Embryonic development of the heart

II. Formation of the epicardium

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The mature heart may be thought of as consisting of three layers, endocardium, myocardium, and an outer investing tissue called the epicardium. During early formation of the tubular heart of chick embryos, at about the 8-somite stage, two tissue layers become clearly discernible with the light microscope: the endocardium and the developing myocardial wall. The outer epicardial layer does not appear until later in development.

It is generally accepted that embryonic heart wall or 'epimyocardium' is composed of muscle and undifferentiated cells. As its name implies, the epimyocardium is thought to give rise to myocardium and epicardium. Kurkiewicz (1909) suggested that the epicardium was not an epimyocardial derivative but rather is formed from cells originating in the sinus venosus region, which migrate over the surface of the heart. Nevertheless, it has become generally accepted that the outer cell layer of the embryonic heart wall differentiates in situ to give rise to the definitive visceral epicardium (Patten, 1953). For reviews, see Romanoff (1960) and DeHaan (1965).

An earlier light and electron microscopic study of cardiogenesis in the chick demonstrated (Manasek, 1968) that by stage 12—(15-somites) the heart wall was composed only of cells that contained myofibrils, and that no epicardial cells were present. It was suggested that the heart wall could not give rise to epicardium unless dedifferentiation occurred, thus supporting Kurkiewicz's (1909) observations. These findings also contradict Bruno's (1918) observations which suggested that epicardial differentiation began at the 13-somite stage.

In the present study, the early epicardium was examined in greater detail, and its relationship to the developing myocardial wall was investigated. Techniques of light and electron microscopy were utilized to elucidate both the cytology of the developing epicardial cells and the histogenesis of this tissue.

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MATERIALS AND METHODS

Fertile white Leghorn eggs were incubated at 38 °C to yield embryos ranging from Hamburger & Hamilton (1951) stage 17+ (approximately 2½ days old) to 11 days of age. A portion of the shell was removed to expose the embryo which was flooded in situ with ice cold glutaraldehyde–formaldehyde fixative (Karnovsky, 1965). The entire embryo was then removed and placed in a dish of cold fixative. The heart was dissected free and placed in fresh fixative at 0 °C for an additional period of 15 min. It was then briefly rinsed in cold 0·2 M cacodylate buffer (pH 7·6) and placed in 1 % OsO₄ for an additional 2 h. Following dehydration in a graded series of alcohols, the heart was embedded in Araldite. For purposes of light microscopy, 0·5–1·0 μ sections were cut with glass knives and stained with 1 % toluidine in 0·2 % borax.

Glycogen was demonstrated at the light microscopic level in this tissue by a modification of the PAS technique of DiBella & Hashimoto (1966). The identification of intracellular PAS positive material as glycogen was made by correlative electron microscopy. A periodic acid solution was made by adding 2·5 ml M/5 sodium acetate and 12 ml distilled H₂O to 0·2 g periodic acid in 30 ml absolute alcohol. This solution was stored in the cold until used. A weak Feulgen solution, consisting of 0·5 g basic fuchsin in 200 ml H₂O, 1·5 g potassium metabisulfite and 10 ml 1 N-HCl was prepared. Plastic sections (0·5–1·5 μ thick) were mounted on glass slides and incubated in the periodic acid solution at 60 °C for 10–20 min. They were then rinsed in running tap water for about 10 min and incubated in the Feulgen reagent for 10 min at 60 °C. After a second tap water rinse the slides were dried and counterstained by gently heating them on a hot plate with a few drops of 0·5 % toluidine blue in 0·05 % borax. The counterstain is not permanent, although the PAS reaction itself is.

Thin sections, for purposes of electron microscopy, were cut with glass or diamond knives, mounted on uncoated copper grids, single-stained with lead citrate (Venable & Coggeshall, 1965) and examined with an RCA EMU 3F electron microscope operated at 50 kV.

RESULTS AND DISCUSSION

During early development, the ventricular myocardial wall contains only developing muscle cells (Manasek, 1968) which differentiate and contain myofibrils by stage 12— (15-somites). The homogeneity of the ventricular wall persists through stage 15, which is the earliest stage illustrated in the present paper. Although there is some variability in their stage of development, the cells in this tissue can be recognized as myocytes because of their myofibrils which are visible in the electron microscope (Fig. 5). The term myocyte is used to refer to cells which contain myofibrils, whereas the term myoblast will be restricted to presumptive muscle cells which contain no fibrils.
By stage 15, the myocardium is no longer a uniformly compact tissue as it was in the earlier embryos (Manasek, 1968). Large intercellular spaces have formed, separating the outermost myocyte layer from the basal layers. Cells of the outer layer generally contain fewer myofibrils than cells more deeply situated (Fig. 5). Although Bruno (1918) and Patten (1953) considered the epicardium to be a derivative of this outer layer of myocardial cells (which were presumed to be undifferentiated cells, i.e. myoblasts) no evidence was obtained in the present study to support this concept.

Shortly before stage 17+, the homogeneity of the myocardial wall is lost
as a result of the appearance of the epicardium. The epicardium is first seen as a simple epithelium that only partially covers the myocardial surface (Figs. 1, 2). As development progresses the area covered by the epicardium increases until by late in the fourth day (stage 24) the entire ventricular surface is covered.

The early simple epithelial epicardium is generally separated from the outer surface of the muscular wall of the heart by a narrow layer of extracellular

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Fig. 3. Section of a ventricle from a 4-day-old (stage 23) chick; the epicardium (E) contains some cuboidal cells, as well as squamous cells. Glycogen is still not demonstrable in the epicardium, although the myocytes contain large amounts of this polysaccharide, which appears black in this light micrograph of a plastic section stained with PAS and toluidine blue. The scale line represents 10 μ. (× 750.)

Fig. 4. In the 7-day-old chick embryo, the epicardium shows an outer epithelial layer and a connective tissue layer containing mesenchymal cells. Neither the epithelium nor the mesenchyme contain demonstrable glycogen. The myocardial wall contains developing blood vessels (arrow) which also lack glycogen pools. Section stained with PAS and toluidine blue. The scale line represents 10 μ. (× 750.)
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material (Figs. 1, 2). As development proceeds, this layer becomes thicker and mesenchymal cells appear within it (Figs. 3, 4).

At the light microscopic level, the epicardium and the developing myocytes of the underlying myocardium differ in that the latter contain large amounts of glycogen. Myocardial glycogen appears in the form of large cytoplasmic pools and scattered granules (Manasek, 1968). Although the isolated glycogen granules are below the resolving power of the light microscope, the larger accumulations are prominent and readily demonstrable with the periodic acid–Schiff reaction. At all stages of development examined in this paper, the PAS technique failed to demonstrate any positive-staining material in the epicardial cells (Figs. 1, 3, 4) although a large increase in myocardial glycogen content is apparent between 2½ days of incubation (stage 17+, Fig. 1) and 7 days (stage 31, Fig. 4). It is interesting to note that glycogen pools are also absent from developing endocardium (Fig. 1) and coronary blood vessels (Fig. 4).

When the developing ventricular wall is examined with the electron microscope, additional differences between embryonic cardiac myocytes and epicardial cells become apparent, and the two types of cells can be readily distinguished on cytological grounds. The developing myocytes of the stage 17+ embryo contain irregularly arranged myofibrils and large amounts of glycogen (Fig. 6). These cells are bound to each other by desmosomes and developing intercalated discs, with the outer myocardial cell layer still demonstrating its epithelial characteristics (Manasek, 1968) by the continued presence of apical junctional complexes (Fig. 6). Sections through the region of the advancing edge of the epicardial layer (Fig. 6) reveal that the uncovered area of the ventricular myocardium is comprised of normally developing myocytes, similar in all respects to those already covered by the epicardium. There is no evidence to suggest that any of these cells are ‘dedifferentiating’ or losing myocardial characteristics to become epicardium. Although at this stage both epicardium and myocardium contain large numbers of free ribosomes, an extensive granular endoplasmic reticulum and scattered lipid droplets, the epicardium is completely devoid of myofibrils. In addition, the electron microscope fails to reveal the presence of glycogen particles in the epicardial cells, confirming the light microscopic observations. Thus, even at stage 17+, the epicardium represents a non-myocardial type, and its development marks the beginning of the heterogeneity of the cell types constituting the wall of the heart (Fig. 7).

The marked differences between early epicardium and the underlying myocardium argue against the concept that the epicardium is derived from the outer myocardial layer. The observation that the epicardium does not develop uniformly over the entire myocardial surface, but is on the other hand initially a discontinuous sheet, also supports this contention. If the epicardium actually did develop in situ from underlying myocytes, one would expect to see transitional cells, demonstrating a progressive loss of differentiated characteristics.
Fig. 5. A low power electron micrograph through the entire ventricular wall of a stage 15 embryo reveals the homogeneous composition of this tissue. All the cells in this section contain myofibrils (arrows) and are thus recognizable as developing cardiac myocytes. The large intercellular spaces (ECS) appear to divide the myocardial wall into two layers and the outer layer of myocytes (upper right) appears to contain fewer fibrils than the deeper layers. The inner surface of the myocardial wall bordering the cardiac jelly is seen in the lower left hand corner.
Fig. 6. In this electron micrograph, of a region similar to Fig. 2, the leading edge of the epicardium is shown (E). The uncovered portion of the ventricle of this heart from a stage 17 + chick embryo consists of developing myocytes (M). Note the prominent apical junctional complex (J) characteristic of this tissue. Glycogen (G) and tangentially sectioned myofibrils (arrows) are seen in the myocytes, but are absent from the epicardium. Lipid droplets, granular endoplasmic reticulum and free ribosomes are present in the epicardial cells. The scale line represents 2 \( \mu \). (\( \times \)13200.)
Fig. 7. The relationship of the early epithelial epicardium to the rest of the ventricular wall is shown in this electron micrograph of a portion of the ventricle of a stage 17+ embryo. In this section, the outer myocardial cell layer (M) is covered by epicardium (E). Note the fibrils (arrows) in the myocytes. The large extracellular space (ECS) separating the outer myocardial cell layer from the remainder of the ventricular wall still persists. Note the basal lamina along the basal surface of the myocardial wall (BL). The scale marker equals 2 μm. (× 12000.)
(myofibrils, intercalated discs) in the outer myocardial cell layer. No such phenomenon was observed in any of the hearts examined. Therefore, in light of the different cytology of epicardium and myocardium, the initially incomplete nature of the epicardial covering and the absence of undifferentiated epicardial precursors, the definitive visceral epicardium appears not to be a derivative of the myocardial wall. Although the outer myocardial cell layer does appear to contain fewer myofibrils than the deeper layers (Figs. 5, 7) this difference is the result of a slower rate of maturation of the outer cells rather than a loss of differentiated characteristics. This relative immaturity of the outer myocyte layer persists even after the surface is covered by epicardium (Fig. 7).

As noted earlier in this paper, the developing embryonic myocardium is generally termed the ‘epimyocardium’ because it was thought to give rise to both the definitive myocardium and its epicardial investment. In light of the present work, it is suggested that this term is a misnomer and the embryonic heart wall should be referred to simply as ‘developing myocardium’. Kurkiewicz (1909) objected to the term ‘myoepicardial mantle’ for much the same reason.

Although the present work demonstrates the non-myocardial characteristics of the epicardium, the origin of this layer remains obscure. In an effort to clarify the topologic features of the growing epicardium, and to determine the source of this tissue, an attempt is under way at the present time to make serial reconstructions of hearts of pertinent developmental stages.

The outer epithelial cells of the epicardium contain many free ribosomes (Fig. 8) and mitochondria scattered throughout their cytoplasm. Profiles of granular endoplasmic reticulum are present and the Golgi apparatus is generally situated close to one side of the pleomorphic nucleus (Fig. 8). The cells are bound together by junctional complexes and membranes of adjacent cells are often interdigitated.

As the embryo matures, the connective tissue portion becomes the major component of the epicardium. This layer is relatively narrow in the earlier stages and is uniformly electron lucent (Fig. 6). Whether this appearance is an artifact of tissue preparation or represents a true characteristic of this early embryonic matrix is not known. Concomitant with the appearance of the epicardial mesenchymal cells, the connective tissue layer widens and the presence of a flocculent material in the extracellular matrix becomes demonstrable (Figs. 9, 10). Collagen bundles can be demonstrated within the extracellular matrix after about the fourth day of incubation. Unfortunately, except for the identifiable collagen bundles, the composition of this material is completely unknown. It may be produced by the epicardial cells which, with their Golgi complex and granular endoplasmic reticulum, have some characteristics of secretory cells. Indeed, it is quite possible that the extracellular matrix receives contributions from the developing cardiac myocytes, cells which also have secretory characteristics (Manasek, 1968).
Fig. 8. The outer epithelial layer of the epicardium of a 4-day-old embryo (stage 23) contains large numbers of free ribosomes and many scattered profiles of granular endoplasmic reticulum. A Golgi complex is shown (G) and multivesicular bodies are present between the Golgi and the nucleus.

The cells are joined by junctional complexes, an example of which is seen near the center of plate. Scale line equals 1 μ. (× 17600.)
Fig. 9. Part of the epicardial mesenchyme consists of phagocytes. In this electron micrograph of the epicardium of a 7-day embryo (stage 31), a phagocyte characterized by large vacuoles is closely applied to the outer myocardial cell layer (left). Many of the vesicles appear empty, but some contain a flocculent material. These cells often contain dead myocytes. The extracellular matrix contains a flocculent material (F) and bundles of collagen (C). Scale line equals 2 μ. (× 17000.)
Fig. 10. This low power electron micrograph illustrates the outer portion of the epicardium of an 8-day-old (stage 35) embryo. The outer cell layer in the region depicted is very thin and the cells appear flattened. The mesenchymal component of the epicardium has become very extensive and the cells have long cytoplasmic processes. These cells are fibroblastic and occasionally a single cilium (arrow) can be seen. A large amount of collagen (C), possibly secreted by the mesenchyme, can be seen in the extracellular matrix. Scale marker equals 2 μ. (× 9100.)
Fig. II. This electron micrograph represents a region of epicardium similar to that of Fig. 10, but shows the area bordering the outer surface of the myocardium (M). The extensive Golgi complexes (G) of the mesenchymal cells, and their long profiles of granular endoplasmic reticulum (arrows) suggest that these cells may be secreting the flocculent extracellular matrix. In addition to the flocculent material, bundles of collagen fibers (C) can be seen. Scale line equals 2 μ. (× 12600.)
The cytoplasm of the irregular mesenchymal cells contains long profiles of granular endoplasmic reticulum and a well-developed Golgi complex (Fig. 11). Nuclear morphology is quite variable and prominent nuclear indentations (Figs. 10, 11) are characteristic. These cells assume the characteristics of fibroblasts as the epicardium develops and they often demonstrate a single cilium (Fig. 10). By about the fifth day of development, phagocytes may be seen comprising part of the mesenchymal cell population. These cells are quite common by the seventh day of development (Fig. 9), and are characterized by autophagic vacuoles and large, apparently empty vacuoles. Occasionally they contain dead myocardial cells (Manasek, 1969). The origin of these phagocyte cells is obscure, and it is not known whether they migrate into the epicardium from a distant source or if they differentiate in situ from the epicardial mesenchyme.

We may conclude that the early functional tubular heart contains only two cell types: myocytes and endocardial cells. The muscular wall contains only myocardial cells and it appears that the variety of cell types seen in the mature heart are not all derived from this embryonic tissue, but rather are added to it.

Although the anatomy of this histologically simple, yet functional organ undergoes progressive development, it is not until late in the second day of incubation that a third component, the epicardium, is seen. By the fourth or fifth day of development, coronary arteries become visible (Spalteholz, 1923), introducing non-myocardial cell types into the wall of the heart. The orderly and sequential addition of various tissue and cell types to the developing heart probably depends upon interactions of tissue types with each other and with the extracellular environment. The elucidation of these processes will be important in our understanding of cardiogenesis.

SUMMARY

1. At stage 17+, the epicardium is a simple epithelium incompletely covering the myocardial surface.
2. As development proceeds, the epicardium covers the heart, and a substantial connective tissue layer is formed between the epicardial epithelium and the outer myocardial surface.
3. Mesenchymal cells appear within this connective tissue layer. Fibroblasts and phagocytes can be identified.
4. Concomitant with the appearance of mesenchymal cells, collagen bundles can be detected in the extracellular matrix.
5. Cells of the epicardium are distinctly different from those of the myocardium. Embryonic epicardial cells do not contain glycogen or myofibrils, two characteristics of myocardial cells.
6. At the time of first appearance of the epicardium, all the muscle cells comprising the wall of the heart have attained a degree of differentiation, i.e. they contain myofibrils.
7. No evidence was obtained to support the concept that the epicardium differentiates in situ from undifferentiated myocardial cells, nor were any myocytes seen to undergo 'dedifferentiation' to form epicardial cells.

8. It is concluded that the epicardium is not a derivative of the myocardial wall and hence, the term 'epimyocardium' is a misnomer when applied to the heart at early stages.

RÉSUMÉ

Développement embryonnaire du coeur. II. Formation de l’épicarde

1. Au stade 17+, l’épicarde est un épithélium simple recouvrant incomplètement la surface du myocarde.
2. Au cours du développement, l’épicarde recouvre le coeur et il se forme une couche substantielle de tissu conjonctif entre l’épithélium épicardique et la surface externe du myocarde.
4. En même temps qu’apparaissent les cellules mésenchymateuses, on peut déceler des faisceaux de collagène dans la matrice extracellulaire.
5. Les cellules de l’épicarde sont distinctement différentes de celles du myocarde. Les cellules épicaudiques embryonnaires ne contiennent pas de glycogène ou de myofibrilles, deux caractéristiques des cellules myocardiques.
7. On n’a pas fait d’observations permettant de supposer que l’épicarde se différencie in situ à partir de cellules myocardiques indifférenciées, et on n’a pas non plus observé de myocytes en cours de "dédifferentiation" pour former des cellules épicaudiques.
8. On conclut que l’épicarde n’est pas un dérivé de la paroi du myocarde; il en découle que le terme d’"épimyocardium" est erroné quand on l’applique au coeur aux premiers stades.

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