Acta Med. Nagasaki 34 : 35－52

Morphogenesis of Aortico-pulmonary Septation Anomaly Induced by Bis-diamine Treatment ——Special reference to vimentin, desmin and fibronectin immunoreactivities——

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Received for publication, December 26, 1987

A part of this study was presented at the 9th Asian-Pacific Congress of Cardiology, 1987, Auckland, New Zealand, at the 76th Annual Meeting of the Japanese Pathological Society, 1987, Tokyo, and 27th Annual Meeting of the Japanese Teratology Society, 1987, Tokyo.

SUMMARY : In order to clarify the morphogenesis of the A-P septum, the conotruncal septum, the myocardial sheath and the smooth muscle cells of the great arteries in the normal hearts and bis-diamine induced cardiovascular anomalies, an immunohistochemical technique using the antibodies for vimentin, desmin and fibronectin was applied. Morphological and immunohistochemical changes in the treated hearts were as follows : (1) the A-P septum which was hypoplastic appeared later than in the normal hearts ; (2) retraction of the myocardial sheath was delayed compared with the normal hearts ; (3) switching from vimentin to desmin in the muscles of the atria and ventricles caused a delay of one day behind that of the normal hearts ; (4) vimentin immuno-reactivity in the smooth muscle cells of the pulmonary trunk was weaker than that of the aorta, and (5) weak reactivity of fibronectin occurred concomitant with weak vimentin reactivity. It is concluded that bis-diamine may induce the morphological anomalies of the A-P septation by inhibiting the production or perturbing the migration of neural crest cells which have been a precursor of the mesenchyme in the anlage of the A-P septum, subsequently by either inhibiting production or changing the nature of extracellular matrix, or by affecting the coordination of intermediate filaments.

INTRODUCTION

The septation process of the aorta and the pulmonary trunk has been controversial for a long time because of the difficulty in tracing the dividing process. Diverse opinions have been caused by the complicated anatomical structure of the outflow tract and the rapid procedure of the septation in every species. There is no consensus to be found in the literature regarding the origin and histology of the A-P septum. Van MIEROP[37] states that the anlage of the A-P septum locates in the wall between the sixth arches and ascending aorta. On the other hand, LOS[23] asserts that the A-P septum does not exist, but is an artifact of two-dimensional histology. Thus the presence

of the A-P septum has remained obscure and no definite evidence for its accurate location has yet been demonstrated.

N, N'-bis- (dichloroacetyl) -diamine, 1, 8-octamethylene diamine (bis-diamine) is an effective inhibitor of spermatogenesis in rats[29], and recently has been used as a model for DiGeorge syndrome[16,17,21,27,30]. But it has not been clearly explained where the bis-diamine specifically affect, or the process of inducing various anomalies. As for the developmental mechanism of DiGeorge's syndrome, several hypotheses have been proposed, such as : (1) a vascular insufficiency associated with disruption of the fourth arch[20] ; (2) truncated pharyngeal development[3], and (3) the possible role of the neural crest[4]. Furthermore, as for the teratogenesity of the bisdiamine, two hypotheses have been proposed as follows : (1) the direct insult to the neural crest and subsequent dysgenesis of both the aortic sac and the pharyngeal pouch derivatives, and (2) interference with the production of extracellular matrix by mesenchyme, particularly hyaluronate-rich glycosaminoglycan.

Considering these viewpoints, the present study was performed to elucidate the morphogenesis of the A-P septum, outflow tract and related mechanisms in the normal and bis-diamine treated embryonic hearts. For this purpose, desmin, vimentin and fibronectin were used as markers in the development of the wall of the A-P septum and outflow tract.

MATERIAL AND METHODS

1. Experimental animals

Female Wistar rats weighing about 250g (Kyudo breeding farm, Saga, Japan) were mated with males overnight and isolated on the morning that a vaginal plug was found (9 : 00 AM was designated as gestation day 0). Both the control and bis-diamine treated females were sacrificed by ether anesthesia on day 12 1/2, 13.0, 13 1/6, 13 1/2, 14.0, 15.0, 16.0 and 17.0 of gestation. The embryos were removed and then fixed in a 10% phosphate-buffered formalin (pH 7.4) or PLP fixative (periodate-lysine-paraformaldehyde) for 12 to 24 hours at 4℃.

2. Administration of the drug

N,N'-bis-(dichloroacetyl)-1,8-octamethylene diamine (Fertilysin, Sigma chemical Co., St. Louis, Missouri, U.S.A.) was blended with a 1% aqueous suspension of gum tragacanth to produce a mixture of 100mg/ml. A single dose of 2ml (200mg of bis-diamine) was administered orally to the pregnant rats on days 9, 10 and 11 consecutively, by means of a stomach-tube. The same quantity of a 1% aqueous suspension of gum tragacanth was administered only to the control rats.

3. Preparation of tissues

After fixation. embryos were embedded in paraffin wax. Serial sections were created and then stained with Haematoxylin and Eosin. Embryos fixed in PLP fixative were washed for 3 hours in 0.05 M phosphate buffer (pH 7.4), then quickly frozen in a mixture of 100% ethanol and dry ice stored at −80℃. Frozen sections were cut serially with a cryostat and then mounted on glass slides coated with albumin.

4. Immunohistochemical staining

Dewaxed sections and frozen sections were rinsed with PBS for 5 minutes, and immersed in 0.03% methanolic hydrogen peroxidase for 20 minutes to block intrinsic peroxidase activity. These sections were then incubated with 5% goat serum in a moist chamber for 10 minutes at room temperature to block non-specific protein binding. After removing excess liquid, anti-vimentin antibody (monoclonal mouse antibody to human : DAKOPATTS) was added to the dewaxed sections and incubated with a 1 : 200 dilution for 1 hour. Anti-desmin antibody (monoclonal mouse antibody to human : DAKOPATTS, 1 : 100 dilution) and anti-fibronectin antibody (rabbit immunoglobulins to human : DAKOPATTS, 1 : 200 dilution) were added to frozen sections and incubated for 1 hour. After all slides were rinsed in PBS, and washed twice in PBS (10 min each), sections incubated with the anti-desmin antibody and anti-vimentin antibody were incubated with biotinylated goat anti-mouse immunoglobulins (BioGenex Laboratories), and sections incubated with anti-fibronectin antibody were

incubated with biotinylated goat anti-rabbit immunoglobulins (BioGenex Laboratories) for 20 minutes. Then, excesses of antibody were removed with PBS, and all the sections were washed twice (10 min each), and then incubated with streptavidin-enzyme conjugate for 20 minutes. After incubation, all the slides were rinsed in PBS and washed twice (10 min each), then were incubated in a 3-3' diaminobenzidine free-base substrate solution (DAB, Sigma) mixed with H_2O_2 for 2-10 minutes. Sections were then quickly washed with distilled water and running water, and subsequently counter-stained with haematoxylin or methyl green solution. Sections were dehydrated in graded ethanol, cleared in xylene and mounted in balsam. All of these procedures were performed at room temperature.

RESULTS

1. Morphogenesis in normal heart

(a) A-P septation

① Gross findings

The A-P septum was not evident on day 12 1/2 and 13.0 of gestation, but was observed on day 13 1/6 at the top of the aortic sac and soon touched with the upper ends of the truncal swellings by day 13 1/2. The great arteries were nearly divided on day 14.0 and completely divided on day 15.0, 16.0 and 17.0 of gestation (Fig. 1).

② Histological findings

On day 12 1/2 and 13.0 of gestation, both the A-P septum and definitive A-P septation were not observed throughout the aortic sac, truncus and conus (Fig. 1-a, b). On day 13, swellings were so prominent in both the distal conus and proximal truncus as to nearly connect with each other (Fig. 1-b).

On day 13 1/6, the A-P septum appeared as a thick mesenchymal globus on the distal truncus, which seemed to originate at the junction between the ascending aorta and the right sixth aortic arch. Fusion of the truncal swellings at the valvar anlages had proceeded more than on day 13.0. Bilateral conus ridges drew closer to each other (Fig. 1-c).

On day 13 1/2, the A-P septation proceeded from the distal truncus to the level of both the aortic and pulmonary valve anlages, the distal conus being just prior to dividing. Septation was not observed at the level of the proximal conus (Fig. 1-d).

On day 14.0, the A-P septation proceeded more downward to the level of the proximal conus (Fig. 1-e).

On day 15.0, the A-P septation had almost completed, and the aortic and pulmonary channels were observed throughout the outflow tract distally to proximally. (Fig. 1-f).

In this way, the aortic channel becomes continuous with the right and inferior portion of the trunco-aortic sac, while the sixth arches shift to the left and the pulmonary channel becomes continuous with the left portion of the trunco-pulmonary sac and sixth arches.

(b) Formation of the myocardial sheath and the tunica media, and arrangement of the mesenchymal cells in the conotruncus

① Formation of the tunica media in the A-P septum

On day 13 1/6 of gestation, the mesenchymal cells that formed concentric globus in the A-P septum resembled the smooth muscle cells of the great arteries in histology (Fig. 3-a). On day 13 1/2, it became difficult to distinguish the cells in the A-P septum from the smooth muscle cells of the great arteries.

② Formation of the myocardial sheath and the tunica media in the truncus

On day 12 1/2 of gestation, the myocardial sheath occupied the outer layer of the distal truncus to the proximal conus. No definitive tunica media was formed within the truncal swellings. The mesenchymal cells in the upper truncus, which were prospective smooth muscle cells, were round or oval and different in shape from the multiform mesechymal cells in the lower conotruncus (Fig. 1-a).

On day 13.0, the myocardial sheath retracted slightly than that of day 12 1/2. In the upper truncus, uniform-size round and oval cells, which were prospective smooth muscle cells, packed densely together without a definitive lamellar structure (Fig. 1-b).

On day 13 1/6, the myocardial sheath retracted to the level of the dividing valvar anlages. The two separated vascular walls above the valvar anlages were densely packed

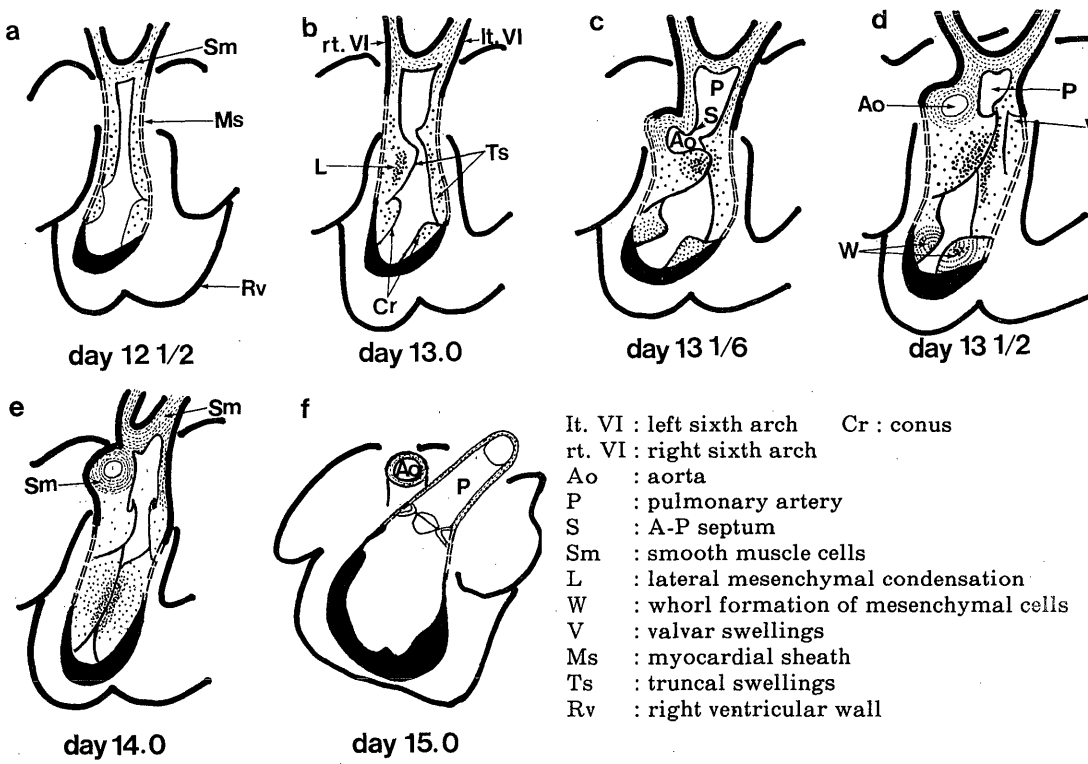

Fig. 1. Schematic diagrams showing the A-P septation, formation of the tunica media of the great arteries and retraction of the myocardial sheath in the normal rat hearts.

a : Day 12 1/2. Conotruncus is a single tube without septation. Tunica media is not formed. The myocardial sheath occupied the outer layer of the distal truncus to the proximal conus. The mesenchymal cells are scattered in the conotruncus with a slight whorl.

b : Day 13.0. The truncal swellings and the conus ridges are well developed. Lateral mesenchymal condensation and whorl formations are prominent in the truncal swellings. The conus ridges are associated with a slight whorl. Prospective smooth muscle cells are irregularly arranged in the great arteries. The myocardial sheath slightly shortens than that of day 12 1/2.

c : Day 13 1/6. The A-P septum appears as a thick globus between ascending aorta and right sixth aortic arch. Upper truncal swellings are fusing to each other with densed lateral mesenchymal condensation. Prospective smooth muscle cells are densely packed in the great arteries. The myocardial sheath shortens up to the valvar anlages.

d : Day 13 1/2. The A-P septation continues near the proximal conus. Tunica media is almost formed. Whorl formations and lateral condensation increase in the truncal swellings and the conus ridges.

e : Day 14.0. The A-P septation nearly completes. Tunica media is regularly formed. The myocardial sheath shortens below valvar tissue.

f : Day 15.0. The A-P septation completes. The myocardial sheath is absorbed into the right ventricle.

with prospective smooth muscle cells which almost formed a regular lamellar structure (Fig. 1-c).

On day 13 1/2, the myocardial sheath was nearly at the same level as on day 13 1/6. The lamellar structure of prospective tunica media was fairly formed in the two divided vascular walls (Fig. 1-d).

On day 14.0, the myocardial sheath retracted to the level below the developing valvar tissue, while the smooth muscle cells of the great arteries progressed down to just above the valve tissue (Fig. 1-e).

The concentric lamellar structure of the

tunica media was completed on day 15.0 (Fig. 1-f). The myocardial sheath was nearly incorporated into the right ventricle on day 16 and 17.

③ Arrangement of the mesenchymal cells in the conotruncus

On day 12.5 of gestation, the mesenchymal cells tended to show slight whorls in the

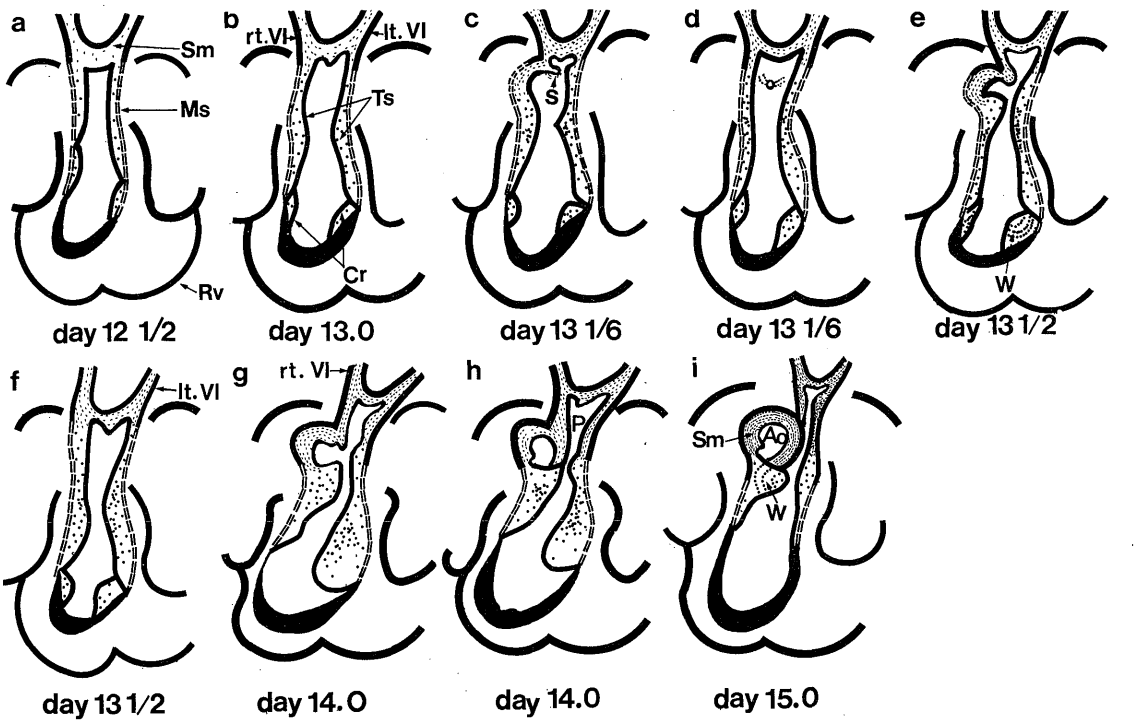

Fig. 2. Schematic diagrams showing formative process of cardiovascular anomalies in the treated rat hearts

a : Day 12 1/2. Conotruncus is a single tube and hypoplastic. The mesenchymal cell density is small.

b : Day 13.0. The truncal swellings and the conus ridges are hypoplastic, where whorl formations of the mesenchymal cells are not so prominent. The myocardial sheath is at similar level to that of day 12 1/2.

c : Day 13 1/6. Small and thin A-P septum appears between ascending aorta and right sixth aortic arch, forming pulmonary hypoplasia. The myocardial sheath slightly shortens than that of day 13.0. Whorl formations are slightly formed.

d : Day 13 1/6. PTA (Type II) is being formed without the A-P septation. The myocardial sheath still does not shorten. The mesenchymal cells are scattered in the truncal swellings and the conus ridges.

e : Day 13 1/2. The A-P septation is progressing to the level of valvar anlages, forming pulmonary hypoplasia. Prospective smooth muscle cells are arranged in vascular walls. Slight whorl formations and lateral mesenchymal condensation is seen in the truncal swellings and the conus ridges.

f : Day 13 1/2. Non-septation case is formed with hypoplsatic trucal swellings and the conus ridges, which is developing to PTA. The myocardial sheath does not retract and is at a level similar to day 12 1/2 and 13.0.

g : Day 13 1/2. Type I of PTA is formed with a small amount of the mesenchymal cells in the conus ridges.

h : Day 14.0. Pulmonary hypoplasia is almost complete with delay of diminution of the conus ridges. Whorl formations and lateral mesechymal condensation is poorly formed.

i : Day 15.0. Pulmonary hypoplasia is complete. The conus ridges are diminutive with a slight whorl formations.

proximal truncus, valvar anlages and conus (Fig. 1-a).

On day 13.0, a large number of the mesenchymal cells arranged in whorls within the proximal truncus and the distal conus. Lateral mesenchymal condensation was observed in each side of the prospective valvar anlages and the distal conus, but not in the proximal conus (Fig. 1-b).

On day 13 1/6, whorl formations of the mesenchymal cells were prominent in the valvar and infra-valvar regions, which associated with the occurrence of lateral mesenchymal condensation. The proximal conus was associated with slight whorls, but not with lateral mesenchymal condensation (Fig. 1-c).

On day 13 1/2, whorl formations and lateral mesenchymal condensation increased further below the valvar region and the proximal conus. Central mesenchymal condensation was observed at the fusing region of the valvar anlages (Fig. 1-d, 4-a, b).

On day 14.0, intense whorl formations were observed in the proximal conus ridges (Fig. 1-e).

Most of the whorl formations disappeared from the truncus on day 15.0 (Fig. 1-f), and whorls remaining only in the proximal conus disappeared on day 16.0.

2. *Expression of vimentin, desmin and fibronectin in the normal developing hearts*

(a) Vimentin

① Vimentin in the smooth muscle cells of the great arteries

Staining of the smooth muscle cells of the great arteries using vimentin began on day 13.0 of gestation and coincided with the time of truncal division and the appearance of the smooth muscle cells (Fig. 3-b).

On day 13 1/2, the smooth muscle cells of the great arteries were most intensely stained with vimentin, and thereafter the immunoreactivity decreased until disappearance on day 16.0 (Table 1.)

② Vimentin in the A-P septum

The A-P septum was depicted as a globular formation with positive vimentin staining on day 13 1/6 of gestation (Fig. 3-b). Thereafter it became difficult to differentiate between the mesenchymal cells of the A-P septum and the smooth muscle cells of the aorta (Table 3).

③ Vimentin in the mesenchymal cells within the conotruncus, semilunar valves and cardiac jelly

Vimentin was stained in the cytoplasm of almost all of the mesenchymal cells in the conotruncus, particularly in the cells within whorl formations, and lateral and central mesenchymal condensation.

On day 13 1/2 of gestation, the mesenchymal cells within central condensation, which were in the process of fusing, showed intense staining for vimentin and its globular cluster was prominent (Fig. 4-a). In lower portions, whorls and lateral mesenchymal condensation were stained with vimentin (Fig. 4-b). But the other mesenchymal cells in the valvar swellings, and cardiac jelly which did not contain the mesenchymal cells, showed no staining with vimentin (Table 3).

④ Vimentin in the myocytes in the atrium, ventricle and the myocardial sheath

Vimentin reactivity in the myocytes of the atrium, the ventricle and the myocardial sheath persisted from day 12 1/2 to 14.0 of gestation,

Table 1. Immunohistochemical properties of the smooth muscle cells in normal rat embryos

Antibody	Site	12 1/2	13.0	13 1/6	13 1/2	14.0	15.0	16.0	17.0
Vimentin	Aorta		(+)	+	++	+*	+*	−	−
	Pulmonary trunk		(+)	+	++	+*	+*	−	−
Desmin	Aorta		−	−	−	−	−	−	+
	Pulmonary trunk		−	−	−	−	−	−	+
Fibronectin	Aorta		−	+	++	++	+*	(+)	−
	Pulmonary trunk		−	+	++	++	+*	(+)	−

\+ : positive staining +* : focal positive staining (+) : faintly positive staining
++ : intensely positive staining − : negative staining

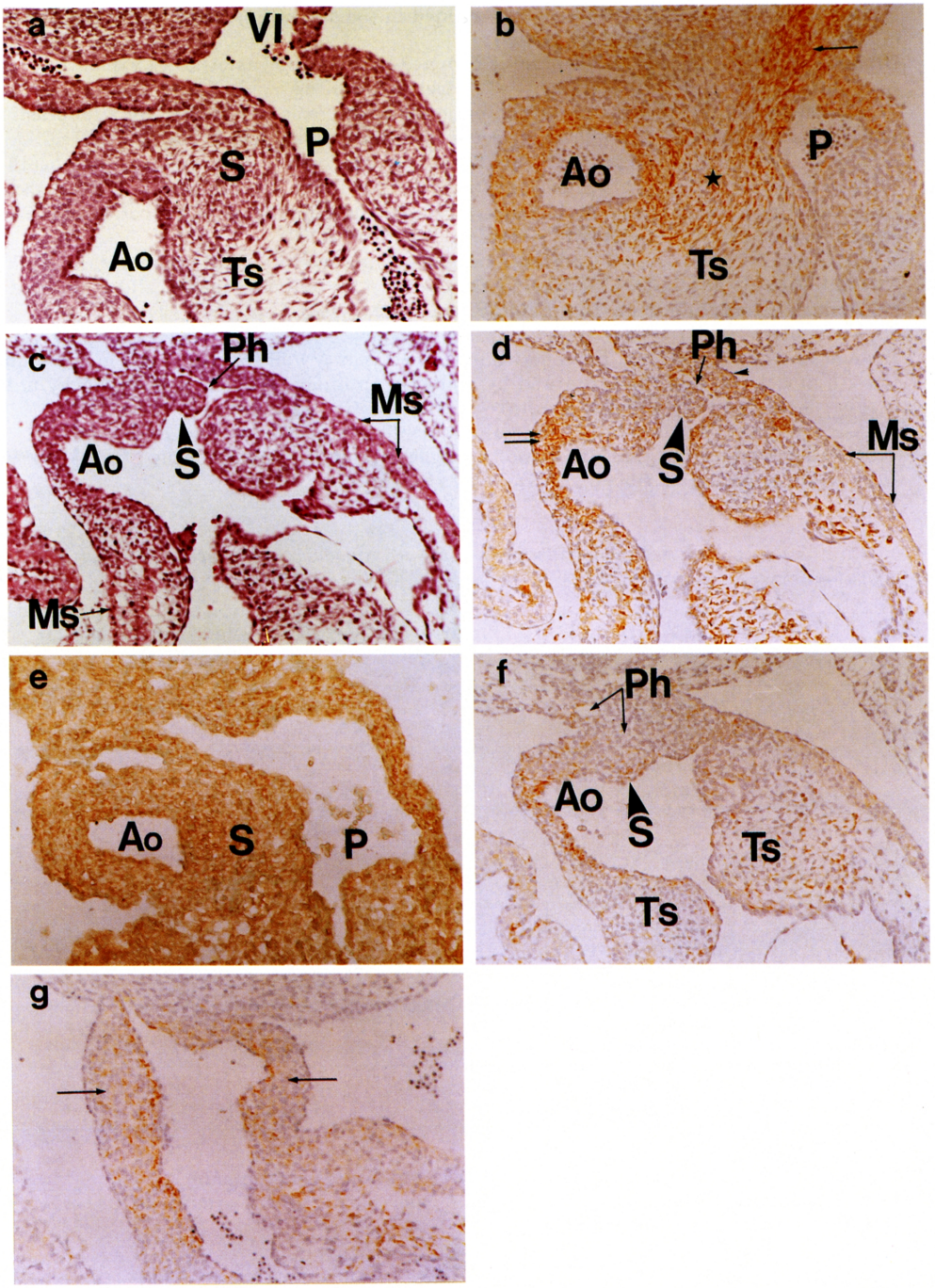

Fig. 3. Light microscopic and immunohistochemical findings of normal and treated rat hearts
a : Normal heart on day 13 1/6. The A-P septum fuses with the upper truncal swellings. Aortic and pulmonary channels are divided by the A-P septum, which forms mesenchymal globus. (H. E stain)
S : A-P septum Ao : aorta P : pulmonary artery Ts : truncal swelling
VI : right sixth aortic arch artery

b : Normal heart on day 13 1/6. Vimentin is stained in the smooth muscle cells of the aorta, pulmonary artery and sixth arches (arrow).
Globular formation of the A-P septum is depicted by vimentin (asterisk). (Vimentin, ×100)
Ao : aorta P : pulmonary artery Ts : truncal swelling

c : Treated heart on day 13 1/2. Aorta and pulmonary channels are divided by hypoplastic A-P septum (S). Pulmonary hypoplasia is seen as slit-like channel. (H. E stain, ×100)
S : A-P septum Ao : aorta Ph : pulmonary hypoplasia Ms : myocardial sheath

d : Treated heart on day 13 1/2. Vimentin is fairly stained in the smooth muscle cells of the aorta (double arrow), while faintly stained in hypoplastic pulmonary trunk (arrowhead). The A-P septum is also faintly stained with vimentin. (Viemtin, ×100)
S : A-P septum Ao : aorta Ph : pulmonary hypoplasia Ms : myocardial sheath

e : Normal heart on day 13 1/6. Fibronectin is stained in the smooth muscle cells of the aorta and pulmonary artery, and the A-P septum.(Fibronectin, ×100)
S : A-P septum Ao : aorta P : pulmonary artery

f : Treated heart on day 13 1/2. Fibronectin is stained faintly and focally in the A-P septum, the smooth muscle cells of the aorta and hypoplastic pulmonary trunk. The mesenchymal cells in the truncal swellings are also stained with fibronectin.(Fibronectin, ×100)
S : A-P septum Ao : aorta Ph : pulmonary hypoplasia Ts : truncal swelling

g : Treated heart on day 13 1/2. The smooth muscle cells in common truncus of PTA are faintly and focally stained with fibronectin (arrow).(Fibronectin, ×100)

Table 2. Immunohistochemical properties of the smooth muscle cells of great arteries in treated rat embryos

Antibody	Cardiovascular Anomalies		12 1/2	13.0	13 1/6	13 1/2	14.0	15.0	16.0	17.0
Vimentin	Non-septation			(+)	(+)	+				
	Ph	Aorta			+	+	+	+	+*	−
		pulmonary trunk			+*	(+)	(+)	(+)	−	−
	PTA	common truncus			+	(+)*	(+)*	(+)*	(+)*	−
		pulmonary trunk			(+)*	(+)*	(+)*	(+)*	−	−
Desmin	Non-septation				−	−				
	Ph	Aorta			−	−	−	−	−	−
		Pulmonary trunk			−	−	−	−		
	PTA	common truncus			−	−	−	−		
		pulmonary trunk			−	−	−	−		
Fibronectin	Non-septation			(+)	(+)*	(+)				
	Ph	Aorta			(+)*	(+)*	+	+	+	(+)
		pulmonary trunk			(+)*	(+)*	+	+	+	(+)
	PTA	common truncus				(+)*	+	+	+	−
		pulmonary trunk				(+)*	+	+	+	−

\+ : positive staining
++ : intensely positive staining
(+) : faintly positive staining
(+)* : faintly and focal positive staining
− : negative staining
Ph : pulmonary hypoplasia
PTA : persistent truncus arteriosus

and then gradually decreased. On day 16.0 and 17.0, vimentin staining disappeared completely from these areas (Table 5).

(b) Fibronectin

① Fibronectin in the smooth muscle cells of the graeat arteries

The smooth muscle cells of the aorta and pulmonary trunk began to express fibronectin from day 13 1/6 of gestation, and intense staining was observed on day 13 1/2 and 14.0. Lamellar structure of the tunica media was clearly revealed by fibronectin (Fig. 4-g). Fibronectin in the smooth muscle cells decreased from day 16.0 and began to localize in the

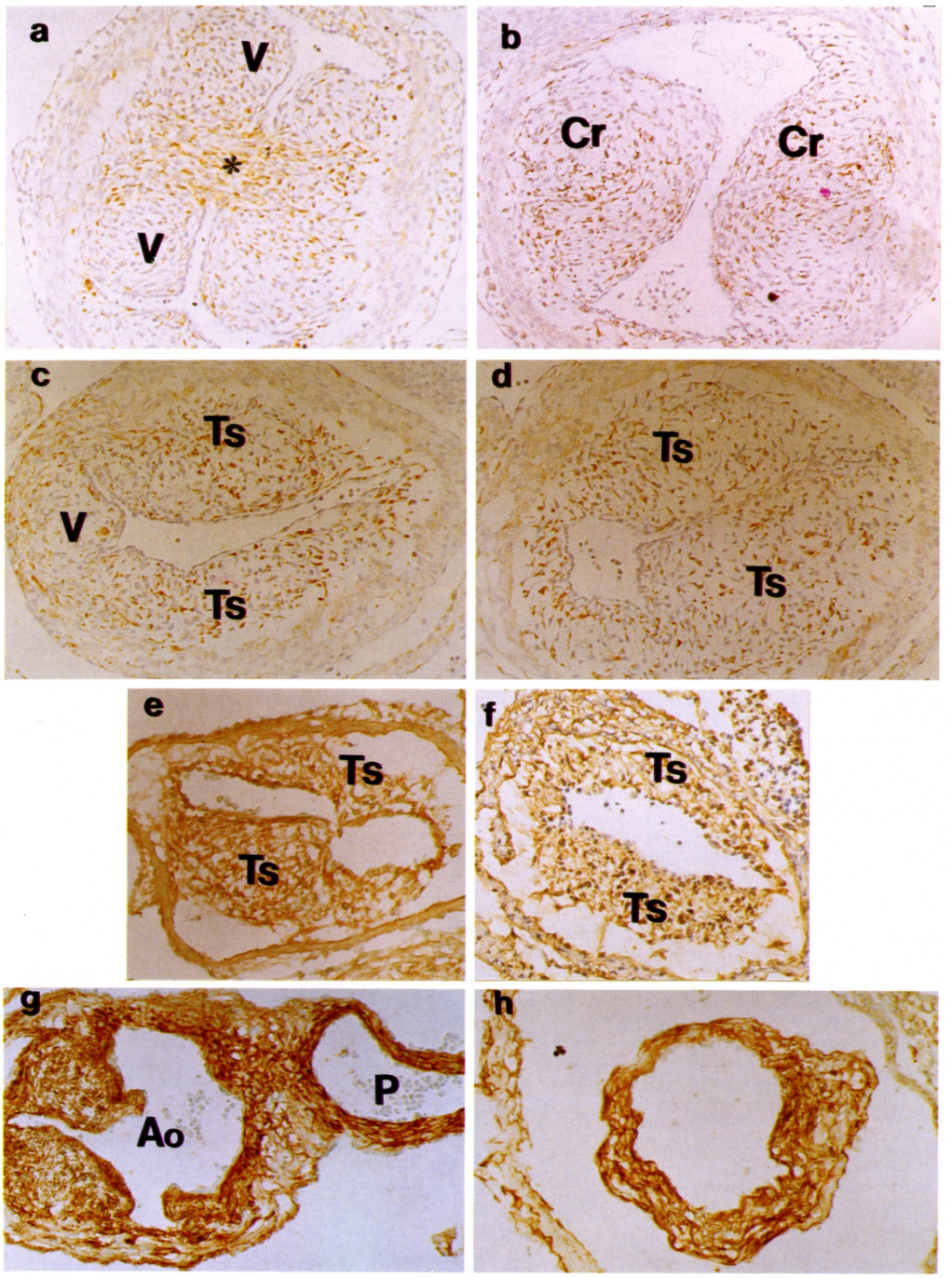

Fig. 4. Immunohistochemical findings of normal and treated rat hearts

a : Normal heart on day 13 1/2. Central mesenchymal condensation shows prominent reactivity with vimentin (asterisk). Vimentin reactivity is not so prominent in the scattered mesenchymal cells in the valvar tissue.
V : valvar tissue

b : Normal heart revealing proximal conus, more downward to Fig. 4-a on day 13 1/2. Mesenchy-

mal whorl formations in the conus ridges are depicted by vimentin staining.(Vimentin, ×100)
Cr : conus ridge

c : Treated heart on day 13 1/2. Scattered mesenchymal cells in the truncal swellings are stained with vimentin. The mesenchymal cells in the aortic intercalated valve swelling (V) are not stained with vimentin. Pulmonary intercalated valve swelling does not develop. (Vimentin, × 100)
Ts : truncal swelling V : aortic intercalated valve swelling

d : Treated heart more downward to Fig. 4-c on day 13 1/2. The mesenchymal cells in the truncal swellings are stained with vimentin, but whorl formations are not so prominent. (Vimentin, ×100 ; compare with Fig. 4-b).
Ts : truncal swelling

e : Normal heart on day 13 1/2. Fibronectin is stained in the mesenchymal cells and extracellular matrix of the truncal swellings, which are in the process of fusing. (Fibronection, ×100 : compare with Fig. 4-a,b)
Ts : truncal swelling

f : Treated heart on day 13 1/2. Fibronectin is stained in the mesenchymal cells and extracellular matrix of the truncal swellings. (Fibronectin, ×100 ; compare with Fig. 4-b, d, e)
Ts : truncal swelling

g : Normal heart on day 14.0. Fibronectin is stained intensely in the tunica media of pulmonary artery, aortic valve and intermediate region between the aorta and pulmonary artery. (Fibronectin, ×100)
Ao : aorta P : pulmonary artery

h : Treated heart on day 14.0. Fibronectin is stained in the tunica media of common truncus of PTA.(Fibronectin, ×100)

Table 3. Immunohistochemical properties of the A-P septum, mesenchymal cells and cardiac jelly in normal rat embryos

Antibody	Site		12 1/2	13.0	13 1/6	13 1/2	14.0	15.0	16.0	17.0
Vimentin	A-P septum				+	+*				
	Mesenchymal cells	conotruncus	+	+	++	++	+	+		
		semilunar valves			−	−	−	−	−	−
	Cardiac jelly		−	−	−	−	−	−		
Desmin	A-P septum				−	−				
	Mesenchymal cells	conotruncus	−	−	−	−	−	−	−	−
		semilunar valves	−	−	−	−	−	−	−	−
	Cardiac jelly		−	−	−	−	−	−		
Fibronectin	A-P septum				+	+*	+*			
	Mesenchymal cells	conotruncus	+	+	+	++	+	(+)	(+)	−
		semilunar valves			(+)	++	+	+	(+)	−
	Cardiac jelly		−	−	−	−	−	−		

\+ : positive staining
++ : intensely positive staining
(+) : faintly positive staining
+* : faintly and focal positive staining
− : negative staining

endothelium of the great arteries, and finally disappeared on day 17.0 of gestation (Table. 1)

② Fibronectin in the A-P septum

The A-P septum was entirely stained with fibronectin on day 13 1/6 of gestation, and partially stained on day 13 1/2 and 14.0 (Fig. 3-e, Table. 3).

③ Fibronectin in the mesenchymal cells within the conotruncus, semilunar valves and cardiac jelly

Fibronectin was stained in the cytoplasm of the mesenchymal cells and extracellular matrix

Table 4. Immunohistochemical properties of the A-P septum, mesenchymal cells and cardiac jelly in treated rat embryos

Antibody	Site		12 1/2	13.0	13 1/6	13 1/2	14.0	15.0	16.0	17.0
Vimentin	A-P septum				+*	+*				
	Mesenchymal cells	conotruncus	+	+	+	++	+	+	+	
		semilunar valves			−	−	−	−	−	−
	Cardiac jelly		−	−	−	−	−			
Desmin	A-P septum				−	−				
	Mesenchymal cells	conotruncus	−	−	−	−	−	−	−	−
		semilunar valves	−	−	−	−	−	−	−	−
	Cardiac jelly		−	−	−	−	−	−	−	−
Fibronectin	A-P septum				−	(+)*				
	Mesenchymal cells	conotruncus	+	+	+	++	+	(+)	(+)	
		semilunar valves				+	++	+	(+)	
	Cardiac jelly			−	−	−	−	−		

+ : positive staining
++ : intensely positive staining
+* : focal positive staining
(+) : faintly positive staining
(+)* : faintly and focal positive staining
− : negative staining

Table 5. Immunohistochemical properties of the atrium, ventricle and myocardial sheath in normal rat embryos

Antibody	Site	12 1/2	13.0	13 1/6	13 1/2	14.0	15.0	16.0	17.0
Vimentin	Atrium	+	+	±*	±*	±*	±*	−	−
	Ventricle	+	+	±*	±*	±*	−	−	−
	Myocardial sheath	+	+	+	±*	±*	−	−	−
Desmin	Atrium	−	−	−	−	(+)	(+)	+	+
	Ventricle	−	−	−	−	(+)	(+)	+	+
	Myocardial sheath	−	−	−	−	−	−	−	−
Fibronectin	Atrium	++	++	+	+	+	(+)	−	−
	Ventricle	++	++	+	+	+	(+)	−	−
	Myocardial sheath	++	++	+	+	(+)	(+)	−	−

+ : positive staining
++ : intensely positive staining
(+) : faintly positive staining
±* : focal positive in diffuse negative staining
− : negative staining

circumscribing the mesenchymal cells within the truncus and the conus all through embryonic stage, and was intensely stained on day 13 1/2 of gestation (Fig. 4-e). Fibronectin reactivity began to decrease from day 15.0 (Table. 3). Cardiac jelly, only in the myxomatous region which did not contain the mesenchymal cells, was never stained with fibronectin throughout the embryonic stage (Table. 3).

④ Fibronectin in the myocytes in the atrium, ventricle and the myocardial sheath

Myocytes of the atrium and ventricle were intensely stained with fibronectin on day 12 1/2 and 13.0 of gestation and its reactivity decreased from day 15.0, completely disappearing on day 16.0 (Table. 5).

⑤ Desmin staining

Desmin was not stained in the heart at any time from day 12 1/2 to 13 1/2 of gestation, but faint and partial staining appeared in both the atrium and ventricle on day 14.0. From day 15.0, desmin was fairly stained in both the

atrium and ventricle as the vimentin staining gradually disappeared (Table 5).

3. Morphogenesis of bis-diamine treated heart

(a) A-P septation in the treated heart

① Gross findings

The A-P septum and the A-P septation was not evident on day 12 1/2 and 13.0 of gestation. From day 13 1/6, pulmonary hypoplasia, type II of persistent common truncus arteriosus (PTA), and non-septation of the outflow tract could be identified (Fig. 2).

② Histological findings

On day 12 1/2 and 13.0 of gestation, the A-P septum and definitive A-P septation was not observed. The swellings of both truncus and conus were hypoplastic compared with those of the normal hearts (Fig. 2-a, b).

On day 13 1/6, pulmonary hypoplasia (two out of eight cases) and PTA (three out of eight cases) were identified. Pulmonary hypoplasia was divided into the aorta and pulmonary channels by a small and thin A-P septum at the level of the prospective semilunar valvar anlages (Fig. 2-c). No definitive septation was observed throughout the outflow tract in three other cases, showing a single tube. They may be the cases developing to the PTA (Fig. 2-d).

On day 13 1/2, out of eight cases examined, three were pulmonary hypoplasia (Fig. 2-e), one case was PTA (type II) and the remaining four showed non-septation of the outflow tract (Fig. 2-f). In the case of the pulmonary hypoplasia, septation of the outflow tract proceeded from the distal truncus to the valvar anlage, while the distal conus was just prior to dividing (Fig. 2-e ; Fig. 3-c, d, f). In the case of the PTA, the upper part of the truncus was hypoplastic without septation (Fig. 2-f, 3-g).

On day 14.0, conotruncal anomalies were nearly completed. Four cases of five were pulmonary hypoplasia and one was PTA (type I : Fig. 2-g). In the pulmonary hypoplasia, the A-P septation proceeded up to the level of the distal conus, and the two proximal conus ridges were prior to fusing (Fig. 2-h).

On day 15.0, three cases of five were pulmonary hypoplasia, one was PTA (type II) and one was a non-septation case (probably PTA). In the case of the pulmonary hypoplasia, the A-P septation proceeded up to the level of the distal conus, while the proximal conus ridges were in a stage just prior to fusing (Fig. 2-i).

On day 16.0 and 17.0, complete malformations were observed.

(b) Formation of the myocardial sheath and the tunica media, and arrangement of the mesenchymal cells within the conotruncus in the treated hearts.

① Formation of the tunica media in the A-P septum

On day 13 1/6 of gestation, prospective smooth muscle cells arranged irregularly within the thin A-P septum in the case of pulmonary hypoplasia (Fig. 2-c). Mesenchymal globular formation was not observed within the A-P septum. On day 13 1/2, it became difficult to distinguish histologically the cells in the A-P septum from the smooth muscle cells in the vascular walls of the great arteries (Fig. 3-c).

② Formation of the myocardial sheath and the tunica media in the truncus

Developmental retraction of the myocardial sheath was delayed in all cases of the pulmonary hypoplasia, PTA and non-septation through all the fetal ages compared to that of the normal hearts. Prospective smooth muscle cells arranged almost regularly within the vascular walls on day 13 1/2 (Fig. 2-e, f).

③ Arrangement of the mesenchymal cells in the conotrucus

On day 12 1/2 and 13.0 of gestation, the mesenchymal cells in the conotrucus showed less cell density and whorl formations compared with a normal one. Neither lateral nor central mesenchymal condensation was observed throughout the conotruncus (Fig. 2-a, b).

On day 13 1/6 and 13 1/2, whorl formations and lateral mesenchymal condensation were observed in the truncal swellings and the conus ridges, but not so prominent as that of the normal hearts. Central mesenchymal condensation was not observed because of a failure in conotruncal fusing. The distal and proximal conus ridges showed a retardation of development (Fig. 2-c, d, f ; Fig. 4-c, d).

On day 14.0, lateral mesenchymal condensation and whorl formations were poor in the conus ridges (Fig. 2-g, h).

On day 15.0, 16.0 and 17.0, the mesenchymal cells in the proximal conus, semilunar valves, and the endocardial cushion in the atrioventricular canal were arranged at random in an edematous stroma, associating with a slight whorl. Diminution of the conus ridges lagged half a day to a day behind that of the normal hearts. In the case of the pulmonary hypoplasia, the mesenchymal cells condensed and made a globular condensation at the subendocardium of the proximal conus. In the case of PTA, almost all the proximal conus ridges departed from the semilunar valves, and also from the cephalic portion of the interventricular septum.

2. Expression of vimentin, desmin and fibronectin in the treated hearts

(a) Vimentin

① Vimentin in the smooth muscle cells of the great arteries

In the case of the pulmonary hypoplasia, vimentin appeared in the smooth muscle cells in the aorta on day 13 1/6 of gestation, and persisted until day 16.0. Disappearance of vimentin from the aorta lagged a day behind that of the normal hearts (Table 2). Interestingly, the smooth muscle cells of the hypoplastic pulmonary trunk were weakly stained with vimentin compared with those of the aorta from day 13 1/2 to day 15 (Fig. 3-d), while the pulmonary trunk showed a similar intensity with the aorta in the normal hearts. In the case of PTA, the smooth muscle cells of both the common truncus and pulmonary arteries were faintly and focally stained with vimentin (Table 2, Fig. 3-g).

② Vimentin in the A-P septum

In the case of pulmonary hypoplasia on day 13 1/6 and 13 1/2 of gestation, the mesenchymal cells within a small thin A-P septum were faintly stained with vimentin (Fig. 3-d). But the cases on other gestation days showed no vimentin staining in the A-P septum (Table 4).

③ Vimentin in the mesenchymal cells within the conotruncus, semilunar valves and cardiac jelly.

There was no difference in vimentin staining of the mesenchymal cells in the conotruncus and semilunar valves between the treated and normal hearts. But whorl formations and lateral mesenchymal condensation which were stained with vimentin were slightly less in the treated hearts (Fig. 4-c, d ; Table 4).

④ Vimentin in the myocytes of the atrium, ventricle and the myocardial sheath

Vimentin reactivity in the atrium, ventricle and the myocardial sheath began to decrease from day 13 1/6 or 13 1/2 of gestation and its disappearance from the ventricle was about two days earlier than in the normal hearts (Table 6).

(b) Fibronectin staining

① Fibronectin in the smooth muscle cells of the great arteries

Prospective smooth muscle cells of the truncus arteriosus in non-septation cases were

Table 6. Immunohistochemical properties of the atrium, ventricle and myocardial sheath in treated rat embryos

Antibody	Site	12 1/2	13.0	13 1/6	13 1/2	14.0	15.0	16.0	17.0
Vimentin	Atrium	+	+	±*	±*	±*	±*	−	−
	Ventricle	+	+	±*	−	−	−	−	−
	Myocardial sheath	+	+	+	±*	−	−	−	−
Desmin	Atrium	−	−	−	−	−	(+)	+	+
	Ventricle	−	−	−	−	−	(+)	+	+
	Myocardial sheath	−	−	−	−	−	−	−	−
Fibronectin	Atrium	++	+	+	+	+	(+)	−	−
	Ventricle	++	+	+	+	+	(+)	−	−
	Myocardial sheath	++	+	+	+	(+)	(+)	−	−

\+ : positive staining
++ : intensely positive staining
(+) : faintly positive staining
±* : focal positive in diffuse negative staining
− : negative staining

weakly stained with fibronectin from day 13.0 to 13 1/2 of gestation (Table 2). In the case of pulmonary hypoplasia, the smooth muscle cells of both the aorta and pulmonary artery showed faint and partial staining of fibronectin on day 13 1/6 and 13 1/2 (Fig. 3-f) and showed fairly staining from day 14.0 to 16.0. Fibronectin in the smooth muscle cells began to disappear on day 18.0 and lagged a day behind that of the normal hearts (Table 2).

In the case of PTA, the smooth muscle cells of both the aorta and pulmonary artery showed faint and focal staining of fibronectin from day 14.0 to 16.0 of gestation (Fig. 4-h). Subsequently, fibronectin disappeared on day 17.0 (Table 2).

② Fibronectin in the A-P septum

The A-P septum was not stained with fibronectin on day 13 1/6 of gestation, but faintly and focally stained with it on day 13 1/2 (Fig. 3-f ; Table. 4).

③ Fibronectin in the mesenchymal cells within the conotruncus, semilunar valves and cardiac jelly

The mesenchymal cells showed almost the same staining of fibronectin as the normal hearts in the conotruncus, semilunar valves and cardiac jelly (Fig. 4-f ; Table 4). However, the time of occurrence of fibronectin lagged 1/3 day behind that of the normal hearts coinciding with the time lag of the formation of the semilunar valves in the treated hearts (Table 4).

④ Fibronectin in the myocytes of the atrium, ventricle and the myocardial sheath

Fibronectin staining in the atrium, ventricle and the myocardial sheath was similar with that of the normal hearts, but the intensity on day 13.0 of gestation was weaker than that of the normal hearts (Table 6).

(c) Desmin

Desmin was not stained in the smooth muscle cells of the A-P septum and the mesenchymal cells in the conotruncus, semilunar valves and cardiac jelly as in the normal hearts, while it began to be stained in the atrium and vetricle, lagging one day behind the normal hearts (Table 6).

DISCUSSION

The present study deals with the abnormal development of the A-P septum, outflow tract and their histochemical properties using antibodies of vimentin, desmin and fibronectin in the bis-diamine treated hearts.

1. Normal morphogenesis of the A-P septum and septation of the conotruncus

Recently, the anlage of the A-P septum is considered to be the mesenchymal cells or the smooth muscle cells of the great arteries, which are driven from branchial mesenchyme. In our study using normal embryonic rat hearts, septation through the distal truncus to the proximal conus was completed in only three days, which is comparable to five and three days in humans and mice, respectively. The dorsal wall of the aortic sac between ascending aorta and right sixth arch invaginates and forms mesenchymal condensation growing proximally to fuse the distal truncal swellings. We refer to this mesenchymal condensation as the A-P septum. However, it soon became difficult to distinguish the mesenchymal globus of the A-P septum from the smooth muscle cells of the great arteries.

SUMIDA *et al.*[32] simply described without detailed findings that the A-P septum appeared as mesenchymal condensation in the distal truncal swellings including the aortic sac. But from the reslut of this study, it is emphasized that the appearance of the A-P septum and septation of the conotruncus begins separately. As beforementioned, the A-P septum is a small vertical tubercle driven from the dorsal wall of the trunco-aortic sac between ascending aorta and right sixth aortic arch. On the other hand, the truncus septum is formed by a fusing of mesenchymal condensation in both the truncal swellings. Then, the proximal conus quickly appears to form mesenchymal condensation. Therefore, central or lateral mesenchymal condensation seems to be formed distally to proximally, concomitant with the truncus septation. It is concluded that the mesenchymal condensation in the truncal swellings and the A-P septum are essentially different and the

A-P septum are soon incorporated into the smooth muscle cells of the great arteries and upper ends of the truncal swellings.

2. *Relationship among the myocardial sheath, mesenchymal arrangement and the septation of the outflow tract*

In the normal hearts, considerable whorls of the mesenchymal cells and lateral mesenchymal condensation appeared to develop simultaneously with the initial retraction of the myocardial sheath and appearance of the A-P septum as THOMPSON *et al.*[34] described. It is suggested that bis-diamine suppresses the formation of mesenchymal condensation and sebsequently delays the retraction of the myocardial sheath, based upon the fact that the lateral mesenchymal condensation in the treated heart is hypoplastic compared with those in the normal hearts, and that the retraction of the myocardial sheath lags behind that of a normal one. Therefore, the formation of the mesenchymal condensation and retraction of the myocardial sheath may be necessary for the septation of the outflow tract.

3. *Expression of vimentin and desmin in the normal hearts and the bis-diamine induced anomaly*

The major subunit proteins of muscle-type and fibroblast-type intermediate filaments are now widely referred to as desmin and vimentin, respectively. Vimentin is an intermediate filament protein of mesenchymal cells, which in many cases coexists with GFAP, keratin and neurofilament protein as well as with desmin. On the other hand, desmin exists in muscular cells and distributes in Z-band of skeletal muscles, bridging myofilaments or between myofilaments and cytoplasmic membrane. NAG *et al.*[24] reported that these vimentin and desmin in embryonic cardiac myocytes showed a gradual increase in concentration as the cell matured in culture. BENNET *et al.*[2] and TOKUYASU *et al.*[35, 36] obtained the same results, that both desmin and vimentin were distributed in immature myotubes, but as the myotubes matured, vimentin gradually disappeared and was not detected after maturation, whereas desmin continued to appear in those which had matured. NAG[24] *et al*, and GARD and LAZARIDES[11] have never observed the disappearance of desmin and vimentin in developing myotubes in culture.

As to the expression of vimentin and desmin in the atrium and ventricle, the author agrees with the results of BENNET[2] and TOKUYASU[35, 36], because vimentin staining in the atrium and ventricle of the normal hearts switches to desmin staining on day 14 of gestation. In the bis-diamine treated embryos, this switching lagged one day behind the normal hearts. Furthermore, vimentin staining in the smooth muscle cells of the pulmonary trunk was less than that of the aorta in pulmonary hypoplasia and PTA. We could not explain the reason for this delayed appearance of desmin and a weak staining of vimentin in the pulmonary trunk of the treated hearts. Bis-diamine may have a direct or indirect suppressing action in delaying the switching and decreasing of the intensity of the staining in the hypoplastic pulmonary trunk.

SUMIDA *et al.*[32] reported in their recent study using chick embryos that the developing tunica media in the truncus did not react to the anti-desmin antibody at the time when cellular condensation in the A-P septum was intensely stained with it, and, on the contrary, staining in the A-P septum was decreasing when the tunica media of the great arteries was stained with anti-desmin antibody as the embryos grew. From these results, they concluded that the cells within the A-P septum are referred to as muscle-type cells, and that they are neither developing smooth muscle cells nor their precursor cells of the tunica media of the great arteries. In the present experiment, however, desmin has never appeared in the smooth muscle cells of the A-P septum, although it did appear in the great arteries of the normal hearts on day 17. This diverse results may have been caused by the difference in the antibody and animal species used.

Recent studies using quail-chick chimeras have shown that the neural crest cells contribute to the formation of the A-P septum and differentiate into the smooth muscle and elastin fibrils of the tunica media of the great arteries[5, 18, 19, 22]. It is a plausible explanation

of the present result that the globular formation depicted by vimentin in the normal hearts is derived from the neural crest, while globular formation is not depicted by vimentin in the treated hearts due to the failure of the neural crest cells' migration.

TOKUYASU *et al.*[35, 36] indicated the presence of desmin in muscle cells and its absence in fibroblast. In this study, the mesenchymal cells in the conotrucus, myocytes in the atrium and ventricle, and the smooth muscle cells of the great arteries show vimentin filaments at the early developmental stages of the hearts, and in the smooth muscle cells of the great arteries the expression of vimentin filaments switching to desmin filaments at the later stages of development. It is suggested vimentin filaments appeared in the immature tissue and acquired muscle-type nature of desmin filaments with maturation. Therefore, we agree partly with the result of SUMIDA *et al.*, yet it can not be denied that the cells within the A-P septum are developing smooth muscle cells or precursors of the smooth muscle cells of the tunica media of the great arteries. On the other hand, loose mesenchymal cells in semilunar valve leaflets were not stained with vimentin at all. This may be due to the acquisition of the mature type of cell nature, such as a fibroblast and/or collagenous nature in preexisting mesenchymal cells of the semilunar valve leaflets not to be stained with vimentin.

It is not known why the mesenchymal cells change their shape into fusiform at the fused region of the conotruncus. But it is conceivable that the intermediate filaments, particularly vimentin filaments, reorient the fused or fusing region, and activate or induce the fusing process of the conotruncus.

Interestingly, vimentin positive areas in the A-P septum and lateral or central mesenchymal condensation in the conotruncus of rats in this study corresponded to the desmin positive areas in the truncus swellings of the chick in the study by SUMIDA *et al.*. Further study is needed to clarify the species differences in the expression of vimentin and desmin as well as the switching mechanisms.

4. The relationship between the development of the normal hearts and the bis-diamine induced anomaly and the immunoreactivity of fibronectin

Fibronectin is a high-molecular-weight glycoprotein believed to play an important role in cell migratory processes, embryonic differentiation and neoplastic transformation in vitro as well as in vivo[6, 25]. Fibronectin was distributed in the areas similar to those with vimentin, and both fibronectin and vimentin increased with the production of the mesenchymal cells. It is suggested that fibronectin may play an important role in the interaction between these mesenchymal cells and the extracellular matrix, and in the process of cellular migration from endocardium toward myocaridum. As to the possible mechanisms, fibronectin may act as a role of contact guidance or as a vectorical component in the cardiac jelly with concentration gradient. But there was no evidence of a concentration gradient in the conotruncus in either normal or treated hearts in the present study. Fibronectin was positive in the tunica media of the great arteries and the semilunar valves during the development in both the normal and treated hearts in this study. However, it is uncertain whether accumulation of fibronectin in the developing tunica media is related to an attraction of the neural crest cells into the tunica media as described by SUMIDA *et al.*. The mesenchymal cells may be involved in the formation of the A-P septum and in the formation of the arterial tunica media as described by ICARDO[13], though the anlage of the semilunar valves was also intensely stained with fibronectin in this study, contrary to the findings of ICARDO[13]. The difference in antibody, method and species may affect the distribution and intensity of fibronectin staining. The fact of the delay in the most intense reactivity of fibronectin in the semilunar valves and the smooth muscle cells of the great arteries, and the weak reactivity in the great arteries and the A-P septum in the malformed hearts suggests that fibronectin is necessary for the development of the tunica media of the great arteries, the semilunar valves and the A-P septum. Thus, an insufficient introduction of the mesenchymal

cells in the anlage of these areas may lead to an insufficient production of fibronectins and result in anomalies of the A-P septation.

Fibronectin has recently been discussed in relation to intracellular actin cables[8], fibronectin receptor[8, 10], a cell surface linkage between fibronectin and cytokeratin, and an anchoring role of cells to the substrate to maintain normal morphology.

It would be necessary to carry out further immunoelectron microscopic study to identify the relationship between induced anomaly and the expression of immunohistochemical markers.

ACKNOWLEDGEMENT

The author would like to gratefully thank Professor T. IKEDA for his valuable guidance and encouragement, and to express my great appreciation to the members in the First Department of Pathology, Nagasaki University School of Medicine, for their excellent technical assistance and cooperation.

REFERENCES

1) AKIMOTO,N. : The study of the truncus artriosus and conus cordis. Hiroshima Med. J. 27 (1) : 103-129, 1979. (in Japanese with English abstract)

2) BENNETT, G.S., FELLIN, S.A., TOYAMA, Y. and HOLOTZER,H. : Redistribution of intermediate filament subunits during skeletal myogenesis and maturation in vitro. *J. Cell. Biol.* **82** : 577-584, 1978.

3) BINDER, M. : The teratogenic effects of a bis (dichloroacetyl) diamine on hamster embryos. *Am. J. Pathol.* **118** : 179-193, 1985.

4) BOCKMAN, D.E., KIRBY, M.L. : Dependence of thymus development on derivatives. *Science.* **223** : 498-500, 1984.

5) BOCKMAN, D.E., REDMOND, M.E., WALDO, K., DAVIS,H. and KIRBY,M.L. : Effect of neural crest ablation on development of the heart and arch arteries in the chick. *Am. J. Anat.* **180** : 332-341, 1987.

6) BOUCAUT,J.C. and DARRIBERE,T. : Fibronectin in early amphibian embryos. Migrating mesodermal cells contact fibronectin established prior to gastrulation. *Cell Tissue Res.* **234** : 135-145, 1983.

7) CARTER,S.B. : Haptotaxis and the mechanism of cell motility. *Nature.* **213** : 256-260, 1967.

8) CHEN,W.T., WANG,J., HASEGAWA.T., YAMADA,S. S. and YAMADA,K.M. : Regulation of fibronectin receptor distribution by transformation, exogenous fibronectin, and synthetic peptides. *J. Cell. Biol.* **103** : 1649-1661, 1986.

9) FANANAPAZIR, K. and KAUFMAN,M.H. : Observation on the development of the aortico-pulmonary spiral septum in the mouse. *J. Anat.* **158** : 157-172, 1988.

10) FRASER, M.B. : An antibody to a receptor for fibronectin and laminin perturbs cranial neural crest development in vivo. Dev. Biol. 117 : 528-536, 1986.

11) GARD, D.L. and LAZARIDES, E. : The synthesis and distribution of desmin and vimentin during myogenesis in vitro. *Cell.* **19** : 263-275, 1980.

12) HAYAKAWA, K., OKISHIMA, T., OHDO, S. and OKAMOTO,N. : Experimental study on pathogenesis and morphogenesis of congenital heart defects—conotruncal abnormality produced by bis (dichloroacetyl) diamine —. *Cong. Anom.* **23** : 267-277, 1983.

13) ICARDO,J.M. : Distribution of fibronectin during the morphogenesis of the truncus. *Anat. Embryol.* **171** : 193-200, 1985.

14) IGAWA, H.H., NAKAMURA, H. and YASUDA, M. : Bis (dichloroacetyl) diamine-induced craniofacial anomalies in Jc. I : ICR and A/J Mice. *Cong. Anom.* **25** : 45-56, 1985.

15) IWASAKI,K. : Bis-diamine induced anomalies of the thymus, parathyroid and thyroid in the rat. *Nagasaki Igk. Z.* **59 (2)** : 154-69, 1984. (in Japanese with English abstract)

16) JUBASHI,T. : Ontogenic development of thymocyte in normal and bis-diamine treated rats—Immuno-and enzymo-histochemical studies —. *Acta. Med. Nagasaki.* **30** : 119-138, 1985.

17) KAWAMOTO, K. : Morphological study on the cardiovascular anomalies and the facial anomalies induced by bis-diamine in rats. *Nagasaki. Igk. Z.* **59 (2)** : 86-105, 1984. (in Japanese with English abstract)

18) KIRBY,M., GALE,T.F. and STEWART,D.E. : Neural crest cells contribute to normal aorticopulmonary septation. *Science.* **220** : 1059-1061, 1983.

19) KIRBY, M.L. and BOCKMAN, D. : Neural crest and normal development : A new perspective. *Anat. Res.* **209** : 1-6, 1984.

20) KUTSCHE,L.M., VAN MIEROP, L.H.S. : Cervical origin of the right subclavian artery in aortic arch interruption : Pathogenesis and signifi-

cance. *Am. J. Cardiol.* **53** : 892-895, 1984.
21) LEE,J.Y. : Morphological studies on the persistent truncus arteriosus, hypoplasia or atresia of the pulmonary trunk and aortic arch anomalies induced by bis-diamine in rats. *Hiroshima. Med. J.* **31(5)** : 751-772, 1983. (in Japanese with English abstract)
22) LE LIEVRE,C.S. and LE DOUARIN,N.M. : Mesenchymal derivatives of the neural crest : analysis of chimaeric quail and chick embryos. *J. Embryol. exp. Morph.* **34** : 125-154, 1975.
23) LOS,J.A. : Cardiac septation and development of the aorta, pulmonary trunk, and pulmonary veins : Previous work in the light on recent observations. Birth defects : *Orig. Art. Ser.* **14 (7)** : 109-138, 1978.
24) NAG,A.C., KREHEL,W. and CHENG,M. : Distributions of vimentin and desmin filaments in embryonic cardiac muscle cells in culture. *Cytobios.* **45** : 195-209, 1986.
25) NEWGREEN,D. and THIERY,J.P. : Fibronectin in early avian embryos : Synthesis and distribution along the migration pathways on neural crest cells. *Cell. Tissue. Res.* **211** : 269-291, 1980.
26) OKAMOTO,N., SATOW, Y., HIDAKA, N., AKIMOTO, N., SUMIDA,H., LEE J.Y. and NAKANO,M. : Congenital cardiovascular malformations— Morphogenesis on the truncoconal anomalies —. *Hiroshima RINMB.* 23 : 135-170, 1982. (in Japanese with English abstract)
27) OKISHIMA, T., OHDO, S., HAYAKAWA, K. and OKAMOTO N. : Pathogenesis of a bisdiamine-induced malformation complex in rat resembling DiGeorge syndrome. *Cong. Anom.* **25** : 29-44, 1985.
28) ORTS-LLORCA, F., PUERTA FONOLLA, J. and SOBRADO,J. : The formation, septation and fate of the truncus arteriosus in man. *J. Anat.* **134** : 41-56, 1982.
29) OSTER, G., SALGO, M.P. and TALEPOROS, P. : Embryocidal action of a bis(dichloroacetyl)-diamine : An oral abortifacient for rats. *Am. J. Obstet. Gynecol.* **119 (5)** : 583-588, 1974.
30) OSTER, G., KILBURN, K.H. and SIEGAL, F.P. : Brief communications Chemically induced congenital thymic dysgenesis in the rat : A model of the DiGeorge syndrome. *Clin. Immun. Immunopath.* **28** : 128-134, 1983.
31) ROBINSON,H.B. : DiGeorge's or the III-IV pharyngeal pouch syndrome : Pathology and a theory of pathogenesis. *Perspect. Pediatric. Pathol.* **2** : 173-206, 1975.
32) SUMIDA,H., NAKAMURA,H., AKIMOTO,N., OKAMOTO N. and SATOW,Y. : Desmin distribution in the cardiac outflow tract of the chick embryo during aortico-pulmonary septation. *Arch. Histol. Jap.* **50 (5)** : 525-531, 1987.
33) SUMIDA,H. : Study of abnormal formation of the aortic arch in rats : By methacrylate casts method and by immunohistochemistry for appearance and distribution of desmin, myosin and fibronectin in the tunica media. Hiroshima. *J. Med. Sci.* **37(1)** : 19-36, 1988.
34) THOMPSON, R.P., SUMIDA, H., ABERCROMBIE, V., SATOW,Y., FITZHARRIS,T.P. and OKAMAOTO, N. : Morphogenesis of human cardiac outflow. *Anat. Rec.* **213** : 578-586, 1985.
35) TOKUYASU,K.T., MAHER,P.A. and SINGER,S.J. : Distributions of vimentin and desmin in developing chick myotubes in vivo. I. Immunofluorescence study. *J. Cell. Biol.* **98** : 1961-1972, 1984.
36) TOKUYASU,K.T., MAHER,P.M. and SINGER,S.J. : Distributions of vimentin and desmin in developing chick myotubes in vivo. II. Immunoelectron microscopic study. *J. Cell. Biol.* **100** : 1157-1166, 1985.
37) VAN MIEROP, L.H.S., ALLEY, R.D., KAUSEL, H. W. and STRANAHAN,A. : Pathogenesis of transposition complexes I. Embryology of the ventricles and great arteries. *Am. J. Cardiol.* 216-225, 1963.
38) WEISS.P. : Guiding principles in cell locomotion and cell aggregation. *Exp. Cell. Res.* **8** : 260-281, 1961.