Carcinogenesis vol.20 no.8 pp.1541–1548, 1999

Effect of nodularin on the expression of glutathione *S*-transferase placental form and proliferating cell nuclear antigen in *N*-nitrosodiethylamine initiated hepatocarcinogenesis in the male Fischer 344 rat

Kye Yong Song, In Kyoung Lim[1,4], Sang Chul Park[2], Seong Oh Lee[1], Hyun Sook Park, Yang Kyu Choi[3] and Byung Hwa Hyun[3]

Department of Pathology, Chung-Ang University College of Medicine, Seoul, [1]Department of Biochemistry, Ajou University School of Medicine, Suwon 442-749, [2]Department of Biochemistry, Seoul National University College of Medicine, Seoul, and [3]Korea Research Institute of Bioscience and Biotechnology, Yusong, Korea

[4]To whom correspondence should be addressed
Email: iklim@madang.ajou.ac.kr

The tumor-promoting effect of nodularin during carcinogenesis was investigated. Male Fischer 344 rats were injected with nodularin for 10 weeks from week 3 after *N*-nitrosodiethylamine initiation without partial hepatectomy. Rats were further maintained for 10 weeks after the cessation of nodularin and were periodically killed. In contrast to the minimal foci in the DEN and nodularin alone groups, treatment with DEN and nodularin produced four kinds of nodules with eosinophilic, clear, mixed and basophilic cells. After the cessation of nodularin, the maximally increased number, but not the area, of glutathione *S*-transferase placental form-positive [GST-P(+)] nodules at week 12 decreased significantly and the appearance of two types of hyperplastic nodules was noted by GST-P immunostaining; homogeneously stained dense nodules (DN) and heterogeneously stained pale nodules (PN), which appeared only after the cessation of nodularin. DN were well circumscribed by enzyme-altered cells, as opposed to poorly in PN. Moreover, normal-appearing hepatocytes replaced the enzyme-altered cells in PN. In contrast to the higher PCNA index in GST-P(+) DN, the background level returned to that of the control at week 15. PCNA indices in DN were significantly higher than in PN, which were still higher than the control, indicating that nodularin affected the PCNA index differentially in the altered and unaltered hepatocytes. However, nodularin without DEN initiation significantly increased the PCNA index through initial cell death and subsequent hepatocyte proliferation. These results suggest that: (i) nodularin has a promoting effect by inducing hepatocyte proliferation in both enzyme-altered hyperplastic nodules and the surrounding parenchyma; (ii) proliferation is transient in background cells but not in enzyme-altered hepatocytes; (iii) GST-P(+) DN can be regarded as progressive and GST-P(+) PN as regressive, revealed by both immunohistochemistry and PCNA index.

Abbreviations: B, basophilic foci; C, clear foci; DEN, *N*-nitrosodiethylamine; DN, GST-P(+) dense nodule; E, eosinophilic foci; GST-P, glutathione *S*-transferase placental form; H&E, hematoxylin and eosin; HCC, hepatocellular carcinoma; HN, hyperplastic nodules; M, mixed foci; PCNA, proliferating cell nuclear antigen; PN, GST-P(+) pale nodule.

Introduction

Nearly all types of primary liver tumors known to occur in humans can be produced by chemicals in laboratory animals, especially in rats (1). The malignant variants of these neoplasms have been classified as hepatocellular carcinoma (HCC), cholangiocellular carcinoma, angiosarcomas and perisinusoidal (Ito) cell sarcomas (2). The sequential cellular and molecular changes preceding these neoplasms have been well documented in experimental chemical hepatocarcinogenesis models (3–5). Preneoplastic foci of altered hepatocytes emerge weeks or months before the appearance of hepatocellular adenomas and HCCs in experimental hepatocarcinogenesis (6–10) and they have also been discovered in humans bearing hepatocellular neoplasms and/or suffering from liver cirrhosis (11). Their early appearance and progression to hepatocellular adenomas and HCCs in experimental animal models and humans offer one of the most promising approaches to the elucidation of the molecular mechanisms of neoplastic conversion of hepatocytes.

It has previously been reported that the incidence of human primary liver cancer in Quidong and neighboring cities, where the people drink pond and ditch water, is about eight times higher than in the other provinces of the People's Republic of China (12) and it has also been pointed out that the drinking water in these areas is one of the high risk factors for primary liver cancer, in addition to other factors such as aflatoxin B1 and hepatitis B and C virus infection, which are highly prevalent in Korea (13–17) and Japan (18–20), respectively. These waters are highly contaminated with the cyanobacteria *Nodularia spumigena* and *Microcystis aeruginosa*, which produce nodularin (21) and the structurally related microcystin-LR (22), respectively. These tumor-promoting hepatotoxins were also identified in mussels (23). When ingested, the hepatotoxin nodularin has been shown to induce death and extensive liver damage (24), therefore representing a significant health hazard to humans and agricultural livestock. Nodularin and microcystin-LR belong to the okadaic acid-type tumor promoters (25), which inhibit protein phosphatase types 1 and 2A (26,27). These phosphatases are known to play significant roles in signal transduction pathways pertaining to cell proliferation, gene expression and neurotransmission. We have recently reported that 1 mM 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 (28) and that i.p injection of nodularin (25 μg/kg) induced expression of these proto-oncogenes in rat liver, which persisted for up to 24 h. These findings suggest that mRNA was stabilized by the inhibition of protein phosphatases 1 and 2A, which constitutes a critical step in tumor promotion (29).

In the Solt–Farber (30) model of multistage hepatocarcinogenesis in the liver, *N*-nitrosodiethylamine (DEN), as a well-known initiator (31,32), and 2-acetylaminofluorene, as a promoting agent, are administered in combination, with partial hepatectomy in the middle of the promotion period. In the

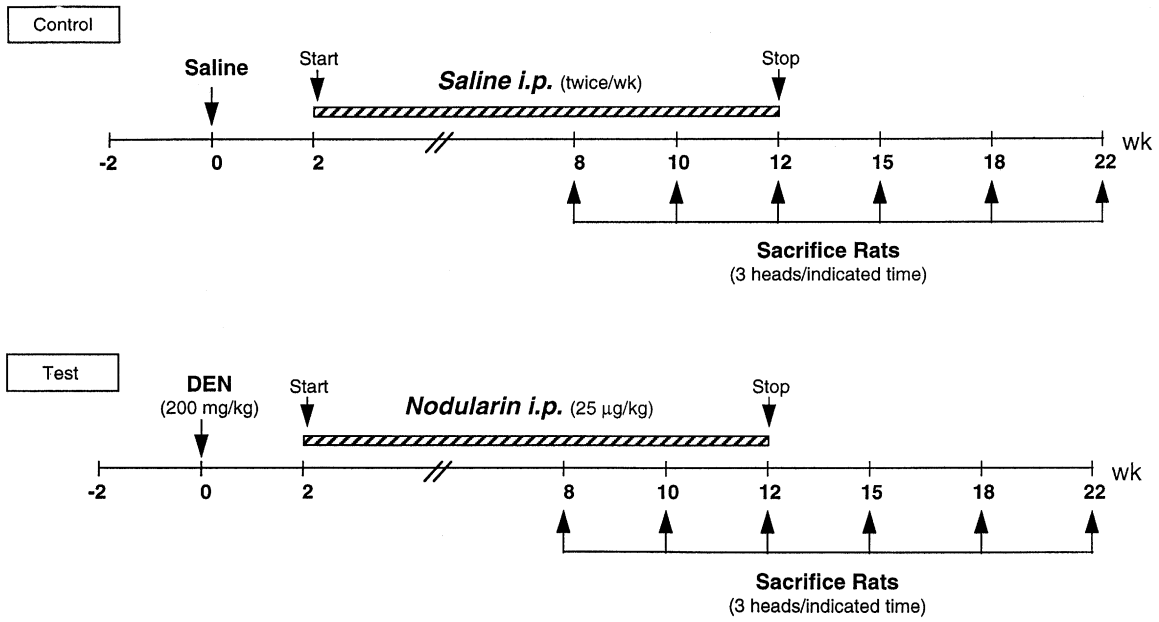

Fig. 1. Experimental scheme of DEN/nodularin-induced hepatocarcinogenesis. Rats were acclimatized for 2 weeks before the initiation and experimental groups were divided into control, nodularin only-, DEN only- and DEN/nodularin-treated groups. A single injection of DEN (200 mg/kg body wt i.p.) was given into 7-week-old male F344 rats of the DEN only- and DEN/nodularin-treated groups; saline was used as a control. From 2 weeks later, the nodularin only- and DEN/nodularin-treated rats were injected bi-weekly with nodularin (25 μg/kg body wt) until week 12. Rats were maintained until week 22 without further nodularin injection. The control and the test rats were killed at the end of weeks 8, 10, 12, 15, 18 and 22.

present study, employing DEN as an initiator and nodularin as a liver-specific promoter, we attempted to demonstrate a tumor-promoting effect of nodularin without partial hepatectomy and raise the following questions: (i) what was the outcome of hyperplastic nodules (HN) after the cessation of nodularin injection; (ii) how active was hepatocyte proliferation in the HN as compared with that in the background parenchyma with or without nodularin treatment. Our experiment demonstrated that nodularin could induce HCCs through selective proliferation of the enzyme-altered preneoplastic hepatocytes with a persistent induction of proliferating cell nuclear antigen (PCNA).

Materials and methods

Materials

DEN, 3,3′-diaminobenzidine and hydrogen peroxide were purchased from Sigma Chemical Co. (St Louis, MO). Nodularin was purified by Dr W.W. Carmichael at Wright State University (Dayton, OH). The polyclonal antibody against glutathione *S*-transferase placental form (GST-P) was prepared in one of our laboratories by the published method (33). The anti-PCNA antibody and LSAB universal ABC kit were purchased from Dako Co. (Copenhagen, Denmark) and hematoxylin and eosin (H&E) were from Merck (Darmstadt, Germany).

Maintenance of animals

Seven-week-old male F344/KIST rats (Genetic Resources Center, Korea Research Institute of Bioscience and Biotechnology, Yusong, Korea) were bred in an inbred colony. All cages and bedding were autoclaved before use and stored in a separate room. The animals were provided with an irradiated and microbe controlled diet (PicoLab Rodent Diet 20, 5053; PMI Feeds) and 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.

DEN/nodularin-induced hepatocarcinogenesis

Experimental groups were divided into control, nodularin only-, DEN only- and DEN/nodularin-treated groups. Initiation was by a single i.p. injection of DEN (200 mg/kg of body wt, D0258; Sigma, St Louis, MO) into 7-week-old F344/KIST male rats; saline was injected into control animals. Two weeks after initiation, the nodularin only and DEN/nodularin groups were injected with nodularin (25 μg/kg body wt twice/week) until week 12. The rats were further maintained until week 22 without nodularin injection (Figure 1). Control and test rats were killed at the end of weeks 8, 10, 12, 15, 18 and 22; animals were killed routinely starting at 10:00 a.m. in order to minimize *in vivo* diurnal variations. Six animals were used for each experiment. Resected livers were fixed in 10% formalin solution for routine histological and immunohistochemical studies.

Immunohistochemical staining

Liver tissues were fixed overnight in 10% formalin solution by a routine procedure for paraffin embedding, then serially sectioned (5 μm) and stained with hematoxylin and eosin (H&E) to verify morphological changes. Immunohistochemical analysis was performed by a standard technique using the ABC (avidin–biotin complex) peroxidase method according to the procedures recommended in the LSAB universal kit (Dako). In brief, sections were incubated with polyclonal antibody to GST-P (1:400), prepared in New Zealand white rabbits using purified GST-P from human placental tissues (33), and monoclonal anti-PCNA antibody (1:3000; Dako), followed by ABC–peroxidase complex. Substrates were hydrogen peroxide with coloring agent 3,3′-diaminobenzidine (yellow brown) for GST-P and aminoethyl carbazole (red) for PCNA. H&E counterstaining was used. Negative controls were made by replacing the antibodies with identical dilutions of non-immune rabbit and mouse IgG, respectively.

Differential counting of GST-P(+) nodules

Areas of eosinophilic (E), clear (C), mixed (M) and basophilic (B) foci and nodules were drawn with an Olympus drawing attachment (model BH2-DW; Olympus, Tokyo, Japan) under an Olympus model BH2 light microscope (×20) at 100 times actual size. Five representative fields of the sections from each group were drawn and total positive area (mm^2/cm^2) of the nodules was calculated after measuring with an area curvimeter (model 360II+; Ushikata, Tokyo, Japan). Criteria for E, C, M and B nodules were as follows: if the cytoplasm was eosinophilic, it was counted as E, or as C if it was clear of glycogen; if it was a mixture of eosinophilic and clear cells with 30–50% of each component it was counted as M; when the cytoplasm was either basophilic or amphophilic, it was counted as B. Areas of GST-P(+) nodules were also drawn by the described method with an Olympus drawing attachment under an Olympus model BH2 light microscope (×20). The frequencies of GST-P(+) nodules were counted as the numbers per cm^2 in the same fields where the areas were measured and were re-calculated as the numbers per cm^2 of the liver sections. Because two kinds of staining characteristics were noted in the nodules, depending on the presence or absence of nodularin injection, we divided the nodules into homogeneously stained GST-P(+) dense nodules (DN) and heterogeneously stained GST-P(+) pale nodules (PN) and differential counting was performed.

Differential counting of PCNA expression

In order to investigate differences in proliferation activity between GST-P(+) nodules and the surrounding hepatocytes, serially sectioned slides were

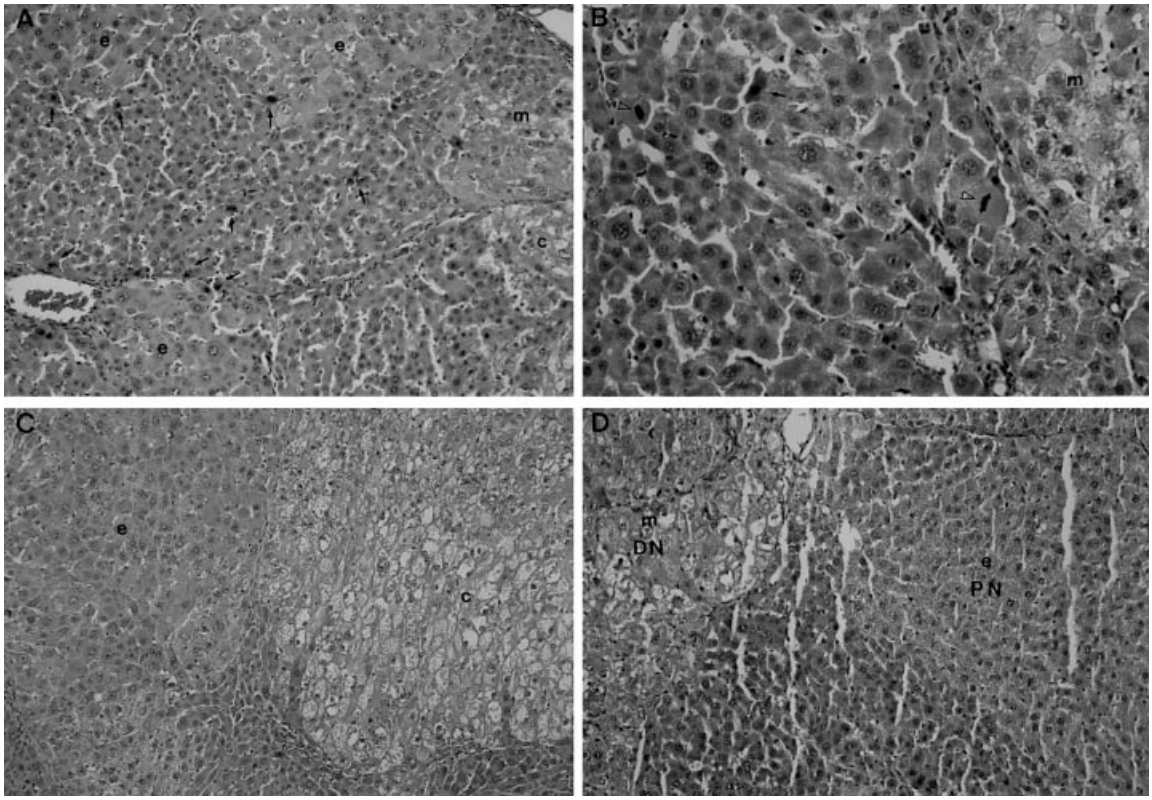

Fig. 2. H&E staining of the rat livers during DEN/nodularin-induced carcinogenesis in male Fischer 344 rats. Small eosinophilic (e), mixed (m) and clear cell (c) nodules are noted with accompanying apoptotic cells (arrow) at week 12 (×100) (**A**). Proliferating hepatocytes with basophilic cytoplasm show a few mitotic (open arrowhead) and apoptotic figures (arrow) at week 12 around the mixed (m) nodule (×200) (**B**). Large nodules composed of eosinophilic or clear cells are also formed by week 12 (×100) (**C**). The large mixed (m) and eosinophilic (e) nodules correspond to the DN and the PN, respectively, when the serial sections were stained with anti-GST-P antibody at week 22 (**D**). Note hepatocytes of the PN which closely resembled normal cells (×100).

examined by immunohistochemical staining with anti-GST-P and anti-PCNA antibodies. PCNA-positive nuclei located in the DN or PN or the surrounding cells were differentially counted with the Optimas 6.2 image analysis program, (Media Cybernetics, Silver Spring, MD) from 10 fields per group and the mean value per 100 hepatocytes was calculated as the PCNA labeling index. Only strongly positive nuclei (bright red by aminoethyl carbazole) were counted as positive.

Statistical analysis

Frequency and area of hyperplastic foci and nodules stained with H&E and anti-GST-P were analyzed by the one-way ANOVA test and the PCNA labeling index was analyzed by the Mann–Whitney *U*-test. Statistical significance was determined by multiple comparisons after the ANOVA test and by two-tailed asymmetric significance after the Mann–Whitney *U*-test.

Results

Experimental schedule

We induced hepatocarcinogenesis in male Fischer 344 rats using DEN as an initiator and nodularin as a promoter without partial hepatectomy. In order to investigate a promoting effect of nodularin, the experimental animals were maintained for 10 weeks after the cessation of nodularin administration, which continued for 10 weeks from week 3 after initiation (Figure 1).

Changes in hyperplastic nodules revealed by H&E staining

Hepatocyte proliferation was very active at week 12 in the DEN/nodularin-treated rat livers. Four kinds of foci and nodules (e, c, m and b) were revealed by H&E staining (Figure 2) and many apoptotic figures as well as mitotic cells were frequently observed around nodules at week 12 (Figure 2A and B). However, after the cessation of nodularin injection, mitotic figures significantly decreased and apoptosis was not apparent either inside or outside the nodules. Nodules observed at week 22, corresponding to 10 weeks after nodularin cessation, were filled with normal-appearing hepatocytes (Figure 2D). They were confirmed as PN by the use of anti-GST-P antibody, whereas cells of the GST-P(+) DN showed hepatocytes of altered shape.

The area of eosinophilic nodules was significantly increased at week 12 as compared with week 10 (Table I) and was not changed significantly after cessation of nodularin injection. Basophilic nodules emerged at week 15, corresponding to 3 weeks after cessation of nodularin treatment.

Table I. Areas of hyperplastic nodules developed during DEN/nodularin-induced hepatocarcinogenesis in male Fischer 344 rats

Duration (weeks)	Areas of foci and nodules examined by H&E staining (mm^2/cm^2 liver section)			
	Eosinophilic	Clear cell	Mixed	Basophilic
8	1.14 ± 0.21	0.37 ± 0.19	0.44 ± 0.19	0
10	3.23 ± 0.64	0.38 ± 0.27	1.18 ± 0.48	0
12	13.43 ± 1.82[a]	0.90 ± 0.49	5.20 ± 2.96	0
15	10.47 ± 3.22[b]	1.54 ± 0.97	5.75 ± 1.92	1.30 ± 0.80
18	13.79 ± 4.08[a]	1.31 ± 0.77	3.01 ± 2.00	2.29 ± 0.84
22	12.06 ± 0.77[a]	1.28 ± 0.94	7.53 ± 2.12	3.92 ± 2.17

Mixed indicates a mixture of eosinophilic and clear cell nodules with 30–50% of each component. All the values represent means ± SE by one-way ANOVA test.
[a] $P < 0.01$ between week 10 versus the test.
[b] $P < 0.05$ between week 10 versus the test.

Changes in GST-P(+) nodules depend on injection of nodularin

Depending on the injection or cessation of nodularin treatment, GST-P(+) nodules in the DEN/nodularin-treated rat livers were of two kinds; homogeneously stained DN and heterogeneously stained PN (Figure 3). The frequency of GST-P(+) DN was maximum at week 12 (124.5 ± 38.3), however, it was significantly diminished at week 18 (60.0 ± 11.4), corresponding to 6 weeks after cessation of nodularin injection (Table II). The frequencies of GST-P(+) nodules in the positive control (nodularin or DEN only groups) remained low during the entire experimental period. After the cessation of nodularin injection, a portion of the GST-P(+) nodules was transformed from DN to PN. Frequencies of PN were 14.5 ± 2.9 at week 15 and 18.0 ± 4.5 at week 22 (Table II).

The total area of the GST-P(+) nodules increased significantly during DEN/nodularin treatment and reached a maximum at week 12 (47.5 ± 13.5 mm^2/cm^2). However, it was significantly decreased 6 weeks after the cessation of nodularin, with concomitant development of PN (Table III). Areas of PN were 4.8 ± 1.1 mm^2/cm^2 at week 15 and 7.8 ± 3.3 mm^2/cm^2 at week 22. These findings indicate that many nodules regressed with cessation of nodularin injection, although some nodules still progressed and continued to increase in size. Again, the areas of the GST-P(+) nodules in the nodularin and DEN alone groups were unchanged (Tables III).

Nodularin-dependent changes in PCNA expression are more sensitive in surrounding hepatocytes

In order to examine the proliferative effect of nodularin during carcinogenesis, we performed immunohistochemistry with step-sectioned slides using anti-GST-P (Figure 3A, C, E and G, ×40) and anti-PCNA antibodies (Figure 3B, D, F and H, ×100). Since proliferation of hepatocytes in both the nodules and the adjacent background increased significantly until week 12, we could not differentiate between the PCNA activity in the DN and that in the surrounding tissue during injection of nodularin (compare Figure 3A and B with C and D). However, after the cessation of nodularin treatment proliferation of hepatocytes in the background parenchyma markedly diminished, with the appearance of GST-P(+) PN (Figure 3E and F versus G and H), as opposed to the remaining high activity in the GST-P(+) DN until week 22.

Nodularin alone without DEN initiation strongly induced hepatocyte proliferation until week 18 as compared with the control rat livers (Table IV). Concurrently, proliferation indices of hepatocytes in the DN, PN and the surrounding background were differentially counted until week 22. PCNA activity markedly increased in the background hepatocytes (43.9 ± 9.8 nuclei/100 hepatocytes) at week 12 as well as in the DN (34.3 ± 5.0) as compared with the control (8.5 ± 1.5). However, hepatocyte proliferation in the surrounding background became minimal at week 15 (11.4 ± 2.9), 3 weeks after the cessation of nodularin, whereas hepatocytes in the DN retained significantly higher activity (30.5 ± 7.3) as compared with the background. Proliferation indices of the PN remained slightly higher than the control until week 22, however, they were much lower than those in the DN. This indicated that the altered hepatocytes consistently proliferated, as opposed to the non-proliferative background parenchyma, after cessation of nodularin injection. Figure 4A and B shows a dramatic change in PCNA expression between the inside and outside of the HN at weeks 12 and 15, respectively (×40). DEN/nodularin treatment stimulated proliferation of the surrounding hepatocytes as well as the nodule itself. Interestingly, the periphery of the GST-P(+) DN showed higher PCNA activity than in the center of the nodule. The size of some GST-P(+) nodules increased markedly at week 22, while PCNA expression returned to the basal level after the cessation of nodularin injection.

Discussion

We reported earlier (34) that, when a synthetic choline-deficient diet containing 0.06% 3′-methyl-4-dimethylaminoazobenzene was fed to Sprague–Dawley male rats for 12 weeks and the animals were maintained for a further 4 weeks after switching to the control diet, epithelial cells lining the bile duct were the predominant transformed cell type a few weeks after feeding of the experimental diet, and cholangiocarcinoma, HCCs and mixed type liver tumors developed in all of the rats tested 16 weeks later. In contrast, as described in this report, with a single i.p. injection of DEN and bi-weekly injections of nodularin to male Fischer 344 rats for 10 weeks from week 3 after DEN initiation without partial hepatectomy, changes were mainly produced in hepatocytes, but not in the lining epithelial, endothelial or perisinusoidal cells of the bile duct until week 22. In the present study, DEN and nodularin treatment induced a large number of GST(+) foci and nodules at week 12, however, the numbers significantly diminished after the cessation of nodularin injection, with a concomitant appearance of heterogeneously stained GST(+) PN (Table II). The areas of the nodules also increased significantly and reached a peak at week 12, thereafter decreasing until week 22, but still maintaining an enhanced level as compared with that in week 10. This indicated carcinogenic progression even after the cessation of nodularin treatment (Table III). However, the cessation of nodularin injection changed the GST-P stainability from homogeneous to heterogeneous (Figure 3E and G). In contrast, injections of either DEN or nodularin alone did not produce any significant changes in the GST-P(+) foci

Fig. 3. Changes in GST-P(+) staining and expression of PCNA on treatment with nodularin. (**A**) A few GST-P(+) foci are noted at week 8 of DEN/nodularin-induced hepatocarcinogenesis (×40). (**B**) The next section to Figure 2A. Strong positive nuclei stained with anti-PCNA antibody are few among the background nuclei, week 8 (×100). (**C**) Enlarged GST-P(+) nodules are noted at week 12. All nodules are homogeneous and stain densely (DN) for GST-P (×40). (**D**) The next section to Figure 2C. Markedly increased strong PCNA-positive red nuclei are noted in a background of DN as well as inside the nodules at week 12 (×100). DN correspond to the same DN shown in Figure 2C (×100). (**E**) Marked changes in GST-P stainability in nodules at week 15, corresponding to 3 weeks after the cessation of nodularin injection. Two kinds of GST-P(+) nodules are noted; homogeneously stained DN and heterogeneously stained PN. Note the disappearance of GST-P staining in many nodules (×40). (**F**) The next section to Figure 2E at week 15 showing the activity of PCNA. Strong positive red nuclei still remained in the GST-P(+) DN, but the frequency was diminished as compared with that at week 12. PCNA expression in the GST-P(+) PN revealed the same level as in the background (×100). (**G**) GST-P(+) nodules at week 22, 10 weeks after the cessation of nodularin treatment. Note the increased size of the GST-P(+) DN and almost complete disappearance of GST-P staining in the PN (x40). (**H**) The next section of Figure 2G at week 22 showing the activity of PCNA. A few PCNA-positive red nuclei are noted in the respective area of the DN. The PCNA activity has disappeared in the respective area of the PN as well as the background hepatocytes (×100).

until week 22. These results suggest that the GST-P(+) DN might be proliferating, while the GST-P(+) PN belonged to regressing nodules. In order to verify this postulate, we compared step-sections of GST-P(+) nodules with their PCNA indices (Table IV) and confirmed the above possibility.

Significant changes in GST-P(+) nodules were observed in the DEN/nodularin-treated group, in which progressive DN and regressive PN developed after the cessation of nodularin injection (Figure 3E–H), and there was also a large difference in PCNA indices between the background and the DN (Table IV). These findings indicate that the effect of nodularin in the DEN/nodularin-treated group differed between the nodal and

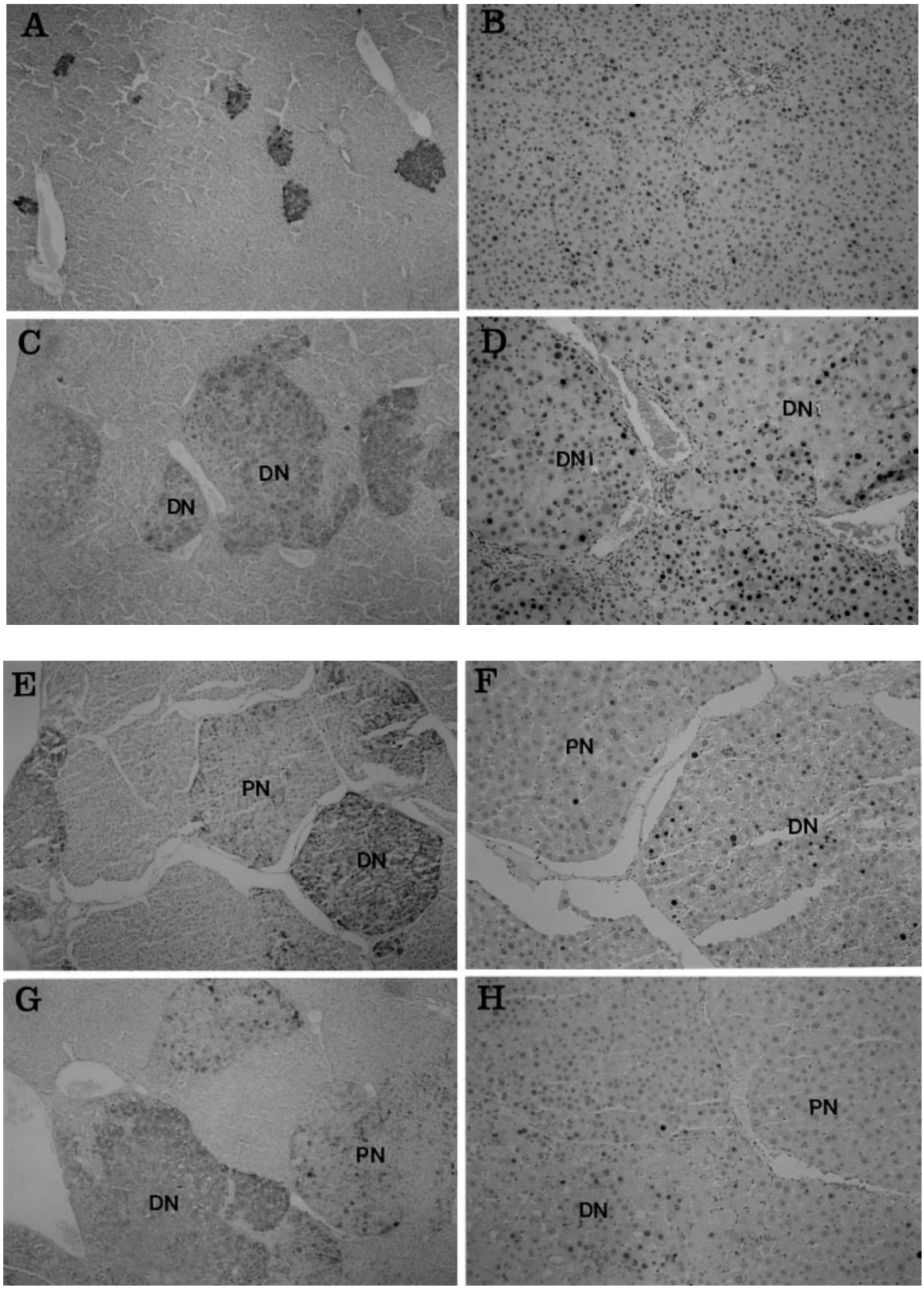

Table II. Frequency of GST-P(+) HN during DEN/nodularin-induced hepatocarcinogenesis in male Fischer 344 rats

Duration (weeks)	GST-P(+) HN frequency (no./cm^2)			
	Nodularin only	DEN only	DEN/nodularin	
			DN	PN
Control	0	0	0	0
8	6.4 ± 2.0	11.4 ± 2.0	71.0 ± 43.0	0
10	6.8 ± 2.0	10.0 ± 2.1	87.8 ± 16.2	0
12	8.5 ± 2.4	9.5 ± 2.0	124.5 ± 38.3[a]	0
15	7.5 ± 1.3	6.9 ± 0.9	83.7 ± 6.8	14.5 ± 2.9
18	5.2 ± 1.0	6.4 ± 1.8	60.0 ± 11.4[b]	13.2 ± 4.5
22	7.2 ± 3.6	7.6 ± 2.2	59.4 ± 17.8[b]	18.0 ± 4.5

Control indicates the value obtained from rats injected with saline for 8 weeks. Duration indicates the time after DEN initiation. All the values represent means ± SD by one-way ANOVA test.
[a]$P < 0.05$ between 8 versus 12 weeks.
[b]$P < 0.05$ between week 12 versus the test.

Table III. Change in GST-P(+) areas during DEN/nodularin-induced carcinogenesis in male F344 rats

Duration (weeks)	GST-P(+) area change (mm^2/cm^2)			
	Nodularin only	DEN only	DEN/nodularin	
			DN	PN
Control	0	0	0	0
8	1.6 ± 0.4	1.2 ± 0.4	3.8 ± 1.5	0
10	1.8 ± 0.3	1.0 ± 0.4	10.6 ± 1.4	0
12	2.9 ± 0.7	1.1 ± 0.3	47.5 ± 13.5[a]	0
15	2.2 ± 0.8	1.2 ± 0.6	33.0 ± 5.4[a]	4.8 ± 1.1
18	2.2 ± 0.5	1.2 ± 0.5	31.5 ± 7.5[a,b]	6.7 ± 2.0
22	2.1 ± 0.2	1.1 ± 0.2	37.45 ± 9.7[a]	7.8 ± 3.3

Control indicates the value obtained from rats injected with saline for 8 weeks. Duration indicates the time after DEN initiation. Homogeneously or heterogeneously stained GST-P(+) nodules were counted under a light microscope at ×20 magnification. All the values represent means ± SD by the one way ANOVA test.
[a]$P < 0.001$ between week 10 versus the test
[b]$P < 0.05$ between 12 versus 18 weeks.

the extranodal liver parenchyma. However, the increased PCNA index in the nodularin alone group returned to the control level at week 22. PCNA indices in HN remained high even after the nodularin treatment was discontinued. In contrast

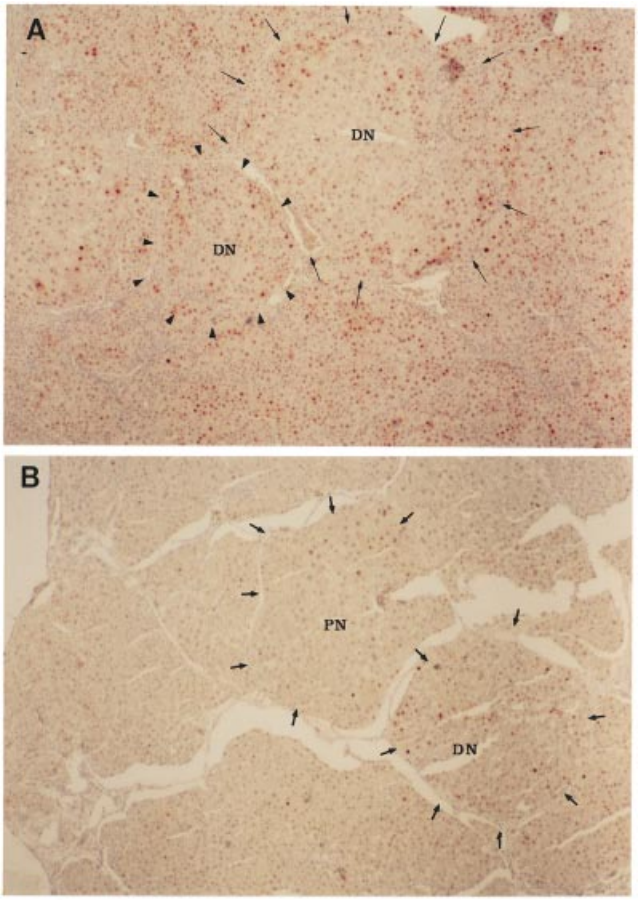

Fig. 4. Proliferation activity of hepatocytes in the HN and their surrounding background depending on the nodularin treatment (×40). (**A**) Numerous positive red nuclei are noted by anti-PCNA immunostaining in the background hepatocytes at week 12 as well as in the DN. Note the higher PCNA activity at the periphery of DN. (**B**) A marked decrease in PCNA-positive nuclei in the PN as well as in the background of nodules at week 15, corresponding to 3 weeks after the cessation of nodularin treatment. GST-P(+) DN also revealed much lower activity of PCNA.

Table IV. PCNA index in the DN and PN and their adjacent background hepatocytes during DEN/nodularin-induced carcinogenesis in male F344 rats

	Number of PCNA(+) nuclei/100 hepatocytes					
	Week 8	Week 10	Week 12	Week 15	Week 18	Week 22
Control			8.5 ± 1.5			
Nodularin only	28.6 ± 2.3[a]	36.8 ± 7.6[a]	38.4 ± 9.2[a]	28.6 ± 9.2[a]	15.6 ± 2.8[a]	10.2 ± 1.8
DEN only	14.5 ± 5.3[b]	15.4 ± 2.4[b]	14.6 ± 5.2[b]	13.0 ± 1.3[b]	10.7 ± 1.1[b]	10.6 ± 3.2
DEN/nodularin						
Background	24.5 ± 3.0[a]	34.1 ± 3.3[a]	43.9 ± 9.8[a]	11.4 ± 2.9	9.5 ± 1.2	10.9 ± 4.0
DN	18.8 ± 2.1[a]	29.7 ± 6.2[a]	34.3 ± 5.0[a]	30.5 ± 7.3[a]	30.9 ± 2.9[a]	32.6 ± 7.1[a]
PN	0	0	0	18.0 ± 4.2[a,c]	17.3 ± 1.3[a,c]	12.2 ± 3.4[c]

Control indicates the means ± SD from rats injected with saline between weeks 8 and 22. All the values represent means ± SD by the Mann–Whitney *U*-test.
[a]$P < 0.01$ between the control versus test.
[b]$P < 0.05$ between the control versus test.
[c]$P < 0.01$ between the DN versus PN at the corresponding week.

to the DEN alone group, multiple injections of nodularin alone induced significant hepatocyte proliferation until week 18, accompanied by focal liver necroses. This indicates that hepatocyte proliferation due to nodularin injection was a consequential phenomenon in liver after the initial cell death. In conclusion, we suggest that nodularin can exert a pressure to replicate on initiated cells through the induction of cell death and subsequent hepatocyte proliferation, resulting in HN formation. However, the extranodal hepatocytes, which are not genetically transformed, resume their normal status after the cessation of nodularin injection.

The observation that expression of PCNA dramatically decreased in the background hepatocytes and the GST-P(+) PN, but not the GST-P(+) DN, after the cessation of nodularin injection (Figure 4) was of a great interest. PCNA is an important factor for the processive polymerase activity of DNA polymerase δ (35–39), the principal replicative DNA polymerase during DNA replication and repair. Significant induction of the regenerative activity of the surrounding hepatocytes might play an essential role in transformation of hepatocytes to enzyme-altered HN. In the present study, the PCNA index of PN was found to be significantly reduced as compared with that in DN (Table IV). Enzyme-altered hepatocytes composed of HN might induce remodeling of the nodules through conversion to or replacement by normal-appearing hepatocytes. Indeed, GST-P(+) PN without a cirrhotic background (Figure 3G) which were not completely circumscribed by enzyme-altered cells clearly demonstrated remodeling of the PN by replacement with normal-appearing hepatocytes (Figure 2D). These findings indicated to us regression of the nodules, a regression from the altered phenotype to normal liver cells.

In summary, the above findings present evidence for an effect of nodularin as a tumor promoter by imposing proliferative pressure on initiated hepatocytes, with the persistent presence of highly proliferating GST-P(+) DN even after its cessation, in non-hepatectomized rat livers.

Acknowledgements

We deeply appreciate Professors Peter Bannasch and Woon Ki Paik for their critical reading of this manuscript and encouraging us during this work. The authors thank Dr Hirota Fujiki for his generous donation of nodularin. This work was supported in part by research grants 96-0403-19-01-3 from the Korea Science and Engineering Foundation to I.K.L. and HMP-96-M-3-0033 from the Korea Ministry of Health and Welfare to I.K.L. and S.C.P.

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Received October 15, 1998; revised April 23, 1999; accepted April 26, 1999