Nodularin, a Potent Inhibitor of Protein Phosphatases 1 and 2A, Is a New Environmental Carcinogen in Male F344 Rat Liver

Tetsuya Ohta, Elsaburo Sueoka, Naoyuki Iida, Atsumasa Komori, Masami Suganuma, Rie Nishiwaki, Masae Tatematsu, Seong-Jin Kim, Wayne W. Carmichael, and Hirotu Fujita

Saitama Cancer Center Research Institute, 818 Komuro, Iwa, Kitadaichi-gun, 362 Saitama, Japan; [T. O., E. S., N. I., A. K., M. S., R. N., H. F.]; Aichi Cancer Center Research Institute, Nagoya 464, Japan [M. T.]; Laboratory of Chemoprevention, National Cancer Institute, Bethesda, Maryland 20892 [S. J. K.]; and Department of Biological Sciences, Wright State University, Dayton, Ohio 45435 [W. W. C.]

ABSTRACT

Nodularin and microcystin-LR are cyanobacterial toxins and environmental hazards. Nodularin inhibits protein phosphatases 1 and 2A with the same potency as does microcystin-LR, which has recently been identified as a potent tumor promoter in rat liver. Our results suggested that nodularin is also a new tumor promoter in rat liver. A two-stage carcinogenesis experiment in rat liver initiated with diethylnitrosamine and without partial hepatectomy revealed that nodularin stimulated glutathione S-transferase plasmid form-positive foci in rat liver more effectively than did microcystin-LR, and that nodularin alone induced glutathione S-transferase plasmid form-positive foci as well as diethylthiorosamine alone. Thus, nodularin itself is a new liver carcinogen, and microcystin-LR is a tumor promoter rather than a carcinogen. Nodularin induced hyperphosphorylation of cytokeratin peptides 8 and 18 in primary cultured rat hepatocytes 20% more effectively than did microcystin-LR, suggesting that nodularin penetrates more easily into the hepatocytes than does microcystin-LR. Nodularin upregulated induction of c-jun, jun-B, jun-D, c-fos, fos-B, and fra-1 mRNA transcripts in rat liver after i.p. administration, and the accumulation of the mRNA transcripts was sustained for over 9 h after treatment. The environmental hazards of cyanobacterial toxins are discussed in relation to the known primary liver cancer in Qldong county in the People's Republic of China. Our results support this hypothesis and indicate the need for prevention measures against cyanobacterial toxins.

INTRODUCTION

Nodularin isolated from the toxic brackish water cyanobacterium Nodularia spumigena is a hepatotoxic and cyclic pentapeptide (1, 2) and is structurally similar to the cyclic heptapeptide microcystin-LR. They both commonly contain Adda (Fig. 1). We reported previously that microcystin-LR and nodularin were similarly potent inhibitors of protein phosphatases present in a cytosolic fraction of mouse liver (3), and that microcystin-LR inhibited PP-1 and PP-2A with the same potency, about 0.1 nM of the 50% inhibitory concentration (4). A recent investigation using computer-assisted molecular modeling of nodularin and microcystin-LR revealed that these two molecules have a similar molecular orientation with respect to the Adda portion, the peptide ring, and the two acidic groups (5). A similar compound, motuporin, was isolated from a marine sponge collected at Motupoa island in Papua New Guinea, and has a valine replacing arginine of nodularin (6). The evidence provides us with important information that nodularin is instead another member of a group of obviously related agents, and that nodularin is a widespread contaminant, occurring in toxic brackish water cyanobacterium as well as marine organisms.

As for interest in microcystin-LR and cancer research, we recently reported that repeated i.p. administrations of microcystin-LR stimulated an increase in the number and percentage areas of GST-P-positive foci in rat liver initiated with DEN in a dose-dependent manner, followed by partial hepatectomy. We concluded that microcystin-LR is a potent liver tumor promoter which mediates its action through inhibition of PP-1 and PP-2A (7). Structural and functional similarity indicate that nodularin might be a liver tumor promoter as microcystin-LR, and that this potency may allow the partial hepatectomy procedure to be deleted from the two-stage carcinogenesis experiments in rat liver treated with DEN plus microcystin-LR. We report here for the first time that repeated i.p. administrations of nodularin induced tumor promotion in rat liver initiated with DEN and without partial hepatectomy, and that a total of about 150 μg nodularin alone per rat induced the number of GST-P-positive foci comparable to the number of foci induced by a total of about 60 mg DEN alone per rat. These results showed that the potency of nodularin that induced similar numbers of GST-P-positive foci was about 400 times stronger than that of DEN, suggesting that nodularin has an initiating activity. Nodularin is thus suggested to be a new class of carcinogens, possessing inhibitory activity against PP-1 and PP-2A. Whether nodularin is mutagenic is not yet known. Inhibition of PP-1 and PP-2A by nodularin resulted in morphological changes in cells because of an increase in various phosphoproteins, two of which were identified in primary cultured rat hepatocytes to be cytokeratins 8 and 18. The i.p. administration of nodularin induced expression of the fos and jun family of proto-oncogenes in rat liver, which is a critical step in tumor promotion. This paper reports carcinogenesis of nodularin with respect to its tumor-promoting and tumor-initiating activities, its mechanisms of action, and its environmental significance, all of which are discussed in relation to the known tumor promoter microcystin-LR.

MATERIALS AND METHODS

Chemicals. Nodularin was extracted from Nodularia spumigena strain L575 isolated from Lake Ellesmere, New Zealand (1, 2). Microcystin-LR was purified from lyophilized cells of laboratory-cultured Microcystis aeruginosa, PCC-7820, and natural bloom material, dominated by Microcystis aeruginosa (8). DEN was purchased from Wako Chemical Co. (Osaka, Japan). A monoclonal anti-cytokeratin antibody, K8.13, was purchased from ICN Biomedicals California (San Diego, CA); jun-B (1.8-kilobase EcoRI fragment) by R. Bravo at European Molecular Biology Laboratory, Heidelberg, Germany (9); fos-B (XbaI/BamHI fragment) by R. Bravo at European Molecular Biology Laboratory, Heidelberg, Germany (9); c-fos (1.7-kilobase EcoRI fragment) by D. Nathans at Johns Hopkins University School of Medicine, Baltimore, MD; c-jun (2.1-kilobase BamHI fragment) by M. Karin at University of California, San Diego, CA; jun-B (1.8-kilobase EcoRI fragment) and jun-D (1.7-kilobase EcoRI fragment) by D. Nathans at Johns Hopkins University School of Medicine, Baltimore, MD; fos-B (XbaI/BamHI fragment) by R. Bravo at European Molecular Biology Laboratory, Heidelberg, Germany (9); 5-end labeled probe was isolated from a marine sponge collected at Motupoa island in Papua New Guinea, and has a valine replacing arginine of nodularin (6). The evidence provides us with important information that nodularin is instead another member of a group of obviously related agents, and that nodularin is a widespread contaminant, occurring in toxic brackish water cyanobacterium as well as marine organisms.
CARCINOGENESIS OF NODULARIN

Molecular Biology Laboratory (Heidelberg, Germany); and fra-1 (1.5-kilobase EcoRI fragment) probes by T. Curran at Roche Institute of Molecular Biology (Nutley, NJ). The cDNA of c fos (1.3-kilobase BamHI fragment) and glyceraldehyde 3-phosphate dehydrogenase (740-base HinfI fragment) were used.

Animals. Male, 6-week-old, Fischer 344 (F344) rats were obtained from Charles River Japan, Inc. (Kanagawa, Japan) and maintained under constant conditions for 1 week on a basal diet prior to the beginning of the experiment, as reported previously (7).

Two-Stage Carcinogenesis Experiment in Rat Liver. Male F344 rats were divided into seven groups. Initiation was conducted in groups 1–3 and 5 by a single i.p. administration of 200 mg DEN/kg body weight, but was not done in groups 4, and 6, which were injected with saline solution only. Tumor promotion was achieved by repeated i.p. administrations of 10 μg nodularin/kg body weight in group 2 and 25 μg/kg in group 3 twice a week from the end of week 12. The livers were excised, fixed in 10% formalin, and processed for embedding in paraffin. Liver sections (4 μm) were cut and stained with hematoxylin and eosin or immunohistochemically with anti-GST-P antibody as described previously (7, 9). The number, area, and volume of GST-P-positive foci were determined by an image analyzer using SalboukeiKesoku program Ver. 2.0 (Nippon Abionikusu Co., Tokyo, Japan) (10, 11). Average numbers of foci/1-cm2 section were evaluated quantitatively and the volume of positive foci per liver was determined by the method of Campbell et al. (12).

Statistical significance of the differences was analyzed using the Student's t test.

Hyperphosphorylation of Cytokeratins 8 and 18 in Primary Cultured Rat Hepatocytes. Hepatocytes were isolated from male F344 rat liver by a collagenase perfusion method, as described previously (11), and were cultured in phosphate-deficient DMEM for 14 h. 32Pi was added to the medium at a concentration of 3.7 MBq/ml and incubated for 3 h. Hepatocytes were further treated for 2 h with either 1 μM nodularin or 1 μM microcystin-LR, and were solubilized in lysis buffer, as described previously (13). Hyperphosphorylated proteins present in the soluble fraction of the cell lysates were immunoprecipitated with the anticytokeratin antibody K8.13, and the immunoprecipitates were absorbed to protein A-Sepharose 4B (Pharmacia, Tokyo, Japan) and then subjected to one-dimensional SDS-PAGE. The radioactivity of cytokeratins 8 and 18 was measured by the Discovery Series (TOYOBO, Inc., Tokyo, Japan). Similar results were obtained by twice repeated experiments.

Expression of Early Response Genes in Rat Liver. Male 9-week-old F344 rats were treated with a single i.p. administration of four different doses of nodularin (25, 50, 100, and 200 μg/kg body weight). Total RNA was prepared from rat liver 3, 6, 9, or 24 h after i.p. administration of nodularin by the method of Chomczynski and Sacchi (14). Ten μg of total RNA for each sample was loaded on a 1% agarose-1.1 m formaldehyde gel and blotted onto a nylon membrane (Schleicher & Schuell) in 20X standard saline citrate buffer (0.15 m NaCl, 0.15 m sodium citrate, pH 7.0). RNA was further treated as described previously (15). Various probes were prepared by Multiprime DNA-labeling systems (Amersham, Tokyo, Japan).

RESULTS

Two-stage Carcinogenesis Experiment in Rat Liver. The tumor-promoting activity of nodularin and microcystin-LR in rat liver initiated with DEN and without partial hepatectomy was determined by induction of GST-P-positive foci and assessment of the number of positive foci/cm2 of the liver, area of foci/liver (mm2/cm2) and volume of foci/liver (v/v %) (Table 1). The two groups treated in a dose-dependent manner with DEN plus nodularin had larger numbers, areas, and volumes of the foci in the liver than did the control groups treated with DEN alone or nodularin alone. In particular, numbers, areas, and volumes of the foci in the group treated with DEN plus nodularin at a dose of 25 μg/kg body weight were 106.0 ± 22.6/cm2, 39.87 ± 10.51 mm2/cm2, and 71.75 ± 18.78%, respectively, whereas those of the foci in the group treated with DEN plus microcystin-LR were 95.7 ± 27.9/cm2, 4.74 ± 2.23 mm2/cm2, and 8.55 ± 4.04%, respectively, indicating that nodularin had a tumor-promoting activity much stronger than microcystin-LR, and that nodularin stimulated clonal growth of the initiated cells more strongly than did microcystin-LR.

It is of great importance to note the effects of nodularin alone in group 4. The repeated i.p. administrations of nodularin alone induced 6.3 ± 7.3 GST-P-positive foci/cm2, and a single administration of

Table 1 Induction of positive foci GST-P by nodularin and microcystin-LR

<table>
<thead>
<tr>
<th>Groups</th>
<th>DEN</th>
<th>Tumor promoter (μg/kg body weight)</th>
<th>Effective no. of rats</th>
<th>No. of foci/liver (μg/cm2)</th>
<th>Area of foci/liver (mm2/cm2)</th>
<th>Volume of foci/liver (v/v %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>+</td>
<td></td>
<td>20</td>
<td>10.0 ± 2.9</td>
<td>0.18 ± 0.07</td>
<td>0.37 ± 0.18</td>
</tr>
<tr>
<td>2</td>
<td>+</td>
<td>Nodularin</td>
<td>19</td>
<td>15.0 ± 8.2</td>
<td>0.25 ± 0.09</td>
<td>0.48 ± 0.16</td>
</tr>
<tr>
<td>3</td>
<td>+</td>
<td>Nodularin</td>
<td>10</td>
<td>106.0 ± 22.6</td>
<td>39.87 ± 10.51</td>
<td>71.75 ± 18.78</td>
</tr>
<tr>
<td>4</td>
<td>+</td>
<td>Nodularin</td>
<td>16</td>
<td>63.3 ± 7.3</td>
<td>0.49 ± 0.89</td>
<td>0.92 ± 1.58</td>
</tr>
<tr>
<td>5</td>
<td>+</td>
<td>Microcystin-LR</td>
<td>18</td>
<td>95.7 ± 27.9</td>
<td>4.74 ± 2.23</td>
<td>8.55 ± 4.04</td>
</tr>
<tr>
<td>6</td>
<td>+</td>
<td>Microcystin-LR</td>
<td>17</td>
<td>1.6 ± 1.4</td>
<td>0.02 ± 0.02</td>
<td>0.04 ± 0.03</td>
</tr>
<tr>
<td>7</td>
<td>-</td>
<td></td>
<td>5</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

a Mean ± SD.
b P < 0.025.
c P < 0.005.
DEN alone induced 10.0 ± 2.9/cm², whereas the repeated i.p. administrations of microcystin-LR alone induced 1.6 ± 1.4/cm², respectively, suggesting that nodularin alone has an initiating activity, as does DEN. Moreover, nodularin alone induced an area of positive foci equal to 0.49 ± 0.89 mm²/cm² and a volume of foci equal to 0.92 ± 1.58%, whereas DEN alone induced 0.18 ± 0.07 mm²/cm² and 0.37 ± 0.18%, respectively, indicating that nodularin significantly stimulated growth of GST-P-positive foci more strongly than did DEN alone. Thus, these results show that nodularin has both initiating activity and tumor-promoting activity, a so-called carcinogenic threat, whereas microcystin-LR has negligible initiating activity but potent tumor-promoting activity. We conclude that nodularin and microcystin-LR induce tumor promotion in the liver through inhibition of PP-1 and PP-2A. How and why their potencies differ will be investigated in the next set of experiments.

Hyperphosphorylation of Cytokeratins 8 and 18 in Primary Cultured Rat Hepatocytes. We reported previously that okadaic acid induced hyperphosphorylation of intermediate filaments through inhibition of PP-1 and PP-2A (16, 17), and that microcystin-LR hyperphosphorylated cytokeratin peptides 8 and 18 in primary cultured rat hepatocytes (13). Fig. 2 shows an autoradiogram of immunoprecipitated cytokeratin peptides 8 and 18 in rat hepatocytes treated with either nodularin or microcystin-LR as a control. Nodularin-lane radioactivities were about 20% higher than those of the microcystin-LR lane. This suggests that there are differences in their uptake into hepatocytes. The hyperphosphorylation of intermediate filaments induced by nodularin was associated with morphological changes of the cells, as was microcystin-LR (3). Thus, the hyperphosphorylation of cytokeratin peptides 8 and 18 in rat hepatocytes is one of the consequences of inhibition of PP-1 and PP-2A in cells affected by the okadaic acid class of compounds (18, 19). In addition to morphological changes of the cells, expression of early response genes concomitantly induced by hyperphosphorylated proteins leads to signaling of tumor promotion.

Expression of Early Response Genes in Rat Liver. It was reported previously that okadaic acid up-regulated expression of the members of the jun and fos gene families (20, 21), and that okadaic acid induced expression of both early and secondary response genes on its application to mouse skin (20). Thus, we studied induction of mRNA transcripts of the different jun and fos family members in rat liver. Maximum expression of c-jun and jun-B genes in rat liver were observed 3 and 6 h after i.p. administration of nodularin (Fig. 3). Fig. 4 shows effects of nodularin on the induction of c-jun, jun-B, jun-D, c-fos, fos-B, and fra-1 mRNA transcripts in rat liver 6 h after treatment. Northern blot analyses revealed that only jun-D was constantly expressed 3 and 6 h after treatment of nodularin at 25 μg/kg body weight, which was the dose administered in the tumor promotion experiment, did not induce the expression of these genes. We think that repeated administrations of nodularin at 25 μg/kg body weight might induce a significant expression of the early response genes that play important roles in cell proliferation. We found recently that nodularin at 25 μg/kg body weight inhibits PP-2A and induces TNF-α mRNA expression in rat liver, indicating that an endogenous tumor promoter, TNF-α, was induced in the liver by nodularin at 25 μg/kg body weight.

Fig. 2. Hyperphosphorylation of cytokeratin peptides (CK) 8 and 18 in primary cultured rat hepatocytes induced by nodularin and microcystin-LR. Cells labeled with 32P for 3 h were further treated for 2 h with 1 μm nodularin (Lane 2) or 1 μm microcystin-LR (Lane 3); Lane 1, nontreated. Immunoprecipitation was performed using anti-cytokeratin antibody K 8.13. The radioactivity of cytokeratin peptides 8 and 18 were measured as described in "Materials and Methods."
binding parts are commonly oriented (22, 23). As for the study of their structure-function relationships, the results with 6(Z)-Adda microcystins-LR and -RR, which are geometrical isomers at C-7 in the Adda portion of microcystins, indicated that the conjugated diene with 4(E),6(E) geometry in the Adda portion is important for receptor binding as well as for interactions with PP-1 and PP-2A (24). This is also applicable to nodularin. However, the main structural difference between nodularin and microcystin-LR is the smaller ring structure of nodularin compared to the larger ring structure of microcystin-LR. Thus, we propose that nodularin is taken into the hepatocytes more easily than is microcystin-LR, resulting in greater cellular effects.

A comment on the initiating activity of nodularin should be discussed here. A single i.p. administration of DEN at a dose of 200 mg/kg body weight induced numbers of foci/cm² comparable to that of the twice weekly administered nodularin at a dose of 25 μg/kg body weight. Each rat received a total of roughly 60 μg DEN and 150 μg nodularin. Thus, nodularin might be expected to have an initiating activity 400 times stronger than DEN. However, nodularin and microcystin-LR did not show any mutagenic activity in the Ames test, probably due to their difficulty in penetrating into the cells (25).

This paper provides the first evidence that nodularin induces expression of the early response genes through inhibition of PP-1 and PP-2A in rat liver after its i.p. administration, suggesting that the okadaic acid class of tumor promoters induces the same biochemical and molecular effects on their incorporation into cells of the target organs (26). Kim et al. (15) reported that okadaic acid enhanced jun-B and jun-D mRNA levels 3- and 4-fold, whereas c-jun mRNA levels were not changed in C2C12 cells, and they suggested that jun-B, jun-D, and c-jun genes are regulated differently by okadaic acid. However, our results showed that expression of the six members in the jun and fos gene families were similarly up-regulated by nodularin in rat liver (Fig. 4). Although nodularin and 12-O-tetradecanoylphorbol-13-acetate both induced expression of early response genes in primary cultured rat hepatocytes, the time course of c-jun and jun-B gene expressions by nodularin was delayed much more than those of 12-O-tetradecanoylphorbol-13-acetate, probably due to their different mechanisms of action. It may be a possibility, especially in the case of c-fos, that increases in mRNA levels after treatment with okadaic acid, probably the microcystins and nodularin, are partly due to messenger stabilization (27, 28). This might in part be induced by hyperphosphorylation of binding proteins to the AU-rich element.

Expression of early response genes in rat liver should be discussed in relation to tumor promotion. As Fig. 4 showed, their expression was observed at a dose of 50 μg nodularin/kg body weight but was not significantly seen at a dose of 25 μg nodularin/kg body weight, the latter dose of which was used for the in vivo two-stage carcinogenesis experiments. Recently we found that nodularin could exhibit its tumor-promoting activity by inducing TNF-α, which is discussed as a potential endogenous tumor promoter and central mediator of carcinogenesis. TNF-α gene was significantly expressed in total RNA, which was prepared from rat liver treated with a single administration of 25 μg nodularin/kg body weight. The results confirmed our previous evidence that TNF-α plays an important role in tumor promotion, such as clonal expansion of initiated cells (29).

The evidence that nodularin is a new liver carcinogen and that microcystin-LR is a potent tumor promoter in rat liver serves to emphasize their environmental hazard, because they have been iso-

---

**DISCUSSION**

A two-stage carcinogenesis experiment without partial hepatectomy showed tumor-promoting activity of nodularin in rat liver initiated with DEN. Two of the main carcinogenic effects produced by nodularin differed from those produced by microcystin-LR: (a) the tumor-promoting activity of nodularin was stronger than that of microcystin-LR; and (b) nodularin slightly but significantly induced GST-P-positive foci in rat liver without initiation, indicating that nodularin itself has an initiating activity. Thus, we think nodularin per se is a new carcinogen, whereas microcystin-LR is a tumor promoter rather than a carcinogen (7). Although nodularin and microcystin-LR are structurally related to each other and have the same specific activity in inhibition of PP-1 and PP-2A, each presents a different potency in tumor-promoting activity. How can the differences in potency be explained by structure? A computer-assisted molecular modeling of nodularin and microcystin-LR revealed that their structures can be superimposed, and that their catalytic and receptor-
lated from toxic cyanobacteria representing widely spread contaminants of drinking water (1). Andersen et al. (30) reported “Netpen Liver Disease,” which is associated with liver damage in Atlantic salmon, and is induced by microcystins (30). In addition to nodularin, the presence of a similar compound, motuporin, indicates problems all over the world (6). We are investigating a specific area in the world where chronic exposure to the microcystins and nodularin in drinking water is one of the leading causes for a high incidence of human liver cancer. Yu et al. (31) reported results of an epidemiological study of human primary liver cancer in Qidong county in the People’s Republic of China where people drink pond and ditch water. The incidence of primary liver cancer in Qidong county was about eight times higher than in other counties where people drink well water (31). Yu et al. (31) suggested that drinking water be added to aflatoxin and hepatitis virus infections as an important third risk factor. Recently, Harada, in collaboration with Yu’s group at the Shanghai Medical University, succeeded in identifying microcystin-LR and 3-desmethyl-microcystin-LR in the collected water reservoirs in Qidong county. More over, we reported that 71% of the administered total radioactivity was taken into the liver by i.p. administration of [3H]dihydropicrocystin-LR after a 1-h treatment, whereas 0.68% of the total radioactivity was present in the liver from 6 to 19 h after p.o. administration (32). Although the liver is the main target organ, the incorporation of microcystin-LR into the liver by i.p. and p.o. administrations differs greatly (32). We are now focusing on the problem of explaining how p.o. administration of cyanobacterial toxins is toxic to humans. This paper provides a prototype study for the investigation of carcinogenic effects from nodularin in conjunction with microcystin-LR, two of the important cyanobacterial toxins.

ACKNOWLEDGMENTS

We thank Mrs. Kiyomi Ebina-Kohyama for her assistance.

REFERENCES


10 Harada et al., personal communication.
Nodularin, a Potent Inhibitor of Protein Phosphatases 1 and 2A, Is a New Environmental Carcinogen in Male F344 Rat Liver

Tetsuya Ohta, Eisaburo Sueoka, Naoyuki Iida, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/54/24/6402

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.