Journal of Oral Science, Vol. 49, No. 2, 107-114, 2007

Original

Cellular origin of microfibrils explored by monensin-induced perturbation of secretory activity in embryonic primary cultures

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(Received 12 January and accepted 27 February 2007)

Abstract: Fibrillin is a primary component of elastin-associated microfibrils. Since microfibrils are distributed rather ubiquitously in embryonic tissues, attention has focused on the types of cells responsible for producing fibrillin. To clarify this issue, we employed monensin-induced perturbation of secretory activity in embryonic primary cultures, as this would allow examination of both the secreted protein and the formation of extracellular fibrils in the same culture. Micromasses of avian limb bud mesoderm, its ectodermal covering and several explants from other sources were cultured in the presence and absence of monensin, and evaluated immunohistochemically using antibodies against fibrillin and cell lineage markers. The results indicated that monensin perturbation induced intracellular accumulation of fibrillin and prevented the formation of microfibrils. It was shown specifically that not only mesodermally derived fibrogenic cells and myogenic cells of skeletal and smooth muscle cell lineage, but also epithelial-type cells such as endothelial and ectodermal cells, are producers of fibrillin. This dual cellular origin of fibrillin at the ectomesenchymal interface is considered significant for understanding the formation and remodeling of microfibrils originating from the basal lamina. (J. Oral Sci. 49, 107-114, 2007)

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Keywords: fibrillin; monensin; microfibrils; elastic fiber; extracellular matrix; development.

Introduction

Microfibrils form endpoint fibrous structures in some adult tissues, such as oxytalan fibers in the periodontal ligament (1) and the suspensory ciliary ligament of the ocular lens (2). However, microfibrils and microfibrillar fibers (MFs) are distributed rather ubiquitously in embryonic tissues (3,4). This suggests that more than one cell population is capable of producing microfibrillar components, and consequently MFs, during embryonic development.

In order to examine the types of cells responsible for producing microfibrillar components, we employed monensin-induced perturbation of secretory activity in embryonic primary cultures. Monensin, a polyether produced by *Streptomyces cinnamonensis*, is a carboxylic ionophore (5) that blocks (or considerably slows down) protein transport through the Golgi stack (5-7). As is the case for ordinary secretory proteins such as digestive enzymes and hormones, components of extracellular fibers are transported from the rough endoplasmic reticulum, through the Golgi complex, to secretory vesicles and eventually discharged into the extracellular space. Perturbation of this cellular pathway by monensin induces intracellular accumulation of secreted components in the Golgi cisternae, and also results in lack of extracellular fiber formation.

In the present study, we focused primarily on cell populations in the chick limb bud, where the temporospatial

distribution of microfibrils and MFs has been well demonstrated immunohistochemically (4). The limb bud consists of an outer layer of ectodermal cells and an inner mass of mesenchymal cells. The latter cells, when placed *in vitro*, retain their intrinsic potential to express and segregate into at least three distinct phenotypes: chondrogenic, myogenic and fibrogenic populations (8-10). Thus, in combination with staining for cell lineage markers, intracellular accumulation of fibrillin and extracellular formation of MFs were visualized in experimental groups (with monensin) and their controls (without monensin), respectively, using FB1 antibody directed against chick fibrillin-2 (4).

Materials and Methods

Tissue source for culture

Fertilized eggs of White Leghorn (*Gallus gallus*) from Ohata Hatchery (Shizuoka, Japan) and Japanese quail (*Coturnix japonica*) from a local experimental animal supplier (Sugito, Saitama) were incubated in a humidified thermogradient incubator (MTI-201A; EYELA). Embryos were removed and staged according to the morphological criteria of Hamburger and Hamilton (11). Chick embryos at stage 20 and at 6 days of incubation and quail embryos at stages 14 and 20 were collected and used as sources for the primary culture.

Micromass culture

High-density micromass culture (12) was prepared from stage-20 chick and quail leg buds as described previously (4). Briefly, leg buds ($n \geq 30$) were gently digested with 0.5% trypsin (Gibco) in Earle's balanced salt solution (EBSS) at 37°C. When the ectodermal jacket became loosened (within approximately 4 min), the leg buds were washed and transferred to ice-cold EBSS containing 2% chicken serum (CS; Gibco), and the ectoderm was removed carefully with forceps. Limb mesoderm after removal of the ectodermal jacket was dissociated completely with 0.5% trypsin in calcium- and magnesium-free phosphate-buffered saline (PBS; pH 7.35) and washed. Cells at a concentration of 2×10^7/ml in 10 µl of α-modified minimal essential medium (αMEM; Gibco) supplemented with 10% CS, ITS premix (Gibco) and antibiotics (100 units/ml penicillin and 100 pg/ml streptomycin; GIBCO) were plated in the center of a 35-mm culture dish and incubated at 37°C in a humidified atmosphere of 5% CO_2 and 95% air.

Ectodermal sheet culture

Leg bud ectodermal jackets removed as described above were used. Because of their convex morphology, one or more incisions were made so that the ectoderm could be placed as a flat sheet on a 35-mm culture dish (precoated with rat tail type I collagen; Becton Dickinson), which had been premoisturized with supplemented αMEM.

Explant culture

Cardiac explants from the stage-14 quail atrioventricular (AV) canal were prepared as described previously (13,14). Tissues removed from day-6 chick gizzard or aortic wall were aseptically divided into small pieces approximately 1×3 mm in size using microscissors. Explants from these three sources were placed on collagen-precoated 35-mm culture dishes, which had been premoisturized with supplemented αMEM for the gut and vascular explants or with similary supplemented M199 medium (Gibco) for the cardiac explants.

Monensin administration

Mesodermal leg bud micromasses, their ectodermal jacket and three other explants from different tissues were fed with the medium referred to above after allowing the cells to attach to the dishes for 3 h. At 18 h of culture, a stock solution of monensin (10^{-4}M; Sigma) in 90% ethanol was added to the culture medium at a final concentration of 10^{-6}M. The concentration at which monensin exerted its optimal effect on the cultures, and the duration of the effect, were determined preliminarily by reference to previous reports (6,7,15). Control cultures were incubated in the absence of monensin. Leg bud mesoderm micromasses were fixed on day 3, and all the other cultures were terminated on day 2.

Immunohistochemistry

Cultures terminated and fixed with 100% ethanol for 30 min at 4°C were rinsed and equilibrated in PBS. After blocking in 1% bovine serum albumin (BSA)-PBS for 1 h, cultures were reacted for 1 h with mouse anti-chick fibrillin-2 IgG_1 (undiluted FB1 hybridoma conditioned medium)(4) and rinsed in PBS. For double staining with anti-desmin (a myogenic cell lineage marker), QH1 (an endothelial cell lineage marker) or anti-fibronectin, cultures were further incubated for 1 h with one of these antibodies diluted 1:100 with BSA-PBS. Rabbit anti-chick fibronectin (AB1946) and rabbit anti-chick desmin (AB907) polyclonal antibodies were both purchased from Chemicon, Millipore Corp. QH1 monoclonal antibody, which is specific to quail vascular endothelial cells (16), was kindly provided by the Developmental Studies Hybridoma Bank (University of Iowa, IA, USA). Controls for immunostaining included omission of these primary antibodies or the use of normal, non-immune mouse or rabbit IgGs.

Cultures incubated with the specific antibodies or control IgGs were subsequently reacted for 1 h with fluorescein-isothiocyanate (FITC)-conjugated goat anti-mouse IgG (Cappel) for FB1, or with tetramethyl rhodamine-isothiocyanate (TRITC)-conjugated goat anti-rabbit IgG (Cappel) for AB1946 and AB907. Fluorochrome-conjugated antibodies were diluted 1:100 in 1% BSA-PBS, to which, in some cases, 4', 6-diamidino-2-phenylindole (DAPI; Sigma) was added at 2 ng/ml for nuclear staining. Since FB1 and QH1 are both mouse IgG_1, their IgG fractions were directly labeled with Cy2 and Cy3 as described previously (4) and used for this combination of double staining.

Immunostained and rinsed cultures were mounted with SlowFade (Light Antifade Kit; Molecular Probes) or 90% glycerol-PBS containing 0.2 M *n*-propylgallate (17) and examined with an epifluorescence microscope (Eclipse E600; Nikon) equipped with a CCD camera (Pro 600ES; Pixera) for digital recording. Double-stained images presented in this report were obtained by either double exposure through B-2A and G-2A filters (Nikon) or by single exposure through a F-R dual filter (Nikon). Images of blue nuclear staining by DAPI were obtained separately through a UV-1A filter and superimposed digitally on the images of the immunostained cells.

Results

To examine the cellular origin of FB1-positive microfibrillar components, or fibrillin, micromass cultures of leg bud mesoderm, its cultured ectodermal sheet, and several explant-type cultures from other sources were prepared and incubated in the presence and absence of monensin. In explant-type cultures, cells growing out from the periphery of the explant were examined, since the explant itself contained preexisting matrices including MFs. In micromass cultures established by high-density plating, source tissue was initially dissociated completely into individual cells and washed, but since multilayered mesenchymal masses were inconvenient for close examination, only cells growing out from the micromass were examined. This kind of observational care was not necessary for ectodermal sheet cultures. Representative examples of reproducible results are illustrated in Figures 1-5; no comparable staining was observed in the immunohistochemical controls in which primary antibodies were omitted or replaced with non-immune immunoglobulins (not shown).

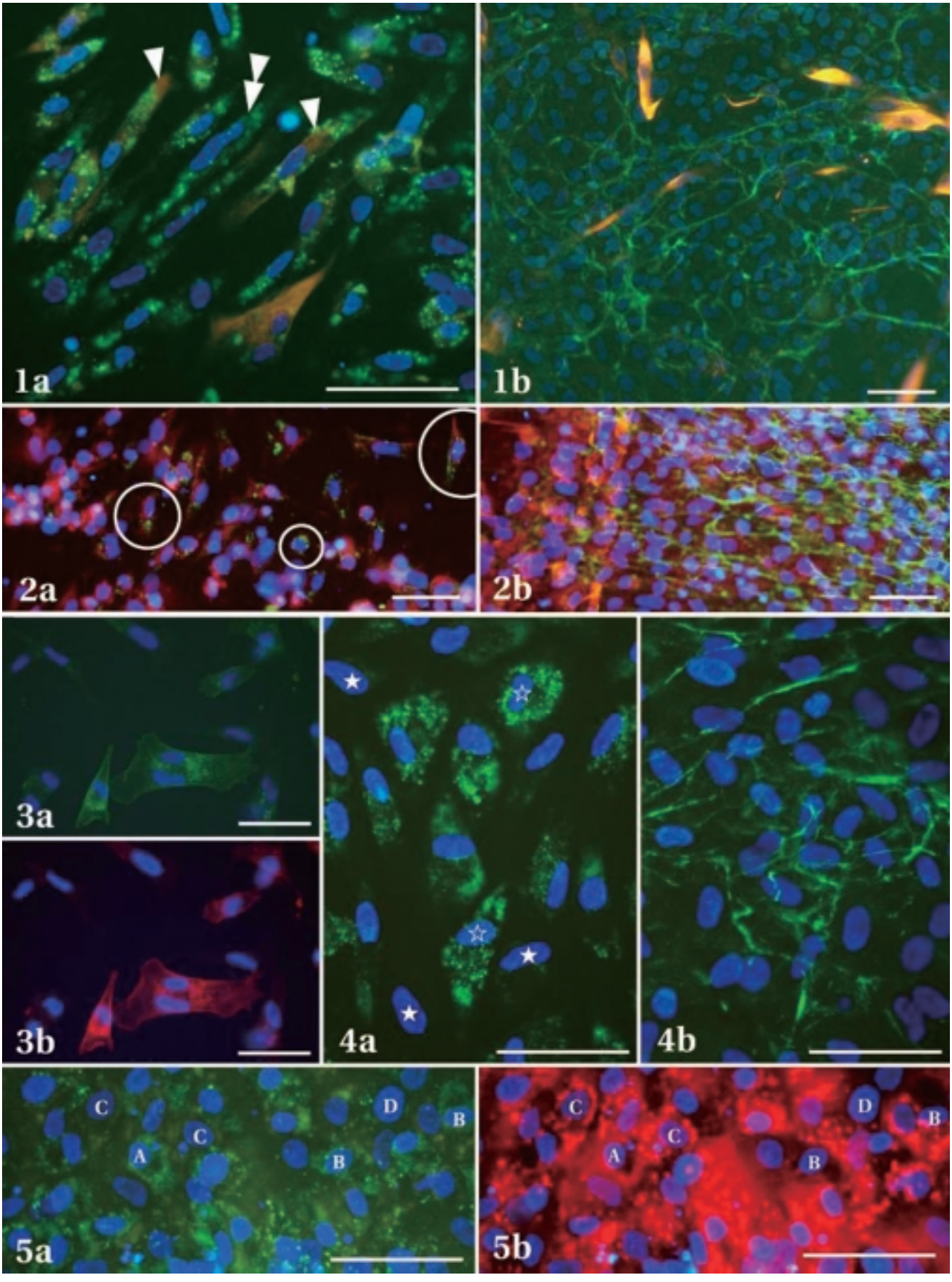
In cultures that included monensin, FB1-positive staining was observed in cytoplasmic vesicles, which were very likely dilated Golgi cisternae, and extracellular MFs were absent (Figs. 1a, 2a and 4a), suggesting that monensin effectively suppressed the secretion of fibrillin. In contrast, intracellular staining for fibrillin was undetectable, and extracellular MFs were distinctively demonstrated in the control cultures that did not contain monensin (Figs. 1b, 2b and 4b). Desmin staining was observed in a fraction of cells in cultures both with and without addition of monensin (Figs. 1 and 2).

Monensin-induced intracellular accumulation of FB1-positive material was found in the majority of limb bud mesenchymal cells that grew out from the micromasses, but a subset of these cells expressed a myogenic cell lineage marker, desmin (Fig. 1a). Since desmin-positive cells in the limb bud mesoderm are known to form multinucleated myotubes characteristic of skeletal muscle cells (9), the ability of smooth muscle cells to synthesize fibrillin was examined separately (Fig. 2). In the presence of monensin, intracellular accumulation of FB1-positive material was observed within desmin-positive visceral smooth muscle cells (Fig. 2a). Similar observations were obtained for vascular smooth muscle cells growing out from aortic wall explants (not shown).

The stage-20 leg bud subjected to micromass culture contains developing vasculature with functional circulation (18). Therefore, quail mesodermal micromasses were analyzed using a QH1 marker specific to quail endothelial cells. Intracellular accumulation of FB1-positive material was clearly shown to reside in QH1-positive cells (Fig. 3), although the number of endothelial cells present in the culture was apparently low. We further examined endothelial cells growing out from cardiac AV explants incubated in the presence and absence of monensin. In the presence of monensin, intense FB1 staining was observed in the majority of cardiac endothelial cells showing a cobblestone-like appearance (Fig. 4a), whereas in the absence of monensin, the cells lacked intracellular staining and showed a well developed extracellular microfibrillar network (Fig. 4b).

Leg bud ectoderm was removed as a continuous sheet and cultured on the dishes. The effect of monensin on secretory activity was examined with respect to both fibrillin and fibronectin proteins, the latter of which is a major extracellular matrix component in the basement membrane. Double immunostaining with FB1 and anti-fibronectin revealed unmatched heterogeneity of fibrillin and fibronectin accumulation; i.e., four different possible combinations of monensin-induced accumulation, +/+, +/-, -/+ and -/- (indicated as fibrillin/fibronectin) (Fig. 5).

Fibrillin producers determined by the presence of monensin-induced intracellular accumulation of FB1-positive material are listed in Table 1 together with other related information.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Discussion

In this study, monensin-induced perturbation was utilized to explore the cellular origin of a microfibrillar component, fibrillin. Microfibrils are composed primarily of fibrillins and other integral and associated molecules, as listed in detail in a recent review (19). The expression patterns of those molecules can be examined by detecting their specific mRNAs, but the present perturbation approach is of significance, because the expression of protein to be secreted and the formation of relevant extracellular fibrils (and fibers) can be observed in the same culture and assessed directly by comparing them with each other.

It is a central conceptual paradigm of matrix biochemistry that matrix molecules such as collagens can self-assemble into fibrils *in vitro* in the absence of cells, but the way in which molecules produced by living cells become organized into extracellular fibers is apparently under cellular control (20,21). Protein expression of matrix fiber components is closely related to the cellular process that governs the formation of normally structured extracellular fibers. For example, macromolecular assembly of collagens is known to proceed in a stepwise manner from initiation through elongation to the stabilization phase, all of which are strictly regulated by cellular involvement (20,22). Likewise, other types of matrix component such as elastic fibers and microfibrils are considered to follow a similar cell-dependent assembly path (22,23).

The present study has shown that embryonic mesenchymal cells are the major producer of microfibrillar components and that their subsets with specific lineage commitment and other embryonic cell populations with advanced phenotypes are also responsible for tissue-specific production of fibrillin. Among the cells identified as producers of fibrillin (Table 1), fibrogenic cells in the leg bud mesenchyme represent a subset without any specific marker such as desmin and QH1, and therefore must contain undifferentiated precursors for distinct cell populations at later stages of development. However, it is unequivocal that the vast majority of these undifferentiated mesenchymal cells are fibrogenic and precursors of so-called fibroblasts, as suggested by their behavior in 3-dimensional collagen lattice culture (9).

Limb bud mesenchyme also contains chondrogenic and myogenic cells. Chondrogenic cells were not analyzed in this study because they are immobile (9,10) and stay within the multilayered micromass. However, since the cartilaginous matrix is completely devoid of fibrillin immunoreactivity (4), chondrogenic cells are unlikely to

Fig. 1 Cell outgrowth from a mesodermal micromass, which was prepared from a stage-20 chick leg bud and cultured in the presence (a) and absence (b) of monensin. Green fluorescence represents intracellular accumulation of FB1-positive material (in a) and a network of extracellular MFs (in b); intracellular accumulation is not detectable in b. Reddish flurorescence represents desmin staining of the myogenic cell population. Cells indicated with a single arrowhead are positive for desmin, while the one indicated with double arrowheads lacks desmin staining. Distribution of nuclei is shown in blue. Bars = 50 μm.

Fig. 2 Cell outgrowth from a gastric explant, which was excised from day-6 chick gizzard and cultured in the presence (a) and absence (b) of monensin. Green fluorescence represents FB1-positive staining, by which intracellular accumulation of fibrillin (a) and extracellular MFs (b) are visualized. Reddish flurorescence represents desmin-positive smooth muscle cells. The latter, as in the ones in circle, contain green vesicles indicating transport perturbation and resulting intracellular accumulation of fibrillin. Distribution of nuclei is shown in blue. Bars = 50 μm.

Fig. 3 Cells migrating away from a mesodermal micromass, which was prepared from a stage-20 quail leg bud and cultured in the presence of monensin. Images of green FITC for fibrillin (a) and red TRITC for a QH1-endothelial marker (b) are taken from an identical area of the double-immunostained culture; in both, nuclei are stained blue. Note that cells expressing the distinct endothelial marker contain accumulated FB1-positive material. Bars = 50 μm.

Fig. 4 Cardiac endothelial cells growing out of an AV explant, which was excised from stage-14 chick heart and cultured in the presence (a) and absence (b) of monensin. Green and blue fluorescence represents fibrillin and nuclear staining, respectively. Fibrillin staining is evident intracellularly in the presence of monensin (a) but shown extracellularly in the form of fibrous structures in the endothelial cells varies from virtually nothing (solid asterisks) to abundant (clear asterisks). Bars = 50 μm.

Fig. 5 An ectodermal cell layer, which was prepared from a stage-20 chick leg bud and cultured in the presence of monensin. These images of green FITC for fibrillin (a) and red TRITC for fibronectin (b) are taken from an identical area of the double-immunostained culture; in both, the distribution of nuclei is shown in blue. All of the four different patterns of intracellular accumulation of fibrillin and/or fibronectin are shown: i.e., +/+ (both fibrillin and fibronectin), +/- (only fibrillin), -/+ (only fibronectin) and -/- (neither) are labeled as A, B, C and D, respectively. Bars = 50 μm.

produce fibrillin. In contrast, myogenic cells in the limb bud mesenchyme, and visceral and vascular smooth muscle cells from two different tissues were all fibrillin producers (Table 1). Considering that myogenic cells detectable with anti-desmin in the limb bud mesenchyme differentiate into multinucleated myotubes (9), embryonic cells in both skeletal and smooth muscle cell lineages appear to be involved in the production of microfibrillar components and subsequent fiber formation.

Subsets of endothelial cells and limb bud ectodermal cells were also fibrillin producers (Table 1). The significance of this finding is two-fold. Firstly, this indicates that not only mesenchymal cells but also epithelial cell populations can be responsible for the production of microfibrillar components and subsequent fibrillogenesis. In the adult epidermis, keratinocytes partially produce dermal extracellular macromolecules such as versican (24), tenascin (25), fibrillin-1 (26) and type XVI collagen (27). We showed that embryonic ectodermal cells do produce a dermal matrix component, fibrillin, similarly. As for endothelial cells, fibrillin secreted by them may form a local matrix associated with microvasculature in the limb bud in concert with relevant smooth muscle cells, but endocardially derived fibrillin, which was abundant as shown in Fig. 4, would contribute significantly to the formation of the early, acellular endocardial cushion (28) which occupies a large spatial volume. The latter interpretation is consistent with previous immunohistochemical observations of cardiac valve development using the JB3 antibody directed against fibrillin (29).

Secondly, the dual cellular origin of the microfibrillar component in the region close to the ectomesenchymal interface would be important for understanding the formation and remodeling of MFs originating directly from the ectodermal basal lamina (4). In comparison with type VII collagen anchoring fibrils, which are believed to maintain the integrity of the dermoepidermal junction (30), MFs constitute a more robust system in the chick embryo, which appears to provide structural and functional connections between the ectoderm and the developing elastic and microfibrillar network in the presumptive dermis (4).

In this context, the heterogeneity of fibrillin accumulation in monensin-treated epithelial cells is intriguing. The difference in the amount of accumulation in endocardial cells (Fig. 4) appears to simply represent variations in the state of the cell cycle and synthetic activity. However, unmatched heterogeneities of fibrillin and fibronectin accumulation in a continuous sheet of limb bud ectoderm (Fig. 5) suggest that these two matrix proteins may be regulated differentially depending on local biological and tissue architectural requirements; for example, whether the ectodermal cells are involved primarily in basal lamina maintenance or in the formation and remodeling of MFs originating from the basal lamina. To obtain definitive answers, further studies are required.

Table 1 Fibrillin producers in the limb bud and other embryonic tissues§

tissue source	cell type	culture method	marker used
leg bud	fibrogenic cell	micromass†	n/a‡
	myogenic cell	micromass†	desmin
	endothelial cell*	micromass†	QH1
	ectodermal cell*	sheet culture	n/a
gizzard	smooth muscle cell	explant†	desmin
aortic wall	smooth muscle cell	explant†	desmin
heart	endocardial cell*	explant†	QH1

§ Cells in which monensin-induced intracellular accumulation of FB1-positive material was observed.

* Quail embryonic tissues were used; others were from chick embryos.

† Cells growing out from the micromass or explant were examined.

‡ Dispersed mesenchymal cells without desmin immunoreactivity were assumed to be fibrogenic.

Acknowledgments

This work was supported by the Sato Fund (KI, YY) and Dental Research Center (KI, YY) at Nihon University School of Dentistry, the Promotion and Mutual Aid Corporation for Private Schools of Japan (KI, YY), and a grant for promotion of multidisciplinary research projects (KI) from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

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