Differentiation of Heart Purkinje Fibres
An immuno- and enzyme histochemical and ultrastructural study

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DIFFERENTIATION OF HEART PURKINJE FIBRES
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The development of the Purkinje fibres, the cells comprising the peripheral part of the cardiac conduction system, has almost exclusively been studied by means of conventional histologic techniques. The descriptions of the identification and mode of differentiation of the cells during development are conflicting. In the present investigation bovine and human Purkinje fibres during development were studied histologically and ultrastructurally and by means of enzyme and immuno-histochemistry.

In both bovine and human fetal hearts the Purkinje fibres were distinguished from the main myocardial mass due to a strong immunofluorescence reaction, in sections incubated with antibodies against the intermediate filament subunit skeletin. Thus, a high content of skeletin, as demonstrated by immunohistochemistry, seems to be a general criterion to identify fetal Purkinje fibres. Furthermore, the Purkinje fibres were identified by their gross morphological features from an early fetal stage of the bovine hearts. In the human fetal hearts the Purkinje fibres were also identified by a high activity of cholinesterase.

By comparison with immunohistochemical observations human fetal Purkinje fibres at midgestation could be identified at the ultrastructural level: The Purkinje fibres but not the ordinary ventricular myocytes were stained in sections incubated with antibodies against MM-creatine kinase. Ultrastructurally subendocardial cells in the ventricles showed dense M-bands. These cells represent Purkinje fibres, as presence of an electron dense M-band correlates with detectability of MM-creatine kinase. In the Purkinje fibres in the bovine fetal hearts, electron dense M-bands were observed and MM-creatine kinase was detected at an earlier stage than in ordinary ventricular myocytes.

During fetal development in the bovine hearts differences appeared between Purkinje fibres and ordinary ventricular myocytes with respect to cell volumes, intercalated disks, myofibrils, mitochondria, amount of glycogen and T-tubules. In all fetal stages of bovine hearts and in the human fetal hearts at midgestation the Purkinje fibres contained a greater amount of intermediate filaments.

The histochemical pattern of Purkinje fibres vs ordinary ventricular myocytes, with respect to enzymes reflecting oxidative and glycolytic activities, changed during fetal development of the bovine hearts. This could be related to the changing enzyme activities in the ordinary myocytes. The staining reactions of these enzymes in the Purkinje fibres seemed to remain unchanged during fetal development. Noticeably the Purkinje fibres showed higher enzyme activities than the ordinary myocytes at early fetal stages, indicating a higher energy demand in the former cells.

The observations show that Purkinje fibres clearly can be identified in fetal hearts and that the use of enzyme and immuno-histochemistry has facilitated this identification. The Purkinje fibres differentiate along a different pathway from ordinary ventricular myocytes. Furthermore, it appears as if the Purkinje fibres have a specialized function already at early fetal stages.

Key words: Heart conduction system, Purkinje fibres, ordinary myocytes, differentiation, skeletin, M-band, enzyme histochemistry, immunohistochemistry, electron microscopy.
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by

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This thesis is based on the following publications and manuscripts, which will be referred to by their Roman numerals:

I. Forsgren, S., Thornell, L-E. and Eriksson, A.  
The development of the Purkinje fibre system in the bovine fetal heart.  

II. Forsgren, S. and Thornell, L-E.  
The development of Purkinje fibres and ordinary myocytes in the bovine fetal heart. An ultrastructural study.  

III. Forsgren, S., Strehler, E. and Thornell, L-E.  
Differentiation of Purkinje fibres and ordinary ventricular and atrial myocytes in the bovine heart - An immunohistochemical study.  
*Histochemical Journal* (in press)

IV. Forsgren, S., Eriksson, A., Kjörell, U. and Thornell, L-E.  
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INTRODUCTION

Aspects on the structure and function of adult Purkinje fibres

In the heart there is a system, the conduction system, which generates the stimulus for the heart beat and conducts the impulse to the different parts of the myocardium. Thereby the proper succession of contractions of the atria and ventricles is ensured. The system consists of the sinoatrial node (SA node), the atrioventricular node (AV node), the atrioventricular bundle (AV bundle, bundle of His), and its branches (the right and left bundle branches). The peripheral part of the bundle branches is called the Purkinje fibre system. The cells comprising this system, the Purkinje fibres, were first discovered in the sheep heart in 1845 by J E Purkinje after whom the cells were termed.

Histologically the Purkinje fibres show wide inter- and intra-species variations. However, in large mammals, e.g. ungulates, the cells are morphologically distinctive: They have about 3-4 times larger diameters than ordinary ventricular myocytes, a vacuolated appearance, sparse myofibrils, and multiple nuclei (1, 17, 50, 75). These cells are often referred to as "typical" Purkinje fibres. Individual cells or bundles of cells are surrounded by a connective tissue sheath which delimits them from the ordinary myocytes. In man and in small mammals, e.g. rat and cat, the cells in the Purkinje fibre system are smaller and do not always have a continuous connective tissue sheath. In these species it is in fact difficult to distinguish these cells from the surrounding ordinary myocytes by means of conventional light microscopy (17, 50, 76). The species variations, with respect to the morphology of the Purkinje fibres, have lead to confusion in the literature regarding the terminology of the cells. Some authors have restricted the term Purkinje fibre to "typical" Purkinje fibres, while others have included the cells in the peripheral ramification of the bundle branches in all higher vertebrates. Some authors have preferred to classify the Purkinje fibres into three main types, mainly based on the diameters of the cells and the extent to which the cells differ from the ordinary myocytes (50, 76). According to Viragh and Challice (83) the morphology of the Purkinje fibres changes at special regions along the distribution of the bundle branches and these authors have classified the cells into Purkinje I, II, and III cells, respectively.

By use of histological stains for glycogen, and enzyme histochemical and immunohistochemical techniques, the Purkinje fibres can be distinguished from the ordinary myocytes. However also with these techniques some interspecies variations are observable. The Purkinje fibres are distinguished from the ordinary myocytes by a higher amount of glycogen, a higher activity of glycolytic and gluconeogenic enzymes and a lower activity of respiratory enzymes (46, 60). These observations, as well as the lower uptake of oxygen and the lower density of capillaries in the Purkinje fibre system than in the ordinary myocardium (18, 53) favour that the Purkinje fibres have a higher rate of anaerobic and a lower rate of aerobic metabolism than the ordinary myocytes. Furthermore, the Purkinje fibre system is distinguished from ordinary myocardium by a high activity of cholinesterase (20, 37). It is, however, not certain to what extent this reflects the rich nervous component of the conduction system or a high activity of the enzyme within the Purkinje fibres (17, 78). Recently, the Purkinje fibres have also been shown to differ from ordinary myocytes by a higher content of the intermediate filament protein skeletin, as seen with immunofluorescence techniques (23).
By electron microscopy clear differences have been observed between Purkinje fibres and ordinary myocytes: The Purkinje fibres have both end-to-end and side-to-side connections (the ordinary myocytes are principally linked end-to-end) including more frequent nexuses, absence of T-tubules, fewer and smaller mitochondria, presence of mitochondria with a doughnut appearance and a larger amount of glycogen granules as compared with the ordinary myocytes (6, 9, 38, 43, 53, 61, 69). In particular, the difference with respect to T-tubules has been stressed as a clear distinguishing feature (61, 63). Furthermore, intermediate filaments are more abundant and the myofibrils are less well organized in the Purkinje fibres (68, 71). Abnormalities in the sarcomeres and myofilament-polyribosome complexes are typical for the Purkinje fibres (51, 67, 68). The differences with respect to mitochondria and glycogen may reflect the proposed difference in cell metabolism (see above). The dissimilarity in amount of intermediate filaments and content of skeleton, as seen immunohistochemically, suggests that Purkinje fibres may need a different type of cytoskeleton from ordinary myocytes. The abnormalities in the sarcomeres of Purkinje fibres are probably due to an imbalance in the synthesis and degradation of myofibrillar proteins (68).

Electrophysiologically, it has been shown that conduction is rapid throughout the bundle branches and the peripheral Purkinje fibres at a rate of 1.5-5.0 m/sec (17, 32). In contrast, the velocity in ordinary ventricular myocardium is 0.3-1.0 m/sec. Numerous attempts have been made to explain these physiological differences. The large diameters of the Purkinje fibres and their aggregation into tightly packed bundles favour a greater conduction velocity in these cells than in ordinary myocytes (63). The nexuses, which are frequent between the Purkinje fibres, probably represent low-resistance pathways (8). The Purkinje fibres are also influenced by both sympathetic and parasympathetic branches of the autonomic nervous system. A direct cellular basis for cholinergic antagonism of the electrophysiological effects of catecholamines on the Purkinje fibres is documented (7). Besides rapid conduction, the Purkinje fibres also have the property of spontaneous depolarization (18). Contractions arising wholly within the ventricles, as in complete AV block, originate in Purkinje fibres.

It is thus clear that in the adult heart the Purkinje fibres differ histologically, ultrastructurally, histochemically, immunohistochemically and electrophysiologically from the ordinary myocytes.

The development of the Purkinje fibres

The origin and development of the different parts of the conduction system has for a long time been the source of controversy. However, it is clear that a conduction system can not be identified morphologically at the stage when cardiac contractions start. Contractions occur soon after the formation of the single heart tube (57) or even earlier, before the two endocardial tubes have begun to fuse (27). The pacemaker region in the tubular heart has been proposed to shift during development, initially being located in the ventricle (55). Others have argued that the impulse from the beginning is generated in the prospective sinoatrial region (45, 80).

With respect to the origin of the conduction system components one theory has been that a new supraventricular growth area in the embryonic heart develops, from which the AV bundle and the bundle branches develop (59, 86). According to this theory the end of the AV bundle is an actively growing tissue. Other authors have contended that the AV bundle (42) and the Purkinje fibres (3, 34, 84) arise in situ in the AV canal and in the ventri-
cles, respectively. Wenink, Anderson and colleagues (4, 87) have proposed that the phylogenetic and ontogenetic origin of the different parts of the conduction system is a series of myocardial rings between segments of the tubular heart. According to these theories the Purkinje fibres develop from the bulboventricular ring, formed between the bulbus and the primitive ventricle. In a recent study these ideas could, however, not be confirmed (42).

The morphological differences between conductive tissue elements and functioning myocardium is so slight in embryonic hearts, that distinction between the two types is extremely difficult at this stage (81). Mainly for that reason there are different opinions about the stage of development at which the different parts of the conduction system can be identified as discrete structures. Most authors, however, agree that the Purkinje fibres appear at a later stage than the more proximal parts of the conduction system.

The development of the "typical" Purkinje fibres, as well as that of the Purkinje fibres of man, has been studied with conventional histologic techniques but not by means of histochemistry or electron microscopy. The criterion for identification of the Purkinje fibres with histologic techniques has been the appearance of cells which, as compared with the ordinary myocytes, are more rounded and larger and have myofibrils only in the periphery. Using such criteria the Purkinje fibres in human hearts can not be distinguished from ordinary myocytes at nine weeks of gestation (40) and are first distinguished at about 14 weeks (86). At later stages of fetal development they become more clearly distinguishable (56, 86). Anderson and Taylor (3), however, describe two cell populations - one subendocardial and one outer layer of cells - which can already be recognized in the ventricles at early embryonic stages in human hearts. These authors propose that the subendocardial cell population forms the Purkinje fibres and the outer layer the ordinary ventricular myocardium. The reason for the divergent descriptions is probably related to the basis for identification of the Purkinje fibres: Although Anderson and Taylor also described a difference in cell diameter it is merely the observation of an extensive layer subendocardially in the ventricles which is their basis for identification. The development of the "typical" Purkinje fibres has been studied in the sheep: The Purkinje fibres are described as distinguishable from ordinary myocardium at 70 mm (49) respectively 100 mm (24) CR-length with conventional histologic techniques.

For clarity it must be pointed out that not all authors studying developing hearts term the conduction cells in the ventricles Purkinje fibres. It must be remembered that the criteria for differentiating Purkinje fibres from ordinary myocytes (see above and Discussion) are valid for adult hearts.

The Purkinje fibres - highly differentiated cells or embryonic remnants?

The degree of differentiation of the Purkinje fibres is a controversial topic: The cells are described to be "embryonic", or to be highly "differentiated". It is also debated whether the Purkinje fibres and ordinary myocytes differentiate along the same route but at different rates or differentiate along different routes (18). Further the description of the three main types of Purkinje fibres, as identified histologically (see above), in terms of differentiation is confusing: The cell types are referred to as fully differentiated, less differentiated or least differentiated (50, 76).

In old literature the Purkinje fibres are said to be cells which remain undeveloped and which resemble embryonic cardiac muscle cells (e.g. 33, 48).
Field (24) contended that the Purkinje fibres were held up in their development. Also in more recent studies the Purkinje fibres have been described as "embryonic" and "retarded" in development. What are the bases for these suggestions? It is a fact that many of the biochemical and metabolic characteristics distinguishing conduction tissue from ordinary myocardium in the adult heart (amount of glycogen, resistance to anoxia, activities of oxidative and glycolytic enzymes) also differentiate embryonic from adult myocardium (18). In addition, there appear to be similarities between the ultrastructure of the adult Purkinje fibres and that of undifferentiated embryonic myocytes (6, 47, 52). This has lead to the conclusion that the Purkinje fibres have an "embryonic organization". Viragh and Challice (82) have further proposed that the myofibrillar development in the Purkinje fibres is "incomplete", as an explanation of the filamentous components in the cells. The "defects" in myofibrillogenesis in the Purkinje fibres (exemplified by e.g. the myofilament-polyribosome complexes) might represent cessation of myofibrillogenesis at an embryonic stage (51). It has also been proposed that the conducting tissue represents remnants of embryonic tissue on the basis of its special physiological properties (41).

Alternatively, the Purkinje fibres and the ordinary myocytes have been described as representing different pathways of differentiation, by which the Purkinje fibres retain the high conductive properties and the spontaneous contractility (29). The appearance of the myofibrillar material in the Purkinje fibres has been attributed to an imbalance in synthesis and degradation of myofibrillar proteins. This imbalance may reflect a process of differentiation in which the development of "nerve-like" properties has taken precedence over the development of contractility (70). Furthermore, the fact that the Purkinje fibres change by age, e.g. increase markedly in size, would favour that the cells are not embryonic remnants (16). Robb et al (56) even proposed that the conduction cells undergo a more extensive change than do the ordinary myocytes, as the conduction cells were histologically more different from ordinary myocytes in human hearts of late fetal stages than of early fetal stages. Bogusch (11) concluded in an ultrastructural study on chicken Purkinje fibres during development that the cells go through a distinct differentiation process. He e.g. observed that typical adult features such as leptomeric fibrils and leptomeric complexes did not appear until after hatching and that the amount of glycogen in the Purkinje fibres decreased during development. However, ultrastructural information on developing Purkinje fibres in the mammalian heart is needed, as there are differences between adult chicken and mammalian Purkinje fibres, e.g. with respect to amount of glycogen.
AIMS OF THE PRESENT INVESTIGATION

The principal aims were to answer the following questions:

Can enzyme and immuno-histochemistry be employed in order to facilitate the identification of fetal Purkinje fibres at the light microscopic level? Is it possible to ultrastructurally define the human fetal Purkinje fibres?

What are the morphological characteristics of fetal Purkinje fibres compared with adult ones?

How do the Purkinje fibres differentiate as compared with the ordinary ventricular and atrial myocytes? Do Purkinje fibres show an "embryonic organization"?
MATERIALS AND METHODS

The original papers are referred to by their Roman numerals. In the original papers further descriptions can be found.

MATERIALS

Fetal bovine hearts (I-III): Twenty-eight fetuses were obtained during routine slaughter of cows. The crown-rump (CR) lengths of the fetuses varied between 5 cm (gestational age about 50 days) and 100 cm (full term, 260-270 days).

The hearts of the fetuses were dissected out immediately after or within 1-2 h of sacrifice. From the former hearts, specimens were fixed in a slightly stretched state in 2.5% glutaraldehyde in Tyrode's buffer and further processed for light microscopy (I) and transmission electron microscopy (II). These hearts were from fetuses with CR-lengths 7-100 cm. The specimens from hearts dissected out with some delay after sacrifice were processed for enzyme histochemistry and immunofluorescence microscopy (I, III). These hearts were from fetuses with CR-lengths 16-100 cm (I) and 5-72 cm (III). The smallest hearts processed for cryo-sectioning (CR-lengths 5 and 8.5 cm) were frozen as whole pieces to allow sectioning in the frontal plane of the hearts (III).

Adult bovine hearts (III): The hearts of three adult cows were obtained during routine slaughter of cows and were dissected within 1-2 h of slaughter. The specimens were processed for enzyme histochemistry and immunohistochemistry. Some specimens were frozen together with fetal specimens from corresponding regions.

Fetal human hearts (IV, V): The hearts of ten human fetuses (gestational age 17-21 weeks), obtained at legal interruptions of pregnancy performed by a prostaglandin instillation procedure (five hearts) or by hysterotomy (five hearts), were investigated. The former hearts were processed for enzyme and immunohistochemistry. These hearts were cut into 3-4 blocks to allow sectioning in the transverse and frontal planes of different regions of the hearts. One heart was not divided to allow serial sectioning in the frontal plane of the entire heart.

The hearts from the fetuses obtained by hysterotomy were immediately removed from the fetuses and within a few minutes perfusion of the hearts with an oxygenated Ringer's solution ad modum Langendorff was started at room temperature. These hearts were processed for transmission electron microscopy.

Adult human hearts (V): Hearts from two human autopsies were obtained. The specimens were processed for enzyme and immunohistochemistry.

Tissues studied (I-V): The tissues studied in the bovine hearts were the Purkinje fibre system (I, II, III), the ordinary ventricular myocardium (I, II, III) and the ordinary atrial myocardium (III). In the human hearts the SA- and AV-nodes, the AV-bundle and bundle branches (IV), the Purkinje fibre system (IV, V), the ordinary ventricular myocardium (IV, V) and the ordinary atrial myocardium (IV, V) were studied. Detailed descriptions of how the specimens were cut can be found in the original papers.
ENZYME AND IMMUNO-HISTOCHEMISTRY

General: The specimens were frozen in freon or propane chilled with liquid nitrogen. Serial sections were cut in a cryostat at -20°C and stained for the demonstration of enzyme activities, the intermediate filament subunit skeletin or the M-line proteins myomesin or MM-creatine kinase (see Discussion). Other sections were treated with control sera or stained with routine histological stains. From several of the specimens a large series of sections was cut. By the serial sectioning procedure it was possible to properly identify the different regions of the conduction system (IV).

Enzyme histochemistry: Sections were stained to demonstrate the activity of myofibrillar ATPase (I, IV), NADH-tetrazolium reductase (NADH-TR) (I, IV), succinic dehydrogenase (SDH) (I, III, IV), menadione-linked α-glycerophosphate dehydrogenase (I, III, IV) or phosphorylase (I). The histochemical methods were according to Dubowitz and Brooke (19). Sections were also stained to demonstrate the activity of cholinesterase (IV, V): The procedures were mainly according to Anderson and Taylor (3), using Gomori's medium (26). Other sections were stained with PAS (I) or van Gieson (I, IV). The sections were viewed in a Leitz Dialux 20 Photomicroscope.

Immunohistochemistry: Rabbit antiserum against bovine Purkinje fibre skeletin (see 22, 23) and against the chicken breast muscle Mr 165,000 M-protein myomesin (see 21, 64, 65) were prepared. Sheep antiserum against human MM-creatine kinase was purchased from Boehringer (FRG). Sera from normal rabbits and sheep served as controls. Sections were incubated with antiskeletin antibodies (I, III, IV, V) or with antibodies against MM-creatine kinase or myomesin (III, V). The sections were examined under a Leitz Orthoplane Photomicroscope equipped with epifluorescence optics.

CONVENTIONAL LIGHT MICROSCOPY AND TRANSMISSION ELECTRON MICROSCOPY

Fetal bovine hearts (II): The glutaraldehyde fixed specimens were cut into small blocks, postfixed in 1% OsO₄, rinsed in buffer and dehydrated. Embedding was carried out in Vestopal W. Survey semithin and fine sections were cut in a LKB Ultrotome I or III. The survey sections were stained with toluidine blue and the fine sections with uranyl acetate and lead citrate. Some of the fine sections were collected on gold grids and stained with periodic acid-thiosemicarbazide-silverproteinate (PA-TSC-SP) (see 66, 69). Light microscopic examination was carried out in a Leitz Dialux 20 Photomicroscope and electron microscopic examination in a Philips EM 300 or in a Siemens Elmiskop 1A.

Fetal human hearts (V): The hearts were initially fixed by addition of 2.5% glutaraldehyde to the perfusion fluid. After the perfusion, the hearts were placed in glutaraldehyde for a further 2 h. The hearts were then rinsed in Tyrode's buffer and selected areas were cut out. Postfixation and further procedures were as described for the bovine hearts.
The observations on the Purkinje fibres are described in relation to the species studied and the techniques used. The tissues studied in parallel are also commented on. Illustrations and further descriptions of the results are found in the original papers.

**COW**

Semithin plastic sections (I): Even at the earliest stage studied (CR-length 7 cm) the Purkinje fibres were distinguishable from ordinary ventricular myocytes. The distinguishing features for the Purkinje fibres were their aggregation into bundles, the connective tissue sheath and the clear cytoplasm of the cells. However, in the late fetal stage the Purkinje fibres were more easily distinguished, due to a difference in cell diameter: They had larger diameters than the ordinary ventricular myocytes at this stage, while in early fetal stages the two cell types had approximately the same diameters. Furthermore, in fetuses with CR-lengths 20 cm and more the Purkinje fibres were distinguished from ordinary ventricular myocytes by a stronger PAS-reaction, while in smaller fetuses the tissues showed a comparable PAS-reaction. The Purkinje fibres seemed to become binucleate more frequently as age increased. Occasional mitotic figures were observed in the Purkinje fibres, while they were more frequent in the ordinary ventricular myocytes.

Immunohistochemistry (I, III): In all the fetal stages studied the Purkinje fibres were distinguished from the ordinary ventricular myocytes by an intense fluorescence in sections incubated with antibodies against the intermediate filament subunit skeletin. The strong fluorescence in the Purkinje fibres emanated from the central part of the cytoplasm of the cells. The nuclei were devoid of fluorescence and the fluorescence was weak at cell borders facing other cells in the bundles.

The M-line protein MM-creatine kinase was detected at an earlier stage in Purkinje fibres and atrial myocytes than in ordinary ventricular myocytes. The other M-line protein, myomesin, could be detected in all three cell types even at the earliest stage studied (CR-length 5 cm). At this stage, however, detectability of myomesin was more obvious in the Purkinje fibres than in the ordinary ventricular myocytes.

Enzyme histochemistry (I, III): The Purkinje fibres showed a higher activity of SDH than the ordinary ventricular myocytes in fetuses with CR-length 32 cm or less. In larger fetuses the activity of SDH seemed to be equal in the two cell populations, while in adult hearts the Purkinje fibres showed a lower activity. The Purkinje fibres showed a higher activity of α-glycerophosphate dehydrogenase (a high activity correlates with glycolytic activities) than the ordinary ventricular myocytes, with the exception that, in hearts of stages 11-32 cm, the ordinary ventricular myocytes eventually exhibited an equal activity. The activity of SDH and α-glycerophosphate dehydrogenase of Purkinje fibres seemed to remain unchanged from early to late fetal stages. The activity of SDH gradually increased for both ordinary ventricular and atrial myocytes. The activity of α-glycerophosphate dehydrogenase was higher in the atria than in the ventricles of the smallest fetuses (CR-length 5 and 8.5 cm), lower in the atria of larger fetuses and low in both tissues of adult hearts.
Electron microscopy (II): In the earliest stages (CR-length 7 and 10 cm) the appearance of the intercalated disks and the myofibrils was quite similar in Purkinje fibres and ordinary ventricular myocytes. With increasing gestational age the latter cells became more packed with myofibrils and attained more step-wise formed intercalated disks. The myofibrillar M-band became more evident with age, the Purkinje fibres clearly preceeding the ordinary ventricular myocytes in this respect. Leptofibrils were observed in both cell types only in old fetuses. Intermediate filaments were more abundant in the Purkinje fibres. Myofilament-polyribosome complexes typical of adult cow Purkinje fibres were not observed. The mitochondria were few and scattered in the cytoplasm in both cell types in early stages. In the oldest fetuses the mitochondria formed longitudinal rows between the myofibrils and perinuclear masses around the nuclei in the ordinary ventricular myocytes, while they were still scattered in the cytoplasm in the Purkinje fibres. In the earliest stages both cell types had abundant glycogen granules, while in later stages the amount was higher in the Purkinje fibres. T-tubules were seen only in the ordinary ventricular myocytes of the largest fetus.

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Immunohistochemistry (IV, V): In sections incubated with antibodies against the intermediate filament subunit skeletin a subendocardial cell population was distinguished from the ordinary ventricular myocardium in the human fetal hearts at midgestation by an intense fluorescence. By serial sectioning it was observed that the bundle branches took contact with this cell population (see below). In fact, parts of the bundle branches were also composed of intensely fluorescing cells. A shift from moderate fluorescence (of comparable magnitude to that of the ordinary myocardium) to intense fluorescence occurred in the initial part of the left bundle branch and in the distal intraseptal part of the right bundle branch. The intensely fluorescing cells are interpreted as the Purkinje fibres.

Also the ordinary ventricular and atrial myocytes showed fluorescence, which emanated from the Z disks and the intercalated disk regions, in sections incubated with antiskeletin antibodies. The fluorescence in the more intensely fluorescing Purkinje fibres was more uniformly spread in the cytoplasm.

The Purkinje fibres and the ordinary ventricular and atrial myocytes of the fetal hearts at midgestation showed fluorescent cross-striations in sections incubated with antibodies against the M-line protein myomesin. The Purkinje fibres were identified in serial sections due to their high skeletin reactivity and high cholinesterase activity (see below). In sections incubated with antibodies against the M-line protein MM-creatine kinase the Purkinje fibres and some atrial myocytes showed a cross-striated pattern, while the ordinary ventricular myocytes did not. In adult hearts striations were evident in the myocytes of all three tissues in sections treated to demonstrate presence of MM-creatine kinase.

Enzyme histochemistry (IV, V): Sections serial to those incubated with antibodies were prepared for enzyme histochemistry. This enabled the identification of the different parts of the conduction system (IV). All parts of the conduction system showed a high activity of cholinesterase.

Electron microscopy (V): Ultrastructurally two cell populations were distinguished in the ventricles in the human fetal hearts: A subendocardial cell population in which the majority of the myofibrils showed dense M-bands and the rest of the myocardium in which the myofibrils showed no dense M-bands.
The subendocardial cell population is interpreted to represent the Purkinje fibres. Also in some regions in the atria the myofibrils showed dense M-bands.

Purkinje fibres formed bundles or loosely arranged cell formations surrounded by abundant connective tissue and few capillaries. Some of them were lying in contact with ordinary myocardial tissue. In general the Purkinje fibres had a more rounded cellular and nuclear shape and larger diameters than the ordinary ventricular and atrial myocytes. The number of specialized cell contacts seemed to be highest between the Purkinje fibres. Intermediate filaments were most frequent in the Purkinje fibres. The myofibrils occupied only parts of the cytoplasm and had a varying appearance in all three cell types studied; however they varied to the greatest extent in the Purkinje fibres. Several features were common to the three cell types: eg. a single nucleus in almost all cells, no well-formed stepwise intercalated disks, presence of electron dense masses (mainly composed of filaments and ribosomes), few and scattered mitochondria, abundant glycogen granules and no T-tubules. In the atrial myocytes there were numerous so called atrial specific granules.
DISCUSSION

THE PURKINJE FIBRES IN THE DEVELOPING HEART

The development of "highly differentiated" ("typical") (bovine) and "less differentiated" (human) Purkinje fibres (cf. 50, 76) has been studied in the present investigation. A subendocardial cell population in the ventricles, interpreted to represent the Purkinje fibres, could be distinguished in both the bovine and human fetal hearts. The accuracy of this interpretation can be discussed in relation to the identification, the terminology and the function of the cells.

Identification

One way to identify the Purkinje fibres in the ventricles is to trace the distribution of the bundle branches peripherally. This was enabled by serial sectioning and by the fact that the bundle branches were clearly distinguished from ordinary myocardium by enzyme histochemistry. For both the bovine and the human fetal hearts it was clearly demonstrated that the bundle branches became distributed into the subendocardial cell populations. Thus these cell populations represent the conduction cells in the ventricles.

Another way to "identify" Purkinje fibres in the ventricles in tissue sections would be to search for cells with "Purkinje fibre-like" morphological characteristics. That was performed in specimens of subendocardial regions of the ventricles and in transverse sections through the ventricles. The characteristics looked for were the ones described to be typical of adult Purkinje fibres, such as their gross morphological features, a high content of skeletin, a high content of glycogen and typical enzyme histochemical characteristics with respect to oxidative and glycolytic activities (cf. 1, 17, 23, 46, 50, 60, 75).

In the bovine fetal hearts, gross morphological criteria for identification of the Purkinje fibres were applicable at the earliest stage investigated (CR-length 7 cm). The distinction was, however, clearer in later stages. The cells were also distinguished from ordinary myocardium by a higher content of skeletin, as seen immunohistochemically. The metabolic criteria for the identification of bovine Purkinje fibres changed during development.

Also in the human fetal hearts, subendocardial cells surrounded by connective tissue were distinguished in the ventricles in semithin plastic sections (unpublished observations). However, it was not possible to unequivocally identify these cells as Purkinje fibres (cf. 40, 56, 86). On the other hand, the Purkinje fibres were identified due to their high content of skeletin, as seen immunohistochemically. Also with other techniques a clear separation between two cell types in the ventricles was possible. The Purkinje fibres, in comparison with the cells in the rest of the ventricular myocardium, showed a higher activity of cholinesterase, were labelled by antibodies against MM-creatine kinase and showed M-bands ultrastructurally. The use of immuno- and enzyme histochemistry had thus enhanced the possibility to identify Purkinje fibres in the human fetal heart at the light microscopic level. In fact, already at ten weeks gestational age an extensive cell population is distinguished subendocardially in the ventricles, due to a high content of skeletin and a high activity of cholinesterase (unpublished observations). Furthermore, it was possible to ultrastructurally identify the
human fetal Purkinje fibres at midgestation as these cells but not the ordinary myocytes in the ventricles had attained electron dense M-bands and contained MM-creatine kinase. Immunohistochemical detection of MM-creatine kinase correlates with the presence of an electron dense M-band (64, 65, 85). The function of the M-line bound MM-creatine kinase is proposed to be a participation in the regulation of the ATP metabolism related to muscular contraction (65, 85).

Terminology

If we conclude that the cells in the subendocardial cell populations are conduction cells and at least partly can be distinguished from ordinary myocardium on the same basis as adult Purkinje fibres, is it accurate to call the cells Purkinje fibres? Clearly the cells in the bovine fetal hearts, from an early fetal stage, can be called Purkinje fibres, mainly as they could be distinguished by gross morphological features and a high content of skeletin. These features are two main criteria of adult Purkinje fibres (e. g. 23, 76).

A high content of skeletin, as demonstrated immunohistochemically (IV), can also be used as a criterion to define human Purkinje fibres (cf. 23). Thus from at least ten weeks gestational age, which is the earliest stage we have studied hitherto, and onwards, conduction cells which merit the name Purkinje fibres are present in human fetal hearts. Furthermore, we observed that the shift into skeletin-rich cells occurred at special levels: In the initial part of the left bundle branch and in the distal part of the intramural right bundle branch. Thus from these levels and further peripherally the conduction cells might be called Purkinje fibres. This is of significance for the human conduction system as the border between bundle branch and peripheral Purkinje fibre cell types for a long time has been discussed and not been determined (cf. 28, 62).

Functional aspects

What do the observations in the present studies imply with respect to the function of fetal Purkinje fibres, as compared with adult ones? Electrophysiologically the behaviour of the human fetal heart is comparable to that of the adult heart (25, 35, 79). Janse et al (35) argued that the electrophysiological maturity of the human fetal heart was produced despite a "morphological immaturity" of the conduction system. However, the present studies indicate that the fetal Purkinje fibres are not "immature". Morphologically it appears that the cells instead have a specialized function already at early fetal stages.

The formation of bundles already at the early fetal stage of bovine hearts may favour that the Purkinje fibres early have fast conduction properties. Whether nexuses are more abundant between Purkinje fibres than ordinary myocytes already in fetal hearts, which is our preliminary opinion (V), must await further studies. The high activity of SDH and a-glycerophosphate dehydrogenase and the presence of dense M-bands in the bovine Purkinje fibres at early fetal stages shows that the cells at these stages are more differentiated than the ordinary myocytes (see below). The high activity of the two enzymes favours that the energy demand is higher in the Purkinje fibres as compared with the ordinary myocytes at early fetal stages. The fact that skeletin is present in large amounts in fetal Purkinje fibres, possibly instead of myofibrillar proteins, suggests that the cells early are not directed to contractility.
The high activity of cholinesterase in the human fetal Purkinje fibres is likely to reflect high activity of the Purkinje fibres themselves, as no nerves were seen with certainty. In the adult human heart, on the other hand, cholinesterase-positive nerves are frequent in association with the Purkinje fibres (37). One interpretation is that the high activity observed in the present study is related to a process of differentiation of the Purkinje fibres (cf. 54). Another interpretation is that it reflects an early step in the development of nerves, as a high cholinesterase activity is detected in a tissue prior to neurogenesis occurs (36). Species differences with respect to cholinesterase exist: The bovine fetal Purkinje fibres, in contrast to the human ones, do not show a clearly higher activity than ordinary myocytes (unpublished observations).

THE DIFFERENTIATION PATTERN OF THE PURKINJE FIBRES

Purkinje fibres, forming an extensive subendocardial population of cells, were already distinguished at early fetal stages of both human (unpublished observations) and bovine hearts. This observation supports the proposal that the Purkinje fibres differentiate in situ in the ventricles (cf. 3, 34, 84). The differentiation pattern of the cells can be discussed in relation to that of ordinary ventricular and atrial myocytes.

Comparison with ordinary ventricular myocytes

The mode of development of the ordinary ventricular myocytes in the bovine hearts conformed to previous descriptions for other species. This includes a gradual maturation of the myofibrillar apparatus and the intercalated disks, a gradually increasing aerobic and decreasing anaerobic metabolism and a comparatively late development of T-tubules. However, there are interspecies differences with respect to myocardial maturation at birth. It seems as if the extent to which the ordinary myocytes in neonates are differentiated corresponds to the degree of general development of the animal. Thus, the newborn calf is born in a quite mature state and the ordinary myocytes are well-developed (e.g. have T-tubules), while e.g. the rat and the mouse are born in a more immature state and the ordinary myocytes are less differentiated (cf. 30, 31, 39).

Both the ordinary ventricular myocytes and, in contrast to previous descriptions (24, 29), the Purkinje fibres, exhibited mitotic figures. Both cell types also divide mitotically in the human fetal heart at midgestation (unpublished observations). In the rat mitotic division of ordinary myocytes is still apparent at birth but has almost completely ceased within the first few weeks of neonatal life (89). Hereafter the ordinary myocytes enlarge but do not proliferate.

The Purkinje fibres in the bovine fetal hearts develop along a quite different pathway from the ordinary ventricular myocytes. Some features were distinguishing through all fetal stages: The bundle formation, the more rounded cellular and nuclear shape and the higher skeletin content of the Purkinje fibres. Other differences between the cell types were manifest only in the late fetal stage: The amount and the orientation of the myofibrils, the arrangement of the intercalated disks and the formation of T-tubules. The two cell types showed quite different differentiation patterns with respect to cell metabolism and development of the myofibrillar M-band.

As evidenced from both enzyme activities and ultrastructural observations the metabolic differences between Purkinje fibres and ordinary ventricular
myocytes in bovine fetal hearts are quite different at various stages of development. It seems as if this is mainly due to the maturation of the ordinary ventricular myocytes. The activities of SDH and α-glycerophosphate dehydrogenase in these cells, but not in the Purkinje fibres, changed considerably during fetal development. Also ultrastructurally (amount of glycogen-granules, arrangement of mitochondria) the changes in the Purkinje fibres were minimal as compared with the ordinary ventricular myocytes. These observations may reflect an early specialization of the Purkinje fibres (see above).

The process of differentiation of cardiac myocytes involves the organization of contractile proteins into characteristic cross-striated myofibrils. Visible M-bands in the sarcomeres is a comparatively late phenomenon and represents a sign of terminal myofibrillar maturation (eg. 2, 5). The presence of dense M-bands and the detection of MM-creatine kinase at an earlier stage in Purkinje fibres than in ordinary ventricular myocytes shows that this part of final myofibrillar maturation proceeds independently and timely separated in the two tissues. The second M-line protein known at present, myomesin, was detected in both Purkinje fibres and ordinary ventricular myocytes at early stages of bovine fetuses. However, the striations were more clearly seen in the Purkinje fibres at these stages. This protein has been reported to be an integral component of the myofibrillar structure and to be present already at the earliest stages of myofibrillogenesis in chicken skeletal muscle cells (21, 65).

Comparison with atrial myocytes

The atrial myocytes in the human fetal hearts at midgestation mainly differed from the ordinary ventricular myocytes by presence of dense M-bands and so called atrial specific granules. Other differences will develop at later stages of development (see III). In the bovine fetal hearts we observed that the atrial myocytes earlier attained myofibrillar M-bands and showed a different pathway of glycolytic activities than the ordinary ventricular myocytes.

With respect to the development of the myofibrillar M-band, the Purkinje fibres are more "atrial-like" than "ventricular-like". In fact, we have observed also other similarities between Purkinje fibres and atrial myocytes in fetal hearts: Darkly stained dense bodies, which appear similar to the atrial specific granules typical of atrial myocytes, are present in Purkinje fibres but not in ordinary ventricular myocytes in human fetal hearts. Furthermore, in early fetal stages of the bovine heart the Purkinje fibres as well as the atrial myocytes show an intense fluorescence in sections incubated with antibodies against atrial myosin (unpublished observations).

In the adult heart there are differences between atrial and ventricular myocytes which, in principal, are comparable to differences between Purkinje fibres and ventricular myocytes: These include less stepwise formed intercalated disks, fewer mitochondria, less developed T-tubulus system, more glycogen and somewhat lower activity of oxidative enzymes in atrial vs ventricular myocytes (12, 15, 31, 38, 44, 74). The differences are, however, probably more pronounced between Purkinje fibres and ventricular myocytes. A problem is that comparisons between atrial myocytes and Purkinje fibres are very few in the literature. However, it is clear that the Purkinje fibres in several respects are more "atrial-like" than "ventricular-like" both during differentiation and in the adult stage. Nevertheless, there are clear differences between Purkinje fibres and atrial myocytes, eg. with respect to
cell shape and volume (cf. 38, 77).

Comment on the proposed "embryonic organization"

The question of whether Purkinje fibres show an "embryonic organization" or not is intimately related to the definition of this "embryonic organization". It is a fact that the myofibrils are less parallelly arranged, the intercalated disks have another configuration (less stepwise formed) and the glycolytic activities are higher in Purkinje fibres than in ordinary ventricular myocytes in the adult heart. These are typical examples of features by which the Purkinje fibres are ascribed to show similarities to undifferentiated ordinary myocytes (see 18, 47). However, what is most important is that the Purkinje fibres do not show an "embryonic organization" in the sense that the cells attain their typical features by a "retardation" of features of undifferentiated ordinary myocytes. Instead the present studies show that the two cell types in several respects are different already at early stages, develop along different pathways and that in fact the Purkinje fibres in some respects earlier show features typical of a mature, differentiated state than the ordinary myocytes. The presence of a myofibrillar M-band and a high activity of oxidative enzymes are such features.

With respect to the proposed "embryonality" of Purkinje fibres comments can also be made on specific organelles. The myofilament-polyribosome complexes typical of adult Purkinje fibres do not represent cessation of myofibrillogenesis at an embryonic stage (cf. 51), as these complexes were not observed in the fetal hearts. Leptofibrils are not embryonic remnants, as previously proposed (13), but develop with age (cf. 11). Recently it was also shown that leptofibrils appear in ordinary myocytes as a result of mechanical unloading (73). The high content of skeletin as seen immunohistochromically and the high amount of intermediate filaments as seen ultrastructurally in the Purkinje fibres is in favour of differentiation and specialization. Skeletin seems to represent a specialized form of intermediate filament protein: During skeletal muscle differentiation there is a shift of synthesis from one type of intermediate filament subunit (vimentin) to the muscle specific type (desmin, skeletin) (10, 88). The abundance of intermediate (sometimes erroneously described as "fine") filaments in adult Purkinje fibres has been one of the features which have been related to an "embryonic organization" (6).

Further studies on the differentiation patterns of the Purkinje fibres, the cells of the proximal parts of the conduction system and the atrial and ventricular myocytes are in progress in our laboratory. One observation is that the myofibrillar M-band, as evidenced by detection of MM-creatine kinase, develops at a later stage in the AV node cells than in the Purkinje fibres and atrial myocytes in the bovine fetal heart. This shows that myofibrillar maturation proceeds differently not only between Purkinje fibres and ordinary myocytes but also between Purkinje fibres and AV-node cells. Another observation is that the bovine Purkinje fibres from being homogeneously stained in early fetal stages become heterogeneously stained in late fetal stages, in sections incubated with antibodies against atrial myosin. Thus, it seems as if the heterogeneity of adult Purkinje fibres with respect to myosin composition (cf. 58, 72) is a result of a differentiation process.
GENERAL SUMMARY AND CONCLUSIONS

The development of Purkinje fibres and ordinary ventricular and atrial myocytes in bovine and human fetal hearts was studied:

1) By serial sectioning and tracing of the bundle branches peripherally the Purkinje fibres were identified. In both bovine and human fetal hearts they could further be identified in sections incubated with antibodies against the intermediate filament subunit skeletin, due to a stronger immunofluorescence reaction than ordinary ventricular myocytes. From early fetal stages and onwards the bovine Purkinje fibres could also be distinguished in semi-thin plastic sections and ultrastructurally due to gross morphological features (aggregation into bundles, a connective tissue sheath, a clear cytoplasm). The Purkinje fibres in the human fetal hearts were not clearly identified by such criteria but could, on the other hand, be discriminated from ordinary myocytes due to a high activity of cholinesterase.

2) Ultrastructurally, the myofibrillar M-band became more evident with age in the bovine fetal hearts, the Purkinje fibres clearly preceeding the ordinary ventricular myocytes in this respect. This was confirmed by presence of MM-creatine kinase, as demonstrated immunohistochemically, at an earlier stage in the former cells. MM-creatine kinase is known to make an essential contribution to the electron density of the M-band. In human fetal hearts at midgestation the Purkinje fibres, but not the ordinary ventricular myocytes, were stained in sections incubated with antibodies against MM-creatine kinase and showed M-bands ultrastructurally.

3) In the human fetal heart at midgestation and in the hearts of small bovine fetuses there were few distinct ultrastructural differences between Purkinje fibres and ordinary ventricular myocytes: Apart from the difference in appearance of the myofibrillar M-band the Purkinje fibres had a greater amount of intermediate filaments. In the late fetal stage of the bovine hearts there were clear differences between the two cell types. These included the amount and orientation of the myofibrils, the formation of the intercalated disks, the arrangement of the mitochondria, the amount of glycogen and the formation of T-tubules.

4) The activities of succinic dehydrogenase (SDH) and α-glycerophosphate dehydrogenase seemed to remain unchanged in the Purkinje fibres from early to late fetal stages of the bovine hearts. The activity of SDH gradually increased for both ordinary ventricular and atrial myocytes, while the activity of α-glycerophosphate dehydrogenase was high at different stages of early fetal development in the two tissues to become low in the adult stage. The activity of SDH of the Purkinje fibres was higher in small fetuses, equal in large fetuses and lower in adult hearts, as compared with that of ordinary ventricular myocytes. In all stages of the bovine hearts the Purkinje fibres showed a high activity of α-glycerophosphate dehydrogenase.

5) In atrial myocytes of both bovine and human hearts electron dense M-bands and detection of MM-creatine kinase was evident at an earlier stage than in ordinary ventricular myocytes. In the human fetal hearts at midgestation so called atrial specific granules were numerous in the atrial myocytes.

The observations show that:
1) Purkinje fibres can be identified already at an early fetal stage.

2) The use of enzyme and immuno-histochemistry has enhanced the possibility to identify the fetal Purkinje fibres. A high content of skeletin, as demonstrated immunohistochemically, seems to be a general criterion to identify not only adult, but also fetal, Purkinje fibres. By comparisons of immunohistochemical and ultrastructural observations it was possible to distinguish the human fetal Purkinje fibres at midgestation at the ultrastructural level.

3) The Purkinje fibres show features of a differentiated state, as formation of bundles, presence of dense M-bands and high activity of oxidative enzymes, at early fetal stages. This may suggest that the cells early have a specialized function.

4) The Purkinje fibres differentiate along a line separate from ordinary ventricular myocytes.

5) The Purkinje fibres do not attain their typical features by a "retardation" of features of undifferentiated ordinary myocytes. The description of an "embryonic organization" of adult Purkinje fibres is thus misleading.
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