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Liver tumor promotion by the cyanobacterial cyclic peptide toxin microcystin-LR

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Summary. Certain waterblooms of toxic cyanobacteria (blue-green algae) are a health threat because of their production of toxic peptides, termed microcystins, which cause liver damage in wild and domesticated animals. The most widely studied microcystin is microcystin-LR, a heptapeptide containing the two L-amino acids, leucine and arginine. The inhibition of protein phosphatase type 1 and type 2A activities by microcystin-LR is similar to that of the known protein phosphatase inhibitor and tumor promoter okadaic acid. We show in this report that microcystin-LR, applied below the acute toxicity level, dose-dependently increases the number and percentage area of positive foci for the placental form of glutathione *S*-transferase in rat liver, which was initiated with diethylnitrosamine. The result was obtained independently through two animal experiments. This observation indicates that microcystin-LR is a new liver tumor promoter mediated through inhibition of protein phosphatase type 1 and type 2A activities. This provides further evidence that the okadaic acid pathway is a general mechanism of tumor promotion in various organs, such as mouse skin, rat glandular stomach and rat liver.

Key words: Microcystin – Tumor promoter – Protein phosphatase

Introduction

Waterblooms of certain planktonic cyanobacteria (blue-green algae), found in lakes, ponds and municipal water supplies, cause intermittent but repeated problems for the maintenance of safe water supplies. In summer, these waterblooms can produce an unpleasant taste and odor in tap water and an odor in the air around water reservoirs. A more serious problem concerns the production of potent cyclic hepatotoxins, termed microcystins, in many of these waterblooms (Botes et al. 1984; Carmichael 1988; Watanabe et al. 1988; Namikoshi et al. 1990). Humans living in areas where the water supply is not well maintained can be continuously exposed to microcystins. Microcystin is a group name for about 23 known monocyclic heptapeptides varying primarily in their two L-amino acids. The nomenclature is based on these two L-amino acids, for example, microcystin-LR contains leucine and arginine (Botes et al. 1985; Carmichael et al. 1988 a). Besides these two variable L-amino acids, microcystins contain three D-amino acids and two unusual amino acids, *N*-methyl-dehydroalanine and 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid (Adda) (Fig. 1). Microcystins have been isolated from toxic strains and species of *Microcystis, Anabena, Oscillatoria* and *Nostoc* (Botes et al. 1984; Carmichael 1988; Watanabe et al. 1988; Namikoshi et al. 1990).

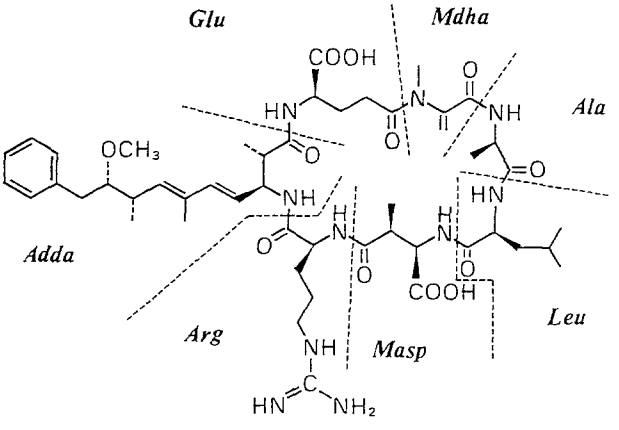

Fig. 1. Structure of microcystin-LR. Besides the two variable L-amino acids, leucine and arginine, the microcystins contain three D-amino acids and two unusual amino acids, *N*-methyl-dehydroalanine (*Mdha*) and 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid (*Adda*)

Abbreviations: DEN, diethylnitrosamine; DMBA, 7,12-dimethylbenz[*a*]anthracene; GST-P, glutathione *S*-transferase placental form

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Microcystin has a unique organotrophy in the liver, with acute lethal i.p. and oral administrations of microcystin to mice or rats causing massive intrahepatic hemorrhages and cellular necrosis within a few hours (Beasley et al. 1989). We have recently demonstrated that microcystin-LR, -YR and -RR inhibit protein phosphatase type 1 and type 2A activities, present in liver cell membrane and cytosolic fractions, as strongly as okadaic acid (Yoshizawa et al. 1990; Suganuma et al. 1988; Fujiki et al. 1992). This inhibition of protein phosphatase activities resulted in an increase in phosphorylation of proteins in the liver cells (Yoshizawa et al. 1990). Microcystin acts on liver cells through a mechanism similar to that of okadaic acid, termed the okadaic acid pathway, which has been reported to promote tumor development in mouse skin initiated with 7,12-dimethylbenz[a]anthracene (DMBA) and in rat glandular stomach initiated with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (Suganuma et al. 1988; Fujiki et al. 1992).

In two experiments, we found that microcystin-LR has a potent tumor-promoting activity in rat liver initiated with diethylnitrosamine (DEN) below the concentrations that do not release aminotransferase (transaminase) from the liver into the blood serum. Microcystin acts on the liver through the okadaic acid pathway and is one of the strongest liver tumor promoters found to date.

Materials and methods

Chemicals

Microcystin-LR was purified from lyophilized cells of laboratory-cultured *Microcystis aeruginosa* PCC-7820 and natural bloom material, dominated by *Microcystis aeruginosa,* collected from a farm pond near Monroe, Wisconsin in 1985 (Krishnamurthy et al. 1986; Galey et al. 1987). Phenobarbital was purchased from Ebisu Pharm. Co. Ltd., Osaka, Japan. DEN and the transaminase CII test kit were purchased from Wako Chemical Co., Osaka, Japan. A vectastain-ABC kit was obtained from Vector Laboratories Inc., Burlingame, Calif., USA. Rabbit antibody against glutathione S-transferase placental form (GST-P) was the generous gift of Prof. K. Sato, 2nd Department of Biochemistry, Hirosaki University (Satoh et al. 1985).

Animals

Male Fischer 344 (F344) rats, 6 weeks old, were obtained from Charles River Japan Inc., Kanagawa, Japan.

Two-stage carcinogenesis experiment in rat liver

The two-stage carcinogenesis experiment in rat liver was based on a variation of the Solt-Farber model (Solt and Farber 1976; Ito et al. 1980; Sato et al. 1984; Farber and Solt 1978). Male F344 rats, 6 weeks old, were maintained under constant conditions for 1 week on a basal diet prior to the beginning of the experiment.

Experiment 1. Male F344 rats, 7 weeks old, were divided into six groups. Groups 1–3 and 6 were i.p. injected with 200 mg/kg body weight DEN as an initiator, and groups 4 and 5 were i.p. injected with saline instead of DEN. Tumor promotion was conducted by i.p. administrations of 1 μg/kg microcystin-LR in groups 2 and 4, or 10 μg/kg microcystin-LR in groups 3 and 5 twice a week from the third week of the experiment, and followed by partial hepatectomy at the end of the third week of the experiment. As a positive control, 0.05% phenobarbital, a well-known liver tumor promoter (Peraino et al. 1971), was given as a diet substitute for microcystin-LR in group 6. Saline was used instead of microcystin-LR in group 1. All animals were sacrificed at the end of week 8. The livers were excised and fixed in 10% formalin, and sections 2–3 mm thick were taken at the maximum diameters of the lobes and then processed for embedding in paraffin. Sections (4 μm) were cut and stained with hematoxylin and eosin or by the avidin-biotin-peroxidase complex method (Hsu et al. 1981) for immunohistochemical demonstration of GST-P. The numbers and areas of GST-P-positive foci were determined by microscope using an eyepiece grid, and foci larger than 50 μm in diameter were counted. Statistical significance of differences was analyzed using Student's *t*-test.

Experiment 2. Male F344 rats, 7 weeks old, were divided into five groups. Groups 1–4 were initiated with DEN as in experiment 1 and group 5 was i.p. injected with saline instead of DEN. Tumor promotion was conducted by two i.p. administrations of 10 μg/kg microcystin-LR in groups 2–5 at the third week of the experiment, followed by partial hepatectomy at the end of the third week, as in experiment 1. After partial hepatectomy, the administered doses of microcystin-LR were 10 μg/kg for group 2, 25 μg/kg for group 3 and 50 μg/kg for groups 4 and 5, twice a week. Saline was used instead of microcystin-LR in group 1. All animals were sacrificed at the end of week 8. Immunohistochemical staining and counting of foci were conducted by the same procedure as in experiment 1.

Measurement of serum aminotransferase level

Microcystin-YR, at a concentration of 80 μg/kg or 800 μg/kg dissolved in 500 μl saline, was i.p. injected into male F344 rats. As a control group, only saline was i.p. injected. Blood serum was collected 1, 3, 6, 9 and 24 h after i.p. injection. Serum aminotransferase levels (alanine aminotransferase and aspartate aminotransferase) were measured using a transaminase CII test kit.

Results

Two-stage carcinogenesis experiment in rat liver

Experiment 1. Repeated i.p. injections of 10 μg/kg microcystin-LR into initiated rats induced an increase in the number and area of GST-P-positive foci in group 3, compared with those of group 5 (Table 1). The numbers and percentage areas of foci were $26.0 \pm 8.1\%$ and $0.73 \pm 0.3\%$, respectively, in group 3. Treatment with 1 μg/kg microcystin-LR in group 2 did not show any significant increase of foci. Groups 4 and 5, treated with microcystin-LR alone, were completely negative. Group 1, treated with DEN alone, gave a basal level of GST-P-positive foci, 16.5 ± 3.9, and $0.59 \pm 0.2\%$ areas of foci. A positive control group given a 0.05% phenobarbital diet showed a significant increase of the foci ($P < 0.005$).

Experiment 2. To confirm the tumor-promoting activity of microcystin-LR in experiment 1, experiment 2 was conducted with various doses of microcystin-LR after partial hepatectomy. Table 2 shows that repeated i.p. injections of microcystin-LR induced a significant increase of both parameters with increasing doses in groups 2–4.

Table 1. Induction of positive foci of glutathione *S*-transferase placental form (GST-P) in rats treated with DEN plus various doses of microcystin-LR[a] (experiment 1)

Group no.	Treatments			No. of rats at week 8	Positive foci of GST-P	
	DEN	Microcystin-LR[b] (μg/kg)	Phenobarbital in diet (%)		No. of foci/liver (No./cm²)	Area of foci (%)
1	+	–	–	15	16.5± 3.9	0.59±0.2
2	+	1	–	16	15.9± 4.1	0.53±0.2
3	+	10	–	15	26.0± 8.1[c]	0.73±0.3
4	–	1	–	10	0	0
5	–	10	–	10	0	0
6	+	–	0.05	9	38.1±10.9[c]	1.09±0.5[c]

[a] Values are means ± SD
[b] i.p. injected
[c] $P<0.005$ compared with group 1

Table 2. Induction of positive foci of glutathione *S*-transferase placental form (GST-P) in rats treated with DEN plus various doses of microcystin-LR[a] (experiment 2)

Group no.	Treatments			No. of rats at week 8	Positive foci of GST-P	
	DEN	Microcystin-LR[b] (μg/kg)			No. of foci/liver (No./cm²)	Area of foci (%)
		Partial hepatectomy				
		Before	After			
1	+	–	–	19	13.4± 4.2	2.7± 3.1
2	+	10	10	15	17.4± 3.8[c]	1.9± 0.5
3	+	10	25	16	32.7±11.1[c]	6.8± 3.8[d]
4	+	10	50	18	44.4±10.3[d]	29.6±12.9[d]
5	–	10	50	14	0.4± 0.3	0.1± 0.2

[a] Values are means ± SD
[b] i.p. injected
[c] $P<0.01$ compared with group 1
[d] $P<0.001$ compared with group 1

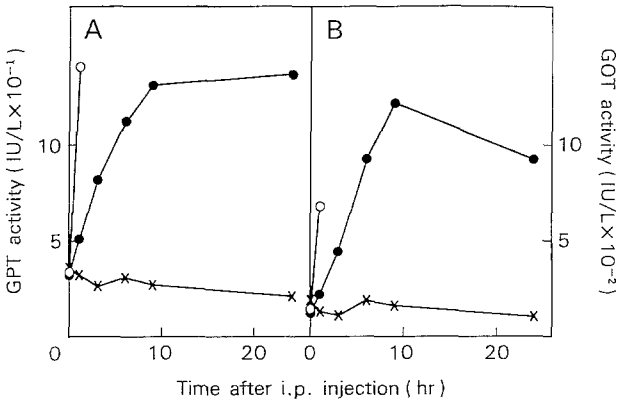

Fig. 2. Effect of microcystin-YR on release of alanine aminotransferase (*GPT*, **A**) and aspartate aminotransferase (*GOT*, **B**) into rat serum. Two different doses, 80 μg/kg and 800 μg/kg, of microcystin-YR dissolved in 500 μl saline were injected i.p. into male F344 rats. Blood serum was collected just before injection, and 3, 6, 9 and 24 h after injection. Serum aminotransferase levels were measured using the transaminase C test kit (Wako Chemical Company, Osaka, Japan). Note that approximately 1 h after injection all rats injected at 800 μg/kg died from intrahepatic hemorrhage. ●, 80 μg/kg microcystin-YR; ○, 800 μg/kg; X, control

The numbers of foci/cm² in groups 2, 3, and 4 were 17.4±3.8, 32.7±11.1 and 44.4±10.3 and percentage areas of positive foci were 1.9±0.5%, 6.8±3.8% and 29.6±12.9%, respectively. The two negative control groups, treated with DEN alone (group 1) or with microcystin-LR alone (group 5), each gave a basal level of GST-P-positive foci. The livers of group 4, treated with DEN plus microcystin-LR (50 μg/kg after partial hepatectomy) had a large number of macroscopic nodules, histologically diagnosed as neoplastic nodules.

Measurement of serum transaminase level

A single i.p. injection of microcystin-YR (80 μg/kg), which has the same hepatotoxicity as microcystin-LR, released alanine aminotransferase and aspartate aminotransferase into the blood serum of the rats in a time-dependent manner (Fig. 2). These aminotransferase levels reached a maximum 9 h after i.p. injection. An i.p. injection of 800 μg/kg immediately released both enzymes, and killed all of the rats through lethal intrahepatic hemorrhaging (Fig. 2). An i.p. injection of up to 50 μg/kg microcystin-YR or -LR, doses that were used in the tumor promotion experiments, showed no increase of the two aminotransferase (data not shown).

Discussion

Microcystin-LR belongs to the okadaic acid class of tumor promoters, because microcystin-LR, -YR and -RR inhibit protein phosphatase type 1 and type 2A activities in liver cell membrane and cytosolic fractions, as strongly as okadaic acid (Yoshizawa et al. 1990). This inhibition of protein phosphatase activities results in an increase in phosphorylation of proteins in the cells. These phosphorylated proteins might play a significant role in the

gene expression involved in cell growth. We previously reported that DNA isolated from skin tumors treated with DMBA plus okadaic acid contains a mutation in the second nucleotide of codon 61 of the mouse c-Ha-*ras* gene (Fujiki et al. 1989). In the present experiments, we did not find any detectable mutation in that position in DNA isolated from rat livers of the group treated with DEN plus microcystin-LR (data not shown).

Liver organotropy of microcystin is unique, because the i.p. and oral administrations of microcystin induce almost exclusively liver damage. At present, the mechanism of microcystin organotrophy is thought to involve preferential uptake of the toxin across the ileum and into the hepatocytes via bile acid carriers (Eriksson et al. 1990). Inside the hepatocytes, a toxin-induced alteration of the cells' microfilaments leads to an aggregation of the microfilaments near the center of the cell. As a result of this loss of cellular support, the hepatocytes become round, resulting in destruction of the sinusoid endothelium.

As a biochemical marker of liver toxicity, we measured the serum aminotransferase level. Since microcystin-YR has the same in vitro activity as microcystin-LR, Fig. 2 shows the results of microcystin-YR alone. Microcystin-YR induced the release of alanine and aspartate aminotransferases at 80 μg/kg, but microcystin-YR and -LR did not show these releases at concentrations of 50 μg/kg or lower (data not shown). Furthermore, we measured their release at the end of these carcinogenesis experiments. The serum aminotransferase level of rats treated with microcystin-LR was almost as low as that of the rats treated with DEN only. This demonstrates that the quantity of microcystin-LR used for this experiment did not show any significant acute or subacute toxic effects on rat liver. Although we think that microcystin-LR has a tumor-promoting activity in rat liver, only a few foci were observed in non-initiated rats. It should be further investigated whether the microcystins have initiating activity.

The only other work on in vivo tumor promotion using a microcystin is by Falconer (1991), who recently reported that orally administered hepatotoxic extracts of *Microcystis* from a natural waterbloom induced tumors on mouse skin initiated with topically applied DMBA. Since these studies did not use a liver-specific initiator, no conclusions could be drawn about liver tumor promotion by microcystin. We have recently demonstrated that microcystin is only slowly transported into epithelial cells of mouse skin or primary human fibroblasts, but it was effective when administered by microinjection into the cells (Matsushima et al. 1990). Because microcystins are preferentially taken up by hepatocytes it is expected that the main health threat as a tumor promoter would be in liver tumor promotion and not promotion of skin tumors.

In addition to phenobarbital, other liver tumor promoters, such as hexachlorocyclohexane, cyproterone acetate and chenodeoxycholic acid have been reported (Schulte-Hermann et al. 1982; Blair et al. 1991). Their tumor-promoting activities are obtained by administration of several milligrams per kilogram per day. By comparison with these other tumor promoters our results would indicate that microcystin is the strongest of the liver tumor promoters found to date. α-Amanitin and phalloidin are also liver toxins but they do not act on liver cells in a manner similar to microcystin (Yoshizawa et al. 1990). We reported previously that the pentapeptide nodularin, isolated from the toxic brackish-water cyanobacterium, *Nodularia spumigena,* exhibits the same in vitro protein phosphatase inhibition as microcystin (Yoshizawa et al. 1990). Because it has a similar monocyclic peptide structure to microcystin (Carmichael et al. 1988 b; Rinehart et al. 1988), nodularin is probably also a liver tumor promoter.

The mechanism of action of microcystin in liver cells is similar to that of okadaic acid, and therefore most likely expressed through the okadaic acid pathway. We have found that the okadaic acid pathway, involving inhibition of protein phosphatase 1 and 2A activities, is a general mechanism of tumor promotion in various organs. Even though the results reported here are based on i.p. dosings of microcystin, they suggest that tumor promotion by microcystin should be considered possible in humans as well. Epidemiological evidence suggests the involvement of human liver cancer by microcystin, which was studied in Qidong County, north of Shanghai, where people drink pond and ditch water. The incidence of primary liver cancer in Qidong County was about eight times higher than in populations who drink well water (Yu 1989). The water of the ponds and ditches of Qidong County is contaminated by high levels of blue-green algae, which might be producing the microcystins (Carmichael, personal communication). These results strongly suggest that drinking water contaminated with the microcystins might induce human liver cancer. If intake of the microcystins is correlated with the development of human liver cancer, it is important to begin some protective measures.

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