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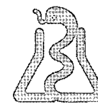

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Morphologic Changes in Microcystin-LR Treated Hepatocytes *In vitro*

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ABSTRACT. Microcystin-LR (MC-LR), a cyanobacterial toxin produced by *Microcystis aeruginosa*, causes severe hepatotoxicity. Here we investigated the morphologic changes of rat hepatocyte spheroid induced by exposure of MC-LR (10^{-6} M) *in vitro*. In addition, to determine the effects of such toxin in the process of hepatocyte spheroid formation, primarily isolated hepatocytes were incubated with MC-LR and the process of spheroid formation was observed. In both hepatocyte spheroid and suspension culture systems, the morphologic changes caused by MC-LR were noticible at 5 min post exposure and were characterized by the loss of microvilli, cytoplasmic vacuolation, the accumulation of lipid droplets, and bleb formation. Especially, the size and numbers of bleb on the cell surface were increased as the incubation time prolonged and the appearance of electron dense bodies were observed in the cytoplasm of hepatocyte at 20 min post exposure. Furthermore, bile canaliculi-like structures in the hepatocyte spheroids were slightly widened and the process of spheroids formation was inhibited in the isolated hepatocytes incubated with MC-LR. These results indicate that morphologic changes in the hepatocyte membrane and organelles seem to be typical events in showing the MC-LR induced hepatotoxic effects and the spheroid culture method might be a useful experimental tool to evaluate hepatoxicity since it reflects the *in vivo* status of hepatocytes.

Keywords: Microcystin-LR, Rats, Hepatocytes, Spheroid and suspension culture, Blebs.

INTRODUCTION

Microcystins are a family of hepatotoxins produced by several genera of cyanobacteria including *Microcystis, Nodularia, Anabaena,* and *Nostoc* (Botes *et al.*, 1984). These organisms form blooms in stagnant and warm waters that is rich in nutrients such as nitrogen and phosphorus that can be accumulated from fertilizers or wastes of livestock and/or human beings (Beasley *et al.*, 1991). Intoxication *in vivo* by these compounds is characterized by widespread hepatic necrosis and haemorrhage in a large number of species (Jackson *et al.*, 1985). In humans, major symptoms caused by toxicity of cyanobacterial blooms are skin irritation, cutaneous rash, fever, vomiting, diarrhea, gastroenteritis, and acute liver damage (Ressom *et al.*, 1994).

Microcystin-LR (MC-LR), the most commonly encountered and the most toxic among the algal cyclic peptide hepatotoxins, has been used as a common model for toxicological studies (Hooser *et al.*, 1989). MC-LR is a potent and specific inhibitor of serine/threonine phosphatases types 1 and 2A, and it leads to marked disorganization of intermediate filaments and actin microfilaments (Wickstrom *et al.*, 1995). MC-LR-induced inhibition of such enzymes results in the increased phosphorylation of numerous cytoplasmic and skeletal proteins, most of which have not been specifically identified (Eriksson *et al.*, 1990). The cell specificity of microcystins may be due to a selective uptake mechanism (Runnegar *et al.*, 1981) and their hepatotoxicity can be caused by the abnormal function of bile acid transport system (Petzinger, 1981). Furthermore, MC-LR has a potent tumor-promoting activity in rat liver induced by diethylnitrosamine (Matsushima *et al.*, 1992) and might mimic the apoptosis-like actions of okadaic acid on hepatocytes (Boe *et al.*, 1991).

In the present study, two different hepatocytes culture models were used to examine the effects of MC-LR *in vitro*. Especially the spheroid culture model can produce similar metabolites as *in vivo*. Furthermore, the isolated primary hepatocytes can reform hepatocyte

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Abbreviations in this paper: MC-LR, Microcystin-LR

spheroids having *in vivo* like cyto-architecture and they can maintain liver-specific function either for a few days or several weeks. Currently, the most convincing application of primary hepatocytes culture is being used to understand the mechanism of various toxins. Here, this study was performed to observe morphologic changes of hepatocytes following MC-LR toxin exposure using suspension and spheroid culture methods.

MATERIALS AND METHODS

Preparation of hepatocytes

Primary rat hepatocytes were obtained from 6 to 8 weeks old male Sprague-Dawley rats (170~220 g) using the two-step collagenase perfusion method as previously described (Seglen, 1976). More than 80% of the cells were used for experiment after measured by trypan blue dye exclusion. The isolated hepatocytes were incubated at 37°C in 5% CO_2 incubator.

Culture of isolated hepatocytes

A. Suspension culture. Isolated rat hepatocytes were seeded at 1×10^6 cells/ml in 12-well culture plates (NUNC, USA). Cells were cultured in Williams' E medium (pH 7.4, Sigma, USA) with the following additions: penicillin (10,000 U/ml), streptomycin (10,000 mg/ml), 10% fetal bovine serum.

B. Three-dimensional (spheroid) culture. Poly 2-hydroxyethyl methacrylate (Poly-HEMA, Sigma, USA) was dissolved in 95% ethanol and 12-well tissue culture plates were coated with 0.8 ml of 2.5% solution of pHEMA. The plates were allowed to dry overnight in a 50°C oven. Isolated rat hepatocytes were inoculated at 5×10^5 cells/ml in complete Williams' E medium. The medium was changed half and every 2 days until 4 days of culture.

Toxin exposure

Freshly prepared hepatocytes and spheroids were incubated with 10^{-6} M MC-LR for 5, 10 and 20 minutes. MC-LR was dissolved in phosphate buffered solution (PBS) and added in 100 μl into each culture dish. The control group was incubated in the medium with the same volume of PBS. To examine the inhibition of spheroid formation by the effects of toxin exposure, isolated rat hepatocytes and 10^{-6} M of MC-LR toxin were co-incubated by day 4 post exposure.

Preparation for Electron Microscopy

For the transmission electron microscopy (TEM), isolated hepatocytes and spheroids treated with microcystine-LR were fixed with 1.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 30 min at 4°C, followed by rinse with 0.1 M phosphate buffer for 5 min. Post-fixation was done using 1% osmium tetroxide phosphate buffer, and then a graded ethanol series was used for sample dehydration. Samples were embedded in epoxy resin at 60°C. Semi-thin sections stained with toluidine blue were examined under a light microscope (Nicon, Japan). Ultra-thin sections were cut and stained with uranyl acetate and lead citrate. Finally samples were observed with a TEM (JEOL JEM 1010, Japan).

For the scanning electron microscopy (SEM), isolated hepatocytes and spheroids were fixed and dehydrated in the same procedure as that of TEM. Samples were transferred into isoamyl acetate and then 98% hexamethyldisilazone (HMDS, Lancaster) for 1 h, and were air-dried. They were then mounted onto SEM specimen using an ion beam sputtered with platinum-palladium (Pt-Pd) before examination with a SEM (JEOL JSM-U3, Japan).

RESULTS

Morphologic Observation

Phase and light microscopy. In suspension culture, isolated primary hepatocytes showed a spherical shape having apparently smooth outline (Fig. 1A). The early morphologic changes of hepatocytes induced by MC-LR treatment (10^{-6} M) were seen in the form of tiny blebs on the surface of hepatocytes at 5 min post exposure. At 10 min post exposure, the blebs were clearly seen on the cell surface and shape of hepatocytes became irregular (Fig. 1B). At 20 min after treatment, the blebs became larger and formed an expanded border around the original cell outline. In addition, co-incu-

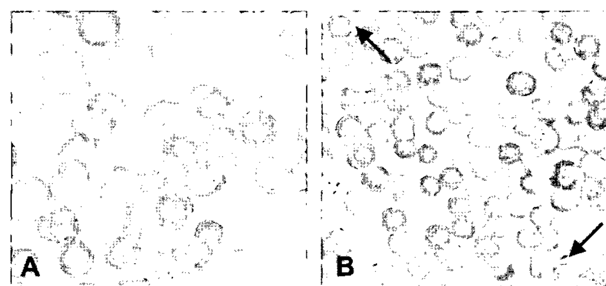

Fig. 1. Phase contrast micrograph of the microcystin-treated hepatocytes. After collagenase digestion of liver, uniformly rounded hepatocytes were isolated. Hepatocytes showed an oval shape and had smooth ontline membrane (A). At 10 min post MC-LR exposure, formation of blebs (arrows) on the surface of hepatocytes was observed (B). The figures show one representative of two independent experiments.

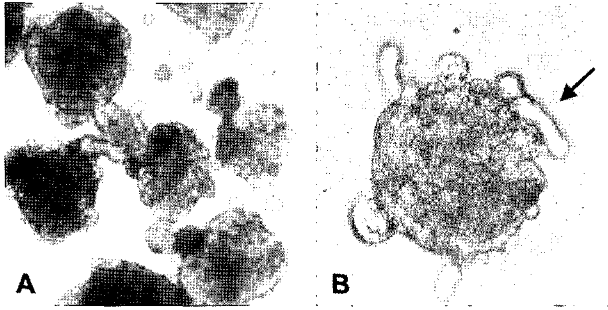

Fig. 2. Phase contrast micrograph of multicellular spheroids exposed to MC-LR. Isolated hepatocytes were seeded into poly-HEMA coated petri dish for 4 days. Such hepatocytes were aggregated and formed spheroids of round-shape feature with various sizes (A). At 20 min post MC-LR exposrue, huge and transparent blebs (arrow) were formed in the outlayer membrane of hepatocytes spheroids (B). The figures are derived from at least two independent experiments.

bation of the isolated hepatocytes with MC-LR inhibited the formation of spheroids and most of the hepatocytes died.

In spheroid culture, isolated hepatocytes were aggregated, and formed spheroids of compact round-shape feature with variable sizes (Fig. 2A). The morphologic changes of spheroids after MC-LR treatment started at 5 min post exposure and the extent of changes was increased in a time-dependent manner. At 20 min after exposure, huge and transparent blebs were formed in hepatocytes spheroids (Fig. 2B) and cells were subsequently detached from spheroids. These results show that exposure of MC-LR resulted in structural changes of hepatocyte spheroid. Such abnormal changes *in vitro* indicate that exposure of MC-LR *in vivo* may also lead to structural changes of liver architecture since hepatocytes were detached from the MC-LR treated spheroids in the current study.

Scanning and transmission electron microscopy. Under the scanning electron microscopy, freshly isolated hepatocytes presented a typical hepatocyte outline with round shape and numerous surface microvilli (Fig. 3A). Hepatocytes exposed to 10^{-6} M MC-LR for 5 min showed the formation of tiny cytoplasmic protrusions or blebs having no microvilli on their surface (Fig. 3B). At 10 min post exposure, most of the microvilli were not observed on the surface of hepatocytes and increased numbers of bleb formation were prominent (Fig. 3C). At 20 min post exposure, the predominant changes were still observed on the cell surface, which the shape of cells became irregular and various sized blebs were scattered on the cell surface (Fig. 3D). The severity of the deformation of cellular outline was increased with the time course of incubation.

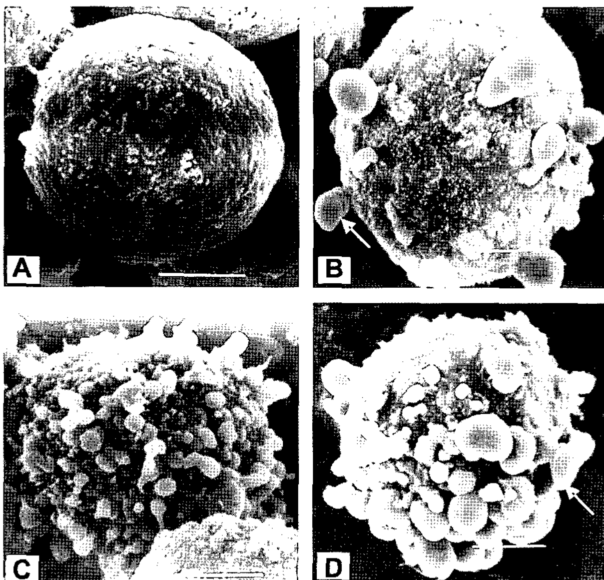

Fig. 3. Scanning electron micrograph of the microcystin-exposed hepatocytes. Control hepatocytes have numerous surface microvilli (A). Hepatocytes exposed to MC-LR resulted in the formation of blebs (arrow) and loss of surface microbilli; hepatocyte 5 min after treatment of MC-LR (B); 10 min after (C); 20 min after (D). The figures are derived from at least two independent experiments (Bar = 5 μm).

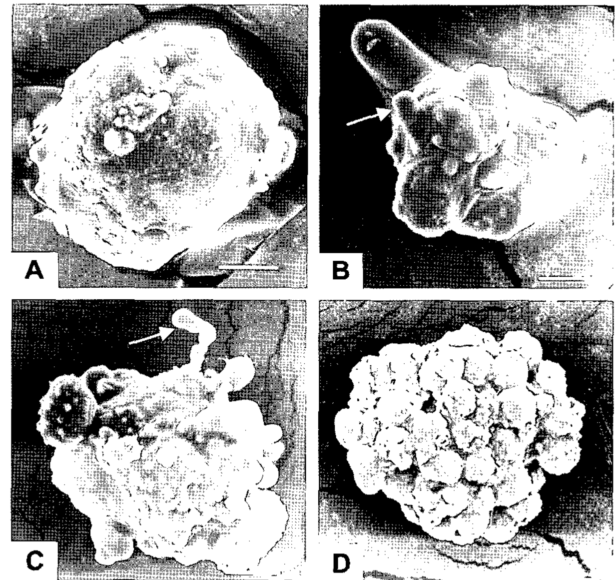

Fig. 4. Scanning electron micrograph of the microcystin-exposed hepatocyte spheroids. Control spheroids did not have blebs (A), but microcystin exposure induced bleb (arrow) formation (B; 5 min and C; 10 min). At 20 min post exposure, hepatocytes lost cell to cell contact and were dissociated from spheroids (D). The figures are derived from at least two independent experiments (Bar = 10 μm).

In spheroid culture, morphologically intact spheroids having microvilli were seen during culture period (Fig. 4A). From 5 min after toxin treatment, formation of huge blebs having no microvilli was evident on surface of spheroids (Fig. 4B and C). At 20 min post exposure, many hepatocytes lost their contact between the cells and finally were dissociated from spheroids (Fig. 4D).

Under the transmission electron microscopy, a large centrally located nucleus, abundant granular rough endoplasmic reticulums, numerous mitochondria, and lipid vesicles which were evenly distributed in the cytoplasm were observed in the isolated hepatocytes. Also highly developed microvilli were present on the cell surface (Fig. 5A). Ultrastructural changes of MC-LR treated hepatocytes were observed starting at 5 or 10 min post exposure. These included the formation of small membrane-bound vacuoles, loss of microvilli, and bleb formation on the plasma membrane. Mitochondria with dilated cristae and shrinkage of nuclear membrane were often found (Fig. 5B). At 20 min after toxin exposure, increased numbers of vacuolizations in the cytoplasm and blebs on the surface were more prominent. The dilation of rough endoplasmic reticulum, swollen mitochondria with dilated cristae, whorling of rough endoplasmic reticulum, and appearance of myelin body were also observed (Fig. 5C and 5D). Finally, membrane-bound blebs were pinched off from the cell surface (Fig. 5E).

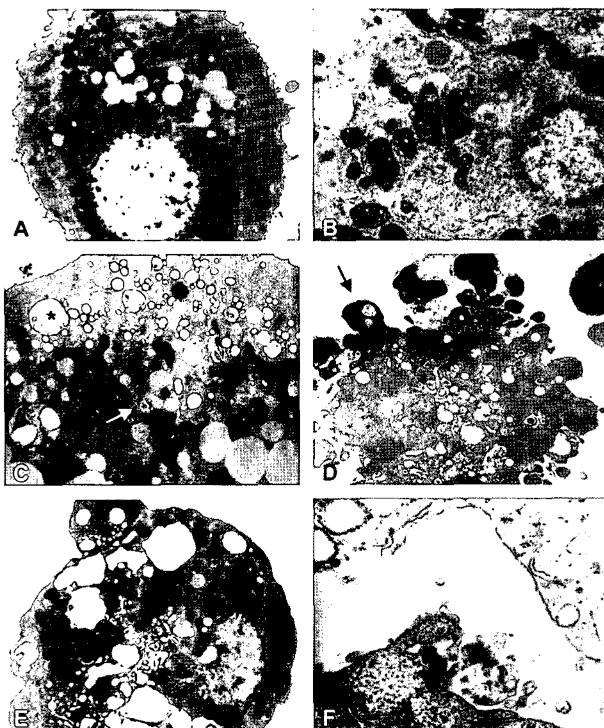

Fig. 5. Transmission electron micrograph of the microcystin-exposed hepatocytes and spheroids. Numerous intact microvilli, mitochondria, and lipid droplets were seen in control isolated rat hepatocyte (A). MC-LR treatment induced morphologic changes of hepatocytes. Mitochondria with dilated cristae and shrinkage of nuclear membrane were observed after 5 min exposure (B). Increased numbers of vacuolizations (*), blebs formation (black arrow), and occurrence of myelin body (white arrow) were noticible in hepatocytes at 20 min MC-LR exposure (C, D). Furthermore, cytoplasmic vacuoles and exfoliated blebs were observed (E). Moderately widened bile canaliculus-like architecture was examined in the hepatocyte spheroids after MC-LR treatment (F). The figures are derived from at least two independent experiments.

In spheroid culture, various sized spheroids were composed of the cell layers which outer and inner layer became thin and cuboidal, respectively. Each hepatocyte had a large round nucleus, abundant cytoplasmic organelles, and lipid droplets. The junctional complexes and bile canaliculus-like structures were identified between the hepatocytes located in near the surface layer. Such structures were similar to those observed *in vivo* hepatocytes, such as the tight junctions and desmosomes. Although most junctions were intact in the MC-LR treated groups, some of them showed the moderately widened bile canaliculus-like architecture (Fig. 5F). The vesiculation of rough endoplasmic reticulum and loss of microvilli were found in the spheroids at 5 and 10 min post exposure. Surface of rough endoplasmic reticulum appeared smooth, presumably due to loss of their ribosomes. At 20 min post MC-LR treatment, the concentric whorl formation of rough endoplasmic reticulum, numerous membrane-bound vacuoles, swollen mitochondria, and myelin body were observed. The hepatocytes were separated to single dead cell, showing swelling of cellular organelles as well as vacuolization in the cytoplasm.

DISCUSSION

In the current study, we observed that MC-LR exposure of isolated hepatocytes resulted in acute cytotoxicity. MC-LR toxin was shown to cause rapid morphologic changes of freshly isolated hepatocytes and cultured hepatocyte spheroids. Such cytotoxic effects of MC-LR were characterized by loss of microvilli, bleb formation, cytoplasmic vacuolation, and loss of cell contact affinity. We interpret these observations to indicate that MC-LR might be an potential hepatotoxins in various species.

The similar effects of MC-LR treatment observed in this study were shown in two different types of cultured hepatocytes. Such effects included the formation of blebs on hepatocyte surface and the consequent loss of

microvilli. Evident morphologic changes of cells after toxin exposure were clearly observed as the previous report that described the morphological alterations in isolated rat hepatocytes (Eriksson *et al.*, 1987). Also, it might be reasonable to propose that the deformation of hepatocytes observed *in vitro* can reflect the hepatocyte damage observed *in vivo* in intoxicated animals (Falconer *et al.*, 1981).

One of the major morphologic changes observed in toxin treated hepatocytes in the current study were the formation of blebs. The size and number of blebs were increased as the incubation time prolonged and the microvilli completely disappeared on the cell surface. Such membrane abnormalities indicate that MC-LR primarily affects hepatocyte membrane. It was reported that bleb formation appeared to be an early and reversible process preceding irreversible membrane damage and cell necrosis (De Vos *et al.*, 1983). Also, *in vitro* toxicity studies using bromobenzene suggested that bleb formation in liver cells might be induced by lipid peroxidation of cell membranes or injury to the cytoskeleton either directly or indirectly by disturbances in extramitochondrial calcium homeostasis (Jewell *et al.*, 1982). However, a previous report showed that the morphological changes induced by MC-LR were not accompanied by an increase in cytosolic Ca^{2+} concentration, and microcystin induced cell deformation could not be inhibited by the protease inhibitors, leupeptin and antipain (Eriksson *et al.*, 1989). However, it was reported that microcystin was potent inhibitors of protein phosphatases 1 and 2A (Honkanen *et al.*, 1990) and that the inhibition of protein phosphatases was associated with hepatocyte deformation due to reorganization of microfilaments (Eriksson *et al.*, 1989). In addition to such effects, MC-LR has also been shown to result in the collapse of intermediate filaments (Falconer and Yeung, 1992) and microtubules in hepatocytes as well as non-hepatocytes (Wickstrom *et al.*, 1993, 1995). Moreover, these cytoskeletal changes were identified as resultant phosphorylation of proteins including cytokeratins 8 and 18 (Eriksson *et al.*, 1990). Therefore, the abnormal bleb formation induced by MC-LR can be explained that increased phosphorylation of several cytosolic and cytoskeletal proteins resulted in disruption of normal cytoskeletal architecture. However, the precise mechanism by which microcystin specifically exerts bleb formation remains unresolved and this issue is under further investigation.

During the incubation of microcystin, the blebs were occasionally detached from the cell surface membrane. Pinching off of the membrane-bound blebs and fragmentation observed in this study were similar to the situation observed in liver of rat given a lethal dose of this toxin (Khan *et al.*, 1995). It is assumed that detached blebs from hepatocyte eventually migrate to the space of Disse and sinusoids, and this may cause the obstruction of micro-vascular complex of terminal hepatic venules *in vivo*. Other studies about MC-LR hepatotoxicity also supported this assumption since intact hepatocytes as well as hepatocellular debris released from the liver could be detected in the pulmonary vasculature and peritubular capillaries of the renal cortex (Hooser *et al.*, 1990).

Current observation suggests that the morphological changes of cell membrane and intracellular microorganelles might be caused by the early cytotoxic effects of MC-LR. Furthermore, considering the effects of the respective components on morphology, cells in the normal three-dimensional organ *in vivo* may resemble those in the spheroids formed by the interaction of isolated rat hepatocytes. Since it conserves cell to cell interaction, the multicellular spheroid culture may provide a useful model for the toxicological study *in vitro*.

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