Variations of Developing Conduction System in Bis-diamine Induced Malformed Rat Heart Recognized by HNK-1 Immunohistochemistry

Hiroshi OHTANI

First Department of Pathology, Nagasaki University School of Medicine

To clarify the abnormal development of the cardiac conduction system in congenital heart anomalies, HNK-1 distribution of bis-diamine induced malformed hearts was studied in rat embryos. All the embryos showed not only conotruncal anomalies, such as persistent truncus arteriosus (PTA) and pulmonary hypoplasia with overriding aorta (comparable to tetralogy of Fallot), but also incomplete absorption of sinus venosus. In the malformed hearts, the middle internodal tract passing through the dorsal wall of the right sinus horn connected the right sinoatrial node (RSAN) to the posterior atrioventricular node (pAVN). Furthermore, an interatrial tract and a posterior tract bypassing the pAVN were observed in a few specimens. These pathways seemed to be equivalent to Bachmann's bundle and James' fiber, respectively. The HNK-1 immunoreactive sites corresponded to the bilateral sinoatrial nodes, middle, posterior, and left internodal tracts, and the pAVN in treated hearts extended caudally. This alteration was associated with incomplete absorption of the sinus venosus, suggesting that these conduction tissues were derived from the primary sinus venosus. The results indicated that HNK-1 immunoreactive sites during cardiac development were closely related to the development of the cardiac structures, and that the distribution of these sites varied according to abnormalities in developing cardiac morphology. We concluded that HNK-1 immunohistochemistry is a useful morphological tool for the study of congenital cardiac malformations and congenital arrhythmias, as well as development of the conduction system.

Key words: HNK-1, bis-diamine, heart conduction system, congenital heart anomalies, rat embryo, sinus venosus

Introduction

The heart conduction system, including both the sinoatrial node (SAN) and the atrioventricular conduction tissues, may be abnormally positioned in the case of congenital cardiac malformation. Clinically, various arrhythmias, such as supraventricular tachycardia and atrioventricular blocks, arise in patients with congenital heart disease. Moreover, reentrant arrhythmia via the bundle of Kent as one of the accessory conduction pathways, i.e., Wolff-Parkinson-White syndrome, has been reported in 10-25% of patients with Ebstein's anomaly. As an experimental heart model, conotruncal anomalies are induced in rats by bis-diamine administration. Experiments using this technique have indicated that these anomalies are caused by a disturbance of the extracellular matrix or migration of neural crest cells.

Since Ikeda et al. first demonstrated that HNK-1 immunoreactive tissue was distributed in the sites corresponding to almost the entire conduction system in rat and human hearts, investigations concerning the development of rat heart conduction system have been performed by HNK-1 immunohistochemistry, using computer graphics for three-dimensional reconstruction. Ito et al. pointed out that incomplete absorption of the sinus venosus was also found in bis-diamine treated hearts and that abnormal distribution of HNK-1 immunopositive areas may occur in association with abnormal morphological development. Previously, James had reported that the primary sinus venosus was the origin of conduction tissues, including the sinoatrial node, the three internodal tracts and the atrioventricular node, in human cardiac embryos. Thus, the sinus venosus has been shown to be a key portion of the heart conduction system.

It is important for surgeons, physicians, and pathologists to recognize abnormal development in the conduction tissue in congenital heart disease. In the present study, the HNK-1 distribution in bis-diamine treated hearts was investigated in detail to demonstrate how the conduction system alters in conjunction with abnormal development of the heart. Variations of the HNK-1 distribution were also studied in the treated hearts. Various alterations of the HNK-1 distribution pattern in association with incomplete sinus venosus are shown in this study.
Materials and Methods

Animals

Female Wistar white rats (Kyudo, Tosu, Japan) were mated with males overnight, and the morning when sperm was observed in the vaginal smears was designated as gestational day 0. On gestational days 9 and 10, bis-dichloroacetyl-diamine (bis-diamine, Sigma Chemical Co., St. Louis, MO, USA) was administered to the pregnant rats by the previously described method. Embryos were removed from both control and bis-diamine-treated females sacrificed by carbon dioxide on gestational day 14.5 (Streeter's stage XIX) because HNK-1 reactive sites topographically corresponded to nearly all the conduction tissue at this stage. For both normal control and bis-diamine-treated heart study, 8 specimens taken from 4 mother rats were used.

Immunohistochemistry

The thoracic regions of the embryos were fixed in 10% buffered formalin (pH 7.4) for 48 hours at 4 °C. The specimens were embedded in paraffin and cut horizontally into complete serial 6 μm sections. Immunohistochemical analysis for HNK-1 was performed by the avidin biotin peroxidase complex method. Briefly, deparaffinized sections were incubated overnight at 4°C with monoclonal Leu-7 (clone HNK-1) antibody (Becton-Dickinson Immunoctometry System, Mountain View, CA, USA) diluted to 1:100. The sections were incubated for 30 minutes with biotinylate anti-mouse IgM (Vector Laboratories, Burlingame, CA, USA) diluted to 1:200, and then incubated for 30 minutes with avidin-biotin-peroxidase complex (Vector Laboratories, Burlingame, CA, USA). Peroxidase activity was visualized using 3,3′-diaminobenzidine tetrahydrochloride. Counterstain was performed with hematoxylin.

Results

Conotruncal anomalies, such as persistent truncus arteriosus and pulmonary arterial hypoplasia with overriding aorta, were induced by bis-diamine administration in all embryos (Fig. 1c). These anomalous hearts also showed incomplete absorption of sinus venosus.

Sinoatrial nodes

The right superior vena cava showed U-shaped immunoreactivity corresponding to the right sinoatrial node (RSAN) at its entrance to the right atrium (Fig. 1a, e). At a more caudal level, the left sinoatrial node (LSAN) was also observed along the left superior vena cava (Fig. 1b, 2c). In bis-diamine treated hearts, the bilateral nodes were poorly developed and deviated caudally toward the inferior vena cava, compared with normal hearts (Fig. 1e, 2c).

Atrioventricular nodes

The right dorsal edge of the superior endocardial cushion strongly expressed HNK-1 antigen in both normal and malformed hearts (Fig. 1b, d). These nodular immunoreactive regions were designated as anterior atrioventricular nodes (aAVN). Just caudal to the aAVN, another small nodular positive region, termed the posterior atrioventricular node (pAVN), was observed (Fig. 2a, c). These nodes were widely separated without connection to each other. The pAVN of the treated hearts was smaller than that of the controls and extended cranially to the caudal edge of the superior endocardial cushion and caudally to the dorsal wall of the sinus venosus (Fig. 2c, d).

Internodal tracts

Developing pectinate muscles and septum spurium in the right atrium showed strong immunoreactivity in the normal and malformed hearts. In these structures, positive areas separated into dorsal and ventral courses, which were designated as anterior tract (1) and antero-lateral tract (2), respectively (Fig. 1a). The two tracts merged both on their right and left sides. The right side was a weakly positive route descending through the lateral wall of the right atrium into the right portion of the RSAN, while the left side was a strongly positive descending route into the aAVN (Fig. 1a, d). In the malformed hearts, the atrial inner structures, including the pectinate muscles and the septum spurium, developed poorly into simple structures compared with those of the controls (Fig. 1d). For this reason, the anterior and the antero-lateral tracts appeared close together and were not completely separated (Fig. 1d). These tracts deviated dorsocaudally. Furthermore, sporadic immunoreactive sites were observed along the lateral wall of the right atrium (Fig. 1e).

The posterior tract (3) originated from the right portion of the RSAN and descended through the right venous valve (Fig. 1b, 2b). Subsequently, the tract passed transversely through the sinus septum and entered the pAVN (Fig. 3a, b). The sinus septum in the bis-diamine treated hearts was located in the extracardiac portion. Through the sinus septum, the posterior tract, which extended caudally to the ventral wall of the right sinus horn, was connected to the caudal extending portion of the pAVN (Fig. 2d, 3c).

In the malformed hearts, we found another immunoreactive route originating from the left portion of the RSAN (Fig. 2c). This route descended and continued a moderately positive but loose course along the dorsal wall.
of the right sinus horn, and then the tract was connected with the pAVN (Fig. 2d). The tract was designated as the middle tract (4). Control hearts also showed a tract-like structure of immunoreactive cells, but this structure was not completely connected to the pAVN (Fig. 2b). The middle tract in the malformed hearts was located caudally compared with similar immunoreactive sites in the normal hearts (Fig. 2b, d).

From the LSAN, a loose immunoreactive region extended to the dorsal mesocardium and to the tissue around the orifice of the common pulmonary vein in both normal and malformed hearts (Fig. 2a, b, c). These immunoreactive sites, termed the left internodal tract (L1), were connected to the pAVN (Fig. 2a, d, 3a). This tract in the treated hearts extended caudally along the dorsal wall of left sinus horn (Fig. 2d, 3c).

Fig. 1 a, b. A 14.5-day normal heart, c, d, e. A 14.5-day bis-diamine treated heart, horizontal section. a The right superior vena cava (RSVC) and septum spurium (SS) strongly express HNK-1 antigen. ×50. b A more caudal section than Fig. 1a. The anterior atrioventricular node (aav) is seen at the dorsal edge of the superior endocardial cushion (asterisk). Note the well developed atrioventricular bundle of His (h) and right (rbb) and left bundle branches (lbb). ×50. c Note the persistent truncus arteriosus and hypoplastic pulmonary artery. ×60. d Same level as Fig. 1b. The right superior vena cava (RSVC) does not yet enter the right atrium. Note that the structure of the septum spurium (SS) is simpler than that of normal hearts, as shown in Fig. 1a. ×50. e A more caudal section than Fig. 1d. The HNK-1 immunoreactivity is scattered along the lateral wall of the right atrium (arrowhead). ×50.
**Fig. 2 a, b.** A 14.5-day normal heart, c, d. A 14.5-day bis-diamine treated heart, horizontal section. 

**a** Weakly immunopositive areas (small arrowhead) are seen around the orifice of the common pulmonary vein (large arrowhead). X100. 

**b** The middle tract (4) is not connected with the left sinoatrial node (lsa). X100. 

**c** The left sinoatrial node (lsa) and the posterior atrioventricular node (pav) are relatively small. Note that the weakly immunoreactive sites at the caudal edge of the endocardial cushion extend to the pAVN (arrowhead). X100. 

**d** Specimen number 1. Various caudally deviated components of HNK-1 positive sites are seen along the sinus venosus. The posterior tract (4) is connected with the left internodal tract (L1) via Bachmann’s bundle (B) and the posterior atrioventricular node (pav). X100.

**Atrioventricular bundle (HIS) and bundle branches**

An atrioventricular bundle (HIS) consisted of a craniocaudal penetrating pathway (pHIS), which showed as strongly immunopositive areas at the ventral edge of the inferior endocardial cushion, and a dorsocaudal extending pathway (eHIS), which showed as weakly positive areas through the right caudal portion of the inferior endocardial cushion (1b, d, 3b, d). pHIS immunoreactive areas extended to the bilateral trabecular muscles, corresponding to the right (RBB) and left bundle branches (LBB) (Fig. 1b, d). The HIS was widely separated from the aAVN and had no connection. The anomalous hearts showed poor development of the ventricular septum and bilateral trabecular muscles, and the pHIS and bilateral bundle branches were hypoplastic (Fig. 1d, e). In particular, the RBB was almost invisible. The eHIS of control hearts was connected to the posterior tract at the left edge of the sinus septum (Fig. 3b), while that of the treated hearts was poorly developed and connected to the caudally deviated pAVN (Fig. 2c). The HIS, LBB and RBB in the malformed hearts were at nearly the same position as in the control hearts (Fig 1b, e).

The characteristics of the malformed hearts are summarized in Table 1 and are illustrated in schematic drawings (Fig. 4).

**Variations of HNK-1 immunoreactivity in the malformed heart**

Among the 8 specimens of malformed hearts, variations of HNK-1 immunoreactive sites were observed in 3 specimens (numbers 1, 3 and 4). The common route of the anterior and antero-lateral tracts faded out and was not connected with the RSAN in 2 specimens of 3 and 4 (not shown). In specimens 1 and 3, the middle tract was connected with the left internodal tract at the dorsal portion of the pAVN. This interatrial, bridging tract was similar to “Bachmann’s bundle” and connected the RSAN with the LSAN (Fig. 2d). Moreover, in specimens 1 and 4, the posterior tract descending through the right venous valve extended caudally and was connected to the eHIS in the inferior endocardial cushion before it entered the sinus septum (Fig. 3d).
Fig. 3 a, b. A 14.5-day normal heart, c, d. A 14.5-day bis-diamine treated heart, horizontal section. a The posterior tract (3, arrowhead) courses transversely through the sinus septum (S) into the posterior atrioventricular node (pav). ×150. b The extending pathway of HIS (eh) is shown as weakly positive areas around the caudal portion of the inferior endocardial cushion (asterisk). Note that the sinus venosus region shows little expression of the HNK-1 antigen, except for the developing sinus septum. ×100. c The sinus septum (S), which is located at the level of the inferior vena cava, is shorter than that of normal hearts. ×150. d Specimen number 4. The posterior tract (3) appears to bypass the posterior atrioventricular node (pav). ×150.

Fig. 4. Schematic representation of a 14.5-day embryonic heart. a normal heart. b bis-diamine induced malformed heart.
Table 1. HNK-1 expression pattern in bis-diamine induced malformed heart.

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-, not deviated; +, caudally deviated; ↓, poorly developed; →, normally developed; n, not evaluated; SAN, sinoatrial node; INT, internodal tract; AVN, atrioventricular node; BBB, bilateral bundle branches

Discussion

In human embryos, the region including SAN, the three internodal tracts (INTs) and AVN are derived from the primitive sinus venosus11,12. Recently, Ito et al10 demonstrated that bis-diamine-induced conotruncal anomalies were associated with insufficient absorption of the sinus venosus, and emphasized that abnormal distribution of HNK-1 immunoreactivity might reflect abnormal morphological development of the heart. In a more recent study, DeRuiter et al14 used the HNK-1 antibody as the marker for the sinus venosus in avian embryos. In the present study, the HNK-1 immunoreactive sites corresponded to the middle, posterior and left internodal tracts, and the pAVN extended caudally to be distributed at the sinus venosus region, suggesting that these components of the rat cardiac conduction system were derived from the primary sinus venosus. On the other hand, we considered that the aAVN, HIS, and bilateral bundle branches did not originate from the sinus venosus, since their positions were nearly normal. Furthermore, hypoplasia of the HIS and bundle branches due to poor development of the ventricular septum and trabecular muscles seemed to be caused by a decrease of venous return into both ventricles.

Since the anterior and antero-lateral tracts pass through developing pectinate muscles and the septum spurius, both tracts may be influenced by their development. It appears that scattered immunoreactive sites along the lateral wall of the right atrium in treated hearts are due to be distributed at the developing pectinate muscles. These immunoreactive tissues in the right atrium may become ectopic focus, causing atrial arrhythmias, such as paroxysmal atrial tachycardia. Spatially, the anterior and antero-lateral tract are distant from the sinus venosus and tightly linked to the aAVN. Therefore, they are probably not derived from the primitive sinus venosus. The two tracts differ from any internodal tracts in the human heart described by James12,13.

The posterior tract corresponds to James' posterior tract, as pointed out previously10. The variation that appeared in specimen numbers 1 and 4, in which the posterior tract connected to the dorsocaudal penetrating pathway of HIS and bypassed the pAVN, appears to be equivalent to "James fiber"12.

To date, the middle tract descending along the dorsal wall of the right sinus horn in malformed hearts has not been described. The middle tract may correspond to James' anterior tract, and in support of this view, it branched and formed the Bachmann's bundle10-like interatrial tract in a few malformed hearts. The discontinuity of immunoreactive sites, such as the middle tract, in normal rat hearts may be caused by transient expression of HNK-1 antigen, visual sensitivity of immunoreactive signals, or anatomical cellular heterogeneity of internodal pathways10.

The present study revealed that abnormalities in the conduction system demonstrated by HNK-1 immunohistochemistry were mainly due to insufficient absorption of the sinus venosus rather than abnormal morphogenesis of the conotruncus. Furthermore, a James fiber-like tract bypassing the pAVN was found in a few specimens. However, it is still unclear whether these abnormal distributions of HNK-1 in embryonic hearts will cause cardiac arrhythmias in the infant and adult, since electrophysiological analysis was not performed in this study.

HNK-1 expression is transient and delicate. It is first observed at the early embryonic stage and fades from the late fetal to the neonatal stage in the developing heart19,20. There were some variations of HNK-1 immunoreactive sites and a newly formed positive route, termed the middle tract, in this study. HNK-1 immunoreactive sites were closely related to the development of internal cardiac structures, such as the pectinate muscles, the septum spurius, the right venous valve, the sinus septum, the interventricular septum, and the trabecular muscles. Moreover, in malformed hearts, these structures with HNK-1 expression altered according to abnormalities of cardiac morphological development caused by incomplete absorption of the sinus venosus. These results suggest that HNK-1 immunohistochemistry may be useful as a marker of heart development, including congenital malformations. Therefore, the experimental heart model induced by bis-diamine administration is invaluable for the study of abnormal development in the heart conduction system, as
was demonstrated by HNK-1 immunohistochemistry, as well as the morphogenesis of congenital heart disease.

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References

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