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TUMOUR PROMOTION BY THE CYANOBACTERIAL TOXIN MICROCYSTIN

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in

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by

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ABSTRACT

The microcystins are a range of cyclic heptapeptide hepatotoxins produced by a number of common freshwater cyanobacteria. They have been shown to be tumour promoters in mouse skin and rat liver. There is also strong epidemiological evidence of their involvement in the high rates of primary liver cancer in certain areas of China due to the consumption of cyanobacterially contaminated drinking water.

The effects of the microcystins are believed to be due to their potent inhibition of protein phosphatases 1 and 2A, enzymes which are intimately involved in the maintenance of cellular homeostasis, and in the regulation of the cell cycle. The Ki for the interaction of microcystin and these enzymes is in the range 0.1 - 1.0 nM. Microcystin binds to the active site and can form a covalent attachment there. Other toxins which target these enzymes, but which do not interact covalently, include nodularin, okadaic acid, the calyculins, and cantharidin. Microcystin and nodularin also differ from the others just mentioned in that they require active uptake by a transport system related to that responsible for bile acid uptake. This confers a high degree of liver specificity, and also means that a high intracellular concentration of toxin can be achieved at relatively low exposure levels.

The present study examined the tumour promoting effects of the microcystins through two independent means. First, a long term *in vivo* dosing study was performed in which cyanobacterial extract containing a range of microcystins was given in drinking water to mice which had been previously treated with the tumour initiator N-nitroso-N-methylurea by gavage. The aim of this study, given that liver cancer is not a major form of neoplasm in western populations, was to determine whether microcystins could promote non-hepatic tumour growth, in particular in the upper intestine. Morbid animals were killed and a careful post-mortem examination was performed. Liver, duodenum and any other affected tissues were examined histologically and tumour growth was quantified using image analysis techniques. No significant differences were found between the two microcystin-treated groups and the control group in terms of survival or degree of development of tumours, nor in the type of tumour engendered.

Second, the effects of pure microcystin-LR were examined in cultured primary hepatocytes from immature mice. The cells were isolated by collagenase perfusion from C3H/HeJ mice and grown in conditions under which proliferation occurred without mitogenic stimulation. Previous studies have used Epidermal Growth Factor (EGF) to induce proliferation in the hepatocytes in culture, however, in this system EGF masked the effects of microcystin-LR. Therefore unstimulated cells were exposed to the toxin. Effective concentrations of toxin were lower in this system than in others which have been reported.

Microcystin-LR was selectively toxic to hepatocytes in the range 1.0 - 100.0 nM. Cellular uptake of tritium-labelled toxin was progressively lost during five days in culture. Therefore, further studies were performed over three days of toxin exposure. A number of outcomes were seen depending upon the toxin concentration and dosing regime used. In an experiment where cells were exposed to a single addition of toxin, 1.0 nM microcystin-LR induced effects consistent with a stimulation of cytokinesis, whereas a concentration of 10.0 nM microcystin-LR induced an inhibition of cytokinesis. Cell-selective toxicity at this higher concentration also caused the enrichment of a more proliferatively active cohort of cells. A reduced rate of cell death, possibly due to interference with the apoptotic process, was also detected at 1.0 and 3.0 nM toxin.

Microcystin-LR did not promote an EGF-like proliferative response. In all other experiments, cells were exposed to multiple changes of medium containing microcystin-LR over three days in order to maintain a more constant exposure. Similar effects to those seen in the single-exposure experiment were observed. However, the effective concentrations were about 10-fold lower. Thus, a stimulation of cytokinesis occurred at picomolar concentrations, whereas an inhibition of this process was apparent at 1.0 nM toxin. Cells surviving a first exposure to 10.0 nM microcystin-LR remained insensitive to further additions of the toxin, instead proliferating at a rate greater than the untreated control.

The interaction of microcystin-LR with the apoptosis inhibitor nafenopin was explored. This compound generally minimised the effect of microcystin-LR at all concentrations. This finding suggests that at picomolar concentrations, microcystin-LR may have had an inhibitory effect on apoptosis, whereas at selectively toxic (nM) concentrations of microcystin-LR, the toxin may have been inducing an apoptotic response in some cells.

The cellular toxicology of microcystin-LR was compared with that of okadaic acid. There was no evidence that okadaic acid had any effect on cytokinesis. Instead okadaic acid inhibited DNA synthesis in a purely dose-dependent manner. The dose-response curves for cell death and DNA synthesis inhibition were of similar shape. However, that for cell death was in the low nM region, whereas that for DNA synthesis inhibition occurred at concentrations about ten-fold lower. This was not the case with microcystin-LR, where DNA synthesis was unaffected in cells resistant to microcystin-LR toxicity. Therefore, the extent of ^{3}H-thymidine labelling of DNA in cells exposed to okadaic acid decreased with increasing toxin concentration, whereas the labelling of DNA in cells exposed to microcystin-LR did not directly correlate with toxin concentration. This suggests that either the differing affinities of these toxins for the protein phosphatases led to different growth regulatory outcomes, or that microcystin-LR exerted a selective pressure on the hepatocyte population such that less toxin-sensitive, proliferatively active cells were favoured.

To further explore the possible effects of microcystin-LR on the various hepatocyte sub-populations, flow cytometric methods were developed in order to be able to differentiate between cells based on their DNA content, that is their ploidy, which directly correlates with the level of differentiation. To validate these techniques, nuclear (DNA) and cytoplasmic (protein) dual fluorescent staining was used to monitor changes in ploidy and the rate of cellular division, respectively, in a mitogen-stimulated hepatocyte population. The patterns of effects seen in these studies compared well with those reported by other workers using different experimental approaches. These techniques were then applied to a study of the cell cycle effects of microcystin-LR. It was found that the acute toxicity of a single dose of microcystin-LR was only minimally selective on the basis of ploidy at concentrations below 10.0 nM, the approximate EC_{50} for acute toxicity in this system. After three days' exposure to microcystin-LR, using the multiple-addition protocol outlined above, the number and proportion of binuclear cells increased with toxin concentration up to 1.0 nM, above which a reversion to control levels was seen. Average nuclear ploidy followed a similar pattern. These results suggest that up to 1.0 nM microcystin-LR, proliferating binuclear cells accumulated in the hepatocyte population. At 10 nM microcystin-LR, where cell-selective acute toxicity occurs, this effect was not seen in the hepatocyte population surviving the first addition of the toxin. Therefore, the cells affected by microcystin-LR at lower concentrations are also those preferentially killed by the toxin at higher doses.

Overall, the results from these hepatocyte culture experiments support the hypothesis that the actions of microcystin-LR are not the same in all hepatocytes in the population. This selectivity may be due to the different rates of uptake of the toxin by the various sub-populations of cells within the hepatocyte population, and also to variations in their cell cycle status at the time of exposure. Therefore, one way in which this toxin might promote tumour growth is to selectively kill the more toxin-sensitive cells in the liver causing a regenerative response in the remaining population. Furthermore, tumourigenic cells are generally diploid, tend to express fewer differentiated characteristics than normal cells, and so are also likely to be less sensitive to microcystin toxicity. Hence, they are likely to obtain a growth advantage during toxin exposure. These effects are likely to play a role at higher exposure levels. When cells were exposed to a single addition of 1.0 nM microcystin-LR, or multiple additions of the toxin at pM concentrations, a promotion of cytokinesis appeared to occur in a significant proportion of the population. This would have the effect of lowering the average ploidy of the hepatocyte population and therefore, possibly, its level of differentiation. Lower ploidy cells, lacking the genetic redundancy of their higher ploidy counterparts, are perhaps more susceptible to the effects of mutagens. An inhibition of apoptosis, also suggested by observations made at these concentrations, could facilitate the fixation of mutations in the genome of affected cells.