Fine structural and functional development of the heart in the chick embryo with special reference to the onset of heartbeat.

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4. contraction 5. electron microscope

ABSTRACT

Fine structural changes of cardiocytes in the chick embryo before and after the onset of rhythmical contraction (Hamburger-Hamilton stages 8 to 17) were studied by light and electron microscopy. The earliest heartbeat was recognized at stage 10 by means of dissecting microscopy and the incidence of beating hearts increased as stage proceeded. In precontracting hearts, cardiocytes contained a small number of actin-like filaments, but thick filaments and primitive myofibrils were not discernible. At stage10, when some hearts are considered to commence rhythmical beating, myosin filaments and primitive myofibrils appeared in a few cardiocytes. The spatial orderly arrangement of actin and myosin filaments appears to play an important role for the onset of the first heartbeat. Desmosomes and intermediate junctions were well developed even in prebeating hearts. Elements of sarcoplasmic reticulum were rarely observed at postheartbeat stage 12 and remained infrequent in later stages of development. Thus these vesicles are unlikely to correlate closely with contraction in young embryonic hearts. It is clearly demonstrated that hearts are able to contract in an immature condition when not all but some cardiocytes contain only a few developing myofibrils.

INTRODUCTION

It is well known that cardiac muscles are specially differentiated cells for contraction. They have highly sophisticated structures characterized by cross striations. These cells differentiate from splanchnic mesodermal cells and obtain contractile function during development. It is interesting to examine the fine structural changes of cardiocytes before and after the onset of heartbeat. Skeletal muscles contract intermittently, whereas heart muscles continue to beat after the onset of first pulsation. Thus it seems rather easy to determine whether or not cardiocytes have acquired contractile function. The development of the chick embryonic heart was reported by Manasek (1968, 1970) and Hiruma and Hirakow (1985). However, analysis with special attention to the fine structural development around the onset of heartbeat has not been fully performed. In this study, we examined the fine strcutural development of cardiocytes in detail to correlate the onset of heartbeat.

MATERIALS AND METHODS

Fertile chicken eggs (Hi-sex brown) were incubated at 37 - 38℃ in an incubator. Chick embryos from stages 8 to 17 (Hamburger and Hamilton, 1951) were used for this study. The number of somites was also described at stages around the onset of heartbeat since this scale was used by some investigators.

An opening was made in the shell and the chick embryo was carefully examined under a dissecting microscope to determine whether or not the heart was beating. After that examination, the embryos were removed and immersed in a solution containing 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) or 3% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4), and the developmental stage was determined. Chick embryos were further immersed in the same fixative overnight at 4℃, postfixed in 1% OsO_4 in 0.1 M phosphate buffer (pH 7.4) for 2 h at 4℃. Most specimens were then rinsed three times with 10% saccharose for a total of 30 min, and stained *en bloc* in 3% uranyl acetate for 1 h at room temperature. This staining procedure for enhancing contrast of specimens resulted in extraction of glycogen. In some samples block staining was omitted to

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avoid loss of glycogen. All specimens were dehydrated in an ascending series of cold ethanol, cleared in propylene oxide and embedded in epoxy resin. After polymerization of the resin, somites were readily observable since somites were more blackened than surrounding tissues by osmification and thus easy to count. The number of somites of whole-mounted embryos was reevaluated under a dissecting microscope and, if necessary, stages were corrected according to Hamburger and Hamilton (1951). In all, 100 embryos were used. The stages and numbers of specimens used are shown in Table 1. Semithin sections of selected specimens were cut coronally or sagittally and viewed until hearts (or their precursors in the case of stage 8) were exposed. Sections for light microscopy (1-1.5 μm) were stained by a 0.5% toluidine blue solution dissolved in either 0.1 M phosphate buffer (pH 7.4) or 0.5% borate. Ultrathin sections were cut and stained with uranyl acetate and lead citrate, and observed under Hitachi H-500 or JEM 1200EX electron microscopes.

Table 1. Stages and numbers of chick embryos examined

stage	8	9	10	11	12
number of embryos	3	7	23	21	15
stage	**13**	**14**	**15**	**16**	**17**

RESULTS

Developmental speed of chick embryos

Some variability was observed among individual embryos. The degree of development was not uniform in terms of incubation period and lagged behind the stages described by Hamburger and Hamilton (1951). Therefore, stage is more reliable than duration of

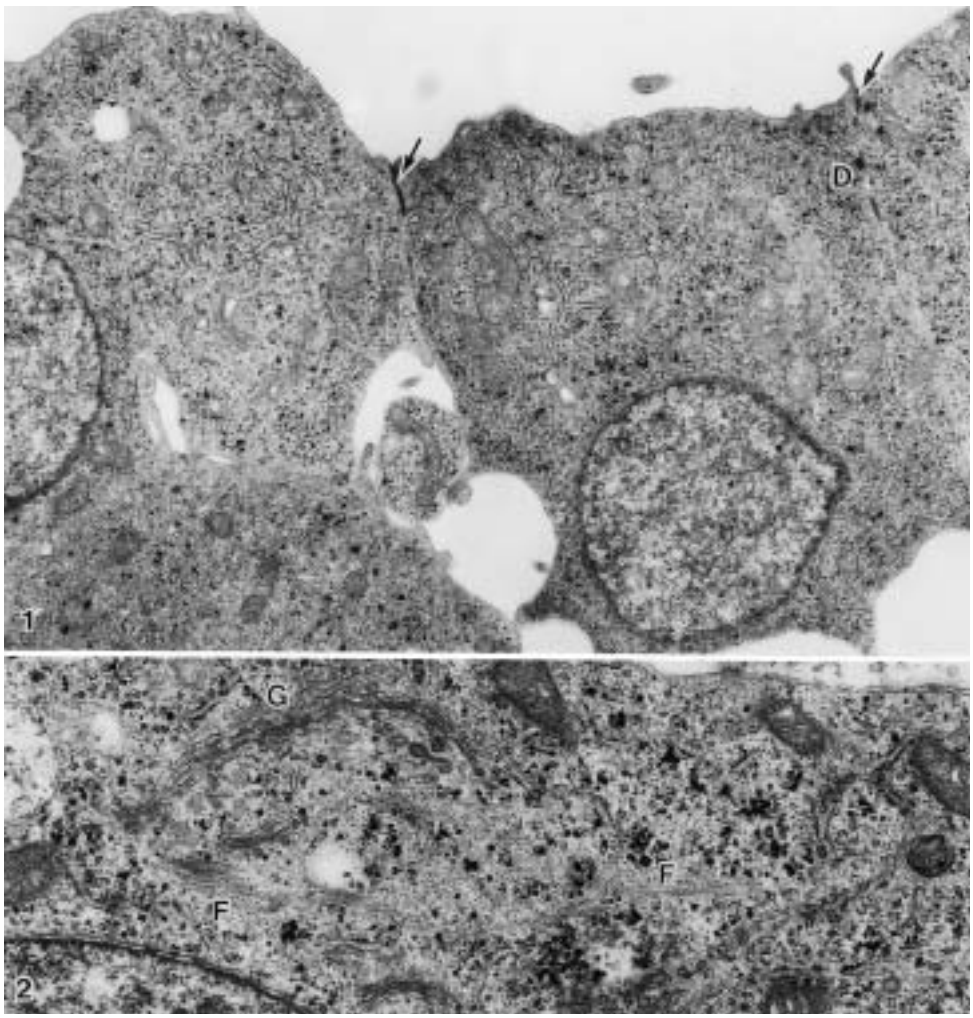

Fig. 1. Destined cardiocytes at stage 9-. Neither myofilaments nor myofibrils are discernible in these cells. Intercellular junctions are seen (arrows). D: desmosome. × 9,000.
Fig. 2. Part of another cardiocyte at stage 9-. Loosely-assembled bundles of filaments (F) are seen. G: Golgi apparatus. ×18,500.

incubation to designate grade of development of the embryos.

Before the onset of heartbeat (stages 8 to 9)

Heartbeat was not recognized in any of these specimens.

At stage 8 (including stages 8-, 8 and 8+; the same inclusiveness applies throughout stages 8 to 14 unless stated otherwise), the right and left primordia of the heart were separated. In addition, no cells contained myofilament-like strands, so we could not identify destined cardiocytes with certainty at the electron microscopic level even with combined use of light microscopy.

At stage 9, the primordia of the heart began to fuse. Cardiocytes were oval or cylindrical in shape with large intercellular spaces. A few cardiocytes contained thin filaments. These filaments, probably actin, usually formed loosely-assembled bundles in the cytoplasm. Thick filaments were not discernible. No primitive myofibrils were recognized (Figs. 1, 2). Numerous free ribosomes, glycogen particles, some mitochondria and elements of rough endoplasmic reticulum were scattered in the cytoplasm. Some Golgi apparatus and one ovoid nucleus were also seen. Intercellular junctions with a relatively well-developed desmosome were found at apical intercellular spaces (Fig. 1).

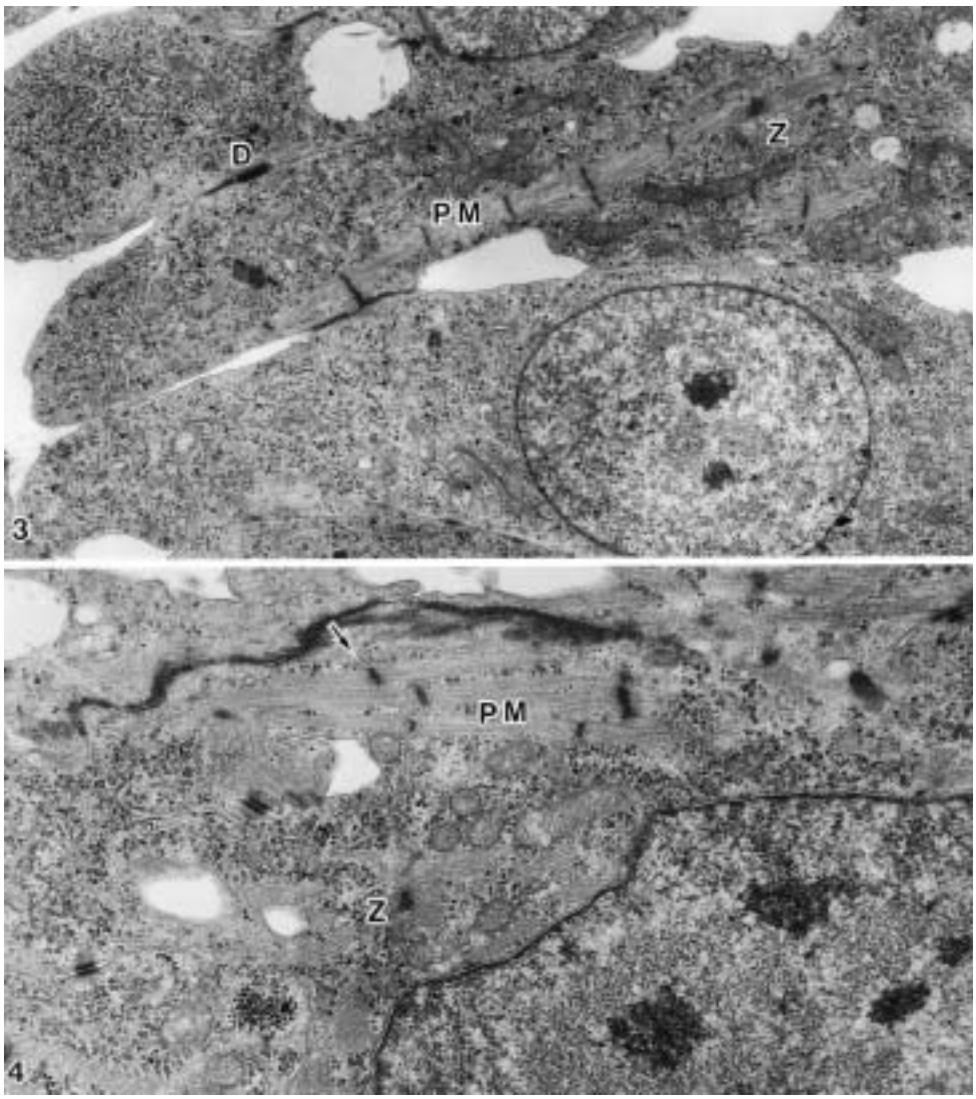

Fig. 3. Cardiocytes at stage 10. The contents of myofilaments and myofibrils vary considerably from cell to cell. A cardiocyte (center) contains a primitive myofibril (PM). D: desmosome. Z: Z band precursor. ×7,500.

Fig. 4. Cardiocytes at stage 12. Myofilaments are considerably increased. Primitive myofibrils (PM) are easily found. A vesicle of possible sarcoplasmic reticulum (arrow) is located near a Z band precursor (Z). ×15,000.

Around the onset of heartbeat (stages 10 to 12)

At stage 10, only 1 of 23 samples was found to beat. Light microscopy revealed that hearts were composed of two circular layers separated by so-called cardiac jelly (extracellular matrix). The interior layer was a single row of endothelial cells. The external layer was one to two cells thick with cardiocytes, with large intercellular spaces among them. Neither epicardium nor mesenchymal cells such as fibroblasts were observed. At the electron microscopic level, most of these cardiocytes contained bundles of thin and thick filaments. These myofilaments were small in amount and usually co-localized in an orderly arrangement. In some instances, however, thick filaments were not evident. Bundles of filaments were either sparsely dispersed in the cytoplasm, radiated from electron dense materials or terminated at plasma membranes in particular desmosomes and intermediate junctions. Electron-dense materials, presumably Z band precursors, were amorphous or linear in shape. Myofilaments spanned regularly situated nascent Z bands forming primitive myofibrils. They were rarely as long as several sarcomeres in length (Fig. 3). Adjacent cells were joined by junctional complexes. Desmosomes were almost as developed as those of adults.

At stage 11, heartbeat was found in 6 of 21 specimens. The heart muscle was composed of cardiocytes two or partly three cells thick. In stage 11- myocardium, myofilaments were scarce and varied in quantity among cells. Occasionally, a number of small Z band precursors were surrounded by thin and thick filaments. Myofilaments, if they existed, tended to be located at cell peripheries and often extended from amorphous Z band precursors or terminated at well developed junctional complexes.

At stage 12, heartbeat was observed in 11 of 15 embryos. Myofilaments and myofibrils were considerably increased in number and length (Fig. 4). Thin and thick filaments ran together in a disoriented fashion. Electron-dense Z band materials were occasionally found and myofilaments radiated from them sometimes in more than two directions. Myofilaments which terminated at intercellular junctions were frequently observed.

After the onset of heartbeat (stages 14 to 17)

At stage 14, all hearts examined were found to beat. The myocardium was composed of two or three cell layers of cardiocytes. Myofilaments were more numerous than in stage 12. Nascent Z bands were often observed. However, primitive myofibrils were rather small in number and A and I bands could be distinguishable only in some samples. In the vicinity of Z bands, vesicles or elements of sarcoplasmic reticulum were rarely found (Fig. 5).

At stages 15 and 16, myofilaments were further increased in number and length and oriented in various directions. Myofibrils were frequently found. Small vesicles were sometimes closely associated with Z bands. Well developed desmosomes and very narrow intercellular contacts, probably gap junctions, were found between adjacent cells.

At stage 17, embryos became considerably larger and heartbeat was apparent. The myocardium was as thick as several cell layers. Blood capillaries were observed in the myocardium for the first time at stage 17.

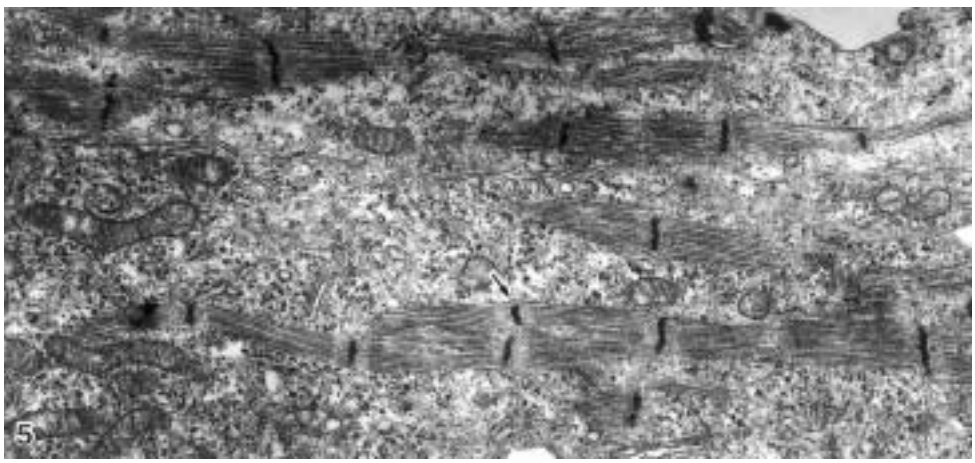

Fig. 5. Part of a cardiocyte at stage 14. Small vesicles (arrow) are infrequently observed in the vicinities of Z bands. ×15,600.

DISCUSSION

The onset of heartbeat of chick embryos is reported to be at stage 10- (9 somites; Fujii et al., 1980) and stage 10 (10 somites; Manasek, 1968; Hiruma and Hirakow, 1985). In the present study, heartbeat was rarely recognized at stage 10 (10 somites), but thereafter the incidence of beating hearts steadily increased. Fujii et al. (1980) used potential sensitive dye combined with an optical recording technique and detected spontaneous electrical activity which was not accompanied by mechanical beating at stage 9 (7 somites). They found the first mechanical heartbeat at stage 10- (9 somites). The optical recording should be more sensitive than dissecting microscopy, so it is plausible that heartbeats start at stage 10- to 10 (9 to 10 somites; Lim et al., 1983) from the right ventricle (Fujii et al., 1980; Hiruma and Hirakow, 1985) and the incidence of beating hearts increases with the advancement of development.

For rhythmical contraction of the heart, the following factors appear to be important: 1) adequate amount of actin and myosin, 2) presence of other proteins which keep actin and myosin filaments in a precise spatial arrangement and control acto-myosin interaction, 3) regulation of ionic calcium by sarcoplasmic reticulum and/or other structures, 4) intercellular junctions for transmission of contractile forces, 5) spontaneous electrical activity and propagation of excitation through a conduction system including gap junctions, and so on.

Lim et al. (1983) reported that actin and myosin comprise 8.53% and 1.84% of total cardiac protein, respectively, at preheartbeat stage 9. As development proceeds to stage 15, actin and myosin represent 10.52% and 3.34% of total protein, respectively. They also reported that α-actinin and tropomyosin remained relatively constant throughout development, while myosin showed a steady increase. In point of fact, myosin filaments were not discernible (the present study) or were sparse (Lim et al., 1983; Hiruma and Hirakow, 1985) at preheartbeat stage 9+ (8 somites), while myosin filaments and primitive myofibrils appeared and were increased at heartbeat stage 10.

The sarcoplasmic reticulum controls ionic calcium in mature cardiocytes, but this cell organelle is not discernible when heartbeat commences. It only rarely appears at stage 10 and becomes more common by stage 12- (Manasek, 1968). In the present study, however, the sarcoplasmic reticulum remained infrequent even at later stages examined. Development of the sarcoplasmic reticulum does not seem requisite for the onset of heartbeat. On the other hand, intermediate junctions and desmosomes are well developed at preheartbeat stage 9 (Shiraishi et al., 1997; the present study) and appear ready for contraction.

Fujii et al. (1981) observed no apparent synchronization of electrical activity in cardiac cells at stage 9 (7 somites). The number of electrically synchronized active cells dramatically increases and electrical activity propagates wider and wider (Fujii et al., 1981) from the right ventricle during stages 9 to 10- (7 to 9 somites), probably through gap junctions. They have also reported that shape of the action potential changes at stages 9+ and 10- (8 and 9 somites) just before the initiation of heartbeat.

Taking these facts mentioned above into consideration, it appears likely that 1) an orderly arrangement of actin and myosin filaments, which accompanies a near simultaneous formation of myosin filaments, and 2) the development of electrical activity and its propagation are involved in the onset of heartbeat. It is also noteworthy that cardiocytes of chick embryos are able to contract in a very immature intracellular structure when myofibrils are very sparse by transmitting the force generated by myofibrils through a network of intermediate filaments (Sugi and Hirakow, 1991). Cardiac and skeletal muscles are similar in principal fine structures as well as in mechanism of contraction. Thus it was suggested that skeletal muscle may be able to contract at early stages of development.

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