Nodal Myosin Distribution in the Bovine Heart During Prenatal Development: An Immunohistochemical Study

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A novel type of cardiac myosin heavy chain, immunologically related to the myosin isoforms expressed during skeletal muscle development, has recently been described in sinoatrial and atrioventricular nodal fibers of the adult bovine heart (Gorza et al., J Cell Biol 1986;102:1758-1766). The tissue-specific expression of this myosin type has been utilized in the present study to investigate the differentiation of nodal fibers during cardiac development. In 4-6-week-old bovine embryos, reactivity for nodal myosin was observed in a cluster of cardiac fibers in the sinus venosus wall, corresponding to the sinoatrial node primordium and in a number of fibers localized in the left atrial wall, especially in proximity to vascular orifices, possibly corresponding to the postulated left-sided sinoatrial node. In contrast, reactivity for nodal myosin was not detected in the atrioventricular node until 12 weeks of gestation. Before this stage, fibers reactive for nodal myosin were also seen scattered in the left atrial wall and interatrial septum, raising the possibility that atrioventricular nodal fibers may derive from the left-sided sinoatrial node. Reactivity for nodal myosin was never seen in normal atrial and ventricular myocardium, nor in the ventricular conduction tissue, indicating that nodal myosin does not represent a primordial myosin form, but is rather a specific marker of a distinct muscle cell lineage. (Circulation Research 1988;62:1182-1190)

The development of the conduction system, and particularly of the nodal conduction tissue, is among the most controversial areas in the study of cardiac muscle development. Open issues include the following: 1) the developmental stage at which sinoatrial (SA) and atrioventricular (AV) nodes are first identified; 2) whether nodal cells belong to the same myoblast lineage as ordinary myocardium cells or to a distinct cell lineage; 3) the postulated existence of a left-sided SA node; 4) the origin of the AV node (in situ versus migrated precursors); and 5) the presence of nodal-like cells in extranodal regions. One of the principal difficulties in answering these questions is the inadequacy of morphological criteria for distinguishing nodal tissue from ordinary embryonic myocardium. The limitations of current histological and histochemical techniques are clearly illustrated by the widely contradictory results obtained by different groups even when the same species and developmental stages were investigated.

Immunohistochemical localization of different myosin isoforms in cardiac muscle represents a useful tool to distinguish among myocyte populations. In the chicken heart, Purkinje fibers were found to express a distinct myosin type that is immunologically related to the myosin isoform present in slow tonic skeletal muscle. This myosin type is not detectable before 10-12 days of egg incubation.

In the mammalian heart, a novel type of cardiac myosin was described recently in nodal conduction tissue: SA and AV nodal fibers of the adult bovine heart stained specifically with antibodies to fetal skeletal myosin heavy chain (MHC). These antibodies do not cross-react with the α- and β-MHCs present in atrial and ventricular myocardium and in the ventricular conduction tissue. These findings point to the existence of a distinct "nodal MHC," antigenically related to the MHC isoforms present in developing skeletal muscle. In the present study, using immunohistochemical procedures, we have investigated the expression of nodal MHC during embryonic development. Our results show that reactivity for nodal MHC is present at the earliest stages examined in SA node primordium but can be detected only in late embryonic stages in the AV node region.

Materials and Methods

Tissue Source

This study was performed on hearts from 16 bovine embryos and fetuses of different ages (Table 1). The gestational age was established by comparison of the crown-rump length of each embryo and fetus with previously reported data on age-length relations.

The whole heart or the atria of 1-16-cm embryos were serially sectioned throughout. In 23- and 40-cm fetuses, only selected regions from atria and ventricles were excised and sectioned. These included the SA and AV nodal regions, the crista terminalis, the coro-
TABLE 1. Gestational Age of All Embryos Examined by Comparison With Crown-Rump Length

<table>
<thead>
<tr>
<th>Animal</th>
<th>Crown-rump length (cm)</th>
<th>Gestational age (days)</th>
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<tbody>
<tr>
<td>1</td>
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<td>28-29</td>
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<tr>
<td>2</td>
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<td>16</td>
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Antimyosin Antibodies

We used polyclonal antibodies raised against bovine atrial and ventricular MHCs (anti-bAm and anti-bVm) and bovine fetal skeletal MHC (anti-bFm) as previously described. Antibodies react selectively with α- and β-MHC, respectively. Anti-bFm reacts with bovine fetal MHC but is unreactive with adult skeletal MHCs and with α- and β-MHCs.

Immunological and Enzymatic Histochemistry

Indirect immunofluorescence and peroxidase-anti-peroxidase (PAP) procedures were performed as previously described. In brief, appropriate dilutions of antimyosin antibodies were applied to freshly cut frozen sections for 30 minutes at 37°C. After several rinses in phosphate-buffered saline, sections prepared for fluorescence were incubated with appropriate dilutions of anti-rabbit immunoglobulins conjugated with FITC fluorochrome (Miles Laboratories, Elkhart, Indiana), mounted in aqueous medium (Elvanol), and

Figure 1. A 1.2-cm bovine embryo. In sagittal cryosections of the whole embryo, the anterior cardiac wall lies on the left side and the posterior wall is on the right side. a: Stained for histochemical demonstration of myosin ATPase to illustrate heart anatomy. Note the common ventricle (V) filled with trabeculae and the primitive atrium (A). b: Higher magnification of area squared in (a) as it appeared in an adjacent serial section stained with PAP immunostaining using anti-bFm. Fibers labeled by anti-bFm are localized in the atrial wall in proximity to sinus venosus cavity. Bar: a, 150 µm; b, 60 µm.
observed with a Leitz microscope equipped with epi-fluorescence optics. The PAP technique was performed by incubating sections treated with antimyosin antibodies with appropriate dilutions of goat anti-rabbit immunoglobulins (Miles Laboratories) for 30 minutes at room temperature and then with rabbit PAP complex (Dakopatts, Glostrup, Denmark). Enzymatic activity was revealed using 1 mg/ml of p-phenylenediamine-pyrocatechol as substrate (Hanker Yates Reagent, Polysciences Inc., Warrington, Pennsylvania) in 0.05 M Tris-HCl, pH 7.6, with 0.01% hydrogen peroxide.

Histochemical demonstration of myofibrillar ATPase activity was performed as previously described and used to identify cardiac muscle fibers.

Results

In the youngest hearts examined (1–1.2-cm embryos), no reactivity with anti-bFm was observed in cardiac myocytes except for a few weakly labeled fibers grouped in the atrial posterior wall and facing the sinus venosus cavity (Figure 1). Looping processes were completed at this age; the primitive atrium was posterior to the bulbus cordis and superior to the primitive ventricle, atrial and ventricular cavities showed no sign of septation, and only slender trabeculae filled the ventricle. At this stage, like the adult bovine heart, anti-bVm stained all ventricular myocytes and only scattered atrial cells. However, unlike the adult heart, anti-bAm stained not only atrial myocytes but also ventricular fibers (not shown).

The localization of the reactivity for nodal myosin within a specific atrial region was more evident in subsequent developmental stages. In the 1.5-cm embryo, two clusters of fibers reactive with anti-bFm were detected in the sinus venosus region: the larger cluster was ring-shaped and surrounded the vein orifice, and the smaller cluster was localized close to the posterior atrial wall. The two reactive foci were more easily localized in the 1.9- and 2.4-cm embryos: the larger cluster was detected at the boundary between the sinus venosus and the right atrium (Figure 2), and the smaller cluster was located in the middle of the left atrial wall in proximity to vascular orifices, probably corresponding to the pulmonary veins (Figures 3A and 3B). Atrial fibers reactive with anti-bFm were always labeled by anti-bAm and variably with anti-bVm, except for nodal fibers that did not react with anti-bVm (Figures 2B and 2C). A number of atrial fibers reactive with anti-bVm were consistently observed at the periphery of the larger cluster (Figure 2). At this developmental stage, ventricular septation was completed and the AV node region could be identified as the origin of the AV penetrating bundle; however, no reactivity for nodal myosin was detected at these sites (Figures 3C and 3D).

Figure 2. A 2.4-cm embryo. Cardiac frontal cryosections: (a) is a PAP immunostaining with anti-bVm that shows the right atrium (RA), the right ventricle (RV) and the aorta (Ao); (b) represents a higher magnification of the area squared in (a), i.e., the superior caval vein orifice (SCV) stained with anti-bVm and (c) is a serial section stained with anti-bFm using PAP. Fibers reactive with anti-bFm are localized around the SCV orifice and surrounded by fibers labeled by anti-bVm. Bar: a, 300 μm; b and c, 150 μm.
In developmental stages from 3.8 to 9.5 cm, fibers reactive for nodal myosin were observed at the SA junction. They were consistently found grouped in a homogeneous cluster (Figure 4A) and surrounded by numerous fibers labeled with anti-βVlm. In contrast, no cluster of fibers reactive with anti-βFm was observed throughout all these stages in the compact AV node region or in the His bundle (Figure 4B and 4C). In contrast, scattered labeled fibers were found in the posterior atrial wall and in the interatrial septum. These became more numerous with age around the tendon of Todaro, at first only in the supratendineous region but later also in the infratendineous portion, always intermingled with unreactive fibers (Figures
FIGURE 4. a: Cryostat section of a 5-cm embryo at level of the SA node region stained with anti-bFm using the PAP procedure. SA node fibers are stained. b and c: Sections at level of the AV junction region; b: higher magnification of the area squared in c. b is a PAP immunostaining with anti-bFm that shows only unreactive fibers in the AV node region; c: PAP immunostaining with anti-bVm that illustrates the origin of the AV bundle. Bar: a and c, 300 μm; b, 150 μm.

5A and 5C). In addition, a number of labeled fibers were seen at all the stages examined in the left atrial wall close to pulmonary vein orifices (Figures 5D–5F). Reactivity with anti-bFm was never observed in the ventricular myocardium except for very rare labeled fibers in the interventricular septum and at the insertion of mitral valve leaflets of hearts 6 and 9.

At subsequent stages (13.0- and 15.0-cm embryos), there was a progressive appearance of labeled fibers in the AV node region. In the 16-cm fetus (Figure 6), a large population of fibers in the AV region, including compact AV node cells, was found to be labeled with anti-bFm. As in the adult heart, no stained fiber was present in the penetrating bundle fibers and in the bundle branches. Numerous fibers labeled by anti-bVm were seen at this stage in the atrial myocardium around the AV node. A similar pattern of reactivity was found in the 23- and 40-cm fetuses.

Discussion

We have previously reported that a population of muscle fibers in the central areas of the SA and AV nodes of the bovine heart is specifically stained by antibodies to fetal skeletal MHC. Ordinary atrial and ventricular fibers, as well as ventricular Purkinje fibers, were not labeled by these antibodies. In the present study we have used this novel type of cardiac MHC as a marker for studying the development of the nodal conduction tissue. The main results are that 1) nodal MHC has a specific and restricted localization in the bovine embryonic heart and 2) nodal myosin can be detected in the AV node region at much later stages than in the SA node.

Reactivity for nodal myosin is limited to few atrial fibers since early developmental stages. Two foci of reactive muscle cells were seen in the 1-month-old embryo: one located in the right atrium at the SA junction, presumably corresponding to the primordium of the SA node, and the other located in the left atrium near the outlet of the pulmonary veins, which may correspond to a primordial left-sided SA node. Based on the immunological similarity between nodal MHC and MHCs present in fetal skeletal muscle, it may be suggested that nodal MHC corresponds to a primordial type of cardiac MHC expressed at early developmental stages. This interpretation is supported by the present findings, showing that most atrial and ventricular muscle cells were unlabeled by anti-bFm in the 1-month-old embryo. At this age, all atrial muscle cells stained for α-MHC and a minor proportion also for β-MHC. In contrast, all ventricular muscle cells stained for both α- and β-MHCs. At subsequent stages, reactivity for α-MHC disappeared progressively in the ventricular fibers. These results indicate that ventricular muscle cells in the 1-month-old embryo have a MHC composition different from that of the adult heart but do not express nodal MHC. However, we cannot rule out the possibility that nodal MHC is expressed in all muscle cells at earlier stages of cardiac development.

In contrast to the SA node, fibers reactive for nodal MHC were not seen until much later stages in the AV node (Figure 7). Although a distinct AV node region could be morphologically identified as early as day 35, sporadic AV node fibers labeled by anti-bFm were first
FIGURE 5. a: Frontal cryosection of a 9.3-cm embryo processed for the histochemical demonstration of myosin ATPase activity to illustrate heart anatomy. Left cardiac cavities lie on the left side of the figure; arrow indicates posterior atrial wall. b and c: serial sections stained in indirect immunofluorescence with anti-bFm that show labeled fibers in the interatrial septum (b) and in proximity of the AV node (c). d: Serial section stained for histochemical myosin ATPase showing pulmonary vein orifices (arrow); adjacent sections (e and f) were stained with anti-bFm in indirect immunofluorescence; fibers labeled with anti-bFm were detected at the pulmonary vein orifices and in the surrounding areas. Bar: a and d, 3.3 mm; b, e, and f, 35 μm; c, 12 μm.

observed by day 70 and a compact area of labeled fibers was only seen by day 88. Parallel changes in MHC composition were observed in the perinodal regions: fibers reactive for β-MHC, which are typically seen at these sites in the adult heart,14 were first detected by day 40 around the SA node and by day 88 around the AV node.

The relatively late appearance of nodal myosin in the compact AV node could result from a differentiation process with a switch from α- to nodal MHC expression in the preexisting nodal fibers. This event might be mediated by the influence of autonomic innervation, as suggested by Navaratnam.28 In fact, although cardiac ganglial cells and axons were described in mammalian embryonic myocardium before the closure of the interventricular communication,29 they were detected in rat AV node region30 and displayed catecholamine fluorescence and cholinesterase activity28,31 only around birth; moreover, cardiac autonomic innervation was demonstrated to be functionally immature in puppies.32 Thus, the adult type of neural control on the AV node is achieved only after birth.
FIGURE 6. Transverse cryosections of SA node (a) and AV node (b) regions of a 16-cm fetus stained with anti-bFm using PAP procedures. SA node reactive fibers are grouped around the superior caval vein orifice, whereas the AV node region is diffusely reactive, from the posterior atrial wall to the origin of the penetrating bundle (arrow), whose fibers remain completely unstained. IVS, interventricular septum. Interatrial septum (c) and pulmonary vein (d) regions labeled with anti-bFm using PAP immunostaining: numerous reactive fibers are detected in the posterior atrial wall and around the orifices of the pulmonary veins (PV). Bar: a and b, 400 μm; c and d, 100 μm.
However, it remains to be determined whether nerves that are morphologically evident and functional contact with SA node fibers earlier than with AV node fibers. Only two histochemical studies demonstrating nerve cholinesterase activity during embryonic and fetal development of human hearts reported differences in nerve fiber density between the SA node and peculiar regions of the AV node: at the earliest stage studied (7-week-old embryos), numerous nerve fibers could be detected in the SA node and in the upper portion of the AV node, whereas the lower portion of the AV node and the His bundle appeared to be nerve free. In the latter regions, nerve fibers were detected only in fetuses older than 20 weeks. Unlike the human heart, several cholinesterase-positive nerves were observed in the AV nodal region and in the His bundle of the bovine embryo already at 8 weeks of gestation (Le.-E. Thornell, unpublished observation), when no reactivity for nodal myosin was found. Thus, the presence alone of nerves does not seem to induce nodal myosin expression in the bovine AV node.

Alternatively, AV node cells reactive for nodal MHC might derive from a distinct muscle cell population present at very early stages of development. It appears unlikely that these cells originate from the SA node: SA node cells reactive for nodal MHC were always grouped around the superior caval vein orifice throughout embryonic development, without any spreading toward the AV node region. On the other hand, AV nodal cells expressing nodal MHC may derive from the loosely grouped labeled fibers of the left atrium because during development an increased number of labeled fibers were seen throughout the left posterior wall, the pulmonary vein orifices, and the interatrial septum. These regions are contiguous to the left sinal horn that may contribute to the formation of the AV node region. In this region, precisely at the junction with the left dorsal atrial wall, Viragh and Challice described in the mouse embryo a cluster of cardiac fibers that mimicked in shape and position the right-sided SA node. They called it "left-sided SA node" and observed that this structure was integrated into the atrial musculature in the subsequent stages of development. Their results were consistent with the distribution of nodal myosin reactivity, although we were not able to discriminate between the left sinal horn and the left pulmonary veins in our specimens since these structures were very close together and presumably both were rich in reactive fibers. In fact, labeled fibers that could be easily distinguished from the caval veins were also observed at the right pulmonary vein orifices. Although the embryological origin of the pulmonary veins is attributed generically to the posterior atrial wall, it is of interest that pacemaker action potentials have been recorded around their orifices in the adult guinea pig heart. Thus, our results confirm previous theories on the existence of nodal-like specialized fibers in the left atrial wall but cannot demonstrate whether these fibers indeed contribute to the development of the AV node.

Several lines of evidence show that the functional specialization of the conduction tissue proceeds asynchronously during prenatal life. In the chicken, specialized action potentials can be recorded from the SA region of the heart tube as early as the 8–13-somite stage 19 whereas they were not recorded before 16–19 somites from the AV canal region. Comparable studies of the developing nodal conduction tissue in mammals are lacking, but several reports indicate that adult AV conduction tissue properties, such as the refractory period and the responsivity to different drugs, were acquired late during prenatal development. Thus, the late expression of nodal myosin in the AV node fibers during bovine embryonic development may correspond to a later appearance of specific nodal cells in the AV region.

References


**KEY WORDS**

- sinoatrial node
- atrioventricular node
- myosin
- cardiac development
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