ORGAN TOXICITY AND MECHANISMS

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Induction and inhibition of testicular germ cell apoptosis by fluoroacetate in rats

Abstract Fluoroacetate (FA), an inhibitor of aconitase, is known to lower the intracellular level of adenosine triphosphate (ATP), which recently has been suggested to be a possible determinant of the form of cell death, apoptosis or necrosis. To investigate which form of germ cell death occurs in FA-induced testicular toxicity, adult Sprague Dawley rats were given a single oral dose of FA (0.5 or 1.0 mg/kg) and euthanized at 3, 6, 12, 24, 48, and 72 h thereafter. Germ cell degeneration was histologically first found in early round spermatids at stage I and in spermatogonia at stages II-IV of seminiferous tubules 6 and 12 h, respectively, after dosing. Degenerating spermatogonia exhibited characteristic features of apoptosis as demonstrated by both electron microscopy and in situ terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL), whereas spermatids did not. At the 24 and 48 h time points, degenerating spermatids were continually present and subsequently formed multinucleated giant cells, while the number of degenerating spermatogonia and TUNEL-labeled spermatogonia was drastically and/or significantly decreased compared to those from the control group, indicating that spontaneous male germ cell apoptosis is inhibited. Coincident with these morphological changes, DNA laddering on gel electrophoresis was apparent only 12 h after dosing. The results demonstrate that FA induces either apoptosis or necrosis of male germ cells in the early stage after dosing and subsequently inhibits spontaneous apoptosis.

Key words Fluoroacetate · Apoptosis · Testis · Toxicity

Introduction

Apoptosis, an active cellular process of gene-directed cell death, has been recognized as an important physiological and pathological mechanism for the removal of unwanted cells, characterized by morphological and biochemical features including cell shrinkage, nuclear condensation, and internucleosomal DNA fragmentation (Hettis 1998). In the testis, apoptosis has been shown to play a significant role in normal development (Rodriguez et al. 1997) and mature spermatogenesis (Yin et al. 1998), as well as in experimental conditions under the influence of various stimuli such as ischemia (Turner et al. 1997), cryptorchidism (Henriksson et al. 1995), hypothermia (Blanco-Rodríguez and Martínez-García 1997), irradiation (Henriksson et al. 1996), and testicular toxicants (Blanco-Rodríguez and Martínez-García 1998; Shinoda et al. 1998, 1999).

Fluoroacetate (FA), a naturally occurring plant toxin and a potent rodenticide known commercially as compound 1080, has been shown to cause testicular toxicity (Sullivan et al. 1979; Al-Juburi et al. 1989). FA enters the tricarboxylic acid (TCA) cycle and is transformed into fluorocitrate, which inhibits the enzyme aconitase (aconitate hydratase; EC 4.2.1.3), leading to the buildup of citrate and limitation of cellular adenosine triphosphate (ATP) production in various tissues with high metabolic rates including the testis. FA-related toxicity and death are generally accepted to be caused by severe impairment of energy production (Bosakowski and Levin 1986; Keller et al. 1996).

Since apoptosis is highly regulated and involves gene expression, it has been thought to be a process with a high energy demand (Richter et al. 1996). Recently evidence has accumulated that its execution requires the maintenance of adequate intracellular ATP levels (Eguchi et al. 1997; Leist et al. 1997). In addition, sev-
eral intensive studies have suggested that intracellular ATP levels might be a possible determinant of the form of cell death, and that intracellular ATP depletion could result in a switch from apoptosis to necrosis (Lieberthal et al. 1998; Feldenberg et al. 1999; Leist et al. 1999).

It is therefore of interest to determine whether cell death after impairment of energy metabolism occurs via apoptosis, necrosis, or both of the processes. There have, to our knowledge, been no specific studies on the processes leading to the cell death caused by FA. We thus assessed germ cell death in the testes of rats given a single oral dose of FA, using histological and electron microscopic examinations, in situ terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL), and DNA gel electrophoresis.

Materials and methods

Animals and treatments

Adult male Sprague Dawley rats at 10-weeks of age obtained from Charles River Breeding Laboratories (Shiga, Japan) were housed in polycarbonate cages with a 12:12 h light/dark cycle, at a temperature of 23 ± 2 °C and humidity of 55 ± 5%, and given MF diet (Oriental Yeast Co. Ltd., Tokyo) and water ad libitum. The animals received a gavage a single dose of 0.5 or 1.0 mg/kg of sodium fluoroacetate (sodium monofluoroacetate, >90% purity; Wako Chemical Co., Tokyo) in distilled water at a concentration of 0.02% (w/v) and were euthanized under ether anesthesia after 3, 6, 12, 24, and 48 h (n = 3, at each time point). The doses were chosen on the basis of the literature (Egekeze and Oehme 1979; Bosakowski and Levin 1986) and data from our preliminary experiments on the induction of testicular toxicity. Control rats (n = 3) received 5 ml/kg distilled water and were euthanized 24 h thereafter. Immediately after killing, bilateral testes from each animal were removed, one of which was immersed in Bouin’s fixative for histological and TUNEL evaluations. The contralateral testes were frozen in liquid nitrogen and stored at −70 °C for subsequent detection of DNA fragmentation by gel electrophoresis, or punctured through the tunica albuginea with a 26-gauge needle followed by infusion with 2.5% glutaraldehyde in phosphate buffer (0.1 M, pH 7.4) for electron microscopic evaluation. In addition to the above-mentioned principal experiments, a supplementary study was conducted only for histological evaluation, in which animals (n = 3) were given 1.0 mg/kg of FA and euthanized 72 h after dosing.

Morphological analysis

Histology and electron microscopy

For light microscopy, testes fixed in Bouin’s fixative were embedded in paraffin followed by sectioning and staining with hematoxylin-eosin (HE). For electron microscopy, cubes of 3- to 5 mm3 from infused testes were immersed in 2.5% glutaraldehyde, post-fixed in 1.0% osmium tetroxide, dehydrated through a graded series of ethanol, and embedded in Epon 812 after several changes of propylene oxide. Ultrathin sections stained with uranyl acetate and lead citrate were examined under a JEOL 1200EX transmission electron microscope.

In situ TUNEL

Histological detection of DNA fragmentation in testes was performed by the TUNEL method using an ApoTag-peroxidase kit (Oncor, Gaitherville, Md., USA). Briefly, sections of 5 μm thickness from testes fixed in Bouin’s fixative and embedded in paraffin were mounted on glass slides, deparaffinized by clearing with xylene, and hydrated through a graded series of ethanol to deionized water. After treatment with proteinase K (20 μg/ml; Sigma) for digestion of nuclear proteins for 10 min and hydrogen peroxide (2%) for inactivating endogenous peroxidase for 5 min, sections were incubated in a solution of terminal deoxynucleotidyl transferase and digoxigenin-dUTP in a humidified chamber at 37 °C for 1 h. Subsequent treatment was with anti-digoxigenin-peroxidase for 30 min at room temperature. Digoxigenin-dUTP end-labeled DNA was visualized by peroxidase detection with diaminobenzidine (0.05%) and hydrogen peroxide (0.02%) for 5 min. Sections were counterstained with hematoxylin. For the quantification of TUNEL-labeled germ cells, the seminiferous tubules were divided into seven groups, i.e., stages I, II-IV, V-VI, VII-VIII, IX-XI, XII-XIII, and XIV, based on the cell types of spermatogonia and spermatocytes according to the description by Hess (1990). The TUNEL-labeled germ cells were identified as spermatogonia, spermatocytes or spermatids by their morphological features and location within the seminiferous epithelium.

DNA gel electrophoresis

The detection of low molecular weight DNA ladders was performed as described by Strauss (1994) with modifications. A whole frozen testis from each animal was minced on dry ice and digested with proteinase K (100 μg/ml; Sigma) for 90 min. After addition of an equal volume of phenol, the preparations were placed on a roller apparatus for 15 min followed by centrifugation at 27,000 g for 15 min. The aqueous phase was extracted with an equal volume of phenol/chloroform (1:1, v/v) for 15 min and subjected to RNase A (10 mg/ml; Sigma) treatment for 60 min followed by repeated phenol/chloroform extraction. The resultant solution was ethanol-precipitated overnight, pelleted, dried and resuspended in TE buffer [10 mM TRIS-HCl and 1 mM ethylenediaminetetraacetic acid (EDTA)]. Following spectrophotometric estimation of the DNA concentration, aliquots of 3 to 5 mg of DNA were loaded onto 1.8% agarose gels and separated by electrophoresis (100 V, 1 h). DNA was stained with ethidium bromide and visualized with an ultraviolet transilluminator (NLM-20 E; UVP, Upland, Calif., USA) at 302 nm. The sizes of resulting DNA bands were estimated by comparison with standard molecular markers (100 bp Molecular Ruler; Takara Co. Ltd., Tokyo).

Statistics

The quantification of TUNEL-labeled germ cells was assessed on > 2000 cross-sectioned seminiferous tubules from each rat (n = 3) and expressed as numbers of TUNEL-labeled germ cells per 100 Sertoli cells in each group of stages. Statistical analyses were performed with one-way analysis of variance and Dunnett’s multiple comparisons. All results were presented as mean ± SE and the level of significance was taken as P < 0.05, compared with the respective control.

Results

Morphological analysis

No remarkable changes were evident in testes from rats given 0.5 mg/kg FA throughout this study. In stage I seminiferous tubules 6 h after dosing of 1.0 mg/kg, germ cell degeneration was first found as early round spermatids with ring-like nuclei formed by peripheral margination of chromatin. This change was also observed in stage I–III tubules at 24 and 48 h after dosing (Fig. 1C), and multinucleated giant cells derived from these spermatids were subsequently formed. At 12 h after dosing, degenerating spermatogonia with pyknotic...