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Developmental Eye Abnormalities in Mouse Fetuses Induced by Retinoic Acid

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Abstract: To clarify the relationship between neural crest cells and various developmental eye abnormalities, pregnant mice were administered an intraperitoneal injection of 12.5 mg/kg retinoic acid (RA) suspended in corn oil on day 7 of pregnancy (RA group). Control mice received an equal volume of corn oil only (control group). The fetuses were removed by laparotomy on day 18 of gestation. The fetal mortality was 46.3% in the RA group and 2.2% in the control group. The live fetuses in both groups were observed grossly, and the eyes were examined histologically in serial sections. In the RA group, gross malformations were observed, including microphthalmos (95.5%), cleft lip and palate (36.4%), and central nervous system anomalies (31.8%). In the control group, these malformations were seen in only 6.7%, 0%, and 2.2%, respectively. Histologic examinations in the RA group revealed microphthalmos (47.7%), anophthalmos (38.6%), faulty closure of the embryonic fissure (36.4%), developmental abnormalities of the vitreous (34.1%), aphakia (22.7%), goniodysgenesis (18.2%), and faulty separation of the lens vesicle (15.9%). They were detected in only 3.3%, 1.1%, 3.3%, 8.9%, 1.1%, 2.2%, and 2.2%, respectively, of the control group. These developmental eye abnormalities arose from abnormal migration of neural crest cells. **Jpn J Ophthalmol 1998;42:162–167**

Key Words: Developmental eye abnormalities, mouse fetus, neural crest cells, retinoic acid.

Introduction

The eye is formed under tissue interactions among surface ectoderm, neuroectoderm including the neural crest, and mesoderm.[1] The neural crest is a specialized population of mesenchymal cells that emigrates from the dorsal margin of the neural folds around the time of neural tube closure.[1] Cranial neural crest cells migrate and differentiate into various ocular tissues, such as the corneal endothelium and stroma, the iris stroma, the trabecular meshwork, the ciliary body stroma, uveal melanocytes, and the sclera.[2,3] The primary vitreous is thought to develop from neural crest cells.[4,5]

Retinoic acid (RA) is the carboxylic acid and the biologically active form of vitamin A (retinol).[6] This acid is regarded as a powerful teratogen that mainly affects the migration of cranial neural crest cells.[7–9]

The developmental mechanisms involved in eye abnormalities have not been well elucidated. In the present study, various eye abnormalities induced by RA were examined histologically to clarify their developmental mechanisms.

Materials and Methods

Animals used in this study were C57BL/6NJcl mice whose body weights ranged from 25 to 35 g and were 12 to 16 weeks of age postbirth. Five animals were kept in each cage, with wood-shaving bedding, and they were given commercial laboratory food and water. The room was kept on a 12-hour light/dark cycle at a constant temperature of 25°C. Pairs of mice, each including an estrous female and a potent male, were caged together overnight. Females that had vaginal plugs the next morning were determined to be in day 0 of pregnancy. Pregnant mice were housed individually in cages. Because the period of organogenesis is considered to be the most vulnera-

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Table 1. Pregnancy Status of Mice

	Retinoic Acid Group	Control Group
No. of pregnant mice	5	5
No. of implantations (mean)	41 (8.2)	46 (9.2)
No. of dead fetuses (%)	19 (46.3[a])	1 (2.2[a])
No. of live fetuses (%)	22 (53.7[a])	45 (97.8[a])
No. of malformed fetuses (%)	21 (95.5[b])	3 (6.7[b])

[a]Percentage of implantations.
[b]Percentage of live fetuses.

Table 2. Gross Malformations

	Retinoic Acid Group	Control Group
Microphthalmos	21 (95.5)	3 (6.7)
Cleft lip and palate	8 (36.4)	0
Central nervous system anomalies	7 (31.8)	1 (2.2)
Low-set ears	6 (27.3)	1 (2.2)
Facial anomalies	4 (18.2)	0
Iris coloboma	4 (18.2)	0
Micrognathia	4 (18.2)	0

Results are expressed as numbers of mice. Numbers in parentheses indicate the percentage of live fetuses in each group.

ble for teratogenesis, we treated pregnant mice on day 7 of pregnancy, the first day of the organogenic period in mice.[10] Retinoic acid (Sigma Chemical Company, St. Louis, MO, USA) was dissolved in corn oil at a concentration of 1 mg/mL just before use. Pregnant mice were administered a single intraperitoneal injection of 12.5 mg/kg of RA on day 7 of pregnancy (RA group). Control mice were given an equal volume of corn oil only (control group). Each group consisted of five pregnant mice. The pregnant mice were sacrificed by cervical dislocation on day 18 of pregnancy. The fetuses were removed by laparotomy and grossly observed under a stereoscopic microscope. All live fetuses were immediately fixed at 20°C in Bouin's solution. Following fixation for 48 hours, tissue blocks of eyes excised from the fetuses were immersed in the same solution for approximately 7 days. The fixed tissue blocks were dehydrated in an ethanol series of ascending concentrations, cleared in xylene, and embedded in paraffin wax. Serial frontal sections of the eye were cut at a thickness of 4.0 μm and mounted on glass slides. After being dewaxed in xylene, sections were hydrated in an ethanol series of descending concentrations. Hydrated sections were stained with Meyer's hematoxylin solution at 20°C for 10 minutes, rinsed in tap water for 15 minutes, immersed in 0.5% eosin solution at 20°C for 10 minutes, dehydrated in an ethanol series of ascending concentrations, cleared in xylene, and then mounted in Harleco synthetic resin solution (Kokusai Shiyaku, Kobe, Japan). The stained sections were examined in detail with a light microscope. For statistical analysis, Student's *t*-tests and chi-square tests were used.

Results

The number of implantations in the two groups did not differ significantly (Student's *t*-test). The fetal mortality was 46.3% in the RA group and 2.2% in the control group (Table 1). The incidence of fetuses with gross malformations was 95.5% in the RA group and 6.7% in the control group. Both the fetal mortality and the incidence of fetuses with gross malformations were significantly higher in the RA group than in the control group (chi-square test, $P < 0.01$).

Figure 1. Microphthalmos observed on gross examination. The eye of the left fetus is normal in size on day 18 of gestation. The eye of the center fetus is smaller than normal. No eye formation is observed in the right fetus.

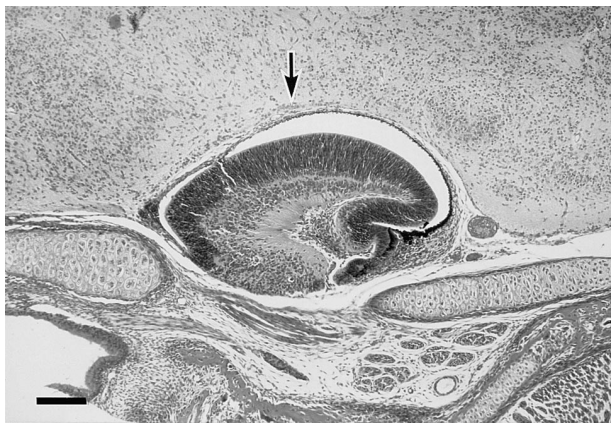

Figure 2. Microphthalmos observed on histologic examination. Rudiments of the inner and outer walls of the optic cup are seen below the cranial tissue (arrow). Bar = 140 μm.

Jpn J Ophthalmol
Vol 42: 162–167, 1998

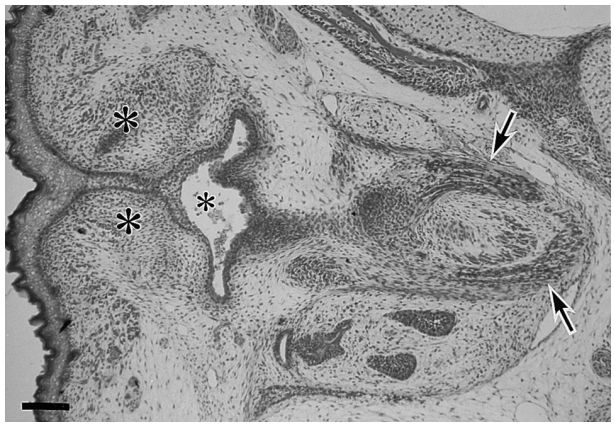

Figure 3. Anophthalmos observed on histologic examination. Although eyelids (large asterisks), conjunctival pouch (small asterisk), and external muscles (arrows) are observed, no rudiments of the anlage derived from the neuroectoderm can be recognized. Bar = 110 μm.

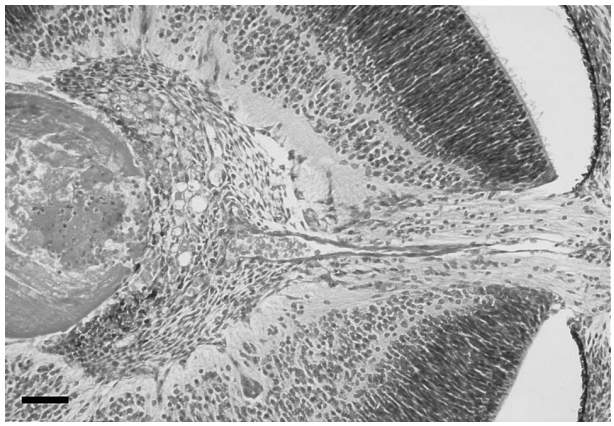

Figure 5. Developmental abnormalities of the vitreous observed on histologic examination. The vitreous cavity is filled with excessive mesenchymal tissue, and the posterior lens capsule is broken. Melanocytes are scattered in the mesenchymal tissue. Bar = 55 μm.

Distinguishing anophthalmos from microphthalmos by gross examinations using a stereoscopic microscope is very difficult. Therefore, any abnormality, ranging from a small eye to no eye formations, was termed microphthalmos on gross examination (Figure 1). In the RA group, gross malformations such as microphthalmos, cleft lip and palate, central nervous system anomalies, and low-set ears were observed frequently (Table 2). In the control group, similar malformations were seen in only a few fetuses. The incidence of each gross malformation was significantly higher in the RA group than in the control group (chi-square test, $P < 0.01$). Histologically, anything ranging from rudiments of the neuroectoderm to a small eye accompanying other ocular abnormalities was termed microphthalmos (Figure 2). The diagnosis of anophthalmos was made when no rudiments of the anlage derived from the neuroectoderm could be recognized in serial sections (Figure 3).

When the inner wall of the optic cup was everted at the margins of the embryonic fissure and an outer wall was absent from the outside of the everted inner wall, we diagnosed a faulty closure of the embryonic fissure (Figure 4). Developmental abnormalities of the vitreous were defined as the presence of excessive mesenchymal tissue in the vitreous cavity (Figure 5). Any eye without tissues derived from the lens in serial

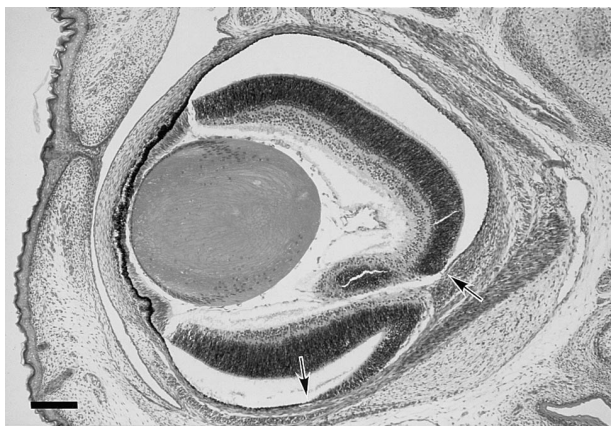

Figure 4. Faulty closure of the embryonic fissure observed on histologic examination. The inner wall of the optic cup is everted at the margins of the embryonic fissure. The outer wall is absent from the outside of the everted retina (arrows). Bar = 140 μm.

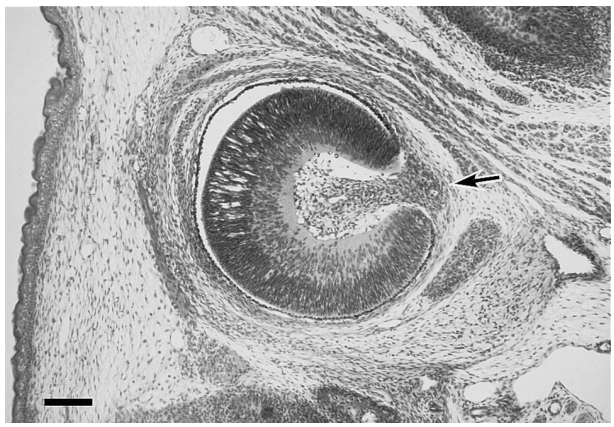

Figure 6. Aphakia by histologic examination. No tissue derived from the lens can be detected in serial sections. The embryonic fissure is not closed (arrow), and the vitreous cavity is filled with mesenchymal tissue. The eye also demonstrates microphthalmia. Bar = 110 μm.

sections represented aphakia (Figure 6). Eyes with any histologic anomalies in the anterior chamber or the chamber angle were diagnosed as goniodysgenesis (Figure 7). When the corneal epithelium was connected with the lens epithelium or remnants of the lens stalk were observed, we diagnosed a faulty separation of the lens vesicle (Figure 8). In the RA group, microphthalmos, anophthalmos, faulty closure of the embryonic fissure, developmental abnormalities of the vitreous, aphakia, goniodysgenesis, and faulty separation of the lens vesicle were observed frequently (Table 3). In contrast, these abnormalities were seen in only a few eyes in the control group. The frequency of each eye abnormality was significantly higher in the RA group than in the control group (chi-square test, $P < 0.01$). In the mesenchymal tissue observed in developmental abnormalities of the vitreous, scattering melanocytes (Figure 5) were detected in 13 (86.7%) of 15 eyes.

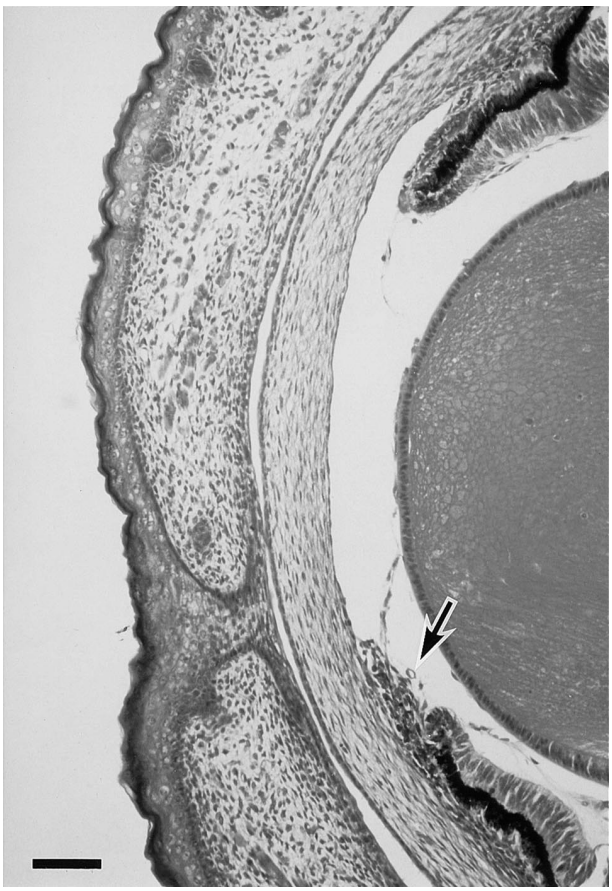

Figure 7. Goniodysgenesis observed on histologic examination. The upper chamber angle is formed normally, and the lower chamber angle space is filled with mesenchymal tissue (arrow). Bar = 70 μm.

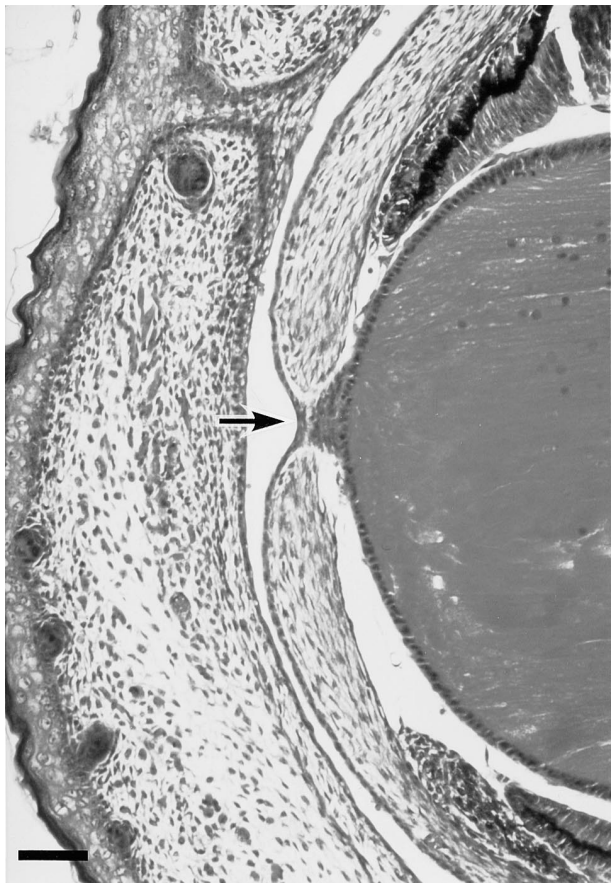

Figure 8. Faulty separation of the lens vesicle by histologic examination. The corneal epithelium is connected with the lens epithelium (arrow), and the continuity of the corneal stroma is interrupted. In addition, development of the chamber angle is poor. Bar = 55 μm.

Discussion

Retinoic acid, first synthesized by Arens and van Dorp[6] in 1946, is a biologically active form of vitamin A (retinol).[11] Retinol is known to induce various biologic effects after its conversion to RA. Kochhar[7]

Table 3. Histologic Eye Abnormalities

	Retinoic Acid Group	Control Group
Microphthalmos	21 (47.7)	3 (3.3)
Anophthalmos	17 (38.6)	1 (1.1)
Faulty closure of the embryonic fissure	16 (36.4)	3 (3.3)
Developmental abnormalities of the vitreous	15 (34.1)	8 (8.9)
Aphakia	10 (22.7)	1 (1.1)
Goniodysgenesis	8 (18.2)	2 (2.2)
Faulty separation of the lens vesicle	7 (15.9)	2 (2.2)

Results are expressed as numbers of eyes. Numbers in parentheses indicate the percentage of eyes of live fetuses in each group.

Jpn J Ophthalmol
Vol 42: 162–167, 1998

administered RA to pregnant rats and mice, and they observed malformations including spina bifida, microphthalmos, and cleft palate in their fetuses, providing evidence of the teratogenic effect of this drug. Wiley et al[9] demonstrated that treating pregnant hamsters with RA disturbed the migration of cranial neural crest cells but did not affect the cells derived from neuroectoderm and mesoderm. In the present study, we evaluated RA as a teratogen that mainly disturbs the migration of neural crest cells. We found that RA frequently induced various developmental abnormalities of the eye. Hence, RA is thought to be one of the most useful teratogenic agents to study developmental eye abnormalities. In a study by Deshmukh et al,[12] the dose of RA given to rats orally was 90% absorbed after 6 hours. The blood level of RA remained at a maximum between 1 and 4 hours after administration, then declined rapidly.[12] Because RA is well absorbed and rapidly metabolized, it is suitable for use in experimental teratology, as in our study. Although we administered RA to mice at the early stage of eye development in the present study, Hyatt and Dowling[13] recently demonstrated that RA given to zebrafish at the later stage of eye development was involved in differentiation and maturation of rod and cone photoreceptors.

Cook and Sulik[14] administered RA to C57BL/6J mice on day 7 of pregnancy, and 62% of fetuses demonstrated a faulty separation of the lens vesicle on day 14 gestation. This finding suggests that faulty separation of the lens vesicle arose from abnormal migration of neural crest cells. From the results obtained in our study, we propose that a faulty separation of the lens vesicle as well as a faulty closure of the embryonic fissure, developmental abnormalities of the vitreous, aphakia, and goniodysgenesis develop from the abnormal migration of neural crest cells.

In the mesenchymal tissue observed in developmental abnormalities of the vitreous, scattering melanocytes were detected frequently. As Shirai[4] demonstrated in his study using ochratoxin A as a teratogen, this result also indicates that the mesenchymal tissue observed in the vitreous cavity is derived from neural crest cells, because melanocyte is confirmed to be of neural crest origin.[15,16]

Clinically, Lammer et al[17] reported that the administration of RA to pregnant women with severe acne caused a characteristic set of birth defects in the tissues derived from cranial neural crest cells. The defects included central nervous system anomalies, facial anomalies, micrognathia, and heart disease. This condition is known as RA embryopathy. Many of the malformations observed in our study were consistent with the birth defects detected in RA embryopathy. Therefore, the mouse fetuses in the present study can be regarded as an experimental model of this embryopathy.

A relationship between the consumption of large amounts of vitamin A during pregnancy and the occurrence of birth defects was first reported by Cohlan[18] in 1953. The teratogenic effect of vitamin A was subsequently confirmed in experimental animals.[19,20] Recently, some pregnant women in the United States had been taking excessive amounts of supplements containing vitamin A, and the number of infants with birth defects was increased.[21] Oakley and Erickson[22] recommended that pregnant women should not take more than 8000 IU of vitamin A daily. We determined the dose of RA given to pregnant mice according to LD_{50} (median lethal dose) of their fetuses. Because 12.5 mg/kg of RA affected 95.5% of live fetuses with an accompanying mortality of 46.3% (nearly 50%), this dose was chosen in our study. This dose of RA corresponds to 400 days of Oakley's recommendation for maximum human consumption. Our study confirms the teratogenicity of RA as well as vitamin A.

Day 7 of gestation in mice corresponds to 2–3 weeks of gestation in humans.[23] Our study demonstrates that exposure to RA or vitamin A during early pregnancy, when women may not realize they are pregnant, can induce various ocular malformations. Women need to be educated about the risk of taking excessive vitamin A during early pregnancy.

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