Identical embryopathogenesis for exencephaly and myeloschisis: an experimental study

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Extensive histological and immunohistochemical studies were performed to elucidate the histopathogenesis of exencephaly induced in chick embryo as an experimental model. The findings were compared with those identified in a chick myeloschisis experimental model and in human autopsy cases. The experimental model of exencephaly in chick embryos was developed by induction with various teratogens including ethylnitrosourea, salicylate, and phenytoin. None of the cases of exencephaly was exposed to a teratogen prior to or within Hamburger and Hamilton stage 12 (45 to 49 hours postincubation), when the anterior neuropore closes. The process of overgrowth in development of exencephaly was identical to that of myeloschisis, and the results suggested neuronal overmaturation in the histological and immunohistochemical studies. Although the late-stage degenerative change with neovascularization over the exposed neural tissue (placode) was more severe in human exencephaly, the present experimental study may suggest a possible common embryopathogenesis of dysraphism. Exencephaly should be regarded as the most severe form of cranium bifidum, as myeloschisis is in spina bifida.

KEY WORDS • myeloschisis • exencephaly • embryopathogenesis • neuronal maturation • chick embryo

The embryopathogenesis of the dysraphic state is still obscure. During the last 100 years, various hypotheses have been seriously considered. With the rapid progress of experimental teratology in recent years, these proposals have mainly been tested in spina bifida animal models. We have also studied a hypothesis for myeloschisis in a chick embryo experimental model, analyzing the stage specificity and immunohistochemical characteristics of the exposed neural plaque (placode). The results revealed that no fetus exposed to a teratogen prior to or within Hamburger and Hamilton stage 12 (45 to 49 hours postincubation), when the neuropore closes, developed dysraphism; however, those affected after neural tube closure demonstrated myeloschisis. Immunohistochemical studies of chick myeloschisis clearly indicated that neuron-specific enolase (NSE)-positive elements were extremely active only in the overgrown placode, corresponding to histological findings in specimens with Klüver-Barrera's special stain. These findings implied the possibility of another mechanism for the embryopathogenesis of myeloschisis: namely, the overgrowth and reopening hypothesis.

Although experimental studies have been extensively performed on the embryopathogenesis of myeloschisis or spina bifida, some experimental models have proved an identical mechanism which can be applied to the development of cranium bifidum. In the present study, we have analyzed stage specificity and histopathogenesis in the development of exencephaly in a chick embryo experimental model in comparison to results from studies of chick myeloschisis. The morphological findings were also compared with those found in human autopsy cases to elucidate the consequential morphology of exencephaly.

Materials and Methods

Chick Experimental Model

Within 24 hours after being laid, 274 fertilized chick eggs (Isa Babcock B 300) were placed in an incubator at a constant temperature of 39.4°C for the first 10 days and 37.5°C thereafter. These were the same conditions as in the study by Hamburger and Hamilton on the normal stages in chick embryo development (H & H stages). Based on knowledge obtained from our study of experimental dysraphism utilizing chick embryo, the teratogens selected and their dosages were: an ethyl-nitrosourea (ACNU (1-(4-amino-2 methyl pyrimidine-5-yl)-methyl-3-(2-chloroethyl)-3-nitrosourea), 1 mg (6

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Fig. 1. Gross appearance of chick exencephaly. An 18-day-old (left) and a 10-day-old (right) chick fetus with exencephaly induced by 2 mg phenytoin injected into the egg at 58 hours postincubation. Arrows indicate the skin defect and exposed neural mass (placode). The placode is surrounded by an intact skin margin mimicking the typical exencephaly form in humans.

mg/ml)); an anticonvulsant agent (phenytoin, 2 mg (20 mg/ml)); and an antipyretic agent (acetylsalicylic acid, 9 mg (90 mg/ml)). Thirty-two eggs (11.7%) were unfertilized, and consequently 48 embryos were treated with ACNU, 88 with phenytoin, and 72 with acetylsalicylic acid. Thirty-four eggs were treated with normal saline solution, 100 µl, as a control group. Each group was further divided into four subgroups. The teratogenic agents were injected into the center of the fertilized eggs on the 1st, 2nd, 3rd, or 4th postincubation days in a dosage of either 200 or 100 µl using a No. 27 needle with a microsyringe infusion pump. The fetuses were sacrificed on postincubation Days 10 or 18 and prepared for gross and histological examination.

Human Autopsy Cases

Three full-term neonates with exencephaly (two males and one female) died within 5 days after birth. The entire body was fixed in 10% buffered formalin. The exposed placode was embedded in paraffin and cut into 4-µm sections. The sections were prepared for histological and immunohistochemical examination.

Immunohistochemical Staining Procedures

Immunohistochemical specimens were prepared as follows. All tissues were fixed in 10% buffered formalin, embedded in paraffin, and cut into 4-µm sections. The avidin-biotin peroxidase complex (ABC) immunohistochemical staining method described by Hou, et al., was used for analysis of NSE. Sections were incubated with primary antibodies at various dilutions (ranging from undiluted to 1:128) at room temperature for 30 minutes in a moist chamber. The sections were then treated with biotin-labeled goat anti-rabbit immunoglobulin G antibodies diluted 1:200, or approximately 50 µg/ml, followed by ABC staining. Fresh ABC was made by incubation with 10 µg/ml avidin and 2.5 µg/ml biotin-peroxidase in Tris buffer (0.05 M, pH 7.6) for 30 minutes at room temperature before use. Control studies for the ABC method were carried out as follows: 1) omission of primary antiserum, or replacement of primary antiserum by nonimmune rabbit serum, and 2) replacement of biotin-labeled antibody by unconjugated antibody. The slides were counterstained with hematoxylin, then dehydrated, cleaned in xylene, and mounted.

Results

Normal and Exencephalic Development

The overall mortality in the chick experimental model was 33.2% and exencephaly was observed in 10. In live fetuses the incidence of exencephaly induced with phenytoin was 10.7%, with ACNU was 7.7%, and with acetylsalicylic acid was 5.4%.

Gross Appearance and Stage Specificity of Exencephaly

In the fetuses that demonstrated exencephaly, three were sacrificed on postincubation Day 10 and the other seven on Day 18 (Fig. 1). All had exposed neural plaques (placodes) at the vertex with a midline frontoparietal durocalvarial defect. Seven fetuses were alive and the other three were dead at the time of exploration. One case demonstrated complete unilateral anophthalmus and severe omphalocele associated with exencephaly.

All these exencephaly models were induced by teratogen introduced on Days 3 or 4 (between 56 to 80 hours, mean 59.9 hours) in the postincubation period, which definitely corresponds to H & H stage 16 or later; by this time, it has been demonstrated that the neural tube is completely closed (Fig. 2). None of the fetuses treated with the teratogens before or at H & H stage 12 (45 to 49 hours postincubation) developed exencephaly.

Human Autopsy Cases

The exposed neural placodes in the three human autopsy cases demonstrated similar findings. The placodes were located in the midline vertex extending close to the nasion anteriorly and the inion posteriorly. The skin defect was oval in shape and filled with protruding reddish thin membranous degenerative tissue which was circumscribed with hair-bearing skin. The tissue was a solid mass, not invested with meninges, which had herniated through a defect in the calvaria and dura mater (Fig. 3 upper left). A midsagittal section and subsequent coronal sections revealed that a degenerative membranous mass continued to the deep brain...
structure without forming any cortical structure of the cerebral hemisphere. The sagittal paramidline sections were prepared for histological and immunohistochemical studies.

**Histological and Immunohistochemical Findings**

All the specimens of chick exencephaly demonstrated nearly identical findings depending upon the H & H stage. In the models sacrificed in the early stage (10 days postincubation), the placode was composed of a dense and highly cellular tissue with disordered cytoarchitecture of the cerebral mantle. Under high power, the hematoxylin and eosin-stained placode was seen to include mainly immature neuroepithelial cells. There were no positive elements in sections prepared with Klüver-Barrera's (K & B) stain or NSE at this stage. In the late-stage model of chick experimental exencephaly (18 days postincubation), remarkable differences in specimens with these special stains were observed between the normal control and exencephaly model. Brain-stem nuclei were stained showing a uniform immature form, and an early myelination process was observed in K & B-treated specimens of the normal control (Fig. 4 upper left and center left). However, the brain stem and basal ganglia of exencephalic specimens were composed of extremely dense myelinated fibers and variously developed neuronal cells. The cytoarchitecture was completely lost, with random neural tract formation and neuronal cell arrangement (Fig. 4 upper right and center right). In the cerebellum, the control specimens demonstrated positively stained Purkinje cells and associated dendrites with developing cortical cytoarchitecture and lamination. The white matter also developed early myelination neural tracts (Fig. 4 lower left). The cerebellum of the exencephaly model revealed developed cortical cytoarchitecture, but the individual layer was composed of highly cellular tissue and abundant stroma. The Purkinje cell layer, as well as the white matter, was extremely densely stained by K & B myelin staining (Fig. 4 lower right). In the cerebrum of the normal control chicks, the germinal matrix layer was still present and K & B staining demonstrated immature stages of cortex formation and myelination process (Fig. 5 upper left and lower left). The K & B-stained specimen of exencephaly placode and underlying cerebral tissue showed completely abnormal cytoarchitecture with extremely dense and highly cellular tissue of the cerebrum and a scanty residual germinal
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Fig. 3. Gross appearance and photomicrographs of placode in human neonatal exencephaly. Upper Left: Gross appearance of the placode. Note the exposed cerebral tissue which is covered by extremely hypervascular tissue. Upper Right: Photomicrograph of the placode revealing remarkable neovascularization and exposed cerebral tissue with formation of multiple islands. H & E, × 13. Lower Left: Same section as shown upper right. KliJver-Barrera, × 13. Lower Right: Immunohistochemical study of the placode. There are no positive elements in the isolated parenchymal tissue of the islands which are also negative for Klüver-Barrera staining shown lower left. NSE, × 13.

matrix layer. The cells were spheroid, oval, starlike, or polar, and of unequal cell size and shape (Fig. 5 upper right). Large starlike cells (probably more mature neuronal cells) were frequently seen in the placode, and the NSE-stained sections disclosed brownish positive elements in the stroma (Fig. 5 lower right).

Examination of the placodes of human exencephaly revealed remarkable neovascularization covering the exposed cerebral tissue, which presented multiple island formations (Fig. 3 upper right). The isolated parenchymal tissue in the islands, however, was not stained by K & B (Fig. 3 lower left) or by NSE (Fig. 3 lower right).

Discussion

Stage Specificity of Dysraphism

Although the embryopathogenesis of dysraphism has been debated for more than 100 years, no definitive theory or specific timing for the development of each dysraphic state has been generally accepted. Classically, several hypotheses proposed by von Recklinghausen,27 Patten,23 Wynne-Davies,28 and Jones, et al.,11 have supported the nonclosure theory for the development of myeloschisis. They proposed different theories, but agreed upon the stage specificity as a “proneurulation disorder,” as described by Lemire and Warkany.13 They considered myeloschisis as a defect occurring at Carnegie stage 12 (3 to 5 mm crown-to-rump length, 26 to 30 gestational days, and 21 to 29 somites), when the posterior neuropore closes. Hypotheses supporting the “reopening” theory are rather few. Gardner8 suggested a hydrodynamic mechanism as the cause of disruption of the neural tube. Padget19 proposed a neural cleft theory and Browne3 supported the theory of fetal compression in utero. Politzer24 and Brouwer2 suggested that cord defects observed in embryos might be traumatic or infectious in origin. In our previous study,15 we suggested that this particular myeloschisis model is a product of the insult only in the postneurulation period and might be due to the phenomenon of reopening.
FIG. 4. Photomicrographs of the brain stem and cerebellum in experimental chick exencephaly compared with a normal control chick on postincubation Day 18. Klüver-Barrera staining. Upper Pair: Sagittal section of chick brain stem and fourth ventricle. Upper Left: Normal control chick revealing uniformly developing brain-stem nuclei and early myelination process. × 13. Upper Right: Exencephaly model demonstrating variously developed neuronal cells and dense myelinated fibers with random neural tract formation and absence of cytoarchitecture. × 13. Center Pair: High-power views of the brain-stem nuclei of normal control (left) and exencephaly model (right) in the same samples as shown (upper pair). × 33. Lower Pair: Sagittal section of chick cerebellar hemisphere in normal control (left) and exencephaly model (right). Note a developing process of Purkinje cells and associated dendrites with some cortical cytoarchitecture formation in the normal control specimen (left). Exencephaly model revealed highly cellular tissue and abundant stroma which were densely stained by Klüver-Barrera myelin staining. × 33.
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the neural tube associated with an overgrown neural placode (in fact, the overgrowth and reopening hypothesis).

Data from our present experimental study have suggested, on the basis of stage specificity, that exencephaly induced by various teratogenic factors can also occur due to the phenomenon of reopening of the rostral end of the neural tube. Therefore, this dysraphic state should be regarded as a "postneurulation disorder" as shown in the myeloschisis model in our previous study. The only difference between the two models was the strain of chicks. Although the stage specificity and teratogens used for the experimental chick dysraphism models were the same, myeloschisis developed only in Broiler Fuji (Arber Acre) chicks, while exencephaly was predominantly induced in B-300 (Isa Babcock) chicks. Strain differences in the teratogenicity have been proved in mice. Finnell, et al., reported that female SWV mice were extremely sensitive to hyperthermic treatment on Day 8.5 of gestation, with 44.3% of their offspring having exencephaly, while other strains (such as SWR/J and C57BL/6J) had less than 14% affected offspring. Naruse, et al., investigated this strain difference using whole embryo cultures. The results indicated that SWV mouse embryos have 1.5 to 3 times the sensitivity of C57 embryos to the embryolethal and teratogenic effects of sodium valproate.

Morphogenesis of Dysraphism

Theories on the morphogenesis of myeloschisis and exencephaly concerning an identical "reopening hypothesis" have been discussed by a few authors. Gardner's hydrodynamic theory suggested that all dysraphic states can be explained by a series of mechanical steps resulting from an inadequate escape of fluid from the central canal or ventricles. Padget's neuroschisis theory dealt with a common embryopathogenetic mechanism for the development of anencephaly and spina bifida.

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Our chick models of exencephaly and myeloschisis demonstrated no hydrocephalus, syringomyelia, Chiari malformation, or possible secondary lesions (such as trauma or infection), but there were identical findings of local neuronal overgrowth in the brain or spinal cord parenchyma under the exposed neural tissue. In recent studies on experimental dysraphism, there have been some models reported in which exencephaly developed after neural tube closure as a “postneurulation disorder.” Schmid, et al.,26 employing a whole-embryoculture technique with continuous morphological observation proved that cadmium chloride (CdCl2)-treated mouse embryo exhibited reopening of the once-closed cranial neural tube. Padmanabhan20,21 reported that the administration of a single dose of cyclophosphamide to Charles Foster rats on Day 12 of gestation (that is, well after neural tube closure) resulted in exencephaly in almost 100% of living fetuses at term. His histological evaluation under a light microscope revealed no overgrowth process, however, although chronological degenerative changes were prominent.21

The present study revealed that the human exencephalic brain is covered by a highly vascular layer of epithelium and very scant residual neural elements. Regarding this, the so-called “anencephalic area cerebrovasculosa” theory has been generally accepted to explain exencephaly as the embryological predecessor of anencephaly in man.4,12,22,26 In animals with relatively short gestational periods, exencephalic fetuses are frequently observed; however, man has a relatively long gestational period, so that destruction of the exposed encephalon may be complete.4 Papp, et al.,22 studied 10 human fetuses with exencephaly and suggested that the exencephaly in humans is converted to anencephaly by degeneration of the exposed neural tissue and in this process the macrophages in fetal circulation and in the amniotic fluid may play a significant role. Thus, human exencephaly may represent degenerative changes of the neural elements together with additional processes, as opposed to exencephaly in animal models.

Overgrowth of Neuronal Component

As indicated in the extensive histological investigation of myeloschisis in early human embryos by Osaka, et al.,17 overgrowth of the neural tissue is always present in lesions seen in such early stages of neural development. Some authors, such as Patten,23 have suggested that the overgrowth of nervous tissue may be a factor prohibiting proper fusion of the neural tube. Barry, et al.,1 reported that two human fetuses with evidence of Arnold-Chiari malformation at 17 to 18 weeks of gestational age showed increases in the volume of the cerebellum and brain stem. They proposed a theory that such overgrowth may cause an Arnold-Chiari malformation. Ellis, et al.,3 observed that fetal rats exposed to benzimidazole fungicide revealed a high incidence of cranioencephalic anomalies and periventricular overgrowth. They speculated that the overgrowth may be a cause for hydrocephalus with secondarily distorted cerebroaqueduct. They also found a fetus with exencephaly, but its relationship to periventricular overgrowth was not mentioned. The present results indicated that the brain stem, cerebellum, and basal ganglia of the exencephaly model consisted of distinctly hypermyelinated fibers and variously overdeveloped neuronal cells. We were not able to determine whether the overgrowth was the result of increased cell proliferation or simple hypermyelination with abnormally advanced neuronal maturation process. Since the stage specificity for this experimental model ranged from H & H stage 16 to 24, cell proliferation might have been disturbed, consequently producing overgrowth. However, our previous study using 5-bromodeoxyuridine failed to clarify this point as a result of technical difficulties (unpublished data). The chick model seems to have limitations using this methodology because of the lack of maternal circulation by which the timing and drug level in the administration of such markers can be easily controlled. It is evident that more work using different methodologies, such as morphological and quantitative analysis of neuropeptides, may be necessary.

Conclusions

Our present experimental studies strongly indicate the possibility of identical embryopathogenesis for exencephaly and myeloschisis, both of which develop with certain mechanisms of neural tube reopening. Although the secondary degenerative change with neovascularization over the placode was more severe in the human autopsy cases, overmaturation of the neuronal components in certain periods of fetal growth shown in the experimental exencephaly placentas may suggest the same concept of embryopathogenesis for myeloschisis. Based on this concept, it should be emphasized that exencephaly can be regarded as the most severe form of cranium bifidum, as myeloschisis is in spina bifida.

References

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