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Abnormal Ventricular Looping and Abnormal Laterality of the Atrial Chambers are the Main Morphogenetic Mechanisms of Cardiac Lesions in Cultured Rat Embryos Treated with Retinoic Acid

To establish the early morphogenetic mechanism in retinoid induced cardiac lesions, we investigated the morphology of the heart in cultured rat embryos treated with retinoic acid (RA) at 9.0 and 9.5 days post coitum (d.p.c). Wistar rat embryos were treated with RA (2×10^{-7} M) for 6 hours from the embryonic day equivalent of 9.0 or 9.5 d.p.c. After further culture in an RA free medium for 2.5 days, embryos were fixed and examined with a stereomicroscope and a scanning electron microscope. Sixty three embryos were treated at 9.0 d.p.c., 14 embryos were treated at 9.5 d.p.c. and 30 embryos were used as control. Abnormal ventricular looping was seen in 31 embryos (49.2%) from the group treated at 9.0 d.p.c., and isomerism of right appendages occurred in 15 (23.8%). Embryos treated with RA at 9.5 d.p.c. showed a low incidence of abnormal ventricular looping (14.3%). We could summarize those abnormal looping as three variants of each looping. The mildest form was hypoplasia of the right ventricle observed in 20 cases. Both the right and left ventricles in the second variant were shifted far to the left or right (10 cases). The third variant was a heart with generalized hypoplasia of both ventricles (3 cases). The incidence of branchial arch anomalies was higher at 9.5 d.p.c. than at 9.0 d.p.c. (71.4% and 30.2%, respectively). Abnormalities in the ventricular looping and the atrial laterality at 9.0 d.p.c. suggest that RA induces derangement in the development of laterality, while at 9.5 d.p.c., the abnormality of the migration of neural crest cells is the principal mechanism.

Key Words : *Retinoic acid; Tretinoin; Cardiovascular abnormalities; Neural crest; Ventricular looping*

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INTRODUCTION

Vitamin A and its active metabolite, retinoic acid (RA), play an important role in the development of vertebrates and in the differentiation of a wide variety of cell types (1, 2). Most craniofacial structures in vertebrates, including the branchial arches and cranial ganglia, have some contribution from cranial neural crest cells (3). Similarly, the cardiac neural crest contributes to the development of the cardiac outflow tract (4-6). Excessive vitamin A in pregnant female rodents resulted in a broad spectrum of embryonic defects in neural crest cell derivatives; these included persistent truncus arteriosus, transposition of the great vessels and aortic arch abnormalities (7).

Similar craniofacial and cardiac abnormalities were induced by RA in murine embryos cultured in vitro (8). It is also known that the teratogenic effect of RA is stage- and region-dependent (8) and it has been reported that the type of cardiac lesion differed according to the time that pregnant rodents were treated with RA (7, 9). To establish the time specific action of RA on cardiac development, we used a whole embryo culture system and excessive RA was applied to embryos at the neural plate stage, when neural crest cells were migrating. Early morphologic change in embryonic hearts was studied by serial histologic sectioning and scanning electron microscopy.

MATERIALS AND METHODS

Rat whole embryos culture

Wistar rat embryos were surgically explanted from cervical dislocated mothers at 9.0 d.p.c. (early neural

plate stage: plug day; 0). Whole embryos were cultured according to a previously described method (8). Briefly, they were placed in 15 ml culture bottles containing 3 ml of culture medium consisting of 100% immediately centrifuged rat serum with 2 mg/ml glucose. The culture bottles were attached to a rotator drum and rotated at 20 revs/min and 37℃ while being continuously supplied with a gas mixture of 5% O_2/5% CO_2/ 90% N_2 for the first 36 h, and subsequently with 20% O_2/5% CO_2/75% N_2 for the remaining culture period. The gas mixture flow rate was increased as necessary.

Retinoic acid treatment

All-trans RA (Sigma) was dissolved in dimethyl sulfoxide (DMSO) to make a stock solution (2×10^{-4} M). A 3 μl aliquot was added to 3 ml of culture medium, and final RA concentration was thus 2×10^{-7} M. Vehicle-control embryos were exposed to the same amount of DMSO for 6 h. After being treated with RA and vehicle, embryos were washed several times with Tyrode solution and transferred to fresh medium for further culture.

Examination of embryos

After culture, embryos were assessed for heart beat, blood circulation in the yolk-sac and whole body, yolk-sac diameter, crown rump length, the number of somites and general morphology. To ascertain whether the culture period itself affected embryonic development, preliminary experiments were carried out in which cultured embryos were compared to those allowed to develop in utero for the same length of time. On the Basis of the number of somites and general morphology, embryos cultured for 72 h from the neural plate stage appeared to be similar to those developed in utero (data not shown). The morphological sequence of the cardiac development in our system was from the neural plate stage, i.e. early ventricular looping, to the four-chambered heart on the 12th embryonic day.

Scanning electron microscopy

For scanning electron microscope (SEM) observations, embryos were fixed in 2.5% glutaraldehyde solution. The samples were then postfixed with 1% osmium tetroxide in 0.05 M cacodylate buffer, dehydrated in graded ethanol, critical point dried (Critical Point Dryer, Hitachi), sputter-coated with Au-Pd, and observed.

General histological observation

For histological observations, embryos were fixed in Bouin solution, dehydrated with ethanol series and embedded in paraffin. Serial frontal sections (5 μm) were mounted on glass slides, stained with hematoxylin-eosin, and observed under a light microscope (Reichert-Jung).

RESULTS

General embryonic development was slightly retarded in embryos treated with RA. The crown-rump length and the number of somites, both in groups treated at 9.0 and 9.5 days, were reduced significantly compared to those in the control group (Table 1).

In RA treated groups, there was a significant increase in the incidence of craniofacial and cardiac abnormalities; the incidence of the latter was 49.2% at 9.0 d.p.c. and 14.3% at 9.5 d.p.c., while in control embryos there was no instance of cardiac abnormalities. In RA treated embryos, the incidence of branchial arch anomalies was highest at 9.5 d.p.c. (71.4%), while at 9.0 d.p.c. and in controls, the corresponding figures were 30.2% and 0.0%, respectively. At 9.5 d.p.c., an open neural tube was more common than at 9.0 d.p.c. (35.7% and 11.1%, respectively).

In embryos with normal atrial laterality and ventricular looping, the heart tube looped to the right of the body axis and the ventricle was brought caudal to the atrium. Soon after this, cardiac contraction and blood flow were evident. The four-chambered heart was formed at the somite 26-28 stage, and the sinus venosus and truncus arteriosus could be seen; the paired dorsal aortae had fused into a single vessel, which extended to the tail fold after giving off the vitelline artery; segmental arteries were seen to issue from the aorta, between the somites. Rat embryos cultured according to our system until the development of a four-chambered heart were clearly comparable to in vivo embryos at 12 d.p.c. (Fig. 1).

Morphological analysis of the junction between the venous component and the appendage of the atrial cham-

Table 1. Development of rat embryos treated with RA for six hours at the neural plate stage and subsequently cultured for 72h[a]

	Number of somites	Yolk-sac diameter (mm)	Crown-rump length (mm)
RA at 9.0 d.p.c. (n=63)	32.3±2.4**	5.27±0.41	4.43±0.35**
RA at 9.5 d.p.c. (n=14)	30.4±1.5**	5.50±0.60	4.40±1.50**
Control (n=30)	33.8±1.6	5.56±0.75	4.81±0.06

[a] Data expressed as mean±standard deviation
** $p<0.01$

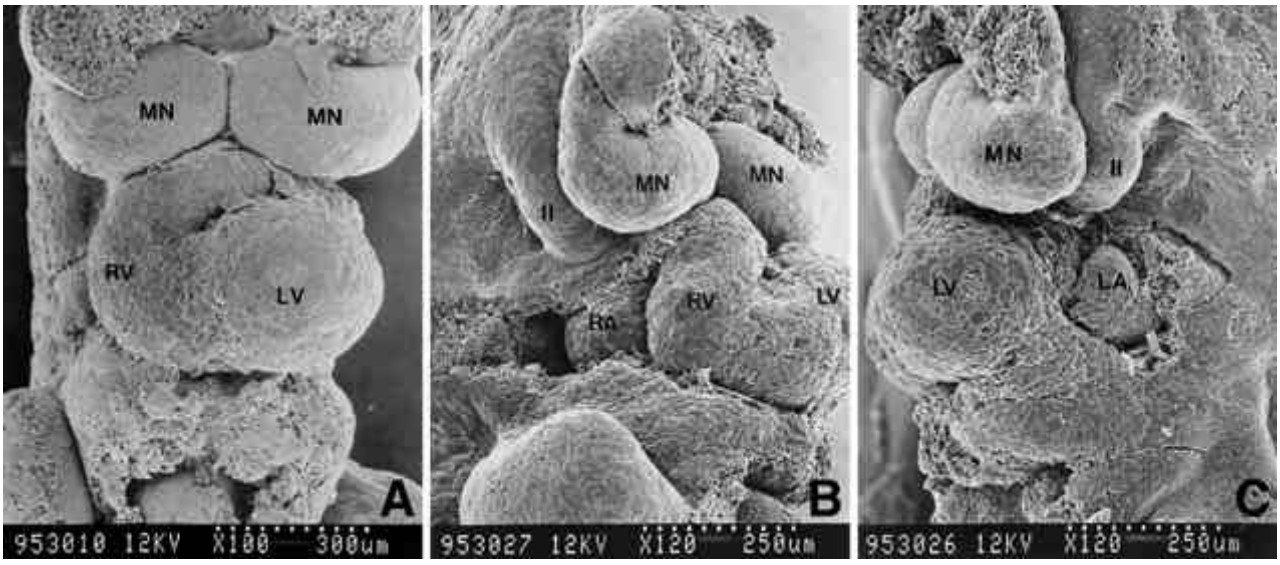

Fig. 1. An embryo with normal atrial laterality and normal d-loop ventricles. A, frontal view; B, right lateral view; C, left lateral view (RA, right atrium; LA, left atrium; RV, right ventricle; LV, left ventricle; MN, mandibular process of the first arch; II, the second branchial arch).

ber was used to determine the laterality of the heart (10, 11). The embryonic right atrium was initially composed of a large conspicuous appendage which was a thin-walled diverticulum at the morphologically right side of the heart tube; as development proceeds, prominent venous valves and the septum spurium appear. In contrast, the embryonic left atrium was initially composed of a less conspicuous appendage, and the major part of the left atrium was the venous component. Sixty of 77 experimental cases showed normal atrial laterality but 17 cases (22.1%) showed right isomerism (Table 2). In embryos with right isomerism, the atrial chambers showed large bilateral appendages, a small venous component and a central connection to the atrioventricular canal (Fig. 2A).

Cardiac ventricular looping was classified according to the location of the morphologically right ventricle, i.e., when the right ventricle was on the right or left, it was d-loop or l-loop, respectively. Our analysis, however, showed three variant types of each looping. The first type, the mildest form of abnormal looping, was hypoplasia of the right ventricle as a variant of l-loop or d-loop (Fig. 2). Both the right and left ventricles in the second variant had shifted far to the left or right, since the atrioventricular canal and the truncus were elongated (Fig. 3). The third variant was a heart with generalized hypoplasia of both ventricles.

Sixteen of 60 RA treated embryos, in which the laterality was normal (26.7%), showed abnormal ventricular looping. Severe hypoplasia of the right ventricle with d-loop or undetermined loop was seen in six cases (10.0%)

Table 2. Cardiac lesions observed in embryos treated with RA for 6 hours at the neural plate stage and subsequently cultured for 72 hours

	RA at 9.0 d.p.c.		RA at 9.5 d.p.c.		Control	Total
	Usual	Right isomer	Usual	Right isomer	usual	
Normal d-loop	32	0	12	0	30	74
Abnormal d-loop	3	6	0	2	0	11
hypoplasia of RV	(3)	(6)		(2)		(11)
Normal l-loop	0	0	0	0	0	0
Abnormal l-loop	7	5	0	0	0	12
hypoplasia of RV		(2)				(2)
ventricles shifted to left	(7)	(3)				(10)
Undetermined loop	6	4	0	0	0	10
hypoplasia of RV	(3)	(4)				(7)
others	(3)					(3)
Total	48	15	12	2	30	107

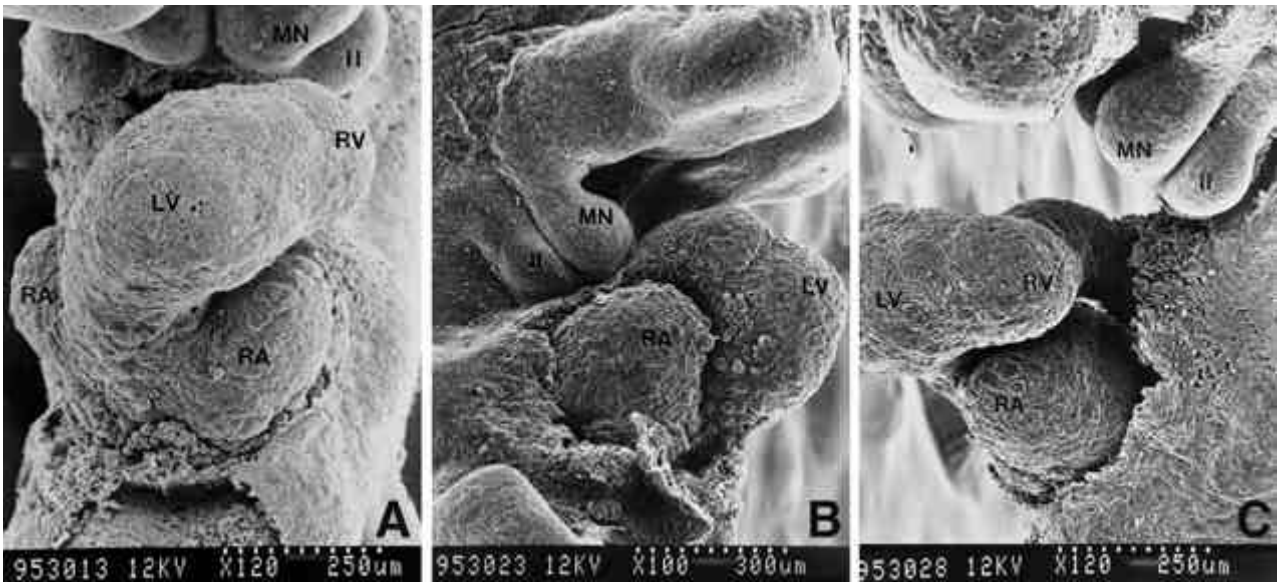

Fig. 2. An embryo with severe hypoplasia of the right ventricle (RV) on the left side (l-loop); both atrial appendages showed right morphology (RA). A, frontal view; B, right lateral view; C, left lateral view (See Fig. 1 for abbreviations).

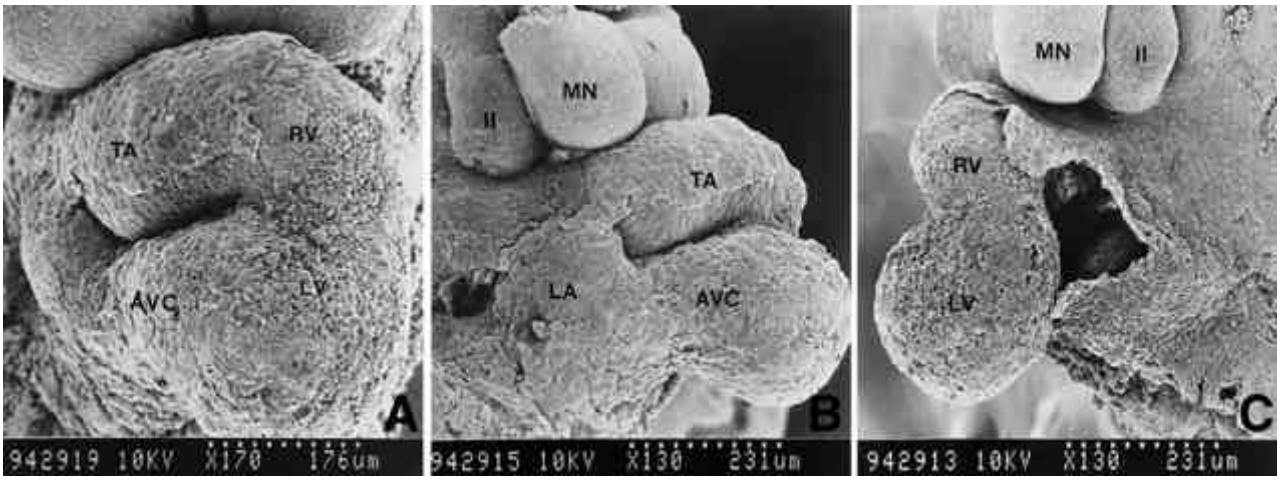

Fig. 3. An embryo with long atrioventricular canal (AVC) and long truncus (TA). Both the right and the left ventricles had shifted far to the left. The mandibular arch (MN) and the second branchial arch (II) can be used to orientate the parasagittal plane. The left-sided atrium is hidden between the shifted left ventricle (LV) and the chest wall as is shown in the left lateral view. A, frontal view; B, right lateral view; C, left lateral view (See Fig. 1 for abbreviations).

(Table 2). Seven cases (11.7%) of RA treated embryos showed elongated atrioventricular and ventriculo-arterial junctions in which the ventricular apex was far to the left lateral side (Table 2, Fig. 3), while poorly developed ventricular looping was present in three cases (5.0%).

In all 17 cases with isomeric right appendages, ventricular looping was abnormal; fourteen cases (82.4%) showed hypoplasia of the right ventricle. The ventricular loop was d-loop in eight cases, l-loop in two and undetermined in four. In three cases (17.6%) both ventricles had shifted to the left.

DISCUSSION

Our experiment clearly showed that in rat embryos cultured in vitro, RA is a potent teratogen, capable of inducing a high frequency of reproducible heart malformations. In 49.2% of cases, RA treatment for only 6 hours on day 9.0 of gestation resulted in defects related to ventricular looping or cardiac laterality. Half the embryos with abnormal looping showed right isomerism of atrial appendages, and in every isomeric case, ventricular looping was abnormal. RA treatment at 9.5 d.p.c., how-

ever, showed a lower incidence of abnormal cardiac looping (14.3%), while the incidence of branchial arch anomalies was much higher at 9.5 d.p.c. (71.4%) than at 9.0 d.p.c. (30.2%). The findings of these embryos at term pregnancy is not available because the use of an in vitro culture system does not permit their growth for longer than 13 d.p.c.

In previous studies, vitamin A and related compounds were administered to several mammalian species and the results varied. Major defects of the heart, seen in vivo models, were so-called conotruncal anomalies exemplified by transposition and aortic arch anomalies. Fantel et al. (12) found transposition of the great vessels in one of 11 offspring of pigtail monkeys treated with serial injections of 10 mg/kg RA on days 20 through 44 of gestation. Excess vitamin A palmitate also induced transposition (TGA), double outlet right ventricle (DORV), overriding aorta, and ventricular septal defect (13). A recent report also showed that in mice, TGA and swelling of the outflow tract are caused by maternally injected RA (14, 15). Shenefelt (16) reported stage-specific RA induced teratogenicity; in particular, TGA and ventricular septal defect in the offspring of hamsters treated relatively early in organogenesis (4, 16). These findings suggest that the morphogenetic mechanism of cardiac defects is related to the abnormal development of the outflow tract and septa (4-6, 8, 17).

Another in vivo study showed stage-specific induction of various types of cardiac lesions. In an experiment involving hamsters, RA treatment on the 7th day showed DORV, TGA and aortic hypoplasia (15.3%) and complex anomalies such as a univentricular heart and right ventricular hypoplasia (8.3%). RA treatment on the next day showed a much higher incidence of outflow tract anomalies (DORV, TGA and aortic hypoplasia: 62.2%) but there was no case of complex cardiac malformation (9). The morphology of the atrial chamber was not, unfortunately, studied. More recent studies by Japanese investigators (7, 14, 15) clearly showed abnormal laterality syndrome in the murine fetus produced by in vivo RA treatment; they did not, however, show the exact time of RA treatment and differences in incidence according to the day of treatment.

In order to compare our data with those of previous studies, it is crucial to understand the characteristics of our experimental system. The most important part of our experiment was the determination of the developmental stage during which RA was administered. Direct visual observation of embryos in both the control and experimental groups enabled us to control the developmental stages during which our experiment started, teratogen was applied and embryos were examined. Another advantage is that we examined early embryonic change (the 12th or the 13th embryonic day) prior to spontaneous abortion. We assume that the incidence and type of anomalies differ according to the day of examination. Most embryos with severely defective looping and laterality are aborted but milder defects such as ventricular septal defect and pulmonary stenosis are not detected until a later stage of pregnancy. The purpose of our experiment, therefore, was to elucidate the early developmental mechanism of major cardiac anomalies induced by RA.

On the basis of our experimental results, we concluded that at this particular stage, before the migration of the neural crest cells, abnormal laterality syndrome and abnormal looping were the main morphological events occurring in murine embryos treated by RA. The resulting cardiac lesions were different from outflow tract anomalies produced during a neural crest cell ablation experiment (17). The developmental mechanism of abnormal laterality and abnormal looping is not known though a recent study suggested that morphogenesis of the developing heart is regulated by transcription factors, such as MEF2C (18). MEF2C deficient mice had defective hearts, particularly hypoplasia of the right ventricle and anterosuperior left ventricle, conditions commonly observed in our RA treated embryos; Lin et al. suggested that this transcription factor might regulate regional heart development, particularly at the looping phenomenon. Because MEF2C is expressed throughout the heart tube early rather than later in embryos, it is possible that RA in some way regulates the function of MEF2C. Whether RA induced heart defect is related to this transcription factor is still undetermined, however. Another possibility is that the septal defect induced by RA at the later stage arises from the targeted mutation of a neurofibromatosis type gene (NF-1), in which a hypoplastic myocardium and severe septal defect were observed, suggesting that NF-1 plays a role in the migration or functional capacity of cardiac neural crest-derived cells (19). TGFβ2 localization in developing heart suggests that this might be involved in the development of outflow tract myocardium and atrioventricular canal in early embryo (8.5 to 9.5 d.p.c.) (20). On the basis of the results of a study of colocalization of expression of retinoid receptor and retinoid-binding protein genes (21), we can speculate that RA is related to the gene expression of TGFβ2. Moreover, double mutant embryos for retinoic acid receptors showed heart malformations occurred in the outflow tract and the great vessels located near the heart (22), suggesting that heart formation is partly regulated by RA related to various genes. The mechanisms involved are not yet fully understood, however.

The morphologic significance of different types of abnormal looping observed in this study is another point

to be discussed. It is clear that reversed looping, i.e. l-looping in normal atrial laterality, is related to discordant atrioventricular connections such as corrected transposition. Hypoplasia of the right ventricle, as seen in our 17 cases, is related to the double inlet left ventricle with rudimentary outlet chamber. It is not clear what the late result for hearts with long atrioventricular canal would be, although twisted atrioventricular connections are likely (23). Secondary remodelling may play an important role in the morphologic outcome.

We believe that in this model, the migrating pattern of neural crest cells is abnormal and that the incidence of branchial arch anomaly (30.2%) reflects the mechanism involved. The incidence of an association between abnormal laterality and abnormal looping (49.2%) was high enough to suggest that in the morphogenesis of RA induced cardiac anomalies, laterality defect is a more important mechanism in the earlier stage of development.

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