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Microcystin-LR induced DNA damage in human peripheral blood lymphocytes

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ABSTRACT

Human exposure to microcystins, which are produced by freshwater cyanobacterial species, is of growing concern due to increasing appearance of cyanobacterial blooms as a consequence of global warming and increasing water eutrophication. Although microcystins are considered to be liver-specific, there is evidence that they may also affect other tissues. These substances have been shown to induce DNA damage *in vitro* and *in vivo*, but the mechanisms of their genotoxic activity remain unclear. In human peripheral blood lymphocytes (HPBLs) exposure to non-cytotoxic concentrations (0, 0.1, 1 and 10 μg/ml) of microcystin-LR (MCLR) induced a dose- and time-dependent increase in DNA damage, as measured with the comet assay. Digestion of DNA from MCLR-treated HPBLs with purified formamidopyrimidine-DNA glycosylase (Fpg) displayed a greater number of DNA strand-breaks than non-digested DNA, confirming the evidence that MCLR induces oxidative DNA damage. With the cytokinesis-block micronucleus assay no statistically significant induction of micronuclei, nucleoplasmic bridges and nuclear buds was observed after a 24-h exposure to MCLR. At the molecular level, no changes in the expression of selected genes involved in the cellular response to DNA damage and oxidative stress were observed after a 4-h exposure to MCLR (1 μg/ml). After 24 h, DNA damage-responsive genes (*p53*, *mdm2*, *gadd45a*, *cdkn1a*), a gene involved in apoptosis (*bax*) and oxidative stress-responsive genes (*cat*, *gpx1*, *sod1*, *gsr*, *gclc*) were up-regulated. These results provide strong support that MCLR is an indirectly genotoxic agent, acting via induction of oxidative stress, and that lymphocytes are also the target of microcystin-induced toxicity.

1. Introduction

Cyanobacteria are well known to produce a variety of toxins, which are released into the environment after cell death. Microcystins (MCs) are the largest group of cyanobacterial toxins consisting of over 80 isoforms, microcystin-LR (MCLR) being the most common and most widely studied congener [1]. Based on acute toxicity studies it is considered as one of the most potent cyanobacterial toxins. In 1998 the World Health Organization (WHO) established a provisional guideline value for MCLR of 1 μg/l in drinking water [2] and recently the International Agency for Research on Cancer (IARC) classified MCLR as a possible human carcinogen (Group 2B) [3].

MCs are highly hepatotoxic and one of the mechanisms of their toxicity is the inhibition of eukaryotic serine/threonine protein phosphatases 1 and 2A (PP1 and PP2A) [4–6] and consequently hyperphosphorylation of cytoskeletal proteins [6]. This leads to disruption of many cellular processes and cytoskeletal damage [7,8]. More importantly, these toxins are potent tumour promoters [9–11]. The tumour-promoting activity of MCLR is supposed to arise from its ability to inhibit PP2A, which regulates several mitogen-activated kinases (MAPK) and these in turn regulate transcription of genes required for cell proliferation [12]. Apart from the tumour-promoting activity there is some evidence that MCs can act as tumour initiators [13]. There is also increasing evidence that MCs are genotoxic [14–20], but the mechanisms by which these toxins induce DNA damage and cancer development are not well understood. Moreover, existing data on their genotoxic potential are contradictory.

Human exposure to MCs is of growing concern due to increasing appearance of cyanobacterial blooms as a consequence of water eutrophication and global warming. The main routes of human exposure are during recreational activity, through drinking of water contaminated with cyanotoxins and by ingestion of food and dietary supplements that may be contaminated with cyanobacteria [1,21–23]. A minor route of exposure to cyanotoxins is the parenteral route through haemodialysis, which nevertheless represents an extremely relevant route of exposure as it results in a high internal dose of the toxins, directly entering the bloodstream [24,25].

For a long time the liver was thought to be the main target organ of MC activities, as these toxins require uptake via active transport by organic anion-transporting polypeptides (OATP), which are

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expressed in the hepatocytes [26]. OATPs are now being increasingly recognized to be expressed not only in the liver but also in the gastrointestinal tract, kidney, and brain [27]. There is evidence that MCLR can be transported across the human blood–brain barrier [26]. When orally ingested, MCs are transported across the ileum into the bloodstream through the bile-acid transport system present in hepatocytes and in cells lining the small intestine [28].

The aim of the present study was to evaluate the induction of DNA strand-breaks and micronuclei (MNi) in human peripheral blood lymphocytes (HPBLs) after exposure to MCLR. In addition, the mechanism of MCLR-induced genotoxicity has been evaluated by use of quantitative real-time-PCR, by examining – with quantitative real-time-PCR – the gene-expression pattern of selected DNA damage-responsive genes and genes involved in oxidative stress and apoptosis.

2. Materials and methods

2.1. Chemicals

Chromosome kit P was from Euroclone, Italy; fetal bovine serum, RPMI 1640, and TRIzol reagent were from Invitrogen, Carlsbad, USA; penicillin/streptomycin, cytochalasin B, histopaque, ethidium bromide, low melting-point (LMP) and normal melting-point (NMP) agaroses were from Sigma, USA; phytohaemagglutinin was from Remel Europe Ltd., England; heparinised vacutainer tubes from Becton Dickinson, USA; Giemsa from Merck, Germany; microcystin-LR from Enzo Life Sciences GmbH, Lausen, Switzerland; Fpg FLARE™ assay kit from Trevigen Inc., USA; total RNA and cDNA High-Capacity Archive Kit, TaqMan Universal PCR Master Mix and Taqman Gene Expression Assays were from Applied Biosystems, USA. All other chemicals were of analytical grade.

2.2. Blood sampling and treatment

Whole blood samples were taken from a healthy male donor (age 28 years; non-smoker) who had not been exposed to ionizing radiation, vaccinated or treated with drugs for a year before blood sampling. Whole venous blood was collected under sterile conditions in heparinised vacutainer tubes (Becton Dickinson, NJ, USA) containing lithium heparin as anticoagulant.

The comet assay and the micronucleus assay were conducted on whole blood, while cytotoxicity and gene expressions were performed on isolated human peripheral blood lymphocytes (HPBLs) cultivated at 37 °C in an atmosphere with 5% CO_2 (Heraeus Heracell 240 incubator, Langenselbold, Germany). HPBLs were isolated by Histopaque-1119 density gradient centrifugation, washed with RPMI medium and centrifuged at 4000 × rpm [give proper "2880 × *g*" value here; Ed.] for 8 min. The pellet of lymphocytes was used for further experiments. The isolated HPBLs were cultured in RPMI-1640 medium supplemented with 14% fetal calf serum and 9 mg/ml phytohaemagglutinin.

The whole blood was treated with 0, 0.1, 1 and 10 μg/ml MCLR for 4, 6 and 24 h for the comet assay and during 24 h for the cytokinesis-block micronucleus (CBMN) assay, while for the mRNA expression the isolated HPBLs were exposed to 1 μg/ml of MCLR for 4 and 24 h. In each experiment a non-treated control and a vehicle control (0.1% ethanol) were included.

2.3. Cell-viability (cytotoxicity) test

Indices of cell viability were established by differential staining of HPBLs with acridine orange and ethidium bromide [29]. A total of 100 cells per replicate were examined with a Zeiss microscope (Göttingen, Germany). Cells were classified as follows: live cells with functional membrane, with uniform green staining of the nucleus, and necrotic cells with uniform orange staining of the nucleus.

2.4. The comet assay

The alkaline comet assay was carried out as described by Singh et al. [30] with minor modifications [29]. Briefly, after the exposure to MCLR, 5 μl of whole blood was mixed with 100 μl of 0.5% LMP agarose and added to fully frosted slides precoated with 0.6% NMP agarose. After solidification, the slides were covered with 0.5% LMP agarose, and lysed (2.5 M NaCl, 100 mM EDTA-Na_2, 10 mM Tris, 1% sodium sarcosinate, 1% Triton X-100, 10% dimethyl sulfoxide, pH10) overnight at 4 °C. After the lysis, the slides were placed in alkaline solution (300 mM NaOH, 1 mM EDTA-Na_2, pH13) for 20 min at 4 °C to allow DNA unwinding, and subsequently electrophoresed for 20 min at 1 V/cm. Finally, the slides were neutralized in 0.4 M Tris buffer (pH 7.5) – three times 5 min – stained with EtBr (20 μg/ml) and analyzed at 250 × magnification using an epifluorescence microscope (Zeiss, Göttingen, Germany) connected through a black-and-white camera to an image-analysis system (Comet Assay II; Perceptive Instruments Ltd., UK). The percent of tail DNA was used to measure the level of DNA damage and a total of 50 randomly captured nuclei were examined from each slide in two independent experiments. The results are shown as box plots.

The analysis of the formation of oxidized purines was performed with the Fpg FLARE™ assay kit (Trevigen Inc., Gaithersburg, USA) with minor modifications. The slides were prepared as described above. For each sample and control, 5 μl of whole blood was mixed with 100 μl of LMP agarose (provided with the FLARE™ assay kit) and placed on the slides. After solidification the slides were covered with 0.5% LMP agarose and then immersed in a pre-chilled lysis solution (provided with the FLARE™ assay kit) and lysed overnight at 4 °C. After lysis, the slides were treated with 100 μl of Fpg enzyme freshly diluted in REC dilution buffer (1:500) or with 100 μl of REC dilution buffer only (control), covered with a cover glass and incubated at 37 °C for 30 min. Subsequently, the slides were immersed in an alkali solution (0.3 M NaOH, 1 mM Na_2EDTA; pH 12.1) for 40 min and electrophoresed for 20 min at 1 V/cm. Finally, the slides were neutralized, stained with EtBr and the comets were analyzed as described above.

2.5. Cytokinesis-block micronucleus (CBMN) assay

The micronucleus assay was performed according to the guidelines by Fenech and Morley [31] with minor modifications [29]. After the exposure (24 h) to MCLR the whole blood (500 μl) was incubated in a Euroclone medium (Chromosome kit P) at 37 °C in an atmosphere of 5% CO_2. Cytochalasin-B was added at a final concentration of 3 μg/ml, 44 h after the culture was started. The cultures were harvested after 72 h. The lymphocytes were fixed in methanol–acetic acid (3:1), air-dried and stained with 5% Giemsa solution (Sigma). All slides were randomised and coded prior to analysis. The binuclear lymphocytes were analyzed under a light microscope (Olympus CX41, Tokyo, Japan) at 400 × magnification. Micronuclei (MNi), nucleoplasmic bridges (NPB) and nuclear buds (NBUD) were counted in 1000 binucleated cells (BNC) per experimental point and were scored according to the HUMN project criteria published by Fenech [32]. Two independent experiments were performed. The cytokinesis-block proliferation index (CPBI) was determined by scoring 1000 cells with one to four nuclei. The CPBI was calculated using the formula $[M_1 + 2M_2 + 3(M_3 + M_4)]/1000$, where M_1–M_4 represent the number of cells with one to four nuclei, respectively, and M_3 and M_4 are equally considered to be in their third cycle [33,34]. Bleomycin at final concentration 0.04 μg/ml and 2 h treatment was used as a positive control.

2.6. mRNA expression

After the exposure to MCLR, the HPBLs were centrifuged at 3000 rpm for 10 min. The pellets were washed with 1 × DEPC-PBS and again centrifuged at 3000 rpm for 10 min. Total RNA from the lymphocytes was isolated using TRIzol reagent, and cDNA synthesized using 1 μg of total RNA and cDNA High-Capacity Archive Kit (Applied Biosystems, USA), according to the manufacturer's protocol. Gene expression of *p53*, *mdm2*, *gadd45α*, *cdkn1a*, *bax*, *bcl*, *cat*, *gpx1*, *gclc*, *sod1* and *gsr* was quantified using real-time quantitative PCR (ABI 7900 HT Sequence Detection System, Applied Biosystems, USA). TaqMan Universal PCR Master Mix and the following Taqman Gene Expression Assays were used (all from Applied Biosystems): *p53* (tumour protein p53), Hs00153349_m1; *mdm2* (Mdm2, 'transformed 3T3 cell double minute 2', p53 binding protein gene), Hs00234753_m1; *gadd45α* ('growth arrest and DNA damage-inducible gene, alpha'), Hs00169255_m1; and *cdkn1a* ('cyclin-dependent kinase inhibitor 1A'), Hs00355782_m1; *cat* (catalase), Hs00937387_m1; *sod1* (superoxide dismutase 1, soluble), Hs00166575_m1; *gclc* (glutamate-cysteine ligase, catalytic subunit), Hs00155249_m1; *gsr* (glutathione reductase), Hs00167317_m1; *gpx1* (glutathione peroxidase 1), Hs01028922_g1; *bax* (BCL2 associated X protein), Hs99999001_m1; *bcl2* (B-cell CLL/lymphoma 2), Hs00608023_m1.

Amplification of the *GAPDH* probe was performed as an internal control. The conditions for PCR were 50 °C for 2 min, 95 °C for 10 min and 40 cycles at 95 °C for 15 s and 60 °C for 1 min. The data obtained from Taqman Gene Expression Assays were analyzed using the ΔΔCt algorithm. The expression levels of target mRNAs were normalized to the GAPDH mRNA level. Two independent experiments were performed, each time in two parallels.

2.7. Statistics

The statistical analyses were performed with GraphPad Prism 5 software. For the comet assay, one-way analysis of variance (ANOVA) was used to analyze the differences between treatments within each experiment. Dunnett's test was used for multiple comparison versus the control; $P < 0.05$ was considered as statistically significant (*). Difference between samples in the micronucleus test and – for the gene expression assays – the statistical significance of the difference between treated groups and controls was determined by Two-tailed Student's *t*-test comparison of the mean; $P < 0.05$ was considered significant.