Three-Dimensional Anatomy of the Conduction System of the Early Embryonic Rabbit Heart

FLORENCE ROTHENBERG* AND IGOR R. EFIMOV
Biomedical Engineering Department, Case Western Reserve University, Cleveland, Ohio

ABSTRACT

The complete embryonic cardiac conduction system is difficult to view in three dimensions, primarily because there has not been a marker of all segments of the normal system throughout all stages of development. Imaging of the conduction system components within the atria has been particularly controversial because different markers reveal different pathways that may or may not represent conduction system components. The conduction system of the adult and embryonic rabbit, however, can be labeled in its entirety with the neurofilament marker, NF-160. The conduction system of rabbit embryos at several stages of development spanning cardiac septation was therefore investigated. Optical mapping of the electrical signature of the conduction system previously revealed a close correlation between the cardiac activation patterns and the anatomy as shown by serial sections. The 3D relationship between the components of the conduction system could only be inferred from the 2D sections. The sections were consequently reconstructed using a commercial software program (AutoQuant). This is the first demonstration of the three-dimensional complete normal rabbit embryonic cardiac conduction system at several stages of development. © 2005 Wiley-Liss, Inc.

Key words: embryo; conduction system; rabbit; three dimensional reconstruction; neurofilament

The embryonic origin of normal and abnormal cardiac conduction has been a focus of recent research with the advent of several new tools, including optical mapping with voltage-sensitive dyes (Rentschler et al., 2001; Reckova et al., 2003; Chuck et al., 2004; Hall et al., 2004; Rothenberg et al., 2005) and transgenic mice that permit labeling of regions that correlate with the anatomical location of the cardiac conduction system (Kupershmidt et al., 1999; Davis et al., 2001; Rentschler et al., 2001; Kondo et al., 2003; Jay et al., 2004). There is some controversy, however, as to whether transgenic markers label true cardiac conduction system cells (Anderson et al., 2004). Large tracts of putative conduction system cells within the atria can be elucidated using the transgenic mouse model that expresses CCS/lacZ in the heart (Jongbloed et al., 2004). These atrial tracts have not been confirmed as being part of the conduction system electrophysiologically or with other histologic methods. In contrast, the neurofilament marker, NF-160, labels all of the components of the cardiac conduction system in the adult rabbit heart (Verheijck et al., 1998; 2003, 2005) and the embryonic rabbit heart (Gorza and Vitadello, 1989; Vitadello et al., 1996). Antibodies against NF-160 and NF-200 have been localized to the atrioventricular node (AVN) and bundle of His in the monkey (Mueller et al., 2003). Antibodies against the neurofilament NAPA-73 have been localized to regions consistent with early conduction system in the ten somite embryonic chick heart (Ciment, 1990). Furthermore, the relationship to impulse conducting cells of the conduction system has been directly confirmed with electrophysiologic recording in the adult rabbit (Dobrzynski et al., 2004).
for the visualization of the atrial and ventricular components of the normal mammalian cardiac conduction system.

Previous work showed that the electrophysiology of periseptation embryonic hearts was clearly correlated with the location of conduction tissue as determined by 2D sections of NF-160-labeled tissue (Rothenberg et al., 2005). While the normal embryonic conduction system appears continuous from these serial sections, its continuity cannot fully be confirmed without detailed 3D reconstructions. Furthermore, 3D reconstructions of the developing cardiac conduction system can be used to analyze its relationship to surrounding structures for the purpose of understanding the contributions of other tissues and developmental signals that may pass between them. Therefore, a three-dimensional understanding of the anatomy of the conduction system with relation to surrounding structures is critical for understanding the signals that may be used to initiate conduction system formation and remodeling during development, as well as the complex interactions that must occur for impulse generation and conduction throughout embryogenesis. We show here for the first time the entire conduction system at three stages of development, reconstructed from serial sections that mark the expression of the neurofilament protein, NF-160, through the entire embryonic rabbit heart. These reconstructions show that the conduction system truly is a continuous structure from sinoatrial node to Purkinje fibers, even in the looped tubular heart prior to cardiac septation.

MATERIALS AND METHODS

Histology

This protocol was approved by the Institutional Animal Care and Use Committee (IACUC) at Case Western Reserve University, Cleveland, Ohio. Pregnant New Zealand white rabbits (gestation days 12, 13, and 15) were euthanized as previously described (Rothenberg et al., 2005) and the embryos harvested. The hearts were extracted and a subset was fixed immediately in 70% ethanol for 10 min, followed by a series through 100%, 70%, 50% saline, 5% sucrose, overnight in 20% sucrose, and embedded in OCT mounting medium (Tissue-Tek, compound 4583; Sakura Finetek, Torrance, CA) for cryosectioning. The hearts were then either stored at -80°C or immediately cryosectioned into 16 μm frontal sections. The serial sections were labeled overnight with anti-NF-160 (1:1,000 mouse IgG; MAB 5254; Chemicon, Temecula, CA) and antisarcomeric antibody (1:1,000 mouse antirabbit IgM; M0874; DakoCytomation, Carpenteria, CA). Secondary antibodies (AlexaFluor 488 and 594) were purchased from Molecular Probes (Eugene, OR). Negative controls using only the secondary antibodies were negative for labeling (data not shown).

Image Processing

The sections were digitally imaged using a Nikon Eclipse upright microscope, and the sections reconstructed using AutoQuant software (AutoQuant Imaging, Waterviet, NY). Sections were imported as TIFF images, automatically aligned, and reconstructed using appropriate dimensions. The original color of the stain (green) was changed by selecting a different color map lookup table to improve clarity of the image. Only sections containing NF-160 label were used in these images. The movie was generated with the AutoQuant software. Three-dimensional images for viewing with red-blue glasses (with the red lens on the left) were generated with Photoshop. Left-eye and right-eye images were selected from frames of the 3D movie prepared as described above. The images were prepared with Adobe Photoshop. Instructions were obtained from the Web (www.savel.org/old/mad/stereo.html).

RESULTS

The video clips (day 12, day 13, and day 15 http://interscience.wiley.com/jpages/1552-4884/suppmat/) reveal the dramatic anatomical changes that took place within the cardiac conduction system over a span of 3 days. Neurofilament labeling in the day 12 heart showed a concentration of marker on the right side of the atrioventricular junction (Fig. 2, panel 1, arrows), a region that was previously the inner curvature of the linear heart tube only a day before. Physiologically, hearts at this stage of development have sequential atrial-ventricular activation despite the fact that the conduction system appears to be concentrated in this single location with little differentiation (Rothenberg et al., 2005). The neurofilament labeling appeared to be equally distributed dorsoventrally.

Only a day later, the day 13 cardiac conduction system appeared much more differentiated morphologically (Fig. 1, panel A; Fig. 2, panel 2, and video clips http://interscience.wiley.com/jpages/1552-4884/suppmat/). It remained a single unit, however the labeled conduction system elongated in a caudal-cranial direction. The primitive sinoatrial node (SAN) and atrioventricular node (AVN) are now more dorsal in the embryonic heart with respect to their connecting pathways. In fact, the conduction system took on a spiral appearance when viewed from the side. Bundle branches formed, and Purkinje fibers took root in the ventricular trabeculae.

Further development revealed an elaboration and refinement of all conduction system components. In the day 15 embryonic heart, the SAN and AVN were now deeply dorsally located within the conduction system axis, whereas the connecting pathways were also more fully developed in a dorsal-ventral axis (Fig. 1, panel B; Fig. 2, panel 3, and video clips http://interscience.wiley.com/jpages/1552-4884/suppmat/). The bridge between the SAN and AVN looped dorsoventrally, while the bundle branches have a characteristic saddle-shaped appearance draped over the interventricular septum.

DISCUSSION

It is becoming clear that anatomy is critical in defining the pattern of cardiac conduction not only in the mature heart, but in the embryo as well. The mouse model has been and remains an unparalleled tool in the quest for understanding the genetic basis for normal and abnormal cardiac conduction system development (Kupersmidt et al., 1999; Davis et al., 2001; Rentschler et al., 2001; Kondo et al., 2003; Wessels et al., 2003; Jay et al., 2004). The precise anatomic location of conduction system cells using markers obtained through transgenic modifications, however, is controversial because not all markers label the same structures. As an example, the CCS/lacZ marker labels a broad region between what is believed to be the...
sinoatrial node and the atrioventricular node (Jongbloed et al., 2004), not seen in the cGATA-6 enhancer transgenic mouse (Davis et al., 2001). The advantage to investigating a normal rabbit is clear: the conduction system is not genetically manipulated and is therefore normal. Labeling with NF-160 is present in all elements of the conduction system in the adult rabbit (Verheijck et al., 1998; Dobrzynski et al., 2003, 2005), and these anatomical regions have been confirmed to be conduction system elements electrophysiologically (Dobrzynski et al., 2003, 2005). NF-160 labeling is likewise present in all conduction system cells in the embryonic rabbit, but is also present throughout development, unlike other markers (Gorza et al., 1988; Gorza and Vitadello, 1989; Vitadello et al., 1996). This argues that there is a fundamental difference in conduction system tissues that, despite the fact that all cardiomyocytes can transmit electrical impulses, makes these labeled cells designed for this purpose. Therefore, while accessory pathways and ectopic arrhythmogenic sites might be capable of transmitting impulses, they are not designed for that as suggested by the limited NF-160 labeling and are therefore potentially more arrhythmogenic. This model system then could be critical in elucidating the signals that turn cardiomyocytes into NF-160 expressing conduction system cells.

A possible disadvantage may be that this marker appears to label all conduction system elements only in the rabbit for reasons that are still unknown. This suggests that inferences about conduction system development derived from these investigations can only be limited to the rabbit. However, it is unlikely that signals important for conduction system induction would be relevant only to the rabbit since NF-160 works only in this setting. Furthermore, an understanding of the basis for this species difference may provide valuable clues to the signals that initiate conduction system formation.

Whether this species difference would confer functional changes as well is also unknown, but should not diminish the power of this model as an investigative tool for several reasons. First, this investigative baseline in the rabbit model system is entirely normal, whereas the more commonly reported markers of the conduction system in the transgenic mouse require a genetically altered baseline, also of unclear relevance to the human heart. Second, with respect to the mouse model of cardiac conduction, the normal physiology of the rabbit appears to be more similar to that of the human in that the resting adult heart rate is approximately 150–200 bpm, as opposed to nearly 600 bpm in the adult mouse. With regard to cardiac electrophysiology, the rabbit again is more similar to the human than the mouse. For example, the action potential in the rabbit has a long plateau phase like the human’s (Valentin et al., 2004), and the rabbit atrioventricular node can contain fast and slow pathways, as seen in humans also (Dobrzynski et al., 2003). Furthermore, the rabbit heart has been shown to be an effective model for testing of potentially proarrhythmic agents due to the high degree of physiologic similarity (Valentin et al., 2004). The rabbit, then, may be a more relevant model system in cardiac physiology investigations. However, the ability to alter genetic components of relevant developmental pathways is still clearly necessary. The power of the transgenic approach is only in its infancy in the rabbit system (Marian et al., 1999; Bosze et al., 2003; Nagueh et al., 2004). All tools at our disposal, therefore, will be valuable in our attempts to understand the signals relevant to conduction system formation.

Whether all elements of the conduction system have been labeled is presently under investigation. Reports have shown that regions not associated with the conduction system such as the outflow tract were positively stained using other markers for the conduction system.
such as HNK-1 and PSA-NCAM in the chick (Chuck and Watanabe, 1997) and rat (Ikeda et al., 1990). Preliminary investigations using these markers in the embryonic rabbit suggest similar staining patterns to the chick and rat (data not shown). It is unlikely that the outflow tract is part of the conduction system. However, the reason for its positive labeling is presently unclear. In addition, evidence from other species suggests the AVN forms from the fusion of two structures in the pre-septation heart (Marino et al., 1983—ferret; James, 1970—human, Arguello et al., 1988—chick) located in the base of the atrioventricular septum and the AV canal. It is possible that the SAN is either not present (has been removed in the dissection) or does not label with NF-160, as it does in the adult. These hypotheses are currently under investigation.

What should be clear is that the combined investigative approach of structure and function in the normal rabbit embryo is an important tool in understanding the normal and abnormal anatomy and physiology of the cardiac conduction system and will provide critical information regarding the relationship between embryonic tissues that must take place for the normal formation of the cardiac conduction system.

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LITERATURE CITED


