Differentiation and innervation of the atrioventricular bundle and ventricular Purkinje system in sheep heart

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Summary

The development of the atrioventricular bundle (AVB) and ventricular Purkinje system and their innervation have been studied in fetal sheep from 27 to 140 days gestation (term is 147 days). The AVB initially consisted of a primordium, which lacked innervation and was composed of small, relatively undifferentiated myocytes. Differentiation of Purkinje-like cells within the AVB began near its distal end and extended towards the atrioventricular node (AVN). Differentiation of the ventricular Purkinje system extended distally from the region of bifurcation of the AVB from cells that were indistinguishable from the working myocardium and continuous with the AVB primordium. Differentiation of Purkinje-like AVB cells was complete by 46 days gestation but Purkinje fibres were still differentiating within the ventricular wall at 60 days gestation. The main morphological changes included a large increase in cell size and organization into strands, development of characteristic glycogen-filled regions containing many intermediate filaments and early development of myofibrillar M lines compared to the working myocardium.

The differentiation of AVB cells and the ventricular Purkinje system preceded their innervation. The AVB became innervated earlier than ventricular Purkinje fibres, intimate contacts between proximal AVB cells and nerve axons being present at 60 days gestation. Nerve fibres were present in the septomarginal band at this time, however, en passant associations with ventricular Purkinje fibres were rarely observed until 140 days gestation and intimate contacts were not present at any stage.

Although the AVB and ventricular Purkinje system of adult sheep are composed of morphologically similar cells, the present study demonstrates that they differ in origin and their mode of differentiation as well as timing and intimacy of innervation. Innervation is not part of the developmental mechanism leading to the differentiation of Purkinje fibres. No primordium of the ventricular Purkinje system could be identified, suggesting that the mechanism of differentiation of ventricular Purkinje fibres involves recruitment from early working myocardium.

Key words: atrioventricular conduction system, Purkinje fibres, heart, sheep, innervation.

Introduction

In spite of agreement on a number of important features in the development of the cardiac conduction system, controversy persists in several areas. It is now established that the atrioventricular conduction system develops \textit{in situ} and not as a result of cell migration or mitotic division from a growth centre (Anderson & Taylor, 1972; Virágh & Challice, 1982). However, some studies have demonstrated a gradual development of the atrioventricular conduction system, beginning at the AVB and with time extending distally (Muir, 1954; Virágh & Challice, 1982), while others have suggested that all parts of the atrioventricular conduction system differentiate simultaneously (Vassall-Adams, 1982a,b).

As well, Purkinje cells are considered to be specialized myocardial cells whose ontogenetic differentiation is distinct from that of the working myocardium (Anderson, Becker, Wenink & Janse, 1976; Bogusch, 1979; Forsgren & Thornell, 1981; Forsgren, Strehler & Thornell, 1983; Forsgren, 1985). However, it is unclear whether the Purkinje system develops from a specialized precursor tissue within
the ventricles (Vassall-Adams, 1982a,b; Anderson et al. 1976; Wenink, 1976), or by differentiation from a common ventricular myocardium (Viragh & Challice, 1977a,b, 1982; Bogusch, 1979; Forsgren & Thornell, 1981).

We have addressed these controversies in an ultrastructural study of fetal sheep heart, a species in which AVB cells are morphologically similar to Purkinje cells and both are clearly distinguishable from the working myocardium (Truex & Smythe, 1965). The aims of this study were to establish the course of early differentiation of Purkinje cells of the AVB and ventricles and to ascertain whether differentiation of Purkinje cells occurs simultaneously in all parts of the ventricles or extends gradually from the AVB. In addition, we have compared the development of innervation of the AVB and ventricular Purkinje system. This aspect has received surprisingly little attention, considering that the conduction system of the adult sheep heart is densely innervated (Canale, Fujiwara & Campbell, 1983).

Materials and methods

Hearts were obtained from fetuses of Border-Leicester cross ewes at the gestational ages (term = 147 days) shown in Table 1.

The pregnant ewes were anaesthetized with intravenous sodium thiopentone (30 mg kg\(^{-1}\)) followed by intravenous \(\alpha\)-chloralose (30 mg kg\(^{-1}\)) as required and the uterus exposed through a midline abdominal incision. The hearts of fetuses aged less than 60 days were removed in toto and immersed in fixative. The 60-day fetuses were fixed by perfusion through an umbilical artery. In fetuses aged 75 days or more, the hearts were fixed by retrograde aortic perfusion using a technique described previously (Smolich et al. 1984). In brief, a cannula was passed within a carotid artery so that its tip lay just above the aortic valve. The cannula was connected through a Y-piece to a blood pressure recorder and a gravity-fed perfusion apparatus. The heart was arrested in diastole with 1 M-KCl (variable volume according to heart size) and cleared of blood with a washout solution consisting of compound sodium lactate (Hartmann’s solution) with added sodium heparin (50–100 i.u. ml\(^{-1}\)), potassium chloride (50 mequiv l\(^{-1}\)) and sodium bicarbonate (to pH 7.3–7.4). Fixation was then carried out at the animal’s previously measured mean arterial pressure (range, 30–80 mmHg) for 10–15 min. For both the immersion and perfusion procedures, the fixative consisted of 2 % paraformaldehyde and 2 % glutaraldehyde in 0.1 M-sodium cacodylate or 0.1 M-phosphate buffer.

The hearts of the 27- and 33-day fetuses were processed and embedded whole. Hearts from fetuses of 35, 37, 40 and 46 days gestation were cut into three pieces; the atria, the right ventricular free wall and the intact septum plus left ventricular free wall. In fetuses of 60 days gestation and older, tissue was taken from the AVB, left and right bundle branches (L&RBB), septomarginal band, false tendons of both ventricles, as well as apical subendocardium, papillary muscle and free wall of the left ventricle. Tissue blocks were postfixed in 1 % OsO\(_4\), dehydrated in acetone and embedded in Epon/Araldite. Light microscopic thick sections were cut on glass knives and stained with 1 % methylene blue. Silver–gold thin sections were cut on a diamond knife, stained with uranyl acetate and Reynold’s lead citrate and then examined in a Siemens Elmiskop 102 or Philips 400T transmission electron microscope.

Results

Atrioventricular bundle

27 days gestation

An AVB primordium was easily identified by light microscopy as an elongated structure, located along the top of the posterior part of the interventricular septum. The lower edge of the posterior part of the AVB was in contact with septal myocardium, while the upper AVB widened and became continuous with cells of the atrioventricular node which formed an overlay on that part of the AVB. The midportion and distal parts of the AVB primordium were circular in cross section and poorly delineated, the constituent cells being continuous with the surrounding septal working myocardium.

In the proximal part of the AVB, primordial cells were small and interconnected by thin cytoplasmic extensions, giving the impression of a meshwork (Fig. 1). In the distal portion, the cells were larger and rounded, with broad areas of sarcoclemmal apposition (Fig. 2). Primordial AVB cells were clearly distinguishable from surrounding working myocardial cells. Both contained abundant glycogen and few myofibrils but glycogen was more prominent and the myofibrils thinner in AVB cells. Also, other organelles such as the Golgi apparatus and mitochondria were more obvious in working myocardial cells (Fig. 3).

33–37 days gestation

Cells in the proximal AVB were unchanged in appearance. However, cells in the distal part of the AVB, in addition to having a similar morphology to

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<th>Days in utero</th>
<th>27</th>
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Table 1. Origins of experimental hearts
cells in the distal AVB at 27 days gestation, were organized into strands (Fig. 4), characteristic of Purkinje cells.

40 days gestation
A larger proportion of the AVB consisted of Purkinje-like cells. Primordial cells were still evident in the midportion of the AVB, confined to a central core, surrounded by larger Purkinje-like cells (Fig. 5). The AVB primordial cells were larger than at earlier ages but exhibited similar morphology. Mitosis was observed only rarely in primordial cells (Fig. 5).

46 days gestation
The entire AVB was composed of cells with Purkinje-like morphology. However, the network arrangement characteristic of primordial cells persisted near the junction of the AVB with the AVN. More distally the AVB cells were arranged into separate strands, aligned with the long axis of the AVB. Gap junctions were observed for the first time but were rare.

60–140 days gestation
For the remainder of gestation, AVB cells underwent morphological changes similar to those in the ventricular Purkinje system (described below). Subsequent morphological development involved gradual increases in cell size, number of myofibrils, intermediate filaments and gap junctions. However, at any one time, AVB cells were smaller than ventricular Purkinje cells and had fewer myofibrils. As well, myofibrillar M lines were absent until 90 days gestation, when they were occasionally observed, and there appeared to be fewer intermediate filaments in AVB cells compared to the ventricular Purkinje cells.

Ventricular Purkinje system
27 days gestation
The left bundle branch (LBB) was evident as short branches arising from the distal AVB, but a right bundle branch (RBB) was not observed. The Purkinje cells of the LBB were mainly composed of glycogen, with the few mitochondria and myofibrils present mostly confined to the cell periphery (Fig. 6).
The myofibrils were thin and had poorly defined I and A bands. Fasciae adherentes junctions between cells were common, desmosomes infrequent and gap junctions were not observed.

33–37 days gestation
The RBB was first noted at 33 days. At this time, Purkinje cells of the L&RBB formed strands over the sides of the septum and blended with the septal myocardium about halfway down the septum. In 35- and 37-day fetuses, a few bundles of early developing Purkinje cells were observed in some left ventricular trabeculae but not within the ventricular walls.

Purkinje cells showed a spectrum of morphologies (Fig. 7A,B). The most-differentiated cells occurred at the junction of the AVB and bundle branches, and the least-differentiated in the distal ends of the septal subendocardial strands. However, all Purkinje cells were arranged into bundles and typically had a rounded cell shape and nucleus, as well as widespread intimate apposition of membranes between cells. Compared with working myocardium (Fig. 7D), myofibrils in Purkinje cells were small, infrequent and appeared randomly orientated. Gap junctions were absent at 33 days and rare at 35 and 37 days (Fig. 8).

Cells with a transitional morphology between Purkinje and working myocardium were observed in the septal subendocardium (Fig. 7C). These cells were smaller and more-irregularly shaped than Purkinje cells and sometimes occurred in large groups of approximately 15–20 cells.

40 days gestation
Purkinje fibres had extended into the septomarginal band and subendocardium of the apex, but not into the ventricular walls. Transitional cells were also observed in the subendocardium of the apex and were similar in appearance to those in the septal subendocardium at 35 and 37 days gestation.

46 days gestation
The morphology of Purkinje cells was unchanged although intermediate filaments were occasionally observed in association with myofibrils and glycogen. Transitional cells were commonly seen in continuity with working myocardium in the apex and papillary muscles.
60 days gestation

Intramural Purkinje fibres were first observed at this age and were smaller and more elongated than subendocardial fibres.

All Purkinje fibres were characterized by heterogeneity in the degree of organization and development of myofibrils. In many myofibrils, I and A bands were, as in younger fetuses, poorly defined. Other myofibrils, present in the same cells, were thicker and possessed well-defined Z, I and A bands (Fig. 9).

Leptofibrils, absent in younger fetuses, were small and rare, occurring both in isolation and in association with the sarcolemma or myofibrils (Fig. 9). Gap junctions were more common than at earlier ages. Most were less than 0.8 μm in length, although the range extended from 0.26 to 1.44 μm. Intermediate filaments were distributed more widely but were still sparse.

A few Purkinje cells of the septomarginal band possessed groups of randomly arranged myosin filaments associated with many ribosomes, typical of ‘myofilament–polyribosome complexes’ (Thornell, 1972; Fig. 10).

75 days gestation

Most myofibrils within Purkinje cells possessed distinct A, I and Z bands. Occasional myofibrils in Purkinje cells also displayed M lines (Fig. 10), which were absent from myofibrils of the working myocardium. There appeared to be an increase in the number of intermediate-sized filaments in association with desmosomes, myofibrils, ribosomes, sarcoplasmic reticulum and sarcolemma.

90 days gestation

Myofibrils were wider and M lines more frequent than at 75 days. As well, myofilament–polyribosome complexes and intermediate-sized filaments were more numerous. A few sarcolemmal outfoldings containing multivesicular bodies (Page, Power, Fozzard & Meddoff, 1969; Rybicka, 1978) were also observed.

99–115 days gestation

The main change during this period was the development of a fibrous connective tissue sheath around Purkinje fibres of the septomarginal band, false tendons and septal subendocardium. By 115 days

Fig. 5. 40 days gestation. A transverse section through the AVB showing relatively more differentiated AVB Purkinje-like cells (P), surrounding a central core of AVB primordial cells. Note the AVB primordial cell in an early stage of mitosis, (mi). Bar, 2 μm.

Fig. 6. 27 days gestation. Purkinje cells of the LBB. Note that the cells are mainly filled with glycogen (g) and contain very few other organelles. Myofibrils (m). Bar, 2 μm.
Fig. 7. The morphological spectrum of ventricular myocardial cells in developing sheep heart at 33 days gestation. (A) Large, glycogen-laden Purkinje cells of the LBB. (B) Smaller Purkinje cells in the subendocardium of the interventricular septum. (C) Transitional Purkinje cells in continuity with working myocardium. Note the more prominent myofibrils and smaller size of these cells compared to Purkinje cells in A and B. (D) Typical working myocardial cells. Note the elongate cell shape and oval nucleus. Myofibrils are prominent. m, myofibrils; d, intercalated disc. Bar (A–D), 3μm.
Fig. 8. A gap junction between Purkinje cells at 35 days gestation (between arrows). Note the fasciae adherentes (f) junctions. Bar, 0.3 μm.

Fig. 9. 60 days gestation. Small and immature leptofibrils (f), in Purkinje cells. Note the presence of myofibrils with ill-defined sarcomeric banding (arrow) as well as more-mature-looking myofibrils (m). Bar, 0.5 μm.

gestation, Purkinje fibres in these locations were ensheathed by wavy bundles of collagen and smaller amounts of elastin. However, Purkinje fibres in the apex and within the ventricular walls possessed little collagenous connective tissue in their sheaths.

By 115 days gestation, intermediate-sized filaments, sarcoplasmic reticulum and mitochondria were found in the glycogen-filled regions of most Purkinje cells.

140 days gestation
The Purkinje fibre connective tissue sheath was well organized and denser than in younger fetuses.

Most myofibrils in Purkinje fibres possessed M lines and were more or less aligned with the long axis of the cell. Occasionally myofibrils were arranged haphazardly or displayed branching sarcomeres and wide, irregular Z bands. The density of intermediate-sized filaments in the glycogen-filled regions increased but remained variable. Sarcolemmal outfoldings containing multivesicular bodies (Fig. 13) were variable in number, sometimes being numerous on one cell but uncommon on another.

Innervation
The three kinds of neuromuscular associations observed in the adult conduction system (Canale, Campbell, Smolich & Campbell, 1986) were also seen during development. These comprised:

(1) intimate contacts, in which the neuromuscular separation was less than 20 nm and lacked an intervening basal lamina;

(2) close associations, in which the neuromuscular separation was less than 100 nm and the intervening space was entirely filled with basal lamina material;

(3) en passant associations, in which the neuromuscular separation was more than 100 nm and the intervening space may have included collagenous connective tissue and fibroblasts.

27–40 days gestation
Nerve fibres were absent from the ventricles and developing AVB.

46 days gestation
Nerve fibres were observed near the proximal part of the AVB but these did not contain any varicosities or form associations with AVB cells.
60 days gestation

Many nerve axons were present in the proximal AVB, separated from AVB cell surfaces by a distance of 0.3 to 0.7 μm. However, vesicle-filled varicosities were rare and, when present, contained a few small clear vesicles. Intimate contacts between nerve axons and AVB cells were few and did not involve varicosities. The distal AVB was less densely innervated and intimate contacts were absent.

Nerve bundles containing over 100 axons were observed in the septomarginal band. Smaller bundles containing less than 50 axons were a few microns from Purkinje fibres in the septomarginal band but no direct innervation of the Purkinje cells was observed.

75 days gestation

The pattern of innervation of the AVB was similar to that at 60 days gestation. Axons forming intimate contacts with proximal AVB cells occasionally contained dense-cored vesicles (Fig. 11). In addition to intimate contacts, a few axons were also observed in close association with AVB cells.

In the septomarginal band and LBB, nerve bundles were present but these did not form any associations with Purkinje cells. Nerve fibres were absent from false tendons, the apex and subendocardium of the left ventricular wall.

90 days gestation

Axon varicosities in the AVB were more common and contained more vesicles than in younger fetuses. Nerve fibres were common in the septomarginal band but infrequent in false tendons. Although nerve fibres occurred in close proximity to ventricular Purkinje cells (Fig. 12), no vesicle-containing varicosities, close associations or intimate contacts were observed. Very few axon bundles were evident near Purkinje fibres in the apex.

140 days gestation

All parts of the AVB were densely innervated and en passant associations were the most common type, occurring on the outer surface of AVB cell bundles and in tunnels running between coupled cells. There were few intimate contacts and close associations and, compared with younger fetuses, these frequently included vesicle-filled varicosities. Varicosities containing small dense-cored vesicles were only occasionally observed.

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Fig. 10. 75 days gestation. Myofibrillar M lines (arrows) and myofilament–polyribosome complexes (•) in a Purkinje cell. Bar, 0.5 μm.

Fig. 11. 75 days gestation. A Schwann cell (S), surrounds an axon (a) containing a few dense-cored vesicles. The axon, Schwann cell and AVB cells are in close contact. Bar, 1 μm.
Many axon bundles, some with vesicle-containing varicosities (Fig. 13), were observed forming en passant associations with ventricular Purkinje fibres in the L&RBB, septomarginal band, false tendons and apical subendocardium. Only small clear vesicles were present in the varicosities. However, no intimate contacts or close associations were observed. Axons bearing vesicle-containing varicosities associated with Purkinje fibres were less common in the apex and false tendons than in the septomarginal band and L&RBB.

Discussion

Our results in the developing sheep heart demonstrate that the AVB developed from a primordium which is morphologically distinct from the working myocardium and that differentiation of Purkinje-like morphology in AVB cells was a gradual process which extended along a distal-to-proximal axis. The latter has not been reported previously and may be a species-related difference. One known difference is that, unlike mice (Viragh & Challice, 1977a,b, 1982), ferrets (Marino, Truex & Marino, 1979; Marino & Severdia, 1983), rats and humans (Truex & Smythe, 1965), the AVB cells of the sheep heart are very similar in morphology to the ventricular Purkinje fibres, even in young fetuses.

In contrast to the AVB, the ventricular Purkinje fibres did not have a morphologically distinguishable primordium. Their development was associated with the appearance of transitional cells which were present at the terminations of the fibres as well as adjacent to them in the LBB. There is agreement that, in some species (Truex, Marino & Marino, 1978; Viragh & Challice, 1982; Marino & Severdia, 1983) including the sheep (Muir, 1954), the AVB primordium becomes distinguishable before ventricular Purkinje fibres differentiate. In spite of the earlier appearance of the AVB primordium in sheep, Purkinje fibres differentiated in the LBB at 27 days gestation, prior to the development of Purkinje-like morphology in the AVB primordium.

The factors involved in the differentiation of Purkinje-like morphology in the AVB and ventricles are unknown. Some have suggested the existence of a Purkinje precursor tissue (Wenink, 1976; Vassall-Adams, 1982a,b), but there was no evidence of a precursor tissue in sheep heart in this study. In agreement with Muir (1954), the distal ends of the
developing Purkinje fibres in sheep were in continuity with working myocardium at all stages of development. This suggests that development of the ventricular Purkinje fibres involves recruitment from early working myocardium, leading to a process of differentiation which is distinct from that of the working myocardium. The development of myofibrillar M lines in myofibrils of Purkinje cells during fetal life prior to their appearance in working myocardium in the sheep, in agreement with similar reports in humans and cattle (Forsgren & Thornell, 1981; Forsgren, Carlsson, Strehler & Thornell, 1982a; Forsgren, Strehler & Thornell, 1982b), suggests Purkinje fibres develop along a line of differentiation distinct from that of working myocardium.

Our results indicate that innervation is unlikely to be important in the process of differentiation or recruitment from the early working myocardium. First, in both the AVB and the Purkinje system, innervation was established after differentiation of a Purkinje-like morphology. Second, nerve fibres first appeared in the proximal AVB, whereas Purkinje-like morphology initially developed in the distal AVB.

The AVB and ventricular Purkinje system differed in timing and intimacy of associations during the development of innervation. Intimate contacts between nerve fibres and proximal AVB cells were established at 60 days gestation. In contrast, no intimate contacts or close associations were observed in the ventricular Purkinje system during the period studied, although en passant associations were established near term and close associations have previously been reported in the adult (Canale et al. 1983).

In summary, although AVB and ventricular Purkinje cells are morphologically similar in the adult sheep, they have different origins and directions of differentiation, temporal differences in innervation and different types of neural associations during development. Differentiation of the Purkinje fibres is not related to innervation.

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References


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