Ethynitrosourea Induces Apoptosis and Growth Arrest in the Trophoblastic Cells of Rat Placenta

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ABSTRACT

Ethynitrosourea (ENU), a well known alkylating agent, induces congenital anomalies in fetuses when it is administered to pregnant animals. In previous studies, we reported that ENU induced apoptosis and growth arrest in fetal tissues and organs immediately after its administration to pregnant rats. In the present study, we investigated the histopathological changes of the placenta after ENU administration to pregnant rats on Day 13 of gestation (GD13) to obtain a clue for clarifying the role of the placenta in the process of fetal developmental disability induced by genotoxic stress. Apoptotic cells increased and DNA-replicating cells decreased in the trophoblastic cells in the placental labyrinth zone of the ENU-treated group by 3 h after treatment. The number of apoptotic cells peaked at 6 h after treatment and returned to control levels at 48 h after treatment. The number of DNA-replicating cells reached minimum levels at 6 h after treatment and returned to control levels at 48 h after treatment. By immunohistochemistry, p53-positive signals were observed in trophoblastic cells in the labyrinth zone of the ENU-treated group from 3 to 6 h after treatment. Significant decreases in fetal and placental weights were observed in the ENU-treated group at 2 days (GD15) and 8 days (GD21) after treatment. A reduction in the thickness of the labyrinth zone was histopathologically significant in the ENU-treated group. These results indicate that ENU induces apoptosis and growth arrest not only in fetal tissues, but also in trophoblastic cells in the rat placental labyrinth zone, and these placental changes may have roles in the induction of fetotoxicity and teratogenicity of ENU. Moreover, a possible involvement of p53 in the induction of apoptosis and growth arrest is suggested.

INTRODUCTION

Placental growth and development are crucial for the development of the fetus, and dysfunction of the placenta may be closely related to fetal development. Apoptosis in human placenta is observed in all placental cell types including trophoblastic cells, endothelial cells, and mesenchymal cells, and apoptosis increases from the first to the third trimester [1]. Thus, placental apoptosis is believed to be a normal aging process. However, some papers have reported that increased apoptosis was observed in placentae of pregnant women with intrauterine growth retardation and other developmental disabilities [2–7]. In experimental animals, apoptosis is induced in the placenta by administration of glucocorticoid [8] or lipopolysaccharide [9, 10]. Placenta is believed to be susceptible to endocrinological abnormality, inflammatory cytokine and oxidative stress, and increased placental apoptosis induced by these insults may cause placental dysfunction, resulting in fetal developmental disability.

Embryos and fetuses are susceptible to genotoxic stresses such as alkylating agents and radiation, and congenital anomalies, including malformation and intrauterine growth retardation, are easily induced [11–16]. These genotoxic stresses induce apoptosis and cell cycle arrest in fetal tissues in a p53-dependent way, and this is recognized as a cause of congenital anomalies [14, 15, 17–20]. However, little is known about the effect of genotoxic stress on placental growth and development. The placenta is a highly proliferative tissue similar to fetal tissue, and a possibility exists that the placenta is also susceptible to genotoxic stress. Placental dysfunction induced by genotoxic stress may play some roles in the pathogenesis of fetal developmental disability.

In the present study, we investigated the histopathological changes of the placenta after administration of ethynitrosourea (ENU), a well known alkylating agent, to pregnant rats, to obtain a clue for clarifying the role of the placenta in the induction of fetal developmental disability induced by genotoxic stress.

MATERIALS AND METHODS

Animals

Sixty pregnant F344/Jcl rats (plug day = Day 0 of gestation) were obtained from Saitama Experimental Animal Co. (Saitama, Japan). They were kept under controlled conditions (23 ± 2°C and 55% ± 5% relative humidity) using an isolator caging system (Niki Shoji Co., Tokyo, Japan) and were fed commercial pellets (MF; Oriental Yeast Co., Tokyo, Japan) and water ad libitum.

Chemicals

N-Nitroso-N-ethylurea (ENU; Sigma Chemical Co., St. Louis, MO) was dissolved in 2 mM sodium citrate buffer (pH 4.5) immediately before treatment, and the concentration was adjusted to 10 mg/ml. 5-Bromo-2′-deoxyuridine (BrdU; Sigma) was dissolved in physiological saline immediately before treatment, and the concentration was adjusted to 5 mg/ml.

Treatments

Experiment 1. Twenty-five pregnant rats were injected with 60 mg/kg of ENU i.p. on Day 13 of gestation (GD13), and 5 dams were killed by heart puncture under ether anesthesia at 3, 6, 12, 24, and 48 h after treatment, respectively. Fifteen pregnant rats were injected i.p. with citrate buffer alone on GD13, and 5 dams were killed in the same way at 3, 6, 12, 24, and 48 h after treatment, respectively. All rats were injected i.p.
FIG. 1. Placenta of the ENU-treated group at 6 h after treatment (a) and of the control group at 6 h after treatment (b). Shown with TUNEL staining. Many TUNEL-positive trophoblastic cells are seen in the labyrinth zone of a. LZ, Labyrinth zone; BZ, basal zone. Bar = 60 μm.

FIG. 3. Placenta of the ENU-treated group at 6 h after treatment (a) and of the control group at 6 h after treatment (b). Immunostaining for BrdU. BrdU-positive cells almost disappear in the labyrinth zone of a. LZ, Labyrinth zone; BZ, basal zone. Bar = 50 μm.

FIG. 2. Apoptotic index of the labyrinth zone. The number of apoptotic cells peaked at 6 h after treatment and returned to control levels at 48 h after treatment. ◆, ENU-treated group; Δ, control group. *P < 0.05. **P < 0.01 (significantly different from controls).

FIG. 4. BrdU-labeling index of the labyrinth zone. The number of BrdU-positive cells reached minimum levels at 6 h after treatment and returned to control levels at 48 h after treatment. ◆, ENU-treated group; Δ, control group. *P < 0.05. **P < 0.01 (significantly different from controls).

Experiment 2. Ten pregnant rats were injected with 60 mg/kg of ENU i.p. on GD13, and 5 dams were killed by heart puncture under ether anesthesia at 2 days (GD15) and 8 days (GD21) after treatment, respectively. The remaining 10 rats were injected i.p. with citrate buffer alone on GD13, and 5 dams were killed in the same way at 2 days and 8 days after treatment, respectively. Collected fetuses and placentae were weighed and fixed in 10% neutral-buffered formalin. The placentae were cut transversely across their central portion. Paraffin sections (4 μm) were stained with hematoxylin and eosin (H&E). These paraffin sections were subjected to in situ detection of fragmented DNA and immunohistochemistry 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 Gavrieli et al. [21] with a commercial apoptosis detection kit (ApopTag In situ Apoptosis Detection Kit; Intergene, Purchase, NY). In brief, multiple fragmented DNA 3'-OH ends on the sections were labeled with digoxigenin-dUTP in the presence of terminal deoxynucleotidyl transferase (TdT). Peroxidase-conjugated antidigoxigenin antibody was then reacted with the sections. Apoptotic nuclei were visualized by peroxidase-diaminobenzidine (DAB) reaction. The sections were then counterstained with methylgreen.

Immunohistochemical Staining for BrdU

To evaluate the proliferative activity of the component cells of the placenta, immunohistochemical staining for BrdU was carried out on the paraffin sections by the labeled streptavidin-biotin (LSAB) method with streptavidin (DAKO, Carpinteria, CA). Mouse anti-BrdU monoclonal antibody (DAKO) was used as the primary antibody. The positive signals were visualized by peroxidase-DAB reaction and then the sections were counterstained with methylgreen.

Immunohistochemical Staining for p53 Protein

Immunohistochemical staining for p53 protein was carried out on the paraffin sections as described in our previous paper [20]. In brief, rabbit anti-p53 polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) was used as the primary antibody, and then incubated with the EnVision polymer reagent (DAKO). The positive signals were visualized by peroxidase-DAB reaction and then the sections were counterstained with methylgreen. As a negative control, some sections were incubated with primary antibody preabsorbed by an excess amount of p53 peptide (Santa Cruz Biotechnology).
PLACENTAL APOPTOSIS INDUCED BY ENU

FIG. 5. Placental labyrinth zone of the ENU-treated group at 3 h after treatment (a) and of the control group at 3 h after treatment (b). Immunostaining for p53. Positive signals for p53 are detected in the nuclei of the trophoblastic cells in the labyrinth zone of a. Bar = 30 μm.

Morphometry

Apoptotic cells in the placental labyrinth zone were counted in 3 randomly chosen placentae from a dam on the TUNEL-stained sections under a light microscope. Three hundred cells were counted in each placenta. BrdU-positive cells were also counted in the same way on the immunohistochemically stained sections. Thickness of the placental labyrinth zone was measured in three randomly chosen placentae from a dam at 2 days and 8 days after treatment on hematoxylin and eosin-stained sections under a light microscope using a micrometer. Thickness of the placental labyrinth zone close to the central portion was measured.

The apoptotic and BrdU-labeling indices (percentages) are expressed as means ± SD of 5 dams for the ENU-treated group and 3 dams for the control group, respectively. Placental and fetal weights and thickness of the placental labyrinth zone are expressed as means ± SD of 5 dams. Statistical analysis was carried out by the Student t-test.

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

RESULTS

Experiment 1

At 3 h after treatment, an increase of apoptotic cells which showed pyknosis on H&E sections and were positively stained by the TUNEL method, was seen in trophoblastic cells in the labyrinth zone of the placenta of the ENU-treated group (Fig. 1). The number of apoptotic trophoblastic cells peaked at 6 h after treatment and returned to control levels at 48 h after treatment (Fig. 2). We could find some apoptotic cells in the basal zone of the placenta of the ENU-treated group, but there was no difference between the ENU-treated and control groups (Fig. 1).

In the immunohistochemistry for BrdU, many positive signals were seen in the trophoblastic cells in both labyrinth and basal zones of the placenta of the control group. However, in the ENU-treated group, BrdU-positive trophoblastic cells significantly decreased in the labyrinth zone (Fig. 3). The number of BrdU-positive trophoblastic cells gradually decreased at 3 h after treatment and reached minimum levels at 6 h after treatment. At 48 h after treatment, the BrdU-labeling index returned to control levels (Fig. 4). There was no difference in the number of BrdU-positive cells in the basal zone between the ENU-treated and control groups (Fig. 3).

Many positive signals for p53 were detected in the nuclei of trophoblastic cells in the placental labyrinth zone of the ENU-treated group from 3 to 6 h after treatment (Fig. 5). On the other hand, only a few positive signals were detected in the nuclei of trophoblastic cells in the basal zone of the ENU-treated group. In the control group, only a few positive cells appeared in both zones (Fig. 5). In the sections incubated with preabsorbed antibody as a negative control, no staining was found (data not shown).

Experiment 2

Significant decreases in the fetal and placental weights were observed in the ENU-treated group at 2 days (GD15) and 8 days (GD21) after treatment (Fig. 6). A reduction in the thickness of the labyrinth zone was histopathologically significant in the ENU-treated group (Figs. 7 and 8).

DISCUSSION

ENU induces brain tumors in offspring when it is administered to pregnant rats [22]. ENU is also known as a teratogen, and it induces congenital anomalies, especially in the central nervous system [11, 16, 23, 24]. GD13 is reported to be the most sensitive period of the rat fetal...
central nervous system to ENU administration [23, 24]. The dose of ENU administered in the present study (60 mg/kg) was widely applied in these studies, and we previously clarified that the administration of 60 mg/kg of ENU to the pregnant rat scarcely influenced the viability of the fetus [16].

In our previous studies, apoptosis and growth arrest were induced in fetal tissues after ENU administration to pregnant rats, especially in the central nervous system [18, 19]. In the fetal central nervous system, the number of apoptotic cells peaked at 12 h after treatment and DNA-replicating cells (BrdU-positive cells) reached minimum levels at 6 h after treatment, and both apoptotic and BrdU labeling indices returned to control levels at 48 h after treatment [19]. In the present study, we observed an increase in the number of TUNEL-positive cells and a decrease in the number of BrdU-positive cells in the trophoblastic cells in the placental labyrinth zone after ENU-administration. These results indicate that ENU induces apoptosis and growth arrest in the trophoblastic cells in the labyrinth zone of the rat placenta almost identically to fetal tissues.

In response to DNA damage, p53 is activated and regulates expression of its transcriptional target genes, which is required for apoptosis and growth arrest [25]. In the present study, the expression of p53 protein in the placental labyrinth zone increased in the early phase of the induction of apoptosis and growth arrest. Therefore, a possible involvement of p53 in the induction of apoptosis and growth arrest is suggested.

Some papers have reported that in humans, increased apoptosis in the placenta is closely related to fetal intrauterine growth retardation and other developmental disabilities [2–7]. Thus, increased apoptosis in the trophoblastic cells in the placental labyrinth zone may play a crucial role in the fetotoxicity and teratogenicity of ENU. Decreases in fetal and placental weights, and reduction in the thickness of the placental labyrinth zone were detected in the ENU-treated group. The labyrinth zone of the placenta plays an important role in the exchange of substances between fetus and dam, so there is a possibility that the dysfunction of the placental labyrinth zone plays some roles in the induction of fetal developmental disability.

ENU induced apoptosis and growth arrest in trophoblastic cells in the labyrinth zone but not in those in the basal zone, and immunoreactivity for p53 was widely detected in the labyrinth zone but was rarely observed in the basal zone. Thus, susceptibility to ENU may be completely different between the labyrinth and basal zones. Further studies are needed to clarify the reason for this different susceptibility.

5-Azacytidine, a DNA hypomethylating agent, induces apoptotic cell death in the fetal central nervous system, similar to ENU [26]. It also induces a significant decrease in placental weight when it is administered to pregnant rats, but it does not induce apoptosis in the placenta [27]. These results suggest that an agent that induces apoptosis in fetal tissues does not always induce apoptosis in the placenta.

In rodents, apoptosis is induced in the placenta by administration of glucocorticoid [8] or lipopolysaccharide [9, 10]. The placenta is believed to be susceptible to endocrinological abnormality, inflammatory cytokine and oxidative stress, and to result in the development of apoptosis. In addition, the present study demonstrates that the placenta is susceptible to genotoxic stress, and induced apoptosis and growth arrest. Placental dysfunction caused by genotoxic stresses may play some roles in the induction of fetal developmental disability.
REFERENCES