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Phagocytosis plays an important role in clearing dead cells caused by mono(2-ethylhexyl) phthalate administration

T.W. Tay [a], B.B. Andriana [a], M. Ishii [b], E.K. Choi [a], X.B. Zhu [a], M.S. Alam [a], N. Tsunekawa [a], Y. Kanai [a], M. Kurohmaru [a,*]

[a] *Department of Veterinary Anatomy, Graduate School of Agricultural and Life Sciences, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-8657, Japan*

[b] *Department of Developmental Neuroscience, Tokyo Metropolitan Institute for Neuroscience, 2-6 Musashidai, Fuchu, Tokyo 183-8526, Japan*

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Abstract

The role of phagocytosis in eliminating apoptotic spermatogenic cells caused by mono(2-ethylhexyl) phthalate (MEHP) was studied. Twenty-one-day-old C57Bl/6N male mice were given a single dose of 800 mg/kg MEHP in corn oil by oral gavage and sacrificed at 1, 3, 5, 7 and 9 days after initial exposure. At the same time, the role of phagocytosis in MEHP related apoptosis was examined using microinjection of annexin V into the seminiferous tubules of living mice. Results showed that mice treated with MEHP had a lower rate of testis weight gain (lower regression line) and a significant TUNEL-positive spermatogenic cell number compared to control. However, this incident was reversible, and the number of TUNEL-positive cells returned to normal after 9 days. Mice microinjected with annexin V and later treated with MEHP showed a large amount of TUNEL-positive cells compared to mice treated with MEHP only. This clearly proves that phagocytosis plays an efficient and highly important role in eliminating dead cells in the injured testis of mice treated with MEHP.

Keywords: Annexin V; Apoptosis; Mono(2-ethylhexyl) phthalate; Phagocytosis; Testis

1. Introduction

Di(2-ethylhexyl) phthalate (DEHP) is a phthalate ester, and one of the most common plasticizer used in consumer products, food packaging materials (Blount et al., 2000), and biomedical devices (Cole et al., 1981). Studies on DEHP have shown that it reduces fertility and induces testicular atrophy in laboratory animals. When administered orally to rodents, DEHP is rapidly hydrolyzed in the gut and other tissues by nonspecific esterases to produce its monoester metabolite, mono(2-ethylhexyl) phthalate (MEHP). MEHP has been found to be the active toxic metabolite of DEHP. Previous researches have shown that young animals are more sensitive to MEHP exposure (Poon et al., 1997) and that the Sertoli cells are the primary targets of MEHP in the testis (Boekelheide, 1993). Loss of spermatogenic cells attached to the seminiferous epithelium and appearance of spermatogenic cells in the lumen are the phenomena frequently observed with several Sertoli cell toxicants, including phthalates (Creasy et al., 1987; Gray and Beamand, 1984).

Although the occurrence of apoptosis in spermatogenic cells at various stages of differentiation was reported (Kerr, 1992), only a limited number of apoptotic spermatogenic cells were detectable when testes sections were examined histochemically. This is most probably due to the rapid elimination of apoptotic cells by phagocytosis, a common fate of cells undergoing apoptosis (Ren and Savill, 1998; Savill and Fadok, 2000). The electron microscopic studies by Miething (1992) and Tay et al. (2007) have shown that degenerating spermatogenic cells in testes are engulfed by Sertoli cells. Phagocytosis occurs when phagocytes bind to apoptosing

* Corresponding author. Tel.: +81 3 5841 5384; fax: +81 3 5841 8181.
E-mail address: amkuroh@mail.ecc.u-tokyo.ac.jp (M. Kurohmaru).

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cells by recognizing phagocytosis markers, which appear on the surface of target cells, using specific receptors (Savill and Fadok, 2000).

The membrane phospholipid phosphatidylserine (PS) is the best characterized phagocytosis marker (Schlegel and Williamson, 2001). The regulatory mechanism that defines the phospholipids localization in the plasma membrane appears to be altered in apoptotic cells (Williamson and Schlegel, 2002), and as a result, PS becomes exposed on the outer leaflet. Investigations into the exposure of PS is facilitated by the finding that annexin V specifically binds to PS (Tait et al., 1989). In short, annexin V can also be used as a phagocytosis inhibitor. By binding to exposed PS, annexin V can inhibit the detection of PS signals by phagocytes.

Therefore, the objective of the current experiment is to clarify whether phagocytosis is involved in eliminating MEHP-caused apoptosis, and also if its function is affected by the phthalate ester.

2. Materials and methods

2.1. Animals and dosing

Twenty-one-day-old C57Bl/6N male mice (Charles River, Co., Japan) were given a single dose of 800 mg/kg MEHP in corn oil by oral gavage. Mice received MEHP in corn oil at a volume equal to 4 ml/kg. Control animals received the same volume of corn oil. MEHP was purchased from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan). Mice were sacrificed at 1, 3, 5, 7 and 9 days after initial exposure. Testes were excised and fixed in 4% paraformaldehyde (PFA) at 4 °C in phosphate-buffered saline (PBS). Thereafter, they were dehydrated in ethanol, cleared in xylene, and embedded in paraffin. Paraffin blocks were cut at 5 μm in thickness.

2.2. Annexin V

The role of phagocytosis in MEHP related apoptosis was checked using annexin V. Microinjection of annexin V into the seminiferous tubules of living 21-day-old C57Bl/6N male mice was carried out according to the method of Ogawa et al. (1997). PBS containing 0.05% trypan blue and 10 μM annexin V were injected through the efferent duct. Trypan blue was included to monitor the distribution of the injected materials in the seminiferous tubules. Then, mice were given a single dose of 800 mg/kg MEHP in corn oil by oral gavage. Control groups were given either corn oil or 800 mg/kg MEHP only. Mice were sacrificed at 3 days after initial exposure. The testes and epididymides were harvested, processed for paraffin embedding, and sectioned at 5 μm in thickness. Sections were later analyzed by the TUNEL analysis. Epididymis sections were also stained with Mayer's hematoxylin (Wako, Tokyo, Japan) and eosin (Wako).

2.3. In situ TUNEL staining

Apoptosis of spermatogenic cells was examined using the TUNEL method according to the protocol of the '*in situ* apoptosis detection kit' (TaKaRa, Tokyo, Japan). Paraffin-embedded sections were deparaffinized, rehydrated, and then predigested with 10 μg/ml protease K for 15 min. Thereafter, sections were incubated in PBS containing 3% H_2O_2 for 15–30 min to block endogenous peroxidase activity. Then, they were incubated with a fluorescein isothiocyanate (FITC)-labeled TdT enzyme in a humidified chamber at 37 °C for 90 min. After washing, the slides were incubated with anti-FITC horseradish peroxidase (HRP) conjugate at 37 °C for 30 min. Finally, TUNEL positive cells were detected by DAB substrate development, counterstained with methyl green dye, mounted, and observed using optical microscopy for counting apoptotic cell number per each seminiferous epithelium. To check for the non-specific reaction, the sections were incubated with PBS alone instead of FITC-labeled TdT enzyme.

2.4. Statistical analysis

Data are expressed as mean ± S.E. Statistical analysis was conducted using the Student's *t*-test. The *p*-values of less than 0.05 were considered statistically significant.

3. Results

Testis weight of control and mice given a single oral dose of 800 mg/kg MEHP from D0 to D9 is shown in Fig. 1. Mice treated with MEHP showed a lower rate of testis weight gain (lower regression line) compared to control.

Figs. 2 and 3 show results of the TUNEL analysis for both control and treated mice. At D1, the number of TUNEL-positive cells in the treated mouse testis was quite significantly different ($p < 0.001$) compared to control. Then the number of TUNEL-positive cells decreased abruptly at D3, but still showed significance ($p < 0.001$). At D5 and D7, the number of TUNEL-positive cells continued to decrease, but in a much slower rate. After more than a week, at D9, the number of TUNEL-positive cells reached the same percentage as that in control.

Observation of the epidydimides in MEHP treated mice at D3 showed spermatogenic cells within the epididymal ducts (Fig. 4). However, these cells were not TUNEL-positive.

Mice treated with MEHP alone showed a quite significant ($p < 0.001$) increase in TUNEL-positive cells compared to control. Surprisingly, mice microinjected with annexin V and later treated with MEHP showed a greater amount of TUNEL-positive cells (Fig. 5). Seminiferous tubules in this mouse were found to be filled with spermatogenic cells, most of which were TUNEL-positive (Fig. 5A). The number of TUNEL-positive cells was significant ($p < 0.001$) compared to mice without annexin V microinjection (Fig. 5B).