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Expression of Extracellular Matrix Components Fibronectin and Laminin in the Human Fetal Heart

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ABSTRACT. **It has been well documented that the extracellular matrix components fibronectin and laminin promote or regulate morphogenesis of the myocardial cells in mammalian heart. However, their chronological change of expression (or localization) in the human heart remains elusive. In this study, fibronectin and laminin in the left ventricle of forty-two human fetuses aged from 8 to 26 weeks gestation and left ventricular tissues obtained from a 2-week old infant and two adults were investigated by Western blot analyses and indirect immunofluorescence technique with monoclonal antibodies. In the fetal heart, fibronectins were present along the endocardium, epicardium, and linings of larger blood vessels. In 14–16 weeks gestation, fibronectin immunofluorescence became stronger but not evenly dispersed in the interstitium. After 24 weeks gestation, they were strongly positive only in the relatively larger blood vessels, as well as those in the infant and adult cardiac tissues. Laminins were strongly positive along the endocardium and basement membrane of the myocardial cells and fibroblasts during fetal life. After birth, laminins formed fine fibrillar network along the basement membrane in association with the transverse tubules of myocardial cell; these morphological characteristics remained in the adult cardiac tissues. These results indicate that fibronectin expression is relatively constant during fetal life but decreases after birth; in contrast, laminin expression is not age-dependent and constant throughout the life.**

Key words: development/fibronectin/human fetal heart/immunohistochemistry/laminin

Cardiovascular development is the net result of a complex genetic program subject to regulation by signals transmitted among cardiac cells and its extracellular environment. Recent data indicate that the cardiac extracellular matrix plays a critical role in growth and development. They significantly contribute to heart development, growth, and cardiac function by direct modulation of the behavior of cells that contact it (6, 23, 34). The role of the extracellular matrix in heart development during embryonic, fetal, and neonatal stages includes signaling necessary for cell migration, cell sorting, adhesion, differentiation, angiogenesis, and valve formation (8, 9). The major components of the extracellular matrix include structural proteins, cell adhesive molecules, and proteoglycans. These may be variable in amount in response to various stimuli to the heart during embryonic, fetal, neonatal, and adult periods.

Cell adhesive molecules, fibronectin and laminin are important non-collagenous glycoproteins. Organization of these two high molecular weight protein network is essential for maintenance of constant and adequate cardiac function (4, 5). Fibronectin is homogeneously distributed throughout the extracellular space in which elements such as the T-tubule network of cardiac myocytes and collagens are located (30); it influences a variety of cell behaviors, including cell growth, adhesion, migration, and wound healing (12). Laminin is the most extensively characterized member of basement membrane component; its function includes mediation of adhesion, migration, growth, and differentiation of cells (11, 14).

Recently, we described fetal age-dependent expres-

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Abbreviations: BSA, bovine serum albumin; ECL, enhanced chemiluminiscence; FITC, fluorescein isothiocyanate; kDa, kilo-dalton; LMP, last menstrual period; PBS, phosphate buffered saline; PLP, periodate-lysine-paraformaldehyde; TBS/T, Tris-buffered saline-Tween 20.

sion of cardiac cytoskeletal proteins in human fetal heart development (19, 20). However, there are few reports in the literature on the structural expression and roles of the extracellular matrix components in the developing human heart. In this respect, we aimed to describe the localization of fibronectin and laminin in the developing human heart as a part of consecutive studies on the molecular mechanism of cardiac dysmorphogenesis in humans by use of immunohistochemical techniques with monoclonal antibodies. We hope that it will facilitate the interpretation of changes observed in congenitally defective or diseased human hearts.

Materials and Methods

Materials

The protocol for this study was reviewed and approved by the Ethics Committee of the Institute of Medical Science, Chung-Ang University. Left ventricular tissues of 42 hearts including the interventricular septum from human fetuses of either sex between 8 and 26 weeks of gestation were investigated (Table I). Fetuses were obtained from therapeutic or spontaneous abortions or from deaths during parturition between April 1, 1989, and January 24, 1996. Fetuses were collected within a few hr after death, and they showed no congenital anomalies or specific findings at autopsy. The gestation age was estimated by the date of the last menstrual period (LMP) or by crown-rump length as previously described (19), if the LMP was not clear. The specimens were rapidly frozen in liquid nitrogen with or without treatment in phosphate buffered saline (PBS; 0.02 M, pH 7.2)-sucrose (10, 15, and 25 vol% in ascending order), and stored in a deep-freezer until use. Left ventricular tissues from a 2-week old infant and two adults (21-year old male and 35-year old female) who had died from an accident were used for the control (or comparison). Tissues were sampled with a biopsy needle during autopsy a few hr after death. These samples were fixed in periodate-lysine-paraformaldehyde (PLP) for 6–8 hr; overnight in 4% paraformaldehyde (pH 7.4, 4°C) and treated with PBS-sucrose for 4 hr.

Table I. HUMAN MATERIALS USED IN THIS STUDY.

	No. of fetuses investigated	CRL[1]
Fetal age (in weeks gestation)[2]		
under 10[3]	8	
10–15	14	23±1.0
15–20	12	141±1.8
21–26	8	189±2.1
Postnatal, 2-week[4]	1	unknown
Adult[5]	2	unknown

[1] Crown-rump length (in mm, mean±s.d.).
[2] Fetuses obtained from legal abortion or death during parturition.
[3] Fetal age estimated only by the LMP (last menstrual period).
[4,5] Obtained from a death by accident.
[5] Obtained from a 25-year-old male and a 35-year-old female.

Monoclonal antibodies to laminin and fibronectin were purchased from Sigma Chemicals (St Louis, USA) or Novocastra (Newcastle upon Tyne, UK); fluorescein isothiocyanate (FITC)-labeled sheep anti-mouse IgG was purchased from Sigma Chemicals or Becton-Dickinson (San Jose, USA).

Western Blot Analysis

Fibronectin and laminin were isolated and analyzed three times each by Western blot. In brief, left ventricular tissues including interventricular septa from the hearts of 8-, 16-, 24-week gestation, infant, and adults were homogenized in a sample buffer (containing 0.0625 M Tris-HCl, 2% sodium dodecyl sulphate, 10% glycerol, 15% 2-mercaptoethanol, pH 6.8) and centrifuged (10,000 rpm for 30 min). Protein concentrations were determined with a commercial kit (Bio-Rad, Hercules, USA). Using Laemmli's buffer system, supernatants, each containing 50 μg of protein, were electrophoresed in a gradient resolving gel (7.5–12.5% for fibronectin; 3–15% for laminin) and electroblotted onto nitrocellulose at 100 V at 4°C for 2 hr. The blots were blocked with Tris-buffered saline-Tween 20 (TBS/T, containing 50 mM Tris, 150 mM NaCl, 0.05% Tween 20, pH 9.0) containing 5% skim milk at room temperature for 1 hr. They were, then, incubated with a 1:1,000 dilution (TBS/T containing 5% skim milk) of mouse monoclonal antibodies to human fibronectin or laminin (B2 chain) at room temperature for 2 hr, followed by incubation in alkaline phosphatase conjugated with anti-mouse IgG (1:1,000). The blots were developed with an enhanced chemiluminiscence (ECL) kit (Amersham, Little Chalfont, UK). Density of each protein band was quantified by computerized image analyzing system (Pias, Osaka, Japan).

Immunohistochemistry

Frozen left ventricular tissue sections, 4 μm in thickness, were fixed in a mixture of cold methanol and acetone (1:2) at −20°C for 10–20 min (PLP-fixed sections excluded this step), rinsed in PBS, and incubated in goat serum (Vector Laboratory, Burlington, CA; 1:30 in PBS-BSA; Boehringer Mannheim, Germany, 0.1 vol%) to block nonspecific reactions. Sections were then incubated overnight with the primary antibodies (1:30 dilution of anti-human laminin (B2 chain) or 1:20 dilution of anti-human fibronectin mouse monoclonal antibodies) in a moist chamber at 4°C and with the secondary antibody (1:30 dilution of FITC-labeled goat anti-mouse IgG) at room temperature for 1 hr; cleared in PBS, mounted with Crystal/Mount (Biomeda, Foster City, CA), and examined with a light microscope (Olympus BHS-2, Tokyo, Japan) equipped with reflected light fluorescence. Photographs were taken with Kodak Tri-X Pan black-and-white negative or Ektachrome color reversal films (ISO 400) with compensa-

tion filters. Frozen sections treated the same way except incubation with the primary antibodies were used for negative controls.

Results

Western blot analysis

Fibronectin and laminin bands were identified by triplicate Western blot analyses with monoclonal antibodies. Figures 1 and 2 show that 230-kDa fibronectin subunits and 200-kDa laminin B2 chain bands are clearly present in the left ventricular samples with little variation. The intensity of the immunoblot reacted with anti-fibronectin antibody tended to remain relatively constant during fetal life but abruptly decrease afterwards (Fig. 1), whereas, that with anti-laminin antibody tended to remain constant (Fig. 2).

Immunohistochemistry

Since there was an overlap of similar localization patterns of extracellular matrix proteins during fetal life, three consecutive periods are described in this study: before 10, 11–19, and after 20 weeks of gestation. All sections without the primary monoclonal antibodies revealed absolutely negative results (data not shown).

Moderate degree of positive reaction with anti-fibronectin monoclonal antibody was present alongside the endo- or epicardium in 8–10 weeks of gestation; strong positive reaction was present around the linings (endothelial cells) of relatively larger blood vessels. However, positive reactions were not found in the myocardial cells or other sites of the interstitium (Figs. 3 and 4). By 14–16 weeks of gestation, positive reaction with anti-fibronectin monoclonal antibody became stronger but not evenly dispersed in the interstitium (Fig. 5). After 24 weeks gestation, strong positive reaction with anti-fibronectin monoclonal antibody was mainly present in relation with the endothelial cells, however, distribution was not significantly different from the previous perid (Fig. 6). In the neonatal left ventricular tissues, strong positive reaction with anti-fibronectin monoclonal antibody was present only in the perivascular regions (Fig. 7); similar findings were observed in the adult left ventricular tissues (Fig. 8). These findings indicate that fibronectin is strongly localized along the coverings (endo- and epicardium) and blood vessels and its density is relatively constantly maintained in the fetal life regardless of gestational age, but expression or localization of fibronectin is considerably decreased after birth.

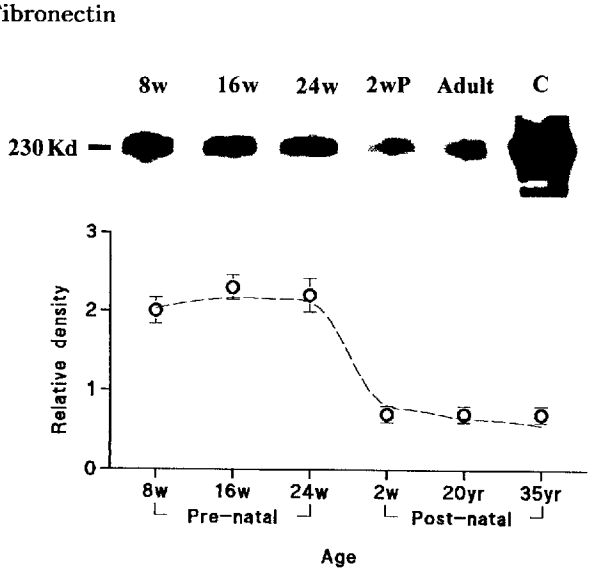

Fig. 1. Western blot analysis of fibronectin. During fetal life (8–24 w gestation), relative densities (mean±s.d.) of fibronectin are not significantly different but abruptly decrease after birth (2wP, 2-week postnatal; and adult). C indicates positive control.

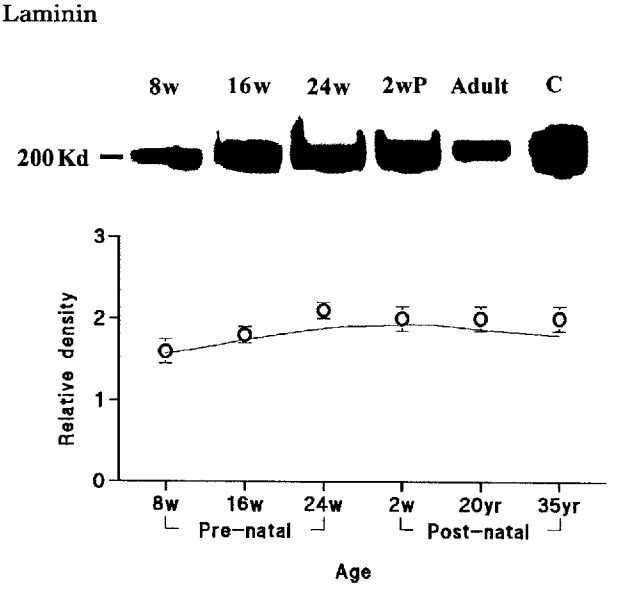

Fig. 2. Western blot analysis of laminin. Relative densities (mean±s.d.) of laminin tended to be constant regardless of age (fetal, neonatal, and adult). C indicates positive control.

Relatively strong positive reaction with anti-laminin monoclonal antibody was found along the endocardium or basement membrane of the myocardial cells in the left ventricular tissues of 8–10 weeks gestation (Fig. 9). Reaction with anti-laminin monoclonal antibody became stronger alongside the basement membrane of the myocardial cells in the left ventricular tissues (Fig. 10) of 14–16 weeks gestation, especially in the papillary muscles (Fig. 11). These morphological characteristics were not significantly different even after 24 weeks ges-

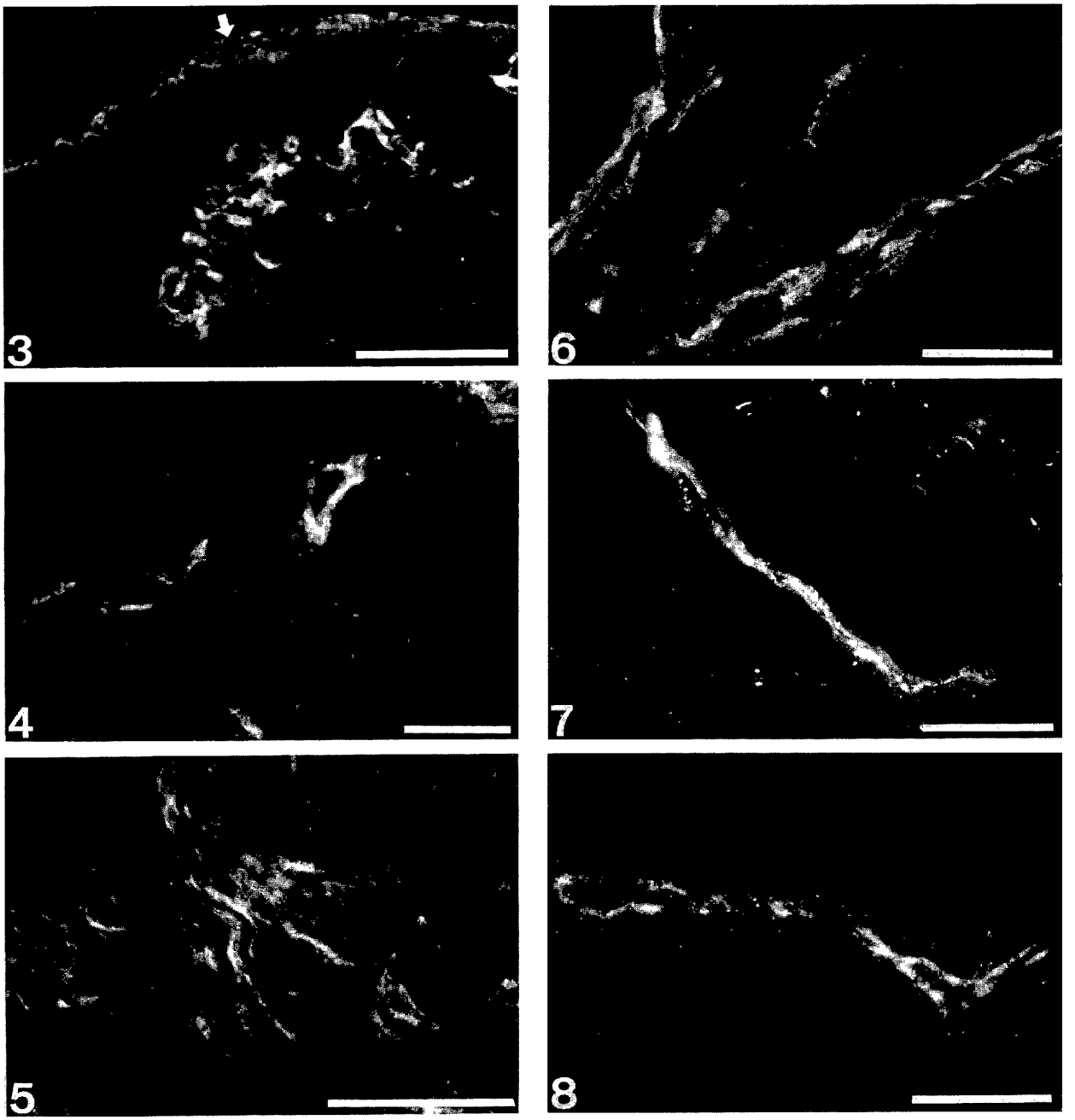

Fig. 3. Fetal left ventricular tissue, 9 weeks gestation. Strongly positive reaction to anti-fibronectin antibody is seen along the endocardium (arrow) or surroundings of blood vessels. Scale bar = 100 μm.

Fig. 4. Fetal left ventricular tissue, 10 weeks gestation. Strongly positive reaction to anti-fibronectin antibody is seen along the surroundings of blood vessels. Scale bar = 50 μm.

Fig. 5. Fetal left ventricular tissue, 15 weeks gestation. Moderately positive reaction to anti-fibronectin antibody is seen along the surroundings of blood vessels. Scale bar = 50 μm.

Fig. 6. Fetal left ventricular tissue, 24 weeks gestation. Relatively strong positive reaction to anti-fibronectin antibody is seen along the surroundings of blood vessels. Scale bar = 50 μm.

Fig. 7. Neonatal left ventricular tissue, 2 weeks after birth. Positive reaction to anti-fibronectin antibody is seen along the surroundings of relatively larger blood vessels. Scale bar = 100 μm.

Fig. 8. Adult left ventricular tissue. Strong positive reaction to anti-fibronectin antibody is seen along the surroundings of relatively larger blood vessels. Scale bar = 100 μm.

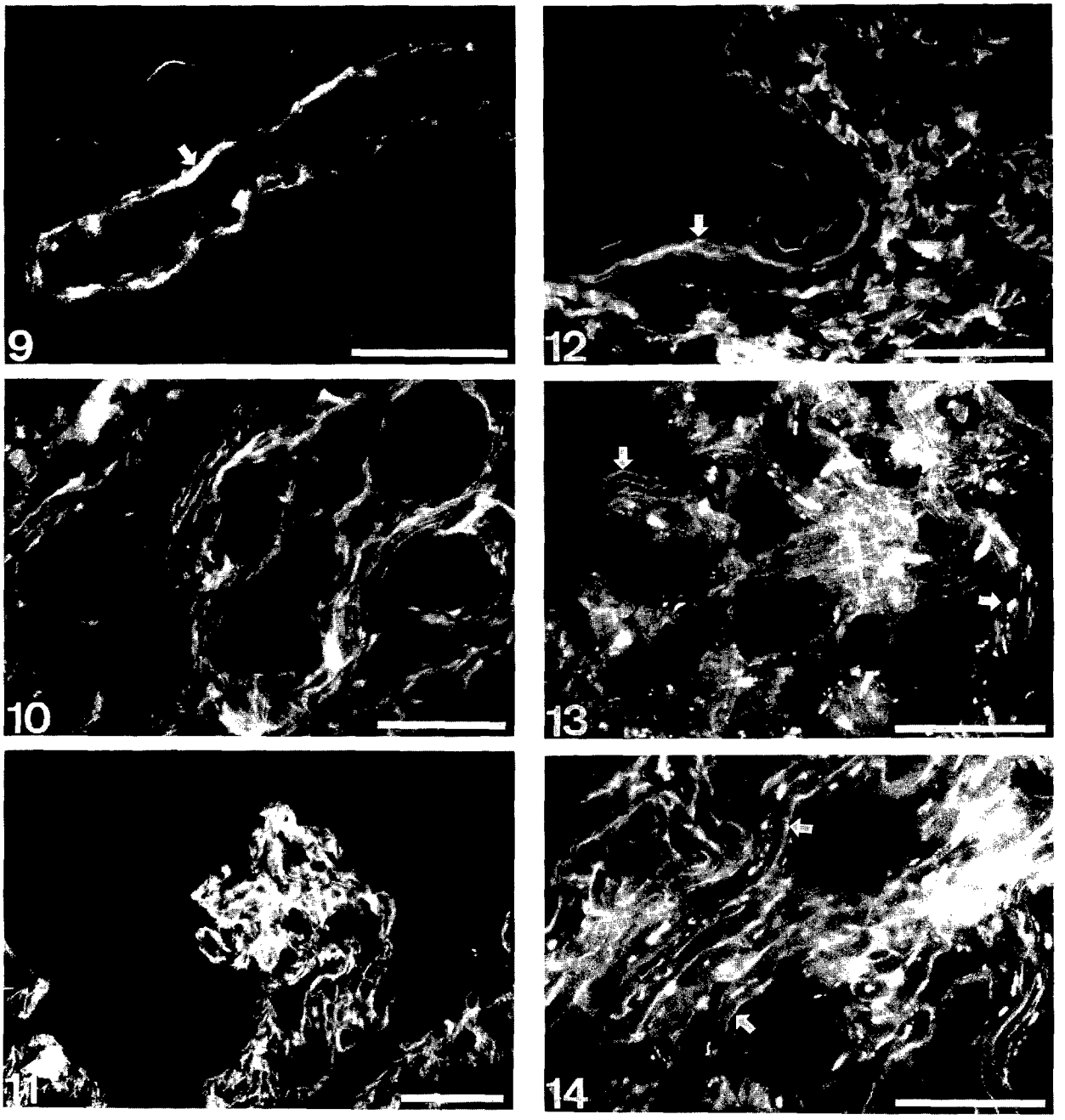

Fig. 9. Fetal left ventricular tissue, 8 weeks gestation. Strongly positive reaction to anti-laminin antibody is seen along the endocardium (arrow). Scale bar = 50 μm.

Fig. 10. Fetal left ventricular tissue, 14 weeks gestation. Strongly positive reaction to anti-laminin antibody is seen along the basement membrane of myocardial cells. Scale bar = 50 μm.

Fig. 11. Fetal left ventricular tissue, 16 weeks gestation. Relatively strong positive reaction to anti-laminin antibody is seen along the papillary muscles. Scale bar = 50 μm.

Fig. 12. Fetal left ventricular tissue, 24 weeks gestation. Relatively strong positive reaction to anti-laminin antibody is seen along the endocardium (arrow) or in the extracellular matrix. Scale bar = 50 μm.

Fig. 13. Neonatal left ventricular tissue, 2 weeks after birth. Positive reaction to anti-laminin antibody is seen along the basement membrane of myocardial cells (arrow); laminin forms fibrillar network. Scale bar = 100 μm.

Fig. 14. Adult left ventricular tissue. As in Fig. 13, positive reaction to anti-laminin antibody is seen along the basement membrane of myocardial cells (arrow); laminin forms fine fibrillar network. Scale bar = 100 μm.

tation (Fig. 12). However, after birth (in the 2-week old infant left ventricular tissues) strong positive reaction was present along the basement membrane of the myocardial cells in association with the transverse tubules and laminin formed a fine fibrillar network (Fig. 13). These structural characteristics were obvious in the adult left ventricular tissues (Fig. 14). These findings indicate that expression or distribution of laminin is not evenly dispersed in the extracellular matrix, but is relatively constantly maintained throughout the life regardless of age (before or after birth).

Discussion

Fibronectin

Fibronectin is composed of two chains that are connected by disulfide bonds at the carboxy-terminal end; it exhibits numerous binding domains for collagen, heparin, fibrin, factor 13a, proteoglycans and for many types of cells (7). The subunits of fibronectins vary in size between approximately 235 and 270 kDa plus carbohydrate. They occur in plasma, body fluids and in tissues in a large number of animal species including all mammals. Therefore, fibronectin may play a key role in linking the extracellular matrix and organ specific cells (23); it is produced by endothelial and smooth muscle cells, fibroblasts and myotubes (28, 29). Streuli and Bissell (32) suggested a feedback mechanism that regulates the synthesis of fibronectin and other extracellular matrix proteins in cell cultures. It may be important to elucidate cellular receptors reacting with the extracellular proteins, since cell receptors possess different affinity which makes possible complicated regulatory mechanisms for synthesis and breakdown of the different components of the extracellular space, although cellular receptors for extracellular proteins are largely identical (33). Fibronectins promote adhesion of cultured cells (17); they influence the organization of tissue in embryogenesis (2); they have a growth regulatory function as shown in the capillary endothelial cells (18); and fibronectin produced by mesenchymal cells in rat myocardium has a regulatory function in embryogenesis and maintenance of tissue structure in adult organs (1). In this study, we found that expression of fibronectin is relatively constant before birth but afterwards it is abruptly decreased; fibronectins are present mainly in association with blood vessels of embryo, fetal, and adult human left ventricular tissues. The pattern of expression of fibronectin in human fetal heart is similar to that in rat. Samuel *et al.* (26) described that during the development of the fetal rat heart fibronectin gene transcription is active and progressively decreases with age. Our results are partly consistent with previous studies (7) in which fibronectins are mainly localized at the vascular endothelium in rat myocardium. These results suggest that fibronectins mediate a tight connection between endothelial cell and proteins of interstitium and basement membrane (33), although we did not examine whole spectrum of left ventricular tissues in fetal period. Fibronectins have been shown to connect collagen and other matrix proteins (31); they interact with the intracellular cytoskeleton through talin-vinculin-actin connection (15), and also play a role in blood coagulation and in would healing (12, 14). In contrast to our results, Speiser *et al.* (30) found a considerable amount of fibronectin in normal adult myocardium, in porcine as well as in human heart. It is not clear why we could not find considerable amounts of fibronectin in the interstitium and why localization of fibronectin is restricted to perivascular area. However, it could be supposed that: 1) fibronectin is weakly attached to myocardial cells, therefore, certain parts of the molecule are highly susceptible to proteolysis during earlier phase of postmortem change; and 2) fibronectin is deposited largely from plasma (16), not principally by mesenchymal cells.

Laminin

Basement membrane (or basal lamina) serves as molecular filters and is part of cell membrane specialization, basal scaffolds that hold together the cells and the extracellular matrix (3). Laminin is the best known member of a family of basement membrane glycoproteins. It is composed of three subunits, the 400 kDa A-chain and two 200 kDa B-chains; it has an extended four-arm cruciform shape with many functional domains, i.e. the neurite outgrowth fragment, receptor mediated cell attachment sites, heparin, and collagen binding sites (3, 11, 21). Different isoforms of laminin exist in different tissues from the same animal (27) and it has been suggested that the different composition of the basal lamina may have functional importance. Panayotou *et al.* (22) and Ekblom *et al.* (10) found that assembly of basement membrane was inhibited by application of anti-laminin antibody. This means that laminin has an important role in cell attachment, spreading, differentiation and growth, and angiogenesis in embryogenesis and tumors.

In this study, regardless of age (before or after birth), laminin was relatively constantly expressed (200 kDa B2 chains were identified by Western blot) and localized in the endo- or epicardium, endothelial cells, fibroblasts, and basement membrane of myocardial cells in the infant and adult left ventricular tissues; it is also present along transverse tubules and formed fine fibrillar network. Our results are partly in agreement with previous studies (10, 30). Speiser *et al.* (30) found that B-chain is present in all basement membranes in associ-

ation with transverse tubules and A-chain especially in blood vessels of adult human myocardium; Ekblom *et al.* (10) found only little laminin A-chain activity in the mouse embryos, but both B-chains were abundantly present. In contrast, report from Borg's laboratory (24) described that distribution of laminin in neonatal rat cardiac tissue is marked by increased absolute amount. This discrepancy may be due to rather differences in the sensitivity of the laminin antigen to the different antibodies than a different composition of laminin. Rescan *et al.* (25) have found that different labeling pattern of laminin was evident for the different antibodies employed. This means that labeling pattern of laminin may vary in different regions even in the same tissue or same animal. However, judging from our results, 1) development and growth of laminin in the human heart are not quite different from those of laboratory animals; and 2) laminin attaches well to myocardial cells as a principal cell adhesive molecule, for maintenance of architecture of the interstitium and for cell differentiation and growth.

Conclusively, these results indicate that the expression of laminin is not age-dependent and constant throughout the life; in contrast, that of fibronectin is relatively constant during fetal life but decreases after birth.

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