPLC for the methylation products. Over 50% of the radioactivity was recovered as NG- [methyl-14C]arginine. As shown in Fig. 5, all three NG-methylarginines were found to be radiolabelled. The ratio of [methyl-14C] group incorporated into NG-monomethyl-, NG,NG-dimethyl- and NG,NG′-dimethylarginine were 12%, 77% and 11%, respectively. Thus, NG,NG′-dimethylarginine (asymmetric) was the most predominantly methylated product.

Discussion

A large number of naturally occurring proteins have been shown to undergo posttranslational methylation on some of the amino acid side chains in vivo (16). Among these, the presence of N-guanidino methyl-substituted arginine residues are limited to a few highly specialized proteins which include MBP, hnRNP proteins, scleroderma antigen, chromosomal high mobility group proteins, and muscle proteins (17).

Since the transmethylation reaction is energy-dependent in the use of S-adenosyl-L-methionine (16) and is catalyzed by a group of highly specific enzymes (18), it is quite logical to assume that the enzymatic methylation of protein-bound arginine residues play an important role in the regulation of the function and/or metabolism of the protein. Unfortunately, however, the
Fig. 1. Analysis of recombinant TIS21 protein on SDS-PAGE. The left lane represents standard protein markers and the right the TIS21 protein.

Fig. 2. Effect of TIS21 protein concentration on protein methylase I activity. Eighteen μg of protein methylase I was employed. Other experimental conditions are described in the Methods section.

The biological significance of protein-arginine methylation is not yet understood. Nevertheless, some of the better elucidated biological systems of protein-arginine methylation include a close parallel relationship between cell proliferation and the protein methylase I activity (19, 20). The possible involvement of MBP-arginine methylation in the integrity and maintenance of myelin (21), the induction of a global change of protein structure by protein-bound arginine methylation (22), and endowment of resistance to intracellular proteolytic enzymes (23) are also in point.

By employing a two-hybrid analysis with yeast and rat liver tissue culture systems, Herschman and his associates demonstrated that GST-TIS protein was conjugated with protein methylase I in vivo and that the protein stimulated the protein methylase I activity in vitro (24, 25). It is proposed that the enzymatic methylation of TIS21 protein-arginines might extend the life-span of the TIS21
Fig. 3. Relationship between the amount of protein methylase I and its enzyme activity. Sixty μg of recombinant TIS21 protein was used. Other experimental conditions are described in the Methods section.

Fig. 4. Effect of incubation period on protein methylase I. Sixty μg of TIS21 protein and 15 μg of partially purified protein methylase I were used. Other experimental conditions are described in the Methods section.
protein by protecting it from intracellular proteolysis (23). Consequently, an elevated level of protein methylase I activity may be maintained by a prolonged presence of the TIS21 protein.

The enzymatically methylated recombinant [methyl-14C]TIS21 protein by rat liver protein methylase I yielded three methylated products; namely, NG-monomethyl-, NG,NG-dimethyl- and NG,N'G-dimethylarginine (Fig. 4). It was previously noted that calf brain MBP-specific protein methylase I yielded NG,N'G-dimethyl- and NG-monomethylarginine with MBP as substrate, while nuclear protein/histone-specific protein methylase I yielded NG,NG-dimethyl- and NG-monomethylarginine, but not NG,N'G-dimethylarginine (11, 26). When synthetic oligopeptides were methylated by rat liver protein methylase I, NG-monomethylarginine was the major product with a trace amount of NG,NG-dimethylarginine (13). The biological significance of these product profiles in relation to the chemical structures of the enzyme and substrate is not yet known.

Acknowledgements

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References

Molecular mechanism of tumor promotion by decreased testosterone during N-diethylnitrosamine/nodularin induced hepatocarcinogenesis in Fisher 344 male rat

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SUMMARY

Change of serum testosterone (T) level and molecular effect on hepatocarcinogenesis were investigated in Fischer 344 male rats. Serum and testicular testosterone levels drastically decreased during the initiation with N-diethylnitrosamine (DEN) injection as well as promotion with nodularin treatment. Employing orchiectomized male rats, effect of T on the expression of Bcl2 family genes was investigated. Nodularin injections after the initiation significantly induced Bax expression, however, expressions of Bcl2 and Bcl-xL were reciprocally varied. Orchiectomy alone reduced Bcl-xL expression during the promotion period, and the DEN/nodularin treatment accelerated reduction of Bcl-xL, as opposed to no significant change of Bcl2. These results implicate that decrease of serum T can enhance apoptosis through the acceleration of BclxL reduction during the DEN/nodularin induced hepatocarcinogenesis in male rats.

INTRODUCTION

Human hepatocellular carcinoma (HCC) is much more prevalent in men than women, and this difference has been suggested to be due to the dif-
ference of sex hormones (Guy and Auslander, 1973; Farrell et al, 1975; Nagasure and Kohno, 1992). Orchiectomized male rats have also been shown to have significantly decreased frequency of chemically induced hepatic tumors (Vesselinovich and Mihailovich, 1967; Yu et al, 1996). Liver is one of the major target organs for male sex hormone, T. In the past few years, we have used DEN as an initiator and nodularin as a promoter in two stage hepatocarcinogenesis model in Fischer 344 male rats (Lim et al, 1999a,b). Nodularin is a microtoxin isolated from the toxic brackish water cyanobacterium Nodularia spumigena (Carmichael et al, 1988) and has been shown to induce tumor promotion through inhibition of protein phosphatase 1 and 2A (Honkanen et al, 1991, 1994).

We have earlier reported that dihydrotestosterone could impose a growth advantage on the initiated hepatocytes through protection of TGF-β1 induced apoptosis in FaO rat HCC cells and through induction of mitosis in HepG2 human hepatoblastoma cell culture systems (Lim et al, 1997). On the other hand, the GST-P(+)HNs which appeared during the DEN/nodularin induced carcinogenesis markedly induced TGF-β1 expression in rat and its induction kinetics was quite different from that in the simple hepatic regeneration after partial heptectomy; TGF-β1 mRNA expression was sustained during carcinogenesis, as opposed to transient in the regenerating cells (Lim et al, 1999a).

While investigating molecular mechanism of higher incidence of HCC in male rats, we observed drastic reduction of serum testosterone level and subsequent acceleration of Bcl-xL decrease during the DEN/nodularin induced carcinogenesis in Fischer 344 male rats.

**MATERIAL AND METHODS**

**Materials**

Nodularin was obtained from Dr. Carmichael and DEN from Sigma Chemical Co. The rest of the chemicals were the highest grade available.

**Treatment of animals with DEN and/or nodularin**

Fischer 344/NKist male rats (Genetic Resources Center, Korea) were

![Fig. 1. Initiation scheme of DEN/nodularin-induced hepatocarcinogenesis in Fisher 344 male rats. Saline was injected in place of DEN or nodularin to the age-matched control rats.](image-url)
housed in specific pathogen free condition. The environment was controlled at a constant temperature (22 ± 1 °C) and humidity (55 ± 5 %), ventilated 17 times/h and illuminated for 12 h/day. The animals were provided with an irradiated and microbial controlled diet (PicoLab Rodent Diet 20, 5053, PMI Feeds, Inc, USA), and water ad libitum. Sacrifice of rats was performed exactly 10:00 AM in order to avoid diurnal variation.

Fig. 2. Decreased T levels in serum (A) and testis (B) during the initiation of DEN/nodularin induced carcinogenesis.
Initiation and promotion of DEN/nodularin induced carcinogenesis

Fig. 1 is the initiation scheme of DEN/nodularin induced carcinogenesis. Seven wk-old male rats were i.p. injected with DEN (200 mg/kg B.W. in corn oil) and the animals were sacrificed 24 hrs later (D) and 2 wks after the initiation (2D). 2DNN indicates daily injections of nodularin (25μg/kg B.W. in phosphate-buffered saline) 2 wks after DEN initiation. Controls (C, 2SN, 2SNN) indicate the age-matched animals injected with saline.

Promotion scheme using DEN/nodularin injections

Animals were treated with nodularin for 10 wks after DEN initiation and then maintained for 10 more wks after the cessation of nodularin injection (fig.3A). The animals were sacrificed from the 4th to 22nd wks at

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**Fig. 3.** Promotion scheme of carcinogenesis in intact or orchiectomized male rats (A). Animals were sacrificed at the indicated times after DEN or saline initiation. Orchiectomy was performed 2 wks before initiation. Decreased serum T level during the promotion period (B). D2Nn indicates all samples of the DEN/nodularin treated rats which were sacrificed at the indicated times.
the indicated times and livers were resected as well as serum preparation. In order to investigate T effect during carcinogenesis, 5wk-old male rats were orchiectomized before puberty, and then initiated at 7wk-old. The other treatments were the same as that described in fig. 3A. Livers were resected at the indicated times and used for investigating changes of Bcl2 family proteins.

**Measurement of serum testosterone level**

Levels of serum testosterone were measured by using an \(^{125}\text{I}\)-testosterone assay kit purchased from New England Nuclear (Boston, MA) according to the manufacturer's instruction. Testicular homogenates in 10 mM Tris-HCl (pH 7.4) were extracted with 8 vol of diethyl ether and the extracts were dried under nitrogen gas in hood. Ether extracted testosterone was resuspended to testosterone zero serum.

**Western blot analysis**

Livers were homogenized in RIPA buffer (50mM TRIS-HCl, pH7.4, 1% NP40, 150mM NaCl, 0.5% deoxycholate, 0.1% SDS, 1mM PMSF, 0.5mM NaN03, 1mg/ml leupeptin), and separated on 12% SDS-PAGE. Electrotransferred nitrocellulose membranes were hybridized with anti-Bax (SC-526), -Bcl2 (SC-492) and -Bcl-xL (Transduction Lab. B22630) antibodies with anti-rabbit Ig and horse radish peroxidase (#NA9340, Amersham) as their secondary antibody.

**RESULT AND DISCUSSION**

**Reduction of testosterone level in serum and testis during the DEN/nodularin induced carcinogenesis in rat**

Serum T level decreased drastically in a day after the DEN initiation (D) and the level recovered somewhat after 2 wks (2D). However, subsequent nodularin injections (2DNN) also significantly reduced serum T level (fig.2A). Fig.2B shows T level in testis during the initiation period. The testosterone level in testis was drastically reduced by a single injection with DEN and somewhat less but significantly reduced by nodularin in the 2DNN. The level of serum T was also significantly reduced during the promotion of carcinogenesis (fig.3B). These results indicate that both DEN and nodularin can reduce serum T level during in vivo carcinogenesis. However, nodularin had somewhat less than DEN (fig. 2,3).

It has been well established that over 95% of T is synthesized in Leydig cells of testis and approximately 90% of the T is degraded in liver (Francis and John, 1994). Precursor for T biosynthesis is supplied as plasma LDL-cholesterol to testicular mitochondria where all the biosynthetic pathways are localized. The above observed reduction of T in either serum or testis during DEN/nodularin-induced carcinogenesis might be due to either increased degradation in liver or decreased biosynthesis in testes.

**Accelerated reduction of Bcl-xL during the DEN/nodularin-induced hepatocarcinogenesis in orchiectomized male rats**

In order to investigate the effect of T on carcinogenesis, we employed
intact and orchiectomized male rats. Nodularin injections after DEN initiation significantly induced \textit{Bax} expression in both animals (fig. 4). Interestingly, orchiectomy alone gradually decreased \textit{Bcl-xL} expression (fig. 5A). However, DEN/nodularin treatment accelerated the reduction of \textit{Bcl-xL} expression (fig. 5B), as opposed to no significant change of \textit{Bcl2} (fig. 5C) by orchiectomy. These results indicate that decrease of \textit{T} during carcinogenesis can enhance apoptotic reaction through accelerating \textit{BclxL} inactivation in the DEN/nodularin treated male rats.

Fig. 4. Induction of \textit{Bax} expression during the DEN/nodularin induced carcinogenesis. Repeated injections of nodularin increased \textit{Bax} expression in the intact (A) and orchiectomized (B) male rats. C and O2, and the DNN and O2DNN indicate the intact and the orchiectomy controls, and nodularin injections (twice/wk) 24 hrs after the DEN initiation to intact and orchiectomized male rats, respectively.

From the above results, we can conclude that the reduction of \textit{T} during the early stage of DEN/nodularin-induced carcinogenesis selectively impose apoptotic pressure to unaltered hepatocytes through decreasing protective effect of dihydrotestosterone against the TGF-\textit{\beta}1-induced apoptosis, thus making them to be apoptosis-proned. Considering the fact that altered hepatocytes express TGF-\textit{\beta}1 but not its receptors, thus to be apoptosis-resistant, decrease of serum \textit{T} can selectively inhibit \textit{T}-induced rescue of unaltered hepatocytes from the TGF-\textit{\beta}1-induced apoptosis. Therefore, drastic reduction of serum \textit{T} within 24hrs after the DEN initiation can provide an environment of selective growth for the
Fig. 5. Accelerated decrease of Bcl-xL expression in the orchiectomized rats. Orchiectomy transiently increased Bcl-xL expression, then gradually decreased until the 22 wk (A). However, DEN/nodularin markedly decreased Bcl-xL expression in the orchiectomized rats (B), as opposed to no change in Bcl2 (C).

altered hepatocytes, whereas it can enhance apoptotic death of unaltered cells during the initiation as well as the promotion of carcinogenesis in male rats.

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Differential expression of $O^6$-methylguanine-DNA methyltransferase during diethylnitrosamine-induced carcinogenesis and liver regeneration in Sprague-Dawley male rats

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Abstract Differential expression of DNA-$O^6$MeG: protein-t-cysteine S-methyltransferase (MGMT) activity and posttranslational modification of the protein during liver regeneration and carcinogenesis were compared in Sprague-Dawley male rats after partial hepatectomy and/or single i.p injection of diethylnitrosamine (DEN, 200 mg/kg). Regenerating hepatocytes after partial hepatectomy induced MGMT transiently within 3 days; however, the induction of MGMT was persistent for 2 weeks after DEN injection, and the combined treatment of DEN and partial hepatectomy maintained the elevated MGMT level for up to 4 weeks. The increased activity was transcriptionally regulated, when analyzed by Northern blot hybridization. The major active form of MGMT protein in the partially hepatectomized or DEN-treated rats was a 26-kDa or 24-kDa species respectively, which was confirmed by Western blot analysis and gel slice assay. The biological significance of the differential induction of MGMT during partial hepatectomy or DEN-induced carcinogenesis is not obvious; however, further studies on possible posttranslational modifications of MGMT protein might shed some light on the functional aspect of MGMT induction.

Key words MGMT modification · DEN carcinogenesis · Liver regeneration

Abbreviations PH partial hepatectomy · DEN diethylnitrosamine · MGMT DNA-$O^6$MeG:protein-t-cysteine S-methyltransferase (EC 2.1.1.63) · rMGMT recombinant rat MGMT protein · $O^6$MeG $O^6$-methylguanine

Introduction

$O^6$-Methylguanine-DNA methyltransferase [DNA-$O^6$MeG:protein-t-cysteine S-methyltransferase (MGMT) EC 2.1.1.63] has high sequence homologies among species ranging from Escherichia coli to human (Morgan et al. 1993) and the methyl-accepting cysteine residue is highly conserved in all species. There has been considerable convincing evidence to indicate that an increased level of MGMT imparts resistance to DNA-damaging agents such as methylating agents (temozolomide and dacarbazine) and chloroethyating agents [1,3-bis(2-chloroethyl)-1-nitrosourea and 1-(4-amino-2-methyl-5-pyrimidinyl)methyl-3-(2-chloroethyl)-3-nitrosourea] in the established cells as well as in tumor cells (Dumenco et al. 1989; Lim et al. 1990a; Gerson et al. 1994; Pegg et al. 1995; Dolan 1997; Pieper 1997). The protective effect of MGMT on dimethylnitrosamine- or diethylnitrosamine(DEN)-induced hepatocarcinogenesis in transgenic mice containing the E. coli ada gene (Nakatsuru et al. 1993), or on tumorigenesis induced by methylnitrosourea in MGMT-knockout mice (Sakumi et al. 1997), has also been of great interest. The enzyme becomes inactivated on alklylation (Foote et al. 1980; Bogden et al. 1981; Waldstein et al. 1982; Harris et al. 1983), and there is a stoichiometric relationship between the amount of alkylated protein and the number of $O^6$-alkylguanine residues repaired (Pegg 1984). Thus, the reaction can be regarded as a titration reaction.

It is, therefore, important for an organism to have an adequate amount of active MGMT to protect it from mutagenesis or carcinogenesis caused by various alkalyating agents. However, the level of MGMT in tumors has been variously reported, as they may be $\text{Mer}^+$ or $\text{Mer}^-$ . The possible mechanism of the change from $\text{Mer}^-$ cells may not only be the selection of a $\text{Mer}^-$ cell from the initial population, but could also be associated with hypermethylation in the promoter region and hypo-methylation of downstream sequences of a gene (Harris et al. 1996). Silber et al. (1998) reported an important
relationship between MGMT and carcinogenesis in the adult glioma, showing that the loss of Mer- cells in the progenitor tissue led to a predominance of Mer+ cells in the tumor through an elevation of MGMT activity during the carcinogenesis.

MGMT cDNA from mouse (Shiraishi et al. 1992; Shiota et al. 1992), human (Hayakawa et al. 1990; Rydberg et al. 1990; Tano et al. 1990) and rat (Rha- den-Staron and Laval 1991; Sakumi et al. 1991) tissues were cloned and the apparent molecular masses of the products were reported to be 24, 22 and 26 kDa respectively. However, transgenic mice containing the bacterial ada gene (Olsson and Lindahl 1980) with the eukaryotic promoter of phosphoenolpyruvate carboxykinase (GTP) (EC 4.1.1.32) revealed multiple bands of the eukaryotic MGMT protein ranging between 20 kDa and 30 kDa (Fig. 5 in Lim et al. 1990b). These findings suggested a possible presence of MGMT isoforms in murine liver or posttranslational modifications of the protein.

In the light of the above, a detailed characterization of the differential induction of MGMT protein during liver regeneration and DEN-induced carcinogenesis in Sprague-Dawley male rats was planned. It was found that MGMT induction in the regenerating liver was transient, whereas that of carcinogenic livers was persistent. Liver regeneration primarily induced a 26-kDa species, whereas DEN treatment strongly induced MGMT proteins of both 26 kDa and 24 kDa, predominantly the latter. This finding very much suggests posttranslational modifications of MGMT protein during DEN-induced carcinogenesis, thus producing multiple bands between 20 kDa and 30 kDa.

Materials and methods

Materials

N-[methyl-3H]-N-Nitrosourea (20 Ci/mmol) and 1µ[n-32P]dCTP (3000 Ci/mmol) were obtained from Amerham Co. (USA). Calf thymus histone II-AS (a mixture of various subfractions), pronase E (from Streptomyces griseus), calf thymus and salmon sperm DNA, EDTA, N-diethylthiourea (DEN, N-0756), dithiothreitol and thrombin were obtained from Sigma Chemical Co. Sprague-Dawley male rats were obtained from the Korea Research Institute of Bioscience and Biotechnology. The random oligolabelling kit, glutathione-Sepharose-4B, and pGEX4T-1 were from Pharmacia Biotech Co., the prpotein assay kit was from Bio-Rad Co., pGEM-T vector from Promega, and enhanced chemiluminescence (ECL) reagent from Amerham, England. The rest of the chemicals were of the highest purity available.

Preparation of carcinogenic and regenerating liver tissues

Seven-week-old Sprague-Dawley male rats were injected intraperitoneally with 100 mg/kg body weight and then maintained for 28 days (Fig. 1). Rats were periodically sacrificed after 1, 2, 3, 5, 7, and 14 days, and the livers were excised for assay (DEN/no partial hepatocytoma, PH). Half of the DEN-injected rats were sacrificed 1, 2, 3, 4, 5, 7, 10 and 14 days after partial hepatocytoma (DEN/PH). Regenerating hepatocytes were obtained periodically from the PH rats (no DEN/PH), and the whole homogenate and supernatant fractions were prepared by centrifugation at 22000 g for 30 min. Age-matched control livers injected with phosphate-buffered saline (PBS) were collected during the experimental periods (no DEN/no PH). Partial hepatocytoma (70%) was performed by the method described previously (Lim et al. 1994a) at about 11:00 a.m. Experiments used four rats at each assay time.

Pronase-based MGMT assay

The MGMT assay was performed by the method previously used in our laboratory (Lim et al. 1996). A 10% tissue homogenate was prepared in 70 mM HEPES, pH 7.8, 1 mM dithiothreitol, 1 mM EDTA and 5% glycerol as a source of MGMT protein, and then calf thymus [methyl-3H]DNA (0.5 mg/ml/50 µl) was added as a substrate. After transfer of the methyl-3H group from O6-MeG-DNA to the cytosine residue in the MGMT protein for 1 h, the methyl-3H-labeled MGMT protein was digested with pronase for 2 h. With the aid of histone as a carrier, unreacted DNA substrate was precipitated with 15% trichloroacetic acid. The radioactivity of 3H-MeG-Hcyxine in the trichloroacetic-acid-soluble supernatant was measured by liquid scintillation counting (Wallac 1210). Protein concentration was determined by a Bio-Rad kit using bovine serum albumin as a standard.

Northern blot analysis

Total cellular RNA was isolated by an established method (Lim et al. 1995) from the livers obtained 2 days after partial hepatocytoma (2PH) and 3 days after DEN injection (3DEN), and 20-µg samples of the RNA were separated on a 1.2% formaldehyde gel for 3 h. The level of mRNA expression was analyzed with the X001 and HindIII fragment of a recombinant MGMT (mYMGMT) cDNA plasmid (Rha- den-Staron and Laval 1991), which was kindly donated by Dr. Laval (France). Autoradiography was performed with a radioactive DNA probe prepared with 3H-dCTP.

Assay by gel slice

In order to analyze the MGMT protein that accepted the methyl group from O6-MeG-DNA, liver homogenates (500 µg) were incubated with calf thymus [methyl-3H]DNA for 1 h at 37°C, and were separated by 12% sodium dodecyl sulfate/polyacrylamide gel

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Maintenance of animals

Sprague-Dawley male rats were maintained in specific-pathogen-free conditions with an irradiated and microbially controlled diet (PicoLab rodent diet 20, 5053; PMI Feeds Inc., USA) and autoclaved water ad libitum. The environmental conditions of the animal housing were controlled at a constant temperature (22 ± 1°C) and humidity (55 ± 5%). The room was ventilated 17 times/h and illuminated for 12 h/day.

Fig. 1. Schedule for the preparation of carcinogenic and regenerating liver tissues. Diethylnitrosamine (DEN, 200 mg/kg i.p.) was injected into 7-week-old rats, which were periodically sacrificed after 1, 2, 3, 5, 7 and 14 days (DEN/no PH). Partial hepatocytoma (PH) was performed on the 14th day after DEN injection and rats were sacrificed 1, 2, 3, 4, 5, 7, 10 and 14 days later (DEN/PH). Regenerating hepatocytes were obtained 1, 2, 3, 4, 5 and 10 days after PH from the rats with no DEN injection (no DEN/PH). Age-matched control livers were collected during the experimental periods (no DEN/no PH).
electrophoresis (SDS-PAGE). The part of the gel containing the 20- to 30-kDa proteins was cut into seven slices of 2.0 mm thickness, and each slice was solubilized by incubation at 65°C for 16 h with 1 ml 30% H₂O₂ after hydration with 100 μl water. Radioactivity from each slice was counted in 5 ml cocktail solution by liquid scintillation counting (Wallac 1210).

Subcloning of pGST-rMGMT

The MGMT expression plasmid was cloned by the polymerase chain reaction (PCR) with primers spanning the translation start and termination sequences: 5'-GGGGAATTCAGCTGATCTGACAAAGC-3' and 5'-CCCCGATCCGGCAGCGGGCTTTGCTG-3', with an EcoRI site and rMGMT cDNA as a template. The amplified DNA were cloned into a pGEM-T vector (Promega) and transformed into DH5α cells (pMGMT, R1). The EcoRI fragment of 627 bp of pMGMT, R1 was subcloned into a pGEX4T-1 vector downstream of the glutathione S-transferase (GST) promoter and thrombin digestion site, and transformed in BL21 (DE3) cells (pGST-rMGMT, Fig. 2).

Purification of rMGMT protein and production of anti-rMGMT IgG

Recombinant GST-rMGMT protein was prepared by the overexpression of pGST-rMGMT in the BL21 (DE3) cells with 0.2 mM isopropyl β-D-thiogalactopyranoside treatment. rMGMT was purified by glutathione-Sepharose-4B affinity (Pharmacia Biotech) column chromatography after removal of the GST fragment by thrombin digestion. The purified rMGMT protein contained five additional amino acids in the upstream region of the rat MGMT protein. The 26-kDa protein banded from the gel was injected into the foot pads and back of New Zealand white rabbits (500 μg/animal). The rabbits were bled after the second booster injection, and the serum containing the highest antibody titer against rMGMT was isolated. IgG bound to protein-A-Sepharose (CL-4B, Pharmacia Biotech) was purified as the anti-rMGMT antibody.

Western blot hybridization

Tissue MGMT proteins were detected by Western blot analysis using anti-rMGMT IgG. Samples of liver homogenates (250 μg) were solubilized in 2 × SDS sample buffer (62.5 mM TRIS HCl, pH 6.8, 2% SDS, 5% 2-mercaptoethanol, 10% glycerol and 0.002% bromophenol blue), and were boiled for 5 min. A part of the tissue lysate (80 μl) was fractionated on 12% polyacrylamide gel. The protein bands were transferred to nitrocellulose membrane (Schleicher & Schüll, Germany), and the blot was hybridized with anti-rMGMT IgG antibody. A 5-ng sample of rMGMT protein was used as a control. ECL reagent (Amersham, England) was applied to reveal the protein bands.

Results

Differential induction of MGMT activity in the regenerating and carcinogenic livers

As shown in Fig. 3, the MGMT level in the liver was highly elevated during regeneration, reaching about 3.5-fold in 2–3 days; however, the level returned to normal after 4 days (no DEN/PH). A single injection of DEN (DEN/no PH) elevated the MGMT level 4-fold within 3 days, and the increased level was maintained until 14 days. However, combined treatment (DEN/PH) with DEN initiation and partial hepatectomy on the 14th day after the initiation significantly sustained the elevated MGMT level during the entire experimental period, an effect much greater than those following the no DEN/PH and the no DEN/no PH treatments.

Transcriptional regulation of MGMT during induction

The higher induction of MGMT protein after DEN treatment and/or partial hepatectomy was transcriptionally regulated. When total cellular RNA was isolated from the regenerating liver 2 days after partial hepatectomy (2PH) and 3 days after a single injection of DEN (3DEN) as well as from the control liver (C), ratios of MGMT mRNA to those of internal standard (CHOb, Lim et al. 1994b) increased about three and five to eight times in the regenerating and carcinogenic tissues respectively, as compared with the control (Fig. 4). Rat CHOb was isolated from the Rat 1 fibroblast library as a constitutively expressed gene (Lim et al. 1994b).
Identification of MGMT proteins and enzymatically active forms during liver regeneration and DEN-induced carcinogenesis

In order to reveal the MGMT proteins found in the partially hepatectomized and DEN-treated livers, Western blot analysis was employed, using a rabbit anti-rMGMT IgG antibodies produced in our laboratory. The MGMT proteins from the control and DEN-treated livers showed two bands of 24 kDa and 26 kDa, and the level was greatly increased in the DEN-treated rat livers (Fig. 5). A further discussion of the identity of the upper band (26 kDa) in the 3DEN sample will be given below. In contrast to 3DEN, the MGMT protein induced in the 2PH rats was predominately a single band of 26 kDa.

By employing the gel slice assay, the possibility that different molecular species of MGMT might be involved in the [3H]methyltransfer reaction during liver regeneration and carcinogenesis was investigated. Freshly obtained liver homogenates were therefore incubated with [methyl-3H]DNA in vitro, proteins were resolved by SDS-PAGE and radioactivity in the sliced gels was measured. It was of a great interest to observe that the MGMT proteins induced in the partially hepatectomized and DEN-treated rats were different, being 26 kDa and 24 kDa respectively (Fig. 6). This experiment was highly reproducible (repeated four times and a representative profile is presented). Although found in the Western blot assay shown in Fig. 5, the 26-kDa MGMT from the 3DEN sample was not detected by the gel slice

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**Fig. 3** Transient/persistent induction of MGMT activities during liver regeneration/DEN-induced carcinogenesis. A single injection of DEN (DEN/no PH) induced DNA repair on the 3rd day that was four to five times that of the group injected with phosphate-buffered saline (no DEN/no PH), and the induced level was maintained until day 14. PH after DEN injection (DEN/PH) maintained the significantly elevated MGMT level for 14 more days, as compared to those rats treated with PH only (no DEN/PH), DEN/no PH and no DEN/no PH. Values indicate the mean ± SD, expressed as pmol methyl group transferred/mg liver homogenate. * The statistical significance, tested by Student's t-test

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**Fig. 4** Induction of MGMT mRNA in the livers of PH and DEN-treated Sprague-Dawley male rats. 2PH, 3DEN livers were obtained 2 days and 3 days after PH and DEN injection respectively. Multiple lanes show individual animal variations. Numbers at the bottom indicate the ratio of induced MGMT mRNA to (CHOb) RNA

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**Fig. 5** Detection of liver MGMT proteins by Western blot analysis. In order to reveal changes in the protein bands, whole homogenates (250 μg) obtained from the livers of the 2PH and 3DEN rats as well as the control were resolved by 12% sodium dodecyl sulfate/polyacrylamide gel electrophoresis with rMGMT (5 ng) as a marker. Migrating proteins were transferred to nitrocellulose membrane, hybridized with anti-rMGMT IgG, and revealed with an enhanced chemiluminescence kit. rMGMT was slightly larger than the tissue MGMT because of an additional five amino acids at the N-terminal end of the MGMT protein. In order to show inter-variation of MGMT proteins induced by 2PH or 3DEN treatment, two lanes are presented for each treatment
Fig. 6. Profile of the gel slice assay. In order to study the active bands of MGMT proteins found in the regenerating and carcinogenic livers, tissue homogenates (500 μg) obtained 2 days after PH (2PH) and 3 days after DEN injection (3DEN), as well as the control, were incubated with [methyl-3H]DNA for 1 h at 37°C. The mixtures were resolved by 12% SDS-PAGE. The part of the gel representing 20–30 kDa was cut into seven 2-mm-wide slices, and each slice was solubilized by incubation at 65°C for 16 h with 1 ml 30% H2O2 after hydration with 100 μl water. Radioactivity from each slice was counted in 5 ml cocktail solution by liquid scintillation counting. Methyl-transfering activity of each fraction was expressed as pmol Me group transferred/mg tissue homogenate.

assay (Fig. 6). A more detailed discussion will be presented later in this paper.

Discussion

In rat livers and rat hepatoma cells, MGMT can be induced by a variety of DNA damage: alkylating agents (Frosina and Laval 1987; Lefebvre et al. 1993), cis-dichlorodiamine-platinum(II), UV and γ irradiation (Habranken and Laval 1991; Lefebvre and Laval 1986), the X-ray mimetic agent bleomycin, and heat (Schmerold and Spath 1986; Lefebvre and Laval 1986; Laval 1990). In addition to the alkylating agents, a variety of hepatotoxins that are not metabolized to alkylating agents, such as aflatoxin B1, 2-acetylaminofluorene, thioacetamide, and carbon tetrachloride (Chu et al. 1981; Cooper et al. 1982; Pegg and Perry 1981), treatment of hypophysectomized or thyroidectomized rats with growth hormone or thyroxine, and partial hepatectomy also restore the level of MGMT in rat liver (Pegg and West 1983).

Of the at least 12 DNA adducts produced by alkylating agents (Pegg 1977; Margison and O’Connor 1981) when the animals were partially hepatopotentized and treated with nitrosamine (Montesano 1981; Pegg 1983), the O\(^\circ\) position in guanine has been suggested to be critical for mutagenic and carcinogenic induction of liver tumors. The repair of O\(^\circ\)EtG by rat liver MGMT is 3.4 times slower than that of O\(^\circ\)MeG, therefore DEN was found to be slightly more potent as a carcinogen to rat liver than dimethylnitrosamine (Pegg 1984). Depletion of MGMT activity after a single exposure to dimethylnitrosamine (20 mg/kg i.p.) and its rapid recovery to a threefold elevated level after 4 days had already been reported by Hall et al. (1990); however, we found that the maximum induction was reached at 3 days following a single DEN injection (200 mg/kg) and the increased methyl-\(^{3}\)H-transferring activity was sustained for 14 days; induction of MGMT by partial hepatectomy returned to the basal level within 4 days. Interestingly, partial hepatectomy after DEN initiation significantly maintained the activity until day 28 (Fig. 3). The induction was accompanied by increased mRNA expression (Fig. 4). Even though not shown, we previously tested whether the maximum induction of MGMT was preceded by maximum incorporation of thymidine during liver regeneration. These findings indicate that the sustained MGMT activity might be a consequence of regenerative growth following cell death and/or normal surveillance against massive DNA insult by the alkylating agent.

By employing Western blot analysis (Fig. 5) and a gel slice assay (Fig. 6), we made the unexpected finding that MGMT protein was synthesized primarily as a 26-kDa species after partial hepatectomy, whereas the protein induced in DEN-injected rats was a mixture of 26 kDa and 24 kDa. However, [methyl-\(^{3}\)H]transferring activity in vitro was mainly carried out by the 24 kDa MGMT species during the carcinogenesis (Fig. 6). We were initially at a loss to explain why the 26-kDa MGMT from the 3DEN sample, detected by Western blotting (Fig. 5), was not detected by gel slice assay (Fig. 6). However, the most plausible explanation might lie in the fact that Fig. 5 represents SDS-PAGE followed by Western blot analysis of the samples obtained from rats who had been treated with DEN for 3 days. Thus, the 26-kDa MGMT might have been alkylated in vivo by DEN, having lost enzymic activity while remaining reactive with anti-rMGMT. On the other hand, the \(^{3}\)Hmethyltransferase reaction of the 3DEN sample shown in Fig. 6 was carried out in vitro, so that only the 24-kDa MGMT activity, but not the in vivo alkylating activity of the 26-kDa species was detectable. The above explanation could be further supported by the observation that C-terminal region of MGMT played an important role in MGMT’s substrate specificity (Morgan et al. 1993): the substrate specificity changed during the conversion from the 26-kDa to the 24 kDa species, suggesting more vigorous removal of the ethyl group from the DEN-treated DNA by the 26-kDa MGMT in vivo.

Since we found a single band of mRNA by Northern blot analysis (Fig. 4), in agreement with others who found a single MGMT cDNA in the rat (Rhaden-Sturon and Laval 1991; Sakumi et al. 1991), we can strongly suggest that the induced 26-kDa MGMT protein is preferentially processed to 24 kDa during the carcinogenesis, and that the processed MGMT protein still retains methyltransfer activity. Since MGMT is inacti-
vated on alkylation, the sustained MGMT activity during the carcinogenesis might be due to a modification of MGMT protein and/or a continued replenishment of the proteins. Of course, the individual time course of MGMT induction by partial hepatectomy or DEN has been well studied by others. However, to the best of our knowledge, MGMT induction patterns under these two conditions have never been compared. Furthermore, no in-depth examination of these two isoforms of MGMT in terms of temporal relationship has ever been attempted. There is, therefore, a great need to investigate the possible mode of posttranslational changes, such as side-chain modification or proteolytic conversion, of the MGMT protein.

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Phosphorylation of methylated-DNA–protein-cysteine S-methyltransferase at serine-204 significantly increases its resistance to proteolytic digestion

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In a previous paper [Lim, Park, Jee, Lee and Paik (1999) J. Cancer Res. Clin. Oncol. 125, 493–499], we showed two major forms of active DNA-6-O-methylguanine:protein-L-cysteine S-methyltransferase (MGMT; EC 2.1.1.63) in the liver with N-nitrosodiethyamine (DEN)-induced carcinogenesis: these were 26 and 24 kDa species. Here we show that a 2 kDa C-terminal fragment was cleaved from the 26 kDa species in vitro by thrombin or microsomal fractions isolated from DEN-treated rat livers. When Ser²⁰⁴ of the 26 kDa protein was replaced with Ala by site-directed mutagenesis, phosphorylation of the protein was completely abolished, indicating Ser²⁰⁴ to be the site of phosphorylation. We also show that the phosphorylation was performed by Ca²⁺-independent protein kinase isoenzymes, and that the phosphorylated rat MGMT protein was resistant to digestion by protease(s) whose activity was increased during DEN-induced hepatocarcinogenesis and also by digestion with endopeptidase Glu-C (V8 protease).

Key words: endopeptidase Glu-C, DNA repair, hepatocarcinogenesis, rat.

INTRODUCTION

Methylated-DNA–protein-cysteine S-methyltransferase (MGMT; DNA-6-O-methylguanine:protein-L-cysteine S-methyltransferase, EC 2.1.1.63) is a ubiquitous protein that repairs O³-alkylguanine via a unique mechanism in that it accepts the alkyl group on a Cys residue in a single-step stoichiometric reaction, thereby itself being inactivated [1,2]. However, it has been reported that the O³-methylguanine repair capacity of H4 rat hepatoma cells can be increased after a single treatment with alkylating agents by a process different from the adaptive response found in Escherichia coli [3]. Indeed, cells capable of repairing O³-alkylguanine are much less susceptible to the cytotoxic effects of N-nitrosourea or N-methyl-N-nitrosoguanidine than MGMT-deficient cells [4,5]. Whereas MGMT is expressed in all normal human cell lines and tissues, approximately 20–30% of human tumours are completely deficient in MGMT expression [6]. The protective effect of MGMT towards carcinogen-induced hepatocarcinogenesis was clearly demonstrated in transgenic mice containing E. coli ada attached to the metallothionein I gene promoter [7] or in tumorigenesis induced by methyl nitrosourea in MGMT knock-out mice [5].

MGMT cDNA species were cloned from mouse [8,9], human [10–12] and rat [13,14] with only a single kind of cDNA; however, the apparent molecular masses of the products were 24, 22 and 26 kDa respectively. When the amino acid sequences of the bacterial Ada, human and rat MGMT proteins are compared, the C-terminal tail of approx. 2 kDa is absent from Ada (Ada C) (Figure 1), and human and rat MGMT proteins do not share any significant sequence similarities at the C-terminal end. In addition, only rat MGMT has a potential Ca²⁺-dependent protein kinase (PKC) phosphorylation site of Ser/Thr-Xaa-Arg/Lys [15,16] at the C-terminal region. These findings strongly suggest a possibility of post-translational processing of the protein such as side-chain modification or proteolytic conversion of the rat MGMT proteins.

During studies on the role of MGMT in N-nitrosodiethyamine (DEN)-induced hepatocarcinogenesis in rat, we observed two major forms of active MGMT proteins, demonstrated by Western-blot analysis and gel slice assay: these had molecular masses of 26 and 24 kDa [17]. To investigate the possible modification of MGMT proteins during hepatocarcinogenesis, the modification and proteolytic conversion of MGMT were examined in vivo and in vitro, with rat recombinant MGMT protein (rMGMT) and its polyclonal and C-terminal peptide antibodies. Here we report for the first time that rMGMT was cleaved off by proteases in vitro, generating a 24 kDa species from the 26 kDa protein, and that the Ser²⁰⁴ residue in the 2 kDa fragment located in the C-terminal region was phosphorylated. Phosphorylation of Ser²⁰⁴ was mediated by PKC isoenzymes. Phosphorylated MGMT protein (MGMT-P) was more resistant to liver microsomal proteases and endopeptidase Glu-C than the unmodified MGMT, whereas its sensitivity to trypsin or chymotrypsin was unaffected. These findings indicate strongly that conformational changes might be induced not only by DNA binding or the alkylation of MGMT protein but also by the phosphorylation of Ser²⁰⁴. However, in contrast to the rapid digestion of MGMT protein by DNA binding or alkylation changes, the phosphorylation of MGMT endows the protein with resistance to proteases such as endopeptidase Glu-C (V8 protease) induced in liver during DEN-induced carcinogenesis.

EXPERIMENTAL

Materials

N-[methyl-³²P]-N-Nitrosourea (20 Ci/mmol), [³²P]ATP (3000 Ci/ mmol) and enhanced chemiluminescence (ECL™) reagent were obtained from Amersham Life Science Products (Little Chalfont, Bucks., U.K.). N-Diethylthiourea (catalogue no. N-0756), diithiothreitol, thrombin (bovine plasma (T-7513)), phos-

Abbreviations used: DEN, N-nitrosodiethyamine; ECL, enhanced chemiluminescence; LV-TLE, low-voltage thin-layer electrophoresis; MGMT, DNA-6-O-methylguanine:protein-L-cysteine S-methyltransferase (EC 2.1.1.63); PKC, protein kinase C; rMGMT, rat recombinant MGMT protein; rMGMT-P, phosphorylated rMGMT.

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Phoamino acids [phosphoserine (P-0753), phosphothreonine (P-1003) and phosphotyrosine (P-5024)], tosylphenylalanylchloromethane ("TPCK")-treated trypsin (T-8642), tosyllysylchloromethane ("TLCK")-treated chymotrypsin (C-3142) and endopeptidase Glu-C (P-6181) were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Glutathione Sepharose 4B was purchased from Pharmacia Biotech (Uppsala, Sweden). Indolocarbazole G6595 (no. 365250), bisindolylmaleimide G6585 and PKC-M (no. 539513) were purchased from Calbiochem-Novabiochem Co. (San Diego, CA, U.S.A.) and protein assay kit from Bio-Rad (Hercules, CA, U.S.A.). Histone H1 was from Boehringer Mannheim (Mannheim, Germany). Sprague-Dawley rats were obtained from the Korea Research Institute of Bioscience and Biotechnology (Daejeon, Chung Nam, Korea). Other chemicals were of the highest purity available.

Maintenance of rats and preparation of microsomal and cytosolic fractions

Male Sprague–Dawley rats were maintained in a specific pathogen-free condition with an irradiated and microbe-controlled diet (PicoLab Rodent Diet 20, 5053; PMI Feeds, Richmond, IN, U.S.A.) and autoclaved water ad libitum. The environmental condition of the animal housing was controlled at a constant temperature (22 ± 1 °C) and relative humidity (55 ± 5%). The room was ventilated 17 times per hour and illuminated for 12 h per day.

Seven-week-old male Sprague–Dawley rats, weighing approx. 200 g, were injected intraperitoneal with DEN (200 mg/kg body weight) and killed 3 days later. The sources of protease were prepared by the published method [18] from DEN-treated rat livers; in brief, 10% (w/v) homogenate in 50 mM Hepes buffer, pH 7.8, containing 0.25 M sucrose were centrifuged at 12000 g for 20 min; the supernatant was further centrifuged at 105000 g for 1 h. The final precipitate was resuspended in 50 mM Tris/HCl, pH 7.8, and used as microsomal proteases in the following experiments.

Various organs from age-matched control rats were excised and the cytosolic fractions were prepared as the source of a non-receptor-type protein kinase in accordance with the published method [19–22]. Thus each organ was homogenized at 10% (w/v) in a solution containing 25 mM Hepes buffer, pH 7.4, 0.25 M sucrose and PMSF as protease inhibitor. The homogenate was centrifuged at 12000 g for 20 min and the supernatant obtained was further centrifuged at 105000 g for 1 h to obtain the cytosolic fraction, which was used as the source of protein kinase.

Preparation of anti-(C-terminal peptide) antibodies

Recombinant MGMT protein and the polyclonal antibodies against rMGMT were prepared as gGSTTrMGMT plasmid by the method described previously [17]. Anti-(C-terminal peptide) antibody (corresponding to residues 194–209) was prepared by the addition of keyhole-limpet haemocyanin to the N-terminus of the SWLKPSFESSFPPKSG (single-letter amino acid codes) peptide synthesized by Research Genetics (Huntsville, AL, U.S.A.). The peptide linked to keyhole-limpet haemocyanin by glutaraldehyde and carbodi-imide was injected into New Zealand White rabbits. After two boosters, the whole serum containing the highest antibody titre against the C-terminal peptide was obtained in 10 weeks, and Protein A-Sepharose 4B (CL-4B)-bound IgG was purified as the antibody against MGMT C-terminal peptide for the following procedures.

Phosphorylation reaction of MGMT proteins

Reaction mixtures for phosphorylation contained rMGMT (3 μg), cytosolic fraction (5 μg), [γ-32P]ATP (9 μCl) (diluted with 0.1 mM unlabelled ATP), 1 mM CoCl2 and 50 mM Hepes buffer, pH 7.4, in a total volume of 60 μl; the mixtures were incubated at 22 °C for 30 min. The reaction was terminated by the addition of 20 mM EDTA. Autoradiography was performed after resolving the total incubation mixture by SDS/PAGE and by exposing the gel to X-ray film for 4 h (or 10–12 h) when liver kinase was used at −70 °C with intensifying screen.

Determination of phospho amino acids

To identify phospho amino acid(s) after the phosphorylation reaction of MGMT, the protein was incubated with [γ-32P]ATP by the procedure described above and the reaction mixture was separated by SDS/PAGE. To elute the phosphorylated rMGMT (rMGMT-P), γ-32P-labelled protein band on SDS/PAGE was cut out and the gels were incubated in 1 ml of 50 mM NaHCO3, 0.5% SDS/5 mg/ml dithiothreitol for 16 h at 20 °C. Eluted
Phosphorylation of rat methylated-DNA-protein-cysteine S-methyltransferase

protein was hydrolysed in 6 M HCl at 110 °C for 90 min after the removal of salt by dialysis against distilled water; the hydrolysate was dried with a Speedvac. The hydrolysate was resuspended in 10 µl of water, and was resolved together with standard phosphoserine, phosphothreonine and phosphotyrosine by one-dimensional thin-layer electrophoresis (LV-TLE; 17 V/cm) [23] on silica-gel plates in a solvent system containing pyridimine/acetic acid/water (1:10:189, by vol.) at pH 3.5. The phosphorylated amino acids on the plate were identified by ninhydrin spray and autoradiography.

Site-directed mutagenesis with mega-primer

To prepare a mutated rMGMTS204A plasmid, mega-primer (132 bp) was first produced by PCR amplification with Fse DNA polymerase (Stratagene, La Jolla, CA, U.S.A.) with S204A primer (5'-GAGTCTTCCGCACCAAAG-3'), pGEX 3'-primer (5'-CCCGGAGCTGCATGTGAAGG-3'; Pharmacia Biotech) and wild-type pGST rGMGT plasmid as template prepared in our laboratory [17]. Another round of PCR amplification was performed with the amplified double-strand mega-primer rGMT upstream primer (5'-GGGGATTCTAGGCTGAGA-TCTGCAA-3') and the wild-type pGST rGMGT plasmid as template; the full-length mutated MGTS204A DNA strands were then inserted into the EcoRI site in the pGEX-4T-1 expression vector. MGTS194A, MGTS199A, MGTS194/199A and MGTS202/203A were also prepared by the same procedure as above, with the use of the primers 5'-TCTGAT-TGGGCCCCTGCTCAA-3' for S194A, 5'-CTCAGGATCCCTGAGGTTG-3' for S199A and 5'-TCTAGGATCCCTGAGGTTG-3' for S202/203A double mutant. The MGTS194/199A double mutant was prepared with the S199A primer and the MGTS194A mutant plasmid as template DNA. Mutations of the prepared plasmids were confirmed by automatic DNA sequence analysis (ABI377; Perkin-Elmer, Foster, CA, U.S.A.).

Effect of PKC Inhibitors on MGMT phosphorylation

To identify which PKC isozenymes were involved in MGMT phosphorylation, a specific PKC inhibitor indolocarbazole Go66976 was employed for inhibiting Ca2+-dependent PKC isozenymes and bisindolylmaleimide Go6850 was employed for inhibiting total PKC isozenymes, including PKC-α, PKC-β1, PKC-γ, PKC-ε, PKC-δ and PKC-ζ [24–26]. Each PKC inhibitor was added, at a final concentration of 20 µM, to the phosphorylation reaction mixture containing [32P]-ATP and spleen cytosolic fraction. The reaction was terminated by the addition of 20 mM EDTA, rMGMT was separated by SDS/PAGE [12% (w/v) gel] and the gel was exposed to X-ray film (4 h with the cytosolic fraction from spleen, but 12 h with the liver fraction) for autoradiography with intensifying screen. As a positive control for PKC inhibitors, up to 2 µM of each PKC inhibitor was added to the incubation mixture containing 5 µg of histone H1, 1 mM of PKC-M (1 unit being defined as the amount of enzyme transferring 1.0 nmol of phosphate to histone H1 per min at 30 °C), 10 µM ATP plus [γ-32P]ATP and 50 mM Hepes buffer, pH 7.4, and the mixture was incubated at 30 °C for 10 min. Histone H1 was separated by SDS/PAGE and the gel was exposed to X-ray film for 4 h with an intensifying screen at −70 °C for autoradiography.

Proteolysis of MGMT protein by thrombin or liver microsomal protease

To investigate the degradation of MGMT proteins, 100 ng of rMGMT was incubated with 5 units of thrombin in PBS, pH 7.4, in a total volume of 120 µl at 25 °C for 12 h. During incubation, aliquots (20 µl) were taken at the indicated times and were separated by SDS/PAGE (12% [w/v) gel]. To confirm whether the C-terminal fragment of rMGMT was cleaved off during carcinogenesis, 200 ng of rMGMT was incubated with 10 µg of the microsomal protease and 10 mM CaCl2 in 50 mM Tris/HCl, pH 7.4, in a total volume of 200 µl at 37 °C. At 0, 1, 2 and 6 h, a 40 µl aliquot was taken for Western-blot analysis and autoradiography. For evaluating the effect of proteolysis on the phosphorylation, the above aliquots were further incubated with rat spleen cytosolic fraction by the method described above in the section on the phosphorylation reaction of MGMT proteins.

Effect of phosphorylation of MGMT on the protease resistance

To test the effect of phosphorylation on protease digestion, the phosphorylated and unphosphorylated rMGMTs (100 ng of each) were incubated with trypsin (enzyme-to-substrate ratio 1:100), chymotrypsin (enzyme-to-substrate ratio 1:100) or Glu-C (enzyme-to-substrate ratios 1:5 and 1:10) in sodium bicarbonate buffer, pH 7.8, at 30 °C for 1 h. Aliquots of the reaction mixture were taken 0, 10, 20 and 60 min later. Degradation of the C-terminal region by proteases was determined by Western-blot analysis with the anti-(C-terminal peptide) antibody and the ECL kit.

Western-blot analysis and autoradiography

MGMT proteins were diluted in 2 × SDS sample buffer [62.5 mM Tris/HCl (pH 6.8)/2% (w/v) SDS/5% (v/v) 2-mercaptoethanol/10% (v/v) glycerol/0.002% Bromophenol Blue], boiled for 5 min and resolved by SDS/PAGE [12% (w/v) gel]. The protein bands were transferred to nitrocellulose membrane (Schleicher and Schuell, Dassel, Germany) and the blot was hybridized with anti-rMGMT IgG antibodies or anti-(C-terminal peptide) antibodies prepared as described above. ECL reagent was applied to reveal the protein bands. [γ-32P]ATP-labelled MGMT proteins were measured by autoradiography after separation by SDS/PAGE [12% (w/v) gel] and exposure to X-ray film at −70 °C.

RESULTS

Conversion of 25 kDa MGMT protein into 24 kDa species in vitro and in vivo

In confirmation of our earlier results [17], MGMT protein in rat liver was significantly induced during the DEN-treated hepatocarcinogenesis; the presence of two MGMT proteins as 26 and 24 kDa species was confirmed by Western-blot analysis with anti-MGMT polyclonal antibody (Figure 2A). Because cancer development is frequently accompanied by increased intracellular protease activity [18], we considered a possible involvement of proteases in the appearance of the protein with two molecular sizes and in the conversion of the 26 kDa species into the 24 kDa species. Therefore, as a first step, rMGMT was incubated with thrombin in vitro and the conversion was followed by Western-blot analysis with an anti-MGMT polyclonal antibody. Indeed, as shown in Figure 2(B), the 26 kDa MGMT protein began to decrease after 1 h of incubation and the decrease continued until 12 h, with a concomitant increase in the 24 kDa species. When rMGMT was pretreated with thrombin for 12 h, during which time almost half of the 26 kDa species was converted into the 24 kDa species (Figure 2B), and the sample was assayed for MGMT activity by reaction with [methyl-3H]J DNA for 1 h at 37 °C to assess remaining activity by the established method [27].
Figure 2. Western-blot analyses showing the conversion of 26 kDa rMGMT into 24 kDa

(A) Induction and proteolytic modification of MGMT protein during DEN-induced carcinogenesis, demonstrated by anti-MGMT polyclonal antibodies. rMGMT in the first lane indicates the recombiant rat MGMT protein (170 ng), control indicates the MGMT level in normal liver, and DEN indicates MGMT protein found in the DEN-treated rat liver. 250 µg of protein was layered in each lane. Note, in the DEN lane, the induced level of the protein and the partial conversion of the MGMT protein from 26 into 24 kDa during the carcinogenesis. (B) Thrombin-induced conversion of 26 kDa rMGMT. rMGMT (100 ng) was incubated with 5 units of thrombin in a total volume of 120 µl at 37°C; aliquots (20 µl each) were taken at the indicated times. The samples were resolved by SDS/PAGE (12% (w/v) gel), then hybridized with anti-MGMT polyclonal antibody. Note the increasing ratio of 24 kDa to 26 kDa rMGMT as the incubation continued.

Figure 3. Phosphorylation of the serine residue of the 26 kDa rMGMT protein

(A) Autoradiography of rMGMTs incubated with [γ-32P]ATP and the cytosolic fractions of various rat organs. Note high kinase activity in brain, spleen and testes; intermediate activity in lung and kidney, and the lowest in liver. (B) Coomassie Blue staining and autoradiogram of [γ-32P]ATP-labeled rMGMTs. Phosphorylation was performed by incubating 3 µg of rMGMT with [γ-32P]ATP and spleen cytosolic fraction as described in the Experimental section. The reaction mixture was resolved by SDS/PAGE (12% (w/v) gel), stained with Coomassie Blue (lane 1) and then autoradiographed (lane 2). Note the phosphorylation signal in the 26 kDa rMGMT only. (C) Analysis of phospho amino acid residue by L-TLE. To identify phosphorylated amino acid residues, 32P-labeled rMGMT was hydrolysed in 6 M HCl; the hydrolysate was resolved by L-TLE procedure and autoradiography was performed. The autoradiogram was aligned with the migration of unlabelled phosphoserine (p-Ser), phosphothreonine (p-Thr) and phospho-tyrosine (p-Tyr) as standards.

the amount of methyl-3H transferred to rMGMT was the same as that in the control, which had not been treated with thrombin (results not shown). This indicated that conversion of the 26 kDa species into the 24 kDa species (Figure 2B) did not diminish the methyl-3H acceptability of the protein; that is, both the 26 and 24 kDa species of MGMT had an equal capacity for accepting a methyl group from [methyl-3H]DNA.

Phosphorylation of serine residue of 26 kDa rMGMT

Because rMGMT protein contains a potential PKC phosphorylation site in the C-terminal region as mentioned above, post-translational phosphorylation of rMGMT protein was evaluated. First, to obtain active protein kinases, cytosolic fractions of several normal rat organs were prepared and were subjected to kinase assay with [γ-32P]ATP and rMGMT. As shown in Figure 3(A), spleen, brain and testes revealed high activity; low activity was found in liver. Next, to answer the question of whether or not both the 26 and 24 kDa species of MGMT protein could be phosphorylated, rMGMT was first incubated with [γ-32P]ATP with the use of the rat spleen cytosolic fraction; the radiolabelled rMGMT was resolved by SDS/PAGE followed by autoradiography. As shown in Figure 3(B), when pretreated with thrombin, subsequent phosphorylation occurred only in the 26 kDa rMGMT species, not in the smaller one (lane 1 shows staining with Coomassie Blue and lane 2 shows autoradiography). To identify the amino acid residue phosphorylated in the 26 kDa rMGMT, 32P-labelled rMGMT was hydrolysed with 6 M HCl and the hydrolysate was resolved by L-TLE as described in the Experimental section. As shown in Figure 3(C), Ser was the only amino acid found to be phosphorylated.

Ser396 phosphorylated by Ca2+-independent PKC isoenzymes

Next, we undertook the definition of the site of phosphorylated serine in the rMGMT-P. To identify the general location of the phosphoserine, 32P-labelled 26 kDa protein was subjected to treatment with CNBr in formic acid. The digest was resolved and autoradiographed at −70°C for 5 h. Of the five peptides produced, arising from the four methionine residues in rMGMT (Figure 1), only the 8 kDa fragment corresponding to residues 139–209 contained 32P label (results not shown). This fragment, which corresponds to the C-terminal region of rMGMT, contained the active site of MGMT protein (PCHR, single-letter amino acid codes) as well as ten Ser residues, two Thr residues and one Tyr residue (Figure 1). Therefore, by means of site-directed mutagenesis, the Ser residues present in the C-terminal region of rMGMT were replaced with Ala to yield the mutants S194A, S199A, S194/199A, S202/203A and S204A. When these MGMT proteins were isolated and reacted for phosphorylation, only the S204A mutant was absolutely inert towards phosphorylation (Figure 4A), thus confirming Ser396 to be the phosphorylation site in the rat MGMT protein. At this
Phosphorylation of rat methylated-DNA—protein-cysteine S-methyltransferase

Figure 5 Degradation of the 26 kDa rMGMT by liver microsomal preparation during DEN-induced carcinogenesis

(A) Autoradiogram of rMGMT-P proteins showing the effect of prior proteolytic processing on the subsequent phosphorylation of rMGMT. The reaction mixture for proteolysis contained 1 μg of rMGMT and 5 μg of microsomal proteins isolated from the liver of DEN-treated rat; the mixtures were incubated at 37°C for the indicated durations. Subsequently, a phosphorylation reaction was performed by the addition of 5 μg of rat spleen cytosolic fraction, 1 mM CcCl4, and 9 μCi of [γ-32P]ATP at 22°C for 30 min. The control (O) contained all the reaction mixture but had no prior proteolysis. The autoradiogram shows that phosphorylation level of the rMGMT. (B) Western-blot analysis with anti-rMGMT polyclonal antibodies. The same blot as used in (A) was hybridized with anti-rMGMT polyclonal antibodies to reveal the proteolytic processing of the 26 kDa MGMT protein by liver microsomal proteins. Because the C-terminal contains the phosphorylation site, the densities of autoradiogram (A) and the 26 kDa MGMT band start to decrease and a new 24 kDa band appears within 1 h of the start of treatment (B).

Figure 4 Phosphorylation of Ser394 by novel PKC (Ca2+-independent) isoenzymes

(A) Autoradiogram confirming the phosphorylation of Ser394 in rMGMT by site-directed mutagenesis. Six Ser residues in the C-terminus of the 26 kDa rMGMT were replaced with Ala by site-directed mutagenesis. The wild-type and mutant rMGMTs were overexpressed in BLC1/DE3 cells and subjected to phosphorylation reaction with [γ-32P]ATP. Only the Ser394 mutant revealed a loss of phosphorylation signal, proving Ser394 to be the only site in rMGMT that is phosphorylated by an MGMT kinase in rat spleen (upper panel) and in liver (lower panel).

(B) Autoradiogram demonstrating the involvement of Ca2+-independent PKC isoforms of spleen (top panel) and liver tissue (middle panel) in the phosphorylation of MGMT. G66850 and G66976 are inhibitors of total PKC isoforms and Ca2+-dependent PKC isoforms respectively. Bottom panel: phosphorylation of histone H1 protein by PKC-M, which is the catalytic subunit of purified PKC, and the inhibition of its phosphorylation by G66850 and G66976, as a positive control.

point it was concluded that the C-terminal 2 kDa fragment of rMGMT was cleaved off by thrombin and that Ser394 was the phosphorylation site.

To delineate the protein kinase isoenzymes that were possibly involved in phosphorylating rMGMT, specific PKC inhibitors were employed. As shown in Figure 4(B), phosphorylation of the rMGMT by spleen or liver cytosolic fractions was inhibited by G66850 at 2 μM, whereas it was not inhibited by G66976 even at 20 μM. The result clearly indicated that phosphorylation of the rMGMT was performed by Ca2+-independent PKC isoenzymes present in the cytosolic fraction of rat spleen and liver tissues [24].

Degradation of 26 kDa rMGMT by liver microsomal preparation during DEN-induced carcinogenesis

To confirm that the MGMT protein could be degraded by proteases induced during hepatocarcinogenesis and that the resulting products could also be phosphorylated ex vivo, rMGMT was first incubated with microsomal proteins prepared from DEN-treated rat livers and phosphorylation was subsequently performed with spleen cytosolic fraction in the presence of [γ-32P]ATP. As shown in Figure 5(A), the phosphorylation of rMGMT was markedly diminished after 4 h of treatment with microsomal fraction (proteolysis). When the samples were hybridized with polyclonal anti-MGMT antibody, a decrease in the 26 kDa species was clearly visible, with a concomitant increase in the 24 kDa MGMT band (Figure 5B). This indicated strongly that the protease(s) that increased during the DEN-induced carcinogenesis cleaved off a peptide fragment that contained a putative phosphorylation site (Ser394) from rMGMT.

Phosphorylated MGMT protein is resistant to digestion with endopeptidase Glu-C and DEN-induced liver proteases

To investigate the biological significance of MGMT phosphorylation, rMGMT was first phosphorylated with spleen cytosolic fraction with or without unlabelled ATP at 22°C for 30 min. Subsequently, the rMGMT-P and unphosphorylated proteins were incubated with microsomal proteins isolated from DEN-treated rat livers at 30°C and analysed by Western-blot hybridization with anti-(C-terminal peptide) antibody. This antibody was expected to quantify MGMT protein with the C-terminal region intact. As shown in Figure 6(A), rMGMT-P revealed more resistance to C-terminal cleavage than the unphosphorylated one. To elucidate the kind of proteases involved in the rMGMT degradation during DEN-induced carcinogenesis in rat, digestions with trypsin, chymotrypsin or Glu-C were performed at 30°C for 60 min. As shown in Figure 6(B), the rMGMTs were relatively resistant to digestions with trypsin and chymotrypsin for 1 h. Moreover, the degree of sensitivity of the MGMT proteins to trypsin and chymotrypsin was independent of phosphorylation. In contrast, as determined with two different
Ada-C underwent conformational changes in its structure on binding to DNA, thereby exposing the active site to the alkaline group on the DNA substrate; the conformational changes consistently activated the protein [38]. Native human MGMT protein was found to have very limited accessibility to proteases in its native state; however, the sensitivity to digestion by trypsin or Glu-C (V8 protease) was significantly increased in response to DNA binding or alkylation [39], indicating that the protein undergoes conformational changes under these conditions. Hubbard et al. [40] suggested that the proteases generally cleave peptide sequences only in the regions that can adopt rather extended conformations. It should be noted that mammalian MGMT protein has also been known to bind to both double-stranded DNA and single-stranded DNA [41-43], and more tightly to the O\(^5\)-methylguanine DNA adduct [44].

In the present study we demonstrated two post-translational modifications of rMGMT during DEN-induced carcinogenesis: the cleavage of a C-terminal 2 kDa fragment containing a potential phosphorylation site at Ser\(^374\), and the phosphorylation of Ser\(^375\), which made rMGMT more resistant to cleavage by proteases induced during DEN-induced carcinogenesis in vitro or by Glu-C. Because amino acid sequence analysis revealed that only rMGMT contained the C-terminal region with a potential PKC phosphorylation site, we explored the best source of PKC activity in normal rat organs and found that brain, spleen and testes had the highest activity, followed by lung and kidney; liver had the lowest (Figure 3A). This pattern of phosphorylating activity in the various rat organs was well correlated with a report by Wetsel et al. [45] on the distribution and concentration of PKC isoenzymes determined by Western-blot analyses. In the present study we therefore chose spleen cytosolic fraction as a source of protein kinase. Because phosphorylation was strongly inhibited by 2 \(\mu\)M G66850, which inhibited the PKC-\(\alpha\), PKC-\(\beta\), PKC-\(\delta\), PKC-\(e\) and PKC-\(\xi\) isoenzymes, but not by 20 \(\mu\)M G66976, which inhibited the Ca\(^{2+}\)-dependent PKC-\(\epsilon\), PKC-\(\beta\) and PKC-\(\mu\) isoenzymes (Figure 4B), it was highly likely that the phosphorylation was performed primarily by Ca\(^{2+}\)-dependent PKC isoenzymes such as PKC-\(\delta\), PKC-\(e\), PKC-\(\xi\) and possibly another uncharacterized kinase in the liver cytosolic fraction [24-26]. A site-directed mutagenesis study (Figure 4A) revealed that the phosphorylation site was absolutely specific; when the six serine residues present in the C-terminal region of rMGMT were mutated to alanine one at a time (S194A, S199A, S204A) or in pairs (S194/199A, S202/203A), only the S204A mutant showed a complete loss of phosphorylation potential. This Ser\(^375\)-Pro-Lys sequence corresponded exactly to PKC consensus sequences reported previously [15,16]; this phosphorylation sequence was present only in rat MGMT protein, not in the human, mouse or Ada-C proteins (Figure 1).

Phosphorylation of the C-terminal fragment that can be cleaved off was confirmed by autoradiography and Western-blot analyses with anti-MGMT polyclonal antibodies (Figure 5). Microsomal proteins isolated from the DEN-treated rat livers significantly degraded 26 kDa rMGMT (Figure 5A) with a concomitant increase in the 24 kDa species during a 6 h incubation (Figure 5B). In addition, the overall level of phosphorylation was significantly diminished when rMGMT was pretreated with the liver microsomal fraction; a 2 kDa peptide fragment was cleaved off from the 26 kDa protein during the pretreatment. This indicated that the C-terminal region of rMGMT was phosphorylated by PKC isoenzymes and that this region could be cleaved off by the microsomal protease that increased in the DEN-treated rat liver. The notion that proteolysis was induced in DEN-treated rat liver was supported by the following findings.

**Figure 6 Western-blot analyses showing the effect of phosphorylation of rMGMT on its sensitivity to various proteolytic enzymes**

rMGMT-P (P) and unphosphorylated rMGMT (N) were prepared as described in the Experimental section and in the legend to Figure 5. Subsequently, the above phosphorylation reaction mixtures at 22 °C were subjected to the actions of the liver microsomal fraction of DEN-treated rat liver at 37 °C (A), or trypsin, chymotrypsin or Glu-C (V8 protease) at 30 °C (B). The final reaction mixtures were resolved by SDS/PAGE (12% acrylamide gel) and transferred to nitrocellulose membrane, then hybridized with anti-C-terminal peptide antibody. The enzyme-to-substrate ratios are indicated in parentheses. Note the significant protection of the rMGMT-Ps against digestion by microsomal proteases and by Glu-C. rMGMT (150 ng) was used for the chymotrypsin assay; 100 ng was used for the others.

Concentrations of Glu-C, rMGMT-P was much more resistant to digestion with Glu-C than rMGMT (Figure 6B).

**DISCUSSION**

The biological significance of MGMT is to remove the O\(^5\)-alkylguanine adduct in DNA formed either by exogenous environmentally important genotoxic alkylating agents or by endogenous S-adenosyl-l-methionine [2,28-30]. If not repaired, the persistent presence of O\(^5\)-methylguanine in DNA could ultimately lead to mutagenesis and/or carcinogenesis by interfering with the G-C hydrogen-bonding in DNA [31-34] or by mismatching owing to its preference for pairing with thymine during DNA replication [35-37].

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The administration of liver tumour promoters to male rats for 1 week induced Ca^{2+}-dependent serine protease activity 3–5-fold and the enzyme was found particularly in the smooth endoplasmic reticulum [18].

Before we investigated the site of hydrolysis of rMGMt by thrombin (Figure 2B), 32P-labelled 26 kDa rMGMt was treated with CNBr in formic acid to define its general location; the digest was resolved by SDS/PAGE followed by autoradiography. Only an 8 kDa fragment corresponding to residues 139–209 was 32P-labelled (results not shown). Thrombin is one of the highly specific proteolytic enzymes to hydrolyse the peptide bond between Lys and Gly present up-stream of the hydrophobic amino acids [46]. On the basis of our results it was therefore highly possible that thrombin cleaved rMGMt at Lys^{167}–Gly^{168} producing a 22-residue peptide. It is of interest that the most likely potential tryptic cleavage site has also been suggested to be Lys^{167}–Gly^{168} in human MGMT [39]. Our finding that thrombin-digested rMGMt retained all the methyltransferase activity is in good agreement with the result of Elder et al. [47] that the rates of methyltransferase activity of full-length and C-terminal truncated human MGMT proteins were virtually identical. Furthermore, full activity was retained in human MGMT mutants with 8 and 31 residues deleted from the N-terminus and the C-terminus respectively [44]. It should again be noted that the cleaved fragment corresponded to the extended C-terminal region of rMGMt, which was absent from yeast, rabbit and bacterial MGMTs [38,46,49]. In contrast, Morgan et al. [49] reported that although both the full-length MGMT and the protein with 28 residues deleted were active at 37° C, only the former was active at 4°C. They also observed different substrate specificities between them, even though the DNA repair activities were the same.

It has been shown in the present study that the phosphorylation of the C-terminal Ser^{344} of MGMT has a significant effect on its resistance to proteases in vivo as well as in vitro (Figure 6). rMGMt-P is clearly more resistant to digestion than the unphosphorylated protein. Therefore, although Glu-C is naturally found in bacteria, this endowment of protease resistance on MGMT by phosphorylation might have some significance during carcinogenesis in liver by alkylating agents. These results indicate that conformational changes are induced not only by the binding of DNA and the allocation of MGMT but also by the phosphorylation of MGMT.

In conclusion, we suggest that the treatment of a rat with alkylating agents might induce Glu-C-like proteolytic enzymes in liver during the carcinogenic process and that the phosphorylation of MGMT can induce a conformational change in the protein, thereby protecting it from digestion by induced proteases. Because alkylated MGMT protein can be ubiquitinated [50] and digested by uncharacterized proteases in vivo for eventual disposal, further studies on this metabolic fate of rMGMt-P in vivo are needed.

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SELECTIVE LEFT-LOBE ATROPHY BY NODULARIN TREATMENT ACCOMPANYED BY REDUCED PROTEIN PHOSPHATASE 1/2A AND INCREASED PEROXISOME PROLIFERATION IN RAT LIVER

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The effect of nodularin on selective atrophy of left lobes in the liver was investigated in F344 rats. Nodularin was injected for 10 weeks from the third week of initiation with saline or N-nitrosodiethylamine (DEN), grouped as S/N and D/N, respectively. Nodularin significantly decreased weights of left (LL) and caudate (CL) lobes but increased right (RL) and middle (ML) lobes in S/N rats. Activity of protein phosphatases (types 1 (PP1) and 2A (PP2A)) was more severely reduced in S/N than in D/N rats, respectively. In LL, compared with RL of S/N rats, activity was significantly inhibited by nodularin treatment from week 4, which corresponded to 2 weeks after nodularin injection. However, nodularin significantly increased cytosomal palmitoyl-CoA oxidase and cyochrome P-450 4A1 expression in S/N compared with D/N rats. An effect of nodularin on apoptosis was evident since expression of Bcl-XL was clearly induced in LL of S/N rats as opposed to various inductions of Bel-XL. However, Bcl-XL in RL was persistently induced, with undetectable Bcl-XL expression. These results demonstrate biochemical evidence of selective atrophy of LL by inhibition of PP1 and PP2A activity, increase of peroxisomal enzymes and induction of Bel-XL expression. In contrast to proliferation of RL in rats treated with nodularin alone. However, nodularin endowed DEN- altered hepatocytes with regenerating power and concomitant restoration of phosphatase activity as well as persistent expression of Bcl-XL in D/N rats.

Key Words: left-lobe atrophy; nodularin; protein phosphatase 1/2A; peroxisome proliferation

Nodularin was first isolated and identified from Nodularia spumigena in ditch water1 and in mussel2 and proved to be one of the tumor-promoting hepatotoxins. When ingested, the hepatotoxic induced death and extensive liver damage,3 thus presenting a significant health hazard to humans and agricultural livestock. It belongs to the okadaic acid type of tumor promoter,4 which inhibits protein phosphatase types 1 (PP1) and 2A (PP2A).5,6 Furthermore, nodularin induced expression of TNF-α as well as protooncogenes of the fos and jun families in primary hepatocyte cultures isolated from F344 rats.7 These findings suggest that nodularin may stabilize proto-oncogene mRNAs by inhibiting PP1 and PP2A, constituting a critical step in tumor promotion.8

We previously reported that treatment by nodularin injection after N-nitrosodiethylamine (DEN) initiation (DEN + treatment) proliferated mainly hepatocytes but not bile duct lining epithelial, endothelial or peri-sinusoidal cells.9 In D/N rats, nodularin injection persistently induced proliferating cell nuclear antigen (PCNA) in the glutathione-S-transferase placentum (GST-P)-positive dense nodules, whereas it was expressed transiently in the surrounding hepatocytes. This indicated that nodularin affected the PCNA index differentially among altered and unaltered hepatocytes and led us to test whether nodularin was one of the possible peroxisome proliferators (PPs) or not.

Peroxisome proliferator is a ubiquitous rodent hepatocarcinogen, known to modulate the activity of xenobiotic-metabolizing enzymes such as GST-P and mixed-function oxidase,10 and treatment of rodents with peroxisome proliferators has resulted in both hepatocyte hyperplasia and peroxisome proliferation.11-13 Elevated levels of hydrogen peroxide resulting from peroxisome proliferation may initiate tumorigenesis by oxidative DNA damage,14 and peroxisome proliferation may also act as a liver tumor promoter by stimulating replicative DNA synthesis.15-17 A highly carcinogenic peroxisome proliferator, WY-14,643, induced hepatic peroxisome proliferation in rat liver regardless of prior treatment with DEN and/or phenobarbital.18 The classical enzyme markers of peroxisome proliferation are enzymes of peroxisomal β-oxidation, such as acyl-CoA oxidase and peroxisomal bifunctional enzyme enoyl-CoA hydratase, and the microsomal cytochrome P-450 4A (CYP4A) family.19-22

Kogure et al.24 compared rat liver with human liver segments and defined 4 lobes: left (LL), middle (ML), right (RL) and caudate (CL). During our continuing study on DEN/nodularin-induced hepatocarcinogenesis in F344 male rats, shrinkage of LL and CL was observed in the group treated with nodularin alone. In contrast, 4 lobes were enlarged by DEN/nodularin treatment. From the above considerations, therefore, to investigate the molecular mechanism of LL atrophy by treatment with nodularin alone, inhibition of PP1 and PP2A, activity of palmitoyl-CoA oxidase, microsomal CYP4A1 expression and changes of Bcl-XL and Bel-XL expression were assessed in liver lobes of S/N as well as D/N rats.

MATERIAL AND METHODS

Material

DEN, phosphorylase b, phosphorylase kinase, caffeine, PMSF, diithiothreitol (DTT), palmitoyl-CoA, 3-amino-1,2,4-triazole, N,N-dimethylformamide and hydrogen peroxide were purchased from Sigma (St. Louis, MO). Horseradish peroxidase was from Boehringer-Mannheim (Mannheim, Germany). 2,7-Dichlorodihydrofluorescein diacetate (H2-DCF) was from Molecular Probes (Eugene, OR). Triton X-100 was from Merck (Darmstadt, Germany). [γ-32P]ATP (3,000 Ci/mmol) was from Amersham Pharmacia Biotech (Uppsala, Sweden). Nodularin was purified by Dr. W.W. Carmichael at Wright State University (Dayton, OH). Polyclonal antibody against CYP4A1 was kindly provided by Dr. B.J. Song at

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NIHAA (Bethesda, MD). Anti-Bcl-X antibody (B22630) was purchased from Transduction Laboratories (Lexington, KY). The rest of the chemicals were obtained from various commercial sources and were of the highest grade available.

**Maintenance of animals**

Seven-week-old F344/KGIST (Genetic Resources Center, Korean Research Institute of Bioscience and Biotechnology, Yuseong, Korea) male rats were bred in an inbred colony. All cages and bedding were autoclaved before use and stored in a separate room. Animals were provided with an irradiated and microbe-controlled diet (PicoLab Rodent Diet 20; PMI Feeds, Richmond, IN) and water ad libitum. Animal housing was controlled at a constant temperature (22 ± 1°C) and humidity (55 ± 5%). The room was ventilated 17 times/hr and illuminated for 12 hr/day.

**Animal experiments**

Hepatocarcinogenesis was induced as described previously? 7-week-old F344 male rats were injected i.p. once with saline or DEN as an initiator (200 mg/kg body weight) and nodularin as a promoter (25 μg/kg body weight, twice/week) for 10 weeks from the third week after initiation. Animals were maintained for 10 more weeks after the cessation of promoter injection and labeled as S/S for control, S/N for saline initiation and then promotion with nodularin and D/N for initiation with DEN and promotion with nodularin (Fig. 1). Here, S, D and N represent saline, DEN and nodularin injection, respectively, as either an initiator or a promoter. Each group consisted of 60 male rats, and an identical experiment was repeated twice. Animals were killed at weeks 4, 8, 10, 12, 15 and 18 after initiation; liver lobes were dissected on ice and weighed, and sliced sections were stored at −70°C until use.

Sections for histological analysis were fixed overnight in 10% buffered neutral formalin, paraffin embedded by a routine procedure. Serial sections (5 μm) were stained with hematoxylin and eosin.

**Preparation of [32P]phosphorylase a for protein phosphatase assay**

The substrate [32P]-phosphorylase a (0.5 nmol, 10μmol protein) was prepared23 by incubating 1 mg of phosphorylase b with 10 μg of phosphorylase kinase and [γ-32P]ATP (40 pmole) in 0.2 ml of buffer A (50 mM Tris-HCl (pH 8.2), 50 mM glycerol-2-phosphate, 10 mM magnesium acetate, 12.5 μM ATP). Following 30 min incubation at 30°C, 0.2 ml of 90% saturated ammonium sulfate was added and centrifuged at 15,000 g for 15 min after incubation at 4°C for 10 min. The precipitate was resuspended in 500 μl of buffer B (50 mM Tris-HCl (pH 7.0), 1 mM EDTA, 50 mM β-mercaptoethanol, 250 mM NaCl) and the suspension was treated with an equal volume of 90% saturated ammonium sulfate. The precipitate was resuspended in 500 μl of buffer B, concentrated with centricon-30 (Amicon, Beverly, MA) and washed with 2 ml of buffer B. The final concentrate was used as a substrate and stored at 4°C until use.

**Assay of PPI and PP2A activity**

A 10% homogenate of liver was prepared in 50 mM Tris-HCl (pH 7.0) containing PMFS (1 mM) and leupeptin (0.5 μg/ml) in a Dounce homogenizer with 10 strokes and incubation with 1% NP-40 on ice for 20 min. Activity of PPI and PP2A was determined by measuring release of [32P]-inorganic phosphate from [32P]-phosphorylase a at 30°C according to the published method25 with some modifications. For assay, 20 μl of liver lysate was diluted in 100 μl of buffer C (50 mM Tris-HCl (pH 7.0), 1 mM EDTA, 1% β-mercaptoethanol, 5 mM caffeine, 0.1% BSA). The reaction was started by adding 5 μl of [32P]-phosphorylase a (>2 × 10^4 cpm), incubated for 15 min at 30°C and terminated by adding 100 μl of 50% TCA. The reaction mixture was centrifuged at 15,000 g for 10 min, and released [32P]-inorganic phosphate was measured using a liquid scintillation counter. One unit of PPI and PP2A represents 1 pmol of [32P]-phosphosphate released/mg protein every 15 min. Activity of protein phosphatase was expressed as the percentage of enzyme activity remaining in the liver of experimental rats compared with control rats.

**Assay of palmityl-CoA oxidase**

Activity of palmityl-CoA oxidase was measured according to the published method24 with a slight modification. A 10% liver homogenate was prepared in 1.15% KCl containing 0.2 mM PMFS and 1 mM DTT and centrifuged at 800 g for 10 min. The supernatant was assayed for palmityl-CoA oxidase by spectrophotometrically measuring the hydrogen peroxide produced, which was coupled to the oxidation of H2DCF in a reaction catalyzed by exogenously added peroxidase: 1 ml assay mixture in a semi-microcuvette contained 0.05 mM H2DCF, 40 mM 3-amino-1,2,4-triazole, 0.02% Triton X-100, 11 mM potassium phosphate (pH 7.4), tissue supernatant and SI medium (10% sucrose with 3 mM imidazole, pH 7.4). After pre-incubating the mixture in the dark for 5 min at 30°C, palmityl-CoA (30 mM) was added and the absorbance change was read at 502 nm.

**Western blot analysis**

For measuring the expression of CYP4A1, a microsomal fraction was prepared from 10% rat liver homogenate in 1.15% KCl containing 0.2 mM PMFS and 1 mM DTT. The homogenate was centrifuged at 10,000 g for 10 min, and then the supernatant was further centrifuged at 100,000 g for 1 hr. The final pellet was resuspended in 1.15% KCl containing 0.2 mM PMFS and 1 mM DTT, and 10% SDS-PAGE was conducted with the microsomal protein (80 μg/lane). In case of Bcl-X, rat liver homogenates (40 μg/lane) were subjected to 12% SDS-PAGE. Proteins on the gel were subsequently transferred to nitrocellulose membranes, and enhanced chemiluminescence was used to monitor the intensity of

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**Figure 1** - Scheme for animal experiments. Seven-week-old F344 male rats were injected once with saline or DEN as an initiator (200 mg/kg body weight) and nodularin as a promoter (25 μg/kg body weight, twice/week) for 10 weeks from the third week after initiation. Animals were maintained for 10 more weeks after the cessation of promoter injection and labeled S/S for control, S/N for saline initiation and then promotion with nodularin, and D/N for initiation with DEN and promotion with nodularin. Here, S, D and N represent saline, DEN and nodularin injection, respectively, as either an initiator or a promoter. Rats were killed at 4, 8, 10, 12, 15 or 22 weeks after DEN or saline initiation.
the protein bands with their primary antibodies and peroxidase-conjugated anti-mouse or anti-rabbit IgG (Vector, Burlingame, CA) as the secondary antibody. The Bel-X antibody could react with both Bel-X\textsubscript{L} and Bel-X\textsubscript{R}.

**Statistical analysis**

The statistical significance of changes in protein phosphatase activity was analyzed by 1-way ANOVA and applied to multiple comparisons using SPSS (Chicago, IL), version 7.1.

**RESULTS**

**Nodularin-induced atrophic change of LL and CL in liver**

As shown in Figure 2, rats injected with nodularin (twice/week) for 8 (S\textsubscript{2}/N\textsubscript{4}) or 10 (S\textsubscript{2}/N\textsubscript{10}) weeks displayed severe shrinkage of LL and CL compared with control (S/S) and D/N-treated rats. The atrophic change started to appear at week 4, which corresponds to 2 weeks of nodularin injection, as yellowish discoloration at the lateral corner of LL in the liver. However, loss of LL could be clearly detected at week 8, which corresponds to 6 weeks after nodularin injection (Table I). Ten weeks after the cessation of nodularin injection, shrinkage of LL and CL in S/N rats was irreversible and remained so for a further 10 weeks. In contrast to the above, RL and ML of S/N rats revealed significant regeneration at weeks 15 and 22, which correspond to 3 and 10 weeks after termination of nodularin treatment, respectively. D/N treatment completely blocked shrinkage of the lobes, with some hypertrophy. Therefore, to investigate whether this was due to an unequal distribution of nodularin in the liver, Tc\textsuperscript{99m}-DMSIDA (500 \mu Ci) was injected i.v. into F344 male rats, and the accumulated radio images in LL and RL were analyzed by a variable-angle \gamma-camera (Elscint, Haifa, Israel); however, we failed to find any significant difference between the LL and RL (data not shown).

**Selective inhibition of PP1 and PP2A in LL by nodularin, but not by D/N/nodularin, treatment in intact rat liver**

Protein phosphatases are classified as PP1a, PP1b, PP2A, PP2B and PP2C depending on their specificity for phosphorylase kinase, substrate, regulators and others.\textsuperscript{25} Phosphorylase a is a good substrate for PP1 and PP2A, and the Ca\textsuperscript{2+}- and Mg\textsuperscript{2+}-free assay system with 1 mM EDTA used in the present study can exclude the activity of both PP2B and PP2C, while detecting only that of PP1 and PP2A. Because of the earlier report on the specific inhibitory effect of nodularin on PP1 and PP2A,\textsuperscript{8} we presently focused our study on these 2 isoforms. To monitor the activity remaining in the liver of S/N and D/N rats, \textsuperscript{32}P-inorganic phosphate released from \textsuperscript{32}P-labeled phosphorylase a by PP1 and PP2A was measured. As shown in Figure 3a, nodularin significantly inhibited phosphatase activity in both the S/N and D/N groups, when expressed as a percentage of control values; however, the inhibition was much more extensive and sustained in S/N compared with D/N rats. Activity began to recover in D/N rats at week 12 up to 52.7% of the control and at week 15 up to 78% of the control, however, it remained significantly low in S/N rats up to 29.3% and 30.1%, respectively. Comparison of LL and RL of S/N rats (Fig. 3b) revealed significant inhibition of phosphatase activity in LL persisting until week 22. PP1 and PP2A activity was evenly distributed in all 4 lobes of control rats (data not shown).

**Figure 2** – Loss of LL in nodularin-treated rats, in contrast to enlargement in D/N/nodularin-treated rats. Injections of nodularin after saline initiation specifically removed LL in S/N rats compared with the enlarged 4 lobes in D/N rats. Atrophic changes caused by nodularin appear to be more apparent in S\textsubscript{2}/N\textsubscript{10} than S\textsubscript{2}/N\textsubscript{4}. 

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure2.png}
\caption{Loss of LL in nodularin-treated rats, in contrast to enlargement in D/N/nodularin-treated rats. Injections of nodularin after saline initiation specifically removed LL in S/N rats compared with the enlarged 4 lobes in D/N rats. Atrophic changes caused by nodularin appear to be more apparent in S\textsubscript{2}/N\textsubscript{10} than S\textsubscript{2}/N\textsubscript{4}.}
\end{figure}
**NODULARIN-INDUCED LEFT-LOBE ATROPHY**

**TABLE 1 - SELECTIVE LOSS OF LL AND CL OF LIVER BY REPEATED INJECTIONS OF NODULARIN IN F344 MALE RATS**

<table>
<thead>
<tr>
<th>Week</th>
<th>Treatment</th>
<th>RL (g)</th>
<th>LL (g)</th>
<th>ML (g)</th>
<th>CL (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>S/S</td>
<td>1.61 ± 0.41</td>
<td>2.25 ± 0.22</td>
<td>2.94 ± 0.08</td>
<td>0.33 ± 0.05</td>
</tr>
<tr>
<td></td>
<td>S/N</td>
<td>1.33 ± 0.28</td>
<td>1.66 ± 0.41</td>
<td>2.40 ± 0.27</td>
<td>0.44 ± 0.08</td>
</tr>
<tr>
<td></td>
<td>D/N</td>
<td>1.21 ± 0.29</td>
<td>1.73 ± 0.30</td>
<td>2.39 ± 0.36</td>
<td>0.25 ± 0.04</td>
</tr>
<tr>
<td>8</td>
<td>S/S</td>
<td>1.83 ± 0.30</td>
<td>2.61 ± 0.04</td>
<td>2.85 ± 0.16</td>
<td>0.47 ± 0.19</td>
</tr>
<tr>
<td></td>
<td>S/N</td>
<td>2.22 ± 0.71</td>
<td>0.90 ± 0.34**</td>
<td>4.47 ± 2.54</td>
<td>0.36 ± 0.14</td>
</tr>
<tr>
<td></td>
<td>D/N</td>
<td>1.72 ± 0.54</td>
<td>1.83 ± 0.45</td>
<td>3.00 ± 0.32</td>
<td>0.30 ± 0.12</td>
</tr>
<tr>
<td>10</td>
<td>S/S</td>
<td>2.04 ± 0.24</td>
<td>3.17 ± 0.23</td>
<td>3.92 ± 0.25</td>
<td>0.56 ± 0.19</td>
</tr>
<tr>
<td></td>
<td>S/N</td>
<td>2.00 ± 1.37</td>
<td>0.45 ± 0.19*</td>
<td>4.97 ± 1.25</td>
<td>0.03 ± 0.04**</td>
</tr>
<tr>
<td></td>
<td>D/N</td>
<td>2.64 ± 0.30</td>
<td>3.60 ± 0.37</td>
<td>3.97 ± 1.02</td>
<td>0.48 ± 0.21</td>
</tr>
<tr>
<td>12</td>
<td>S/S</td>
<td>2.04 ± 0.24</td>
<td>3.17 ± 0.23</td>
<td>3.92 ± 0.25</td>
<td>0.56 ± 0.19</td>
</tr>
<tr>
<td></td>
<td>S/N</td>
<td>3.23 ± 1.52</td>
<td>0.81 ± 0.88*</td>
<td>4.38 ± 0.13</td>
<td>0.08 ± 0.11**</td>
</tr>
<tr>
<td></td>
<td>D/N</td>
<td>3.29 ± 0.37</td>
<td>3.44 ± 0.09</td>
<td>4.55 ± 1.24</td>
<td>0.42 ± 0.18</td>
</tr>
<tr>
<td>15</td>
<td>S/S</td>
<td>2.49 ± 0.28</td>
<td>3.10 ± 0.49</td>
<td>3.54 ± 0.56</td>
<td>0.44 ± 0.05</td>
</tr>
<tr>
<td></td>
<td>S/N</td>
<td>3.37 ± 0.19**</td>
<td>0.25 ± 0.17**</td>
<td>6.26 ± 0.31</td>
<td>0.16 ± 0.05</td>
</tr>
<tr>
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<td>D/N</td>
<td>3.25 ± 0.08</td>
<td>3.09 ± 0.67</td>
<td>4.46 ± 1.07</td>
<td>0.27 ± 0.20</td>
</tr>
<tr>
<td>22</td>
<td>S/S</td>
<td>2.08 ± 0.50</td>
<td>3.34 ± 0.53</td>
<td>3.85 ± 0.78</td>
<td>0.76 ± 0.24</td>
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<tr>
<td></td>
<td>S/N</td>
<td>4.10 ± 1.24**</td>
<td>0.67 ± 0.23*</td>
<td>7.33 ± 0.51*</td>
<td>0.07 ± 0.10**</td>
</tr>
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<td></td>
<td>D/N</td>
<td>3.11 ± 0.57</td>
<td>4.53 ± 0.90</td>
<td>5.79 ± 1.42</td>
<td>0.48 ± 0.13</td>
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</tbody>
</table>

1Week indicates duration of time after initiation with either saline or DEN. - S/S, D/N and S/N indicate that saline (S) or DEN (D) by single injection was used as initiator and twice/week injections of saline (S) or nodularin (N) as promoter of carcinogenesis 2 weeks afterinitiation. - *p < 0.01 and **p < 0.05 vs. S/S by ANOVA.

**FIGURE 3** - (a) Significant inhibition of PP1 and PP2A activity in S/N compared with D/N rats. Rat livers were dissected at the indicated weeks, and 20 μg of each liver lysate were used for activity assay. Error bar represents an average of duplicate assays from 3 independent experiments. Asterisk indicates a significance of probability between S/N and D/N rats by independent ANOVA. (b) Selective inhibition of PP1 and PP2A activity in LL of S/N rats compared with RL. LL and RL were separately dissected at the indicated times from S/N rats. Twenty micrograms of each liver lysate were subjected to the assay. Error bar represents an average of duplicate assays from 3 independent experiments. Asterisk indicates a significance of probability between LL and RL by independent ANOVA.

**Nodularin-induced peroxisome proliferation in liver**

To investigate the effect of nodularin on peroxisome proliferation, activity of palmitoyl-CoA oxidase, one of the marker enzymes in peroxisome proliferation, was assayed by measuring H₂O₂ production in a reaction catalyzed by exogenously added peroxisomes. As shown in Table II, activity in S/N rats was clearly elevated from week 4 compared with controls. Moreover, activity in S/N rats was significantly higher than in D/N rats from week 8 to week 15. It returned to the control level at week 22, which corresponds to 10 weeks after the cessation of nodularin injection. This indicated that induction of palmitoyl-CoA oxidase was highly specific for nodularin injection.

To further confirm the nodularin effect on peroxisome proliferation, CYP4A1 expression was also measured by Western blot analysis. As shown in Figure 4 (upper panel), the level in both LL and RL was significantly enhanced in S/N compared with S/S; however, the level in D/N rats represented some variation. The level of CYP2E1 expression (Fig. 4, lower panel) failed to show any significant changes in control and test rats.

**Apoptotic changes in LL of S/N rats by nodularin treatment**

To elucidate the effect of nodularin on apoptosis in LL and RL of S/N rats, expression of Bcl-X-related gene products was measured by Western blot analysis. As shown in Figure 5a, expression
of Bcl-X<sub>L</sub>, a pro-apoptotic protein, was clearly induced in the LL from week 4 to week 15. A slight increase appeared at weeks 8, 10 and possibly 12. However, expression of Bcl-X<sub>L</sub>, an anti-apoptotic protein, was transiently increased at week 4 and then returned to the control level from week 10. Each lane in Figure 5 reveals a representative animal variation at the indicated times. However, the anti-apoptotic protein Bcl-X<sub>L</sub> was persistently increased in RL of S/N rats during the experimental period, as opposed to no induction of Bcl-X<sub>L</sub> (Fig. 5b). The above results clearly represent molecular evidence of apoptosis in LL of S/N rats, in contrast to the proliferation of RL by treatment with nodularin alone.

**Nodularin effect in LL and RL of S/N rat livers and D/N rats**

As shown in Figure 6, nodularin induced peri-central necrosis in RL of S/N rats (S<sub>N</sub> and S<sub>N</sub>), however, it was rapidly regenerated 1 week after the cessation of nodularin injection (S<sub>N</sub>) and then returned to normal 10 weeks later (S<sub>N</sub>). The same treatment in S/N rats led to LL atrophy with broad fibrosis (S<sub>N</sub>-LL), as opposed to adenoma formation (D<sub>N</sub>-D) in D/N rats. The size of the liver was markedly diminished in LL compared with RL of S/N rats.

**DISCUSSION**

Peroxisome proliferators represent a class of non-genotoxic carcinogens and a diverse class of chemicals, including the hypolipidemic drugs, herbicides, plasticizers, solvents, hormones (DHEA sulfate) and naturally occurring agents. Peroxisome proliferators cause liver tumors and liver enlargement, due to both hepatocyte hyperplasia and peroxisome proliferation, in rodents. Promotion of surviving hepatocytes by peroxisome proliferators can fix DNA damage into the genome and allow clonal expansion of initiated cells. However, peroxisome proliferators failed to induce DNA strand breaks and cytotoxicity as monitored by lipid peroxidation or any increase of free radical production in the liver due to insufficient DNA damage. The molecular mechanism of mitogenesis by peroxisome proliferators has been suggested to have synergistic action with EGF/TGF-α through EGF receptor expression in spontaneously initiated rat hepatocytes. Carcinogenic peroxisome proliferators increased phosphorylation of EGF receptor, suggesting that this class of non-genotoxic carcinogens could perturb hepatocyte growth regulation by altering signals through the phosphorylated growth factor receptor pathway. However, liver growth is controlled by the balance between cell birth by mitosis and cell death by apoptosis.

Nodularin belongs to the peroxisome proliferators, as assayed by palmitoyl-CoA oxidase activity and CYP4A1 induction (Table II, Fig. 4), and it regulates cell death through differential induction of Bcl-X<sub>L</sub> and Bcl-X<sub>L</sub> in LL and RL (Fig. 5), respectively, resulting in severe atrophy of LL of S/N rats as opposed to adenoma formation in D/N rats (Figs. 2 and 6, Table I). Peroxisome proliferators significantly induce mRNAs of peroxisomal acyl-CoA oxidase and microsomal CYP4A1. Peroxisome proliferator-activated receptor α (PPARα) is a peroxisome proliferator-activated receptor that transcriptionally transduces these, thereby, it directly controls lipid metabolism through activation of genes encoding enzymes for fatty acid β-oxidation (acyl-CoA oxidase), fatty acid ω-hydroxylation (CYP4A1) and fatty acid binding protein. In contrast to CYP4A1, there was no significant difference in CYP2E1 expression in S/N compared with controls and D/N rats (Fig. 4). This is in good agreement with the report that CYP2E1 expression by peroxisome proliferators was tissue-specific and highly induced in kidney but not in liver. These findings strongly support the concept that nodularin-induced peroxisome proliferation, when measured by the activity of palmitoyl-CoA oxidase and CYP4A1 expression, is a specific change. There was no significant difference in peroxisome proliferation between LL and RL; however, it was significantly higher in S/N than controls or D/N rats (Table II, Fig. 4). Nodularin significantly inhibited the activity of PPI and PPIA in LL compared with RL of S/N rats (Fig. 3). These data strongly indicate that peroxisome proliferation itself failed to drive hepatocytes to proliferate, whereas it was successful in altered hepatocytes. It is very well supported by our previous report on the PCNA index, which was persistently high only in the GST-P-positive liver nodules of D/N rats as opposed to a transient increase in rat livers treated with nodularin or DEN alone. However, it is necessary to discover the molecular targets of PPI and PPIA, which might be deeply involved in the selective atrophy of LL of S/N rats.

A heterogeneous lobe response in hepatocarcinogenesis was observed variously as CCl<sub>4</sub>-induced centrilobular damage in ML and RL and dimethylthiourea-induced damage in LL and RL. Dimethylnitrosamine-induced damage in LL and RL of rat lobe is due to a higher dimethylnitrosamine dehydroxylase activity in the left lobe. Also, incidence of HCC is high in DEN (40 ppm in drinking water) was complete in the left liver compared with 50% in the right anterior lobe, the difference being partially explained by high concentrations of O<sub>2</sub>-ethylthymidine adduct in the left liver, which is pro-mutagenic mainly for the newly synthesized DNA strand during replication.

It has been well established in vivo and in vitro that peroxisome proliferators stimulate hepatocyte division; however, no increase in growth rate of cells was seen in response to nafenopin, as measured by up-regulation of the inducible markers despite the ability of some cells to respond to the drug. Rat hepatoma cell line FaO, derived from Reuber H4IIEC3 hepatoma cells, was recommended as a useful model for assessing the effects of peroxisome proliferators due to its differentiation and sensitive response, as measured with CYP4A1 and PPAR. Since inhibition of PPI and PPIA by nodularin was associated with hepatotoxicity, nodularin-induced apoptosis was further investigated by examining expression of Bcl-X<sub>L</sub> and Bcl-X<sub>L</sub> which have pro-apoptotic and anti-apoptotic functions, respectively. As shown in Figure 5, in contrast to no expression of Bcl-X<sub>L</sub> in RL of S/N rats, the induction of Bcl-X<sub>L</sub> in LL might have driven hepatocytes to apoptosis, whereas the persistent induction of Bcl-X<sub>L</sub> in RL allowed hepatocytes to resist nodularin-induced apoptosis. In contrast, however, in D/N rats, marked proliferation of all 4 lobes could counteract the cytotoxic effect of nodularin by rapidly regaining PPI and PPIA activity (Fig. 3) and concomitant induction of peroxisome proliferation. Microscopic examination revealed that l.p. injection of nodularin caused broad fibrosis and severe atrophy in LL by S/N treatment (S<sub>N</sub>-LL); however, RL was rapidly regenerated from the peri-central necrosis and returned to normal (S<sub>N</sub>-LL) in the same animal. However, D/N treatment produced adenomas with regenerating nodules (D<sub>N</sub>-D) instead of hemorrhagic necrosis or atrophic change.

<table>
<thead>
<tr>
<th>Time (weeks)</th>
<th>Palmitoyl-CoA oxidase (mmol/min/mg protein)</th>
<th>S/N</th>
<th>D/N</th>
<th>S/N</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>0.41 ± 0.18</td>
<td>0.89 ± 0.45</td>
<td>0.81 ± 0.27*</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>1.20 ± 0.35*</td>
<td>2.22 ± 0.43**</td>
<td></td>
<td></td>
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<tr>
<td>10</td>
<td>1.29 ± 0.33*</td>
<td>2.19 ± 0.36**</td>
<td></td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>1.22 ± 0.46*</td>
<td>2.91 ± 0.16**</td>
<td></td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>0.70 ± 0.12*</td>
<td>0.21 ± 0.27**</td>
<td></td>
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</tr>
<tr>
<td>22</td>
<td>0.65 ± 0.17</td>
<td>0.79 ± 0.14</td>
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<td></td>
</tr>
</tbody>
</table>

1Time indicates duration after initiation with either saline or DEN.--25/S, D/N and S/N indicate that saline (S) or DEN (D) by single injection was used as initiator and twice/week injections of saline (S) or nodularin (N) as promoter of carcinogenesis 2 weeks after initiation.--Data indicate means ± SD by 1-way ANOVA.--*p < 0.01 vs. S/N. --**p = 0.000 S/N vs. D/N.
**NODULARIN-INDUCED LEFT-LOBE ATROPHY**

**Figure 4**—Significant induction of CYP4A1 expression vs. no change of CYP2E1 expression in S/N rat livers. Microsomal fractions of LL and RL from S/S, D/N and S/N rats were prepared. Eighty micrograms of the fraction were subjected to Western blot analysis using anti-CYP4A1 (upper panel) and anti-CYP2E1 (lower panel) antibodies. Note the significant induction of CYP4A1 in S/N rats compared with controls, however, treatment with nodularin did not change CYP2E1 expression in either D/N or S/N rat livers compared with controls.

**Figure 5**—Differential induction of Bcl-X expression in LL and RL of S/N rats. Forty micrograms of liver lysate of LL and RL from S/N rats on each lane were subjected to Western blot analysis with anti-Bcl-Xₐₕₕ antibodies. Six rats each week were separately tested for individual variation, and only 2 representatives are shown. Numbers at the top indicate the week after saline initiation. **(a)** Bcl-Xₐ was induced in the LL of S/N rats only during nodularin treatment until week 12, whereas Bcl-Xₐ expression was transient during the early phase of the experiment. **(b)** Induction of Bcl-Xₐ expression in the RL of S/N rats was persistent but extensive; however, expression of Bcl-Xₐ was undetectable in the RL.

PPARα forms heterodimers with ubiquitous factor retinoid X receptor; the heterodimer binds to peroxisome proliferator response element, which is located upstream of the genes associated with peroxisome proliferation. Also, PPARα could induce hepatocarcinogenesis through induction of DNA synthesis and suppression of apoptosis in hepatocytes by treating rats with peroxisome proliferators. In conclusion, nodularin might be a PPARα ligand that can force normal hepatocytes to die and altered hepatocytes to proliferate through regulation of apoptotic gene expression as well as protein phosphatase activity. Further studies on the characterization of nodularin receptor, marked inhibition of PP1/2A and preferential induction of apoptosis in LL of S/N rats should be pursued.
Figure 6 - Comparison of pathologic changes between LL and RL of S/N and D/N rats. Nodularin induced mild to moderate peri-central necrosis in RL of S/N rat livers until week 12 (S2N10-LL, ×100) and S2N10-LL, ×40); however, it was rapidly regenerated 1 week after the cessation of nodularin injection (S2N10-LL, ×40), then returned to normal. 10 weeks later (S2N10-LL, ×40). However, LL of S/N rats showed marked loss of hepatocytes with broad fibrosis and a few regenerating nodules at week 12 (S2N10-LL, ×40), as opposed to adenoma formation (D2N10, ×40) by D/N treatment.
ACKNOWLEDGEMENTS

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REFERENCES

43. Lawson TA, Pound AK. The different susceptibility of rat liver lobes