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Cardiac Laterality and Ventricular Looping in Retinoic Acid-treated Rat Embryos

To determine the ventricular looping pattern in relation to cardiac laterality, we studied rat embryos treated with retinoic acid (RA). A total of 243 Wistar rat embryos from an in vivo treated group (a single dose of 20-40 mg/kg all-trans RA administered to pregnant rats on day 6.5 to 9.5) and 29 control embryos were examined on day 13 of gestation. Twenty-nine embryos from the in-vitro treated group (treated by all-trans RA at 2×10^{-7}M for 6 hr on day 9.0 or 9.5 during the entire embryo culture for 72 hr) and seven control embryos were examined on day 12 of gestation. Abnormalities in cardiac laterality and ventricular looping were found in the in-vivo groups treated on day 8.5 and 8.75 and in the in-vitro group on day 9.0. Among 25 animals with abnormal laterality, right isomerism was the most common feature (22 cases), while the type of ventricular looping varied. Cases with normal laterality had a low incidence of abnormal looping (1.4%). In rat embryos treated with all-trans RA, normal cardiac looping was expected when cardiac laterality was normal. But in cases with abnormal laterality, the type of abnormal ventricular looping was unexpected.

Key Words : *Situs inversus; Dextrocardia; Etretinate; Retinoids*

Jung-Sun Kim, Jeong-Wook Seo, You Mie Lee*, Je G. Chi

Department of Pathology, Seoul National University College of Medicine
Department of Toxicology, Korean Food and Drug Administration*, Seoul, Korea

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Address for correspondence
Je G. Chi, M.D.
Department of Pathology, Seoul National University College of Medicine, Seoul 110-799, Korea
Tel : +82.2-760-3540, Fax : +82.2-745-7966

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INTRODUCTION

Cardiac laterality and ventricular looping are two basic steps in the development of normal and abnormal hearts (1). Clinical examples of abnormal laterality syndrome suggest that body sidedness plays an important role in cardiac development (2). Human cardiac lesions of double inlet ventricle or those with malalignment between the atrial and ventricular septa are understood to be lesions derived from abnormal ventricular looping. The determination of cardiac laterality and ventricular looping should, therefore, be an important step used in the studies of embryos with probable cardiac anomalies. Cardiac laterality and ventricular looping are, moreover, closely related processes, but the morphogenetic mechanism involved and the mode of association still need to be elucidated (3, 4).

Retinoic acid (RA), an active metabolite of vitamin A, plays an important role in the development and differentiation of wide varieties of cell types (5). Excess RA in humans and mammals has resulted in various embryonic defects particularly in the heart (6-10). The classic example of RA embryopathy showed conotruncal malformations, but recently abnormal laterality syndrome in certain animals could be produced with RA treatment (11-14).

We used RA-treated rat embryos to evaluate the relationship between atrial arrangement and ventricular looping, and studied the cellular mechanism of abnormal looping, particularly in regard to changes in cellular proliferation in RA-treated cases.

MATERIALS AND METHODS

Retinoic acid treated rat embryo

The morning after having been placed with a male the night before, female Wistar rats were examined for vaginal plugs. The day of plug detection was designated embryonic day (ED) 0.

In-vivo study

All-trans RA (Sigma, U.S.A.) was dissolved in sesame oil and 70% ethyl alcohol to make a solution (concentration: 10 mg/mL; all-trans RA : ethyl alcohol : sesame oil = 50 mg : 0.8 mL : 4.2 mL). Pregnant rats were given a single dose of 20, 30, or 40 mg per body weight (kg) all-trans RA by gavage on ED 6.5, 7.5, 7.75, 8.5, 8.75, or 9.5. On ED 13, embryos were delivered by caesarian section. A control preg-

nant rat was given only a mixture of sesame oil and ethyl alcohol on ED 8.5, and the other did not receive any treatment.

In vitro culture

Wistar rat embryos were explanted on ED 9.0 or on ED 9.5. Embryos were placed in 15 mL culture bottles containing 3 mL of culture media 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, i.e., 5% O_2/5% CO_2/90% N_2 for the first 36 hr, and subsequently with 20% O_2/5% CO_2/75% N_2 for remaining culture period. The flow rate of gas was increased as necessary. All-trans RA 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, such that final RA concentration was adjusted to 2×10^{-7}M. Embryos were exposed to all-trans RA for the first 6 hr and control embryos were exposed to the same amount of DMSO for the same period. After being treated with RA or vehicle, embryos were washed several times with Tyrode's solution and transferred to fresh medium for further culture. After whole embryo culture had been continued for an additional 66 hr, embryos were observed on ED 12.

Stereomicroscopic examination

Using a stereomicroscope, embryos were examined for external anomalies including closure of the neural tube or branchial arches. After elimination of the thoracic wall of the embryo, the morphology of the heart was examined. Ventricular looping was determined from the superoanterior view, and atrial arrangement from the inferoposterior view. The latter was defined by the shape of the atrial appendage, as observed from both the inferior and lateral aspects. The right appendage was large and a wide junction to the venous component was seen, while the left atrial appendage was small and the left primary atrial segment was conspicuous (15). Ventricular looping was defined by the location of the right ventricle (RV) to the left ventricle (LV): D-loop if the RV was right of the LV, A-loop if the RV was anterior to the LV, L-loop if the RV was left of the LV, intermediate D-loop if the RV was right and anterior, and intermediate L-loop if the RV was left and anterior.

Microscopic examination

For histologic observation, embryos were fixed in 10% formalin, dehydrated with ethanol series and embedded in paraffin. Serial transverse sections (5 μm) were mounted on glass slides, stained with hematoxylin-eosin, and observed under a light microscope.

Immunohistochemical staining for proliferating cell nuclear antigen (PCNA)

Serial sections of paraffin embedded embryos were deparaffinized with xylene and series of alcohol, quenched with hydrogen peroxide, blocked with normal swine serum and processed overnight for application of a PCNA antibody (DAKO, Denmark) at a dilution of 1:200 at 4℃. Immunoreactivity was detected using an ABC kit (DAKO, Denmark). Diaminobenzidine (DAB) was used to visualize the reactive product and the sections were counterstained with hematoxylin.

Statistical analysis

Using statistical package SAS, the proliferation index of the RA-treated group was compared with that of the control group. The Median test was used to determine the probability of significant differences; a p-value of less than 0.05 was considered statistically significant.

RESULTS

Incidence of anomalies and mortality

Among the 243 rat embryos observed on ED 13 after treatment with all-trans RA in vivo (Table 1), those treated between ED 6.5 and 7.75 showed a mortality of less than 16.6% and no cardiac anomalies. Though the mortality of embryos treated on ED 9.5 was more than 50%, there were no cardiac anomalies. Only embryos treated on ED 8.5 or ED 8.75 had cardiac anomalies with the mortality rate set at 37.1%.

Abnormalities of cardiac development, including abnormal atrial arrangement and ventricular looping, were found in 24.8% of live embryos treated in-vivo with all-trans RA on day 8.5 or 8.75, especially when the dose was more than 30 mg/kg. In cases treated with all-trans RA, extracardiac anomalies such as neural tube defects or branchial arch anomalies were also seen.

Forty-five percent of embryos cultured in vitro and treated with all-trans RA on ED 9.0 showed abnormal cardiac morphology, and 70% extracardiac anomalies (Table 2). Those treated on ED 9.5 did not show any cardiac or extracardiac abnormalities.

Pattern of abnormal cardiac development

Atrial morphology (Fig. 1)

The right atrium had a large appendage which extended below the inflow tract, while the left appendage was small and did not extend downwards. In 25 of 109 live embryos

Table 1. Numbers of live and total embryos and their anomalies after treatment with all-trans retinoic acid in vivo

Embryonic day (day)	Dose (mg/kg)	No. of dams	No. of embryos (live/total)	No. of live embryos with anomalies (cardiac/extracardiac/total abn. embryos)
6.5	30	1	10/12	0/0/0
7.5	30	1	14/14	0/0/0
7.75	30	1	14/15	0/0/0
8.5	20	2	23/26	1/1/2
	30	2	15/21	8/4/9
	40	4	31/52	12/16/16
8.75	20	2	28/31	0/0/0
	30	1	5/12	5/4/5
	40	1	7/14	1/3/3
9.5	20	1	7/15	0/0/0
	40	2	13/31	0/1/1
Control 1*	-	1	14/15	0/0/0
Control 2†	-	1	13/13	0/0/0

*Control 1: used a mixture of sesame oil and ethyl alcohol only on embryonic day 8.5, †Control 2: no treatment

Table 2. Number of embryos and their anomalies after treatment with all-trans retinoic acid in vitro

Embryonic day (day)	Dose (M)	No. of embryos	No. of live embryos with anomalies (cardiac/extracardiac/total abn. embryos)
9.0	2×10^{-7}	20	9/14/16
9.5	2×10^{-7}	9	0/0/0
Control*	-	7	1/0/1

*Control : treated with DMSO only

(23%) treated in-vivo with all-trans RA on ED 8.5 and 8.75, abnormal atrial arrangement was found (Table 3). In 22 animals, right isomerism was detected, with large appendages extending downwards on both sides. In the other three cases, atrial arrangement was of left isomerism, mirror-imaged or type-undetermined. In all control group embryos, the usual atrial arrangement was present.

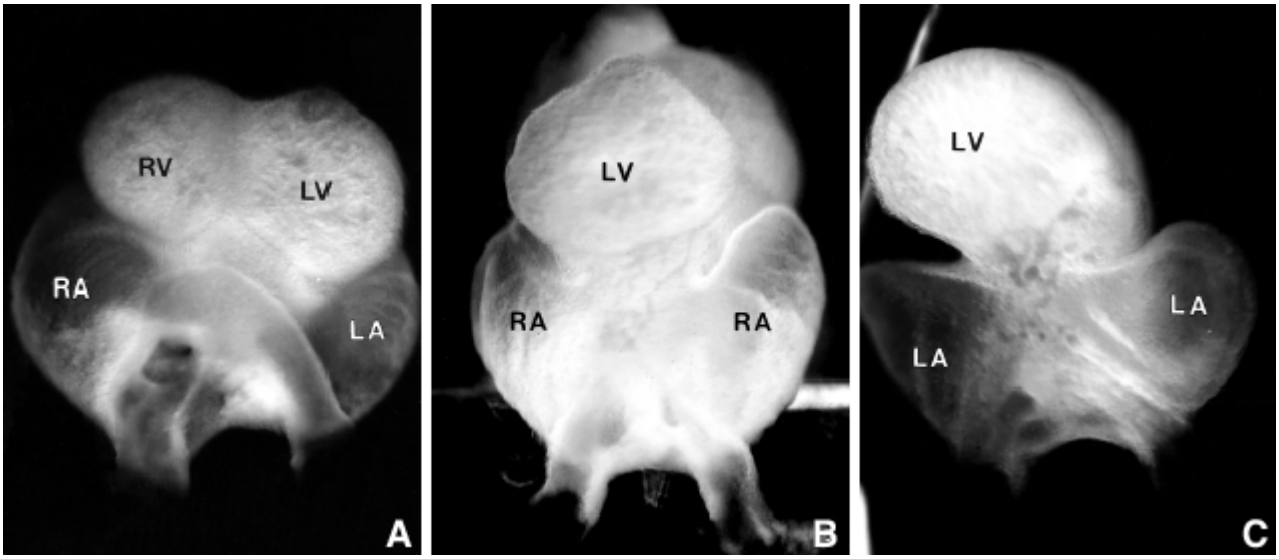

Fig. 1. Posteroinferior view of the heart on embryonic day 13 showing variable atrial morphologies. A, usual arrangement; B, right isomerism; C, left isomerism. LA indicates left atrium; LV, left ventricle; RA, right atrium; RV, right ventricle.

Table 3. Atrial arrangement and ventricular looping of live rat embryos treated with all-trans retinoic acid in vivo on embryonic day 8.5 and 8.75

Embryonic day (day)	Dose (mg/kg)	Atrial arrangement					Ventricular looping*				
		Usual	Mirror - imaged	Isomerism			D	D'	A	L'	L
				right	left	?					
8.5	20	22	0	1	0	0	22	0	1	0	0
	30	7	0	8	0	0	10	1	0	1	3
	40	21	1	7	1	1	22	1	0	1	7
8.75	20	28	0	0	0	0	28	0	0	0	0
	30	0	0	5	0	0	1	2	0	2	0
	40	6	0	1	0	0	6	1	0	0	0
Total	(109)	84	1	22	1	1	89	5	1	4	10

*D, D-loop; D', intermediate D-loop; A, A-loop; L', intermediate L-loop; L, L-loop

Table 4. Ventricular looping of rat embryos treated with all-trans retinoic acid in vitro

Embryonic day (day)	Dose (M)	Ventricular looping*					
		D	D'	A	L'	L	total
9.0	2×10^{-7}	11	6	0	2	1	20
9.5	2×10^{-7}	9	0	0	0	0	9
Control	-	6	1	0	0	0	7

*D, D-loop; D', intermediate D-loop; A, A-loop; L', intermediate L-loop; L, L-loop

Ventricular looping (Fig. 2)

Abnormal ventricular looping was found in 20 in-vivo embryos, 18.3% of those treated with all-trans on ED 8.5 or 8.75 (Table 3). These cases comprised five intermediate D-loop, one A-loop, four intermediate L-loop, and ten L-loop.

Nine of 20 embryos (45%) cultured in vitro with all-trans RA on ED 9.0 revealed abnormal ventricular looping (Table 4). The numbers of cases with intermediate D-, A-, intermediate L-, and L-loop were 6, 0, 2 and 1, respectively.

All embryos in the control group showed D-loop, except

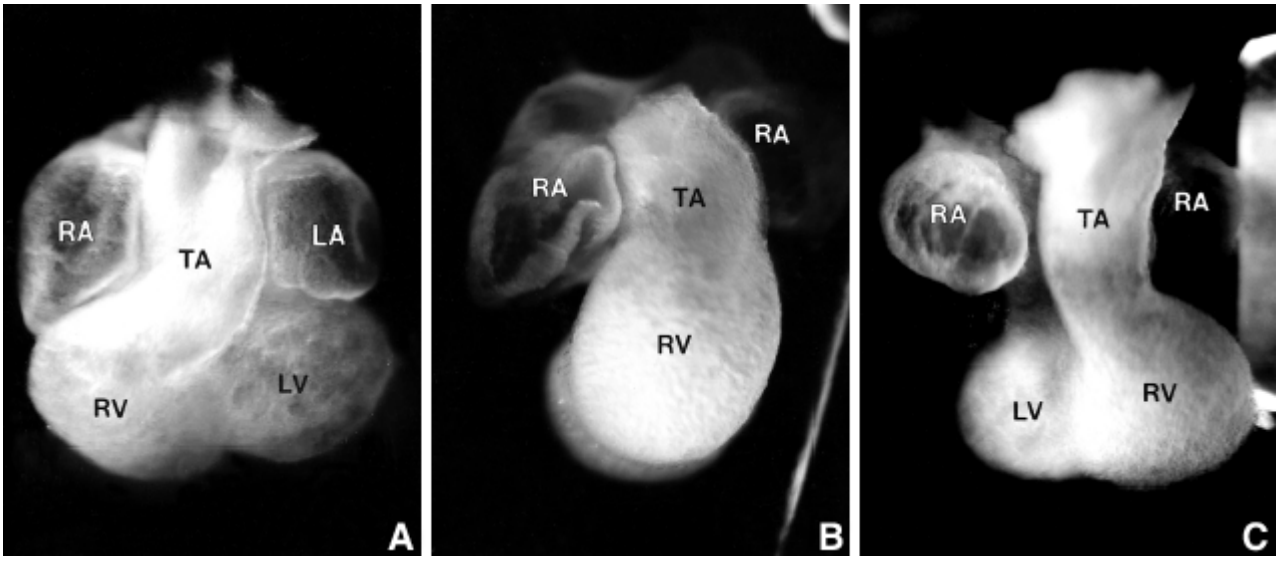

Fig. 2. Anterosuperior view of the heart on embryonic day 13 showing variable patterns of ventricular looping. A, D-loop; B, A-loop; C, intermediate L-loop. LA indicates left atrium; LV, left ventricle; RA, right atrium; RV, right ventricle; TA, truncus arteriosus.

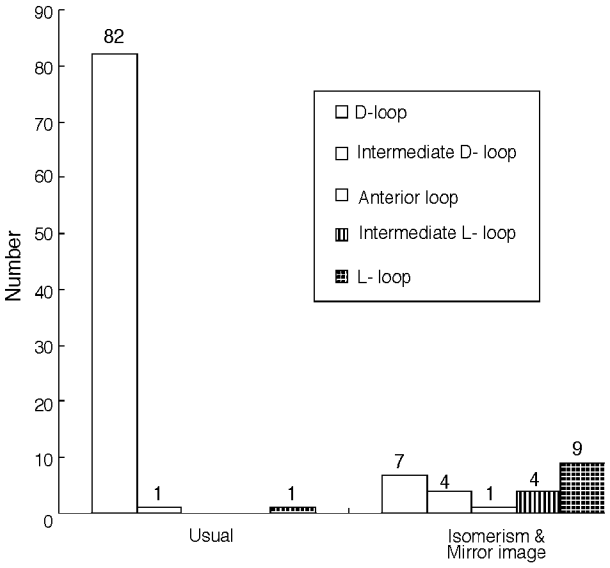

Fig. 3. Correlation between the atrial arrangement and the ventricular looping in embryonic heart treated with all-trans retinoic acid in vivo.

one cultured in vitro, which had intermediate D-loop and generally underdeveloped compared to the other control embryos.

Relationship between atrial arrangement and ventricular looping

In in-vivo treated group, there were only two cases of abnormal looping among 84 animals with normal atrial laterality; one was intermediate D-loop and the other, L-loop. In contrast to cases with normal atrial morphology, cases with abnormal atrial arrangement had a wide range of ventricular looping: D-, intermediate D-, A-, intermediate L, and L-loop accounted for 7, 4, 1, 4, and 9 cases, respectively (Fig. 3).

Internal morphology

Serial histologic sections of rat embryos on ED 13 revealed internal and sectional aspects of embryonic cardiac defects induced by RA. The prominence of the right (or lateral) venous valve was easily identified, marking the morphologically right atrium (Fig. 4A), while, the morphologically left atrium, in contrast, had a wide primary atrial segment without pectinate muscle. Hearts of isomerism showed bilateral presence of morphologically right or left atrial appendage (Fig. 4C). Sections in the four chamber plane demonstrated connections between atrial chambers and ventricles. In the control group, normal heart on ED13 showed four almost-septated four chambers (Fig. 4A); the right atrium was connected to the right ventricle, and the left atrium to the left ventricle. Except for a small opening, interatrial and interventricular septa were attached to fused endocardial cushion tissue. All RA-treated embryos, including those with usual atrial arrangement and D-loop revealed a common atrioventricular canal with no fusion of the endocardial cushion tissue and incomplete septation (Fig. 4B, C).

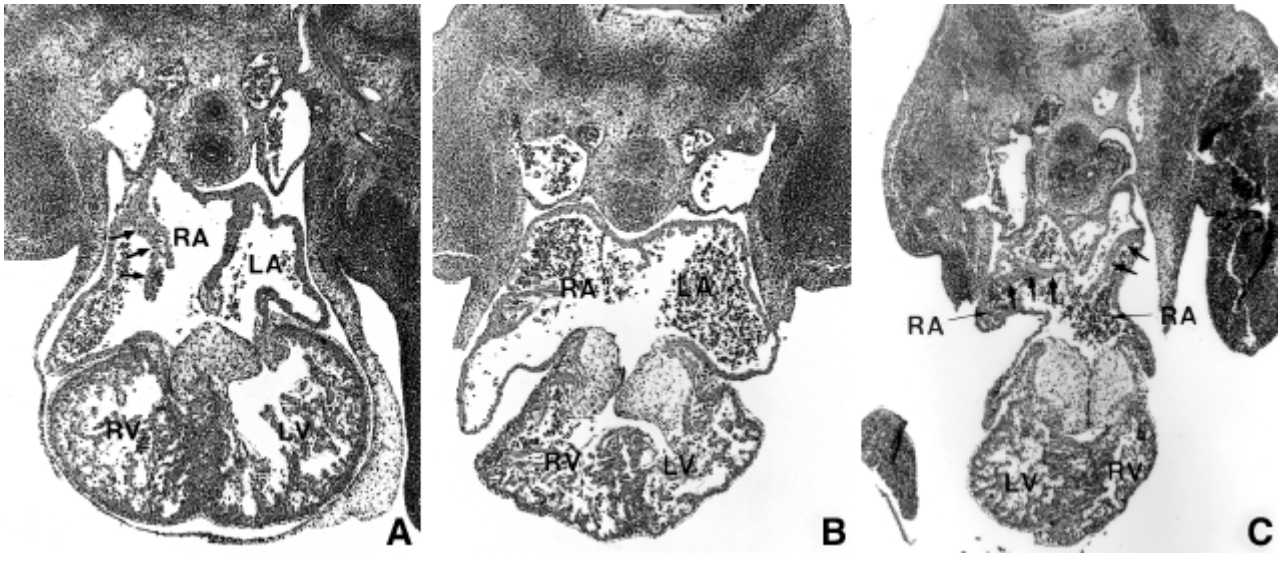

Fig. 4. Microscopic sections of the embryonic heart on embryonic day 13 . A: Normal heart of a control embryo shows four almost septated chambers with only a small opening of the atrial septum, and a prominent venous valve in the right atrium (×40). B, C: Two all-trans RA-treated embryos reveal a common atrioventricular canal among atria and ventricles, with incomplete interatrial and interventricular septa (×40). B, with normal atrial arrangement; C, right isomerism. LA indicates left atrium; LV, left ventricle; RA, right atrium; RV, right ventricle; arrows, venous valve.

Table 5. Proliferation index (PI) at each cardiac segment of embryos treated with all-trans retinoic acid in vitro

Cardiac segment	PI of RA-treated group (Mean±SEM)	PI of control group (Mean±SEM)	*p*-value†
Right atrium	3.93±1.99	9.74±1.72	0.074
Left atrium	4.30±0.41	10.37±4.73	0.014*
Atrioventricular canal (Endocardial cushion)	11.10±2.85	11.33±3.19	0.456
Atrioventricular canal (Myocardium)	4.73±2.61	7.37±1.11	0.307
Left ventricle	6.10±2.61	8.27±1.56	0.307
Right ventricle	7.03±1.47	8.69±2.60	0.683
Ventriculoarterial area (Endocardial cushion)	9.38±1.45	16.47±4.91	0.014*
Ventriculoarterial area (Myocardium)	4.75±0.91	8.00±1.53	0.014*

†A *p*-value of less than 0.05 was considered statistically significant (*).

Regional cell proliferation index

Using PCNA labeling on serial sections of rat embryos of ED 12, the ventriculo-arterial junction and left atrium showed a statistically significant reduction of cellular proliferation in the treated group ($P<0.05$). The ventriculoarterial cushion area of the treated and normal control group had mean proliferation indices of 9.38 and 16.47, respectively, and in the myocardium of the same area, treated and control group indices were 4.75 and 8.00, respectively. The proliferation index of the left atrium was 4.30 in the treated group compared to 10.37 in the normal control group; mean right atrium index was lower in the treated group than in control, though the difference was not statistically significant. The right atrium index was 3.93 in the treated group and 9.74 in the control group. No difference between the treated group and control group was seen in either ventricle or atrioventricular junction (Table 5).

DISCUSSION

The determination of body sidedness is one of early steps in mammalian development and plays an important role in the subsequent processes of cardiac development (16, 17). When the body situs is normal or "solitus" in humans, cardiac lesions is rare or mild, but in hearts with abnormal laterality, the occurrence of complex cardiac anomalies is the rule (2, 18, 19). A similar association of abnormal laterality with complex cardiac lesions has been reported in animals (20, 21). Ventricular looping was initially understood to be a reflection of situs, but cases in which there is a discrepancy between situs and looping are common. In order to understand the role of body sidedness in the development of a normal or abnormal heart, a study of the looping process in hearts with abnormal laterality is therefore essential.

Retinoic acid is a biologically active retinoid, which exerts a broad spectrum of effects in the development of vertebrates (5). Following the clinical use of RA as a therapeutic agent against some types of cancer and chronic skin disorders (22), there have been reports of congenital anomalies induced by RA administered just before or during the early stage of pregnancy (9, 10, 23). It is also known that abnormal development of mammalian embryos has been caused by excessive retinoid (6, 7, 24-26). Current understanding of the cardiovascular anomalies produced by RA is mainly focused on the conotruncal area and the aortic arch (7, 8, 27-31). Recent experiments, however, have demonstrated RA-induced heterotaxy syndrome in some animals (11-14). The purpose of this study is to elucidate the morphogenetic mechanism of abnormal laterality and looping produced by RA, using in-vivo and in-vitro models.

Our study successfully produced abnormal laterality syndrome in 24.8% of embryos treated in-vivo with all-trans RA on day 8.5 or 8.75, especially at a dose of more than 30 mg/kg. Interestingly, right isomerism accounted for 88% of cases with abnormal laterality syndrome. The overall incidence of abnormal laterality syndrome and the proportion of right isomerism were comparable to those found in a study by Kim et al. (13), who used mouse embryos treated by etretinate on ED 7. These data suggest that at this particular stage, the teratologic effect of RA plays a consistent role in the determination of laterality in rat embryos.

RA's mechanism of action in determining right-left axis is still to be studied; several studies have suggested that it probably plays a role in the specification of the anteroposterior axis during normal development (32, 33). Retinoic acid has been shown to be an essential factor in the expression of genes involved in anteroposterior determination, such as *Hox* genes. Exogenous RA administered to murine embryos in utero induced abnormal expression of certain *Hox* genes, thus causing changes in segmental patterning (34, 35). A few recent reports have shown that determination of left-right asymmetry is linked to dorsal-anterior development, but is not an independent process (36), which might explain the occurrence of abnormal left-right asymmetry in RA-

treated animals. More recently, it has been reported that extrinsic RA altered the asymmetry of cardiac matrix protein in the precardiac field in such a way that the variable direction of heart looping may be predicted (3, 4). Retinoic acid might be able to mediate interactions between cardiocytes and their environment to induce normal or abnormal cardiac laterality and looping.

Ventricular looping is an early morphological manifestation of body asymmetry. Definitively abnormal looping is defined as an anteriorly displaced right ventricle and in this experiment was diagnosed as an intermediate loop or anterior loop. In this study, unusual ventricular looping either intermediate or anterior, occurred in 18.3% of in-vivo RA-treated embryos on ED 8; most instances were associated with isomeric atrial arrangement. These results suggest that the morphogenetic process of cardiac looping in this model treated with all-trans RA is related to cardiac laterality, but that in cases with abnormal laterality, the type of ventricular looping is randomized. In our experiment, mild hypoplasia of the embryonic right ventricle associated with mild looping abnormality was often seen. This feature has been reported in the normal developmental process (1).

One possibility for the final common pathway of looping and laterality is differential proliferation of cardiac cells (37). Our study of the proliferation index therefore suggested a possible molecular pathway involved in the basic cardiac development of RA-treated embryos. Using PCNA labeling, we found that in the treated group, the left atrial chamber and ventriculo-arterial junction showed a marked reduction of cellular proliferation. It could be expected that in RA embryopathy, decreased cellular proliferation in the outflow portion and left atrium of the embryonic heart affect the development of cardiac abnormality and the determination of laterality. It is also possible that these changes are a secondary effect following the interaction between cells and extracellular matrix (38, 39).

In abnormal laterality syndrome, the most common and clinically important intracardiac lesion is the atrioventricular septal defect (20). Since loss of spatial information regulates endocardial cushion formation and remodelling, the atrioventricular junction may be directly influenced by a disturbance of laterality (40). The endocardial cushion plays a critical role in the construction of the atrioventricular septum through interaction between the cardiac myocytes and mesenchymal cells (41). Morphologic change in the atrioventricular junction of RA-treated chick embryo may imply that cardiac looping disturbance leads to the malalignment of septal components (30). Our results showing maldevelopment of the atrioventricular canal, even in RA-treated heart with normal atrial arrangement and variable changes in atrioventricular cushion growth (42, 43), suggest, however, that the development of atrioventricular septal defect in RA-induced isomerism may be explained by another mechanism.

There are other experimental models of abnormal laterality, each with a different spectrum of laterality. Retinoic acid was the major cause of right isomerism. Approximately 20% of non-obese diabetic (NOD) mice showed right isomerism in approximately 20% but in about 17% of such mice, other types occurred (44). It has also been shown that maternal hyperthermia can lead to visceroatrial heterotaxy syndrome, mainly left sided in about 70-80% of murine fetuses (45). Genetic models of heterotaxy have included the *iv/iv* mice, about 50% of which revealed situs inversus and 5%, bilateral right or left sidedness (21, 46). All the *inv* mice were of situs inversus (47).

Abnormal laterality type depends not only on the type of teratogen but also on the date of application. The optimal developmental stage for RA-induced abnormal laterality syndrome in the rat was late ED 8 (in-vivo) or early ED 9 (in-vitro). This period is the neural plate stage, when the cardiogenic plate consist of precardiac mesoderm. It is interesting that rat embryos develop heterotaxy syndrome, especially left isomerism, only when exposed to heat stress on ED 9 (45).

In summary, heterotaxy syndrome, especially right isomerism, was induced in rat embryo by RA treatment at a specific stage. Changes in cardiac looping in this model was closely related to change in cardiac laterality, but in cases with abnormal laterality, ventricular looping was a random process. Reduced cellular proliferation in the ventriculoarterial area and left atrium of the developing heart was associated with the development of cardiac and laterality-related lesions in RA embryopathy.

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