Histol Histopathol (2000) 15: 707-711

http://www.ehu.es/histol-histopathol

Histology and Histopathology

Cellular and Molecular Biology

Ethylnitrosourea (ENU)-induced apoptosis in the rat fetal tissues

K. Katayama, N. Ishigami, K. Uetsuka, H. Nakayama and K. Doi

Department of Veterinary Pathology, Graduate School of Agricultural and Life Sciences, The University of Tokyo, Bunkyo-ku, Tokyo, Japan

Summary. Ethylnitrosourea (ENU), a well known DNA alkylating agent, induces anomalies in the central nervous system (CNS), craniofacial tissues and male reproductive organs. In this study, pregnant rats were treated with 60 mg/kg ENU at day 13 of gestation, and their fetuses were examined from 1 to 48 hours after treatment (HAT) to find a clue for clarifying the mechanisms of the ENU fetotoxicity and teratogenicity. From 3 to 12 HAT, the moderate to marked increase in the number of pyknotic cells was detected in the fetal CNS, craniofacial mesenchymal tissues, gonads and so on. These pyknotic cells had nuclei positively stained by the TUNEL method, which is widely used for the detection of apoptotic nuclei, and they also showed electron microscopic characteristics identical to those of apoptotic cells. The present results strongly suggest that excess cell death by apoptosis in the fetal CNS, craniofacial tissues and gonads may have a close relation to the later occurrence of anomalies reported in these tissues following ENU-administration.

Key words: Apoptosis, Ethylnitrosourea, Fetus, Rat

Introduction

Ethylnitrosourea (ENU), a well known DNA alkylating agent, is one of the powerful carcinogens and has been given to thousands of carcinogenic experiments. ENU is also known as a teratogen, and it induces anomalies in the central nervous system (CNS) (Druckrey et al., 1967; Pfaffenroth et al., 1974; Bosch, 1977a; Hallas and Das, 1978, 1979), craniofacial tissues, limbs and male reproductive organs (Druckrey et al., 1967; Ohnishi, 1989; Nagao et al., 1996). These ENU-induced anomalies are also reported in the whole embryo culture (Ohnishi, 1989; Faustman et al., 1989). However, except for several reports indicating that ENU causes death and growth arrest in the neuroepithelial cells of the fetuses (Bosch, 1977b; Fujiwara, 1980; Oyanagi et al., 1988, 1998), there are only a few reports concerning the mechanisms of ENU-induced fetotoxicity and teratogenicity.

This study was carried out to find a clue for clarifying the mechanisms of ENU-induced fetotoxicity and teratogenicity.

All procedures including animals were performed in accordance with the protocol approved by the Animal Care and Use Committee of Graduate School of Agricultural and Life Sciences, the University of Tokyo.

Offprint requests to: Dr. Kei-ichi Katayama, Department of Veterinary Pathology, Graduate School of Agricultural and Life Sciences, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-8657, Japan. Fax: +81-3-5841-8185

Materials and methods

Animals

Thirty-nine pregnant Jcl:F344 rats were obtained from Saitama Experimental Animal Co., Saitama, Japan. They were kept under controlled conditions (temperature, 23±2 ºC; relative humidity, 5525%) using an isolator caging system and were fed commercial pellets (MF, Oriental Yeast Co., Tokyo, Japan) and water ad *libitum.*

Chemical

N-Nitroso-N-Ethylurea (ENU) (Sigma, St. Louis, MO) was dissolved in 2 mM sodium citrate buffer (pH4.5) immediately before the treatment, and the concentration was adjusted to 10 mg/ml.

Treatments

Thirty pregnant rats were injected with 60 mg/kg of ENU intraperitoneally (i.p.) at day 13 of gestation, and five dams were sacrificed by heart puncture under ether anesthesia at 1, 3, 6, 12, 24 and 48 hours after treatment (HAT), respectively. The nine remaining pregnant rats were injected i.p. with citrate buffer alone at day 13 of gestation, and three dams were sacrificed in the same way at 3, 12 and 48 HAT, respectively.

Histopathology

Fetuses were collected by Caesarian section and fixed in 10% neutral-buffered formalin. Paraffin sections (4μm) were stained with hematoxylin and eosin (HE). Some of the paraffin sections were subjected to in situ detection of fragmented DNA as mentioned below.

In situ detection of fragmented DNA

DNA fragmentation was examined on the paraffin sections by the modified TUNEL method first proposed by Gravieli et al. (1992),with commercial apoptosis detection kit (ApopTag In situ Apoptosis Detection Kit; Oncor, Gaithersburg, MD). In brief, the procedure was as follows: multiple fragmented DNA 3'-OH ends on the sections were labeled with digoxigenin-dUTP in the presence of terminal deoxynucleotidyl transferase (TdT). Peroxidase-conjugated anti-digoxigenin antibody was then reacted with the sections. Apoptotic nuclei were visualized by peroxidase-diaminobenzidine (DAB) reaction. The sections were then counterstained with methylgreen.

Electron microscopy

Small pieces of fetal tissues were fixed in 2% glutaraldehyde in 0.1M phosphate buffer (pH 7.4) and postfixed in 1% osmium tetroxide in the same buffer, and embedded in Epok 812 (Oken, Tokyo, Japan). Semithin sections were stained with toluidine blue for light microscopic survey. Ultrathin sections of the selected areas were double-stained with uranyl acetate and lead citrate and observed under a JEOL 1200 EX electron microscope (Nippon Denshi, Tokyo, Japan)

Results

Neither death nor resorption of fetuses were observed throughout the experimental period.

Histopathologically, at 1 HAT, there was no significant difference in the histology of fetuses between ENU-treated and control groups. At 3 HAT, a marked increase in the number of pyknotic cells was detected in neuroepithelial cells of CNS, as well as in mesenchymal cells of craniofacial tissue, limb bud, tail bud and mandible. Although somewhat less prominent, the

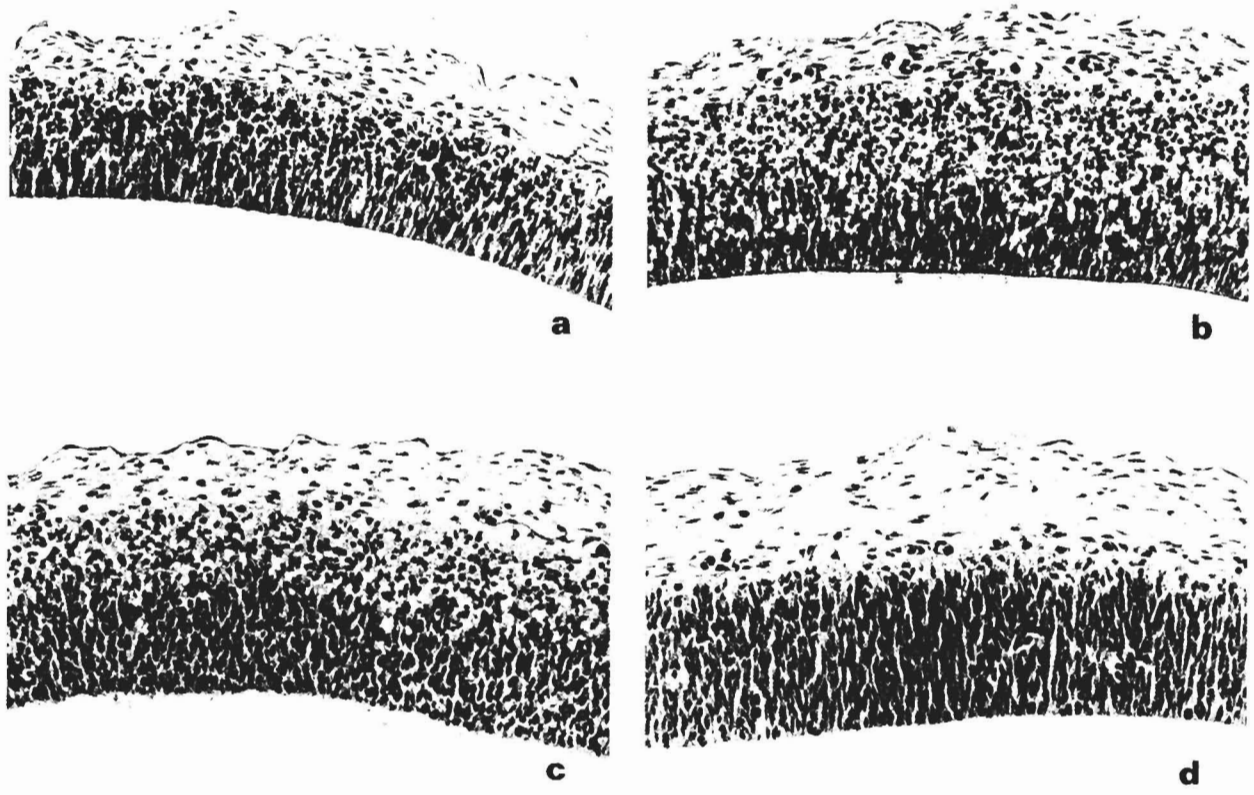

Fig. 1. Pyknotic cells in the telencephalic wall of fetuses of ENU group at 6HAT **(a)**, 12HAT **(b)**, 24HAT**(c)** and a fetus of control group at 12HAT**(d)**. HE. x 185

increase in the number of pyknotic cells was also seen in the spinal ganglia, hematopoietic progenitor cells of the liver, epithelial cells of the alimentary tract, and germ cells of the gonads. Few pyknotic cells were seen in these tissues of the control fetuses throughout the experimental period.

The number of pyknotic cells in the mesenchymal tissues, liver, alimentary tract, and gonads remained unchanged until 12 HAT, but decreased to the control level at 24 HAT (Table 1). In the CNS, the number peaked at 12 HAT, gradually decreased towards 24 HAT, and was similar to that in control group at 48 HAT (Table 1, Fig. 1). Pyknotic cells in CNS were mainly observed in the neuroepithelial cells beneath the external limiting membrane of the ventricular layer. Almost all of the nuclei of these pyknotic cells were positively stained by the TUNEL method (Fig. 2).

Table 1. The incidence of pyknotik cell in the rat fetal tissues obtained from dams treated with ENU at day 13 of gestation.

	1HAT	3HAT	6HAT	12HAT	24HAT	48HAT
CNS	±	++	+++	+++	++	±
mesenchyme	±	++	++	++	±	±
spinal ganglia	±	+	+	+	±	±
liver	±	+	+	+	±	±
alimentary tract	±	+	+	+	±	±
gonads	±	+	+	+	±	±

±: no significant increase; +: mild increase; ++: moderate increase; +++: marked increase.

Electron microscopically, the pyknotic cells were characterized by shrinkage of the cell body, condensation of nuclear chromatin and/or margination of condensed chromatin along the nuclear membrane. Some nuclei were fragmented into small pieces, which were frequently ingested by adjacent macrophages (Fig. 3).

Discussion

Almost all of the nuclei of pyknotic cells observed in the fetal tissues after ENU-treatment to the dams at day 13 of gestation were positively stained by the TUNEL method which detects fragmented DNA in situ and is widely used for the evaluation of apoptotic cells. In addition, electron microscopic features of these cells fulfill the morphological characteristics of apoptotic cells. Therefore, it is reasonable to consider that these pyknotic cells are apoptotic ones.

In CNS, apoptosis was most frequently seen in the neuroepithelial cells beneath the external limiting

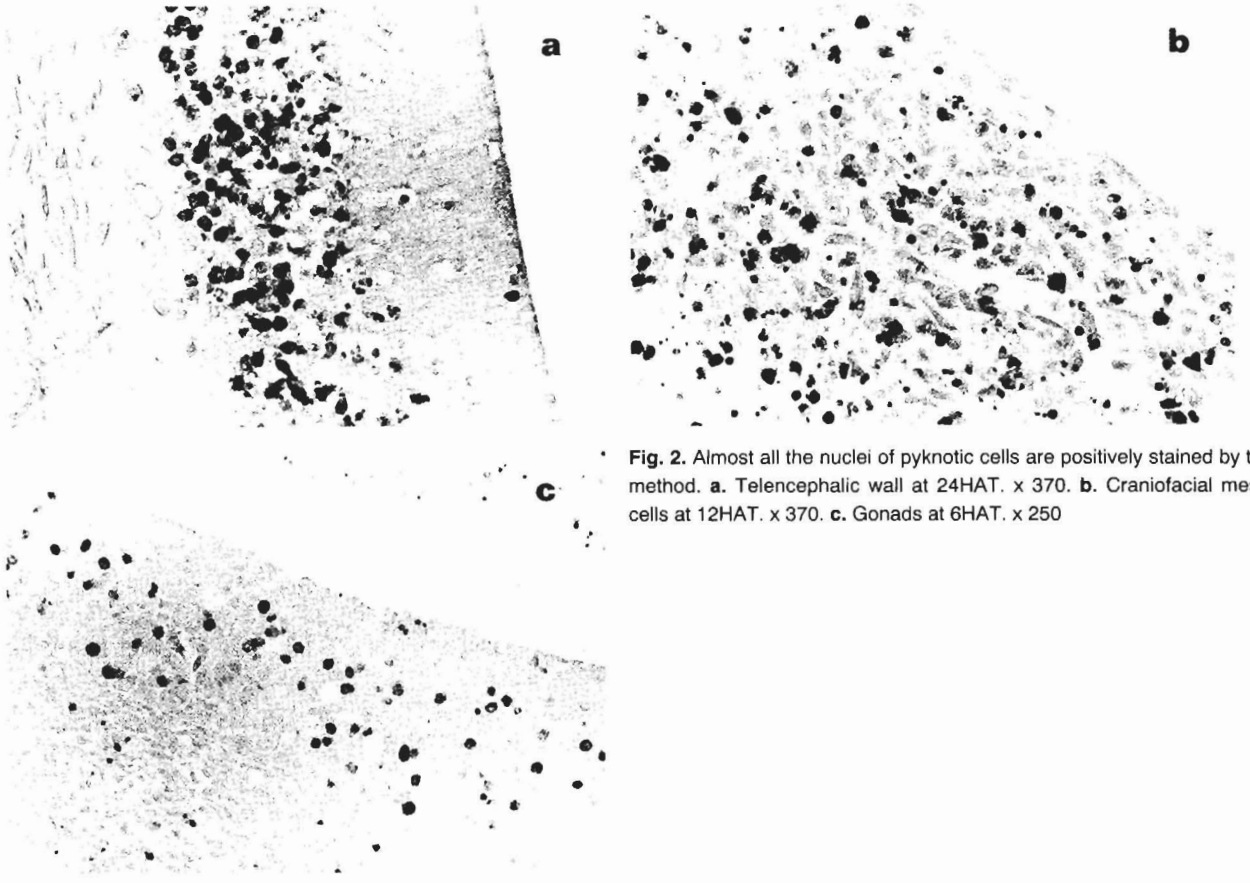

Fig. 2. Almost all the nuclei of pyknotic cells are positively stained by the TUNEL method. **a.** Telencephalic wall at 24HAT. x 370. **b.** Craniofacial mesenchymal cells at 12HAT. x 370. **c.** Gonads at 6HAT. x 250

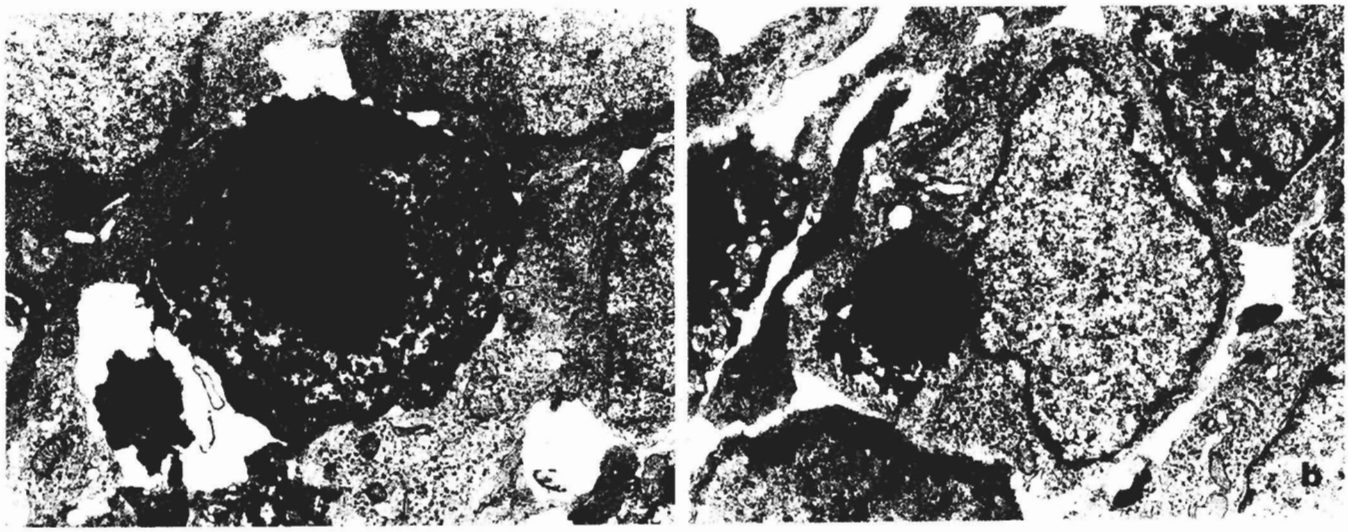

Fig. 3. Electron micrograph of pyknotic cells in the CNS. Condensation of nuclear chromatin and fragmentation into small pieces **(a)** and apoptotic bodies ingested by macrophages **(b).** x 7,000

membrane of the ventricular layer. It is said that the neuroepithelial cells beneath the external limiting membrane actively synthesize DNA (Langman et al., 1966), and that ENU damages cellular DNA synthesis by alkylation of the bases (Swann and Magee, 1971; Itze et al., 1979). Thus ENU seems to affect mainly the S-phase cells even in fetuses and induces apoptosis.

Similar enhancement of apoptotic cell death in the fetal CNS was also reported in rats following administration of 5-Azacitidine, a DNA hypo-methylating agent, and γ-radiation (Borovitskaya et al., 1996; Lu et al., 1998), and these findings suggest that the fetal CNS might be highly sensitive to such genotoxic stimuli and easily cause apoptosis.

In addition to CNS, the clear enhancement of apoptotic cell death was detected in craniofacial tissues including mandible, limb bud, and gonadal tissues in the present study. These tissues well correspond to those in which anomalies are frequently reported in the offsprings following ENU administration to their dams (Druckrey et al., 1967; Pfaffenroth et al., 1974; Bosch, 1977a; Hallas and Das, 1978, 1979; Ohnishi, 1989; Nagao et al., 1996). This strongly suggests that excess cell death by apoptosis may bring about lack of cell populations required for the later normal histogenesis and organogenesis, resulting in anomalies. To clarify this point, further studies on neonates from dams treated with ENU are now in progress.

Apoptotic cell death was also observed in lymphocytes and hematopoietic cells in the spleen of dams treated with ENU (data not shown). Similar findings are also reported in T-2 toxin-treated dams and fetuses. Namely, Shinozuka et al. (1997, 1998) reported that T-2 toxin induced apoptosis in mouse lymphoid and hematopoietic tissues, and Ishigami et al. (1999) reported that the same chemical induced apoptosis in mouse fetuses. These findings suggest that at least a part of ENU-induced fetotoxicity and teratogenicity may be secondary to maternal toxicity, though such toxicities of ENU are generally considered to be direct effects on fetuses based on the data of experiments using whole embryo culture (Faustman et al., 1989; Ohnishi, 1989).

The number of chemicals with teratogenic potency is increasing year by year in the environment surrounding humans and animals, and therefore the mechanisms of fetotoxicity which may be induced by these chemicals are much of interest in these days. This study may provide a clue for elucidating the mechanisms of fetotoxicity and teratogenicity induced by genotoxic agents.

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Accepted February 11, 2000