Development of the Cardiac Conduction Tissue in Human Embryos Using HNK-1 Antigen Expression
Possible Relevance for Understanding of Abnormal Atrial Automaticity

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Background—Abnormal atrial automaticity in young patients with structurally normal hearts is often located around the pulmonary veins and in sinus venosus–related parts of the right atrium. We hypothesize that these ectopic pacemaker sites correspond to areas of embryonic myocardium with an early phenotypic differentiation, as indicated by differences in antigen expression during normal cardiac development.

Methods and Results—In human embryos ranging in age from 42 to 54 days of gestation, the development of the cardiac conduction system was studied with the use of HNK-1 immunohistochemistry. HNK-1 stains the developing atrioventricular conduction system, ie, the bundle branches, His bundle, right atrioventricular ring, and retroaortic ring. In addition, the myocardium around the common pulmonary vein showed transient HNK-1 antigen expression. In the right atrium, 3 HNK-1–positive connections were demonstrated between the sinoatrial node and the right atrioventricular ring. An anterior tract through the septum spurium connects the sinoatrial node with the anterior right atrioventricular ring, and 2 posterior tracts connect the sinoatrial node with the posterior right atrioventricular ring through the right venous valve (future crista terminalis) and sinus septum, encircling the coronary sinus. The medioposterior part of the right atrioventricular ring connected to the His bundle and the medioanterior part form 2 node-like structures.

Conclusions—In patients with abnormal atrial automaticity, the distribution of left and right atrial pacemaker foci correspond to areas of the embryonic myocardium that temporarily express the HNK-1 antigen. (Circulation. 1999;99:800-806.)

Key Words: morphogenesis conduction arrhythmia atrioventricular node

Abnormal atrial automaticity is a rare form of supraventricular tachycardia in (usually) young patients with otherwise normal hearts. Although the pathophysiology is not known, electrophysiological studies and radiofrequency catheter therapy have demonstrated that these ectopic pacemaker foci are often located around the pulmonary veins in the left atrium or in sinus venosus–related structures in the right atrium, like the crista terminalis and the orifice of the coronary sinus. Abnormal atrial automaticity corresponds to parts of the embryonic myocardium with an early phenotypic differentiation, as indicated by differences in antigen expression during normal cardiac development.

The use of immunohistochemical markers to study the developing cardiac conduction system has provided important new data, although none of these markers are unique for the cardiac conduction tissue. With the use of a neural tissue antigen (GIN2), it was demonstrated that the developing atrioventricular conduction tissue in human embryonic hearts, including the compact atrioventricular node, develops from one specialized ring between the primitive ventricles. Very similar to GIN2, HNK-1 (Leu-7) antigen expression has been used to study the developing cardiac conduction tissue in different species. The monoclonal antibody HNK-1, originally used to identify human natural killer cells, was first described as a marker of neural crest cells during embryonic development. Later transient HNK-1 expression has been reported both in the developing atrioventricular conduction system and in the sinus venosus myocardium of different species, including humans.

Although it is generally believed that the sinoatrial node is derived from the sinus venosus or sinoatrial transition, the role of the sinus venosus in the developing cardiac conduction system has not been clarified in human embryos. In this study, we investigated whether the HNK-1 antigen could be used as a marker to study the developing cardiac conduction...
system in human embryonic hearts, with special reference to the sinus venosus myocardium.

Materials and Methods

Five human embryos ranging in age from 42 to 54 days of gestation were studied. The embryos were obtained after legal abortion and the studies were approved by the medical-ethical committee of the Leiden University Medical Center. The developmental stage of the embryos was determined and they did not show malformations. Whole embryos were fixed at room temperature in 4% phosphate-buffered formalin solution, embedded in paraffin, serially sectioned (6 μm) transversely and washed in PBS. This was followed by 15 minutes in PBS with hydrogen peroxide to block the endogenous peroxidase activity; the embryos were then washed again. Alternate sections were incubated with the monoclonal antibody HNK-1 (Hybridomabank, Iowa City, Iowa) and the monoclonal anti-muscle actin antibody HHF-35 (Dako M635, Carpinteria, Calif), diluted in PBS with 1% ovalbumin and 0.05% Tween-20. After overnight incubation, the sections were rinsed in PBS. Rinsing was followed by incubation for 2 hours with 1:200 diluted rabbit anti-mouse conjugated to horseradish peroxidase (Dako, P260, Carpinteria, Calif), 2-hour incubation with 1:50 goat anti-rabbit immunoglobulin (Nordic, Tilburg, Netherlands), and 2 hours with 1:500 rabbit peroxidase anti-peroxidase (Nordic, Tilburg, Netherlands). After washing, the staining reaction was performed with 0.04% diaminobenzidine tetrahydrochloride (Sigma, D5637, St. Louis, Mo) in 0.05 M trismaleic acid (pH 7.6) and 0.006% hydrogen peroxide for 10 minutes; this was followed by washing. The slides were counterstained with Mayer’s hematoxylin for 10 s. Graphic 3D reconstructions were made to obtain a better cognition of the distribution of HNK-1–positive myocardium and its relationship to other cardiac structures.

Results

Stage 18 (42 to 46 Days, Crown-rump [CR] Length 14 mm)

In this youngest embryo, the separation of the aorta and pulmonary trunk at orifice level is complete. Both right and left portions of the atrioventricular canal are present and the ventricular and atrial septation have progressed. The sinus venosus has been incorporated into the atrium, and the entrance of the superior caval vein is guarded by 2 large valves, the right and left venous valves. Dorsocranially, these valves fuse to become the septum spurium, a marked structure that runs anteriorly through the roof of the right atrium.
HNK-1 staining is present in the ring of myocardium around the right part of the atioventricular canal, the so-called right atioventricular ring (RAVR). The medioanterior and, in particular, the medioposterior part of this ring form 2 compact HNK-1–positive areas. The distance between these 2 areas is relatively large with thick cushion tissue separating them. The medioposterior part is connected with HNK-1–positive myocardium in the top of the interventricular septum, ie, the His bundle which divides into both bundle branches. HNK-1 antigen expression is also detected in parts of the sinus venosus myocardium, whereas the myocardium of both atria and the septum primum remain HNK-1 negative. HNK-1 is present in the sinoatrial node (SAN), located in front of the superior caval vein. Three tracts of HNK-1–positive myocardium converge in this area. One tract runs anteriorly in the roof of the right atrium through the septum spurium to make contact with the HNK-1–positive mass of the anterior part of the RAVR. A second tract runs posteriorly and through the base of the right venous valve, around the orifice of the coronary sinus to the base of the atrial septum. A third tract leaves the SAN and runs posteriorly and through the base of the left venous valve and the base of the atrial septum. These 2 posterior bands are still remote from the posterior RAVR and the His bundle. In addition, the myocardium around the orifice of the common pulmonary vein is still HNK-1–positive. This region is continuous with the HNK-1–positive myocardium of the sinus venosus in the right atrium through the base of the atrial septum (Figure 1). A graphic 3D reconstruction of the distribution of the HNK-1 antigen expression is shown in Figure 2.

Stage 19 to 20 (47 to 51 Days, CR Lengths 17,19, and 21 mm, Respectively)

At these stages, the ventricular septation is well advanced. The sinus venosus has become further incorporated into the right atrium. By now, the septum spurium is less marked, but the left venous valve is still a large structure. The 3 embryos that have been studied have similar HNK-1 staining patterns.

The RAVR remains HNK-1–positive and is continuous anteriorly with an HNK-1 band of myocardium behind the aorta, the so-called retroaortic ring. The distance between the medioanterior and the medioposterior node–like parts of the RAVR has become much smaller. The latter remains connected with the His bundle and bundle branches, and the medioanterior part is continuous with the retroaortic ring.

The SAN forms a semicircular HNK-1–positive structure in front of the superior caval vein. The same 3 HNK-1–positive myocardial bands connected to this area can be easily recognized. The anterior tract through the septum spurium is still continuous with the medioanterior RAVR, thus connecting the SAN with the primitive atioventricular conduction tissue. The second HNK-1 band remains present in the base of the right venous valve (around the coronary sinus to the base of the atrial septum) and surrounds the orifice of the inferior caval vein. The third band of HNK-1–positive myocardium leaving the SAN is present in the left venous valve and continues in the base of the atrial septum. These 2 posterior HNK-1–positive bands are adjacent but not connected to the posterior RAVR and His bundle. HNK-1 staining around the common pulmonary vein is still in continuity with the HNK-1–positive myocardium of the sinus venosus in the right atrium through the base of the atrial septum (Figures 3 and 4).

Stage 22 (54 Days, CR Length 23 mm)

The ventricular septation is complete and the atioventricular insulation has progressed. The septum spurium is difficult to recognize as a separate structure in the roof of the right atrium. At this stage, the left venous valve is reduced in size. HNK-1 is only faintly present in both bundle branches and His bundle. The His bundle runs through the fibrous skeleton where it meets the medioposterior node–like structure which consists of strongly HNK-1–positive myocardial cells and which is still part of the posterior RAVR. Also, the medioanterior part of the RAVR is still a compact HNK-1–positive area. By now, the distance between these 2 node-like structures has become minute, although they have not fused. The staining has disappeared in the left venous valve and around the common pulmonary vein. The SAN, now located at the lateral margin of the superior caval vein, is only faintly positive. The anterior HNK-1 tract through the roof of the right atrium (septum spurium) toward the medioanterior part of the RAVR is faint but still present.
Importantly, the posterior HNK-1 band running from the SAN through the right venous valve is connected to the medioposterior RAVR. Also, the sinus septum and the base of the atrial septum are HNK-1–positive and continuous within this same region (Figures 5 and 6).

**Discussion**

In humans, as in other species, the development of the cardiac conduction system and the origin of its different elements remains a topic of controversy. Although the use of immunohistochemical markers for the conduction system such as neurofilament, nodal MHC, HNK-1 (Leu-7), GIN2, and others has resulted in new insights, none of these markers are specific for conduction systems only, which make the interpretation of these results difficult.4–11

With the so-called ring theory, Wenink14 and Anderson15 proposed that the cardiac conduction system was derived from 4 separate rings of specialized myocardium found between the primitive segments of the heart and partially disappearing during development. According to this theory, the sinoatrial node is derived from the sinoatrial ring, the AV node from the atrioventricular ring, and the His bundle and bundle branches from the interventricular ring. Later, Wessels et al4 demonstrated in human embryos, with the use of a neural tissue antigen (GIN2), that the so-called retroaortic ring, the right atrioventricular ring, the compact AV node, the His bundle, and bundle branches all originate from one ring between the primitive ventricles, the interventricular ring. The composition of the AV node and the role of the sinus venosus in the development of the cardiac conduction system remains undescribed.

In human embryonic hearts, and presumably in those of other species, the GIN2 antigen expression seems identical to the HNK-1 expression with regard to the developing atrioventricular conduction system.4–5 Transient HNK-1 expression is also present in sinus venosus myocardium of birds and mammals (including humans).5,7,9,16 Many studies have been published that use HNK-1 (Leu-7) as a marker of the developing cardiac conduction system in rat embryos. They mention 3 tracts of HNK-1–positive myocardial cells that connect the HNK-1–positive sinoatrial node with 2 atrioventricular node primordia that fuse later.5,7,8 In our depart-
ment. DeRuiter et al. demonstrated that the entire sinus venosus, as well as the orifice of the common pulmonary vein, expressed the HNK-1 antigen in chicken embryos at early stages.

In the present study on human embryos, we demonstrate that the HNK-1 antigen is transiently expressed in parts of the myocardium of the sinus venosus and around the common pulmonary vein, whereas the myocardium of both atria and septum primum remain HNK-1 negative. Surprisingly, we identified, in the youngest embryo, an anterior HNK-1 tract that runs anteriorly through the roof of the right atrium in the septum spurium; this tract was found between the sinoatrial node and the medioanterior part of the right atrioventricular ring. At a later stage, the sinoatrial node and medioanterior part of the right atrioventricular ring show connections of HNK-1–positive myocardial bands, in the base of the right venous valve and sinus septum, running laterally and mediately to the coronary sinus. These posterior connections seem to be the result of the incorporation of the sinus venosus into the atrium and the invagination of the sulcus tissue.

In addition, transient HNK-1 staining appeared present in the myocardium around the orifice of the common pulmonary vein. The HNK-1–positive right atrioventricular ring forms medioanteriorly and medioposteriorly 2 node-like structures that approach each other but remain separated at the stages examined. The medioposterior part, forming a large node, is in continuity with the His bundle and bundle branches at all stages examined, and the medioanterior part is continuous with the retroaortic ring. We have not been able to confirm fusion of these areas as reported by Aoyama, and there are studies that demonstrate that the anterior “primordium” always remains separated from the posterior node and His bundle by fibrous tissue.

The function of the HNK-1 antigen has not been clear up to now. What the known glycoproteins carrying this epitope have in common is that they all play a role in cell-cell or cell-substrate interaction. Although HNK-1 is present in all parts of the developing conduction system, including the sinoatrial node, it is difficult to appoint a functional role to the HNK-1–positive sinus venosus myocardium. Experiments in chicken embryos have demonstrated that the sinus venosus segment is electrophysiologically distinct from the embryonic atrial myocardium at early stages of development. This seems to correspond to the transient HNK-1 staining of the entire sinus venosus myocardium in chicken embryos at these stages.
In the present study on human embryos, we show that HNK-1 staining is present in parts of the sinus venous myocardium. In the postnatal heart, the crista terminalis, the myocardium medial and lateral to the coronary sinus, and the junction of the medial wall of the right atrial appendage (septum spurium) correspond to the HNK-1–positive parts in the right embryonic atrium. Electrophysiological studies and radiofrequency ablations have shown that abnormal right atrial automaticity in children and adults with structurally normal hearts cluster along the crista terminalis, around the coronary sinus, or near the right atrial appendage.1–3 Corresponding to the areas of transient HNK-1 expression, although the right atrial appendage develops from the embryonic atrial segment (HNK-1 negative), we speculate that the automatic foci often reported at the junction of the right atrial appendage could relate to the HNK-1–positive septum spurium.

In the normally developed heart, the relevance of the embryonic presence of HNK-1–positive connections between the developing sinoatrial node and the atrioventricular conduction tissue is unknown. The tracts are very similar to the HNK-1–positive internodal connections as reported in rat embryos;5,7,8 they also resemble the tracts of pale myocytes in human fetal hearts as described by Gittenberger-de Groot and Wenink,19 suggesting the fetal presence of cells from the sinoatrial ring. The distal parts of these HNK-1 bands also seem to correspond with the localization of 3 atrial conduction tissue.1–3 They converge and become continuous with the atrioventricular node as described in dogs.20 However, these bundles end in the atrial myocardium and do not connect the 2 nodes. From the present study, we cannot decide the debate on the presence or absence of specialized “internodal tracts.” In the human heart, 3 internodal tracts have been proposed by Sherf and James21: one posterior pathway running along the crista terminalis and the middle and anterior pathway, along the posterior and anterior aspect of the atrial septum. One of the described pathways, the posterior, corresponds exactly to our HNK-1 tract through the right venous valve. The other two HNK-1–positive tracts have a slightly different position as compared to those described by Sherf and James.22 Other authors have not been able to demonstrate discrete tracts of specialized myocytes between the nodes.22,23 Furthermore, it is generally agreed that the lack of insulation of these internodal pathways from the surrounding myocardium is an important histological criterion against the existence of true specialized internodal conduction tracts.24,25

Another important finding is the HNK-1 staining around the orifice of the common pulmonary vein, which is continuous with the positive myocardium of the sinus venosus through the base of the atrial septum. Although the staining pattern suggests that this HNK-1–positive myocardium originates from the same segment of the heart, younger embryos are required to demonstrate this. Different theories exist on the development of the pulmonary vein in different species. Questions remain as to whether the common pulmonary vein originates from the sinus venosus segment of the heart16,26 or if it is always discrete from the sinus venosus.27,28 The present study cannot bring a resolution to this controversy. The myocardium that encloses the pulmonary vein is of special interest in abnormal atrial automaticity. In adult rats, nodal-like cells have been described in this region29; and in experiments with guinea pigs, Cheung30 demonstrated pacemaker activity of the myocardium of the pulmonary vein propagating action potentials to the right atrium. Furthermore, Gorza et al.10,11 described this same area around the pulmonary vein with the use of nodal myosin heavy chain expression in bovine embryos and anti-neurofilament antibodies in rabbit embryos as markers of conduction tissue.

Electrophysiological studies and radiofrequency ablations in young patients with ectopic atrial tachycardia have shown that the foci are located mostly around the orifices of the pulmonary veins,1 and recently it has been reported that arrhythmogenic foci around or even inside the pulmonary veins sometimes play a role in atrial fibrillation.31 As in the right atrium, abnormal atrial automaticity in the left atrium corresponds to the transient HNK-1 antigen expression of the embryonic myocardium around the common pulmonary vein, as shown in our study.

We have demonstrated that during normal cardiac development, specific areas of embryonic myocardium with an early phenotypic differentiation might provide the foci of abnormal atrial automaticity, as seen in patients without structural heart disease.

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_Circulation_. 1999;99:800-806
doi: 10.1161/01.CIR.99.6.800

_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

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