Undifferentiated Cells in the Snail Myocardium Are Capable of DNA Synthesis and Myodifferentiation

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**Abstract.** Cellular mechanisms of heart-muscle growth in the snail *Achatina fulica* have been studied using cytophotometry and electron microscopic autoradiography. Cytophotometric DNA measurements showed that the snail cardiomyocytes are mononucleated cells with diploid nuclei. Ultrastructural analysis of the snail myocardium revealed that, in addition to mature myocytes, it contains small roundish undifferentiated cells (UCs) and poorly differentiated muscle cells. EM autoradiography detected silver grains over the nuclei of UCs 2 h after injection of tritiated thymidine ([3H]Tdr), while the nuclei of both mature and poorly differentiated myocytes remained unlabeled. In EM autographs of the myocardial tissue fixed 14 days after [3H]Tdr administration, labeled myonuclei were evident, which may suggest some myodifferentiation of prelabeled UCs. Many labeled UCs persist for 14 days after a single [3H]Tdr injection, suggesting that not all UCs undergo myodifferentiation after passing through the cell cycle, and that those that do not can enter the next cycle. UCs in the snail myocardium presumably provide not only reserve but also stem cells for myocytes. Thus, the heart muscle of the adult snail consists of mononucleated diploid myocytes with blocked proliferative activity and a renewable population of precursor myogenic cells. The results obtained suggest that the growth of this muscle involves a myoblastic mechanism of myogenesis; this mechanism differs from that of vertebrate cardiac muscle growth, which is non-myoblastic—that is, based on proliferation or polypliodization of cardiomyocytes. Evolutionary aspects of cellular mechanisms of the heart-muscle growth are discussed.

**Introduction**

In vertebrates, developing and mature cardiac muscles lack a pool of morphologically undifferentiated myoblasts. This is in contrast with skeletal muscle, which possesses an obvious pool of satellite cells. Differentiated cardiomyocytes of vertebrates retain their ability to synthesize DNA, and vertebrate heart muscle grows non-myoblastically, through hyperplasia or hypertrophy of myofibril-containing cells (reviewed by Rumyantsev, 1991).

In many respects the molluscan heart resembles that of vertebrates. This striking convergence involves myogenic automatism, high heart volume, beat frequency, and some other physiological features. In addition, such morphological peculiarities as the location of the heart in the pericardial cavity, a chambered heart construction, the availability of valves and trabeculae, and a rich supply of nerve endings are worth mentioning (Martin, 1980). Some cephalopods have a relatively closed circulatory system (Kling and Schipp, 1987; Schipp, 1987), as well as a pacemaker zone in the heart and coronary vasculature (Wells and Smith, 1987). As in the vertebrate myocardium, the cellular unit of molluscan heart muscle is a mononucleated muscle cell linked to its neighbors by intercalated discs (North, 1963; Watts et al., 1981; Okland, 1982; Kling and Schipp, 1987). In other invertebrates, the heart muscle is formed either by multinucleated muscle fibers, as in arthropods (Nylund, 1981; Nylund et al., 1986; Martynova et al., 1986); or by myoepithelial cells, as in brachiopods (Martynova and Chaga, 1997), annelids (Hama, 1960; Jensen, 1974), pogonophorans (Jensen and Myklebust, 1975), hemichordates (Lester, 1982; Balser and Ruppert, 1990), and ascidians (Martynova and Nylund, 1996).

Although the molluscan heart muscle has been the subject of many ultrastructural and physiological studies, little is known so far about the cellular mechanisms that underlie its growth.
In our previous ultrastructural study of molluscan heart muscle (Bystrova et al., 1996), the presence of a population of undifferentiated cells (UCs) was revealed in the bivalve Crenomytilus grayanus, the gastropod Helix aspersa, and the cephalopod Rossia macrosoma. These cells were assumed to be myogenic stem cells involved in the growth of the molluscan heart muscle. The goal of the present work was to validate this assumption and attempt to understand the mode of heart-muscle growth in molluscs. For this purpose we examined the DNA-synthesizing capacity of UCs by means of electron microscope autoradiography with tritiated thymidine as a precursor and followed the fate of labeled UCs in tests with delayed fixation. We also studied the ploidy levels of cardiomyocytes in the snail heart.

Materials and Methods

Experimental animals

We used a gastropod mollusc, the land snail Achatina fulica, which we reared in the laboratory. The snails were kept at room temperature in large glass vessels and fed a vegetable diet.

Cytophotometry

Tissue samples from the atria and ventricles of three mature snails (about 40 g body weight) were fixed in 10% formalin and then treated with 50% KOH. Smears of isolated cells were postfixed in Carnoy solution and stained by the Feulgen technique. DNA was measured using a Morphoquant scanning cytophotometer (Germany) at a wavelength of 550 nm on 200 muscle nuclei from each heart chamber. The quantity of DNA in the blood cell nuclei available in the same smears served as a standard of the diploid DNA value.

Scintillation counting

A Beckman LS-8100 β-scintillation counter was used to determine the time of [3H]Tdr (tritiated thymidine) circulation in the body fluid. After a single [3H]Tdr injection at a dose of 10 μCi/g live weight, 0.1 ml of hemolymph was taken at regular intervals (each time from a different animal), placed on a paper filter, and left to dry. The filters were then put into standard glass vials with the scintillation liquid, and the radioactivity was measured. The results were expressed as percent clearance of radioactivity as related to the first sample taken immediately after the isotope injection.

Autoradiography

[3H]Tdr (State Institute for Applied Chemistry, St. Petersburg, Russia) with a specific activity of 16 Ci/μmole was injected at a dose of 10 μCi per gram of body weight into the pericardial cavity of small 2-week-old snails (about 0.2 g body weight) through a small hole drilled in the shell. In 2 h (test I) or 14 days (test II), the heart tissues were fixed in a cacodylate-buffered solution of 2.5% glutaraldehyde, pH 7.4, postfixed in 1% OsO4, dehydrated in a series of ethanol, and embedded in a mixture of Epon and Araldite. Four animals were used in each test for EM autoradiography. EM autographs were prepared by the method of Larra and Droz (1970) with an exposure time of 60 days. Observations were made with a JEM 7A electron microscope.

For two animals in each test (four animals in all), the percentage of [3H]Tdr-labeled nuclei from the total number of nuclei of undifferentiated cells (UCs) was calculated from EM autoradiographs. Over 100 UC nuclei from each animal were examined. From the same autoradiographs, the percentage of UC nuclei related to the total population of myocardial nuclei (namely, muscle nuclei plus those of UCs) was estimated. The average number of myocardial nuclei counted for each of four animals was 1000.

Results

Ultrastructure of the snail heart

The snail Achatina fulica has a two-chambered heart. The atrial and ventricular walls consist of an outer single-cell layer of coelomic epithelium (epicardium), a myocardium, and an inner discontinuous layer of endothelial cells (Fig. 1). The myocardium is intensely penetrated by nerve fibers (Fig. 2), which are particularly numerous in the atrium. The myocardium is formed of a compact part that underlies the epicardium and a spongy trabecular part that faces the lumen. In the atrium, the compact part is less expressed, and the trabeculae are thinner and more loosely arranged than in the ventricle. Mature cardiomyocytes (Fig. 2) are tightly packed with obliquely striated myofibers and contact each other by small desmosomes and intercalated discs (Fig. 3). Dense bodies and attachment plaques form Z-material. The nuclei are normally located in the lateral luminal protrusions of the sarcoplasm. The karyoplasm is filled with condensed chromatin blocks, located predominantly under the plasma membrane, and with finely dispersed chromatin. Sometimes a poorly developed Golgi complex and centrioles are seen near the nucleus.

Small oval cells devoid of myofilaments were observed in the myocardium beside the muscle cells and in close association with them (Fig. 4). These undifferentiated cells (UCs) were usually tightly adjacent to the luminal surface of the myocardial trabeculae. Sometimes spot desmosomes were between UCs and adjacent myocytes (Fig. 4, inset). These cells were quite common, and in the heart muscle of 4-week-old snails made up as much as about 10% of the total population of myocardial nuclei. Each UC consists of a single nucleus surrounded by scanty cytoplasm. A set of cytoplasmic organelles includes a few mitochondria, ribo-
somes, and a number of small vesicles. UCs were easy to distinguish from other nonmuscle cells in the heart, such as endothelial cells, gliointerstitial cells accompanying nerve fibers, and blood cells, by this extreme paucity of cytoplasmic organelles and the presence of a spherical “empty” nucleus whose chromatin is not dispersed but clumped immediately beneath the nuclear envelope. These peculiar nuclei were commonly surrounded by fragments of evidently destroyed cytoplasm, which may presumably result from occasional damage of UCs during fixation. A high fragility of UCs probably accounts for the fact that these were missed in many earlier relevant publications on the ultrastructure of the molluscan heart.

Sometimes small muscle cells were seen containing only a few myofilaments randomly arranged and embedded into some amorphous material (Fig. 5). Moderately differentiated myocytes were also seen (Figs. 6, 7). Thus, all morphological intermediate stages may be observed in the molluscan heart—from small oval cells with no or only a few myofilaments to well-differentiated cardiomyocytes—and a series of cells representing the process of transformation of undifferentiated cell into mature muscle cell can be identified.

We failed to observe any mitotic figures in UCs or myocytes.

**Ploidy analysis**

Cytophotometric measurements of DNA contents in KOH-dissociated, Feulgen-stained cells showed that both atrial and ventricular myocytes of the snail heart are exclusively mononucleated, with the nuclei being diploid.

**Scintillation counting**

The data on [3H]Tdr availability are presented in Figure 8. Of the amount of [3H]Tdr injected at a dose of 10 μCi/g into the body cavity of the snail, about half was consumed within the first 10 min, and by 2 h almost all of the isotope was used up. Then the curve reached a plateau, which may indicate the presence of some other labeled compounds into which [3H]Tdr could be converted or of blood cells that also might incorporate thymidine. So, [3H]Tdr is available for only about 2 h.

**Electron microscope autoradiography**

**Test I.** The first series of experiments was aimed at examining the DNA-synthesizing capacity in UCs and myocytes of the *Achatina* heart. Electron microscope autorgraphs of the heart tissue fixed 2 h after [3H]Tdr injection showed labeling of some nuclei in UCs (Fig. 9). The labeled UC nuclei composed 4.2% and 4.3% (for each animal) of the total population of UC nuclei. No silver grains were detected over the nuclei of either poorly or well-differentiated muscle cells. In addition to the UCs, the nuclei of some epicardial, endothelial, and gliointerstitial cells in the heart tissue demonstrated [3H]Tdr incorporation.

**Test II.** To follow the fate of [3H]Tdr-labeled UCs, the heart tissue was examined 14 days after the isotope injection. Electron microscope autorgraphs showed the appearance of labeled nuclei in both moderately differentiated (Fig. 10) and mature myocytes (Fig. 11). It is noteworthy that, along with labeled myocytes, labeled UCs were also observed. For one snail, the number of [3H]Tdr-labeled nuclei composed 13.0% of the total UC population; labeled muscle and UC nuclei made up 9.5% and 3.5%, respectively. For another snail, the number of labeled nuclei amounted to 17.2% of the total UC population; labeled muscle and UC nuclei made up 9.5% and 3.5%, respectively. The nuclear labeling was also seen in some endothelial, epicardial, and gliointerstitial cells. Similar patterns of labeling were observed in both the atrium and ventricle.

**Discussion**

The electron microscope study has shown that the snail myocardium contains a population of undifferentiated cells (UCs) and a number of poorly and moderately differentiated myocytes. All these myocardial cells can be arranged successively into a series of stages representing a process of myodifferentiation, which indicates that the UCs in the snail...
heart are myogenic. The autoradiographic experiments supported this view. As evident from test I with a short \[^{3}\text{H}]\text{Tdr-pulse\text{-}}\text{pulse, UCs are capable of DNA synthesis, but muscle cells, including those at the earliest stages of myodifferentiation, are not. The appearance of labeled myonuclei in test II with a delayed fixation indicates that UCs underwent mitosis and myodifferentiation within the first several hours after \[^{3}\text{H}]\text{Tdr injection. Furthermore, the labeled UCs were also present in the heart tissue fixed 14 days after isotope injection. Since \[^{3}\text{H}]\text{Tdr was available in hemolymph for only 2 h, it may be that not all UCs differentiated into myocytes after DNA synthesis and mitosis: that some daughter UCs might remain undifferentiated and compose a defined population. Thus, in the snail heart, UCs could produce a renewable population and eventually function not only as a reserve but also as stem cells for the myocytes. The counts of labeled nuclei showed that their numbers increased by a factor of 4 over 2 weeks, suggesting that, for a given time, the prelabeled UCs could undergo about two cell cycles. Other nonmuscle cells of the snail
heart do not demonstrate any morphological indications of transformation into myocytes, so they could not be serving as stem cells for muscle differentiation.

The present study of the Achatina heart muscle provided no evidence about whether the examined UCs are remnants of the original, embryonic myoblasts or represent new cells of another origin, which invaded the heart only later in its development. Recent data on the participation of bone-marrow-derived cells in cardiac muscle regeneration in Duchenne dystrophic mice demonstrate that adult vertebrate myocardium has a source of myogenic stem cells located outside the organ (Bittner et al., 1999).

A population of undifferentiated cells was found also in the myocardia of bivalve and cephalopod molluscs (Bystrova et al., 1996). It may be assumed that the mosaic distribution of myogenic cells is a characteristic feature of the molluscan myocardium shared by representatives of different classes. This shared feature enables us to classify the molluscan heart muscle as a special muscle type consisting of discrete mononucleated diploid myocytes and, at the same time, possessing myogenic UCs. Such a cellular organization makes it possible to differentiate the molluscan heart muscle from all other muscle types so far described in either vertebrates or invertebrates.

It is well known that in warm-blooded vertebrates—birds (Anatskaya et al., 2001), mammals (Kudryavtsev et al., 1997), and humans (Adler, 1991)—polyploidization of cardiac myocytes is an essential component of heart-muscle growth. The mere presence of undifferentiated myogenic elements in the snail myocardium does not preclude the possibility that polyploidy may occur in molluscan cardiac myocyte nuclei. In many adult tissues of gastropod molluscs, such as nervous tissue (Bulloch, 1985; Moffett, 1995), connective tissue, various gland and gonad tissues, gut and skin epithelia (Anisimov, 1999), the occurrence of undifferentiated stem cells is surprisingly accompanied by a high level of ploidy in their differentiated cells. Even though the cardiomyocytes of adult snails do not synthesize DNA, polyploidization might be able to occur at earlier stages of development. To check this possibility, we examined nuclear DNA content in snail cardiomyocytes and found that the snail myocardium was composed exclusively of mononucleated diploid myocytes. This is another proof for repression or irreversible loss of DNA-synthesizing capacity in the molluscan cardiomyocytes.

In vertebrate cardiomyocytes, syntheses of DNA and cell-specific proteins are not mutually exclusive. Myogenic stem cells are absent from the vertebrate myocardium (Rumyantsev, 1991). In contrast, all protostome invertebrates so far examined in this respect demonstrate the pres-
ence of myogenic precursor cells in the heart muscle and the absence of DNA synthesis in myocytes, even though the cellular organization of heart muscles in different groups of invertebrates varies significantly. Thus, brachiopod myocardium consists of alternating myoepithelial and epithelial cells, the latter being myogenic (Martynova and Chaga, 1997). Crustacean heart muscle, like vertebrate skeletal muscle, is composed of multinucleated myofibers and mononucleated undifferentiated satellite cells, which function as myogenic stem cells (Martynova, 1993). Crustacean heart myonuclei have no DNA synthetic activity. In deuterostome invertebrates such as ascidians, the myocardium consists of a homogeneous population of myoepithelial cells and contains myogenic precursor cells localized in a region of transition from pericardium to myocardium. At the same time, myoepithelial cells of the ascidian myocardium are capable of DNA synthesis (Martynova and Nylund, 1996). Thus, it can be suggested that DNA blocking in muscle cells, combined with the occurrence of a pool of undifferentiated myogenic cells, is the common feature of the heart.

Figure 9. Electron-microscopic autoradiograph of a labeled undifferentiated cell from the Achatina heart fixed 2 h after injection of tritiated thymidine. m, mature cardiomyocytes. Arrow points to centriole. Scale bar = 1 μm.

Figure 10. Electron-microscopic autoradiograph of a labeled moderately differentiated cardiomyocyte from the Achatina heart fixed 14 days after injection of tritiated thymidine. Scale bar = 1 μm.

Figure 11. Electron-microscopic autoradiograph of a labeled mature cardiomyocyte from the Achatina heart fixed 14 days after tritiated thymidine injection. n, nerve ending, e, endothelial cells. Scale bar = 1 μm.
muscle in protostomes (the myoblastic mechanism of myogenesis), whereas the cardiomyocytes capable of DNA synthesis developed only in deuterostomians and as early as the emergence of ascidians. Further development of the cardiac muscle in deuterostomians was accompanied by an ultimate loss of undifferentiated myogenic elements in vertebrates (the non-myoblastic mechanism of myogenesis). The principal difference in cellular mechanisms of heart-muscle growth between Deuterostomia and Protostomia may be accounted for by different origin and development of cardiogenic mesoderms in these two main phylogenetic taxa.

To gain an insight into the behavior of myogenic stem cells, it is necessary to identify components involved in regulation of their activity. It has been well established that in vertebrate skeletal muscle myogenic stem cells (i.e., satellite cells) participate not only in growth but also in regenerative processes. The injury of skeletal muscle fibers causes reactive proliferation of satellite cells (Groups and McGeachie, 1989; Roberts et al., 1989). Our attempts to activate DNA synthesis in molluscan UCs by mechanical injury of the heart were not successful. The only observed reaction was the filling of the wounded heart wall with a cluster of blood cells (unpubl. data). A similar reaction to mechanical injury was observed in the crayfish heart (Martynova and Khaitlina, 1991). Thus, the regenerative process in crustacean and molluscan heart muscles, unlike that in vertebrate skeletal muscle, does not trigger proliferation of precursor myogenic cells. DNA synthesis and myodifferentiation of satellite cells in the crayfish heart was initiated by ecdysterone, an arthropod molting hormone, both intrinsically and applied exogenously (Martynova, 1999, 1997). Furthermore, ecdysteroids have been detected in snails (Romer, 1979; Whitehead and Sellhreyer, 1982; Nolte et al., 1986), and the presence of ecdysteroid receptors was recently demonstrated in the heart of Helix aspersa (Bugaeva et al., 1999). These data suggest that ecdysteroids are probably a trigger factor activating mitoses and myodifferentiation of myogenic stem cells in the molluscan myocardium. Experiments are in progress on the effect of ecdysterone on biosynthetic processes in cells of the snail heart.

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