Compositional and structural heterogenicity of the cardiac jelly of the chick embryo tubular heart: a TEM, SEM and histochemical study

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SUMMARY
The hearts of chick embryos of stages 9–13 were subjected to SEM, TEM and histochemical studies to ascertain possible regional differences in the structure and composition of the cardiac jelly. Two distinct regions, the cardiac jelly filling the space located between the myocardium and the endocardium (MECJ) and the cardiac jelly filling the dorsal mesocardium (EECJ), were distinguished by their structural and histochemical properties. MECJ is formed by amorphous and fibrillar material arranged between the endocardial and myocardial layer. The amount of its components increases when cetylpyridinium chloride is introduced into the fixative, and it appears intensely stained by ruthenium red and alcian blue at low concentrations of MgCl₂. The amount and arrangement of its components increase during the beginning of the looping process of the heart tube. The EECJ is very rich in ruthenium-red-positive basal-lamina-like material and the addition of cetylpyridinium chloride to the fixative does not modify its appearance. It also appears poorly stained by alcian blue at low concentrations of MgCl₂ and its arrangement undergoes modifications closely associated with the events of endocardial fusion. The possible significance of these results in the early morphogenesis of the heart is discussed.

INTRODUCTION
Numerous recent papers show that the embryonic extracellular matrix plays an important role in morphogenesis and differentiation of tissues and organs (Bernfield, Cohn & Banerjee, 1973; Manasek, 1975; Overton & Collins, 1976; Toole, 1973). The morphogenetic role of the extracellular matrix is dependent on the kind, amount and distribution of its components.

During the early stages of heart development the extracellular matrix, 'cardiac jelly' (CJ) (Davis, 1924), constitutes the major component of this organ. Among the different components of the CJ, collagen (Johnson, Manasek, Vinson & Seyer, 1974; Hurle & Ojeda, 1977), glycoproteins (Manasek, 1976b, 1977), and cellular detritus (Ojeda & Hurle, 1975) and mucopolysaccharides

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(glycosaminoglycans) are very abundant (Manasek, 1970; Markwald & Adams Smith, 1972; Ortiz, 1958) and abnormal development of the heart occurs when the synthesis of mucopolysaccharides is altered (Overman & Beaudoin, 1971; Satow & Manasek, 1977). Biochemical analysis of the chick embryo CJ showed the presence of the following mucopolysaccharides: hyaluronate and chondroitin (Manasek et al., 1973; Orkin & Toole, 1978); undersulphated chondroitin sulphate (Manasek et al., 1973) chondroitin 4- or 6-sulphate (Gessner, Lorincz & Boström, 1965; Manasek et al. 1973) and heparin/heparan sulphate (Gessner et al. 1965; Satow & Manasek, 1977). However, it must be noted, as Manasek pointed out (1976a), that more components could be present since most of the studies are synthetic analyses rather than total compositional analyses.

Temporal and regional differences in the composition and arrangement of the CJ would explain some of the proposed functions for the CJ (see Manasek, 1976c, and Hurle & Ojeda, 1977). However, most of the studies focused on that problem have been made in the period in which the CJ is invaded by mesenchymal cells (Markwald, Fitzharris & Manasek, 1977; Markwald, Fitzharris, Bank & Bernake, 1978) while earlier stages have received less attention although during these stages important morphogenetic events occur, such as the fusion of the paired cardiac primordia and the beginning of the looping process of the tubular heart.

In the present paper we report the results of a structural and histochemical study of the CJ in the early stages of heart morphogenesis. The structure of the CJ was studied by scanning electron microscopy which gives three-dimensional information on the arrangement of the CJ. In addition, to assess possible regional differences in the composition of the CJ, ruthenium-red staining was employed in transmission electron microscopy and alcian blue staining at the ‘critical electrolyte concentration’ (Scott & Dorling, 1965) in light microscopy. This technique gives an approximate analysis of the composition in sulphated mucopolysaccharides by-passing the difficulty of the biochemical studies derived from the small amount of material available. In addition it allows detection of the regional distribution of the sulphated mucopolysaccharides impossible in biochemical analysis.

**MATERIAL AND METHODS**

Fertile White Leghorn eggs were incubated at 38 °C to yield normal embryos ranging between stage 9 and 13 (Hamburger & Hamilton, 1951) and the structure of the CJ was studied by the following techniques:

**Scanning electron microscopy (SEM)**

The embryos were fixed for 3–5 h in 4% cacodylate-buffered glutaraldehyde with and without 1% cetylpyridinium chloride and then transferred into cacodylate buffer in which the specimens were transversely sectioned throughout
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The fragments were dehydrated through a series of acetones dried by the critical-point method (Anderson, 1951) and gold sputter-coated. The specimens were viewed using a Philips SEM 501.

Although the addition of cetylpyridinium chloride to the fixative produces alterations of the cell membranes (Morris & Solursh, 1978) it was employed here because it has also been reported that it retains abundant extracellular components of the CJ which are extracted by conventional fixative fluids (Markwald et al. 1978; Pratt, Larsen & Johnson, 1975).

Transmission electron microscopy (TEM)

Ruthenium-red staining was carried out according to the Luft (1971) procedure. The embryos were immersed in 3% glutaraldehyde buffered in 0.1 M sodium cacodylate pH 7.3 containing 0.1% ruthenium red, and quickly transversely sectioned through the heart for better penetration of the dye. After 4–10 h of fixation, the specimens were washed in cacodylate buffer, postfixed for 2 h in 2% osmium tetroxide containing 0.1% ruthenium red and processed for electron microscopy according to the usual procedure.

Ultrathin sections either unstained or counterstained with uranyl acetate and lead citrate were examined with a Philips EM 201 electron microscope.

Histochemistry

Embryos were fixed in Carnoy’s fluid or Newcomer’s solution, embedded in paraplast and serial sectioned at 8 or 30 μm. Sections were processed to detect sulphated mucopolysaccharides as follows:

(a) Differential sulphated mucopolysaccharide staining by the ‘critical electrolyte concentration’ method of Scott & Dorling (1965). Sections were stained in solutions of 0.1% alcian blue 8GX (G. T. Gurr Ltd) in 0.025 M acetate buffer (pH 5.8) plus MgCl₂ in concentrations ranging from 0.1 to 1 M. Sections were rinsed in MgCl₂ solutions of the appropriate molarity before and after staining and examined microscopically after dehydration, clearing and mounting in DPX. For a better evaluation of the staining intensity, in addition to subjective evaluation, the sections were analyzed with the help of the grey scale of a Micro-Videomat (Zeiss, Oberkochen). This technique allows an approximate differentiation of the sulphated mucopolysaccharides owing to their different critical electrolyte concentrations (at which the anionic polymers change from binding dye to binding Mg²⁺).

(b) Hyaluronidase digestion. To distinguish chondroitin sulfate A/C from chondroitin sulphate B (dermatan sulphate), heparin and Keratan sulphate, deparaffinized sections were incubated overnight with bovine testicular hyaluronidase at 37 °C in pH 6 citrate buffer prior to staining (Leppi & Soward, 1965).

(c) Digestion in methanol-CIH. Control of specificity of the alcian-blue staining for sulphated mucosubstances was made by treating the sections with
0.1 M ClH in absolute methanol at 60 °C for 4 h. This treatment abolishes sulphate-specific staining (Quintarelli, Scott & Dellovo, 1964).

Sections of human umbilical cord were included in all staining tests to ensure validity of dye reactions and enzyme digestion.

**OBSERVATIONS**

*Scanning electron microscopy*

During the stages studied in the paper the formation of the tubular heart takes place by the fusion in the midline of two lateral primordia (for details see Ojeda & Hurle, 1975). During this fusion process, the heart is a very simple organ consisting of three concentric tissue layers. It has an outer wall, termed the myocardial layer (after Manasek, 1969; and Ho & Shimada, 1978), which is open at the dorsal side forming the dorsal mesocardium. The innermost structure is the endocardium. Between the endocardium and the myocardium there is a large extracellular compartment filled by an acellular matrix, the cardiac jelly (CJ). This extracellular matrix is also observed in the dorsal mesocardium arranged between the ventral foregut endoderm and the endocardium. As can be seen in Fig. 1 two morphologically different regions can be distinguished within the CJ: the CJ located between the myocardium and the endocardium (MECJ) and the CJ located between the endoderm and the endocardium (EECJ).

The MECJ appears as a meshwork of extracellular material arranged between the basal surfaces of the endocardium and the myocardium. A progressive modification in the structure of this zone of the CJ can be observed. Initially (stages 9–10) it appears to be formed by radially arranged fibrillar material associated with abundant fine granular material (Fig. 2). The total amount of material at these stages is small, and numerous spaces which lack material are observed. In the next stages the amount of material increases and the amorphous material appears as rounded masses, 0.5–1 μm in diameter (in the coated material) closely associated with the fibrillar material (Fig. 3). By stage 12–13 the endocardium and the myocardium are closer to each other, diminishing the space filled by MECJ. In these stages the MECJ is very rich in material consisting mainly of thick strands of fibrillar material with a clear radial arrange-
Fig. 5. SEM view of the endocardial wall of a stage-13 embryo showing the dense fibrillar sheet located under the endocardial surface.

Fig. 6. Panoramic view of the MECJ of a stage-10 embryo fixed in glutaraldehyde-cetylpyridinium chloride. Note the abundant fine granular material retained and compare with Fig. 2. Endocardium (E). Myocardium (M).

Fig. 7. EECJ of a chick embryo heart after fusion of the endocardial tubes. This CJ consist of amorphous and fibrillar material and contains cell fragments (C). Ventral foregut endoderm (F). Myocardium (M). Endocardial tube (E).

Fig. 8. EECJ of a chick embryo heart during fusion of the endocardial tubes (E). This CJ appears to be formed by two sheets (arrows) that are continuous with the basal lamina of the ventral foregut endoderm (F).
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...ment, joining one another by delicate strands of fibrils (Fig. 4). Amorphous material is not abundant at these stages and on the basal surfaces of the myocardium and endocardium a sheet of close-packed fibrils is observed (Fig. 5).

The addition of cetylpyridinium chloride to the fixative results in the preservation of abundant fine granular material within the MECJ which makes it difficult to recognize the fibrillar component of this zone of the jelly (Fig. 6).

The EECJ fills the extracellular space of the dorsal mesocardium and in addition to fibrillar material the amorphous material is a major component of this zone of the CJ. Rounded cell fragments are frequently found associated with the extracellular material (Fig. 7). The arrangement of this zone of the CJ also shows differences between the studied stages but, as has been previously described by us (Hurle & Ojeda, 1977), these modifications appear to be closely related with the fusion of the endocardial tubes. Before and especially after the fusion of the endocardial tubes EECJ appears as thick chains of closely packed extracellular material joining the endoderm with the endocardium (Fig. 7). During the process of fusion both endocardial tubes are located lateral to each other and the EECJ appears as two sheets of dense basal lamina-like material occupying the limited space between the fusing tubes (Fig. 8). These sheets are continuous dorsally with the basal lamina of the endoderm (Fig. 8). The addition of cetylpyridinium chloride to the fixative does not produce gross differences in the appearance of this zone of the CJ.

Ruthenium-red staining

Ruthenium red (RR) penetrates well into the heart tissues and gives a strong positive reaction both with the cell surfaces of all the heart tissues and with the extracellular material of the CJ (Figs. 9, 10). As can be seen in Fig. 9, the MECJ appears in the sections as a loose meshwork of RR-positive fibrils. This fibrillar material is more abundant in the proximity of the myocardium where the fibrils are often seen to contact the RR-positive coat of the developing myocardial cells (Fig. 9). The EECJ appears composed mainly of an amorphous RR-stained mass associated with RR-positive fibrils among which crossbanded collagen fibrils can be recognized (Fig. 10). Large amounts of EECJ are often observed filling invaginations of the endocardial cells of the midline in the stages in which the endocardial tubes undergo fusion (fig. 11). This location of the extracellular matrix seems particularly interesting since most of these cells are detached towards the bloodstream and it could be a mechanism of elimination of the EECJ when it is no longer necessary.

No gross modifications in the staining pattern of the CJ were observed during the studied stages.

Alcian-blue staining

In all the studied embryos CJ appears strongly stained with alcian blue (AB) when MgCl₂ is added at concentrations lower than 0·2 m (Fig. 12a, b). However,
Fig. 9. Panoramic view of the MECJ stained with RR. Note the relation of the extracellular fibrils with the RR-positive coat of the myocardial cell basal surface (arrows).

Fig. 10. Electron micrograph showing the EECJ stained with RR. This zone of the CJ consist of strongly RR-positive amorphous material and fibrillar material. Endocardium (E).

Fig. 11. Electron micrograph showing abundant RR-positive extracellular material filling a large invagination of a midline endocardial cell.

the AB-positive material does not appear homogeneously distributed within the CJ sleeve. The MECJ is richer in stained extracellular material than EECJ. Furthermore the distribution of the AB-positive material does not appear homogeneous within the MECJ itself. Most of the stained material appears arranged as two laminae associated with the basal surface of the myocardial and the endocardial layers (Fig. 12a). A less-intense stained material appears radially arranged between the endocardium and the myocardium.

When the concentration of MgCl₂ is increased at 0.5 M the staining pattern is similar to that described above but the intensity of staining is drastically reduced. At concentrations of 0.6 M MgCl₂ a small amount of AB-positive material is retained which is mainly associated with the basal surface of the myocardium (Fig. 12c, d). The staining practically disappears at concentrations
Fig. 12. Two consecutive sections of a stage-11 embryo stained with AB plus MgCl₂, 
(a) Staining with 0·2 M MgCl₂. Note that most of the stained material is associated 
with the basal surfaces of the myocardium and endocardium and very little with 
EECJ (arrow). (b) Same section observed in the micro-videomat at the appropriate 
grey level to discriminate the AB-staining. (c) Staining with 0·6 M MgCl₂. Note that 
some dye can be recognized at the basal surface of the myocardium. (d) same section 
observed in the micro-videomat at the same grey level as that of (b). (Note the same 
level in both grey scales –G–.)

over 0·8 M MgCl₂. The amount of stained material at concentrations of MgCl₂ 
greater than 0·5 M increases in the older stages.

Hyaluronidase digestion drastically reduces the AB-staining at concentration 
of 0·1–0·2 M MgCl₂ but it does not significantly affect the staining at concentrations 
over 0·5 M MgCl₂. Treatment of the sections with methanol/CIH 
resulted in complete inhibition of the staining at all the concentrations of MgCl₂.

DISCUSSION

Structure of the CJ

Since the early description of Davis (1924) an intriguing question about the 
CJ has been the divergence between its physical properties in vivo and the 
amount of materials detectable by light and electron microscopy. Our SEM 
observations show that the CJ is very rich in solid materials supporting the 
hypothesis which gives important mechanical functions to this structure (Barry,
The general appearance of the chick CJ under the SEM appears similar to that observed in rat and chick embryos of older stages (Markwald, Fitzharris & Adams Smith, 1975; Markwald et al. 1977; Markwald et al. 1978) and to other embryonic extracellular matrices (Overton & Collins, 1976; Tosney, 1978).

The present observations confirm our previous ultrastructural study (Hurle & Ojeda, 1977) showing that two distinct regions can be distinguished within the CJ. The MECJ located between the myocardium and the endocardium appears as the major component of the CJ and comprises mainly RR-positive unbanded fibrillar material. The fact that the amount of matrical components increases when cetylpyridinium chloride is introduced into the fixative suggests that hyaluronate is an important component of this zone of the CJ (Pratt et al. 1975). The presence of hyaluronate in this zone of the CJ can be related to the invasion of the CJ by mesenchymal cells as has been pointed out by other authors (Markwald et al. 1978; Orkin & Toole, 1978). Our observations show that the fibrillar components of the MECJ tend to appear radially arranged between the endocardium and the myocardium. This is accentuated in the latest studied stages in which the looping process of the heart begins. This observations supports the hypothesis of Nakamura and Manasek (1978), who have suggested that CJ has an intrinsic shape which could be responsible for the looping process of the heart tube. Contrary to our observations, it has been reported (Markwald, Fitzharris, Bolender & Bernake, 1979) that the CJ of the atrio-ventricular cushions does not display a radial arrangement until it is invaded by mesenchymal cells. This discrepancy can be explained by the difference in the stages studied, or by the fact that the SEM observations of those authors are based on material fixed in glutaraldehyde plus cetylpyridinium chloride. In these conditions the large amount of fine granular material retained makes it difficult to recognize the arrangement of the fibrillar material. An alternative explanation is that the differences can be due to artefacts produced either by the omission or by the addition of cetylpyridinium chloride. Recently Morris & Solursh (1978) have reported gross ultrastructural artefacts due to the addition of cetylpyridinium chloride to the fixative.

The EECJ fills the dorsal mesocardium and it can only be observed during the stages in which this structure is present in the heart. Recent studies of Manasek (1976b) show that this zone of the jelly is particularly rich in fucose-containing glycoproteins probably elaborated by the endoderm. In our study this zone appears very rich in RR-positive amorphous material similar to the basal lamina material, and hyaluronate does not seem to be very abundant since the addition of cetylpyridinium chloride to the fixative does not produce gross modifications in its appearance. On the other hand the fact that the arrangement of this zone of the CJ is modified during the fusion of the endocardial tubes suggests that it may be involved in that process (see Hurle & Ojeda, 1977, for a detailed discussion).
AB-staining of the CJ reveals the presence of abundant sulphated mucopolysaccharides as previously reported by numerous authors (Gessner & Boström, 1965; Gessner, Lorincz & Boström, 1965; Manasek, 1970; Markwald & Adams Smith, 1972; Ortiz, 1958). Manasek et al. (1973) in a synthetic analysis made in cultured chick embryos, showed that the synthesis of sulphated mucopolysaccharides undergoes changes related to myocardial differentiation; before stage 11, only undersulphated chondroitin sulphate is produced in the heart, while during the stages 11 to 13−, chondroitin 4- or 6-sulphate is also produced. In our observations the disappearance of most of the AB-staining at concentrations over 0.5 M MgCl₂ and the hyaluronidase lability of this staining indicate that most of the stained material is chondroitin sulphate (Leppi & Stoward, 1965; Scott & Dorling, 1965) but it can not be ascertained whether it corresponds to undersulphated or to 4- or 6-sulphate. However, it should be noted that a weak staining, labile to methylation and resistant to hyaluronidase digestion, remains at concentrations of 0.6 and even at 0.8 M MgCl₂. This suggests that other strongly anionic mucopolysaccharides such as heparin or dermatan sulphate and keratan sulphate might be present. These results are in agreement with similar studies made in rat embryos (Markwald & Adams Smith, 1972). Heparin has been detected in the CJ of older embryos (Gessner et al. 1965; Satow & Manasek, 1977) but other sulphated mucopolysaccharides have never been detected in synthetic biochemical studies. These observations suggest that the turnover of those mucopolysaccharides, if present, must be very slow.

One of the principal purposes of this study was to find out possible regional differences in the distribution of sulphated mucopolysaccharides which could explain some of the proposed functions for the cardiac jelly. In this regard we found that the EECJ, despite being very rich in ultrastructural components, appears very poor when stained with AB, suggesting that sulphated mucopolysaccharides are not the main components of this zone of the jelly. This observation supports the view that the two zones of the CJ have different origins and functions (Manasek, 1976b; Hurle & Ojeda, 1977). On the other hand the MECJ is very rich in sulphated mucopolysaccharides and most of these are located in the basal surfaces of the myocardium and endocardium. This arrangement can represent a gradient in the diffusion of those materials since they are produced both by the myocardium (Kosher & Searls, 1973; Manasek, 1970) and by the endocardium (Gross, Challice & Schrével, 1974; Johnstone & Comar, 1957; Markwald et al. 1975) and it is consistent with an involvement of this extracellular matrix in the cytodifferentiation of both tissues as suggested by other authors (Manasek, 1976b; Markwald et al. 1975).

We conclude from this study that the CJ is not a homogeneous material either in its ultrastructure, its tridimensional arrangement or its histochem-
istry. On the contrary, our observations suggest that CJ has a precise structure and composition possibly related to the morphogenetic events taking place in the heart during these early stages (fusion and looping). It seems therefore erroneous to us to consider this cardiac component as a passive structure playing only unspecific mechanical functions. The recent experimental study of Nakamura & Manasek (1978) constitutes a nice demonstration of this hypothesis.

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REFERENCES

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Nakamura & Manasek (1978 in Morphogenesis and Malformation of the Cardiovascular System, G. C. Rosenquist and D. Bergsma (ed.), pp. 229-250. Alan R. Liss: New York) have recently published a SEM study of the EECJ of chick embryos of the same stages studied here and their results are in agreement with our present observations.