Indole-3-Acetic Acid Induces Microencephaly in Rat Fetuses

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ABSTRACT

Indole-3-acetic acid (IAA), known as natural auxin, induces cleft palate in rodents. However, there has been no report about the neurodevelopmental toxicity of IAA in rats. In the present study, we found microencephaly in the fetuses from the rats exposed to IAA. The purpose of this work was to examine the effects of IAA administration in pregnant rats on neuroepithelial cells in the embryos/fetuses. IAA was administered at 500 and 1,000 mg/kg on gestation days (days) 12, 13, and 14, and then embryos/fetuses were harvested on days 14.5, 15, 16, and 21. Cleft palate was induced at 1,000 mg/kg. The brain in treated groups exhibited reduction in the size and weight on day 21 in a dose-dependent manner. Histopathologically, apoptotic cells were observed mainly in the medial and dorsal layer of the neuroepithelium at 500 and 1,000 mg/kg on day 14.5. On day 15, they were observed in the medial and dorsal layer of the neuroepithelium, and preplate at 1,000 mg/kg. On day 16, they existed in the dorsal layer of the neuroepithelium and intermediate zone in the embryos from 1 dam at 1,000 mg/kg. On day 21, an increase in cell proliferative activity was observed in the neuroepithelium at 500 and 1,000 mg/kg. The reduction of the cortical plate, the enlargement of the neuroepithelium and a slight increase in neuron density in the intermediate zone were observed at 1,000 mg/kg. These findings indicated IAA might induce the neuronal apoptosis in the S phase and lead to microencephaly.

Keywords. Apoptosis; fetus; indole-3-acetic acid; microencephaly; neuroepithelium; rat; teratogen.

INTRODUCTION

Vulnerable periods during the development of the central nervous system (CNS) are sensitive to environmental insults, because they are dependent on the temporal and regional emergence of critical developmental processes, such as proliferation, migration, differentiation, synaptogenesis, myelination, and apoptosis (Rice and Barone, 2000). In normal neocortical development, the neuroepithelial cells in the dorsal layer of the neuroepithelium actively synthesize DNA. The postmitotic neurons migrate out of the neuroepithelium and form the preplate. The neurons accumulate within the preplate and form the cortical plate, which splits the preplate into the marginal zone and subplate. The neurons of the cortical plate are generated in an inside-out gradient and differentiate into the layers (O’Leary and Koester, 1993; Gressens, 2000). This process is highly sensitive to various physical, chemical, and biological agents. The neuronal migration disorders are major causes of brain malformation in experimental animals and humans in utero exposure to several environmental factors (Sun et al., 2002). Induced-brain malformations are reported in fetal rats following exposure to γ- and X-radiation (Hoshino and Kameyama, 1988), administration of ethanol (Miller, 1986), 1-β-D-arabinoferanosylcytosine1 (Ara-C) (Yamauchi et al., 2003), 5-azacytidine (5AzC) (Lu et al., 1998; Ueno et al., 2002a, 2002b), ethylnitrosourea (ENU) (Katayama et al., 2000a, 2000b), mercury compounds (Monnet-Tschudi, 1998), 5-methyl-D-aspartate (Takai et al., 2003), and methyla-zoxymethanol (Cattabeni and Di Luca, 1997; Ferguson and Holson, 1997) etc. Most of them induce excessive neuron death and result in microencephaly. Excessive neuron death is one of the most important mechanisms that induce the disturbance of neuronal migration in developing cerebral cortex.

Indole-3-acetic acid (IAA), a plant hormone known as natural auxin, regulates the plant growth (Bandurski et al., 1995) and is obtained by mammals from a diet rich in vegetable stems (Gordon et al., 1972). It is identified in extracts from embryos of several species of animal and may play a role in the mechanisms of cell growth in animals (Ichimura and Yamaki, 1975). It is also known as a teratogen and induces cleft palate in rats and mice (John et al., 1979). Moreover, other malformations including exencephaly, ablepharia, dilated cerebral ventricles, and crooked tail were observed in mice. However, there has been no report about the neurodevelopmental toxicity of IAA in rats.

In the present study, we found microencephaly in the fetuses from the rats exposed to IAA during gestation days 12–14, early stage of cerebral cortex developing. To clarify the relationship between the effects on neuroepithelial cells and the development of IAA-induced microencephaly, we examined the effects of IAA administration in pregnant rats on neuroepithelial cells in the embryos/fetuses.

MATERIALS AND METHODS

Animals

A total of 48 pregnant specific pathogen-free Wistar Hannover GALAS rats (CLEA Japan, Inc., Japan), at approximately 10–12 weeks of age, were purchased. The animals were single-housed in wire-mesh cages in an air-conditioned room (22 ± 2°C; humidity, 55 ± 10%; light cycle, 12 hours/day). Feed (Oriental Yeast Co., LTD., Japan) and water were available ad libitum.
Experimental Design

Day 0 was designated as the day when the presence of vaginal plugs was identified. IAA (Kanto Chemical Co., Inc., Japan) (Figure 1) was suspended in olive oil and administered orally (1 ml/100 g body weight of rats). The pregnant rats were randomly allocated to 3 groups of 16 rats each. Animals were treated daily at dose levels of 0 (olive oil only), 500, or 1,000 mg/kg during days 12–14. The dose level of 1,000 mg/kg was the maximum dose in the rat teratology study and that of 500 mg/kg was previously reported to induce cleft palate by the treatment during days 7–22 in rats (John et al., 1979). All treatments were made between 10 and 11 am. Four rats from each group were sampled on days 14.5, 15, 16, or 21. Animals were weighed, euthanized by exsanguination under diethyl ether anesthesia, and necropsied. The embryos/fetuses were removed from the uterus, weighed, and examined for external malformation with the aid of a dissecting microscope. The embryos/fetuses were fixed in 10% neutral buffered formalin. The brains of 4 fetuses from each dam in each group on day 21 were weighed. These experiments were conducted according to the Guidelines for Animal Experimentation, Japanese Association for Laboratory Animal Science, 1987.

Histological Examination

Five embryos/fetuses were randomly selected from each litter. Three embryonic brains including eyeballs on days 14.5, 15, and 16, and 4 fetal brains on day 21 were trimmed transversely for histopathological evaluation. In addition, 2 embryonic brains on days 14.5, 15, and 16 were trimmed longitudinally in order to bring out the distribution of TUNEL-positive cells in CNS. Their brains were trimmed in the way shown in Figure 2, embedded in paraffin, sectioned at a 4-µm thickness, and stained routinely with hematoxylin and eosin (H&E). The nomenclature for the layers of developing...
brain used was in accordance with that used by O’Leary and Koester (1993), Ueno et al. (2002a) and Bayer and Altman (2004) (Figure 2). Immunohistochemical staining involving proliferating cell nuclear antigen (PCNA) (PCNA, PC10: Dako Cytomation Co. LTD., Japan), gliarial fibrillary acidic protein (GFAP) (GFAP: Dako Cytomation Co. LTD., Japan), and in situ TUNEL (ApopTag in situ Detection Kit Wako, Wako Pure Chemical Industries, Japan) were performed according to the avidin-biotin complex (ABC) method (VECTABLAST ABC Kit, Vector Laboratories Inc., Canada). The incidence of residues of pyknotic cells on days 14.5, 15, and 16 was counted in each transverse anterior and posterior section in a TUNEL reaction, by light microscopy with a ×40 objective. For PCNA labeling index (LI%) of the neuroepithelial cell on day 21, labeled nuclei and total nuclei for the neuroepithelium were scored in 4 areas in the anterior sections of each fetus by light microscopy with a ×60 objective, with the aid of an image analyzer (IPAP; Image Processor for Analytical Pathology: Sumika Technoservice Co., Japan). PCNA LI% of individual animals was determined by dividing the total number of PCNA-labeled neuroepithelial cells by the total number of neuroepithelial cells counted. GFAP immuno-staining was performed to identify the cells in the cerebral cortex on day 21.

Electron Microscopic Examination

Small pieces of the telencephalic wall from the 1 formalin-fixed embryo on day 15 at 1,000 mg/kg were postfixed in 1% solution of osmium tetroxide, dehydrated in ethanol, and embedded in Epon 812. Ultrathin sections were stained with uranyl acetate and lead citrate.

Statistical Analysis

Means and standard error (SE) were calculated. Continuous data were analyzed with the Bartlett test. When variances were homogeneous, the Dunnett’s multiple comparison test was performed. The Steel’s multiple comparison test was employed, when variances were not homogeneous or the data, such as the number of TUNEL-positive cells, was non-parametric.

RESULTS

The declining body weight gains of dams were statistically or biologically significant on day 15 at 500 mg/kg and during days 13–21 at 1,000 mg/kg, compared to control group. The body weight suppression ratio at 500 mg/kg on day 15 and 1,000 mg/kg on day 21 were 95% and 93% of control on the same time point, respectively. The body weight gains at 500 mg/kg returned to control values by day 16. All dams did not show any changes in signs.

Table 1 shows the numbers and weights of examined live embryos/fetuses and placental weights at each sampling time point, and the incidence of cleft palate on day 21. The embryo weights on days 15 and 16, and the placental weights on day 16 at 1,000 mg/kg were statistically lower than that of control group. Cleft palate was significantly induced in 61.9% of the fetuses at 1,000 mg/kg on day 21. No other external malformation was observed in all groups. The brain in treated groups exhibited reduction in the size and weight in a dose-dependent manner (Table 2, Figure 3). A significant absolute and relative brain weight reduction was seen at 1,000 mg/kg, in comparison with the control group.

Histological Examination on Gestation Days 14.5, 15, and 16

The neuroepithelial cells characterized by pyknosis or karyorrhexis and phagocytosis were scattered in the myelencephalon of the embryos on day 14.5 at 500 mg/kg, and in the telencephalon, diencephalon, mesencephalon, metencephalon, and myelencephalon of the embryos on days 14.5, 15, and 16 at 1,000 mg/kg. However, on day 16 at

<p>| TABLE 2.—Fetal brain weight on gestation day 21. |
|-----------------|-----------------|-----------------|-----------------|</p>
<table>
<thead>
<tr>
<th>Dosage (mg/kg)</th>
<th>No. of pregnant animals</th>
<th>Absolute brain weight (g)a</th>
<th>Relative brain weight (% B.W.)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>4</td>
<td>197.1 ± 8.3</td>
<td>4.06 ± 0.16</td>
</tr>
<tr>
<td>500</td>
<td>4</td>
<td>169.2 ± 8.4</td>
<td>3.66 ± 0.20</td>
</tr>
<tr>
<td>1000</td>
<td>4</td>
<td>124.8 ± 11.4***</td>
<td>2.85 ± 0.25**</td>
</tr>
</tbody>
</table>

Mean ± SE.

aMean of individual litter values.

**Significantly different from control at the p < 0.01, p < 0.001, respectively (Dunnett’s test).
FIGURE 3.—Gross appearance of fetal brain on gestation day 21. The fetal brains in treated groups exhibit reduction in the size in a dose-dependent manner.

1,000 mg/kg, they were only detected in the embryos from 1 dam and there was no apparent morphological change in the embryos from other dams. Most of these cells were stained by TUNEL method. As shown in Figure 4, a statistically or biologically significant increase in the number of the TUNEL-positive cells in CNS was detected in the posterior sections on day 14.5 at 500 mg/kg and both of the anterior and posterior sections at on days 14.5, 15, and 16 at 1,000 mg/kg. On days 14.5 and 15, they were observed mainly in the medial and dorsal layer of the neuroepithelium (Figure 5). In addition, they were also seen in neural retina, and in preplate with width reduction at 1,000 mg/kg on days 14.5 and 15, respectively (Figure 5). On day 16, they were observed in the dorsal layer of the neuroepithelium and intermediate zone with width reduction in the embryos from 1 dam at 1,000 mg/kg (Figure 6). There was no morphological apparent change in the control group.

**Histological Examination on Gestation Day 21**

In comparison with the anterior sections in control, the reduction of the cortical plate, the enlargement of the neuroepithelium, and a slightly increase in the cell packing density consisting of GFAP-negative cells, which were considered to be neurons, in the intermediate zone were observed at 1,000 mg/kg (Figure 6). The number of PCNA-positive cells increased significantly in the neuroepithelium at 500 and 1,000 mg/kg (Figures 6 and 7). There was no apparent morphological change in the posterior sections of all groups.

**Electron Microscopic Examination**

Pyknotic cells in the medial and dorsal layer of neuroepithelium exhibited cell death characterized by shrinkage of the cell body with high electron density of fragmented nuclear chromatin along the nuclear membrane, termed “apoptotic bodies.” Macrophages ingested fragmented apoptotic bodies. The surrounding neuroepithelial cells were intact (Figure 8).
FIGURE 5.—Distribution of TUNEL-positive cells in neuroepithelium and eyeball on gestation days 14.5 and 15. Bar = 100 µm. NE: Neuroepithelium; VL: Ventricular layer; ML: Medial layer; DL: Dorsal layer; PP: Preplate. a–c: TUNEL stain of control (a), 500 mg/kg IAA (b), and 1,000 mg/kg IAA (c). Neuroepithelium in anterior section on day 14.5. TUNEL-positive cells are present in the medial and dorsal layers of the neuroepithelium of the 1,000 mg/kg rat. d–f: TUNEL stain of control (d), 500 mg/kg IAA (e), and 1,000 mg/kg IAA (f). Neuroepithelium in posterior section on day 14.5. TUNEL-positive cells are present in the neuroepithelium of the 500 and 1,000 mg/kg rat. g: TUNEL stain of 1,000 mg/kg IAA eyeball on day 14.5. TUNEL-positive cells are observed in neural retina (>). h–j: TUNEL stain of control (h), 500 mg/kg IAA (i), and 1,000 mg/kg IAA (j). Neuroepithelium in anterior section on day 15. TUNEL positive cells are present in medial and dorsal layer of neuroepithelium and preplate with width reduction at 1,000 mg/kg.
FIGURE 6.—Distribution of TUNEL-positive cells in neuroepithelium on gestation day 16. Morphological changes (H&E stain) and distribution of PCNA-positive cells in neuroepithelium of cortex cerebrum on gestation day 21. Bar = 100 µm. NE: Neuroepithelium; VL: Ventricular layer; ML: Medial layer; DL: Dorsal layer; IZ: Intermediate Zone; SP: Subplate; CP: Cortical plate; MZ: Marginal zone. a–c: TUNEL stain of control (a), 500 mg/kg IAA (b), and 1,000 mg/kg IAA (c). Neuroepithelium in anterior section on day 16. TUNEL-positive cells are present in the dorsal layer of the neuroepithelium and intermediate zone with width reduction in the 1,000 mg/kg rat. d–f: H&E stain of control (d), 500 mg/kg IAA (e), and 1,000 mg/kg IAA (f). Cerebral cortex on day 21. Reduction of the cortical plate, the enlargement of the neuroepithelium, and a slightly increase in the cell packing density are observed in the intermediate zone of the 1,000 mg/kg rat. g–i: PCNA stain of control (g), 500 mg/kg IAA (h), and 1,000 mg/kg IAA (i). Neuroepithelium in cerebral cortex on day 21. The number of PCNA-positive cells increases in the neuroepithelium of the 500 and 1,000 mg/kg rats.
FIGURE 7.—PCNA labeling index (%) in neuroepithelium on gestation day 21. Each value represents mean ± SE. *Significantly different from control at \( p < 0.05 \) (Dunnett’s test). The number of PCNA-positive cells increases in the neuroepithelium at 500 and 1,000 mg/kg.

DISCUSSION

The results of our experiments indicated that IAA treatment during days 12–14 evoked the neuroepithelial cell death in the CNS of embryos in the dose-dependent manner. It was confirmed that this cell death was apoptosis, as demonstrated by the TUNEL method, which detects fragmented DNA in situ, and in addition, electron microscopic observation of condensed nuclear fragments within macrophages. The excessive death of newborn neuron by apoptosis might have inhibited the developing of cerebral cortex and induced microencephaly.

As mentioned in the introduction, some substances cause apoptosis in the neuroepithelial cells and in turn induce neuronal migration disorders in developing cerebral cortex. Ara-C, a cytidine analog (Yamauchi et al., 2003) and ENU, a DNA alkylating agent (Katayama et al., 2000a, 2000b), induce the neuronal apoptosis mainly in the medial and dorsal layer of the neuroepithelium with a single treatment on day 13 in rats. It appears that the S phase cells are affected through inhibition or damage of DNA synthesis, and subsequently lead to apoptosis in embryos. It is known that the both substances bring about microencephaly by treatment into pregnant rats (Ritter et al., 1971; Katayama et al., 2000a). On the other hand, 5AzC, a cytidine analog, induces the neuronal apoptosis in the preplate or intermediate zone (Hossain et al., 1995; Ueno et al., 2002b) and brings about hypoplasia of the dorsal forebrain in mice (Schmahl et al., 1984). It affects the post-mitotic neurons and induces apoptosis after leaving the cell cycle (Ueno et al., 2002a, 2002b). It has been hypothesized that 5AzC is incorporated into DNA during the S phase and is supposed to exert its toxicity by its hypomethylating effect on DNA or by the direct inhibitory effect on DNA function.

In our study, IAA-induced apoptotic cells were observed mainly in the medial and dorsal layer of the neuroepithelium on days 14.5 and 15, although they existed in the dorsal layer of the neuroepithelium and intermediate zone on day 16 at 1,000 mg/kg. The location of IAA-induced apoptosis at 24 hours after the last treatment, corresponding to day 15 was quite similar to that of Ara-C or ENU-induced apoptosis at the same time point. Therefore, we suspected that IAA might selectively affect the S phase neurons, by inhibition or damage of DNA synthesis. On the other hand, the increase in the number of apoptotic cells at 500 mg/kg was only observed on day 14.5. It might be caused by the IAA-elimination rate related to the dose-dependent manner, although we did not examine the IAA level in the dam’s serum and embryo/fetus.

The enlargement of the neuroepithelium, the mitotic activity elevation, and an increase in the packing density of

FIGURE 8.—Electron micrograph of pyknotic cell in neuroepithelium on gestation day 15 at 1,000 mg/kg. Bar = 5 \( \mu \)m. High electron density of fragmented nuclear chromatin, termed “apoptotic bodies” ingested by macrophages.
neurons were observed in the intermediate zone of the cerebral cortex on day 21 at 1,000 mg/kg. Similar changes were observed in the rat fetuses obtained from the dams exposed by ethanol (Miller, 1986). It is known that a proportion of neuroepithelial cells of rat fetal neocortex at the neurogenesis stage return to neuroepithelial cell proliferation stage in ENU treated fetuses (Oyanagi et al., 1998). Although the number of apoptotic cells on days 14.5, 15, and 16 at 1,000 mg/kg was greater compared with that at 500 mg/kg, it is not evident that PCNA LI% on day 21 was comparable at 500 and 1,000 mg/kg. However, since the enlargement of the neuroepithelium at 1,000 mg/kg was greater compared with that at 500 mg/kg, the total number of proliferative cells in the neuroepithelium increased in the dose-dependent manner. Therefore, it was suggested that these changes might be the compensatory reactions to the excess neuron death by IAA. Moreover, since neuroepithelial cells have the cell proliferative ability, IAA did not injure the radial glia in the ventricular layer of the neuroepithelium irreversibly.

IAA is found in the urine of humans as a product of tryptophan metabolism and is present in cerebrospinal fluid (Hu and Dryhurst, 1997), blood (Martinez et al., 1983), and several organs such as lung, kidney, liver, and brain (Tusell et al., 1984; Weissbach et al., 1959). IAA is predominantly formed as a result of the monoamine oxidase-mediated oxidative deamination of tryptamine, a putative neurotransmitter or neuromodulator in CNS (Artigas et al., 1983). IAA is welltolerated in humans (Mirsy and Diergott, 1956) and has not been known to induce apoptosis in normal tissue in vivo or to affect CNS in rats (John et al., 1979). However, previous studies indicate that IAA induces cytotoxicity in fibroblasts (Folkes et al., 1999), neutrophils (De Mello et al., 2004), and several cancer lineages (Greco et al., 2002) in vitro. The photoproducts of IAA sensitized by riboflavin (Edwards et al., 1999) and the combination of IAA and horseradish peroxidase (HRP) (Folkes and Wardman, 2001; Greco et al., 2002; Kim et al., 2004) enhance cellular oxidative stress and induce apoptosis in these cells. IAA activated by HRP produces free radicals, such as indolyl, skatole, and peroxyl radicals. Although these radicals induce membrane lipid peroxidation, it is not an important component of IAA/HRP-induced cytotoxicity. On the other hand, the incubation of activated IAA with plasmid DNA in a cell-free system results in the formation of DNA adducts and strand breaks, indicating that DNA damage could be involved in the observed cell death (Folkes et al., 1999). Therefore, it is possible that IAA is activated in the neuroepithelial cells of the rat embryo brain by unknown factors. Activated IAA might inhibit or damage the DNA function during the S phase of the neurons in the neuroepithelium, which in turn leads to apoptosis. From the present study, we could not prove that IAA-induced free radicals caused the neuronal apoptosis in rat embryos. Further detailed investigations of the activated genes in relationship with IAA-induced apoptosis are necessary to clarify the mechanisms of neuronal apoptosis.

In conclusion, IAA administration in pregnant rats during days 12–14 might selectively affect the S phase neurons and leads to their apoptosis in the neuroepithelium. Although the enlargement of the neuroepithelium and the increase in proliferative neuroepithelial cells occurred as the compensatory reaction, the excessive death of newborn neuron might inhibit the developing of cerebral cortex and induce microencephaly in the dose-dependent manner. This study is the first report that IAA induces microencephaly in rats.

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