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Developmental Stage Dependent Expression of the Endothelial Stress Fibers and Organization of Fibronectin Fibrils in the Aorta of Chick Embryos

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ABSTRACT—Organizational relationships between endothelial stress fibers and fibronectin fibrils in the developing chick abdominal aorta, from 5th day embryos to 3rd day young chicks, were studied with immunofluorescence and electron microscopy. Stress fibers, axially aligned parallel to the longitudinal cell axis, were expressed in the largely elongated endothelial cells, in embryos older than 8th day of incubation. Fibronectin fibrils in the aortic basal lamina, changed its organizational pattern from the network-like form to the straight bundles arranged parallel to the vessel's longitudinal axis after 9th day of incubation. Such axial alignment was dominant in the matrix beneath the elongated cells containing stress fibers, suggesting the existence of stress fibers may possibly modify the fibronectin's organizational pattern. The vinculin-containing dense plaque, which shaped like as the adhesion plaque in the cultured cells, was located at the ends of or lateral associating sites of stress fibers in embryos older than 8th day stage. The expression of stress fibers, as well as the formation of stress fiber's end plaques, may closely relate to the alignment between the stress fiber and fibronectin fibrils in the extracellular matrix.

Key words: stress fiber, fibronectin, endothelial cell, aorta, chick embryo

INTRODUCTION

The stress fiber is a cable like structure consisting mainly of actin microfilaments and is supposed to enhance the cell adhesion to substrate (Byer *et al.*, 1984; Geiger *et al.*, 1984; Burridge, 1986; Gotlieb *et al.*, 1991). Vascular endothelial cell is the typical cell which expressed prominent stress fibers both *in vitro* and frequently in tissues. The formation of stress fibers is supposed to be one of the morphological responses demonstrated by the endothelial cells to the mechanical stresses produced by the laminar blood flow (Byer *et al.*, 1984; White and Fujiwara, 1986; Kim *et al.*, 1989b). The high hemodynamic force exerted upon the endothelium is one of the possible reasons why the cells elongated (Flaherty *et al.*, 1972; Levesque and Nerem, 1985; Kim *et al.*, 1989a) or the cells expressed stress fibers (Franke *et al.*, 1984; Wechezak *et al.*, 1985; Kim *et al.*, 1989b; Girard and Nerem, 1993). High frequency in the stress fiber distribution observed in the aortic endothelium of spontaneous hypertensive rats (Gabbiani *et al.*, 1975; White *et al.*, 1983), may also explain the positive influences of high

blood shear stress on the induction of stress fiber expression.

Although the physiological states of blood vessels including hemodynamic forces is supposed to change in the processes of the embryonic development, it is difficult to detect in the embryonic tissues. In our previous morphological study using the mesenteric artery of young chicks, we showed that the expression of stress fibers closely related to the cell length which was roughly proportional to vessel diameter (Jinguji and Fujiwara, 1994). As an other view point we focused our attention on the distribution of fibronectin fibrils in the aortic basal lamina, because the stress fiber may possibly enhance the cell-to-substrate adhesion (Byer *et al.*, 1984; Burridge, 1986; Gotlieb *et al.*, 1991). The arrangement and components of extracellular matrix are supposed to be modified following the expression of stress fibers in the overlaying cells. In our previous study, we showed that the fibronectin organizational pattern in the basal lamina changed as the stress fibers were expressed and/or the cells elongated in the artery (Jinguji and Fujiwara, 1994). Sugimoto *et al.* (1997) suggested in their study on rat embryonic aortae, that fibronectin fibrils in the basal lamina may change its organizational pattern depending on the size of vessels. In these studies using the developing blood vessels, however, the relations between the localization of actin

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cytoskeletons and extracellular matrix components has not been enoughly analysed. In the present study, the organizational pattern of actin microfilaments or fibronectin fibrils were examined in relation to the size of aorta or endothelial cell shape using the abdominal aorta obtained from 5th day embryos to 3rd day young chicks.

MATERIALS AND METHODS

Biological materials

The aorta was obtained from 5th to 20th day White leghorn chick embryos and 3rd day old young chicks. The posthatch chicks were anesthetized by the intraperitoneal injection of 0.2 ml of 5 mg/ml pentobarbital sodium water solution (nembutal, Abbott Co., USA) and the blood vessels were fixed by perfusion via the ventricle. The fixatives used are described below with a description of microscopy procedures.

Antibodies and fluorescent reagents

Rhodamine- or fluorescein-conjugated phalloidin was purchased from Molecular Probe, Inc. (Eugene, OR, USA). Rabbit antibody to bovine fibronectin was obtained from Advance (Tokyo, Japan). Mouse monoclonal antibody (IgG) to chicken gizzard vinculin was obtained from Sigma Chemical Co. (St. Louis, Mo, USA). Rhodamine-conjugated goat anti-rabbit IgG and rhodamine-conjugated rabbit anti-mouse IgG were obtained from Cappel Laboratories (Malvern, PA, USA). An in-house preparation of mouse monoclonal antibodies (IgM) against unknown antigens in the human platelet were used to visualize endothelial cells shape since they stained the entire cytoplasm of the cells (Jinguji and Fujiwara, 1994). Protein-A gold (5 nm) were from Amersham (Amersham International plc, Amersham, UK).

Immunofluorescence microscopy

Blood vessels of chick embryos were fixed by perfusion with 2% paraformaldehyde in phosphate-buffered saline (PBS) for 10 min and the aorta was then excised. En face whole-mount preparations were used for studies at the fluorescence microscopic level.

After fixation, the aorta was opened longitudinally using fine-tipped scissors to exposed the luminal surface and were then cut into small pieces. The vessel pieces were washed in PBS, and incubated in PBS containing 5% non-fat milk for 30 min to block nonspecific antibody binding. After a brief rinse in PBS, the vessel pieces were treated with primary antibodies for overnight in a refrigerator. Anti-fibronectin IgG was diluted $\times 200$ and anti-vinculin IgG $\times 50$ with PBS. The vessel pieces were washed in PBS for 1 hr with several changes of PBS and stained for 1 hr at room temperature with rhodamine-conjugated secondary antibodies diluted $\times 100$ with PBS. Fluorescent phalloidins were diluted $\times 100$ with PBS before use and the vessel pieces were stained for 20 min at room temperature.

Fluorescent-labeled vessel pieces were placed on slides, mounted using 60% glycerol in PBS and observed using a Zeiss Axiophot equipped for epifluorescence microscopy with $\times 40$ (Plan-NEOFLUAR, N.A. 0.75) and $\times 63$ (Plan-APOCHROMAT, N.A. 1.4) lenses. Fluorescence images were recorded on Kodak T-Max 100 films. A confocal laser scanning unit MRC-600 (Bio-Rad, Cambridge, UK) mounted on a Zeiss Axiophot was used to obtain confocal fluorescence images.

The length of endothelial cell was examined using the immunofluorescence micrographs obtained from tissues stained with mouse monoclonal antibodies IgM (Jinguji and Fujiwara, 1994), which uniformly labeled the cytoplasm of endothelial cells, or with fluorescent phalloidin.

Electron microscopy

Blood vessels were fixed by perfusion as described above using a fixative containing 2% paraformaldehyde, 2.5% glutaraldehyde and 0.1% tannic acid in 0.1 mol/L Na-cacodylate buffer (pH 7.4) for 5 min. The vessels were excised from chicks, cut into small pieces and further fixed in the fresh fixative for 2 hr or more at room temperature. After being washed in the buffer, vessel pieces were post-fixed with 0.5% OsO₄ for 1 hr on ice, washed in distilled water, dehydrated with graded ethanol solutions and embedded in epoxy resin. Ultrathin sections were stained with uranyl acetate and lead citrate and then examined using a JEOL JEM 2000 FX electron microscope.

Immunoelectron microscopy

The pieces of blood vessels were fixed with a mixture of 1% paraformaldehyde and 0.25% glutaraldehyde in PBS for 10 min. After washing in PBS on ice, tissue pieces were treated with 0.05% polyethylene glycol tert-octylphenyl ether (Triton X-100) for 5 min and washed again in PBS. The tissue pieces were treated with 0.02 mol/L glycine in PBS for 20 min and then with 5% non-fat milk in PBS for 30 min. After being rinsing in PBS they were treated with anti-fibronectin IgG diluted $\times 200$ with PBS for overnight in a refrigerator. The tissues pieces were washed in PBS and then treated with Protein A gold (5 nm) for 6 hr in a refrigerator. They were washed in PBS, refixed with 2.5% glutaraldehyde in PBS and then prepared for electron microscopy described above.

RESULTS

General morphologies of embryonic and posthatched chick aortae

Table 1 shows that the average length and diameter of aorta increased largely as the embryos became older. The outer diameter of aorta increased roughly ten-times during 5th day embryonic through 3rd posthatched day. The comparisons of morphological parameters among the developmental stages showed that the endothelial cells rapidly elongated in the period when the vessels showed a relatively large growth (Tables 1 and 2). Aortic endothelial cells largely elongated along the vessel's longitudinal axis in embryos after 6th day of incubation, showing maximum length, approximately 64 μm at 8th day of incubation. This was 1.7 times longer than that of 5th day embryos. In chicks staged from 17th day embryonic through 3rd day posthatched,

Table 1. Length and outer diameter of chick abdominal aorta.

Age (days)	length (mm, mean \pm S.D)	outer diameter (mm, mean \pm S.D)
5E	7.7 \pm 0.3 (n=9)	0.2 \pm 0.1 (n=10)
6E	7.8 \pm 0.2 (n=8)	0.4 \pm 0.1 (n=10)
8E	9.0 \pm 0.2 (n=8)	0.5 \pm 0.1 (n=10)
11E	15.0 \pm 0.6 (n=8)	0.6 \pm 0.1 (n=10)
17E	19.4 \pm 1.0 (n=9)	1.2 \pm 0.1 (n=10)
20E	24.0 \pm 1.1 (n=9)	1.6 \pm 0.1 (n=10)
3P	30.7 \pm 1.4 (n=9)	2.2 \pm 0.4 (n=10)

n, number of subjects; E, embryonic days; P, posthatched days. Length of aorta was measured between the heart and bifurcation point of iliac arteries. Outer diameter was measured at midlevel of abdominal aorta.

Table 2. Cell length and major location of actin cytoskeleton in endothelial cells of chick abdominal aorta.

Age (days)	cell length (μm , mean \pm S.D)	major locations of actin cytoskeleton
5E	36.2 \pm 5.0 (n=200)	cell periphery
6E	43.8 \pm 9.8 (n=160)	cell periphery, stress fiber
8E	63.9 \pm 6.3 (n=170)	stress fiber
11E	62.3 \pm 10.2 (n=105)	stress fiber
17E	55.6 \pm 8.7 (n=102)	stress fiber
20E	55.8 \pm 11.1 (n=190)	stress fiber
3P	54.0 \pm 4.9 (n=127)	cell periphery, stress fiber

n, number of cells measured; E, embryonic days; P, posthatched days. Cell length was measured using immunofluorescence images.

endothelial cell length decreased again to approximately 55 μm that is shorter than the length observed in 8th day embryos (Table 2).

Observations of 5th and 6th day embryos

Fig. 1a shows endothelial cells of the aorta obtained from 6th day embryo, stained with rhodamine-phalloidin.

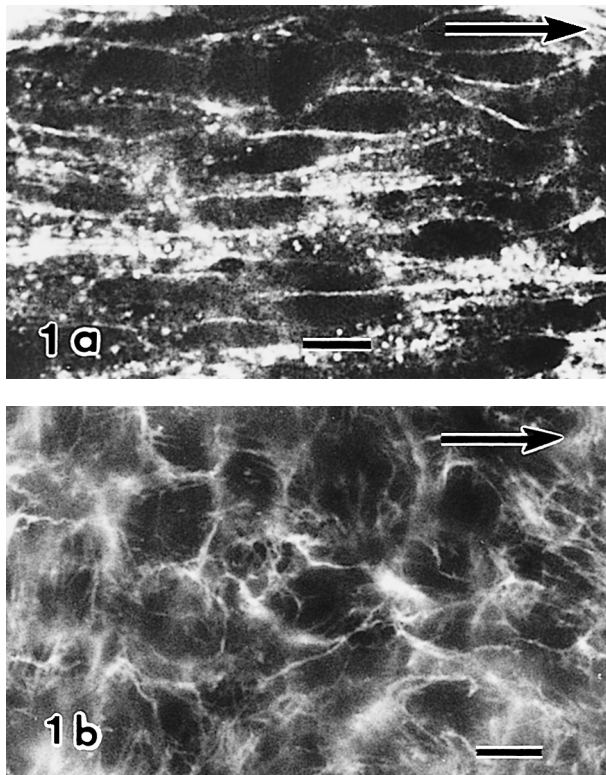


Fig. 1. Fluorescence images of 6th day embryonic chick aortae stained with rhodamine-phalloidin (a) or with anti-fibronectin (b). (a) Confocal image of endothelial cells is oval and contain a few or no visible stress fibers. Lateral cell border was intensely stained with rhodamine-phalloidin. Small particles distributed on the cell surface are blood cells remained in the preparation. (b) Bundles of fibronectin fibrils in the subendothelial space are organized in a network-like pattern. Arrows in (a) and (b) indicate the blood flow direction. (a), \times 790. Bar: 10 μm ; (b) \times 800. Bar: 10 μm

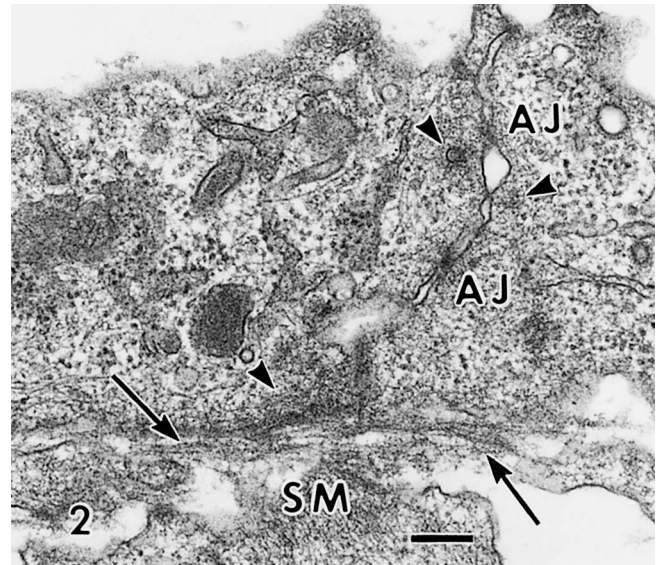


Fig. 2. Electron micrograph of the aortic wall of 6th day embryo. The aorta was longitudinally cut along the vessel axis. Thick bundles of microfilaments (arrowheads) are located in the cell periphery, often attach to the adherens junction (AJ). The basal lamina is a patch-like mass of fibrillar materials (arrows). Smooth muscle cells (SM) located close proximity to endothelial cells. \times 26000. Bar: 0.3 μm

The endothelial cells were ellipsoidal or oval in shape and lay parallel to the direction of general blood flow. The lateral cell border was strongly stained by fluorescent phalloidin, suggesting the microfilament bundles were localized beneath the cell-cell boundary. In 6th day embryos, the stress fiber expression was in a low level or not discernible in endothelial cells with fluorescence microscopy. When cells contained thin and wispy stress fibers they also showed the distinct staining at lateral cell border with fluorescent phalloidin. Many rhodamine-phalloidin positive apical spots, which correspond to the microvilli-like structure, were present in this stage. Such spots were not commonly observed in endothelial cells containing prominent stress fibers. Immunofluorescence microscopy using anti-fibronectin revealed that fibronectin fibrils in the subendothelial space were organized in a network-like pattern (Fig. 1b).

Electron micrographs of 6th day embryos showed that thin bundles of microfilaments were localized in the basal region as well as in lateral cell border (Fig. 2). The aortic basal lamina was primitive in shape, consisting of patch-like masses of fibrous materials. The internal elastic lamina was not discernible in these small aortae. Medial smooth muscle cells of the aortic wall were often located proximity to the endothelial cells when the basal lamina was primitive in shape.

Observations of 8th day embryos

Endothelial cells of the abdominal aorta shaped an elongated spindle-like and reached its maximum length in this stage (Table 2). These highly elongated cells generally

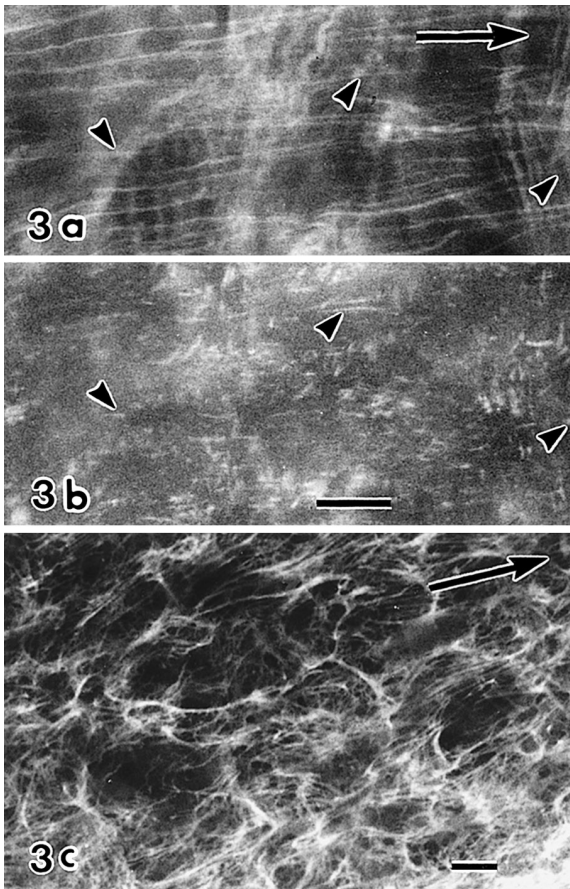


Fig. 3. Fluorescence micrographs of aortic endothelia of 8th day chick embryos stained with both fluorescein-phalloidin (a) and anti-vinculin (b), or with anti-fibronectin (c). (a) Prominent stress fibers (arrowheads) are arranged parallel to the longitudinal cell axis or blood flow direction (arrow). In cells expressing stress fibers, cell border is not visible in the fluorescence image. (b) Vinculin-positive spots (arrowheads) are located at the ends of, as well as along, stress fibers. In a pair of images (a) and (b), arrows indicate the corresponded points. (c) Fibronectin fibrils in the basal lamina are organized in a network-like pattern. Arrow indicates the blood flow direction. (a) and (b), $\times 950$. Bar: $10 \mu\text{m}$; (c), $\times 620$. Bar: $10 \mu\text{m}$

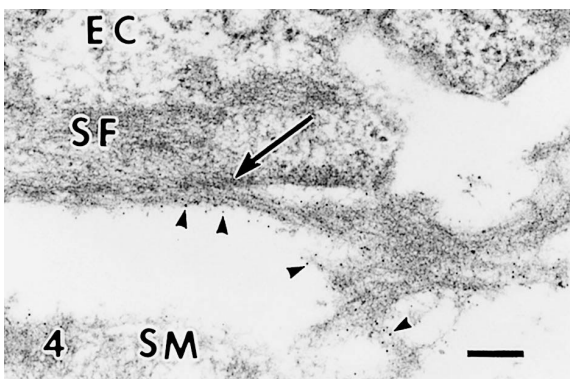


Fig. 4. Immunoelectron microscopical localization of fibronectin in the aortic basal lamina of 12th day chick embryo labeled with anti-fibronectin and then with 5 nm gold-protein A. Fibrous masses in the subendothelial space were decorated with 5 nm gold particles (arrowheads). EC, endothelial cell; SF, stress fiber; arrow, stress fiber end plaque; SM, smooth muscle cell. $\times 45000$. Bar: $0.2 \mu\text{m}$

contained linear, but thin stress fibers. The staining of lateral cell border with fluorescent phalloidin was not distinct in situ when cells expressed thicker and better defined stress fibers. Both the major cell axis and the stress fibers were parallel to the direction of blood flow (arrow in Fig. 3a). Vinculin is a protein found in the adhesion plaque of tissue cultured cells including vascular endothelial cells (Geiger *et al.*, 1984; Burridge, 1986). By double staining with anti-vinculin IgG and fluorescein-phalloidin, discrete positive fluorescent spots with anti-vinculin IgG staining were demonstrated to

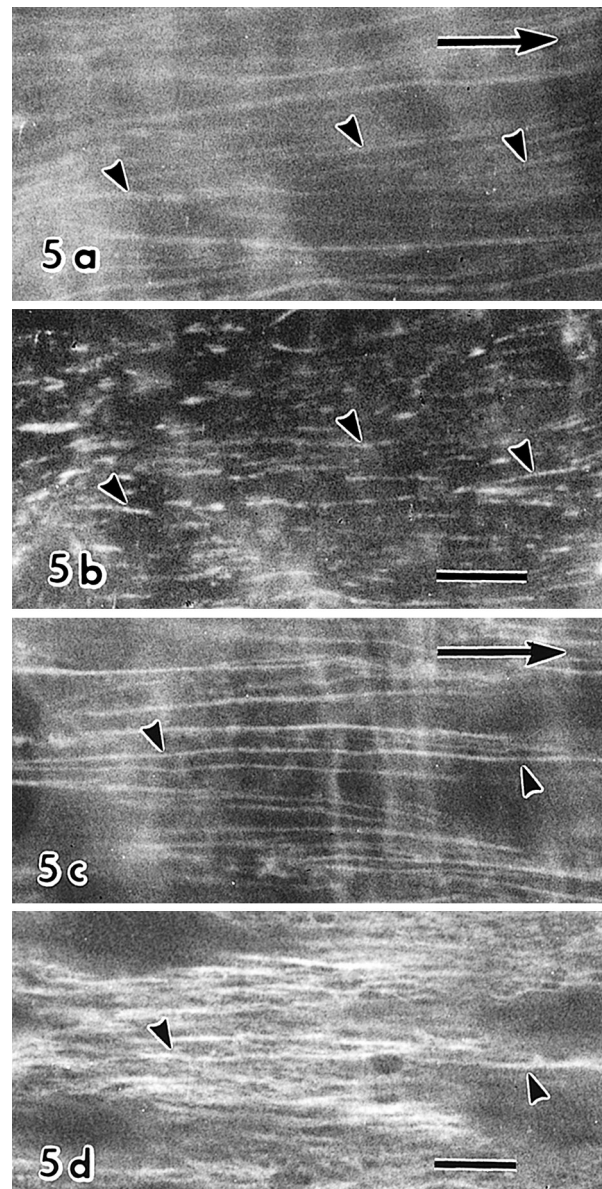


Fig. 5. Fluorescence micrographs of 12th day chick embryonic aortae stained with rhodamine-phalloidin (a, c), anti-vinculin IgG (b), and anti-fibronectin (d). Thick bundles of fibronectin fibrils (arrowheads in d) are aligned parallel to stress fibers in overlying cells (arrowheads in c). Arrowheads in (a) and (b), or in (c) and (d) also indicate the correspond points in the respective fluorescence image pairs. Arrows in (a) and (c) indicate the blood flow direction. $\times 1100$. Bar: $10 \mu\text{m}$

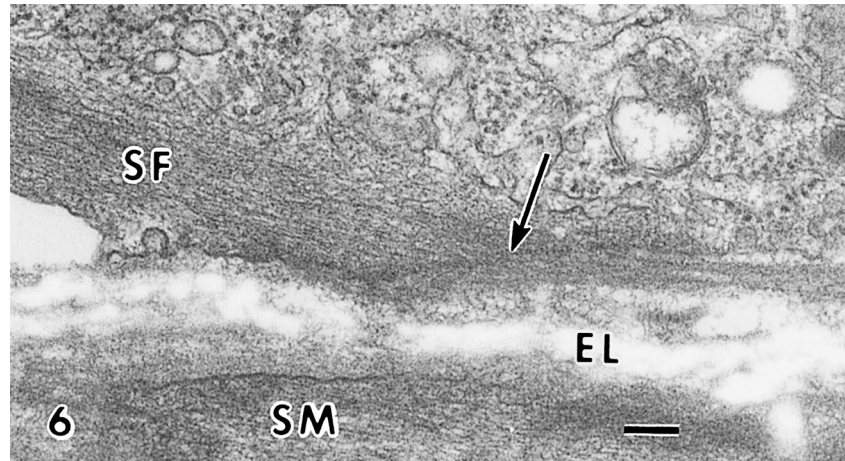


Fig. 6. Electron micrograph of the aortic intima of 18th day chick embryo longitudinal cut along the vessel axis. One end of stress fiber (SF) terminated on an electron dense adhesion plaque-like structure formed on the cytoplasmic side of the plasma membrane (arrow). The amorphous blocks consisting of electron light materials in the subendothelial space are the internal elastin layer (EL). SM; smooth muscle cell. $\times 35000$. Bar: $0.2 \mu\text{m}$

be located at the ends of, as well as along, stress fibers (Fig. 3a, b). Fibronectin fibrils arranged parallel to the longitudinal axis of overlaying endothelial cells were increased in the basal lamina, but the dominant arranging pattern was a network-like (Fig. 3c).

Using electron microscopy, the majority of stress fibers in endothelial cells were observed to be contiguous with the basal plasma membrane. At the site of stress fiber terminus on the plasma membrane the formation of an electron dense adhesion plaque-like structure was distinct. Much of fibrous materials including fibronectin fibrils attached to the basal cell surface opposite to the stress fiber terminus as was shown in an immunoelectron micrograph labeled with anti-fibronectin IgG and then protein A gold (Fig. 4).

Observations of 9th to 20th day embryos

The average length of aortic endothelial cells showed a gradual shortening in this developmental stage (Table 2). Thick and long stress fibers were generally observed in the endothelial cells (Fig. 5a). In the preparation stained with fluorescein-phalloidin and anti-vinculin IgG, vinculin positive fluorescent spots were located at the ends of, as well as along, stress fibers (Fig. 5a, b). The dominant pattern of fibronectin organization in the basal lamina changed from unaligned to axially aligned as vessel caliber increased. Fibronectin-containing thick fibrils were arranged being parallel to the longitudinal axes of overlaying endothelial cell and/or stress fibers located above them. When closely compared in double labeled specimens using anti-fibronectin IgG and fluorescein-phalloidin, the stress fiber and the thick fibronectin fibrils could often be superimposed on each other (Fig. 5c, d), indicating the close association between these two structures.

In both terminal ends, stress fibers attached on or laterally associated with the basal plasma membrane via an electron dense area (Fig. 6). Since the distribution pattern of

such stress fiber terminus observed with electron microscopy and with immunofluorescence microscopy using anti-vinculin IgG were remarkably similar to those of the adhesion plaque of cultured cells (Geiger *et al.*, 1984; Burridge, 1986), these dense area or spots are the supposable adhesion plaques in the aortic endothelia (Fig. 5a, b). Electron microscopy showed that the aortic basal lamina was a thick layer of fibrillar bundles containing the bolder elements, the internal elastic lamina (Fig. 6).

Observations of 3rd day young chicks

The average length of endothelial cells was in a level similar to that observed in 17th to 20th day embryos (Table 2). Endothelial cells were rather short and often shaped elliptic-form than those in the previous embryonic stages. Generally, the staining of stress fibers with fluorescent phalloidin was substantially thinner and wispy in short cells than those in the more elongated cells (Fig. 7). In the endothelial

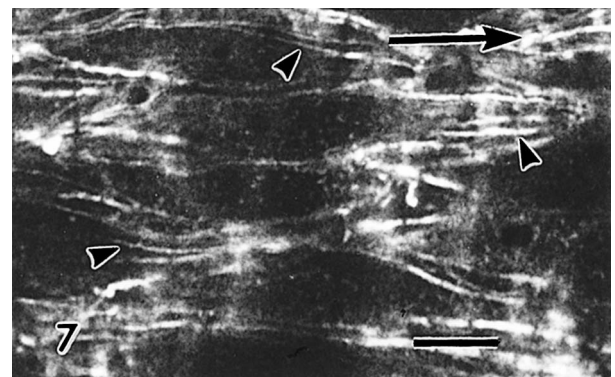


Fig. 7. Confocal fluorescence micrograph of the aortic endothelium of 3rd day young chick stained with rhodamine-phalloidin. The lateral cell border of endothelial cells is clearly stained by rhodamine-phalloidin. Some cells contain wispy stress fibers (arrowheads). Cells are still elongated and aligned parallel to blood flow direction (arrow). $\times 1100$. Bar: $10 \mu\text{m}$

cells, dominant staining of actin microfilaments with fluorescent phalloidin was observed in the lateral cell border rather than stress fibers (Fig. 7, Table 2). These morphological features were similar to those observed in young stages such as 6th day embryos (e.g., Fig. 1a). In the basal lamina examined with fluorescence microscopy, fibronectin fibrils were arranged being parallel to the vessel's longitudinal axis even beneath the elliptic-form endothelial cells (data not shown).

DISCUSSION

Endothelial cell shape and stress fiber expression

Results of the present study showed that the major sites of actin cytoskeletal organization in the endothelial cells changed from the lateral cell border to stress fibers in the developmental stages. The axially aligned stress fibers were prominent in the largely elongated cells, while, in the cells shaped elliptic form contained no or few thin stress fibers. In the mesenteric artery of young chicks examined in our study, elongated endothelial cells in the proximal segment contained prominent stress fibers, while short cells in the more distal segments contained a peripheral actin filament layer rather than stress fibers (Jinguji and Fujiwara, 1994). The difference of stress fiber expression observed in our previous and the present studies, is clearly depended on the cell length. In the chick embryonic aorta examined in the present study, as the embryos grew endothelial cell elongation became evident (Table 2). Thus, the cell elongation in the direction parallel to the blood flow direction or vessel's longitudinal axis may stimulate the stress fiber expression in the embryonic aortae. Considerable evidences has been accumulated indicating that the endothelial cell elongation might be a cellular response to a high level of local fluid shear stress *in vivo* (Flaherty *et al.*, 1972; Kim *et al.*, 1989a) or *in vitro* (Franke *et al.*, 1984; Levesque and Nerem, 1985). It has been also supposed that the cell elongation or the high blood shear stress could stimulate the stress fiber expression (White and Fujiwara, 1986; Kim *et al.*, 1989b; Sugimoto *et al.*, 1997). The elongation of endothelial cells and the following expression of axially organized stress fibers, observed in the present study, might be induced by the increased blood shear stress in the processes of embryonic development.

In published results including our previous study, endothelial cells located in areas where nonlaminar flow was expected, for example, near the branch points of small arteries, were polygonal in shape and did not express the discernible stress fibers (White *et al.*, 1983; White and Fujiwara, 1986; Jinguji and Fujiwara, 1994). These morphological results clearly showed that the laminar blood flow directing parallel to the vessel's axis is an essential factors for inducing the stress fiber expression or the cell elongation.

In the relatively short cells observed in the aorta of 3rd day old chicks, major sites of actin microfilaments distribu-

tion were found again in the region beneath the plasma membrane at cell-cell boundary (Fig. 7). The reasons why endothelial cells became short again after 17th day has not been cleared in this morphological study. In the study using the uterine veins of pregnant rats, Sago *et al.* (1993) supposed that the decreasing of mechanical tensions previously exerted on the vessel wall may cause the cell shortening and the disappearance of stress fibers from the endothelia after delivery. Details of the relations between the cell shortening and the modification of blood shear stress are not yet cleared even in their study. The change of cell proliferation rate might be a considerable point of view dealing the reasons why endothelial cells became short. Concerning this possibility, we examined the endothelial cell proliferation rate of the chick aortae using the vital labeling with bromodeoxyuridine and immunofluorescent detection with anti-bromodeoxyuridine IgG (Jinguji and Nomura, 1998). We revealed that the rate of cell division decreased in the developmental stages; the rate downed from approximately 3.7% in 10th day embryonic to 1.4% in 3rd day young chicks. We, however, have not yet examined the time length of cell cycle which may closely relate to the expanding rate of endothelial surface areas in these developmental stages. Thus, in the chick embryonic aortae, the participation of cell proliferation on either the increasing of endothelial surface areas or the change of endothelial cell length is not yet cleared.

Organizational relationship between the stress fiber and fibronectin fibrils

Results of the present immunohistochemical study showed that fibronectin fibrils changed its organizational pattern in the primitive aortic basal lamina during embryonic development. Fibronectin is a component of the extracellular matrix and is supposed to regulate the cell adhesion, spreading, migration, and proliferation in a variety of cells *in vivo* or *in vitro* (Clark *et al.*, 1982, 1986; for reviews, Hynes and Yamada, 1982; Hynes, 1990; Risau, 1997). For examples, in the sites of new capillary formation, including the processes of angiogenesis and vasculogenesis, the distribution of fibronectins increased in the vessel wall and which could act as scaffolds guiding the morphogenesis (D'Amore and Thompson, 1987; Risau and Lemmon, 1988). Hynes (1990) reported that the distribution of fibronectin was rich in the extracellular matrix of embryonic tissues rather than in matured tissues. Results of these published studies suggested that the components and the distribution density of extracellular matrix may change as the maturation or differentiation of tissues proceeded. In the present study, many fibronectins were present in the basal lamina of the embryonic aortae, which may indicate that the blood vessels are in the way of histological maturation. In the mechanisms of angiogenesis, fibronectin's role in the cell adhesion is thought to be mediated by some transmembrane proteins, for examples, integrins (Hynes, 1987; Fujimoto and Singer, 1988; Albelda and Buck, 1990; Risau, 1997). The ultrastruc-

ture of stress fiber ends in embryonic aortae, revealed in the present study, was highly reminiscent of the adhesion plaque in tissue cultured cells and vinculin, an adhesion plaque protein, was also present at the ends of, as well as along, stress fibers (Fig. 5a, b). Although the present study did not deal the mechanism how can stress fibers connect with fibronectin fibrils via the transmembrane proteins, the principal adhesion mechanism employed by embryonic endothelia is supposed to be the same as that observed *in vitro* (for examples, Burridge, 1986; Hynes, 1987) or in adult arteries (White and Fujiwara, 1986; Kano *et al.*, 1996). The expression of well organized stress fibers including the adhesion plaques formed at their ends may participate to maintaining the histological integrity of endothelia when the tissues were exposed to the relatively high mechanical forces produced by blood stream (Davis, 1993).

The existence of axially aligned fibrils in the subendothelial space has been reported in large arteries (Buck, 1979; Davis, 1993), which are most likely to contain fibronectins. In the embryonic aortae examined in the present study, thick bundles of fibronectin fibrils were generally aligned parallel to the vessel's longitudinal axis. Since the fibronectin bundles widely superimposed on the stress fibers of overlaying endothelial cells in immunofluorescence images, the fibronectin's axial organization may possibly associate with the stress fiber expression. Similar colineage between the orientation of stress fiber and fibronectin fibrils was observed in rat aortae (Sugimoto *et al.*, 1997) or in cultured cells (Burridge, 1986; Madri *et al.*, 1983; Wechezak *et al.*, 1985). In our previous study using the mesenteric artery of young chicks (Jinguji and Fujiwara, 1994), both the axial alignment of fibronectin fibrils and the expression of stress fibers were prominent as vessel diameter becomes larger and endothelial cells become longer. We also showed that such axial arrangement of fibronectin fibrils was highly disturbed in the opening of arterial branches particularly in the region downstream to the "flow divider" area. In this region, endothelial cells shaped round or elliptic form containing no stress fibers under which fibronectin fibrils were organized in a network-like pattern (Jinguji and Fujiwara, 1994). Results of these published studies and the present study may point to the conclusion; the fibronectin axial alignment is determined by the presence of stress fibers in the overlaying cells. The majority of fibronectin fibrils in the matrix beneath the cells containing no visible stress fibers maintained their axial arrangement in 3rd day young chicks. Although the actual mechanisms involving in the induction of fibronectin's regular arrangement are not clear in this morphological study, the observed dynamic changes in the fibronectin's arranging pattern was limited to the early embryonic stages, from 6th to 9th day of incubation. In these developmental periods, endothelial cells showed relatively rapid elongation. Large elongation of the overlaying endothelial cells and the following expression of the axially aligned stress fibers may participate in the axial alignment of fibronectin fibrils.

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REFERENCES

- Albelda SM, Buck CA (1990) Integrins and other cell to adhesion molecules. *FASEB J* 4: 2868–2880
- Buck RC (1979) The longitudinal orientation of structures in the sub-endothelial space of rat aorta. *Am J Anat* 156: 1–14
- Burridge K (1986) Substrate adhesion in normal and transformed fibroblasts: Organization and regulation of cytoskeletal, membrane and extracellular matrix components at focal contacts. *Cancer Rev* 4: 487–525
- Byers HR, White GE, Fujiwara K (1984) Organization and function of stress fibers in cells *in vitro* and in situ. A review. *Cell Muscle Motil* 5: 83–137
- Clark RAF, Pelle PD, Manseau E, Lanigan JM, Dvorak HF, Colvin RB (1982) Blood vessel fibronectin increases in conjugation with endothelial cell proliferation and capillary ingrowth during wound healing. *J Invest Dermatol* 79: 269–276
- Clark RAF, Folkvord JM, Nielsen LD (1986): Either exogenous or endogenous fibronectins can promote adherence of human endothelial cells. *J Cell Sci* 82: 263–280
- D'Amore PA, Thompson RW (1987) Mechanisms of angiogenesis. *Ann Rev Physiol* 49: 453–464
- Davis E (1993) Endothelial cell connecting filaments anchor endothelial cells to the subjacent elastic lamina in the developing aortic intima of the mouse. *Cell Tissue Res* 272: 211–219
- Flaherty JT, Pierce JE, Ferrans VJ, Patel DJ, Tucker WK, Fry DL (1972) Endothelial nuclear patterns in the canine arterial tree with particular reference to hemodynamic events. *Circ Res* 30: 23–33
- Franke RP, Grafe M, Schnittler H, Seiffge D, Mittermayer C, Drenckhahn D (1984) Induction of human vascular endothelial stress fibers by fluid shear stress. *Nature* 307: 648–649
- Fujimoto T, Singer SJ (1988) Immunocytochemical studies of endothelial cells *in vivo*. II. Chicken aortic and capillary endothelial cells exhibit different cell surface distribution of the integrin complex. *J Histochem Cytochem* 36: 1309–1317
- Gabbiani G, Badonnel MC, Rona G (1975) Cytoplasmic contractile apparatus in aortic endothelial cells of hypertensive rats. *Lab Invest* 32: 227–234
- Geiger B, Avnur Z, Rinnerthaler G, Hissen H, Small VJ (1984) Microfilament-organizing centers in areas of cell contact: Cytoskeletal interactions during cell attachment and locomotion. *J Cell Biol* 99: 83s–91s
- Girard PR, Nerem RM (1993) Endothelial cell signaling and cytoskeletal changes in response to shear stress. *Frontier Med Biol Engng* 5: 31–36
- Gottlieb AI, Langille L, Wong MKK, Kim DW (1991) Biology of disease. Structure and function of the endothelial cytoskeleton. *Lab Invest* 65: 123–137
- Hynes RO (1987) Integrins: A family of cell surface receptors. *Cell* 48: 549–554
- Hynes RO, Yamada KM (1982) Fibronectins: Multifunctional modular glycoproteins. *J Cell Biol* 95: 369–377
- Hynes RO (1990) *Fibronectins*. Springer-Verlag, New York
- Jinguji Y, Fujiwara K (1994) Stress fiber dependent axial organization of fibronectin fibrils in the basal lamina of the chick aorta and mesenteric artery. *Endothelium* 2: 35–47
- Jinguji Y, Nomura T (1998) The distribution of dividing endothelial cells of chick embryonic aortae studied by vital labeling with

- bromodeoxyuridine. *Zool Sci* 15: 213–216
- Kano Y, Kato K, Masuda M, Fujiwara K (1996) Macromolecular composition of stress fiber-plasma membrane attachment sites in endothelial cells in situ. *Circ Res* 79: 1000–1006
- Kim DW, Gotlieb AL, Langille BL (1989a) In vivo modulation of endothelial F-actin microfilament distribution by experimental alterations in shear stress. *Arteriosclerosis* 9: 439–445
- Kim DW, Langille BL, Wong MKK, Gotlieb AL (1989b) Patterns of endothelial microfilament distribution in the rabbit aorta in situ. *Circ Res* 64: 21–31
- Levesque MJ, Nerem RM (1985) The elongation and orientation of cultured endothelial cells in response to shear stress. *J Biomech Eng* 107: 341–347
- Madri JA, Williams SK, Wyatt T, Mezzio C (1983) Capillary endothelial cell cultures: phenotypic modulation by matrix components. *J Cell Biol* 97: 153–165
- Sago H, Sugimoto K, Fujii S, Inuma K, Yamasita K, Kitagawa M, Terasima Y (1993) Dynamic changes in stress fiber expression in rat uterine vein endothelial cells associated with pregnancy. *Microvascu Res* 46: 178–189
- Sugimoto K, Fujii S, Takemasa T, Yamasita K (1997) Factors inducing codistribution of marginal actin fibers and fibronectin in rat aortic endothelial cells. *Am J Physiol* 272: H2188–H2194
- Risau W, Lemmon V (1988) Changes in the vascular extracellular matrix during embryonic vasculogenesis and angiogenesis. *Develop Biol* 125: 441–450
- Risau W (1997): Mechanisms of angiogenesis. *Nature* 386: 671–674
- Wechezak AR, Viggers RF, Sauvage LR (1985) Fibronectin and F-actin redistribution in cultured endothelial cells exposed to shear stress. *Lab Invest* 53: 639–647
- White GE, Gimbrone MA, Fujiwara K (1983) Factors influencing the expression of stress fibers in vascular endothelial cells in situ. *J Cell Biol* 97: 416–424
- White GE, Fujiwara K (1986) Expression and intracellular distribution of stress fibers in aortic endothelium. *J Cell Biol* 103: 63–70

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