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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

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ABSTRACT

The pathogenesis of left or right aortic arch with aberrant subclavian artery was studied by making methacrylate casts and by immunohistochemistry using antidesmin, antimyosin and antifibronectin antibodies in the rat. Maternal rats were administered a total dose of 400 mg of N,N'-bis-(dichloroacetly)-diamine (bisdiamine) on day 9-10 after conception. It was demonstrated by statistical analysis that on day 18 after conception, the absorption rate was not significantly different between the controls and the group treated with bisdiamine. Left or right aortic arch with aberrant subclavian artery was seen in about 95% of the surviving embryos from the treated group. It was shown by the casts that the period of obliteration of the dorsal aorta in the embryos from the treated group was half day earlier than that of the control embryos. In the control embryos, the left 7th intersegmental artery moved to the proximal dorsal aorta to form the left subclavian artery during development. In the embryos from the treated group, such movement of the 7th intersegmental artery was retarded. Antimyosin staining demonstrated certain cells, presumably neural crest cells, migrating to the tunica media of the dorsal aorta. After these cells reached the developing tunica media, the immunoreactivities to the antimyosin and antidesmin antibodies appeared in the tunica media. The period of appearance of the immunoreactivities in the left dorsal aorta corresponded to the period of obliteration of the right dorsal aorta in the control embryos. In the embryos from the treated group, the appearance of immunoreactivities to the antidesmin and antimyosin antibodies was half to one day later than that of the control embryos. Antifibronectin staining demonstrated that fibronectin accumulated in the developing tunica media and disappeared from the tunica media. In the embryos from the treated group, however, the disappearance of fibronectin was one day earlier than that of the control embryos. From these results, it is suggested that the obliteration of the dorsal aorta is regulated by certain cells, probably neural crest cells and that the beginning of differentiation and function of the tunica media is regulated by these cells. Bisdiamine might inhibit the action or migration of the cells.

Key words: *Abnormal aortic arch, Methacrylate cast, Desmin, Myosin, Fibronectin*

In human cases of aberrant subclavian artery, the subclavian artery arises from the descending aorta as the fourth branch of the aortic arch and passes behind the esophagus. In cases of right aortic arch, the aorta passes backward to the right of the trachea and the esophagus (see review by Alley and Van Mierop[4], Stewart et al[53]). The pathogenesis of both anomalies has been explained by abnormal obliteration of the dorsal aorta (see review by Alley and Van Mierop[4], Nishimura and Okamoto[45]). It has been basically confirmed that the dorsal aorta between the 4th aortic arch and the 7th intersegmental artery, and the left dorsal aorta are abnormally obliterated in cases of aberrant subclavian artery and of right aortic arch, respectively. However, no information on the details and period of abnormal obliteration compared with that in normal development, is available because an experimental model which can frequently produce these abnormal aortic arches, has not yet been established.

N,N'-bis-(dichloroacetly)-diamine,1,8-octa methylene diamine (bisdiamine) produces cardiovascular anomalies in a high incidence, including aberrant subclavian artery and right aortic arch, in rat embryos following oral administration to the mother[20,25,37,38,46,47]. The rat experimental model using bisdiamine is very useful for analysis of mor-

phogenesis of abnormal aortic arch because the shape of the rat aortic arch and its branches is very similar to that of man. The author, therefore, attempted to analyze the morphogenesis of left or right aortic arch with aberrant subclavian artery by making methacrylate casts of the aortic arches at various developmental stages of rat embryos treated with bisdiamine.

Anomalies induced in rats by bisdiamine, such as aortic arch anomalies, persistent truncus arteriosus and hypoplasia or aplasia of the thymus, are produced in chick embryos by inhibition of the neural crest cell migration[2,7,29-31,44]. This finding suggests that bisdiamine may inhibit the migration of neural crest cells[47]. Since neural crest cells migrate into the tunica media of the aortic arch[2,39], neural crest cells may play an important role in tunica media formation and/or morphogenesis of the aortic arch. Thus, it is suggested that abnormal development of the aortic arch of the rat embryo treated with bisdiamine is attributable to failure of neural crest cell migration. However, light microscopic observation has not demonstrated any differences in the developing tunica media of the controls and of embryos treated with bisdiamine (see results in the present study). Another purpose of this study is to examine the differences in protein level between the tunica media of the controls and that of the embryos from the treated group.

Desmin, one of intermediate filament proteins, is usually found in muscle cells (see review by Lazarides[35,36]. In the chick embryo, desmin appears in the tunica media of the great arteries after division of the truncus arteriosus and is also found in cells within the aortico-pulmonary septum[54]. Since the cells within the aortico-pulmonary septum are also derived from the neural crest[2,29-31], the neural crest cells migrate into the truncus arteriosus and the aortic arches may differentiate into a type of cell exhibiting the presence of desmin. If migration failure of the cells is related to abnormal formation of the aorta, the period and/or amount of desmin appearance in the tunica media may be different between the controls and the embryos from the treated group.

The function of the tunica media may be different between the controls and the embryos from the treated group. The smooth muscle of the tunica media has contractile ability against blood pressure[51]. The contraction of either muscle and non-muscle fibers is due to association of myosin to actin (see review by Alberts et al[3], Darnell et al[12]). Thus, appearance of myosin in the tunica media can show contractile ability of the tunica media.

Fibronectin is a component glycoprotein of the extracellular matrix. Fibronectin promotes migration of neural crest cells and is distributed through the pathway of neural crest cell migration[13,14,43,56]. Fibronectin is also a cell-substratum adhesion molecule[18,22,59]. As fibronectin is well distributed in the chick embryonic tunica media, it may be one of the important molecules implicated in tunica media formation[23,24].

Today, information is poorly available on the appearance and distribution of the above proteins in the developing tunica media. To know the information, the author examined the appearance and distribution of desmin, myosin and fibronectin in the tunica media of the developing aortic arches of the controls and treated embryos using indirect immunofluorescence or immunoperoxidase.

MATERIALS AND METHODS

Donryu strain rats were used. Primipara females with body weight of about 250 g were caged overnight with matured males. Day 0 of pregnancy was presumed by detecting sperm in the vaginal smear at 10:00 AM on the following morning. Two hundred mg of N,N'-bis-(dichloroacetyl)-diamine, 1,8-octa methylene diamine (Sigma, USA) suspended in 1% aqueous solution of gum tragacanth was administered orally on days 9 and 10 of gestation. Non treated rats were used as controls. Offspring were sacrificed at either day 12, 13, 13.5, 14, 15, 16 or 18 of gestation.

Stereomicroscopy

Eighty two 18-day-old fetuses from 6 maternal rats treated with bisdiamine and 44 fetuses of the same age from 3 control rats were used. The thoracic wall of the specimens was removed in Ringer's solution under a stereomicroscope. The heart of the specimens was perfused from the inferior vena cava with 4% formaldehyde in 0.1M phosphate buffer. After perfusion, the specimens were fixed with the same fixative described above for more than 24 hr at room temperature. The heart and great vessels of the specimens were observed under a stereomicroscope. Aortic arch anomalies observed in this experiment were classified into left and right aortic arches with or without aberrant subclavian artery.

Methacrylate injection

Used in this study were 12 to 16-day-old embryos. The heart of the specimens was perfused with Ringer's solution from the inferior vena cava. Methacrylate (Mercox; Japan Vilene Co. Ltd., Japan) was injected into the left ventricle with a capillary connected to a 5 ml disposable syringe. During injection, the specimens were kept in Ringer's solution. Methacrylate overflowing from the embryo was removed with a small pipette prior to polymerization. The specimens were kept in Ringer's solution for 3 hr and then immersed in 15% KOH for over 72 hr at room temperature. The methacrylate casts were washed with distilled water.

Protein extraction

Desmin was roughly purified from adult chicken gizzard by a modified method of Hubbard and Lazarides[21]. The following procedure was performed at 0 — 4°C. The smooth muscle from about 200 g of chicken gizzards was homogenized with 140 mM KCl, 10 mM EGTA, 0.5 mM PMSF and 10 mM Tris (pH 7.5), and centrifuged at 6,500 rpm for 20 min. Soluble proteins were extracted by mixing precipitation with 140 mM KCl, 1 mM EGTA, 0.5 mM PMSF and 10 mM Tris. After centrifugation as described above, precipitation was mixed with 1 M acetic acid for 6 hr and centrifuged. Supernatant was neutralized with 1 M NaOH. Desmin was obtained as precipitation.

Proteins including vimentin were extracted by an altered method of Geisler and Weber[17]. Fresh bovine lenses were obtained from a local slaughterhouse. The lenses were homogenized with 50 mM Tris, 5 mM $MgCl_2$, 10 mM 2-mercaptoethanol and 0.5 mM PMSF, and centrifuged at 15,000 rpm for 30 min. After washing with the same solution, precipitation was mixed with 50 mM Tris, 8 M urea, 5 mM $MgCl_2$, 5 mM DTT and 0.5 mM PMSF for overnight. After centrifugation as described above, supernatant was used.

Myosin was purified from adult chicken gizzards by the method of Takano-Ohmuro et al[55].

Purified fibronectin from human plasma was purchased from Biomedical Technologies Inc., USA.

Immunoblotting

SDS-PAGE electrophoresis, using 10% slab gels, was carried out by the method of Laemmli[33]. Immunoblotting was performed by an altered method of Towbin et al[58]. Proteins were transferred from gels onto nitrocellulose sheets. For immunoreactions, the nitrocellulose sheets were incubated with PBS containing 3% bovine serum albumin and 0.2% sodium azide. After rinsing with PBS containing 0.05% Tween 20 (T-PBS), the nitrocellulose sheets were incubated with either antidesmin (Bioscience, Switzerland), antimyosin (Advance, Japan) or antifibronectin (Transformation, USA) rabbit IgG for 1 hr at room temperature. After rinsing with T-PBS, the nitrocellulose sheets were incubated with peroxidase labeled anti-rabbit IgG $(ab')_2$ fraction (Tago, USA) under the same condition as in the case of the primary antibodies and were then developed with 0.075% diaminobenzidin — 0.002% H_2O_2 for 10 min at room temperature.

Immunofluorescence

In this examination 12 to 16-day-old embryos were used. The specimens were fixed with 4% formaldehyde in 0.1 M phosphate buffer for 6 hr at room temperature. After dehydration with graded ethanol, the specimens were embedded in paraffin. Deparaffinized sections about 5 μm in thickness were preincubated with 0.1% trypsin in 0.1% $CaCl_2$ for 20 min at 37°C. This treatment has been reported to increase the sensitivity for the detection of antigens in tissues embedded in paraffin[19]. The following procedures was performed at room temperature. The sections were blocked with 20% normal goat serum for 20 min, and were incubated with 50—80 times diluted antidesmin or antimyosin antibodies for 1 hr at room temperature. For controls, non-immune rabbit IgG or the antibodies preincubated with the antigens overnight at 4°C were used instead of the primary antibodies. After rinsing, the sections were incubated with FITC-labeled anti-rabbit IgG $(ab')_2$ fraction (Tago, USA) for 1 hr. The sections were mounted with glycerol/PBS (1/1) solution.

Immunoperoxidase

The specimens and fixation were the same as those for immunofluorescence. The sections were blocked with 3% H_2O_2 for 10 min. After rinsing, the sections were incubated with 70 times diluted antifibronectin antibody. The control procedure was the same as those for immunofluorescence. After rinsing, the peroxidase conjugated streptoavidin — biotin system (Stravigen; BioGenex Lab., USA) was applied. Incubation time of biotinated antirabbit IgG and streptoavidin was each 20 min. The sections were developed with 0.075% diaminobenzidin — 0.002% H_2O_2.

RESULTS

Frequency of aortic arch anomalies

Because the animal care system was improved after our previous report in 1984[46], types and frequency of aortic arch anomalies induced by bisdiamine were re-examined. In the new system, temperature and humidity have been constantly kept at 24°C ± 2°C and 50% ± 10%, respectively, light has been automatically controlled for 12 hr of light and 12 hr of darkness per day, and sterile cages have been used.

Table 1. Frequency of aortic arch anomalies

Left aortic arch				Right aortic arch			
normal		with aberrant RSA		without aberrant LSA		with aberrant LSA	
3 (3.7%)	*2 (*4.1%)	72 (87.8%)	*30 (*62.5%)	1 (1.2%)	*0 (*0%)	6 (7.3%)	*16 (*12.5%)

RSA: right subclavian artery
LSA: left subclavian artery
* Data from the previous report[46]

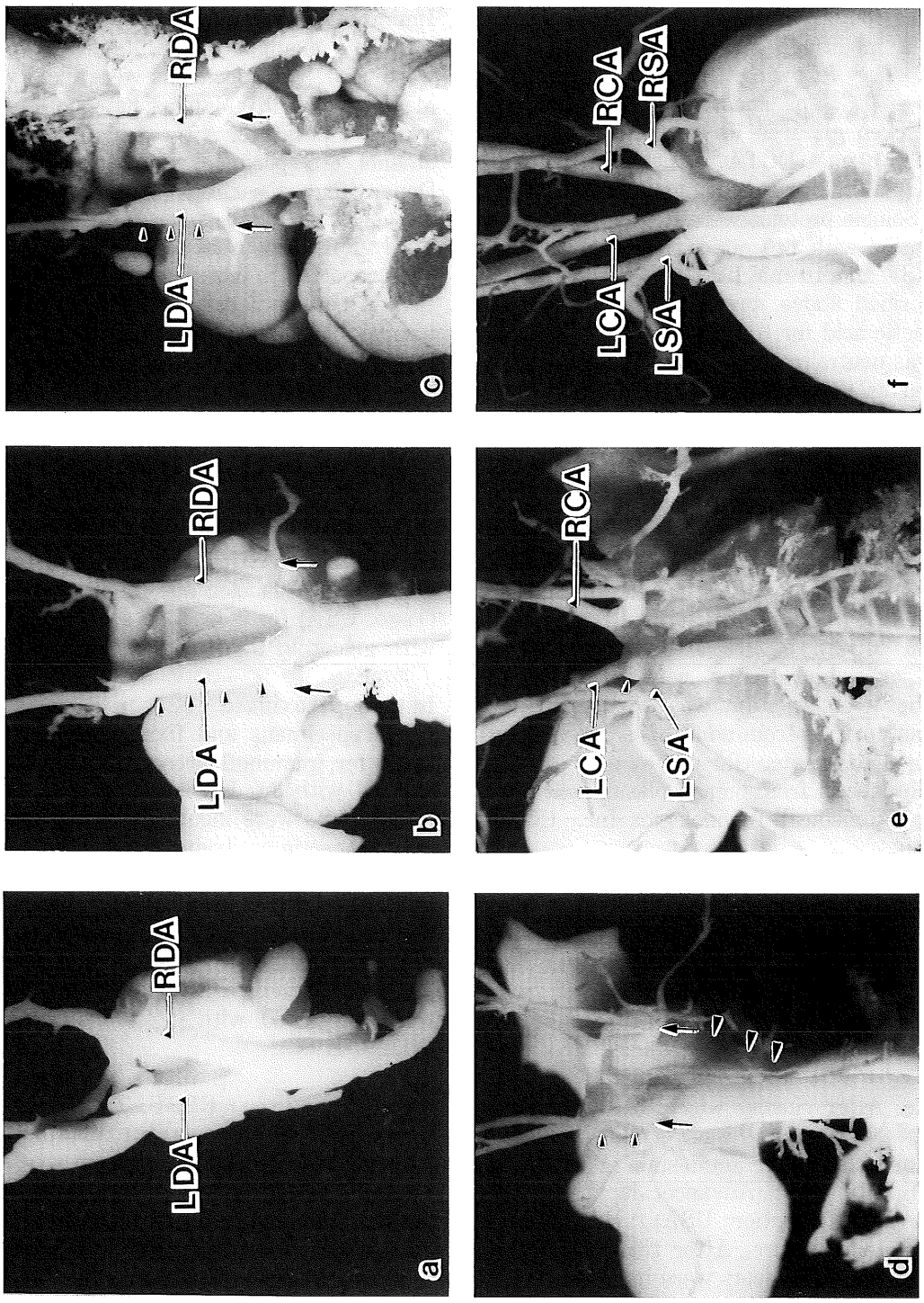

Fig. 1. Dorsal view of the methacrylate casts of the aorta in 12-day-old (a), 13-day-old (b), 13.5-day-old (c), 14-day-old (d), 15-day-old (e) and 16-day-old (f) control rat embryos. In the 13.5-day-old embryo (c), the diameter of the right dorsal aorta is smaller than that of the left. In the 14-day-old embryo (d), the right dorsal aorta distal to the 7th intersegmental artery (arrows) is obliterated (large arrowheads). Shortening of the dorsal aorta between the 4th arch artery and the 7th intersegmental artery can be seen (arrowheads). The right and left 7th intersegmental arteries are situated on the same transverse plane during development. RDA: right dorsal aorta, LDA: left dorsal aorta, RCA: right common carotid artery, LCA: left common carotid artery, RSA: right subclavian artery, LSA: left subclavian artery.

Survivors from the control and maternal rats treated with bisdiamine were 44 (100%) of 44 implants and 82 (94.2%) of 87 implants, respectively. No significant difference in numbers of survivors and absorptions between the control and the treated groups was shown by the chi-square test at a 5% significance level ($\chi^2=0.675$, $p>0.05$). This result was similar after Yate's correction ($\chi^2=0.234$, $p>0.05$). To examine the litter effects on the survival rate among 6 maternal rats treated

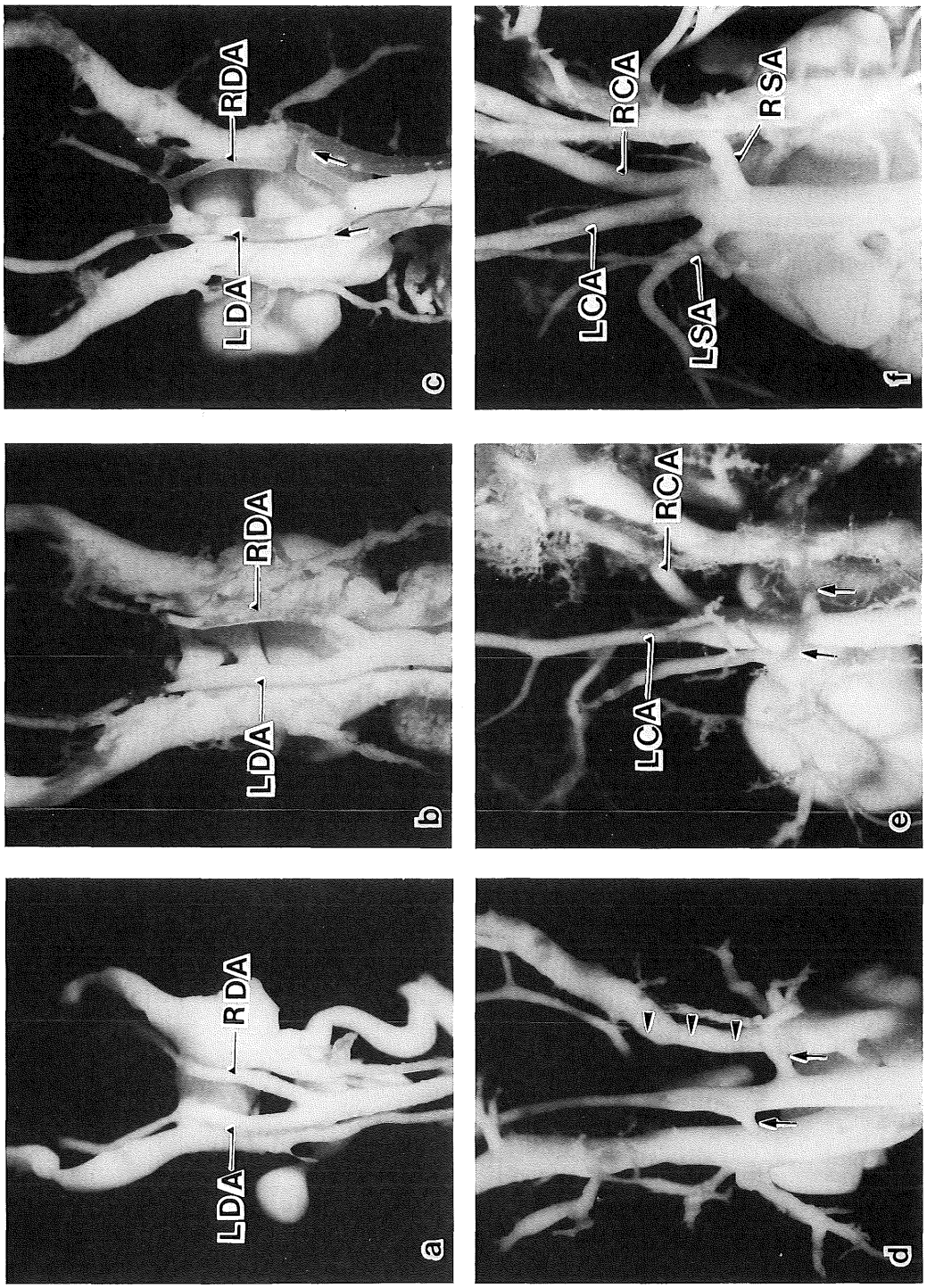

Fig. 2. Dorsal view of the methacrylate casts of the aorta in 12-day-old (a), 13-day-old (b), 13.5-day-old (c), 14-day-old (d), 15-day-old (e) and 16-day-old (f) rat embryos from the treated group, showing morphogenesis of the left aortic arch with aberrant right subclavian artery. In the 13-day-old embryo (b), the diameter of the right dorsal aorta is smaller than that of the left. In the 13.5-day-old embryo (c), the right aorta between the right 4th arch artery and the right 7th intersegmental artery is obliterating. In the 14-day-old embryo, the right dorsal aorta is obliterated (large arrowheads in d). Shortening of the left dorsal aorta between the left common carotid artery and the left 7th intersegmental artery is retarded compared with that of the controls. The right and left 7th intersegmental artery (arrows) are not situated on the same transversal plane. RDA: right dorsal aorta, LDA: left dorsal aorta, RCA: right common carotid artery, LCA: left common carotid artery, RSA: aberrant right subclavian artery, LSA: left subclavian artery.

with bisdiamine, a 2 × 6 contingency table of the number of survivors and absorptions from 6 maternal rats was designed. As a result, no significant difference was revealed by the chi-square test at a 5% significance level ($\chi^2 = 4.229$, $p > 0.05$).

Aortic arch anomalies were observed in 79 embryos (96.3%) of survivors from the treated group, and classified as shown in Table 1. To compare the

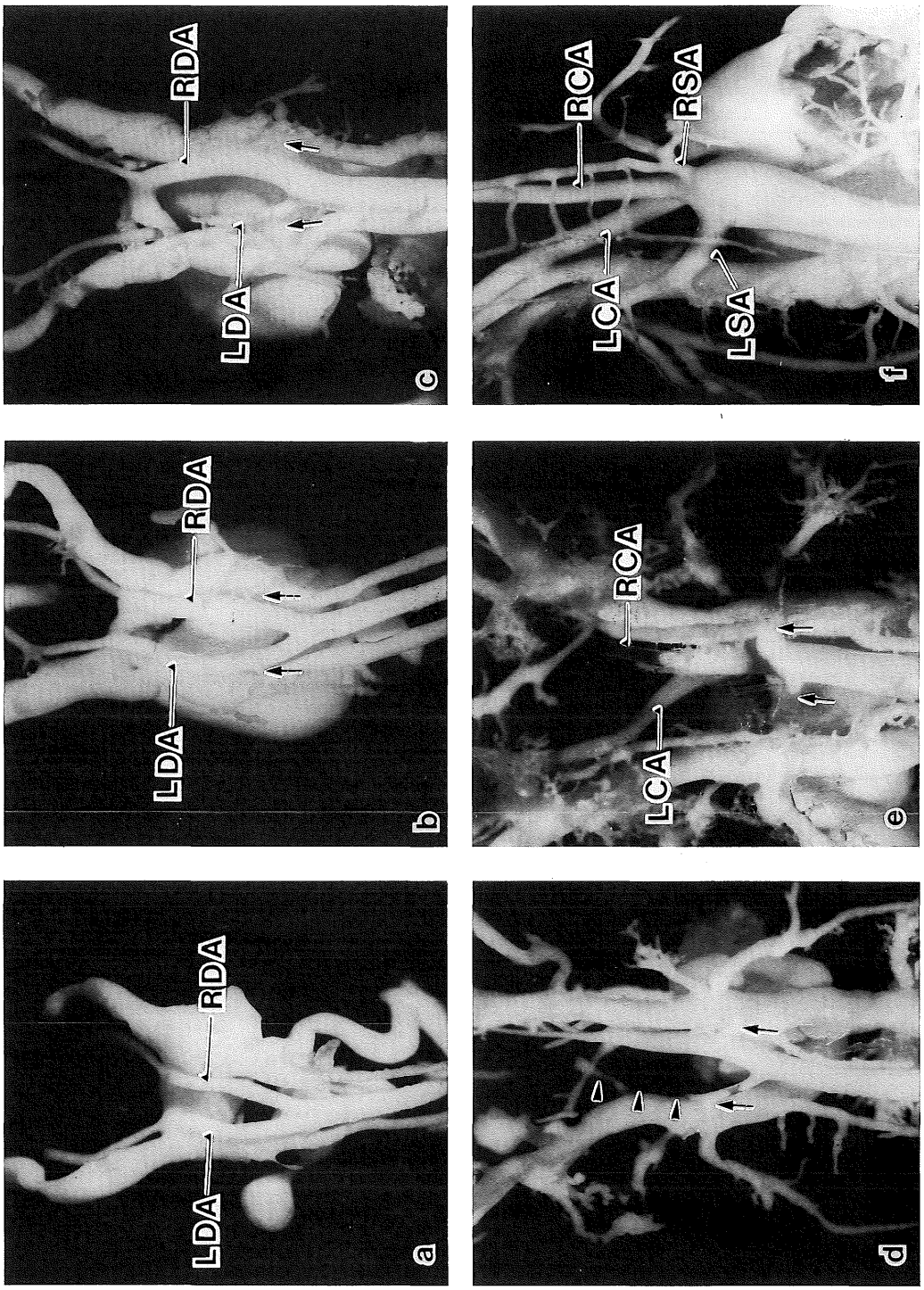

Fig. 3. Dorsal view of the methacrylate casts of the aorta in the 12-day-old (a), 13-day-old (b), 13.5-day-old (c), 14-day-old (d), 15-day-old (e) and 16-day-old (f) rat embryos from the treated group, showing morphogenesis of right aortic arch with aberrant left subclavian artery. Process of formation of right aortic arch with aberrant left subclavian artery is a mirror image of formation of left aortic arch with aberrant right subclavian artery (Fig. 2). The photograph same as Fig. 2a is used for Fig. 3a, because no specimens from the treated group at this stage show any indication of future aberration in the aortic pattern. RDA: right dorsal aorta, LDA: left dorsal aorta, RCA: right common carotid artery, LCA: left common carotid artery, RSA: right subclavian artery, LSA: aberrant left subclavian artery.

results with the previous data (* in Table 1), a 2 × 4 contingency table was designed for the chi-square test. The result showed a significant difference between the new and previous data at a 5% significance level ($\chi^2=8.379$, $p<0.05$).

Morphogenesis of the aorta

Morphogenesis of the aorta of the controls, of left aortic arch with aberrant right subclavian artery and of right aortic arch with aberrant left subclavian artery was demonstrated by methacrylate

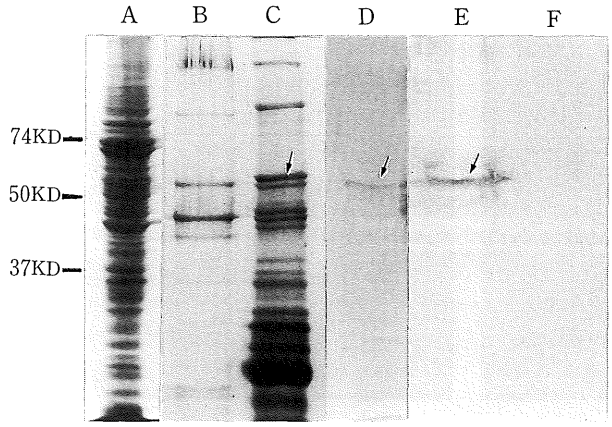

Fig. 4. Immunoblotting analysis for specificity of the antidesmin antibody. Line A: Coomassie blue staining of the total proteins from a 15-day-old rat embryo. Line B: Coomassie blue staining of roughly purified desmin from the adult chicken gizzards. Line C: Coomassie blue staining of the extract including vimentin (arrow) from the bovine lenses. Line D: Transferred protein of line A to nitrocellulose sheet. Binding of the antibody to an about 50 KD protein (arrow) can be seen. Line E: Transferred protein of line B to nitrocellulose sheet. Binding of the antibody to desmin (arrow) is recognized. Line F: Transferred protein of line C to nitrocellulose membrane. The antibody does not bind to any protein from the bovine lens.

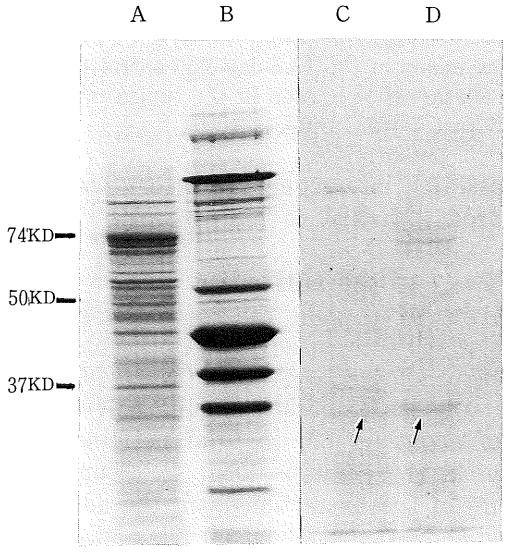

Fig. 5. Immunoblotting analysis for specificity of the antimyosin antibody. Line A: Coomassie blue staining of the total proteins from a 15-day-old rat embryo. Line B: Coomassie blue staining of roughly purified myosin from the adult chicken gizzards. Lines C and D: Transferred proteins of lines A and B to nitrocellulose sheet, respectively. Some nonspecific binding is observed. Arrow shows presumable light chain of myosin.

casts of the aortic arches of 12 to 16-day-old embryos (Figs. 1-3). In the 12-day-old control embryos, both the right and left dorsal aorta were found to

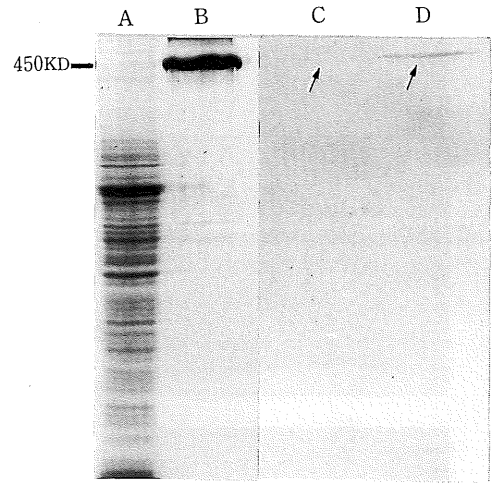

Fig. 6. Immunoblotting analysis for specificity of the antifibronectin antibody. Line A: Coomassie blue staining of the total proteins from a 15-day-old rat embryo. Line B: Coomassie blue staining of purified fibronectin from human plasma. Lines C and D: Transferred proteins of line A and B, respectively. Weak immunoreactivity of the antibody to a 450 KD protein (arrow in line C) is seen in line C. The antibody binds to fibronectin (arrow in line D) from human plasma.

have the same diameter (Fig. 1a). In the 13-day-old control embryos, the developing 7th intersegmental artery was observed with casts. The diameters of the right and left dorsal aortae were same (Fig. 1b). In the 13.5-day-old control embryos, the diameter of the right dorsal aorta was smaller than that of the left. In the 14-day-old control embryos, the distal region of the right dorsal aorta was obliterated (Fig. 1c). The diameter of the remaining right dorsal aorta was much smaller than the left. In histological sections, the vestigial right dorsal aorta was observed to be obliterating (Fig. 9d). The 7th intersegmental artery was localized in a more cephalic region by shortening of the dorsal aorta. In the 15 to 16-day-old control embryos, the morphological pattern of the aortic arch and its branches was developed into the adult type. The shortening of the dorsal aorta between the left common carotid artery and the left subclavian artery was remarkable (Fig. 1e, f). During development, the right and left 7th intersegmental arteries were localized at the same transverse plane.

In the 12-day-old embryos from the group treated with bisdiamine, the diameters of both the right and left dorsal aortae were almost same (Fig. 2, 3a). In the 13-day-old embryos from the treated group, the diameter of either the right (Fig. 2b) or left (Fig. 3b) dorsal aorta was smaller than that of the dorsal aorta on the opposite side. In the 13.5-day-old embryos from the treated group, abnormal obliteration of the dorsal aorta between the 7th intersegmental artery and 4th arch artery was observed with the casts (Fig. 2, 3c). In histological sections of the 13.5-day-old embryos from the treat-

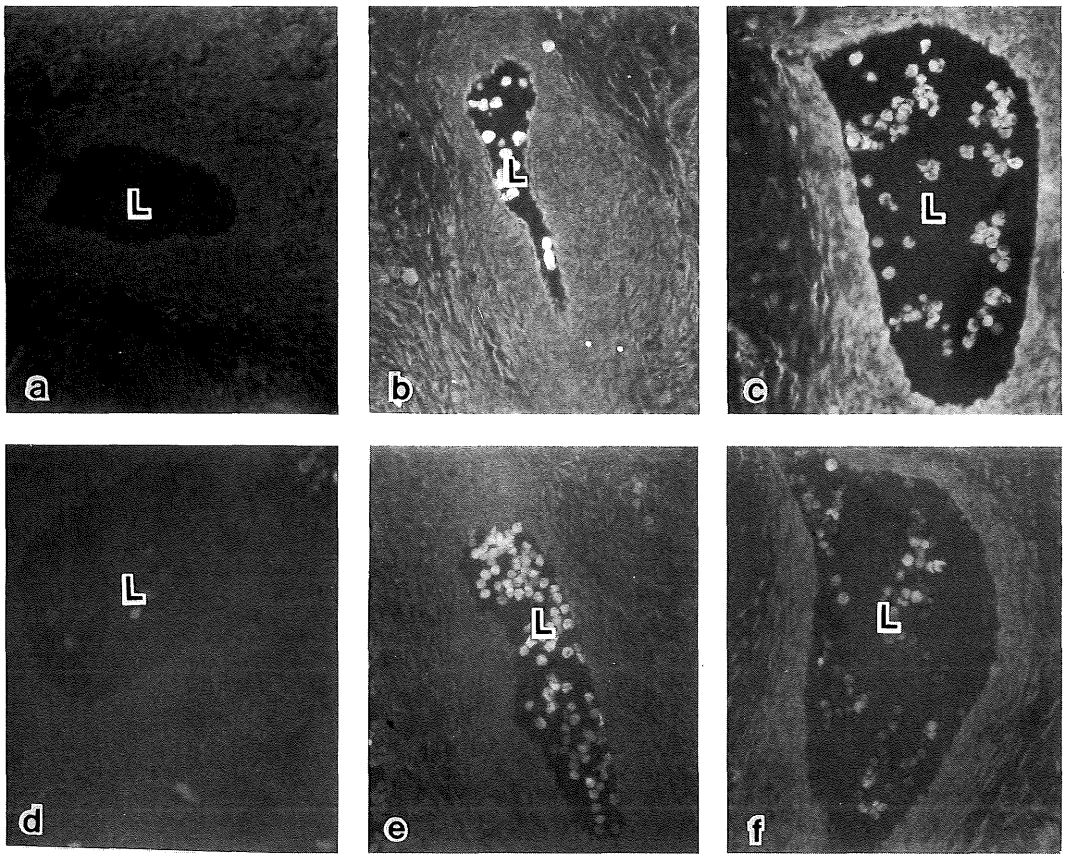

Fig. 7. Fluorescence micrographs of the antidesmin staining for the tunica media of the left dorsal aorta slightly proximal to the arising portion of the 7th intersegmental artery in 13.5-day-old (a), 14-day-old (b) and 15-day-old (c) control embryos, and in 13.5-day-old (d), 14-day-old (e) and 15-day-old (f) embryos from the treated group. Weak immunoreactivity is seen in the tunica media of the 13.5-day-old control embryo (a) but not of an embryo from the treated group (d). Intense fluorescence is seen in the tunica media in 14 (b) and 15-day-old (c) control embryos. L: lumen of the dorsal aorta. ×400.

ed group, the obliterating vestigial dorsal aorta was recognizable (Fig. 10c).

In cases of left aortic arch with aberrant right subclavian artery, the right dorsal aorta between the 4th arch artery and right 7th intersegmental artery was completely obliterated in the 14-day-old rat embryos (Fig. 2d). The right 3rd arch artery was forming the right common carotid artery, arising from the aortic arch proximal to the arising portion of the left common carotid artery. On the other hand, in cases of right aortic arch with aberrant left subclavian artery in the 14-day-old rat embryos (Fig. 3d), the left dorsal aorta between the left 4th arch artery and 7th intersegmental artery was obliterated. The developing right common carotid artery was arising from the aortic arch distal to the arising portion of the left common carotid artery.

In cases of right or left aortic arch, the period of obliteration of the dorsal aorta was almost same, being half day earlier than that of the controls.

During development in the treated group, the right and left 7th intersegmental arteries were not localized at the same transverse plane. Shortening of the dorsal aorta between the common carotid artery and 7th intersegmental artery was retarded compared with that of the controls.

Specificity of the antibodies

In order to examine the specificity of the antibodies which were used in the present study, immunoblotting was performed (Figs. 4-6).

The antidesmin antibody bound only to one protein of about 50 KD in total proteins from the 15-day-old rat embryos (Fig. 4). Coomassie blue staining of the extraction from the adult chicken gizzard showed that about 50 KD and 42 KD proteins were roughly purified. The antidesmin antibody bound only to an about 50 KD protein. The antidesmin antibody did not bind to any extract proteins including vimentin from the bovine lens.

The antimyosin antibody reacted to several proteins in the total proteins from the 15-day-old rat embryo (Fig. 5). Several proteins were observed in the extract including roughly purified myosin from the adult chicken gizzard with Coomassie blue staining. The antimyosin antibody bound not only to about 20 KD protein, probably light chain of myosin, from both 15-day-old rat embryo and adult chicken gizzard but also to some proteins from the

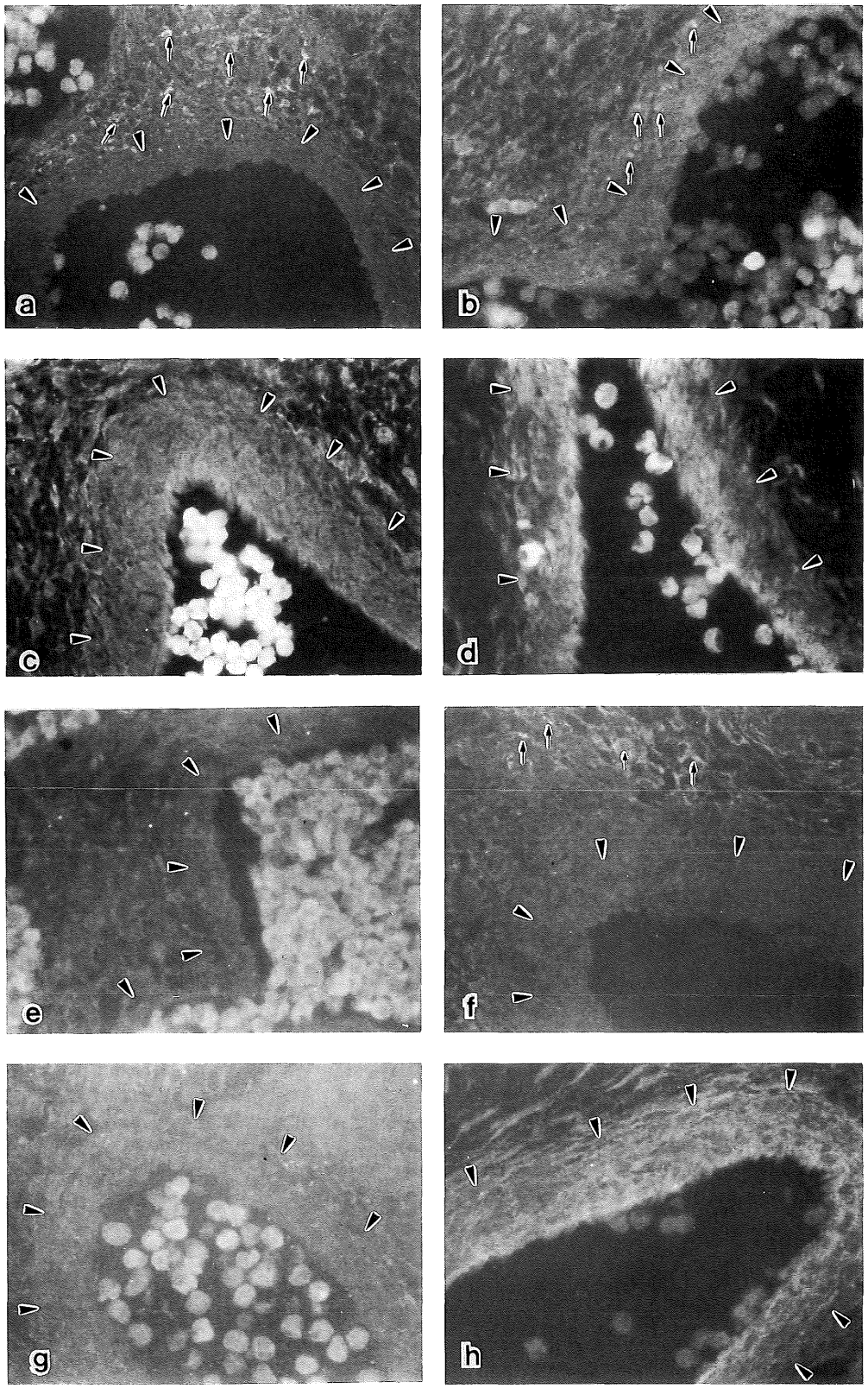

Fig. 8. Fluorescence micrographs of the antimyosin staining for the tunica media of the dorsal aorta distal to the arising portion of the 7th intersegmental artery in 13-day-old (a), 13.5-day-old (b), 14-day-old (c) and 15-day-old (d) control embryos, and in 13-day-old (e), 13.5-day-old (f), 14-day-old (g) and 15-day-old (h) embryos from the treated group. In the 13-day-old control embryo (a), the antibody positive cells (arrows) are scattered around the dorsal aorta shown by arrowheads, but few such cells are seen in the 13-day-old embryo from the treated group (e). In the 14-day-old (c) and the 15-day-old control embryos (d), intense fluorescence is observed in the tunica media. ×700.

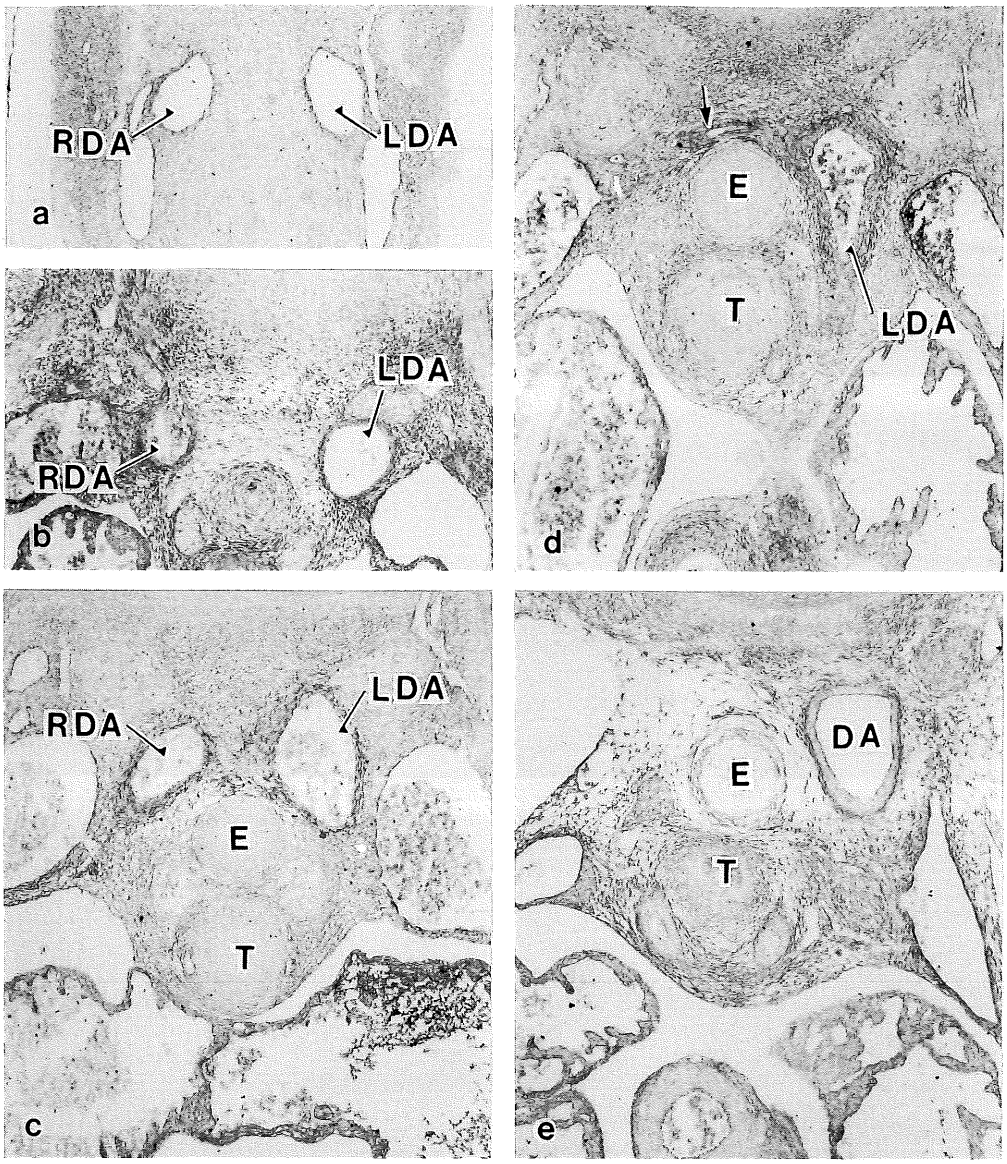

Fig. 9. Micrographs of the antifibronectin staining in 12-day-old (a), 13-day-old (b), 13.5-day-old (c), 14-day-old (d) and 15-day-old (e) control embryos. In (c), the diameter of the right dorsal aorta is smaller than the left, but not smaller than that in the 13-day-old control embryo (a). In the 14-day-old control embryo (d), obliterating right dorsal aorta is seen (arrow). Immunoreactivity to the antifibronectin antibody is intense in the tunica media of the 13 (b) to 14-day-old (d) embryo, but it is weak in the 15-day-old embryo (e). RDA: right dorsal aorta, LDA: left dorsal aorta, DA: descending aorta, E: esophagus, T: trachea. ×250.

chicken gizzard.

The antifibronectin antibody bound only to about 450 KD protein from the 15-day-old rat embryo (Fig. 6). This reaction was very weak, but the antifibronectin antibody revealed binding to purified fibronectin from human plasma.

Appearance and distribution of desmin, myosin and fibronectin

The appearance and distribution of desmin and myosin, the component proteins of the cytoskeletal filaments, were examined by immunofluorescence.

In the 13.5-day-old control embryos, weak immunoreactivity to the antidesmin antibody appeared in the tunica media of the dorsal aorta (Fig. 7a). In the 13-day-old control embryos, no fluorescence was observed in the tunica media. In the 14 and 15-day-old control embryos, intense fluorescence was observed in the tunica media of the dorsal aorta (Fig. 7b, c). In contrast, in the 13.5-day-old embryos from the treated group, no immunoreactivity to the antidesmin antibody was demonstrated in the tunica media of the dorsal aorta (Fig. 7d). In the 14-day-old embryos from the treated group, fluorescence was recognizable in the tunica media, but it was much weaker than that of the control embryos of the same age (Fig. 7e). In the 15-day-old embryos from the treated group, immunoreactivity to the antidesmin antibody was relatively apparent

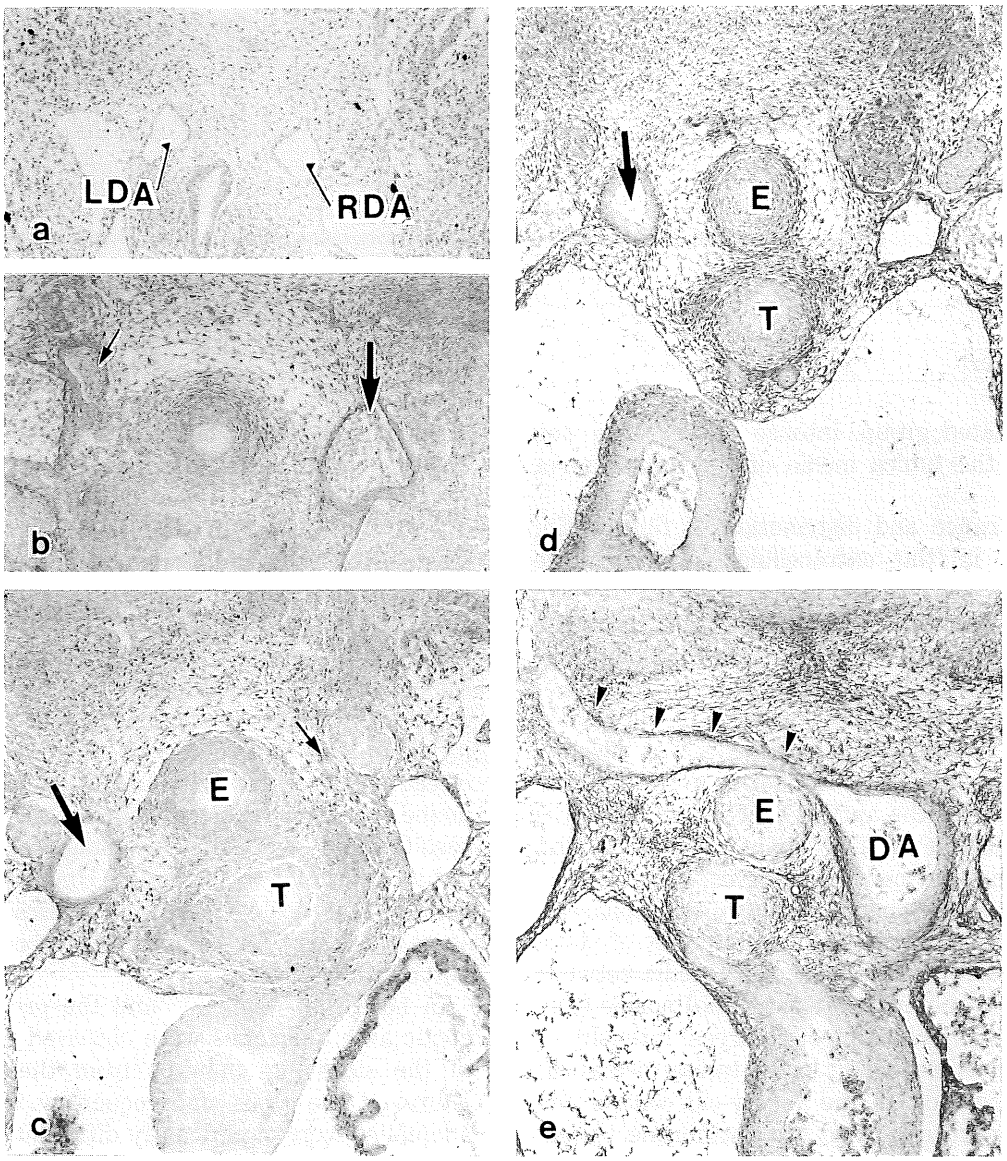

Fig. 10. Micrographs of the antifibronectin staining in 12-day-old (a), 13-day-old (b), 13.5-day-old (c), 14-day-old (d) and 15-day-old (e) embryos from the treated group. Immunoreactivity to the antifibronectin antibody is seen in the tunica media in the 13 (b) and 13.5-day-old (c) embryos from the treated group, but it is weak in the 14 (d) and 15-day-old (e) embryos from the treated group. Large arrows show the developing dorsal aorta, and small arrows show the obliterating dorsal aorta. Arrowheads show aberrant subclavian artery. RDA: right dorsal aorta, LDA: left dorsal aorta, DA: descending aorta, E: esophagus, T: trachea. ×250.

(Fig. 7f).

The appearance and distribution of myosin was also different in the tunica media of the controls from that of the embryos from the treated group. In the 13-day-old control embryos, no fluorescence was observed in the tunica media of the dorsal aorta, but intense fluorescence was scattered outside the tunica media of the dorsal aorta (Fig. 8a). In the 13.5-day-old control embryos, the scattered fluorescence was located closer to the dorsal aorta, and weak fluorescence was observed in the tunica media of the dorsal aorta (Fig. 8b). In the 14 and 15-day-old control embryos, intense fluorescence was observed in the tunica media of the dorsal aorta (Fig. 8c, d).

In the 13-day-old embryos from the treated group, the fluorescence outside the tunica media of the dorsal aorta which could be recognized in the controls was difficult to recognize (Fig. 8e). In the 13.5-day-old embryos from the treated group, however, the immunoreactivity to the antimyosin was observed around the tunica media of the dorsal aorta (Fig. 8f). In the 14-day-old embryos from the treated group, immunoreactivity to the antimyosin antibody was recognizable in the tunica media of the dorsal aorta, but the immunoreactivity was weaker than that of the control embryos of the same age (Fig. 8g). In the 15-day-old embryos

Table 2. Immunoreactivity to the antibodies in the tunica media

	Embryonic age			
	day 13	day 13.5	day 14	day15
Control				
Desmin	−	±	+	+ +
Myosin	−	+	+ +	+ + +
Fibronectin	+ +	+ +	+ + +	+
Treated				
Desmin	−	−	±	+
Myosin	−	±	+	+ +
Fibronectin	+ +	+ +	+	+

from the treated group, intense fluorescence was observed in the tunica media of the dorsal aorta (Fig. 8h).

The appearance and distribution of fibronectin, glycoprotein in the extracellular matrix, were demonstrated using immunoperoxidase. In the 12-day-old control embryos, immunoreactivity to the antifibronectin antibody was observed around the lumens of the dorsal aorta (Fig. 9a). At this stage, the tunica media was not yet formed. In the 13-day-old control embryos, the tunica media was developing around the lumens of the dorsal aorta, and immunoreactivity to the antifibronectin antibody was observed in the developing tunica media (Fig. 9b). In the 13.5-day-old control embryos, immunoreactivity to the antifibronectin antibody was apparent. No difference in distribution and intensity of staining was demonstrated in the tunica media between of the left and obliterating right dorsal aortae (Fig. 9c). In the 14-day-old control embryos, intensity of the antifibronectin staining in the tunica media was maximum (Fig. 9d). In the 15-day-old control embryos, immunoreactivity to the antifibronectin antibody in the tunica media decreased, though intense staining of the antibody was observed outside of the tunica media (Fig. 9e).

In the 12-day-old embryos from the treated group, immunoreactivity to the antifibronectin antibody was weak (Fig. 10a). In the 13-day-old embryos from the treated group, fibronectin positive layer was accumulating around the lumens of the dorsal aorta (Fig. 10b). In the 13.5-day-old embryos from the treated group, immunoreactivity to the antifibronectin antibody in the tunica media was maximum. No difference in distribution and intensity of the staining was demonstrated in the tunica media between the developing side and obliterating side of the dorsal aorta (Fig. 10c). In the 14 and 15-day-old embryos from the treated group, immunoreactivity to the antifibronectin antibody decreased in the tunica media but was observed around the tunica media (Fig. 10d, e).

All cases stained by the control procedure revealed negligible staining in the tunica media.

A summary of the results of immunohistochemistry applied to the developing tunica media is shown in Table 2. Because the tunica media was not yet formed, the results obtained from the 12-day-old embryos were omitted from the table.

DISCUSSION

Absorption rate, and type and frequency of aortic arch anomalies

Bisdiamine has a high lethal effect on chick embryos[10], but a low lethal effect of bisdiamine on rat embryos was reconfirmed by statistical tests in the present study. Bisdiamine has no dose specificity from 150 to 200 mg of treatment for lethal effect[37]. Stage specificity for lethal effect of bisdiamine has been reported[38]. In contrast, a previous result[20] has suggested no stage specificity for lethal effect of bisdiamine. Under the current dosage and dosing period, significant dose and stage specificity for lethal effect of bisdiamine may not be detectable.

In both the previous[46] and the present studies, aortic arch anomalies were observed in about 95% of the surviving embryos from the treated rat. However, the types and frequencies of aortic arch anomalies were significantly different between the previous and the present studies. Animal care system was changed after the previous report in 1984[46]. Environmental factors might have affected the results of the bisdiamine experiment. Lots of chemicals and/or genetic changes of the animals might also have changed the results of the experiment.

Although right aortic arch without aberrant subclavian artery had not been observed following administration before day 9.5 after conception[20,46,47], in the present study, only one case of this anomaly was observed. Probably, the period of abnormal obliteration of the right dorsal aorta distal to the 7th intersegmental artery is later than that of abnormal obliteration between the 7th intersegmental artery and the 4th arch artery.

Morphogenesis of the aorta

The methacrylate casts revealed the detailed aspects of aortic formation. The principal findings obtained with the methacrylate casts in the present study were as follows: 1) In the controls, the diameter of the right dorsal aorta decreased in the 13.5-day-old embryos, and in the 14-day-old em-

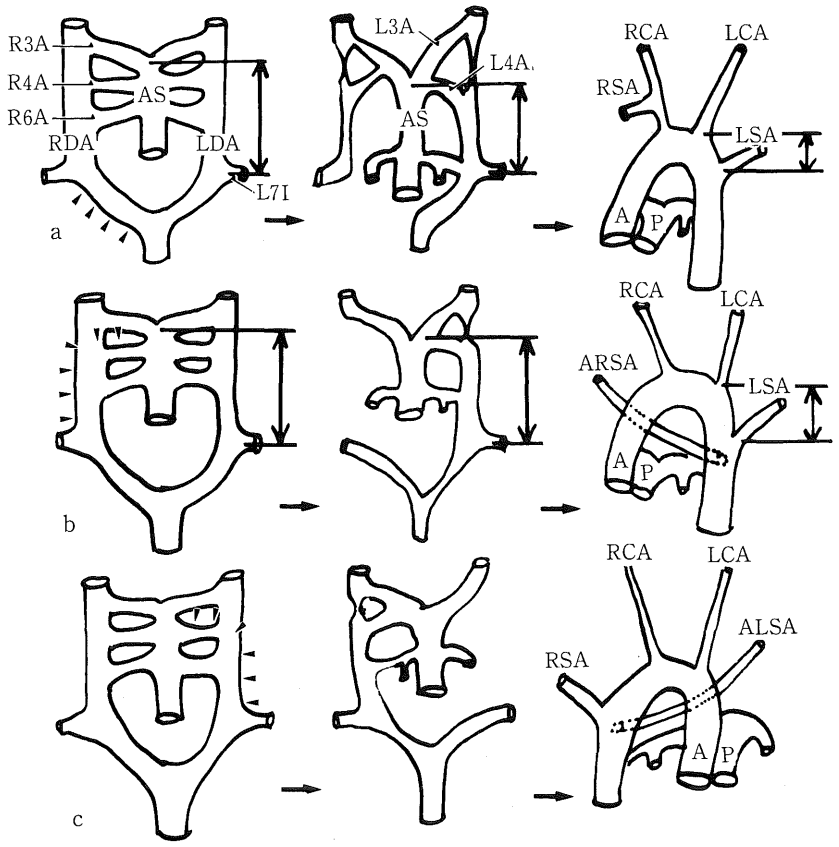

Fig. 11. Schemes showing the morphogenesis of the normal aortic arch (a), left aortic arch with aberrant subclavian artery (b) and right aortic arch with aberrant left subclavian artery (c). In normal development, the right dorsal aorta (RDA) distal to the right 7th intersegmental artery (arrowheads) obliterates. During development, the distance (double headed arrow) between the rising portions of the left 3rd arch artery and of the left 7th intersegmental artery decreases, resulting from development of the aortic sac (AS) to the caudal direction. In cases of left aortic arch with aberrant right subclavian artery (b), the right dorsal aorta between the right 4th arch artery and the right 7th intersegmental artery (arrowheads) obliterates. In cases of right aortic arch with aberrant left subclavian artery (c), the left dorsal aorta between the left 4th arch artery and the left 7th intersegmental artery obliterates. In this manner, the left common carotid artery (LCA) arises from the aortic arch proximal to the right common carotid artery (RCA). In cases of right or left aortic arch with aberrant subclavian artery (b, c), the decrease in the distance between the rising portions of the 3rd arch artery and of the 7th intersegmental artery is retarded from failure in development of the aortic sac. R3A: right 3rd arch artery, R4A: right 4th arch artery, R6A: right 6th arch artery, L3A: left 3rd arch artery, L7I: left 7th intersegmental artery, RSA: right subclavian artery, LSA left subclavian artery, ARSA: aberrant right subclavian artery, ALSA: aberrant left subclavian artery, A: aorta, P: pulmonary trunk.

bryos, the right dorsal aorta distal to the right 7th intersegmental artery was obliterated. In the embryos from the treated group, however, the process of obliteration of the dorsal aorta was half day earlier than that of the controls. 2) The dorsal aorta between the 4th arch artery and the 7th intersegmental artery was shortened during development. In the embryos from the treated group, the shortening of the dorsal aorta was retarded. 3) The 7th intersegmental artery rapidly developed by day 13. In the controls, the right and left 7th intersegmental arteries were situated on the same transversal plane during the development, but not in the embryos from the treated group.

Two possible causes for the earlier obliteration of the dorsal aorta in the embryos from the treated group than the controls can be considered. First, bisdiamine activated a factor promoting obliteration. Second, bisdiamine inhibited or changed the actions of a factor which negatively regulates obliteration. As a phenomenon similar to the obliteration of the dorsal aorta, the ductus arteriosus closes after birth. One of the factors promoting closure of the ductus arteriosus is oxygen[41]. The ductus arteriosus is closed by contraction of the tunica media and thickening of the intima[26]. This aspect of the closure, however, is different from the histological findings of the obliteration of the dorsal aorta. In the present study, the dorsal aorta obliterated before completion of the tunica media formation and no proliferation of any cells was observed in the obliterating dorsal aorta.

On the other hand, the hemodynamic theory has suggested that the direction of blood flow may be

related to the morphogenesis of the aorta[8,27,49]. Furthermore, it has been suggested that interruption or coarctation of the aorta is caused by hemodynamic factors[42,50]. In fact, interruption of the aorta is produced by experimental alterations of the blood stream in the chick embryo[11]. The hemodynamic theory has suggested that vessels with blood flow of sufficient magnitude remain but not the vessels with reduction of blood flow. A primary defect, such as disintegration of the aortic sac[6,46], by bisdiamine could change the direction of the blood flow. However, failure of the aortico-pulmonary septation is not a cause of the hemodynamic changes because the period of the abnormal obliteration is earlier than the period of the aortico-pulmonary septation[46]. Certainly, aberrant right subclavian artery is frequently complicated with aortic arch interruption[32]. According to the hemodynamic theory, narrowing of the vessels is located downstream of reducing blood flow. It was shown by the methacrylate casts in the present study that abnormal obliteration occurred in the dorsal aorta proximal to the 7th intersegmental artery, though the dorsal aorta of the downstream distal to the 7th intersegmental artery remained. Thus the hemodynamic theory is not plausible in explaining of abnormal obliteration of the dorsal aorta.

Barry[5] has described in detail shortening of the dorsal aorta in the aortic arch formation. Observations of the methacrylate casts agree with this description, but the mechanisms of the shortening of the dorsal aorta remain unclear. No cytological information about the shortening of the dorsal aorta, such as cell death, is available from previous studies[15,28]. The 7th intersegmental artery is localized distal to the ductus arteriosus, and the subclavian artery derived from the 7th intersegmental artery is finally localized proximal to the ductus arteriosus. This aspect of shortening of the dorsal aorta is difficult to explain by cytological and hemodynamic theories. Binder[6] has demonstrated in his bisdiamine experiment in hamster embryos that the heart develops caudally by elongation of the aortic sac. This relative movement of the heart against the pharyngeal pouch might result in lifting up of the 7th intersegmental artery. The relative movement of the rising position of the 7th intersegmental artery to the cephalic direction might give an impression of shortening of the dorsal aorta, that is, no histological shortening (Fig. 11a). Thus, it can be considered that retardation of the movement of the 7th intersegmental artery in the treated group results from developmental failure of the aortic sac[6,46] (Fig. 11b).

The situation of the right and left 7th intersegmental artery on the same transversal plane during development indicates that the lifting up force is even between the right and left. In early development, the 7th intersegmental artery arises from the region cephalic to the bifurcation of the right and left dorsal aorta. The obliteration of the dorsal aorta between the 4th arch artery and 7th intersegmental artery results in the rising of the developing 7th intersegmental artery from the region of the bifurcation. Thus, the aberrant subclavian artery arises below the normal subclavian artery which has arisen from the opposite side of the dorsal aorta (Fig. 11b, c)[5].

In the process of formation of right aortic arch, the left dorsal aorta between the left 7th intersegmental artery and 4th arch artery was obliterated. By this manner, it is resulted that the right common carotid artery arises from the aortic arch distal to the arising portion of the left common carotid artery (Fig. 11c). This morphological pattern of the branches is a criterion of the diagnosis for right aortic arch. Thus, in 14-day-old rat embryos, the diagnosis of right or left aortic arch can be made.

Specificity of the antibodies

The antidesmin and antifibronectin antibodies show a high specificity to bind to the antigens by immunoblotting. The molecular weight of desmin, protein of muscle type intermediate filament, is about 50 KD[35,36,52]. The antidesmin antibody had uni-binding specificity to an about 50 KD protein in total proteins from the 15-day-old rat embryos. The antibody reacted to desmin purified from the chicken gizzard. Desmin is molecularly similar to vimentin which is also one of the intermediate filament proteins[52], and is codistributed with vimentin in the tunica media[16,40]. The antidesmin antibody did not bind to vimentin extracted from the bovine lens.

The molecular weight of fibronectin is about 450 KD, composed by two subunits of about 220 KD protein[18,22,59]. The antifibronectin antibody reacted only to an about 450 KD protein in total proteins from 15-day-old rat embryos. The antibody was recognized to react to purified fibronectin from human plasma.

Several bands were observed in line for purified myosin from the adult chicken gizzard by SDS PAGE analysis. Some proteins, one of which is probably actin, might have been contaminated in the supernatant including myosin. The antimyosin antibody reacted to several proteins in total proteins from 15-day-old rat embryos. Although the control cases with the antibody preincubated with roughly purified myosin showed negative staining in the tunica media, it is not correct to assume that positive staining of the antimyosin antibody reveals presence of only myosin.

Appearance and distribution of desmin, myosin and fibronectin in the tunica media

Desmin is a component protein of the muscle type intermediate filament which is usually found in the muscle cells[35,36]. Some adult smooth muscles of

the aorta lack desmin[36]; in the present study, however, the appearance of desmin was demonstrated in the tunica media of the 14-day-old rat embryo. Desmin is densely distributed in the tunica media of the aorta in adult chickens[16]. In the chick embryo, desmin appears in the tunica media of the aorta at the period after division of the truncus arteriosus[54]. In 14-day-old rat embryos, division of the truncus arteriosus is almost complete[1,46]. Thus, the period of desmin appearance in the tunica media of the chick and rat embryos is similar.

Desmin also appears in the cells within the developing aorticopulmonary septum in the truncus arteriosus[54]. The cells within the aorticopulmonary septum are derived from the neural crest[2,29-31]. A preliminary study by the author et al, (abstract: Sumida et al, 1987. Develop. Growth and Differ. 29: 388) showed that, except the myocardium, the distribution of the cells exhibiting desmin in the truncus arteriosus corresponds to the distribution of the cells derived from the neural crest in the chick embryo. Since the cells in the tunica media of the aortic arch are derived from the neural crest[2,39], the neural crest cells may exhibit desmin themselves and/or have a key to express desmin in the tunica media of the aortic arch as well as in the aortico-pulmonary septum.

It has been suggested from the types of anomalies that bisdiamine inhibits the migration of the neural crest cells[47]. Bisdiamine produces persistent truncus arteriosus, a result of failure in development of the aortico-pulmonary septum, and hypoplasia or aplasia of the thymus besides aortic arch anomalies. In fact, these anomalies are produced by ablation of the premigratory neural crest in the chick[2,7,29-31,44]. In the treated embryo, desmin appeared in the tunica media of the aortic arch about one day later than the controls. The delay in appearance of desmin may depend on the failure of neural crest cell migration.

Immunoreactivity to the antimyosin antibody appeared in the tunica media in the 13.5-day-old control embryos but in the 14-day-old embryos from the treated group in the present study. The dominantly distributed cells in the tunica media are smooth muscle cells[48]. The smooth muscles in the tunica media contract against blood pressure[51]. The contractile ability in the muscles is due to rotation of the headpiece of myosin binding to actin[3,12,57]. Thus, it can be assumed that the tunica media in the controls gained contractile ability at day 13.5 but that from the treated group half day later.

In the 13-day-old control embryo, the immunoreactivity to the antimyosin antibody was observed outside the tunica media. In the 13.5-day-old control embryos, the immunoreactivity was observed closer to and in the tunica media. On the other hand, in the 13-day-old embryos from the treated group, the immunoreactivity of the antimyosin antibody outside the tunica media was rarely observed. In the 13.5-day-old embryos from the treated group, the immunoreactivity was observed outside of the tunica media but not in the tunica media. Thus, about half day delay of appearance of the immunoreactivity of the antimyosin antibody was also recognized outside the tunica media in the embryos from the treated group. Myosin is localized also in the non muscle fiber and provides migration ability to the cells[9,34]. The immunoreactivity to the antimyosin antibody which is observed outside the tunica media might demonstrate migrating cells into the tunica media. Bisdiamine might inhibit migration of these cells. Half day delay of appearance of immunoreactivity to the antimyosin antibody in the tunica media may be due to delay of migration of these cells.

In the 4-day-old chick embryo, neural crest cells migrate around the arch arteries, and form the tunica media of the aorta by the 6th day of incubation[29]. Since these developmental stages of chick embryo correspond to 13 to 14-day-old rat embryos, the migrating cells to the tunica media in the 13 to 13.5-day-old control embryos are presumably neural crest cells.

Fibronectin is a glycoprotein in the extracellular matrix[18,22,57]. Fibronectin has been demonstrated to be distributed in the developing tunica media and suggested to be implicated in tunica media formation[23,24]. The result of the present study agree well with distribution of fibronectin in the developing tunica media. Furthermore, the present study demonstrated that fibronectin disappears from the tunica media at a later stage. Fibronectin is well distributed through the migration pathway of neural crest cells[13,32,56]. Accumulation of fibronectin in the developing tunica media may be related to attraction of neural crest cells into the tunica media.

Distribution of fibronectin was not differed in the tunica media between the developing and obliterating dorsal aortae. This result shows that fibronectin does not regulate obliteration of the dorsal aorta.

In the embryos from the treated group, fibronectin disappeared from the tunica media one day earlier than the controls. The mechanisms of the earlier disappearance of fibronectin are still unknown. Disintegration of distribution of such an extracellular protein might induce abnormal morphogenesis. In contrast, some changes of function of cells producing fibronectin in the tunica media might result in an earlier disappearance of fibronectin.

Finally, the findings can be summarized as follows: Normal obliteration of the dorsal aorta was observed in the 13.5 to 14-day-old control embryos, and certain cells were migrating toward the developing tunica media in the 13 to 13.5-day-old con-

trol embryos. The certain cells were strongly presumed to be neural crest cells. The tunica media began to develop in the 13-day-old control embryos as well as those from the treated group. The immunoreactivities to desmin and myosin appeared in the tunica media in the 13.5 to 14-day-old control embryos and were intense in the 15-day-old control embryos. In the embryos from the treated group, obliteration of the dorsal aorta and disappearance of fibronectin was half to one day earlier than the controls. On the other hand, appearance of desmin and immunoreactivity of the antimyosin antibody in the tunica media was half to one day later than the controls. From the time of obliteration, appearance of migrating cells, and appearance of proteins as described above, the author suggests that: 1) formation of the aortic arch is regulated by neural crest cells; 2) the neural crest cells, migrating to the tunica media, not only form the tunica media but play a role in regulating the differentiation and function of the tunica media; and 3) one of the causes of abnormal formation of the aortic arch is inhibition of action and/or migration of the neural crest cells.

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