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Role of nitric oxide in the genotoxic response to chronic microcystin-LR exposure in human–hamster hybrid cells

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ABSTRACT

Microcystin-LR (MC-LR) is the most abundant and toxic microcystin congener and has been classified as a potential human carcinogen (Group 2B) by the International Agency for Research on Cancer. However, the mechanisms underlying the genotoxic effects of MC-LR during chronic exposure are still poorly understood. In the present study, human–hamster hybrid (A_L) cells were exposed to MC-LR for varying lengths of time to investigate the role of nitrogen radicals in MC-LR-induced genotoxicity. The mutagenic potential at the *CD59* locus was more than 2-fold higher ($p < 0.01$) in A_L cells exposed to a cytotoxic concentration (1 μmol/L) of MC-LR for 30 days than in untreated control cells, which was consistent with the formation of micronucleus. MC-LR caused a dose-dependent increase in nitric oxide (NO) production in treated cells. Moreover, this was blocked by concurrent treatment with the NO synthase inhibitor N^G-methyl-L-arginine (L-NMMA), which suppressed MC-LR-induced mutations as well. The survival of mitochondrial DNA-depleted (ρ^0) A_L cells was markedly decreased by MC-LR treatment compared to that in A_L cells, while the *CD59* mutant fraction was unaltered. These results provided clear evidence that the genotoxicity associated with chronic MC-LR exposure in mammalian cells was mediated by NO and might be considered as a basis for the development of therapeutics that prevent carcinogenesis.

Introduction

The distribution of toxic cyanobacterial blooms in eutrophic rivers, lakes, reservoirs, and recreational waters worldwide has increased in recent years to become a global problem threatening human health and ecosystem safety. Cyanobacterial toxins are diverse in their chemical structure and are assigned to different substance classes such as hepatotoxins (microcystins and nodularins), neurotoxins (saxitoxins and anatoxins), cytotoxins (cylindrospermopsin), and irritant toxins (lipopolysaccharides) (Pearson et al., 2010). Of more than 100 variants, microcystin-LR is the most common and toxic congener, and has been classified as a potential human carcinogen (Group 2B) by the International Agency for Research on Cancer (Grosse et al., 2006). The general population is exposed to microcystin-LR (MC-LR) through natural water and food consumption and is vulnerable to the risks associated with long-term exposure (Chen et al., 2009). Experimental studies and epidemiologic data have linked MC-LR exposure to the development of liver

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and colorectal cancers (Yu, 1995; Zhou et al., 2002), possibly caused by an MC-LR-induced increase in the expression of tumor necrosis factor-α (Fujiki and Suganuma, 2011).

Various quantitative measurement of genotoxicity such as DNA strand breaks and mutations have been used to assess the carcinogenic potential of MC-LR, which remains controversial. MC-LR had no effect on the induction of micronuclei (MNs) in CHO-K1 cells (Lankoff et al., 2006b) and on the frequency of chromosomal aberration in lymphocytes (Lankoff et al., 2006a). Meanwhile, another study found that MN frequency was approximately 3.8-fold higher in human lymphoblastoid thymidine kinase (TK) 6 cells exposed to MC-LR at a concentration of 80 μg/mL for 24 hr than in control cells (Zhan et al., 2004). In human lymphocytes, the mitotic index was increased by MC-LR treatment in a dose- and time-dependent manner (Lankoff et al., 2004b). MC-LR-treated WRL-68 cells exhibited chromosomal instability and a high incidence of aneuploidy (Xu et al., 2012), and the potential carcinogenicity of MC-LR has also been suggested from *in vivo* studies (Ito et al., 1997). Mutagenic studies of MC-LR using the reverse Ames assay in tester strains TA98, TA100, and TA102 and the *Bacillus subtilis* multigene sporulation test using the 168 and hcr-9 strains have shown that MC-LR is inactive or only weakly active (Repavich et al., 1990). However, in another study, MC-LR induced a base substitution in codon 12 of *K-Ras* and ouabain resistance mutations in human RSa cells at concentrations greater than 15 μg/mL (Suzuki et al., 1998), as well as the loss of heterozygosity at the *TK* locus in human lymphoblastoid TK6 cells at greater than 20 μg/mL (Zhan et al., 2004). It should be noted that the concentration of MC-LR in hypereutrophic water bodies is less than 50 ng/mL (Nasri et al., 2007), but is nearly 1 μg/g in freshwater mussels due to bioaccumulation (Kann et al., 2010). Although alterations in microRNA expression in human WRL-68 cells were shown to play a critical role in MC-LR-induced carcinogenesis during chronic exposure (Xu et al., 2012), the underlying mechanisms are not well understood.

In addition to the well-documented interaction of protein phosphatases PP1 and PP2A, oxidative stress induced by MC-LR is considered as a key determinant of MC-LR-mediated genotoxicity (Zegura et al., 2008). The involvement of reactive oxygen species (ROS) and the protective effects of antioxidant enzymes such as superoxide dismutase, catalase, and glutathione-S-transferase have been investigated in various cell lines and in the animal liver, heart, and reproductive system (Li et al., 2008; Xiong et al., 2010). ROS, especially the hydroxyl radical ($OH^{\cdot}$), directly or indirectly damage neighboring biomolecules such as DNA and membrane lipids (Ziech et al., 2010). There is also evidence that MC-LR induces oxidative DNA damage in HepG2 cells (Zegura et al., 2003), as well as the formation of 8-oxo-7,8-dihydro-2′-deoxyguanosine in primary cultured rat hepatocytes and liver (Maatouk et al., 2004). $OH^{\cdot}$ is a intracellular oxidant likely induced by MC-LR, but is so highly reactive that it can only diffuse a short distance from the site of formation; others may include reactive nitrogen species (RNS). MC-LR stimulates nitric oxide (NO) production in a dose-dependent manner in rat insulinoma (INS-1) cells (Ji et al., 2011). In the presence of superoxide anion ($O_2^{\cdot-}$), NO may form the more reactive and toxic peroxynitrite, which contributes to the nitration of proteins, hydroxylation or nitration of DNA, and mutations (Juedes and Wogan, 1996; Ducrocq et al., 1999). Although it has been postulated that NO and ROS cooperatively mediate the toxic effects of MC-LR, the origin of these species and the pathways involved in the generation of other secondary radicals remain to be elucidated.

Human–hamster hybrid (A_L) cells, which contain a full set of hamster chromosomes and a single copy of human chromosome 11, are sensitive to mutagens, such as ionizing radiation and certain chemical agents, that induce large multilocus deletions (Waldren et al., 1986; Hei et al., 1998). Since only a small part of 11p15.5 is required for the viability of A_L cells, chromosomal deletions up to 140 Mb can be detected in these cells. In the present study, A_L cells were exposed to a range of doses of MC-LR for varying time periods and then assayed for genotoxicity-related endpoints. Mitochondrial DNA encodes a nitric oxide synthase (NOS) that is a major source of NO (Giulivi et al., 1998), the role of mitochondria in MC-LR-induced mutagenesis was assessed in mitochondrial DNA-depleted (ρ^0) A_L cells.

1. Materials and methods

1.1. Cell culture

A_L hybrid cells, which contain a set of CHO-K1 chromosomes and a single copy of human chromosome 11, were used in the experiments. Chromosome 11 encodes cell surface markers that confer A_L cells with sensitivity to the cytotoxic effects of specific monoclonal antibodies in the presence of rabbit serum complement (Merck, Darmstadt, Germany) (Hei et al., 1998). Antibody E7.1 specific to the *CD59* antigen was produced from hybridoma cultures. A_L cells were cultured in Ham's F-12 medium supplemented with 8% heat-inactivated fetal bovine serum (FBS), 25 μg/mL gentamicin, and 2×10^{-4} mol/L glycine. ρ^0 A_L cells were generated by treating A_L cells with the chemotherapeutic drug ditercalinium over a period of 3–4 months to deplete mitochondrial DNA by 95% (Liu et al., 2005). ρ^0 A_L cells were cultured in Dulbecco's Modified Eagle's Medium/F12 (1:1) supplemented with 15% heat-inactivated FBS, 2.7 g/L glucose, 584 mg/L glutamine, 50 μg/mL uridine, 25 μg/mL gentamicin, and 2×10^{-4} mol/L glycine. All cells were maintained at 37°C in a humidified 5% CO_2 incubator and passaged every 3 days.

1.2. MC-LR preparation and chemical treatment

A stock solution of MC-LR (Alexis Biochemicals, Lausanne, Switzerland) was prepared at a concentration of 50 μmol/L with doubled-distilled water and sterilized by passage through a 0.22-μm pore size syringe filter. Working solutions were diluted from the stock solution with complete F-12 medium. A_L and ρ^0 A_L cells in the exponential growth phase were incubated with 0.01–1 μmol/L MC-LR in 60-mm diameter petri dishes for varying time periods, with untreated time-matched control cells grown concurrently. Treated cells were subcultured every 3 days for up to 30 days with fresh medium containing MC-LR. After treatment, cells were washed twice with balanced salt solution and processed for various endpoints. In experiments involving nitric oxide synthase (NOS) inhibition, N^G-methyl-L-arginine (L-NMMA, Molecular Probes, Inc., Eugene, OR, USA) dissolved in doubled-distilled water and filter-sterilized was added to cultures concurrently with MC-LR for 1 or 3 days; during the 30-day treatment, confluent cells were subcultured with fresh medium containing MC-LR and L-NMMA every 3 days. After treatment, cells were collected for further analysis.

1.3. Cell survival assay

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to evaluate the cytotoxicity of MC-LR (Mosmann, 1983). A_L and ρ^0 A_L cells in the exponential