Shimane J. Med. Sci., Vol.30 pp.1-10, 2013

Histological Changes in the Developing Heart of Human Fetuses

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(Received February 6, 2013; Accepted February 6, 2013)

Histological changes of human fetal hearts were observed in fetuses from the 3rd to 7th month of gestation to analyze the myocardium, endocardium, and epicardium as well as Purkinje fiber cells. In the 3rd month, cardiac muscle cells were irregular in shape and arrangement. In the 7th month, they were more homogenous in shape, longer than in 3rd month, and typically arranged in parallel. These changes became evident from the 6th month, and by the 7th month their histological features had become almost the same as those in adults. There were no significant regional differences in the morphology of the cardiomyocytes among the parts of the heart. In both the endocardium and epicardium, a relatively rich underlying connective tissue layer was observed in the 3rd month, whereas it decreased significantly thereafter. Subendocardial Purkinje fibers were not clearly identified in the 3rd month, but they were identified from the 4th month. The present findings show normal morphological development of human fetal heart and suggest the corresponding functional maturation.

Key words: Human fetus, cardiac muscle cell, Purkinje fiber, endocardium, epicardium, histology

INTRODUCTION

There has been an explosion of work on the steps involved in the formation of the primary heart tube and the formation of cardiac chambers and arterial trunks during heart development [1, 2]. In recent studies using experimental animals such as mice, rats, and chicken, expression patterns of transcription factors in the developing heart muscle cells have been evaluated and electrophysiological characteristics have been analyzed in association with early cardiac development [3, 4]. It has also been shown that the oriented clonal cell growth of myocardial cells plays an important role in the cardiac morphogenesis [5, 6]. However, much less has been described about the histological development of the cardiac muscle cells and the Purkinje fiber cells in human fetuses. Previous reports have lacked detailed descriptions of the morphological changes in cardiac muscle during the human fetal period when histological differentiation and maturation occur [7]. Significant advances have been made in understanding the developmental biology of the fast conduction network - the His-Purkinje system in the cardiac ventricle and septum. Recent work has revealed the comparability of the molecular mechanisms of the patterning of the cardiac conduction tissues in the developing heart in humans and mice [8]. Recent studies also have elucidated that growth factor signaling from both the endocardium and epicardium is responsible for stimulation of myocardial proliferation following cardiac looping [9, 10]. While these advancements have largely been made using animal models such as chicken and mice, descriptions of histological changes during the fetal period in humans are still lacking. Therefore, the findings gained from humans are not only useful to correlate cardiac development between human and animals, but also may help to understand the etiology of human cardiac diseases, as well as lead to new treatment strategies [11].

In this study, we report histological development of the cardiac muscle and Purkinje fiber cells together with the endocardium and epicardium of human fetuses from the 3rd to the 7th month of gestation as observed by light microscopy.

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MATERIALS AND METHODS

Human fetuses and histological sections of the heart

The present study was approved by the Ethics Committee of Shimane University Faculty of Medicine and was conducted in accordance with the World Medical Association and the Declaration of Helsinki. Twenty-seven normal human fetuses ranging from the 3rd to 7th month of gestation, all belonging to the Kyoto Collection of human embryos [12-17], were used in the present study (Table 1). They were obtained through cooperation with the Japanese obstetricians in accordance with the Law for the Protection of Mother's Bodies. Therefore, these fetuses were mostly from healthy parents and therefore can be considered representative of the normal Japanese intrauterine population. This is in contrast to most previous studies which have been based on naturally aborted fetuses, so that abnormalities in the fetuses could not be excluded. Upon removal, the fetuses were fixed and preserved in 10% formalin. Organs including the heart of well preserved representative fetuses were dissected and prepared for histological sections using a routine paraffin-embedding method. The hearts were cut transversely (vertically to the baso-apex axis), and sections of 5-μm thickness were stained by hematoxylin eosin (HE) staining. These histological sections of human fetal organs including the heart have been preserved as a part of the collection at the Department of Developmental Biology, Faculty of Medicine, Shimane University. We selected for the present study heart sections of the 27 fetuses from the 3rd to 7th month of gestation (lunar month) [13] (crown-rump length (CRL) 76mm - 295mm, body weight 30g - 2205g, Table 1) and observed them under a binocular microscope. We observed morphological changes in cardiac muscles and Purkinje fiber cells comparatively among the right and left ventricles and the interventricular septum, as well as developmental changes in the endocardium and epicardium from the 3rd and 7th months. Characteristic morphology was described when it was constant in consecutive sections of a sample and in corresponding sections of multiple samples at the same lunar month.

Table 1. Fetuses used in the present study

Lunar month	CRL (mm)	BW(g)	Number
III	76	30	1
IV	57.6-113	14-85	3
V	80-176	25-340	9
VI	148-218	235-690	9
VII	165-295	750-2205	5
Total			27

RESULTS

Development of the heart muscle cell morphology in human fetuses

We observed cardiac muscle cells from the ventricles and septa of human fetuses (Fig. 1). Cardiac muscle cells could be identified by striations, branching fibers, centrally located nuclei and intercalated discs. We first compared cardiac muscles at the 3rd and 7th months, i.e., the youngest and oldest examined in the present study, to identify the characteristic histological changes. Cardiac muscle cells of the 3rd month were irregular in shape, round-shaped or rod-shaped, and variable in diameter. By the 7th month, the cardiac muscle cells were gener-

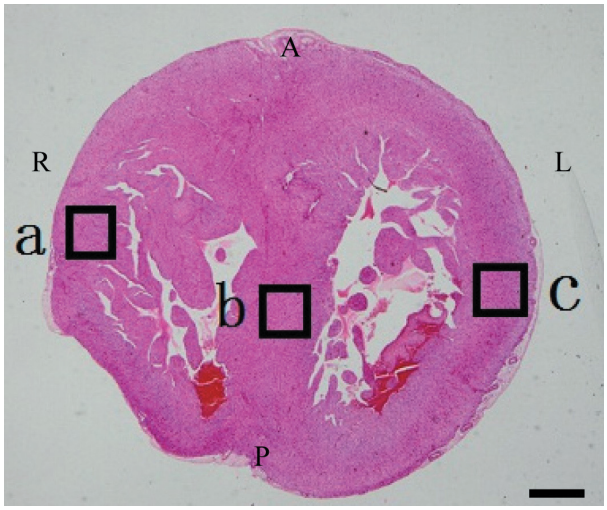

Fig. 1. A transverse section from a 3rd month human fetus. We observed the cardiac muscle cells from the ventricles and septa of the human fetuses. The rectangles represent the parts of which magnified views are shown in the subsequent figures. R: right, L: left, A: anterior, P: posterior. Scale bar: 500 μm.

ally more uniform, arranged parallel in the same direction and were longer than those in the 3rd month. The morphology of the ventricular wall cells resembled that of the septum cells, and they were both immature, containing mitotic cells, in the 3rd month (Fig. 2). Cross-striations in cardiac muscle cells were observed more clearly in the 7th month than in the 3rd month (Fig. 3). Also, the nuclei in the 7th month appeared larger than those in the 3rd month, whereas they were centrally located in both months.

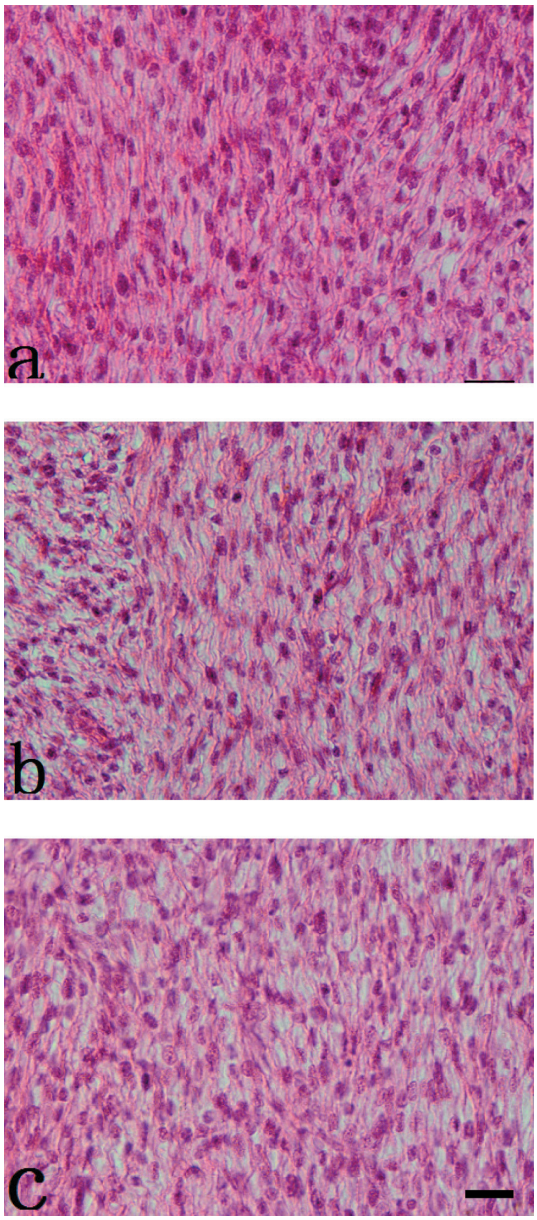

Fig. 2. Heart muscle cells in the ventricle and septum. Light micrographs of the heart muscle cells of the 3rd month stained with HE staining. The locations of (a), (b), and (c) are as shown in Fig. 1. Scale bar: 20 μm.

The perinuclear region of the cardiac muscle cell appeared empty by HE staining. In the 7th month the fibers were observed to branch clearly. We followed individual fibers to find branching points of the cardiac muscle cells, focusing carefully up and down with fine control to locate the intercalated discs. In many sections in the 3rd month the intercalated discs were difficult to see, whereas in the 7th month they were stained darkly and easily observed in those areas sectioned longitudinally (Fig. 3b'). Since morphological differences between 3rd and 7th month cardiac muscle cells were so apparent, we further analyzed when these changes became apparent using sections from the 4th, 5th, and 6th months (Fig. 4). In the 4th month, the cells were generally arranged in the same direction, but the cells were still irregular in shape, and the nuclei were different in size, as in the 3rd month. In the 5th month, the cell orientation had not changed significantly, while cell shape was still irregular but larger than in the 4th month. In the 6th month, the cell shape changed significantly, the cells were arranged parallel in the same direction, and the shape and size of the cylindrical nuclei were basically homogenous and regularly arranged as with those in the 7th month. Intercalated discs also became more and more clearly observed from 4th to 6th month (data not shown).

Developmental changes in the endocardium and epicardium in human fetuses

Recently, it has become evident that the epicardium and endocardium both play important roles in myocardial development and the control of myocardial function [9, 18, 19]. We observed changes in the epicardium of the left ventricle and the detailed organization of the tissues from the 3rd to 7th month (Fig. 5). There were significant differences in the epicardium between the 3rd and 7th months. In the 3rd month pericardial cells and the underlying loose connective tissue were clearly observed, and the epicardial layer was much thicker than in the 7th month. In the following months the epicardium gradually decreased in thickness mainly due to a decrease in the connective tissue (Fig. 5). The endocardium of the heart consists of an endothelial layer supported by subendothelial connective tissue. We focused on the endocardium of the left ventricle and

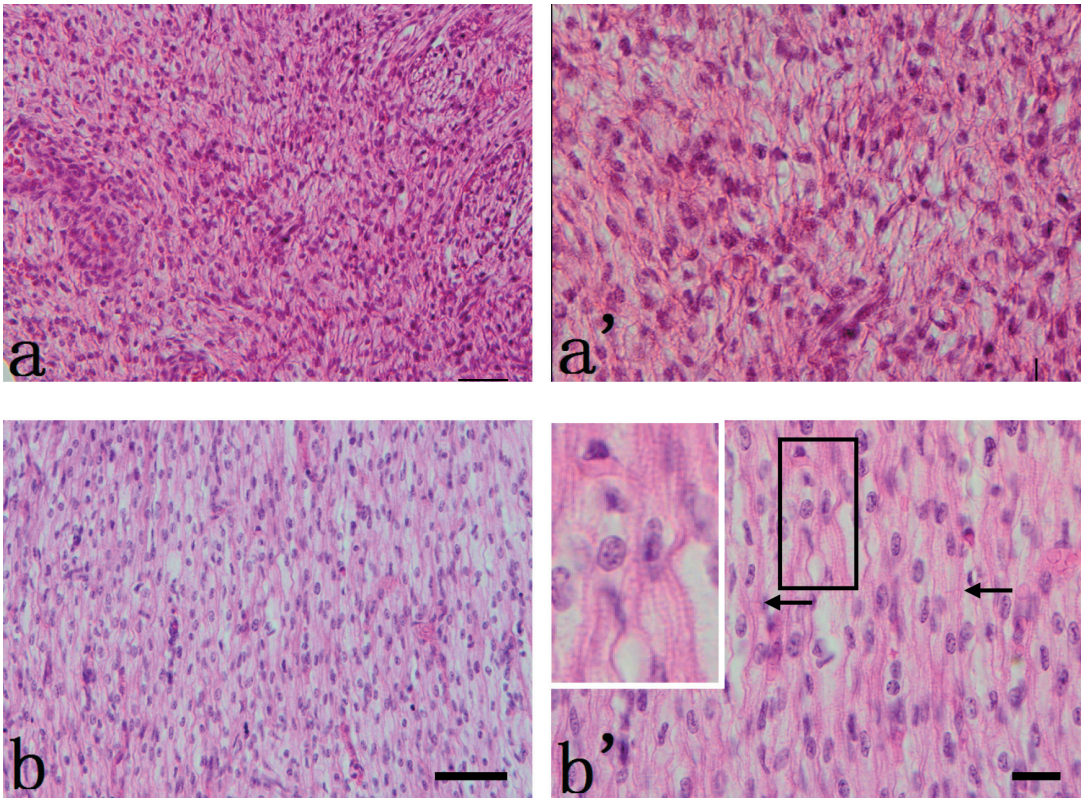

Fig. 3. Heart muscle cells in the ventricle. （a. a'） the 3rd month.（b. b'） the 7th month. We focused on the muscle cells that were cut in longitudinal orientation, as in the above photos. The inset is the magnified view of the boxed part to show cross-striation. Arrows indicate intercalated discs. Scale bars: a, b: 50 μm; a', b': 20 μm.

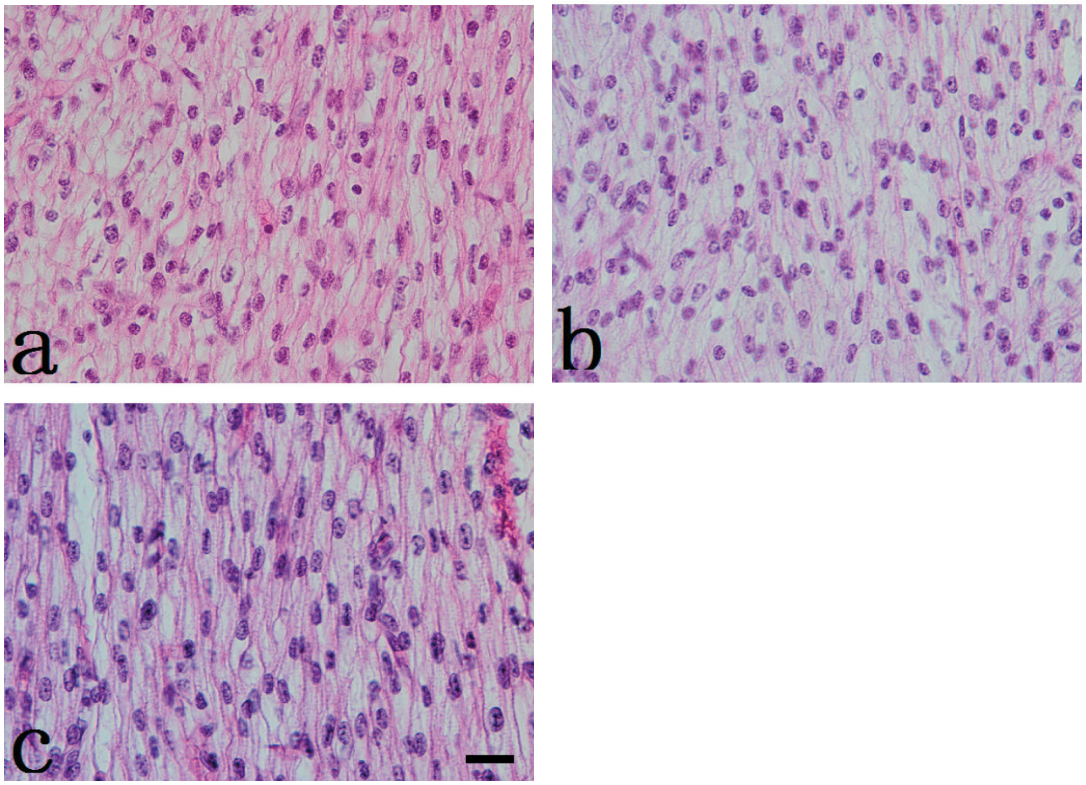

Fig. 4. The cardiac muscle cells in the 4th month（a）, 5th month（b） and 6th month（c）. Scale bar: 20 μm.

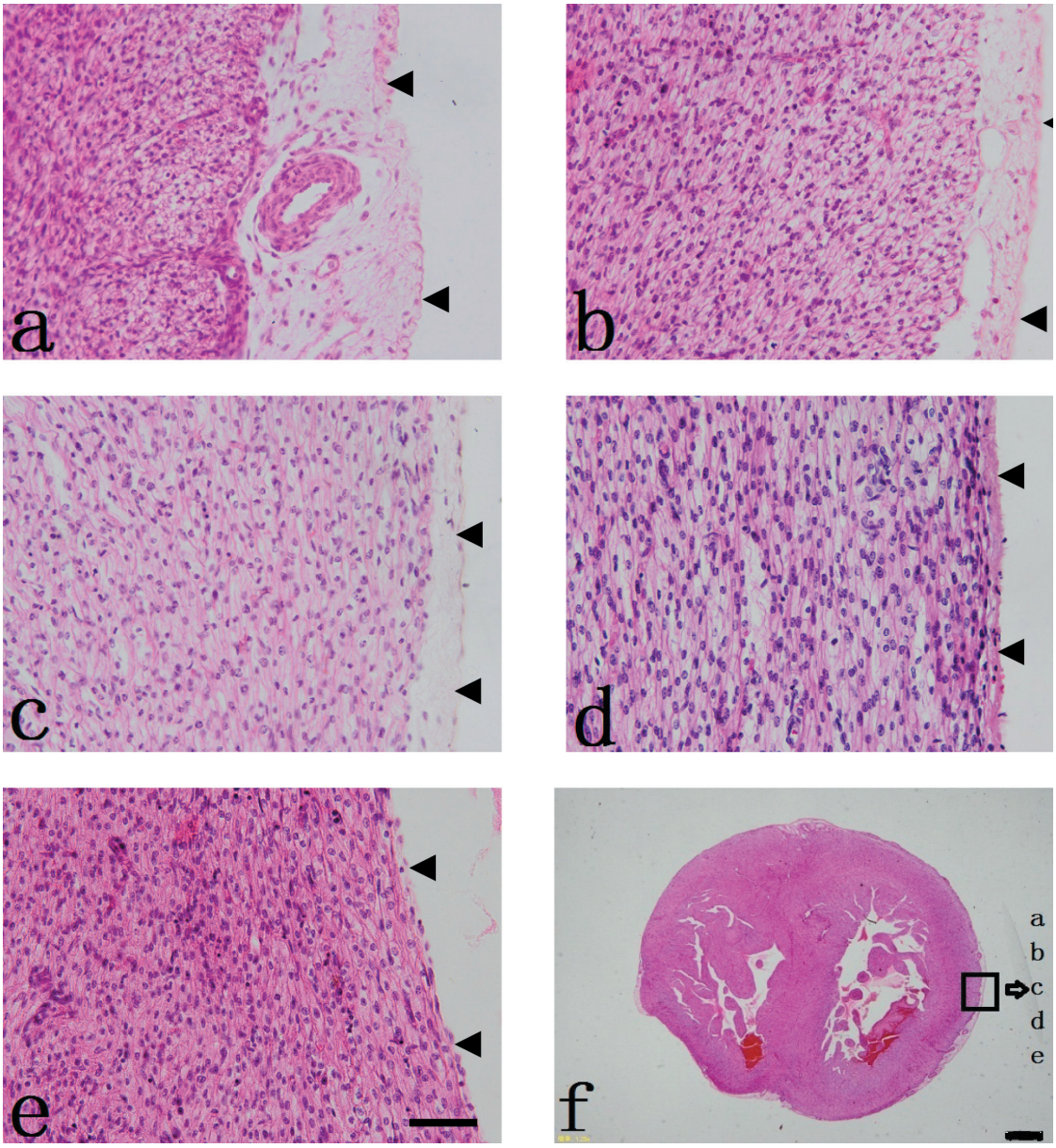

Fig. 5. Development of the epicardial layer in the 3rd month (a), 4th month (b), 5th month (c), 6th month (d) and 7th month (e) from the left ventricle of the heart. Arrowheads indicate the outer surface of the left ventricle. Scale bar: 50 μm.

observed the changes from 3rd to the 7th month (Fig. 6). In the 3rd month, the endothelial surface was irregular and connected with the myocardium by connective tissue. Cells beneath the endothelium were irregular in shape, size, and orientation. From the 4th to 7th month, the endothelial surface became flat and the connective tissue decreased significantly. Cells beneath the endothelium and cardiac myocytes became aligned in the same direction. The endothelial cells were smaller and the nuclei were also smaller than in normal myocardial cells. The endocardial area became smaller in the 7th month (Fig. 6), and the developing myocardial cells occupied most areas of the entire heart.

Development of the Purkinje fiber cells in human fetuses

The conduction system includes the central conduction system and peripheral conduction system. In the peripheral conduction system, components include the left and right septal bundle branches, the subendocardial Purkinje fibers. While another sub-

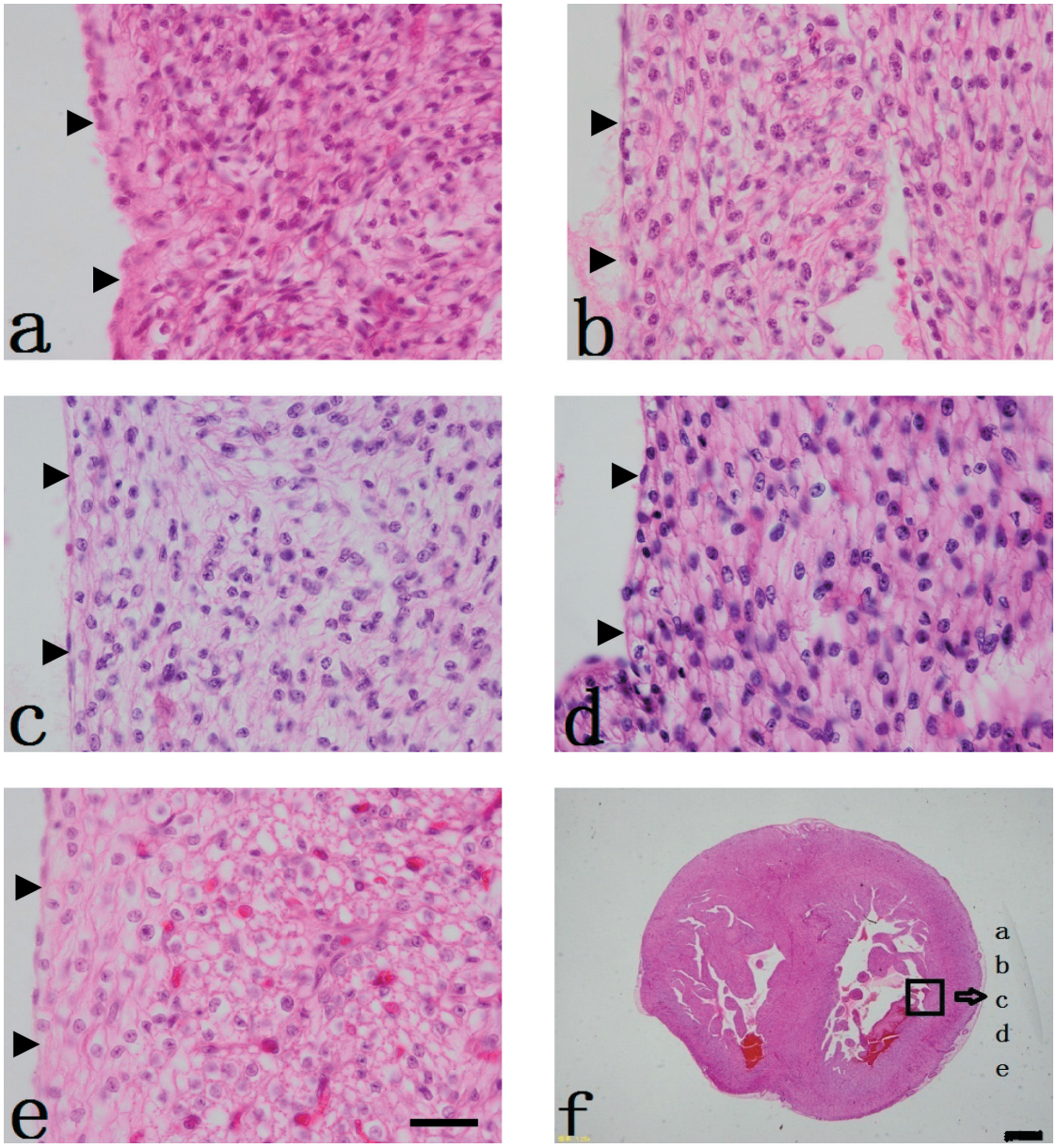

Fig. 6. The development of the endocardial layer from the left ventricle of the heart in the 3rd month (a), 4th month (b), 5th month (c), 6th month (d) and 7th month (e). Arrowheads indicate the inner surface of the left ventricle. Scale bar: 50 μm.

class, i.e. periarterial Purkinje fibers, has been documented in the peripheral conduction system of birds, no such fibers have been identified in mammals [9, 11]. One of the bundles of the His-Purkinje system distributed to the right ventricle and the other to the left ventricle, so the periphery of the ventricular wall and septum were covered with Purkinje fibers (Fig. 6). The Purkinje fiber cells in the fetal hearts were distinguished from cardiac muscle cells by their less differentiated and less organized structure. While these fibers are bundles of specialized heart muscle cells and are larger than ordinary cardiac muscle fibers, they also have other features of heart muscle cells, including a centrally located nucleus and intercalated disks. In the 3rd-month human fetal hearts in this study, Purkinje fibers were not yet clearly identified in the HE-stained sections, probably due to their poor histological differentiation from cardiac muscle cells. However, they were observed in the sections from the 4th to 7th months (Fig. 7), and as myocardial features became evident as described above, Purkinje fibers became more

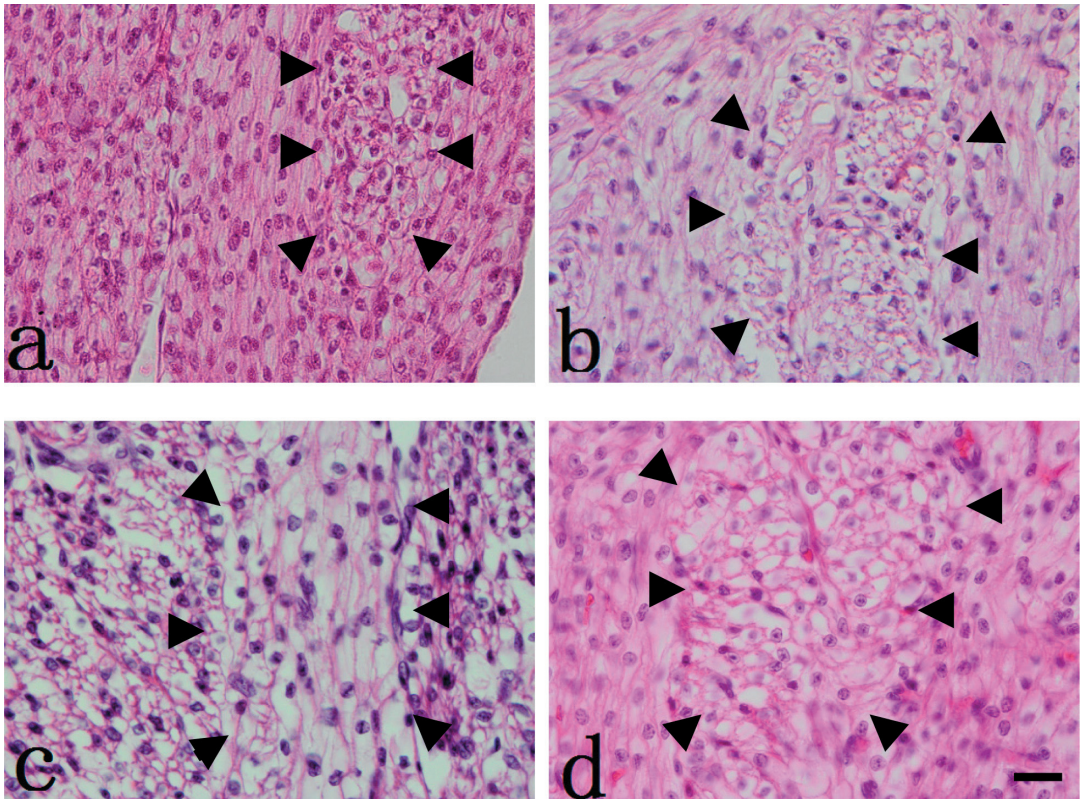

Fig. 7. Development of the Purkinje fiber cells (arrowheads) in the 4th month (a), 5th month (b), 6th month (c) and 7th month (d). Note that they are much larger and more lightly stained than regular heart muscle cells. Scale bar: 20 μm.

and more clearly observed beneath the endocardium. The fibers were surrounded by loose connective tissue. There were no significant differences in Purkinje fiber cell density between the ventricles and septum, and developmental changes in the Purkinje fiber cells from the 4th to 7th month were not clearly observed.

DISCUSSION

The cardiovascular system is the first to function among the developing human embryo's organ systems [1, 2, 9, 13]. Cardiac muscle is a type of striated muscle found only in the myocardium of the heart, and, during prenatal development, muscular cells in the heart differentiate into cardiac myocytes and conduction system cells including Purkinje fiber cells [7, 13]. The cells may be mononuclear or binuclear and branch so that they connect together tightly at the junctions called intercalated discs. Whereas early specification of multiple highly specified myocardial lineages, including the conduction system, has been extensively investigated [9], much less is known about the later maturation of these cells from the late fetal stages onward. Likewise, the histological differentiation of the cardiac myocytes or Purkinje fiber cells during the human fetal period has not been described in detail. In this study, therefore, we observed the development of normal cardiac muscle cells and Purkinje fiber cells as well as the endocardium and epicardium in the heart of human fetuses by light microscopy.

In the present observation, 7th month cardiac muscle cells in the ventricles showed two distinct morphological differences from those in 3rd month: namely, cylindrical cell shape and parallel cell alignment. There are gap junctions at the intercalated disks, irregularly spaced dark bands that act as a communication channels situated between the cardiac muscle cells. In longitudinal sections of cardiac muscles, the intercalated discs between the cells were evident in the 7th month, whereas their presence was unclear in the 3rd month. These are purely morphological features, but they do suggest

maturation of human cardiomyocytes during the period between the 3rd and 7th month of gestation. In the current study, 6th and 7th month cardiac muscle cells were morphologically similar to those in the adult heart [13], again suggesting the maturation of fetal cardiomyocytes by these months. Development of heart muscle cells from the 3rd month to 7th month may be closely correlated with the functional changes of the human fetus heart during this period.

Two different lineages of heart progenitors are known to contribute to the formation of the heart, at least in avian embryos [2]. The left ventricle arises from the lateral-most region of the heart-forming mesoderm, whereas the right ventricle arises from entire region of the heart-forming mesoderm. This difference in the progenitor lineage might predispose the cells to distinct histological differentiation. However, in the present observation, no significant histological differences were noticed between the regions of different lineages, suggesting histological differentiation occurs similarly in cells of both lineages.

Recent studies have shown that the myocardium, from the time of its formation, is not only a regionalized but also a polarized tissue and that its oriented clonal cell growth plays an important role in cardiac chamber morphogenesis [5, 6]. In the present observation, dividing muscle cells were observed in the hearts of human fetuses ranging through the ventricles and septum, including the oldest fetus examined in this series, and cardiomyocytes gradually became enlarged and arranged in parallel from the 3rd to 7th month. Whereas the present observation is at a later period than the cardiac morphogenesis, the findings are consistent with the notion that the heart develops based on polarized and oriented cell growth.

A previous paper reported that specialized conducting tissue can be recognized in human embryos as small as 8 mm (approximately 38 days of gestation) [7]. His-Purkinje system development is responsible for the coordination of ventricular contraction. From here the impulse is distributed rapidly around the ventricles via bundles of specialized large heart muscle cells called Purkinje fibers. According to a previous paper based on a total of 15 human embryos and fetuses ranging from 8 mm to 150 mm, Purkinje fibers of typical appearance can be first identified in the upper part of the left limb at the 105 mm (14 weeks, 4th month) stage [7]. In the present study, Purkinje fibers were not clearly identified in the 3rd month fetus (CRL 76 mm), whereas they were observed in the sections from 4th month fetuses (CRL 57.6 mm-113 mm), and as myocardial features became evident, Purkinje fibers became more and more clearly observed. These findings are consistent with those in a previous report [7]. Recent studies have revealed the myogenic but not neurogenic origin of the cardiac conduction system [20]. A series of retroviral lineage studies showed that the cardiac conduction system originates from cardiomyocytes and no other cardiac cell types including those of the neural crest origin. Furthermore, an in-growth rather than outgrowth developmental mechanism of the conduction system has also been elucidated [9, 20].

The endocardium and epicardium have been shown to play important roles in the development of cardiac cell lineages [9]. Viral lineage-tracing studies in chick embryos showed that conduction cells are progressively recruited from cardiomyogenic cells preceding recruitment to the peripheral components of the network, subendocardial Purkinje fibers, and an analogous recruitment has been suggested in rodents [9, 11]. In mouse embryos, neuregulin which is secreted by endocardium can recruit myocardial cells into the Purkinje fiber lineages [21]. Cells from the proepicardium migrate onto the early looping heart to give rise to the epicardium, and epicardial cells undergo epithelial-mesenchymal transformation and delaminate from the epicardium to enter the myocardium and give rise to cells of the coronary vasculature and interstitial fibroblasts [22]. Further, embryonic fibroblasts have recently been shown to promote myocardial proliferation [23], and at least *in vitro*, it has been suggested that proepicardial lineages have the capacity to be directed toward a myocardial cell fate [24], whereas later-stage epicardial cells may have lost this ability. As with the epicardium, growth factor signaling from the endocardium to the myocardium is responsible for stimulation of myocardial proliferation following cardiac looping [12]. Fibroblastic growth factor (FGF) signaling from either the epicardium or endocardium stimulates myocardial proliferation, and

endocardially derived FGF signals regulate myocardial proliferation during mid-gestation heart development [18]. These studies have focused on the early stages of heart development including endocardium and epicardium, whereas much less is known about the endocardial and epicardial layer development from such late fetal stages as those examined in the present study. In the present study, a relatively rich underlying connective tissue layer was observed both in the endocardium and epicardium at the 3rd month, whereas it significantly decreased thereafter. These histological changes appear to be consistent with the proliferation-stimulatory roles of the endocardium and epicardium that were reported in animals.

A limitation of this study was that it is based on formalin-fixed human materials, and that it was a purely morphological observation at the histological level and did not focus on microstructure and the development mechanisms. However, we here observed the histological changes during the fetal development of heart muscle cells and described the cell features from the 3rd to the 7th month of gestation. The present findings would be useful in evaluating normal morphological development of human heart and correlating it with the functional maturation of human fetal heart in the future.

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