The repair of gamma-radiation-induced DNA damage is inhibited by microcystin-LR, the PP1 and PP2A phosphatase inhibitor

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The genotoxic activity of microcystin-LR (MC-LR) is a matter of debate. MC-LR is known to be a phoshatase inhibitor and it may be expected that it is involved in the regulation of the activity of DNA-dependent protein kinase (DNA-PK), the key enzyme involved in the repair of radiation-induced DNA damage. We studied the effect of MC-LR on the repair capacity of radiation-induced DNA damage in human lymphocytes and human glioblastoma cell lines MO59J and MO59K. A dose of 0.5 μg/ml of MC-LR was chosen because it induced very little early apoptosis which gives no false positive results in the comet assay. Human lymphocytes in G0-phase of the cell cycle were pre-treated with MC-LR for 3 h and irradiated with 2 Gy of gamma radiation. The kinetics of DNA repair was assessed by the comet assay. In addition the frequencies of chromosomal aberrations were analysed. The pre-treatment with MC-LR inhibited the repair of radiation-induced damage and lead to enhanced frequencies of chromosomal aberrations including dicentric chromosomes. The results of a split-dose experiment, where cells were exposed to two 1.5 Gy doses of radiation separated by 3 h with or without MC-LR, confirmed that the toxin increased the frequency of dicentric chromosomes. We also determined the effect of MC-LR and ionizing radiation on the frequency of γ-H2AX foci. The pre-treatment with MC-LR resulted in reduced numbers of γ-H2AX foci in irradiated cells. In order to elucidate the impact of MC-LR on DNA-PK we examined the kinetics of DNA repair in human glioblastoma MO59J and MO59K cells. Both cell lines were exposed to 10 Gy of X-rays and DNA repair was analysed by the comet assay. A strong inhibitory effect was observed in the MO59K but not in the MO59J cells. These results indicate that DNA-PK might be involved in DNA repair inhibition by MC-LR.

Introduction

Cyanobacterial toxins are responsible for the death of domestic and wild animals and human illness (1). More importantly, these toxins are potent tumour promoters (2–4) and there is an indication that they may also act as tumour initiators (5). The most abundant in the environment and best studied toxin, related to tumour promotion, is microcystin-LR (MC-LR), produced by several genera of Cyanobacteria, including Microcystis, Anabaena, Oscillatoria (Planothrix) and Nostoc (6). It is supposed that one possible mechanism for cancer promotion by MC-LR is the inhibition of protein phosphatase PPI and PP2A (7) which leads to hyperphosphorylation of some cellular proteins and results in uncontrolled cell proliferation (8). It is also believed that MC-LR may directly induce gene mutations. Ding et al. (9) used four strains of Salmonella typhimurium to demonstrate with the Ames test that MC-LR induced point mutations. The results obtained by Mankiewicz et al. (10) also indicate that MC-LR had a genotoxic effect on Escherichia coli (strain PQ37). Zhan et al. (11) reported that MC-LR specifically induced loss of heterozygosity at the TK locus, but not point mutations in human lymphoblastoid TK6 cells. It was also noted that MC-LR induced DNA damage in mouse embryo fibroblasts and BHK-21 cell line (12), as well as in primary cultured rat hepatocytes (9). Recently Zegura et al. (13) demonstrated with the comet assay that MC-LR induced DNA strand breaks in human hepatoma HepG2 cells. The breaks were transiently present and probably arose during the repair of other types of DNA damage. Contrary to those results Repavich et al. (14) and Grabow et al. (15) reported that MC-LR, with and without metabolic activation, did not have any mutagenic properties. Our previous studies revealed that microcystin did not induce any chromosomal damage in CHO-K1 cells as well as in human lymphocytes treated with MC-LR (16). We also showed that the DNA damage observed by the comet assay in cells treated with MC-LR was related to the early stages of apoptosis and not to the genotoxicity of the toxin (17).

Our preliminary results indicate the MC-LR suppressed the repair of radiation induced DNA damage (17). This result is in line with the observation that MC-LR may be involved in the regulation of the activity of DNA-PK, which is involved in the non-homologous end joining (NHEJ) of radiation-induced breaks (18).

Considering this and the inconsistent data regarding the genotoxicity of MC-LR we decided to examine in greater detail its effect on the capacity of repair of damage induced by ionizing radiation in human lymphocytes and human glioblastoma cell lines MO59J and MO59K. The kinetics of DNA repair was assessed by the alkaline version of the comet assay. The level of residual DNA damage was determined by the chromosome aberration assay. The formation of γ-H2AX repair foci was determined by the immunocytochemical method. Apoptosis was analysed with the Annexin method using flow cytometry.

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Materials and methods

The studies on human blood were made according to the permit issue from the local bioethical committee no. 74/2004.

Cells

Human venous blood was collected from three healthy donors (in two repetitions) into heparinized (10–20 U/ml) Greiner bio-one tubes. Peripheral blood mononuclear cells were isolated by Histopaque-1077 density gradient centrifugation, washed twice with phosphate-buffered saline (PBS) and centrifugated at 900 × g for 10 min. The pellet containing lymphocytes was suspended at a density of 5 × 10^5 cells/ml in RPMI 1640 medium supplemented with 20% fetal bovine serum (FBS), 10 μg/ml phytohemagglutinin (PHA), 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. The cultures were incubated at 37°C and 5% CO2. Human glioblastoma cell lines MO59K and MO59P were purchased from ATCC. These cell lines differ in their sensitivity towards ionizing radiation: the radiosensitive MO59I cells are deprived of a catalytic subunit of DNA-dependent protein kinase (DNA-PKcs), and the radiosensitive MO59K cells have a normal level of DNA-PKcs. Cell lines were cultured in DMEM:F12 (1:1) medium, supplemented with 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C and 5% CO2.

MC-LR extraction and purification

Microcystis aeruginosa PCC 7813 (Institut Pasteur, Paris) was cultivated in controlled laboratory conditions and toxics were extracted from the harvested and lyophilized cells with 100% methanol. The extract and the medium were filtered through 110 mm GF/C discs (Whatman, UK) and enriched by the solid phase extraction (SPE) procedure (19). The eluate from the SPE was evaporated to dryness at room temperature under reduced pressure and dissolved in water. The characteristic microcystin UV spectrum was dried, dissolved in methanol:water (30:70, v/v) and filtered through a 0.45 mm PVDF-D membrane filter (Millipore, USA).

The toxic fraction from the ion exchange step contained two main peaks: without the methyl group on methyl-aspartic acid (D-Asp-L-MC-LR (~30%)) and the second, MC-LR (~60%), which eluted very closely to each other. The sufficient separation of these compounds was accomplished by HPLC (20). The MC-LR fraction was collected manually by repeated separations, desalted on a Zorbax SB-C18 column (9.4 × 250 mm, 5 μm) (Agilent) using 100% methanol for elution, dried and dissolved in water for HPLC. The determinations were carried out using a Waters HPLC system consisting of a 600E multisolvent-delivery system, a 717plus autosampler, a 996 photodiode array detector (PDA), Millennium^™ SS software and a Jetstream 2 plus column thermostat. The modified method of Lawton et al. (21) was applied using a Nova-Pak C18 (4.6 × 250 mm, 4 μm) column. A multilevel calibration curve was obtained using the commercial MC-LR (Sigma). The identification of the peak of MC-LR was based on the retention time and the spectrum of the standard. Additionally, multichannel spectrometry analysis of MC-LR was performed by the commercial VICTOR (22). The chromatographic purity was evaluated as the percentage of the toxin peak area in the total area of all peaks on the 239.4 nm chromatogram extracted from the PDA data. Tripple analysis was performed to determine the purity and the concentration of the toxin. Dried MC-LR was dissolved in PBS and was used for further experiments.

Flow cytometric analysis of apoptosis

In the first part of our experiment human lymphocytes were treated with MC-LR for 3 h at a dose of 0.5, 1 and 2 μg/ml. The next set of experiments was performed in parallel with the comet assay experiments and comprised the determination of apoptosis in lymphocytes following treatment with 0.5 μg/ml of MC-LR for 3 h and irradiation with a dose of 2 Gy. The frequencies of apoptotic and necrotic cells were then determined with the annexin V-FITC apoptosis detection kit I (BD Pharmingen, USA), according to Darzynkiewicz et al. (22). Briefly, cells were washed twice with cold PBS and then re suspended in a 1× binding buffer at a concentration of 1 × 10^5 cells/ml. Aliquot of 100 μl of cell suspension was incubated with 5 μl of annexin V-FITC and 5 μl of propidium iodide (PI) at room temperature for 15 min in the dark. The cells were resuspended in 400 μl of a 1× binding buffer. The fluorescence was determined using a FACScan flow cytometer Becton Dickinson.) A computer system (CellQuest Pro, Becton Dickinson) was used for data acquisition and analysis. Data from 20000 events were stored. A cell gate containing lymphocytes was established on the basis of forward and side light scatter. Four different populations of cells are detected with the annexin V-FITC kit: normal cells that are annexin negative and PI negative (lower right panel) and that expressed green fluorescence, late apoptotic/necrotic cells that are annexin positive and PI negative (upper right panel) and that expressed green and orange fluorescence, necrotic cells that are annexin negative and PI positive (upper left panel) and that expressed orange fluorescence (Figure 1).

MC-LR treatment and irradiation

Freshly isolated human lymphocytes were cultured for 2 h and treated for further 3 h with MC-LR at a dose of 0.5 μg/ml. Then, cells were cooled on ice and irradiated with 2 Gy of gamma radiation (60Co, Siemens Theratron Elite 80). The exponentially grown human glioblastoma cell lines MO59K and MO59P were treated for 2 h with MC-LR at a dose of 0.25 μg/ml. Then, culture plates were cooled on ice and irradiated with 10 Gy of X-rays. MC-LR was not removed from the medium. After irradiation, the lymphocytes and human glioblastoma cell lines were placed back into the incubator (37°C) and the kinetics of DNA repair was assessed by the alkaline comet assay.

Analysis of DNA repair

After 0, 30, 60 and 120 min post-exposure to ionizing radiation the alkaline comet assay was performed according to Singh et al. (23). Briefly, 200 μl of cell suspension containing ~100 000 cells was mixed with 200 μl of 2% low melting temperature agarose at 37°C and then placed on a slide pre-coated with a thin layer of 0.5% normal melting agarose. The cell suspension was immediately covered with a coverglass and the slides were kept at 4°C for 5 min to allow solidification of the agarose. After removing the coverglass, the cells were lysed in a lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris and 1% Triton X-100, pH 10) for 1 h. After washing in re-distilled water the slides were placed on a horizontal gel electrophoresis chamber. The chamber was filled with cold (4°C) electrophoretic buffer (1 mM EDTA and 300 mM NaOH, pH 13) and slides were incubated in this buffer for 40 min at 4°C to allow the DNA to unwind. Electrophoresis was performed for 30 min (150 mA, 300 mA). After electrophoresis, the slides were washed three times with the neutralization buffer (0.4 M Tris, pH 7.5). All preparative steps were conducted in darkness to prevent additional DNA damage. The slides were stained with 1 μM 4,6-diamidino-2-phenylindole (DAPI) for 24 h and analysed with a fluorescence microscope (NIKON Eclipse 400) equipped with a CCD-4230 A video camera. Digital images were obtained using the MultiScan software (Poland). At least 50 images per point were analysed and Olive Tail Moment (OTM) was determined by CASP software (24).

Analysis of chromosome aberrations and mitotic indices

The first experiment was to correlate the cytogenetic data with the comet assay results. Freshly isolated human lymphocytes were cultured for 2 h, treated for 3 h with 0.5 μg/ml MC-LR and irradiated with gamma radiation as described above. After irradiation, 5-bromo-2-deoxyuridine (BrdU; 10 μM final concentration) was added to each experimental group to allow identification of the first division metaphases. Lymphocytes were placed back into the incubator (37°C and 5% CO2) and cultured for 50 h. Colcemid (0.15 μg/ml) was added for the final 2 h. Cells were harvested by the standard cytogenetic technique (16), dried and stained by the Fluorescence-plus-Giemsa method (25). Mitotic indices (MI) were calculated by counting 1000 cells per point. The analysis of chromosome aberrations was performed on a minimum of 100 metaphase cells per point. Structural chromosome aberrations were categorized as acentric fragments (b: chromatid-type, and B: chromosome-type), dicentrics (dic) and ring chromosomes (rings) according to Savage (26).

The second experiment with MC-LR was performed to verify the impact of MC-LR on DNA double-strand break (DSB) rejoicing following irradiation. In this study a split-dose experiment was done on freshly isolated human lymphocytes from one donor in three repetitions. Briefly, one experimental group was pre-treated with MC-LR (0.5 μg/ml) for 3 h and then exposed to two 1.5 Gy doses of ionizing radiation, separated by 3 h. MC-LR was not removed from the medium during this time (3 h of MC-LR + 1.5 Gy + 3 h of MC-LR + 1.5 Gy). The second experimental group was exposed to two 1.5 Gy doses of radiation, separated by 3 h without treatment with MC-LR (1.5 Gy + 3 h + 1.5 Gy). After the last irradiation, BrdU (10 μM final concentration) was added to each experimental group. Cells were placed back into the incubator (37°C and 5% CO2) and cultured for 50 h. Colcemid (0.15 μg/ml) was added for the final 2 h. Lymphocytes were harvested and stained as described above. The frequency of dicentrics chromosomas was calculated from a minimum of 100 cells in the first mitotic division counted per point.

Immunofluorescence labeling of γ-H2AX foci

Freshly isolated human lymphocytes were cultured for 2 h and treated for further 3 h with 0.5 μg/ml MC-LR. Then, cells were cooled on ice and irradiated with 1 Gy of gamma rays. Then, MC-LR was removed from the medium and cells were incubated for 30 and 120 min, washed with PBS and placed on slides pre-coated with poly-γ-lysin and left to attach for 15 min. Then cells were fixed with methanol at −20°C for 5 min and rehydrated by incubating in PBS for 30 min (PBS was changed three times). The slides were
blocked for 1 h in blocking buffer [2% BSA, 10% dried milk powder, 0.1% Triton X-100 in KCM buffer (120 mM KCl, 20 mM NaCl, 10 mM Tris–HCl, 0.5 M EDTA and 0.1% Triton X-100, pH 8.0)] and thereafter incubated with mouse anti-phospho-Histone H2A.X(Ser139)-FITC conjugated antibody (2 μg/ml) of blocking buffer for 1 h. The slides were washed three times in 0.1% Triton–PBS and counterstained with 2 mM DAPI. At least 30 images of cells were randomly collected from each experimental group and time point and the number of foci was determined optically under a fluorescent microscope at a (1000) magnification.

Chemicals
Histopaque-1077, RPMI 1640, L-glutamine, Agarose-Type VII, Agarose-Type I, EDTA (Ethylendiaminetetraacetic acid disodium salt dihydrate), Tris (Tris-hydroxymethyl-aminomethane), Triton X-100, DAPI, trypan blue, BrdU, Giemsa stain, bisBENZIMIDE, Colcemid, PBS, NaCl, KCl, Tris, HCl, NaOH were obtained from Sigma-Aldrich (USA). Penicillin, streptomycin, PHA, FBS, Dulbecco’s modified Eagle’s medium (DMEM) and Fi2 were obtained from Gibco (Germany). SPE cartridge packed with reversed phase octadecylsilica (40 μm) and trifluoroacetic acid (TFA) were obtained from J.T.Baker, pentfluoropropanoic acid (PFPA) from Merck and HPLC solvent (methanol, water and acetonitrile) were obtained from Lab-Scan. QAE-Sephadex A-25 to ion exchange step was obtained from Pharmacia.

Statistical analysis
Data were evaluated by Kruskal–Wallis one-way analysis of variance (ANOVA) on ranks followed by Dunnet’s method (results of flow cytometry) and by ANOVA followed by Mann–Whitney rank sum test (all other data) and statistical significance was tested at \( P < 0.05 \) as the critical value. Correlations were determined using the Pearson product moment. \( P \)-values <0.05 were considered statistically significant.

Results
Flow cytometric analysis of apoptosis
Apoptosis was measured in lymphocytes treated with different doses MC-LR. The aim of this experiment was to establish an optimal dose of MC-LR for experiments with ionizing radiation. As shown in Figure 1, cells treated with 1 and 2 μg/ml of MC-LR showed typical features of early and late apoptotic cells, respectively. Following treatment with 0.5 μg/ml MC-LR, the level of early apoptosis was very low and this concentration was chosen for further experiments.
To exclude the possibility of false positive results in the comet assay, apoptosis was also determined in parallel with the comet assay experiments in lymphocytes following treatment with MC-LR and irradiation after various repair times (0, 30, 60 and 120 min). As presented in Table I, the results showed no statistically significant differences dealing with the percent of late apoptosis (a possible confounding factor) in cells treated with both MC-LR and irradiation as compared with the control group and microcystin-treated group. However, a marked increase of early apoptosis (which gives no false positive results in the comet assay) was observed in lymphocytes treated with MC-LR and with both MC-LR and irradiation as compared with the control group.

**Analysis of DNA repair capacity by the comet assay in human lymphocytes pre-treated with MC-LR and irradiated with gamma radiation**

The repair of DNA damage was analysed comparatively in gamma-irradiated lymphocytes (IR) and lymphocytes pre-treated with MC-LR and exposed to gamma radiation (MC-LR + IR). Results are shown in Figure 2A. No DNA damage was observed in cells treated with MC-LR alone. In general, the pre-treatment with MC-LR resulted in increased values of residual DNA damage as compared with the IR group. When the results are expressed as percent of initial damage (Figure 2B) it is evident that treatment with MC-LR inhibits the repair of radiation-induced damage (54 versus 32% after 60 min and 38 versus 15% after 120 min, respectively).

**Analysis of chromosome aberrations and MI**

Chromosomal aberrations were analysed in human lymphocytes pre-treated with MC-LR and exposed to 2 Gy of gamma radiation in order to see if the inhibition of DNA repair could be detected on the level of cytogenetic damage. As presented in Figure 3, MC-LR had no significant influence on the frequencies of spontaneous chromosome aberrations in lymphocytes. Irradiation of human lymphocytes increased the total frequencies of chromosome aberrations as compared with non-treated cells (21.0 ± 9.2 versus 1.0 ± 1.52). Pre-treatment with MC-LR increased the total frequencies of gamma-radiation-induced chromosome aberrations (33.1 ± 11.8 versus 21.0 ± 9.2) including chromosome-type aberrations (18.2 ± 4.2 versus 11.3 ± 3.3) and dicentric chromosomes (13.6 ± 3.5 versus 8.3 ± 2.7). MI were analysed on the same slides that were used for the comet assay experiments in lymphocytes following treatment with MC-LR and exposed to gamma radiation (MC-LR + IR). Results showed no statistically significant differences dealing with the percent of late apoptosis (a possible confounding factor) in cells treated with both MC-LR and irradiation as compared with the control group. However, a marked increase of early apoptosis (which gives no false positive results in the comet assay) was observed in lymphocytes treated with MC-LR and with both MC-LR and irradiation as compared with the control group.

Data are expressed as mean values ± SD from six independent experiments and evaluated by Kruskal–Wallis one-way ANOVA on ranks followed by Dunnet’s method.

*Denotes a statistically significant difference versus the control group.

$^a$Denotes a statistically significant difference versus the MC-LR group. $P$-values <0.05 were considered as significant.

**Table I.** Percentage of early and late apoptotic cells following treatment with microcystin-LR (MC-LR, 0.5 μg/ml) and ionizing radiation (IR, 2 Gy) after various repair times (0, 30, 60 and 120 min)

<table>
<thead>
<tr>
<th>Repair time (min)</th>
<th>Apoptosis</th>
<th>Control</th>
<th>MC-LR (0.5 μg/ml)</th>
<th>IR (2 Gy)</th>
<th>MC-LR + IR</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Early (annexin+/PI−)</td>
<td>1.66 ± 0.3</td>
<td>10.96 ± 1.6*$^a$</td>
<td>1.56 ± 0.5$^b$</td>
<td>12.10 ± 1.6*$^b$</td>
</tr>
<tr>
<td></td>
<td>Late (annexin+/PI+)</td>
<td>0.42 ± 0.2</td>
<td>0.58 ± 0.1</td>
<td>0.48 ± 0.2</td>
<td>0.60 ± 0.2</td>
</tr>
<tr>
<td>30</td>
<td>Early (annexin+/PI−)</td>
<td>1.75 ± 0.3</td>
<td>14.98 ± 2.1*$^a$</td>
<td>1.70 ± 0.5$^a$</td>
<td>14.58 ± 1.2*$^a$</td>
</tr>
<tr>
<td></td>
<td>Late (annexin+/PI+)</td>
<td>0.38 ± 0.1</td>
<td>0.54 ± 0.1</td>
<td>0.48 ± 0.2</td>
<td>0.52 ± 0.2</td>
</tr>
<tr>
<td>60</td>
<td>Early (annexin+/PI−)</td>
<td>1.73 ± 0.4</td>
<td>17.75 ± 1.5*$^a$</td>
<td>1.56 ± 0.3$^b$</td>
<td>17.08 ± 1.5*$^a$</td>
</tr>
<tr>
<td></td>
<td>Late (annexin+/PI+)</td>
<td>0.44 ± 0.2</td>
<td>0.62 ± 0.1</td>
<td>0.54 ± 0.2</td>
<td>0.62 ± 0.2</td>
</tr>
<tr>
<td>120</td>
<td>Early (annexin+/PI−)</td>
<td>1.66 ± 0.4</td>
<td>18.60 ± 1.2*$^a$</td>
<td>1.55 ± 0.6$^b$</td>
<td>17.71 ± 1.5*$^a$</td>
</tr>
<tr>
<td></td>
<td>Late (annexin+/PI+)</td>
<td>0.44 ± 0.1</td>
<td>0.45 ± 0.1</td>
<td>0.46 ± 0.2</td>
<td>0.66 ± 0.2</td>
</tr>
</tbody>
</table>

![Fig. 2.](https://academic.oup.com/mutage/article-abstract/21/1/83/1405576) Fig. 2. (A) DNA repair kinetics in human lymphocytes treated with 0.5 μg/ml MC-LR for 3 h and irradiated with a dose of 2 Gy ionizing radiation. Data are presented as mean ± SD from six independent experiments. a: Difference to the irradiated group value, statistically significant with $P < 0.05$. (B) Curves of repair kinetics with damage expressed as percent of initial damage in human lymphocytes treated with 0.5 μg/ml MC-LR for 3 h and irradiated with a dose of 2 Gy of ionizing radiation. Data are presented as mean ± SD, from six independent experiments. a: Difference to the irradiated group value, statistically significant with $P < 0.05$. 

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were used for the estimation of the chromosome aberrations. Treatment with MC-LR had no effect on the MI values of non-irradiated (control: 7.23 ± 0.5, MC-LR: 7.45 ± 1.2) and irradiated cells (IR: 5.05 ± 1.2, MC-LR + IR: 5.03 ± 1.4).

The results of the split-dose experiment revealed that the frequency of dicentric chromosomes was significantly increased in lymphocytes exposed to MC-LR and two 1.5 Gy doses of radiation separated by 3 h [3 h of MC-LR + 1.5 Gy + 3 h of MC-LR + 1.5 Gy] (38.2 ± 9.2) as compared with lymphocytes exposed to two 1.5 Gy doses of radiation separated by 3 h without the drug [1.5 Gy + 3 h + 1.5 Gy] (23.5 ± 6.6).

Correlation between the capacity of DNA repair and the frequencies of chromosome aberrations in human lymphocytes
To determine whether the results obtained with the comet assay correlate with the cytogenetic results we compared the capacity of DNA repair (expressed as the slope of repair kinetics up to 60 min post-exposure) and the frequency of chromosome aberrations. We observed a significant negative correlation between both endpoints (Figure 4) indicating that lymphocytes with inhibited DNA repair showed higher frequencies of chromosome aberrations.

Analysis of γ-H2AX focus formation
The formation of γ-H2AX foci was analysed in human lymphocytes pre-treated with MC-LR and exposed to 1 Gy of radiation and compared with irradiated only cells. As shown in Figure 5, the pre-treatment of lymphocytes with MC-LR resulted in a decreased level of γ-H2AX foci formation. The difference was significant both at 30 and 120 min post-exposure.

Analysis of DNA repair in MO59J (DNA-PKcs-deficient) and MO59K (DNA-PKcs-proficient) cell lines
The impact of MC-LR pre-treatment on the repair of radiation-induced DNA damage in MO59J and MO59K cell lines was analysed in order to verify if MC-LR inhibits DNA-PK. As shown in Figure 6, without MC-LR treatment the repair of radiation-induced DNA damage was impaired in MO59J cells as compared with MO59K cells (29.5 versus 45.7% after 30 min, 53.8 versus 74.6% after 60 min and 74.9 versus 84.4% after 120 min). Pre-treatment with MC-LR led to an inhibition of DNA repair in the MO59K cells as compared with the irradiated MO59K cells (20.5 versus 45.7% after 30 min, 43.6 versus 74.6% after 60 min and 70.8 versus 84.4% after 120 min). The repair of DNA damage was not statistically impaired in MO59J cells.
are presented as mean ± SD from three independent experiments. a: Difference the MO59J-IR to the MO59K-IR group value; b: difference the MO59K-MC-LR + IR to the MO59K-IR group value difference the MO59J-MC-LR + IR to the MO59J-IR group value, statistically significant with P < 0.05.

Discussion

The aim of this study was to determine the effect of MC-LR on the repair of DNA damage induced by ionizing radiation. Because ionizing radiation causes a broad spectrum of DNA lesions such as alkali-labile lesions, single-strand breaks (SSBs) and DSBs (27) we used the alkaline version of the comet assay which is able to detect all these types of DNA damage of individual cells (28). We and others have shown that apoptosis may be a confounding factor in the analysis of DNA damage and repair by this assay (29,17). Therefore, experiments were necessary to determine a dose of MC-LR that did not induce apoptosis at a level that would confound the results of DNA repair. That MC-LR is taken up efficiently by human lymphocytes was shown by us previously (30). Hence we hypothesize that MC-LR is taken up efficiently by human lymphocytes for 3 h with with 0.5, 1 and 2 μg/ml MC-LR and irradiated with a dose of 10 Gy X-rays. Data are presented as mean ± SD from three independent experiments.

Fig. 6. Curves of repair kinetics with damage expressed as percent of initial damage in human glioblastoma cell lines MO59J and MO59K treated with 0.25 μg/ml MC-LR for 2 h and irradiated with a dose of 10 Gy X-rays. Data are presented as mean ± SD from three independent experiments. a: Difference the MO59J-IR to the MO59K-IR group value; b: difference the MO59K-MC-LR + IR to the MO59K-IR group value difference the MO59J-MC-LR + IR to the MO59J-IR group value, statistically significant with P < 0.05.

Potent inhibition of PP1 (IC50 2.5 nM). An inhibited activity of PP2A (IC50 1.68 nM) and PP2A (IC = 0.04 nM) by MC-LR was reported by Dawson (41). However Borthwick et al. (42) observed a strong inhibition activity of MC-LR against PP5 (IC = 2.5 nM). An inhibited activity of phosphatases leads to hyperphosphorylation of many regulatory proteins (43). Douglas et al. (18) reported that phosphorylation-induced loss of the protein kinase activity of DNA-PK was restored by the addition of protein phosphatases PP1 and PP2A and this reactivation was inhibited by MC-LR. Experiments performed by Wechsler et al. (44) suggested a strong interaction between PP5 and DNA-PK and showed that the impaired phosphorylation or premature dephosphorylation of DNA-PK by PP5 leads to an increased radiation sensitivity. Recent studies also revealed that treatment of cells with the protein phosphatase inhibitor okadaic acid enhanced DNA-PKs phosphorylation and reduced DNA-PK activity in vivo (45).

To confirm our assumption that DNA-PK might be involved in MC-LR-decreased DNA repair capacity we examined the kinetics of DNA repair in human glioblastoma cell lines MO59J that lack the catalytic subunit of DNA-PK and in their isogenic counterpart MO59K that is proficient in DNA-PK (46). If DNA-PK is the repair enzyme inhibited by MC-LR, then no effect of MC-LR should be seen in the irradiated lymphocytes. Our results show that MC-LR significantly increased the frequency of gamma-radiation-induced aberrations including dicentric chromosomes and chromosome-type breaks. The split-dose experiment revealed that the frequency of dicentric chromosomes was significantly increased in lymphocytes exposed in G0-phase of the cell cycle to MC-LR and two 1.5 Gy doses of radiation separated by 3 h as compared with lymphocytes exposed only to two 1.5 Gy doses of radiation separated by 3 h. These results confirmed the hypothesis that MC-LR-decreased DNA DSB rejoining is due to alterations in NHEJ. The comparison of the DNA repair capacity with the frequency of chromosome aberrations in lymphocytes pre-treated with MC-LR revealed a clear negative correlation suggesting a causative relationship between both phenomena. A similar relationship between a high level of chromosome aberrations and low repair capacity was reported by Blaise et al. (34) in gamma-irradiated human B-chronic lymphocytic leukaemia cells.

There is evidence that dicentric chromosomes involve two radiation-produced DSBs (35,36). A sensitive and selective signal for the existence of a DNA DSBs in cells treated with ionizing radiation is the formation of phosphorylated H2AX (γ-H2AX) nuclear foci (37,38). To elucidate whether the increased level of dicentric chromosomes in lymphocytes pre-treated with MC-LR correlates with the number of DSBs we treated human lymphocytes in the same manner as for the analysis of DNA repair and determined the frequencies of γ-H2AX foci. Our results show that pre-treatment with MC-LR indeed reduced the frequencies of radiation-induced γ-H2AX foci.

Our experiments were performed with freshly drawn human lymphocytes that are in the G0 phase of the cell cycle (39). Therefore, the effect of MC-LR on DNA repair and the formation of γ-H2AX foci is related to the NHEJ repair pathway. NHEJ operates in the G0 and G1 phases of the cell cycle and involves a DNA end-binding heterodimer of the Ku70 and Ku80 proteins, the catalytic subunit of the DNA-PK, DNA ligase IV and its co-factor XRCC4 (40). We assume that the observed inhibition of NHEJ by MC-LR results from the inhibition of PP2A-like protein phosphatases by this toxin. Potent inhibition of PP1 (IC50 = 1.68 nM) and PP2A (IC = 0.04 nM) by MC-LR was reported by Dawson (41). However Borthwick et al. (42) observed a strong inhibition activity of MC-LR against PP5 (IC = 2.5 nM). An inhibited activity of phosphatases leads to hyperphosphorylation of many regulatory proteins (43).

MC-LR-decreased DNA repair capacity may be a confounding factor in the analysis of DNA damage and repair by this assay (29,17). Therefore, experiments were necessary to determine a dose of MC-LR that did not induce apoptosis at a level that would confound the results of DNA repair. That MC-LR is taken up efficiently by human lymphocytes was shown by us previously (30). Hence we treated human lymphocytes for 3 h with with 0.5, 1 and 2 μg/ml MC-LR. Apoptosis was determined with the annexin-V assay which detects the translocation of phosphatidylserine from the inner to the outer leaflet of the cytoplasmic membrane, as an early event in the apoptotic pathway (31). Lymphocytes treated with 1 and 2 μg/ml MC-LR at presented typical features of apoptotic cells. However, following treatment with 0.5 μg/ml MC-LR, the level of early apoptosis was very low and this concentration was chosen for further experiments.

The analysis of DNA repair kinetics in irradiated lymphocytes not treated with MC-LR revealed a progressive decrease of residual DNA damage that is consistent with the model of DNA repair presented by others (32). Lymphocytes pre-treated with MC-LR and irradiated showed a similar trend in kinetics to that found in irradiated only lymphocytes. However, the capacity of repair was markedly decreased.

It is believed that unrepaired or misrepaired DNA may lead to chromosome rearrangements (33). To investigate whether the decreased DNA repair capacity observed in the comet assay correlates with the chromosome rearrangements we determined the frequencies of chromosome aberrations in human
MO591 cells. Our results clearly show that pre-treatment with MC-LR inhibited the repair in MO57K cells but not in MO591. These data indicate that DNA-PK—a key enzyme of the NHEJ DSB repair system—could be the main target of MC-LR, although the influence of the poison on other DNA repair enzymes or signaling pathways, including ATM (Ataxia Telangiectasia Mutated) is also possible.

In conclusion, our studies revealed that MC-LR decreases the capacity of NHEJ, enhanced the frequencies chromosomal aberrations and diminished the formation of γ-H2AX foci in cells exposed to ionizing radiation. Our results also suggest that DNA-PK might be involved in MC-LR inhibited NHEJ. Further experiments are necessary to confirm this hypothesis.

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References


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