J. Fd Hyg. Safety 15(1), 41–45 (2000)

Apoptosis of Rat Embryonic Midbrain Cells in Ochratoxin A-induced Microcephaly

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ABSTRACT – Induction of DNA fragmentation of rat embryonic midbrain cells was studied to see whether apoptosis plays a role in OTA-induced microcephaly observed in cultured rat whole embryos during embryogenesis. We first cultured whole embryos (prepared from day 9.5 gestation rats) for 48 hrs with OTA and found that OTA induced microcephaly in cultured rat whole embryos. We also examined whether the microcephaly seen in cultured whole embryos is partially related to the increase of apoptosis of undifferentiated embryonic midbrain cells. Embryonic midbrain cells were prepared from day 12 gestation rat embryos, and cultured in the mixture media of Dulbecco's modified eagle's medium nutrient and Ham's F12 (1 : 1) containing 10% Nuserum, 100 μg/ml of streptomycin and 100 units/ml of penicillin for 96 hrs. Induction of DNA fragmentation was increased by 0.25-1 μg/ml OTA in a dose dependent manner in the embryonic midbrain cells. We also tested whether increase of apoptosis by OTA would be associated with change of apoptosis-related proteins (TNF-α and P^{53}) level in embryonic midbrain cells. OTA also increased TNF-α and P^{53} levels. These results show that OTA induced microcephaly in cultured whole embryos and this effect may be at least a part due to the induction of apoptosis and apoptosis-related protein levels of undifferentiated embryonic midbrain cells.

Key words □ Ochratoxin A, Microcephaly, Embryonic midbrain cell death.

The mycotoxin ochratoxin A (OTA) is produced by several species of fungi such as Aspergillus and *Penicillium* genera.[1] OTA is a natural contaminant of feed and food, and has been found in the blood of animals and humans after consumption of contaminated food.[1-3]

OTA is suspected to play a critical role in the etiology of Balkan endemic nephropathy and associated urinary tract tumors.[2] OTA has also been known to cause immunotoxic, genotoxic, carcinogenic effect.[4-6] OTA could cross placenta and induced maternal toxicosis, teratogenicity and/or embryotoxicity in the experimental animals.[7-9] The developing brain appears to be the most susceptible targets to environmental chemicals at the early organogenesis as observed in teratogenic studies.[10,11]

It has been reported that OTA induced highly frequency of microcephaly in rodents.[12,13] We also found that OTA reduced head length (It may come from the result of the formation of microcephaly) in cultured rat whole embryos.[14] However, the mechanisms by which OTA-induced microcephaly have not been definitely answered yet. The relationship between teratogenicity of several teratogenic chemicals and changing of cell behaviors such as cell death and differentiation in the organogenesis of embryos has been proposed.[15] It was found that OTA caused brain necrosis in mouse fetuses exposed transplacentally.[9,13] OTA-induced cell death was found in hamster kidney and HeLa cells.[16]

In the present study, we therefore tested whether induction of rat embryonic midbrain cell death and cell death-regulatory protein (P^{53} and tumor necrosis factor-α) levels during the organogenesis is involved in the OTA-induced microcephaly.

Materials and Methods

Whole embryo culture

Whole embryos were cultured by the method of

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New.[17] Pregnant rats were anethesized on the afternoon of the 9th days of gestation (sperm positive morning=day 0 of gestation) and their embryos were removed from maternal tissue and Reichert's membrane. Embryos exhibiting normal appearance were used for whole embryo culture. Two or three embryos were cultured in 3 ml of rat immediately centrifuged serum for 48 hrs at 37°C with 95% O_2 and 5% CO_2 using the whole embryo culture system (Ikemoto Rika Co., Tokyo, Japan). Several different concentration of OTA (0.1 to 20 μg/ml) dissolved in Tyrode's solution were exposed to embryos. The presence of malformation including the induction of microcephaly was determined by the method described by Malele-Fabry et al.[18]

Embryonic midbrain cell culture

Embryonic midbrain cells were cultured by the method of Flint and Orton.[19] Midbrain was isolated from embryos prepared from day 12 gestation rats. Embryonic midbrain tissues were dissociated into individual cells by successively digestion with Ca^{2+}, Mg^{2+}-free Dulbecco's phosphate buffered saline (Gibco, Gaithersburg, MD, USA) containing 1% trypsin. Cells were then resuspended in the mixture media of Dulbecco's modified eagle's medium nutrient and Ham's F12 (1 : 1 mixture, Sigma Co., St. Louis, MO, USA) containing 10% Nuserum, 100 μg/ml of streptomycin and 100 units/ml of penicillin. Cells were then adjusted to give 5×10^6 cells/ml, and allowed to attach for 2 h at 37°C. A 2 ml of culture medium containing appropriate concentration of OTA was added to each well and cultured for up to 4 days.

Biochemical parameters assay

Protein content was measured by the method of Bradford. TNF-α and P^{53} protein levels were determined with each of ELISA kit by the product company's specifications (Oncogene Res. Products, Cambriges, MA, USA).

Agarose gel electrophoresis and quantitation of fragmented DNA

For the apoptosis assay, embryonic midbrain cells were exposed for 4 hrs with OTA after 92 hrs culture. Cultured embryonic midbrain cells were transferred to microcentrifuge tubes, and centrifuged at 200×g for 10 min. Cell pellets were lysed with lysis buffer (5 mM Tris buffer, 20 mM EDTA and 0.5% Triton X-100, pH 7.4) for 15 min at room temperature. Cell lysates were then centrifuged at 13,000×g for 10 min and supernatants containing fragmented DNA were separated from the pellets. DNA fragments were precipitated with 2 volume of absolute alcohol and 0.1 volume of 3 M sodium acetate at −20°C overnight. The DNA samples were then resuspended in TE solution (10 mM Tris, 1 mM EDTA, pH 7.4) and were analyzed by electrophoresis at 30 V for 2 hrs on an 1.6% agarose gel. For the quantification of the percentage of fragmented DNA, DNA in the pellet or supernatant was determined by the diphenylamine method described elsewhwere.[14] The percentage of DNA fragmentation was calculated as: % of DNA fragmentation=(optical density of supernatant×100)/optical density of pellet +optical density of supernatant)

Statistics

Data were analyzed using one-way analysis of variance followed by Bonferroni's test as *post hoc test*. Differences were considered significant at $p<0.05$.

Results

Effect of ochratoxin A on rat embryo development in vitro

Day 9.5 rat embryos were exposed to OTA in culture for 48 hrs. Less than 0.2 μg/ml OTA did not show any toxic effects on embryo development, however 0.5 μg/ml OTA started the induction of several developmental defects. Typical OTA-induced microcephaly was depicted in Figure 1. The head length and somite number were significantly decreased with over 1 μg/ml OTA. Heart and tail development were also severely retarded. More than 8 μg/ml OTA was very toxic, and could not induce normal growth at all (data not shown).

Apoptosis of cultured embryonic midbrain cells

We next tested whether embyrotoxic effect (microcephaly) resulted from the increase of apoptosis of undifferentiated embryonic midbrain cells since apoptosis is important aspect of cell behavior in embryo-

Fig. 1. OTA-induced micrpcephaly in cultured whole embryos.
Embryos were prepared from rats on gestation 9.5 days, and cultured in the absence (left) or presence (right) of OTA for 48 hrs as described in the materials and methods.

genesis. DNA fragmentation, an indicator of apoptosis was increased by OTA in cultured embryonic midbrain cells (Fig. 2A). Induction of DNA fragmentation by OTA was dose-dependently increased (Fig. 2B).

TNF-α and P^{53} levels

The protein levels of TNF-α and P^{53} were measured in cultured embryonic midbrain cells exposed two

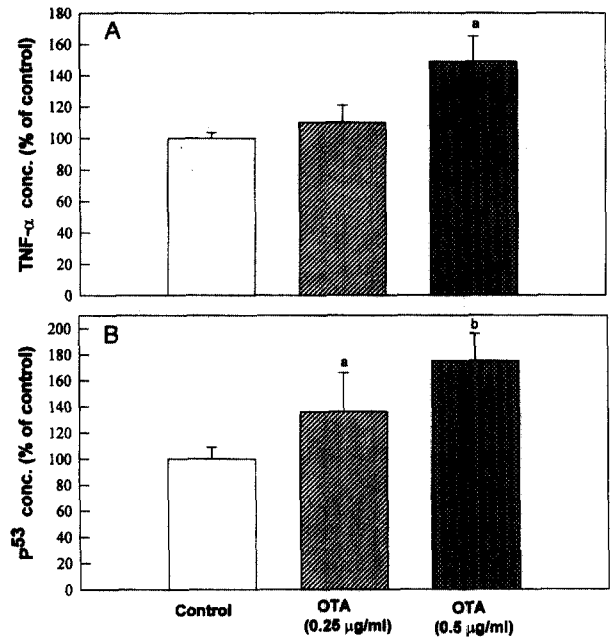

Fig. 3. Effect of OTA on the TNF-α (A) and P^{53} levels (B) in the cultured embryonic midbrain cells.
Embryonic midbrain cells were prepared from rats on gestation 12 days, and cultured in the presence of two doses (0.25 of 0.5 μg/ml) of OTA of 96 hrs. TNF-α and P^{53} were measured by ELISA method as described in the materials and methods. Values are mean ± standard error of four experiments, with triplicates of each experiments. Values with super- scripts are significantly different.

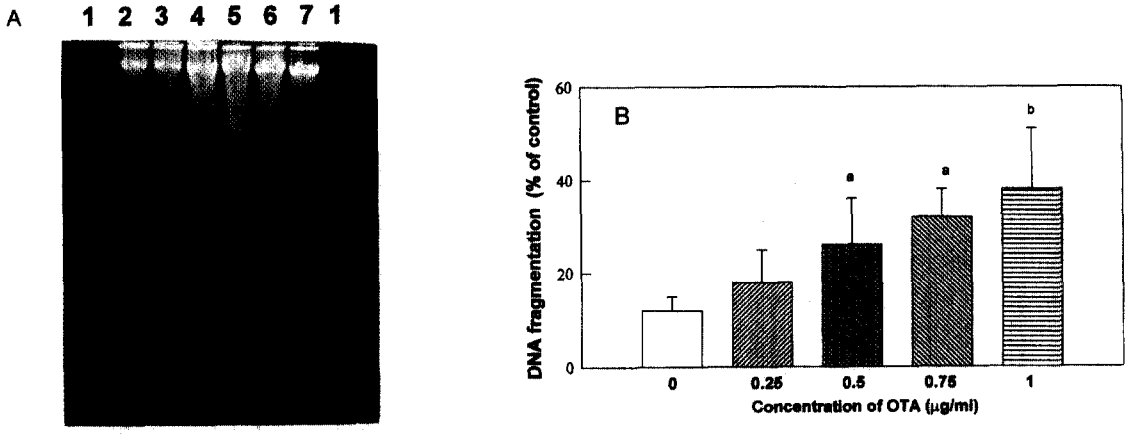

Fig. 2. DNA fragmentation by OTA in the cultured embryonic midbrain cells. Embryonic midbrain cells were prepared from rats on gestation day 12, and cultured in the presence of OTA for 4 hrs.
DNA fragmentation were determined as described in the materials and methods. Values are mean ± standard error of four experiments, with triplicates of each experiments. Lane 1; 1kb DNA ladder and size marker, lane 2; untreated control cells, lane 3; 0.125 μg/ml OTA, lane 4; 0.25 μg/ml OTA, lane 5; 0.5 μg/ml OTA, lane 6; 0.75 μg/ml OTA, lane 7; 1 μg/ml OTA. A; DNA fragmentation pattern, B; % of DNA fragmentation.

doses of OTA (0.25 or 0.5 μg/ml) by ELISA method to see whether induction of cell death may be related to any change of these protein levels. Levels of TNF-α and P^{53} were significantly increased by 0.5 μg/ml OTA (Fig. 3).

Discussion

In this study, we found that OTA induced microcephaly in cultured whole embryos. We also found that OTA increased the induction of apoptosis and apoptosis-related proteins, TNF-α and P^{53} levels in cultured embryonic midbrain cells in a dose dependent manner.

Similar to several in vivo other studies demonstrating that OTA induce microcephaly during organogenesis,[12-13] we found that OTA induced microcephaly (Figure 1) and other abnormalities in cultured rat whole embryos.[14] The post implantation whole embryo culture system has been applied to screen potential toxic chemicals of their teratogenic effects since data got from this system has been demonstrated to be well correlated with those from in vivo studies.[18] Therefore, the induction of microcephaly by OTA might be the same consequence as found in vivo of other studies. Inhibition of the synthesis of macromolecules (DNA and protein) may be accounted for the toxico-mechanism of OTA-induced microcephaly.[14]

Although cell death has been known to be a common feature found in embryogenesis after exposure to a variety of teratogens such as alcohol, ionizing radiation, retinoic acid, there have not been reported that OTA induced cell death in cultured embryos and in cultured embryonic midbrain cells. Therefore, in separated experiments, we examined the effect of OTA on apoptosis of embryonic midbrain cells during differentiation of embryonic midbrain cells to neuron cells to see whether OTA induce apoptosis and change of apoptosis can play a role in the induction of microcephaly. We cultured undifferentiated embryonic midbrain cells extracted from rat embryos on gestation 12 days. Induction of DNA fragmentation (apoptosis) of cultured embryonic midbrain cells was increased by OTA in a dose-dependent manner. Aspect of cell behaviors such as cell proliferation and cell differentiation is important in embryogenesis. Apoptosis is also known to play a crucial role in cyto-architectural organization of cells during organogenesis. Thus, the increase of apoptosis during the organization of undifferentiated midbrain cells may play at least a role in the OTA-induced embryotoxicity (induction of microcephaly). The degree of DNA fragmentation increased about 23% at 0.5 μg/ml and 35% at 1 μg/ml of OTA. The dose inducing DNA fragmentation is higher than that induces cytotoxicity and cell differentiation.[14] The difference in the exposure time may cause different sensitivity to OTA. We exposed OTA to cells for 4 hrs in the DNA fragmentation study, however, cell cytotoxicity or differentiation were determined after 96 hrs exposure. In addition, apoptosis could be induced at a relatively higher dose of chemicals than other cellular functions such as cell proliferation. The molecular mechanisms of OTA-induced cell death remain unclear. We found that OTA increased TNF-α and P^{53} levels which are well known to be molecules involved in the processes of cell death. The increase of the level of these two proteins may be related with the induction of apoptosis. Several lines of evidence have shown that oxygen radicals are implicated in TNF-α mediated cell death.[20-22] Many of chemicals are capable of inducing P^{53}-mediated apoptosis are also known to evoke oxygen reactive radicals.[23] Involvement of oxidative damage of macromolecule such as lipid peroxidation was also well studied in the toxico-mechanism of OTA.[24-25] It is therefore that OTA-induced apoptosis may be related to the production of oxidative damages of macromolecules (such as DNA). Further studies showing that direct relationship between the production of reactive oxygen species and OTA-induced apoptosis are needed.

In summary, the present study showed that increase of apoptosis in embryonic midbrain cells may play at least a part role in OTA-induced microcephaly in cultured rat whole embryos.

Acknowledgements

This work supported by the research fund from Korea Food and Drug Administration (1997).

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