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Pathogenesis of retinoic acid-induced spina bifida in rat embryos

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Key words
retinoic acid, spina bifida, neuroepithelium, rat embryo

Abstract

In a previous study, we showed that the intrauterine exposure of rat embryos to retinoic acid (RA) in the organogenesis period (8-11 days of gestation) induced various malformations according to the dose and timing of RA administration[1]. Analysis of the characteristics of RA-induced malformations showed that many of them arose from the lumbosacrocaudal region of the axial skeleton[2]. Based on these results, the present study was focused on the elucidation of the pathogenesis of spina bifida. All-trans RA was administered at a dose of 60 mg/kg to pregnant DA rats at 10 days of gestation, and its influence on the posterior neuropore of embryos was examined serially by morphological methods. As a result, neural plate elevation was impaired at 6-9 h after RA administration, and neuroepithelial eversion occurred 12 h after administration. Thereafter, neuroepithelial eversion due to neuroepithelial overgrowth occurred, presumably leading to the development of spina bifida.

1. Introduction

Retinoic acid (RA) is present in the body in minute amounts, and essential for the differentiation of generalized epithelial tissues and the retina, as well as for reproductive function. For many years, RA has been shown to regulate cell differentiation and growth, and has come to be applied clinically in the treatment of various skin diseases and tumors[3-6]. Meanwhile, the excessive administration of RA has been reported to cause a variety of congenital malformations in mice[7-16], rats[7,15,16], and hamsters[14,17-20]. In our previous studies, when a single oral dose of RA was administered to rats on gestation day 8, 9, 10, or 11, the highest incidence of malformations occurred in the embryos of rats administered RA on gestation day 10[1], and many of them were characterized by their origin in the lumbosacrocaudal region of the axial skeleton, typified by spina bifida. In this study, we focused our attention on spina bifida, and conducted experiments to elucidate its etiology and pathogenesis.

2. Materials and Methods

2-1. Animals used

Inbred DA rats with uniform genes were used to preclude inter-strain variation. Six-week-old DA rats weighing 160-180 g were purchased from Japan SLC. Male rats were caged individually, and female rats were housed in groups of 4 to 5, with free access to rat chow (OA-2, CLEA Japan, Inc., Tokyo) and drinking water. Eight-week-old female rats in heat were selected and cohabitated with single male rats (1:1) for 16 h (16:00-8:00 h), and vaginal smears were

examined microscopically the next morning. The detection of sperm was regarded as an indication of pregnancy, and this date was defined as day 0. The rats were kept in an animal room under the following conditions: lighting hours, 7:00-19:00; temperature, 23.4℃; and humidity, 50±5%.

2-2. Drug administration

All-trans RA (Sigma-Aldrich Corp.) was suspended in olive oil. RA at a dose of 60 mg/kg of RA was orally administered to dams using a stomach tube at 9:00 am on gestation day 10. Olive oil (10 ml/kg) was similarly administered to a control group of dams.

2-3. Specimen preparation

Dams that had been administered RA or olive oil were sacrificed by cervical dislocation under deep ether anesthesia after 3, 6, 9, 12, 18, or 24 days. Untreated dams were similarly sacrificed at 9:00 a.m. on gestation day 10. After laparotomy, the uterus was removed, and the embryo together with its embryonic membranes was excised. The Reichert, yolk, and amniotic membranes were removed in normal saline to recover the embryo, which was then fixed for 2-3 h in 3% glutaraldehyde (in 0.1% phosphate buffer) that had been kept on ice at 4℃. The number of somites of the embryo in the fixative solution was counted under a stereomicroscope, and the fixed embryo was washed in 0.1 M phosphate buffer, and post-fixed in 1% osmium tetroxide in 0.1% phosphate buffer at 4℃ for 1.5 h. Subsequently, embryos were divided into two groups. In one group, the tail was dehydrated in alcohol, embedded in Epon, and used as a specimen for light and transmission electron microscopy. In the other group, the embryo was dehydrated in alcohol, then in isoamyl acetate, subjected to critical-point drying, and used as a specimen for scanning electron microscopy. The Epon-embedded specimen for light microscopy was cut into 1-μm-thick serial sections from the distal end at right angles to the body axis, and the sections were stained with 0.5% toluidine blue and examined microscopically. The specimen for transmission electron microscopy was cut into 0.05-μm-thick sections, which were double-stained with uranyl acetate and lead citrate, and examined under a Hitachi HU-12A electron microscope. Specimens were examined, with a focus on areas showing the most marked changes, and compared with similar areas in control specimens.

3. Results

The number of somites in the embryo increased steadily with time, and did not significantly differ between the RA-administered and control groups, although embryonic development tended to be somewhat slower in the RA-administered group.

On gestation day 10 at 9 a.m. when RA was administered, the embryo had 5-6 somites, and the anterior and posterior neuropores were still open on scanning electron microscopy (Fig. 1). Light microscopic examination of the posterior neuropore showed that the shape of its cross-section gradually changed from a U- to V- and finally to O-shaped as the sections moved caudally to cranially.

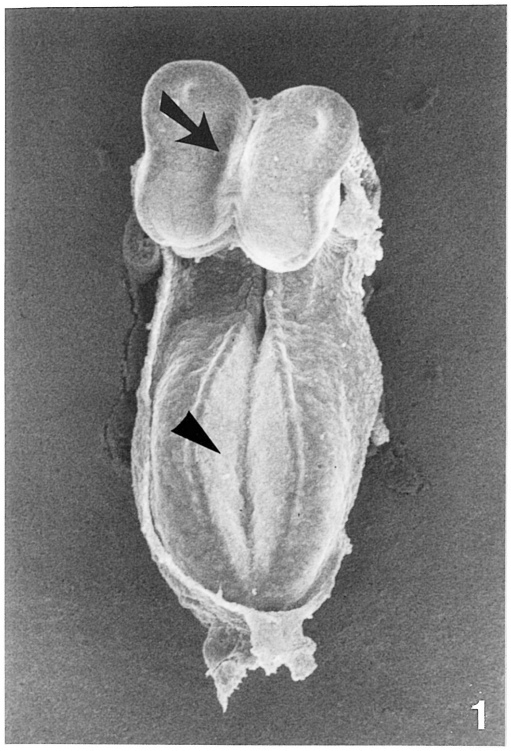

Fig.1 Scanning electronmicrograph of an embryo at the time of administration of RA or vehicle on day 10 of gestation Anterior neuropore (arrow) and posterior (arrowhead) were still open. ×94

3-1. Posterior neuropore 3 h after administration

3-1-1. Scanning electron-microscopic findings

In the control and RA administration groups, the state of embryonic development had changed little from that at the time of administration (on gestation day 10 at 9 a.m.), except that the surface ectoderm was beginning to slightly hang over the edge of the neuropore inward (to the neuroepithelial side). On cross-section, the neural groove consisted of portions with a U- or V-shape, and the caudal one-third and cranial two-thirds of the neuropore had U- and V-shapes, respectively (Figs. 2a and 3a). The neuroepithelial surface had numerous undulations with fine processes and microvilli.

3-1-2. Light-microscopic findings

In the RA-administered and control groups, the neuroepithelial cells in the neuropore portion showing a U-shaped cross-section were relatively sparse and had a wide intercellular space. In the mid-portion of the neuroepithelium, the boundary between the neuroepithelial and underlying mesenchymal cells was indistinct, and the former were often in contact with the latter cells, with dividing cells migrating to the latter cells. There was no difference in the distribution density of mesenchymal cells between the control and RA administration groups (Figs. 4Aa and 4Ab).

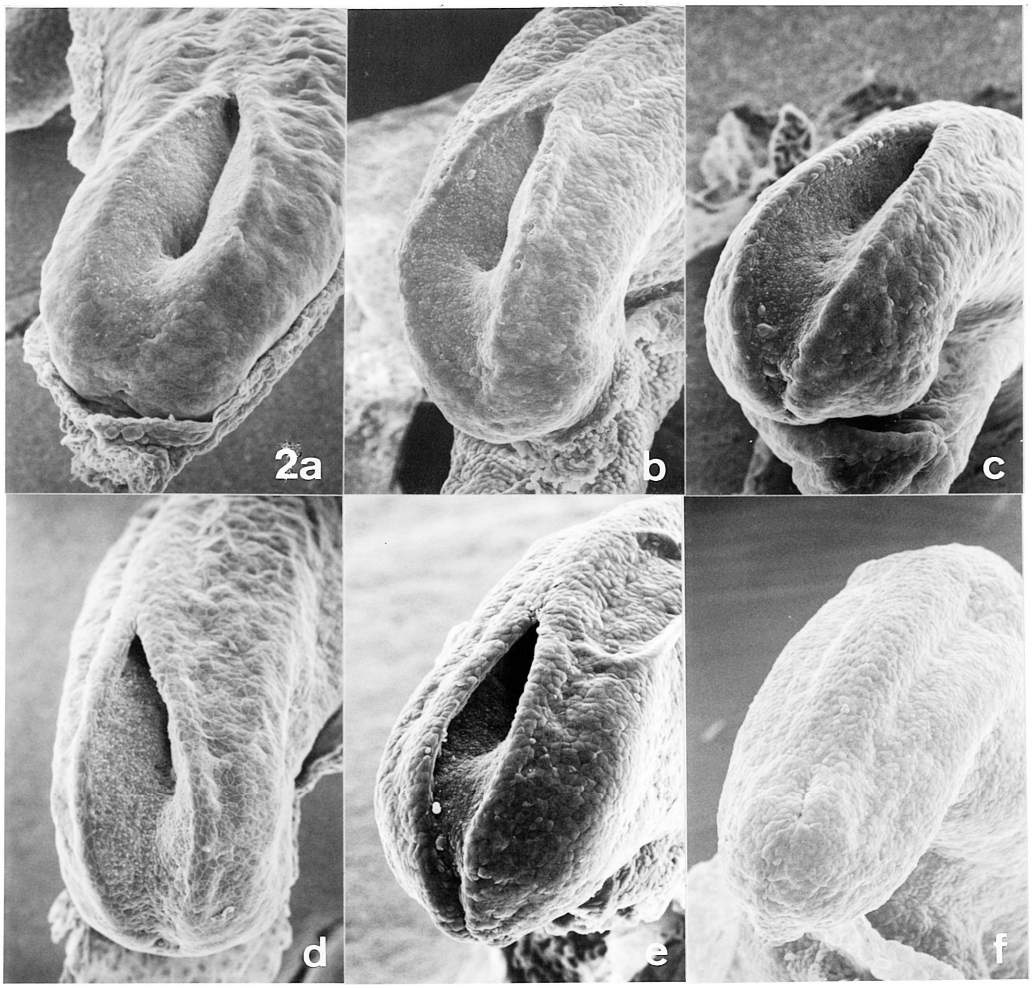

Fig.2 Scanning electronmicrographs of the posterior neuropore of embryos treated with vehicle (control)×120
a 3 hours after the administration
b 6 hours after the administration Neuropore is in the process of fusion
c 9 hours after the administration
d 12 hours after the administration
e 18 hours after the administration
f 24 hours after the administration Neuropore was closed

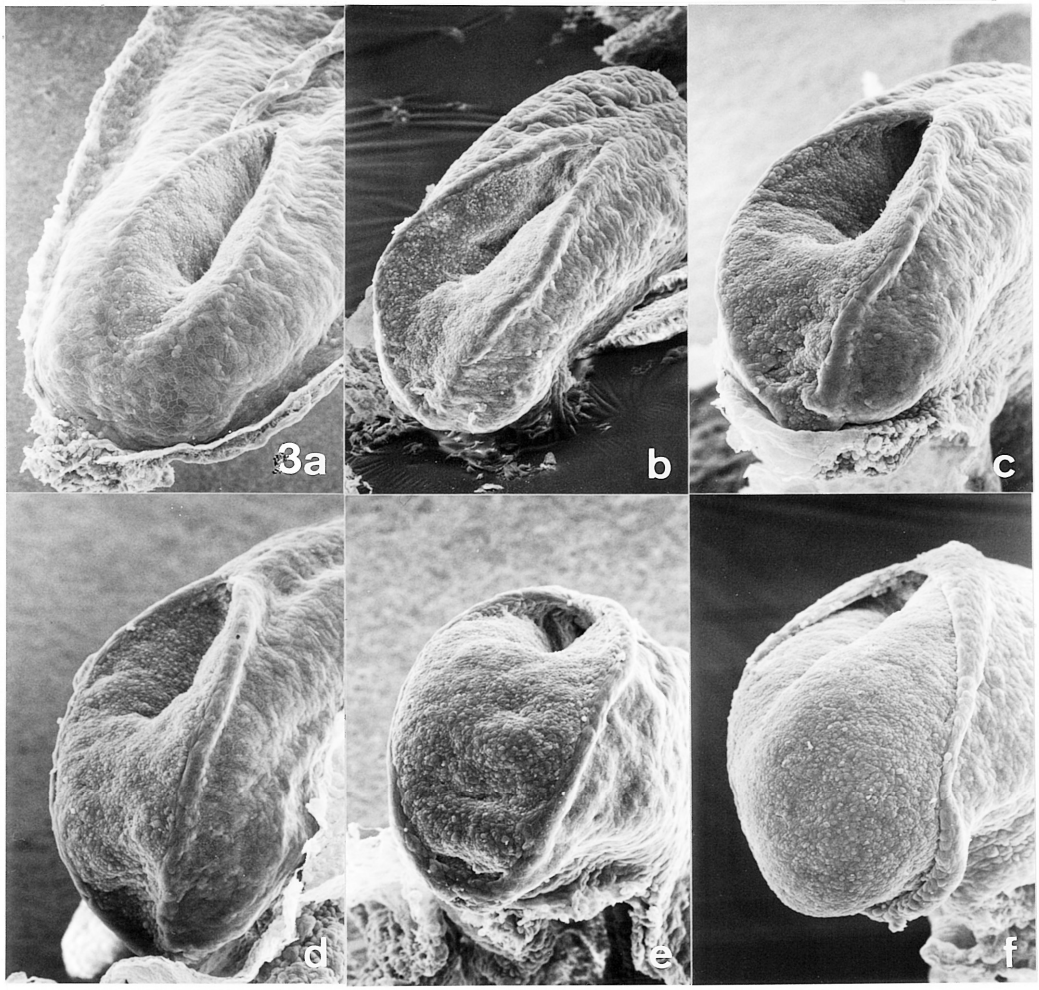

Fig.3 Scanning electronmicrographs of the posterior neuropore of embryos treated with RA×120
a 3 hours after the administration
b 6 hours after the administration Neuropore was widely open.
c 9 hours after the administration
d 12 hours after the administration
e 18 hours after the administration
f 24 hours after the administration
Note that the neuroepithelium of the neuropore was completely everted.

3-1-3. Transmission electron-microscopic findings

In the control and RA administration groups, intermediate junctions were seen at the apical surface of the neuroepithelium, along with many microfilaments in their vicinity. These findings were not significantly different between the two groups. However, in the control group, vascular endothelial continuity in the mesenchymal cell compartment was maintained (Fig. 5a). In contrast, in the RA 60 mg/kg administration group, vascular endothelial continuity was disrupted, resulting in a communication between the inside and outside of the vessel (Fig. 5b), suggesting an influence on angiogenesis.

3-2. Posterior neuropore 6 h after administration

3-2-1. Scanning electron-microscopic findings

In the control embryos, the mid-portion of the posterior neuropore was slightly narrowed by its edges, indicating the progression of neuropore closure. The portion with a U-shaped cross-section had extended, and that with a V-shaped cross-section had shortened in the body-axis direction, compared with that in the control group 3 h after administration (Fig. 2b). In addition, processes and microvilli were present on the surface of the neuroepithelium (Fig. 6a). In contrast, in the RA-exposed embryos, the posterior neuropore was open wider than that 3 h

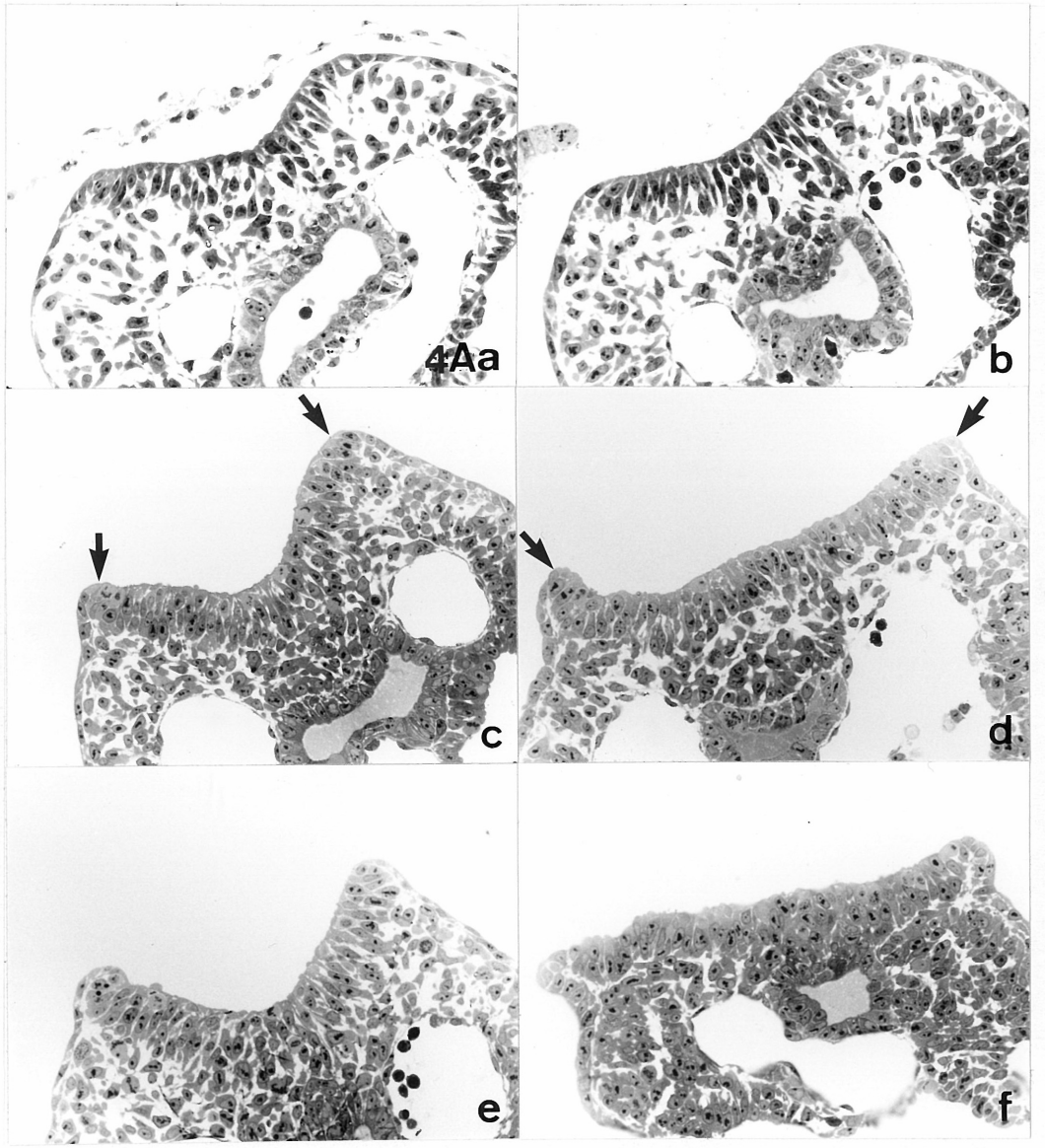

Fig.4A Transverse sections through the posterior neuropore of embryos 3-9 hours after the administration of vehicle or RA
a 3 hours after the administration of olive oil (control)×150
b 3 hours after the administration of RA×150
c 6 hours after the administration of olive oil (control)
At the tip of neural folds, surface ectoderms were turned inside of neuropore (arrows).×150
d 6 hours after the administration of RA Neural plate was not elevated, and surface ectoderms were remained on lateral side of neuroepithelium (arrows).×150
e 9 hours after the administration of olive oil (control)×150
f 9 hours after the administration of RA Neural plate was flat and the neuroepithelial cells were rounder than those of controls.×150

after administration, particularly from the caudal end to the groove portion with a U-shaped cross-section, and the U-shaped depression was shallow (Fig. 3b).

3-2-2. Light-microscopic findings

In the control embryos, the neural groove became deep, and epithelial cells increased in number (Fig. 4Ac). In the RA administration group, the U-shaped cross-section of the neuroepithelium was shallower and flatter in the center than in the control group (Fig. 4Ad). Further, anterior to this site, this shallow and flat-bottomed U shape of the cross-section gradually became V-shaped, rising from both ends of the neural folds, and finally fused as in the control group. In addition, the neuroepithelium itself was thinner than in the control group. On the flat site of the

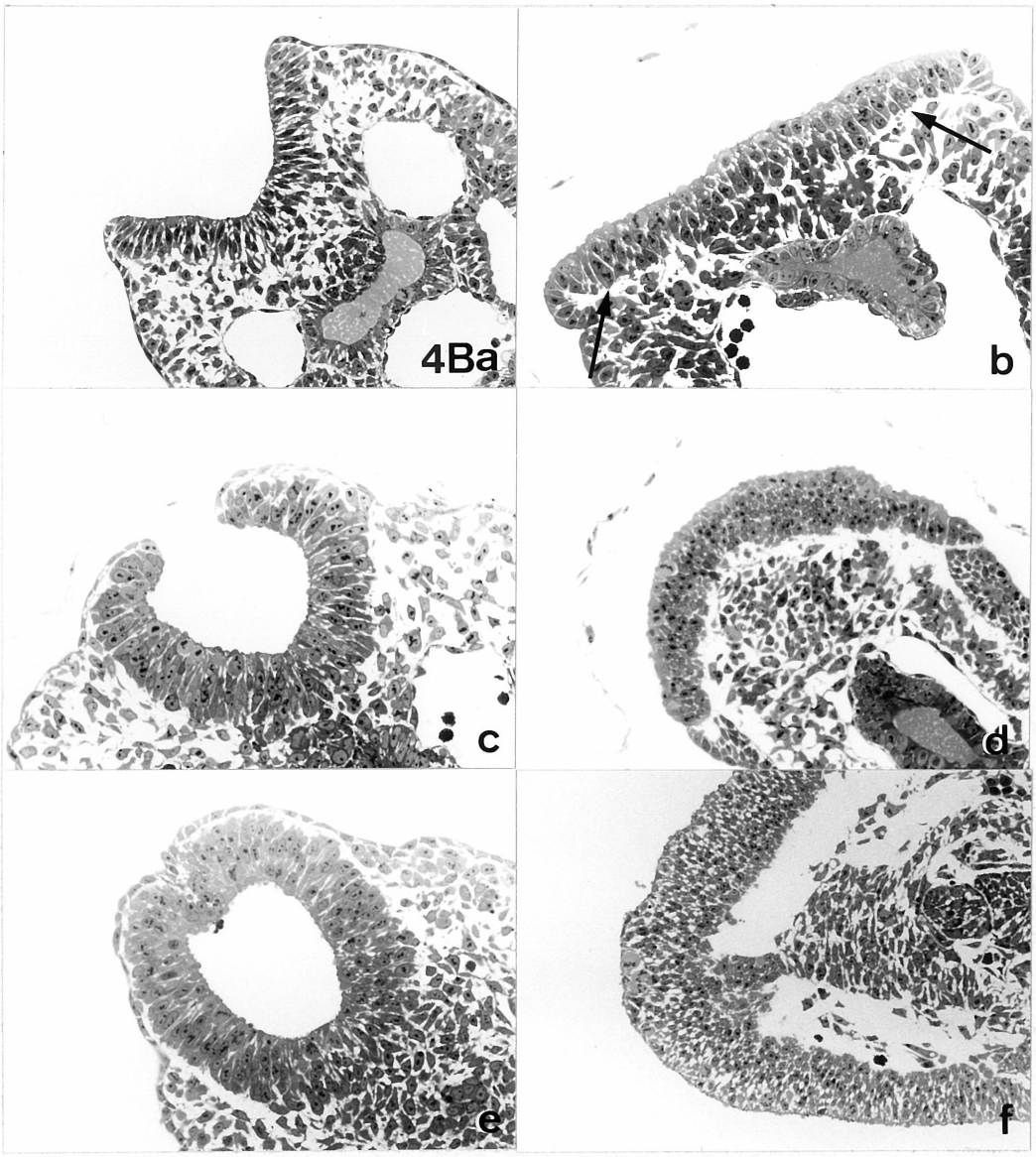

Fig.4B Transverse sections through the posterior neuropore of embryos 12－24 hours after the administration of vehicle or RA

a 12 hours after the administration of olive oil (control)×100
b 12 hours after the administration of RA×150
Neuroepithelium at the peripheral areas had no contact with mesenchymal cells and clear cell free spaces (arrows) were seen.×150
c 18 hours after the administration of olive oil (control)
Neural folds were opposite each other.×150
d 18 hours after the administration of RA
Neuroepithelium was completely everted, and neuroepithelial cells were abnormally proliferated.×150
e 24 hours after the administration of olive oil
Opposite neural folds had fused together.×150
f 24 hours after the administration of RA
Intercellular spaces of neuroepithelial cells were enlarged.×150

neuroepithelium, the number of mesenchymal cells was smaller than that in the control group, and the embryo was flat overall on cross-section. At the tip of the neural folds, the surface ectoderm was directed inward in the control group (Fig. 4Ac), but, in the RA administration group, it terminated there (Fig. 4Ad).

3-2-3. Transmission electron-microscopic findings

As shown in Fig. 7a, in the control embryos, the apical portions of neuroepithelial cells were compressed, slim, and elongated, and adjacent cells were joined by junctional complexes. At a higher magnification, these cells showed endocytosis in

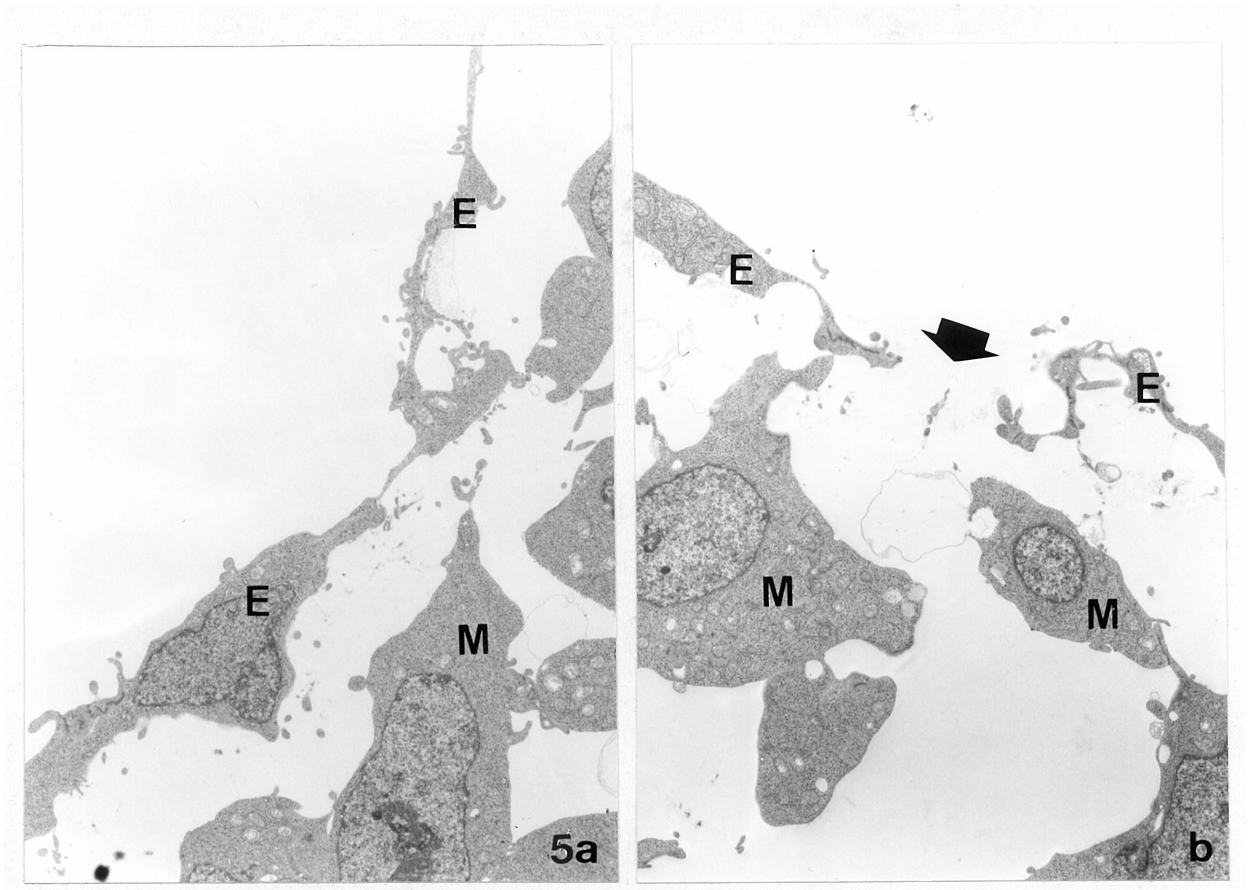

Fig.5 Transmission electronmicrographs of the mesenchymal region of posterior neuropore 3 hours after the administration of olive oil and RA.
a Endothelial connection of control embryo×2700
b Endothelial connection of RA treated embryo×2700
The endothelial connection was disrupted (arrow).
E : endothelial cell
M : mesenchymal cell

places and many microtubules (Fig. 7b), had centrally or basally located nuclei, and were tapered toward the apical surface. In contrast, in the RA administration group, the nuclei were located closer to the apical surface of neuroepithelial cells (Fig. 7c), which showed no apical constriction and fewer microtubules (Fig. 7d).

In the control embryos, on the basal side of the neuroepithelium, many cells were observed to migrate from the neuroepithelium toward mesenchymal tissue. These cells had a polygonal nucleus containing one or two large nucleoli, and clusters of chromatin granules in the karyotheca and karyoplasm, suggesting that they were neural crest cells.

3-3. Posterior neuropore 9 h after administration

3-3-1. Scanning electron-microscopic findings

In the control embryos, the surface ectoderm at the caudal end of the posterior neuropore was directed further inward, and the neural groove was slightly narrower than that in the control group 6 h after administration (Fig. 2c). The surface of the neuroepithelium portion with a U-shaped cross-section showed irregularities caused by epithelial cells, as well as cell processes and microvilli. In the RA administration group, the neuropore was open wider than that observed 6 h after administration. In particular, the portion caudal to the site, where the V-shaped cross-section of the neuroepithelium endes, was elevated dorsally, and the pore was flattened, which apparently caused its widening (Fig. 3c). In addition, many epithelial cell processes were seen to project from the surface of this portion.

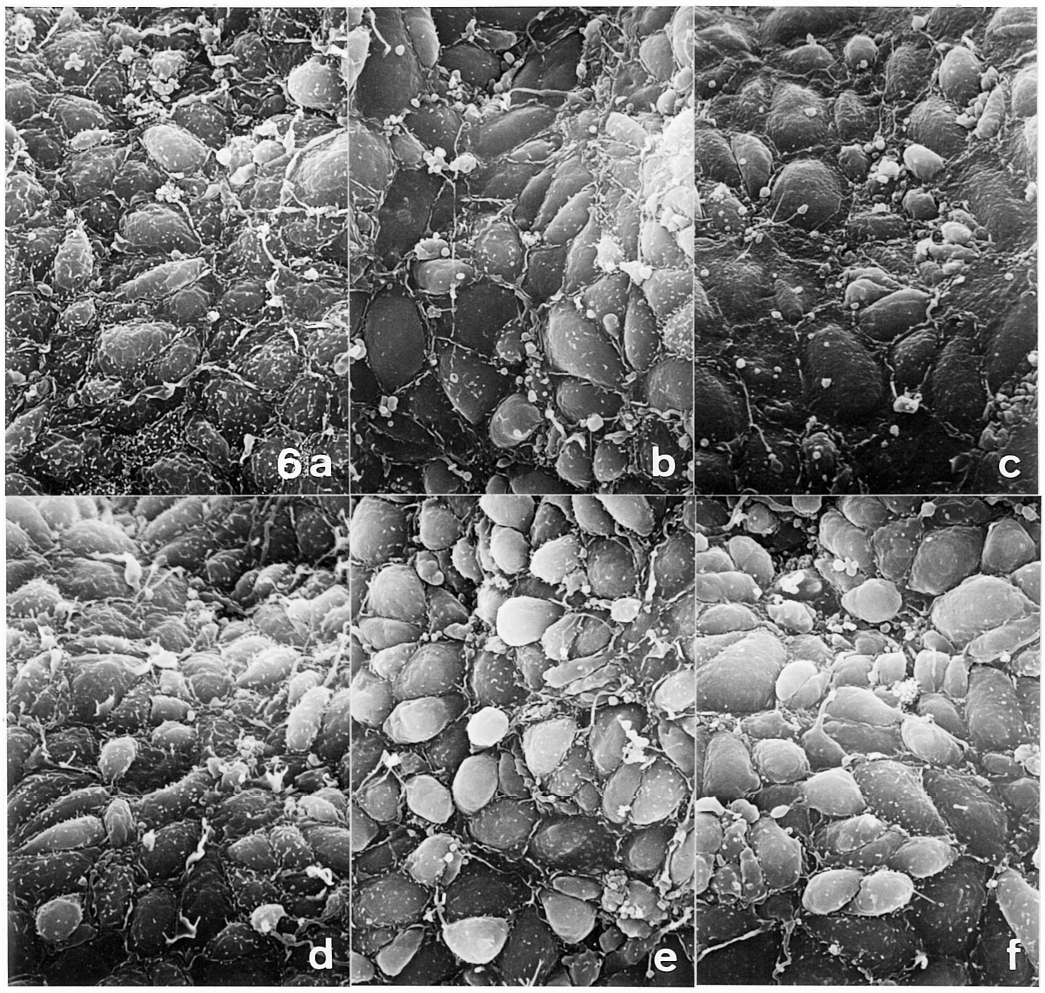

Fig.6 Scanning electronmicrographs of the bottom of posterior neuropore (apical surface of the neuro - epithelium)
- a 6 hours after the administration of olive oil (control)
  A number of microvilli and threadlike processes were senn.×2000
- b 12 hours after the administration of olive oil (control)
  The number of microvilli was reduced.×2000
- c 18 hours after the administration of olive oil (control)
  Microvilli were almost invisible.×2000
- d 6 hours after the administration of RA
  No marked difference from controls.×2000
- e 12 hours after the administration of RA
  Many epithelial cells protruded from the apical surface, ×2000 but the number of microvilli was not reduced.
- f 18 hours after the administration of RA
  Note the cellular protrusion of the apical surface, and microvilli were still present.×2000

3-3-2. Light-microscopic findings

In the control embryos within this time period, what appeared to be neural crest cells were observed to migrate from the neuroepithelium toward mesenchymal cells (Fig. 4Ae). This was seen at the periphery as well as the center of the neuroepithelium. Individual neuroepithelial cells were elongated at right angles to the apical surface, and had relatively wide intercellular spaces. On the other hand, in the RA administration group, the neuroepithelial cells were flatter and thinner with narrow intercellular spaces than those in the control group, as evident from the scanning electron microscopic findings (Fig. 4Af). The individual cells were not as elongated as those in the control group, but were round and not in contact with mesenchymal cells except in the center, and no cells were observed to migrate from the neuroepithelium toward mesenchymal cells (Fig. 4Af). In addition, the mesenchymal cells had narrower intercellular spaces and higher cell densities than

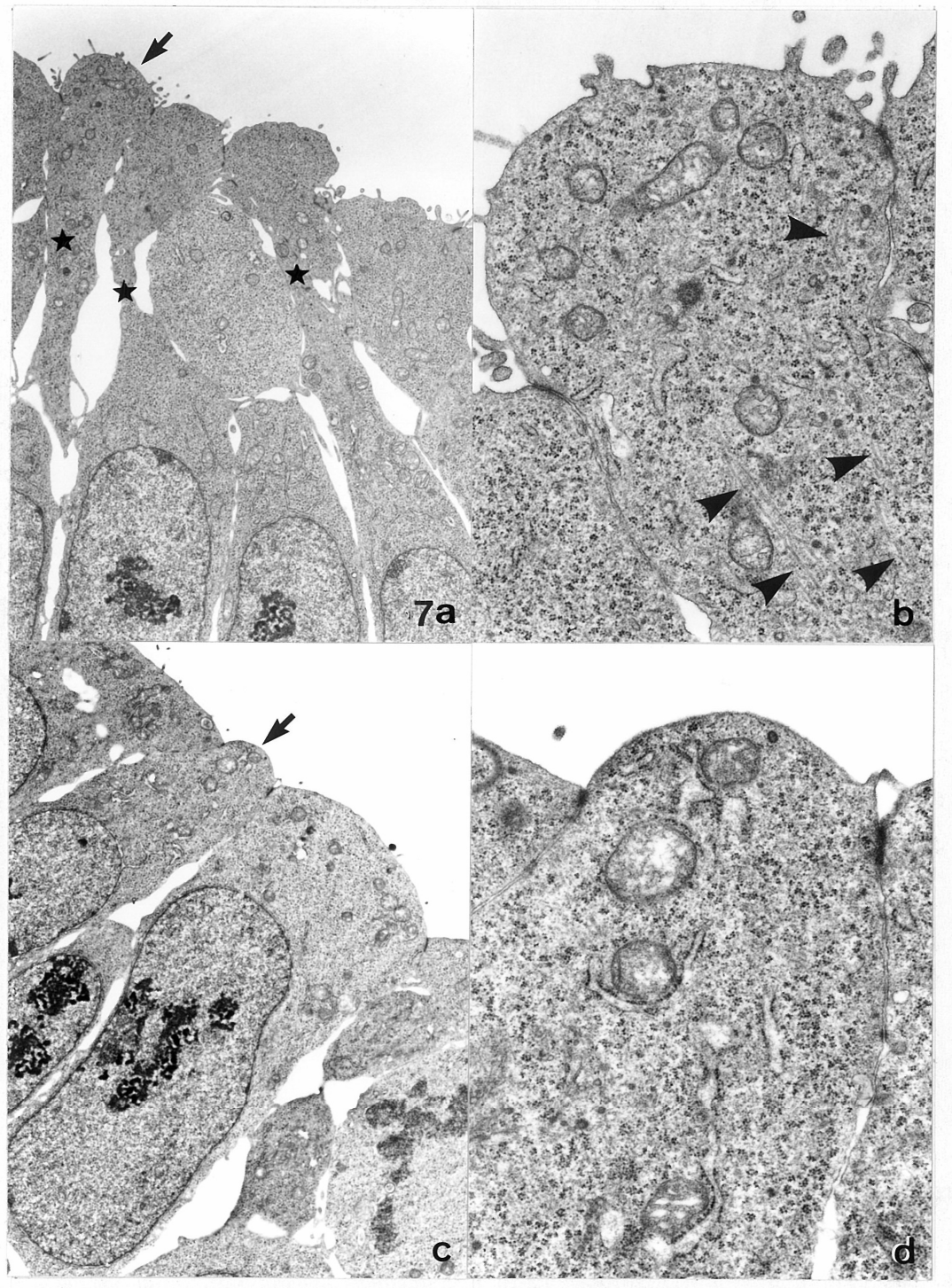

Fig.7 Transmission electronmicrographs of the neuroepithelium (apical side)
a 6 hours after the administration of olive oil (control)
Many apical contractions were seen (asterisks.)×3200
b Magnification of the apical side indicated by the arrow in Fig.7a Note the bundle of microtubules (arrowheads).
×16000
c 6 hours after the administration of RA
Apical contractions were not senn.×3200
d Magnification of the apical side indicated by the arrow in Fig.7c The bundle of microtubules had disappeared.
×16000

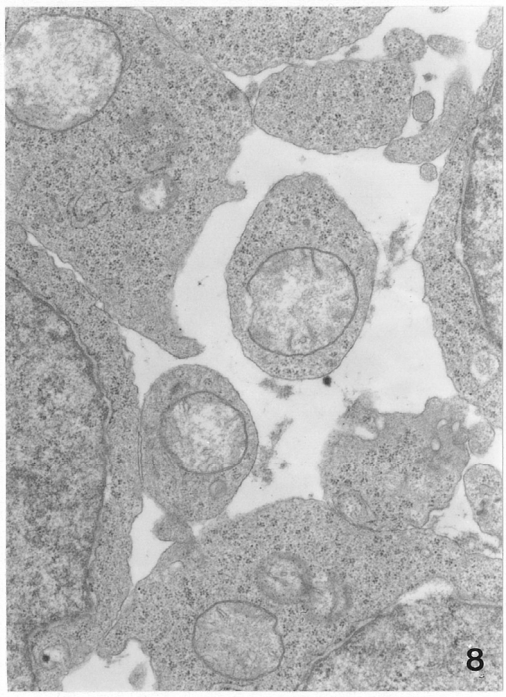

Fig.8 Transmission electronmicrograph of the neurospithelium of RA treated embryo (9 hours after aministration)
Note large mitochondria in the neuroepithelial cells.
×13680

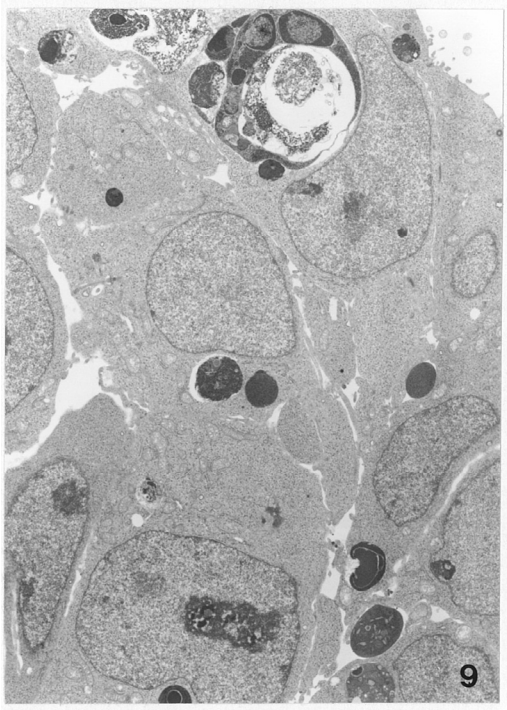

Fig.9 Transmission electronmicrograph of the mesenchymal cells of RA treated embryo (12 hours after administration)
Many necrotic cells are seen in the mesenchyme.
×2700

those in the control group.

3-3-3. Transmission electron-microscopic findings

The changes observed in the control embryo were essentially the same as those 6 h after administration. However, cells containing many apparent glycogen granules appeared in the neuroepithelium. In the RA administration group, such cells were not observed, and fewer microtubules were observed at the apical surface of the neuroepithelium than in the control group. Swollen mitochondria were found in some neuroepithelial cells (Fig. 8).

3-4. Posterior neuropore 12 h after administration

3-4-1. Scanning electron-microscopic findings

In the control embryos, the posterior neuropore width became narrower, and the groove portion with a U-shaped cross-section was elongated in the caudal direction (Fig. 2d). At a higher magnification, many cell processes and microvilli were observed on the neuroepithelial surface, but their numbers were smaller on the floor than on the sides (Fig. 6b). On the other hand, in the RA administration group, the portion that should have a U-shaped cross-section was everted and swollen caudally (Fig. 3d), and microvilli, which were decreased in the control group, were not decreased at the center, and were evenly distributed (Fig. 6e).

3-4-2. Light-microscopic findings

In the control embryos, the number of neuroepithelial cells at the posterior neuropore was increased, individual cells and nuclei were elongated at right angles to the apical surface, and many of them were in contact with mesenchymal cells on the basal side of the neuroepithelium (Fig. 4Ba). In the RA administration group, the neural groove was not U-shaped on cross-section, but was slightly elevated in the center, and the epithelial cells and their nuclei were rounder than those in the control group (Fig. 4Bb).

Mesenchymal cells were in contact with the neuroepithelium at the center, but not at the periphery.

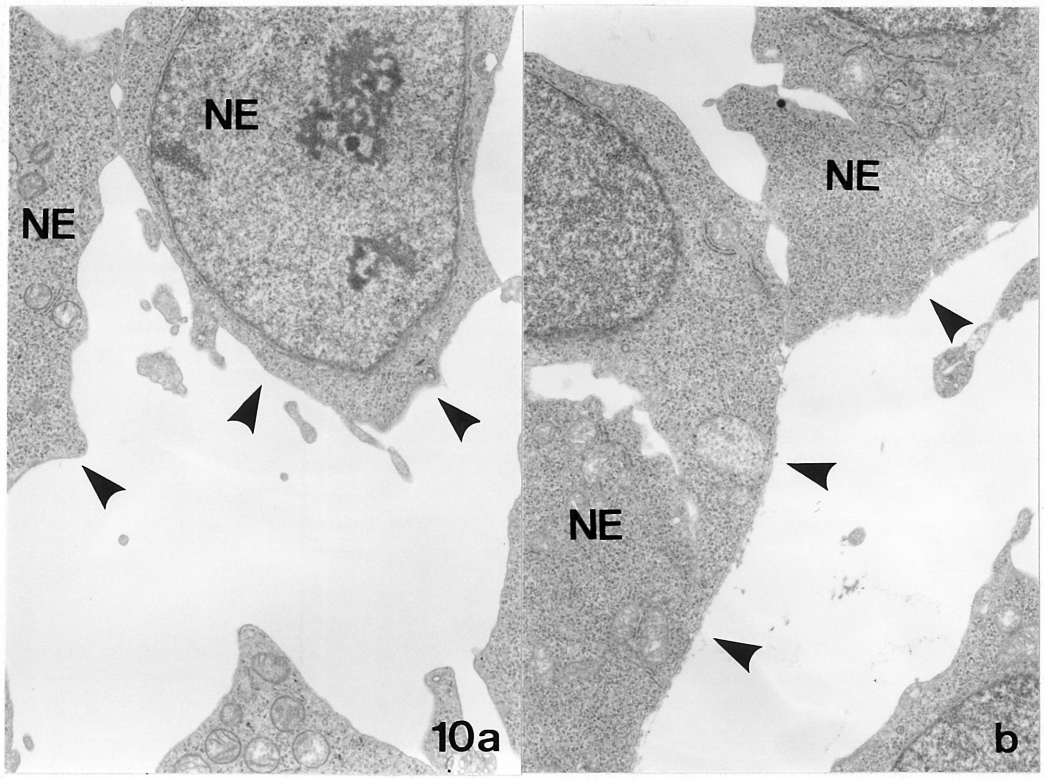

Fig.10 Transmission electronmicrographs of the basal side of the neuroepithelium (12 hours after administration)
a The neuroepithelial cells of a control embryo The basal lamina was not seen at the basal side of neuroepitheium (arrowheads).×7500
b The neuroepithelial cells of a RA treated embryo The basal lamina was present at the basal side of neuroepitheium (arrowheads).×7500
NE：neuroepithelial cell

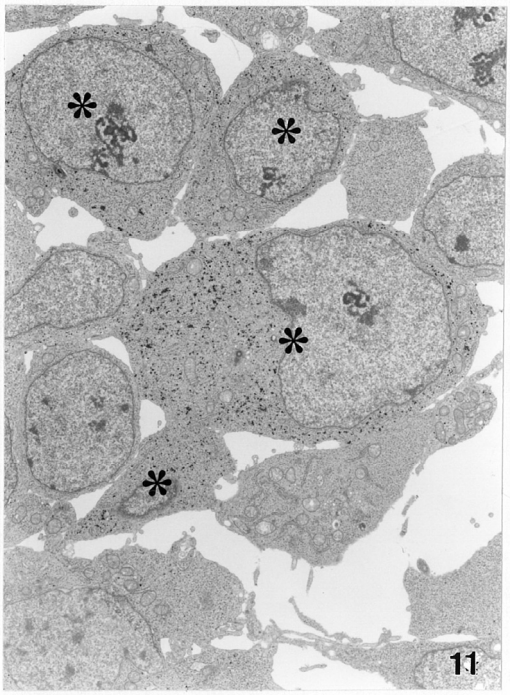

Fig.11 Transmission electronmicrograph of the mesenchymal cells of control embryo (18 hours after administration)
Note the abundance of glycogen granules in the neural crest cells (asterisks).×2700

3-4-3. Transmission electron-microscopic findings

In the control embryos, the changes in the neuroepithelium were essentially the same as those 9 h after administration. In the RA administration group, the neuroepithelial cells were rounder and had fewer microtubules than those in the control group. Many necrotic mesenchymal cells were observed (Fig. 9). No basal lamina was present throughout the basal side of the neuroepithelium in the control group, whereas, in the RA administration group, it was observed at the periphery where the neuroepithelium was not in contact with mesenchymal cells (Fig. 10a, b).

3-5. Posterior neuropore 18 h after administration

3-5-1. Scanning electron-microscopic findings

In the control embryos, the posterior neuropore was narrower in width and shorter in the body-axis direction (Fig. 2e). The neuroepithelial surface was

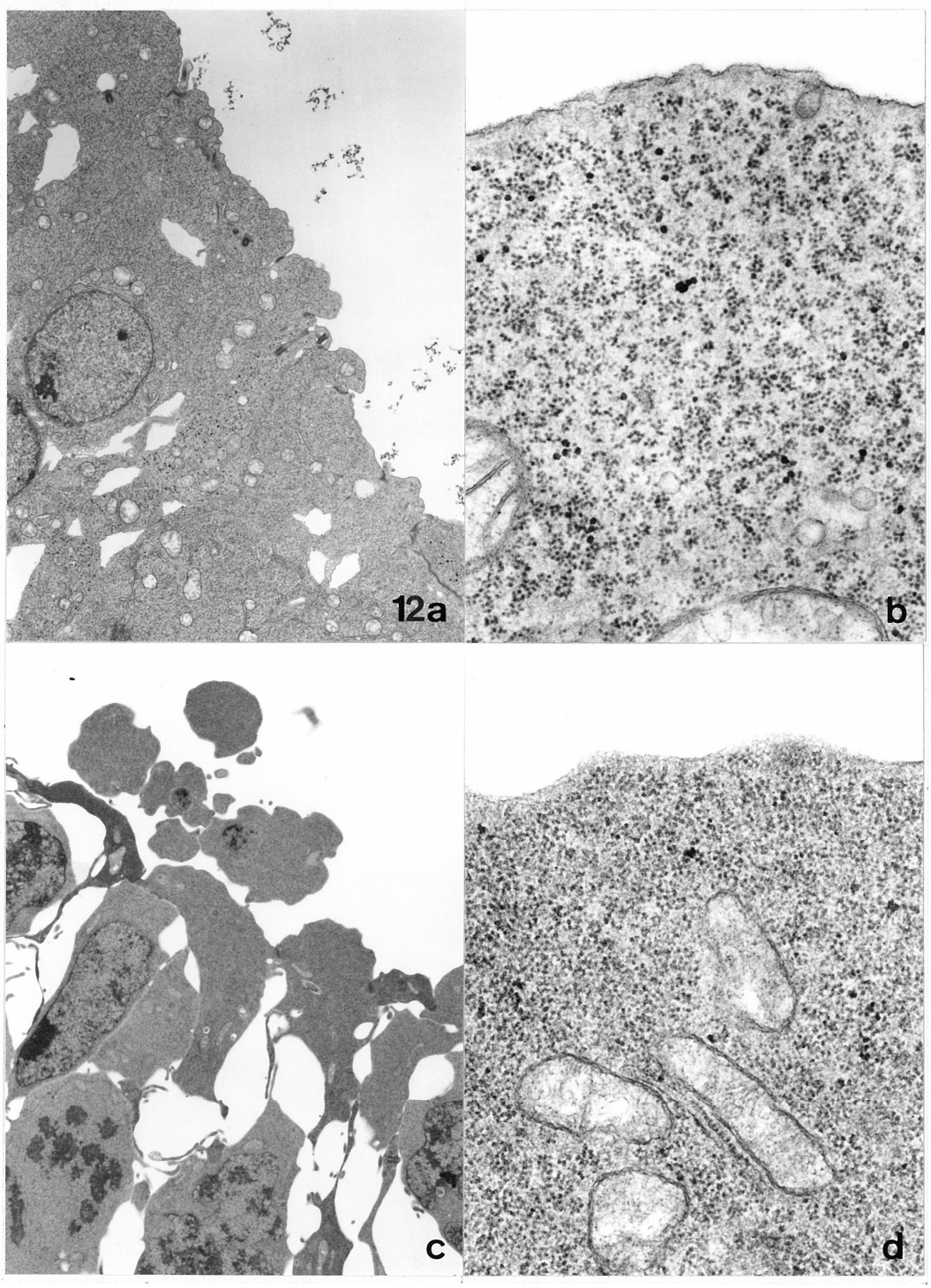

Fig.12 Transmission electronmicrograph of the apical side of neuroepithelium
a 24 hours after the administration of olive oil (control)
The area of cell to cell contact was large, and the apical surface was relatively smooth.×3200
b High-power electronmicrograph of the sample shown in Fig.12a Ribosomes were clustered as polysomes.×24400
c 24 hours after the administration of RA The area of cell to cell contact was smaller than that of controls, while the intercellular spaces were larger. The apical surface was rough due to cytoplasmic protrusion.×3200
d High-power electronmicrograph of the sample shown in Fig.12c Ribosomes were dispersaed as monosomes.
×24400

relatively smooth, and showed some vesicular and filamentous processes and few microvilli (Fig. 6e). In contrast, in the RA administration group, the portion that should normally have a U-shaped cross-section

was completely everted, and the opening was widened (Fig. 3e). The everted portion occupied the caudal three-quarters of the neuropore. Unlike in the control group, epithelial cells with many microvilli remained protruding from the neuroepithelial surface, and the number of vesicular or filamentous processes was increased compared with that in the control group at 18 h (Fig. 6f).

3-5-2. Light-microscopic findings

At the posterior neuropore of the control embryos, all parts of the two edges of the neuroepithelium were bent inward to face each other (Fig. 4Bc). The gap between the neuroepithelial edges became narrow, approaching the shape of a neural tube. Only the central portion of the neuroepithelium was in contact with mesenchymal cells. In contrast, in the RA administration group, the neuroepithelium was completely everted, and the epithelial cells were small and round, and were increased in number (Fig. 4Bd). Necrosis was observed in some mesenchymal cells. Unlike in the control group, neuroepithelial cells were apparently not in contact with mesenchymal cells.

3-5-3. Transmission electron-microscopic findings

In the control embryos, cells apparently containing glycogen granules appeared in some areas of neuroepithelial and mesenchymal tissues from 9 h after administration, subsequently increased in number with time, and reached the highest number at 18 h after administration (Fig. 11). However, no such cells were observed in any of the RA-exposed embryos. Neuroepithelial cells in the RA administration group had wider intercellular spaces, resulting in smaller areas of contact between adjacent cells. In the control embryos, the basal lamina was first observed on the basal side at the periphery of the neuroepithelium.

3-6. Posterior neuropore 24 after administration

3-6-1. Scanning electron-microscopic findings

As shown in Fig. 2f, the posterior neuropore in the control embryos was closed completely, leaving a raphe at the site of neural fold fusion. On the other hand, in the RA administration group, the neuroepithelium bulged further outward, pushing the surface ectoderm to the ventral side (Fig. 3f).

3-6-2. Light-microscopic findings

In the control embryos, the neural folds were completely closed to form the neural tube, which was in contact with mesenchymal cells ventrally (Fig. 4Be), and encircled by the surface ectoderm, with which about half of the neuroepithelium was in direct contact. In the RA administration group, many small cells formed the neuroepithelium, and their intercellular spaces were thick and large. The neuro-epithelium was in contact with mesenchymal cells at the center, but mesenchymal cell-free spaces were observed under most areas of the neuroepithelium (Fig. 4Bf). Blood cells were present in clusters extravascularly, forming hematomas in some areas.

3-6-3. Transmission electron-microscopic findings

In the control embryos, the neural tube was complete, and the apical surface of part of the neuroepithelial cells was seen to be constricted tangentially to the neural tube, and many intermediate junctions were formed between neighboring cells, resulting in smaller intercellular spaces (Fig. 12a). However, in the RA administration group, the area of contact between neuroepithelial cells was relatively large until 12 h after administration, but became small at 18 h after administration. At 24 h, epithelial cells had small, complex cell processes, which were in contact with neighboring cells, resulting in a very small area of contact via such processes (Fig. 12c). The intercellular spaces of neuroepithelial cells were wider than those in the control group. Furthermore, cytoplasmic ribosomes were in the form of polysomes in the control group, whereas, in the RA administration group, they were free ribosomes (monosomes) without ribosomal rosette formation (Fig. 12b, d).

4. Discussion

All-trans RA was administered at a dose of 60

mg/kg to pregnant DA rats at 10 days of gestation, and its influence on the posterior neuropore of embryos was examined serially by morphological methods. Externally, elevation and apposition of the neural folds occurred with time, and the posterior neuropore of the control embryos had closed almost completely at 24 h after administration, i.e., on gestation day 11. In contrast, in the RA-treated embryos, neural fold elevation was impaired from 6 h after administration onward. Histologically, a marked increase in the number of neuroepithelial cells occurred from 18 h after RA administration, resulting in neuroepithelial eversion, leaving bulging neural tissue behind on the neuroepithelium. We speculate that this state was maintained while the embryo continued to grow until the end of pregnancy, leading to the development of open spina bifida.

At 3 h after administration, no external difference was noted between the two groups, but, at the transmission electron-microscopic level, vascular endothelial disruption was observed in the RA-treated embryos (Fig. 5b). However, there was no difference in epithelial cells or other tissues between the two groups, suggesting that the vascular system at the posterior neuropore is first affected by RA. At 24 h after RA administration, hematomas were present in the tail of many embryos at the same site as that of the origin of spina bifida, suggesting that vascular disruption is related to the development of spina bifida. However, from 6 h after administration onward, no vascular injury was noted, and no hematomas were observed at 6-12 h when neuroepithelial elevation was inhibited, making the direct effect of RA unlikely. Further, transplantation experiments using chickens concluded that vascular lesions and hematoma formation do not lead to malformations of the secondary neural tube21). Therefore, hematoma formation is unlikely to have directly caused spina bifida, which presumably developed secondarily.

At 6 h after administration, neural fold elevation was markedly inhibited in the RA-treated embryos (Fig. 3b). In the control embryos, the apical constriction of neuroepithelial cells was frequently observed, and the development of many microtubules in a direction orthogonal to the apical surface was seen (Fig. 7b). In the RA administration group, no apical constriction was observed, and neuroepithelial cells had fewer microtubules than those in the control group (Fig. 7d). These findings suggest that RA affected microtubules, that is, damaged the cytoskeleton system, and thereby inhibited the shape change of neuroepithelial cells required for neural fold elevation, with the result that neuroepithelial cells had a smooth apical surface. However, Peters and Dormans reported that, in trypan blue-induced rat spina bifida, the intercellular space of mesodermal tissue widened in early embryos22). Similarly, using rats, Taniuchi et al. concluded that a trypan blue-induced large cyst in the mesoderm secondarily inhibited the normal growth of the neuroepithelium, resulting in the development of spina bifida23). In the present study, no large cysts appeared, and the intercellular space of mesodermal cells did not widen, suggesting that trypan blue and RA induced spina bifida by different mechanisms of action.

Until 12 h after administration, at the posterior neuropore of the control embryos, epithelial cells were observed to migrate from the entire basal side of the neuroepithelium into the mesenchymal tissue (Fig. 4Aa, c, e, 4Ba). It was not until 18 h after administration that basal lamina formation was observed at the periphery of the neuroepithelium. However, in the RA administration group, at 6-12 h after administration, the neuroepithelial cells at the basal side were flatter than those in the control group, the basal lamina was formed at the periphery of the neuroepithelium, and no cell migration was observed (Fig. 4Ad, f, 4Bb). Although the role of the basal lamina has not been fully elucidated, it is known to act as a lining to reinforce the neuroepithelium. In this time period when neural fold elevation occurs, the basal side of the neuroepithelium needs to expand. The formation of the basal lamina with a reinforcing role suggests that it prevented the neuroepithelium from bending.

In the RA-exposed embryos 18 h after

administration onward, neuroepithelial cells clearly overproliferated, as observed by light microscopy. RA is known to have the ability to elevate ornithine decarboxylase activity and DNA synthesis in the neuroepithelium24) and thereby induce its hyperplasa25). Scanning electron microscopy of the apical surface of neuroepithelial cells showed that they had already protruded from the surface 9 h after RA administration. We speculate that this protrusion was due to weakened intercellular adhesion at the apical surface or an increase in the intraepithelial pressure associated with cell proliferation. Similarly, there were more filamentous or vesicular processes on the apical surface than those in the control group, which was in agreement with a report that, in a whole-embryo culture experiment, RA increased microvilli and cytoplasmic processes on the apical surface of the neuroepithelium26).

Regarding cell proliferation, Angel et al. reported that RA addition to a rabbit renal epithelial cell culture increased thymidine uptake and DNA and protein synthesis, thereby influencing the cell cycle27). We speculate that, in this study, too, RA influenced the neuroepithelial cell cycle, resulting in a marked increase in neuroepithelial cell proliferation 18 h or later after RA administration, leading to neuroepithelial eversion, that is, dorsal bulging of the neuroepithelium.

A study reported that when all-trans RA was orally administered to hamsters at 8 days of gestation, the blood RA level peaked within 1 h, thereafter rapidly decreasing to below the limit of detection, and that its metabolites, 13-cis RA and all-trans-4-oxo RA, appeared soon after RA administration, remaining in the dam's blood for up to 12 and 24 h after administration, respectively 28). Although the action of these metabolites cannot be excluded, it is reasonable to speculate that the initially administered all-trans RA passed through the placenta to the embryo and acted directly on the neuroepithelium, because it is the most teratogenic.

A study reported that RA induced the dissociation of polysomes into monosomes in neuroepithelial cells in defective cranial neural tube closure in mice29). Similarly, in this study, the conversion of polysomes to monosomes was clearly observed at the posterior neuropore of rat embryos 24 h after RA administration, suggesting its influence on the protein synthesis system.

Various studies in rats have demonstrated that the tail bud is formed by secondary neurulation at 11 days of gestation or later, followed by the formation of the secondary neural tube, which fuses and becomes continuous with the primary neural tube, and that the tail bud-derived tissue is caudal to the lower lumbar vertebrae30-32). Alles and Sulik reported that physiological cell death, which normally occurs in the tail bud or mesenchymal tissue, was excessively induced by RA, resulting in the development of spina bifida in mice33). Other studies reported that spina bifida presumably resulted from the abnormal development of the tail bud or the presence of cysts or hematomas34,35). Another study noted that the RA-induced anomalous formation of tail bud tissue, and therefore the secondary neural tube, was not due to vascular disruption, but due to the direct action of RA on tail bud tissue21). In this study, RA was administered at 10 days of gestation, before tail bud formation; therefore, it is unlikely that RA induced tail bud malformation by its direct action. Indeed, tail bud malformation was observed, but the posterior neuropore at the caudal end of the primary neural tube first underwent changes resulting in the development of spina bifida. Thus, we speculate that the main causes of spina bifida in this study were the inhibition of neurulation at the posterior neuropore and neuroepithelial eversion. Hypoplasia of the tail bud developed late, apparently leading to hypoplasia of the sacrococcygeal vertebrae.

5. Conclusions

All-trans RA was administered at a dose of 60 mg/kg to pregnant DA rats at 10 days of gestation, and its influence on the posterior neuropore of embryos was examined serially by morphological methods. Although no external changes were observed

3 h after administration, neural plate elevation was inhibited 6-9 h after administration. Neuroepithelial eversion occurred at 12 h. We speculate that RA acted directly on embryonic neuroepithelial cells, damaged the cytoskeletal system, inhibited the shape change of neuroepithelial cells required for neural fold elevation, induced early formation of the basal lamina on the basal side of the neuroepithelium, and thereby inhibited neural fold elevation. However, at 12 after administration or later, RA caused neuroepithelial overgrowth, resulting in neuroepithelial eversion, leading to the development of typical spina bifida.

Literature cited

1）Konishi H : The effects of intrauterine exposure to retinoic acid on development of rat fetuses. Annual Reports of Shijonawate Gakuen University. 1: 7-13, 2005

2）Konishi H : Expression pattern of malformations induced by prenatal exposure to retinoic acid in rats. Annual Reports of Shijonawate Gakuen University. 2: 9-17, 2006

3）Strauss JS, Windhorst DB, Weinstein GD. Oral retinoids : A workshop. J Am Acad Dermatol. 6:573-832, 1982

4）Peck GL, Retinoids. Therapeutic use in dermatology. Drugs. 24:341-351, 1982

5）Goureia J, Mathe G, Hercend T, et al. Degree of bronchial metaplasia in heavy smokers and its regression after treatment with a retinoid. Lancet. 1:710-712, 1982

6）Kessler JF, Mevskens F1 JR, Levine N, et al. Treatment of cutaneous T-cell lymphoma(mycosis fungoiders) with 13-cis-retinoic acid. Lancet. 1:1345-1347, 1983

7）Kochhar DM. Teratogenic activity of retinoic acid. Acta Pathol Microbiol Immunol Scand. 70 : 398 -404, 1967

8）Kochhar DM, Aydelotte MB. Susceptible stages and abnormal morphhogenesis in the developing mouse limb, analysed in oegan culture after transplacental exposure to vitamin A (retinoic acid). J Emnryol Exp Morphol. 31:721-734, 1974

9）Kwasigroch TE, Kochhar DM. Production of congenital limb defects with retinoic acid.:phenomenological evidence of progressive differentiation during limb morphogenesis. Anat Embryol. 161:105-113, 1980

10) Newall DR, Edwards JRG. The effects of vitamin A on fusion of mouse palates. □.Retinyl palmitate, retinal, and retinoic acid in vitro. Teratology. 23:125-130, 1981

11) Kochhar DM, Penner JD, Tellone CL. Comparative teratogenic activities of two retinoids:Effects on palate and limb development. Teratogenesis Carcinog Mutagen. 4:377-387, 1984

12）Johnston MC, Sulik KK, Webster WS, et al. Isotretinoin embryopathy in a mouse model : Cranial neural crest involvement. Teratology. 32:26A-27A, 1985

13）Javis BL, Johnston MC, Webster WS, et al. Isotretinoin-induced ear malformations in mice. Teratology. 32:27A, 1985

14）Wiley MJ, Tibbles LA, Wong M. A comparative study of the effects of retinoic acid on the morphology of caudal tissues in mouse and hamster embryos during the 24 hours after maternal treatment. Teratology. 32:49A, 1985

15）Kistler A. Teratogenesis of retinoic acid in rats:susceptible stages and suppression of retinoic acid-induced limb malformation by cycloheximide. Teratology. 23:25-31, 1981

16）Steele CE, Trasler DG, New DAT. An vivo/invitro evaluation of the teratogenic action of excess vitamin A. Teratology. 28:209-214, 1983

17）Shenefelt RB. Morphogenesis of malformations in hamsters caused by retinoic acid:relation to dose and stage at treatment. Teratology. 5:103-118, 1972

18）Taylor IM, Wiley MJ, Agur A. Retinoic acid-induced heart malformations in the hamster. Teratology. 21:193-197, 1980

19）Wiley MJ. The pathogenesis of retinoic acid-induced vertebral abnormalities in goldenhamster fetuses. Teratology. 28:341-353, 1983

20）Wiley MJ, Cauwenbergs P, Taylor IM. Effects of

retinoic acid on the development of the facial skeleton in hamsters:early changes involving cranial neural crest cells. Acta Anat. 116:180-192, 1983

21) Griffith CM, Wiley MJ. Direct effects of retinoic acid on the development of the tail bud in chick embryos. Teratology. 39:261-275, 1989

22) Peters PW, Dormans JA. An experimental study of the morphogenesisi of spina bifida. Acta Morphol Neerl-Scand. 19:1-20, 1981

23) Taniuchi M, Nakata K, Onomura T. Pathogenesis of experimental lumbosacral agenesis in rats. Cong Anom. 31:285-296, 1991

24) Connor MJ, Lowe NJ. The induction of ornithine decarboxylase and DNA synthesis by retinoids. Cancer. 43:5174-5177, 1983

25) Michael JC, Richard EA, Nicholas JL. A comparative study of the induction of epidermal hyperplasia by netural and synthetic retinoids. J Pharmacol Exp Ther. 237:31-35, 1985

26) Robert ES, Craig H, Daniel LL. Morphological differences elicited by two week acid, retinoic and valproic, in rat embryos grown in vitro. Teratology. 43:133-150, 1991

27) Angel A, Norbert EK, Paul H. Retinoic acid affects the cell cycle and increases total protein content in epithelial cells. Kiney Int. 36:954-949, 1989

28) Howard WB, Willhite CC, Omaye ST. Comparative distribution, pharmacokinetics and placental permeabilities of all-trans-4-oxo-retinoic acid and 9-cis-retinal in hamsters. Arch Toxcol. 63:112-120, 1989

29) Yasuda Y, Konishi H, Kihara T. Developmental anomalies by all-trans- Retinoic acid in fetal mice Ⅱ. Induction of abnormal neuroepithelium. Teratology. 35:355-366, 1987

30) Schoenworf GC. Effects of complete tail bud extirpation on early development of the posterior region of the chick embryo. Anat rec. 192:289-296, 1978

31) Schoenworf GC. Tail,(end)bud contributions to the posterior region of the chick embryo. J Exp Zool. 201:227-246, 1977

32) Schoenworf GC, Chandler NB, Smith JL. Analysis of the origins and early fates of neural crest cells in caudal regions of avian embryos. Dev Biol. 110:467-479, 1985

33) Alles AJ, Sulik KK. Retinoic acid-induced spina bifida：evidence for a pathogenetic mechanism. Development. 108:73-81, 1985

34) Lendon RG. The embryogenesis of trypan blue-induced spina bifida aperta and short tail in the rat. Dev Med Child Neurol. 17:3-10, 1975

35) Rokos J, Cekanova E, Kithierova E. Pathogenesis of trypan blue-induced spina bifida. J Pathol. 118:25-34, 1976