Visualisation of endocardial cells in the cardiac jelly of quail using Mab QH1

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SUMMARY

Cardiac jelly is a basement membrane located between the endocardium and myocardium, which is seeded by fibroblastic cells in the atrioventricular canal and outflow tract.

It has been demonstrated that this process begins with the emission of endocardial cell prolongations followed by cell seeding in the cardiac jelly, with the subsequent transformation into mesenchyme by myocardial induction of specific molecules at stage 16 HH (Hamburger and Hamilton, 1951) in chicken embryos.

In this work, using the monoclonal antibody QH1 (a specific marker for cell surface determinants on quail vascular endothelial cells and haemopoietic cells), we observed the appearance of free non-QH1-expressing cells in cardiac jelly at stage 9+ HH; at stage 13 HH we only observed QH1-positive cells and these were mixed with non-QH1-expressing cells, increasing the number of QH1-possitive cells at stage 16 HH. Furthermore, clear links between the myocardium and endocardium have been observed in the three stages studied.

The data suggest that in quail mesenchyme formation in cardiac jelly is proportionally earlier than that described for chickens, and that the first mesenchymal cells do not express QH1. This suggests clear links between the endocardium and myocardium in the stages studied. The appearance of links between the myocardium and the endocardium occurs earlier than stages 16-17 HH in chicken embryos, when mesenchymal tissue formation starts.

INTRODUCTION

The heart is the first organ formed in vertebrates. Normal heart development arises from paired heart-forming primordia to a primitive tubular structure and, later, by the action of cardiac cushions, resulting in a multichambered organ.

The cardiac cushions are located in the atrioventricular canal (AVC) and in the proximal outflow tract (OFT), in a space between the myocardium and endocardium termed cardiac jelly (CJ). CJ is considered to be myocardial basement membrane. It is an acellular and avascular space that persists from the beginning of development until stages 16-17 HH (Hamburger and Hamilton, 1951) in chicken embryos, when mesenchymal cells invade these zones to form the cardiac cushions (Krug et al., 1985; Markwald et al., 1990).

The theory defending the myocardial origin of mesenchymal tissue in CJ was first proposed by Chang (1932) and Adams Smith (1963). Later, using TEM, Argüello et al. (1978) observed the differentiation of myocytes in fibroblastic phenotype cells in the OFT at stage 26 HH (4.5-5 days) in the chicken embryos. In 1984, Arrechedera et al., using the same technique, observed the loss of cellular junctions of myocytes in AVC at stage 29 HH (6 days), which were later observed in lateral cushions.

Controversially, De la Cruz et al. (1972), Bolender et al. (1979), Markwald et al. (1990), Sinning et al. (1992), Isokawa et al. (1994), Wunsch et al. (1994), Eisenberg and Bader

(1995), Sinning et al. (1995), Fishman and Chien (1997) have proposed the endocardium as the origin of mesenchymal tissue in AVC chicken embryos, where some endocardial cells move into the cardiac jelly to form the cushion tissue.

Furthermore, Bolender and Markwald (1979), Markwald et al. (1990), Isokawa et al. (1994), Wunsch et al. (1994), Eisenberg and Bader (1995), Sinning et al. (1995), and Krug et al. (1995) have suggested that the only function of the myocardium in mesenchyme formation is to secrete glycoproteins (fibronectin, transferrin and ES proteins) into the CJ in order to induce the formation of mesenchymal tissue from the endocardium, because endocardial cells are unable to transform themselves (Mjaatvedt et al., 1991; Rezaee et al., 1993; Sinning and Hewitt, 1996). Therefore, these cells could play complementary functions by segregating cell differentiation molecules belonging to the TGF-ß family, which are also secreted by osteoblasts, fibroblasts and neural crest cells (Ghosh and Brauer, 1996).

Thus, in avian embryos, CJ is possibly a mixture of cell populations, because although it is known that cells from the endocardial layer are present in the OFT (Markwald et al., 1975, 1990; Noden et al., 1991) and in the AVC (Markwald et al., 1990). Other types of cells, such as neural crest cells, can be found in the OFT (Sieber-Blum and Ito, 1995; Kirby and Waldo, 1995; Noden et al., 1995) and cells from the myocardium have already been described in the OFT at stage 9+ HH and in the bulboventricular region at stage 10 HH by Martín-Rodríguez et al. (1993).

Here, we attempted to study the time of appearance of these cells in CJ, and their potential endocardial lineage. We used quail embryos from stages 9+, 13 and 16 HH and used MAB QH1 (Pardanaud et al., 1987), which is specific for cell surface determinants in quail vascular endothelial cells and haematopoietic cells.

MATERIALS AND METHODS

Fertile eggs of Japanese quail (Coturnix coturnix japonica) were examined at stages 9+, 13 and 16 HH (33, 50 and 54 hrs of incubation) (at least three eggs per stage). The embryonic stages were determined according to the criteria used to stage the development of chicken embryos (Hamburger and Hamilton, 1951), equivalent to stages 7+, 11 and 14 of Zacchei (1960). Embryos were incubated at 37°C in a humidified atmosphere and were removed from the shell at the stages indicated above, washed in phosphate-buffered saline (PBS) (ph:7.4 ± 0.2) and fixed in Bouin solution (5% acetic acid, 24% formaline in saturated picric acid solution) at room temperature. Specimens were dehydrated in a graded

alcohol series and embedded in paraffin. Serial transversal sections were obtained at 7 µm thickness.

Dewaxed sections were washed in PBS, incubated with 1:50 diluted supernatant from cultured hybridoma cells of MAB QH1 (Developmental Studies Hybridoma Bank. Iowa. USA) for 2 hours at room temperature in a humid chamber, washed in PBS and incubated in the darkness for 30 minutes with the second antibody (anti-mouse Ig, biotinylated species-specific whole antibody from sheep, AMERSHAM) after dilution at 1:100.

The third antibody (Streptavidin-Biotinylated Horseradish Peroxidase complex, AMERSHAM) was added for 30 minutes after dilution at 1:1000. Sections were then washed in PBS for 30 minutes and developed with diaminobenzidine (DAB) (250 ml PBS + 250 µl H_2O_2 +33 µg DAB), washed in distiled water for 5 minutes, dehydrated and mounted with Eukitt (SIGMA).

Finally, sections were studied by light microscopy (Nikon Optiphot).

RESULTS

Stage 9 + HH

At stage 9+ HH, the endocardium is clearly marked, as are the cells located between the dorsal part of the foregut and the neural tube beside the notochord that later develops into the dorsal aorta artery.

Endocardial tubes are remodeled at the cephalic (Fig. 1a) and caudal poles (Figs. 1c, 1d, 1e and 1f) and are fused at the middle level (Fig. 1b), forming the bulbus and a single primitive ventricle. Next to the aortic sac (Fig. 1a), QH1-positive cells are located between the foregut epithelium and the splanchnopleura.

CJ is found between the endocardium and myocardium throughout embryonic heart, except for the dorsal-most zone, where the endocardium is very close to the foregut (Figs. 1a to 1f).

At this stage, CJ shows free QH1-negative cells at both the cephalic pole (Fig. 1a) and in the middle-caudal portion (Fig. 1d).

The inner surface of the myocardial wall throughout the cardiac tube is not flat, and shows irregularities in the form of triangular projections towards the endocardium. Very often, these are linked to the endocardium (Figs. 1a, 1d, 1e and 1f).

The endocardial wall is a monolayer, with some triangular-shaped cells whose apex is oriented towards the myocardium (Fig. 1c), giving the impression that they are oriented towards the myocardial triangular projections (Figs. 1b and 1f). There are only a few cells in

the remaining CJ, all of which are negative for QH1 antibody (Figs. 1a and 1d).

Stage 13 HH

At stage 13 HH, the myocardial wall is thicker, with an irregular inner face owing to the trian- gular QH1-negative prolongations, which are oriented towards the endocardium and in con- tact with it (Fig. 2a).

At this stage, the first QH1-positive cells are seen, isolated or forming small groups inside the CJ (Figs. 2b and 2e), or near the myocardium (Figs. 2c and 2d). In the AVC, endocardial cells reach the myo-
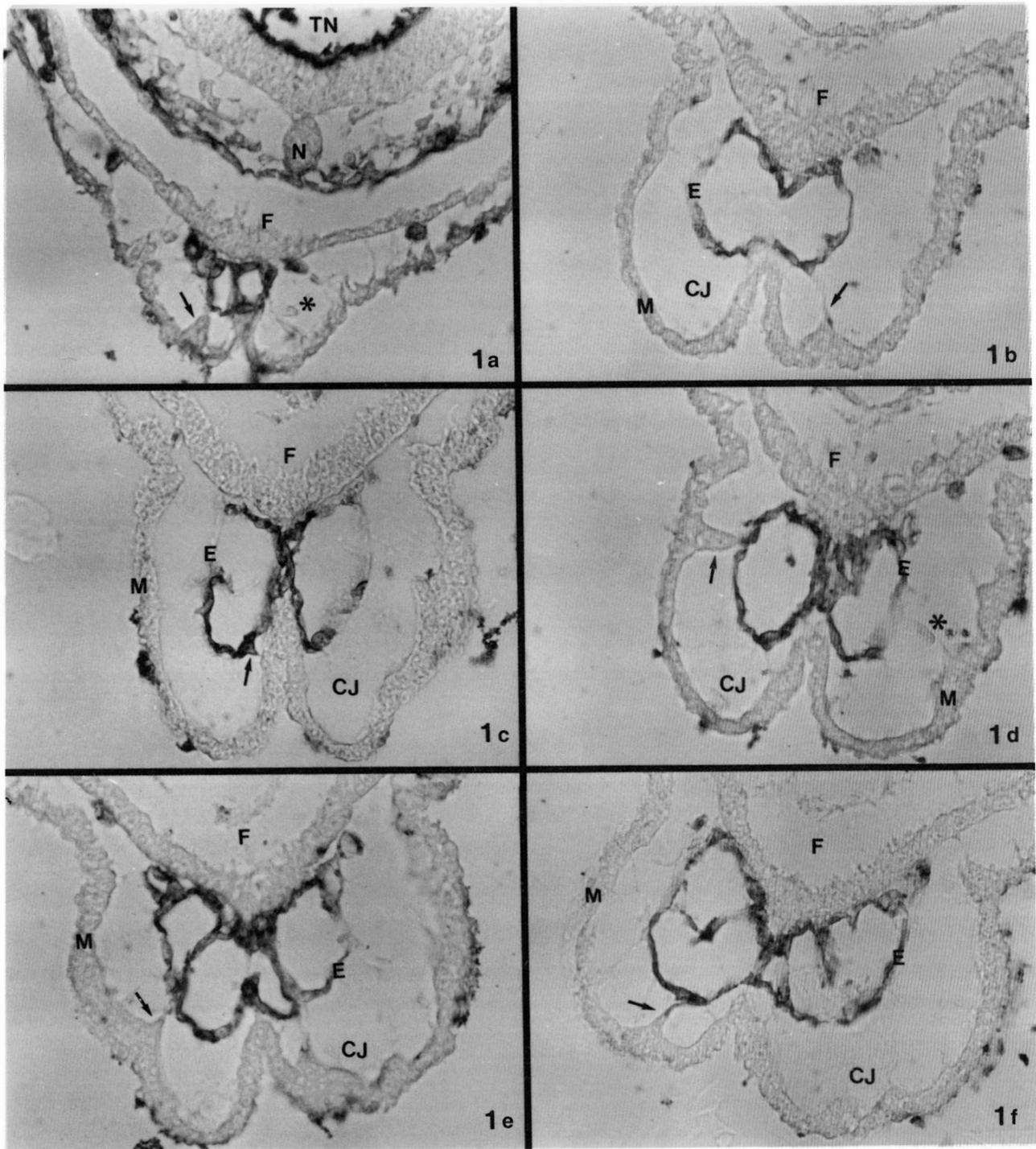
Fig. 1.– Stage 9+ HH. Transversal sections. 250x.
    a: Aortic sac. Arrow shows a QH1-negative bridge between the endocardium and the myocardium. Asterisk shows QH1-negative cells in the cardiac jelly (CJ).
    b: Bulbus-primitive ventricle. Arrow shows a QH1-negative myocardial prolongation oriented towards the endocardium.
    c: Primitive auricle. Arrow shows a QH1-positive endocardial triangular-shaped cell linked to the myocardium.
    d, e and **f:** Sinus venosi level. Arrow shows QH1-negative bridges and links between the endocardium and the myocardium. Aste- risk shows QH1-negative cell in the CJ.

cardium, forming QH1-positive bridges (Fig. 2f). No free QH1-negative cells are seen at this stage.

Stage 16 HH

At stage 16 HH, a spectacular increase in CJ cell numbers occurs (Figs. 3a to 3d). In the OFT,

a mixture of QH1-positive and QH1-negative cells forms a mesh resembling large bridges, linking the myocardium and endocardium in this region (Fig. 3a).

At this stage, the endocardium is thicker at certain points where the deeper cells seem to accumulate next to the CJ and form a multilayer-
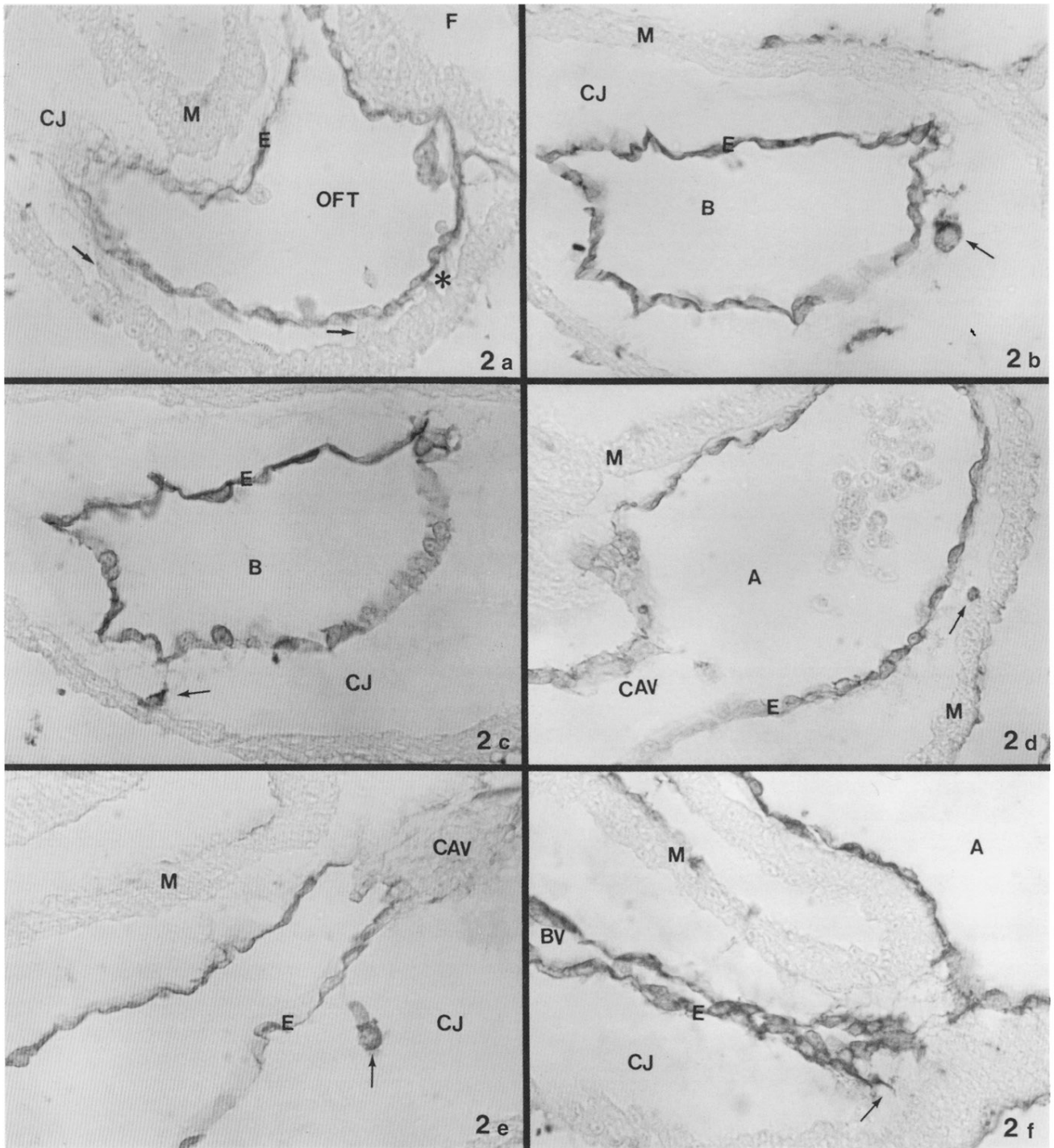
Fig. 2.– Stage 13 HH. Transversal sections. 100x.
    a: Outflow tract. Asterisk shows links between the myocardium and the endocardium. Arrows show QH1-negative myocardial prolongations that do not reach the endocardium.
    b: Bulbar region. Arrow shows a QH1-positive cell in the CJ.
    c: Bulbar region. Arrow shows a QH1-positive cell next to the myocardium.
    d: Primitive auricle. Arrow shows a QH1-positive cell next to the myocardium.
    e: Atrioventricular canal. Arrow shows QH1-positive cells in the CJ.
    f: Atrioventricular canal. Arrow shows QH1-positive bridges between the endocardium and the myocardium.

like endocardial tube in the area that later beco-
mes atrioventricular cushions (Figs. 3b and 3d).
Next to these areas, other small groups of two or
three QH1-positive cells are free inside the CJ
(Figs. 3b and 3d).

At bulbus level, small groups of QH1-nega-
tive cells and myocardial bridges with positive

or negative reactivity to the antibody are seen
(Fig. 3c).

The inner face of the myocardial wall is flat-
ter and more regular than in early stages throug-
hout the heart, except in the OFT, where the
bridges and meshes are located (Fig. 3a).
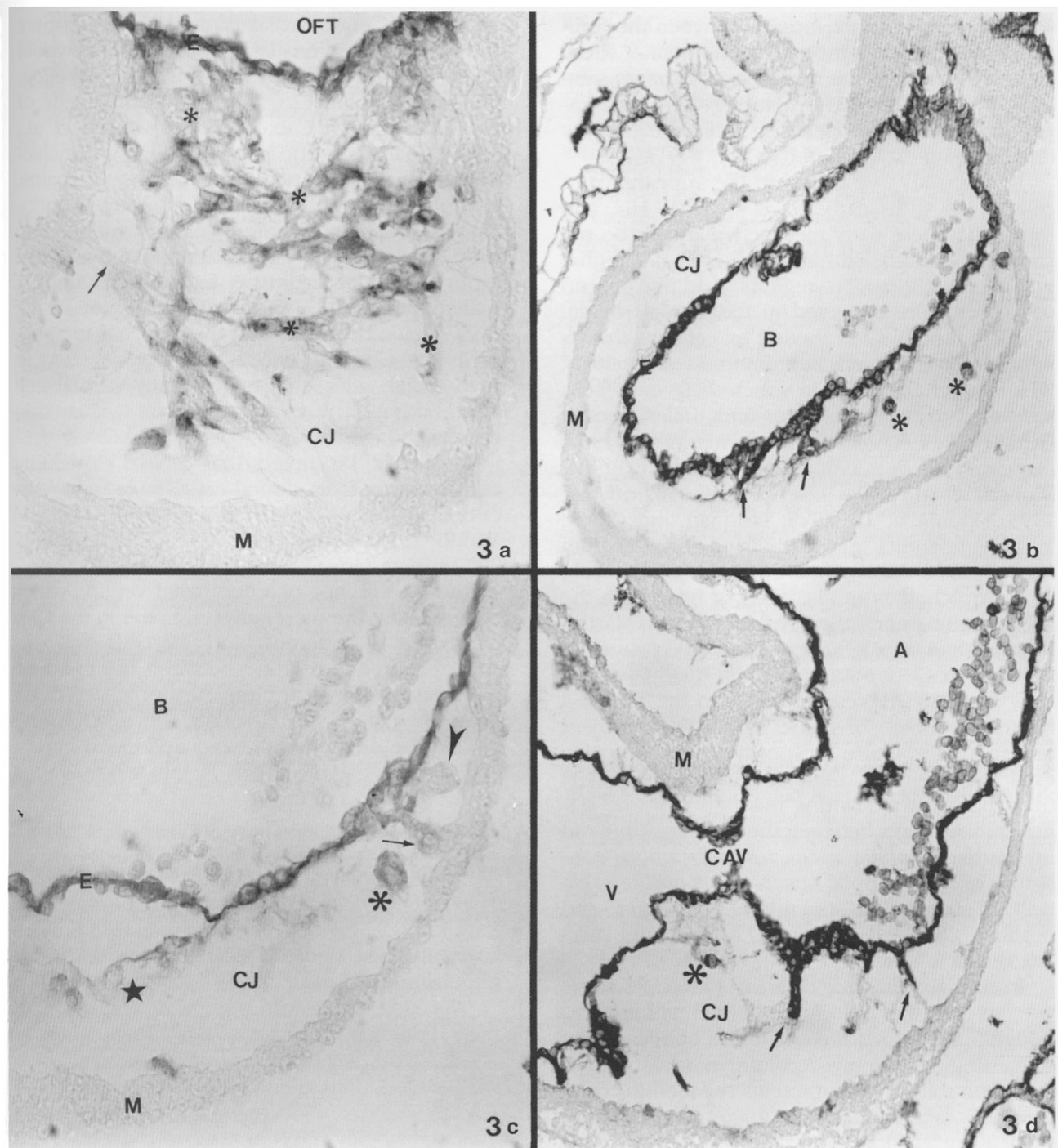
Fig. 3.– Stage 16 HH. Transversal sections.
    a: Outflow tract. Asterisks show a mesh of QH1-positive and QH1-negative cells in the CJ forming cellular links (arrow). 100x
    b: Bulbar region. Arrows show small groups of QH1-positive cells. Asterisks show free QH1-positive cells in the CJ. 50x
    c: Bulbar region. Asterisk shows a QH1-positive cell. Star shows negative cells. Arrow shows a positive and negative bridge bet-
ween the endocardium and the myocardium. Arrowhead shows a group of QH1-negative cells next to the endocardium. 100x
    d: Atrioventricular canal. Arrows show small groups of QH1-positive cells. Asterisk shows a free QH1-positive cell in the CJ.
50x

DISCUSSION

We have studied the presence of cells in the CJ at stages 9+, 13 and 16 HH by using MAB QH1 (Pardanaud et al., 1987) in order to determine when the first cells appear in the CJ and to gain insight into their potential endothelial lineage.

At stage 9+ HH, endocardial tubes begin to show signs of remodeling. Next to the aortic sac QH1-positive cells are located between the foregut epithelium and the splanchnopleura. These cells are embryonic angioblasts that move ventrally around the pharynx during the initial stages of heart morphogenesis, while the outflow tract is being established (Noden, 1991).

In our study, we observed the appearance of cells in the CJ as early on as stage 9+ HH, and found that these early mesenchymal cells do not show the endothelial marker used in the cephalic and middle cardiac regions. Additionally, no cell types were observed in the caudal region. Therefore, quail CJ is seeded by cells much earlier on than in the chicken embryo (stages 16-17 HH, Krug et al., 1985), in which these early cells are believed to be of either endothelial lineage (Krug et al., 1985; Fitzharris and Markwald, 1982; Markwald et al., 1990; Isokawa et al., 1994; Wunsch et al., 1994; Eisenberg and Markwald, 1995; Sinning et al., 1995; Fishman and Chien, 1997) or could proceed from myocardium (Chang, 1932; Adams Smith, 1963; Argüello et al., 1978; Arrechedera et al., 1984) or from both the myocardium and the endocardium (Martín-Rodríguez et al., 1993).

The first QH1-positive cells in quail CJ appear at stage 13 HH, earlier still than the situation described above for chicks. From this moment onwards, a mixture of positive and negative QH1 cells remains until at least stage 35 HH (data not shown). During this period in the stages studied, links between the endocardium and myocardium appear in the form of bridges consisting of a mixture of reactive and non-reactive cells. At stage 16 HH, the number of positive and negative QH1 cells in the CJ, bridges and meshes is much greater.

In sum, the first free cells in CJ are QH1-negative, as are the myocardial prolongations and bridges, which are linked to the endocardium, suggesting that the myocardium, in addition to its induction role by glycoproteins released into cardiac jelly (Markwald et al., 1990; Wunsch et al. 1994; Krug et al., 1995), could also induce the endocardium through the links between the two layers. Above all, this demonstrates that non-endocardial cells are part of mesenchymal tissue in CJ as from the early stages.

It has been suggested that during cardiac morphogenesis, at least some lateral plate mesoderm cells may share the characteristics of the endocardial and myogenic phenotypes during this time period (Linask and Lash, 1993). Also, the common origin of some endocardial and myocardial cells has been demonstrated by culturing QCE-6 cardiac mesoderm cells (Eisenberg and Bader, 1995 and 1996). Recent studies have demonstrated that, in culture, mesenchymal cells may show double staining: one for endocardial phenotype (QH1), and other for myocardial phenotype (MF20) (Sugi and Markwald, 1996).

That the endocardial layer does not display the same characteristics throughout the whole heart has been shown by JB3 antibody labeling, which is assumed to stain endocardial cells of the AVC and OFT exclusively (Wunsch et al., 1994). Using specific antibodies for adult chicken atrial and ventricular myosin heavy chains, De Jong et al. (1990) have also reported endocardial cells that bear the isomyosin epitope and have speculated about their possible myocardial origin and later migration into the CJ. Additionally, at stage 9 HH Martín-Rodríguez et al. (1993) have described that the first cells to appear in the CJ show a cell phenotype related to myocardial cells, suggesting a myocardial cell contribution to mesenchymal tissue formation in the early stages.

It should be noted that HNK-1-expressing cells, coming from neural crest, have been described in the CJ (Luider et al., 1993; Kirby and Waldo, 1995; Noden et al., 1995).

Our data indicate that the first cells to appear in the CJ are not of endothelial origin, which suggests that the mesenchymal tissue in the CJ is a mixture of different kinds of cells and that there are frequent links between the endocardium and the myocardium. Further in-depth studies are required to elucidate the relationships among the different cell types addressed here.

REFERENCES

ADAMS SMITH WN (1963). The site of action of trypan blue in cardiac teratogenesis. *Anat Rec*, 147: 507-524.

ARGÜELLO C, DE LA CRUZ MV and SÁNCHEZ C (1978). Ultrastructural and experimental evidence of myocardial cell differentiation into connective tissue cells in embryonic chick heart. *J Mol Cell Cardiol*, 10: 307-315.

ARRECHEDERA H, STRAUSS MC, ARGÜELLO C, AYESTA C and ANSELMI G (1984). Ultrastructural study of the myocardial wall of the atrioventricular canal during the development of the embryonic chick heart. *J Mol Cell Cardiol*, 16: 885-895.

BOLENDER D and MARKWALD RR (1979). Epithelial-mesenchymal transformation in atrioventricular cushion tissue morphogenesis. *Scanning Electron Microscopy*, 3: 313-321.

CHANG C (1932). On the reaction of the endocardium to the bloodstream in the embryonic heart, with special reference to the endocardial thickenings in the atrioventricular canal and the bulbus cordis. *Anat Rec*, 5: 253-265.

DE JONG F, GEERTS WJC, LAMERS WH, LOS JA and MOORMAN AFM (1990). Isomyosin expression pattern during formation of the tubular chicken heart: a three-dimensional immunohistochemical analysis. *Anat Rec*, 226: 213-227.

DE LA CRUZ MV, MUÑOZ-ARMAS S and MUÑOZ-CASTELLANOS L (1972). Development of the chick heart. The Johns Hopkins University Press. London.

EISENBERG CA and BADER D (1995). QCE-6: A clonal cell line with cardiac myogenic and endothelial cell potentials. *Dev Biol*, 167: 469-481.

EISENBERG CA and BADER D (1996). Establishment of the mesodermal cell line QCE-6. *Circ Res*, 78: 205-216.

EISENBERG LM and MARKWALD RR (1995). Molecular regulation of atrioventricular valvuloseptal morphogenesis. *Circ Res*, 77: 1-6.

FISHMAN MC and CHIEN KR (1997). Fashioning the vertebrate heart: earliest embryonic decisions. *Development*, 124: 2099-2117.

FITZHARRIS TP and MARKWALD RR (1982). Cellular migration through the cardiac jelly matrix: a stereoanalysis by high-voltage electron microscopy. *Dev Biol*, 92: 315-329.

GHOSH S and BRAUER PR (1996). Latent transforming growth factor-β is present in the extracellular matrix of embryonic hearts in situ. *Dev Dyn*, 205: 126-134.

HAMBURGER V and HAMILTON HL (1951). A series of normal stages of the chick embryo. *J Morphol*, 88: 49-92.

ISOKAWA K, REZAEE M, WUNSCH A, MARKWALD RR and KRUG EL (1994). Identification of transferrin as one of multiple EDTA-extractable extracellular proteins involved in early chick heart morphogenesis. *J Cell Biochem*, 54: 207-218.

KIRBY ML and WALDO KL (1995). Neural crest and cardiovascular patterning. *Circ Res*, 77: 211-215.

KRUG EL, REZAEE M, ISOKAWA K, TURNER DK, LITKE LL, WUNSCH AM, BAIN JL, RILEY DA, CAPEHART AA and MARKWALD RR (1995). Transformation of cardiac endothelium into cushion mesenchyme is dependent on ES/130: temporal, spatial, and functional studies in the early chick embryo. *Cell Mol Biol Res*, 41 (4): 263-277.

KRUG EL, RUNYAN RB and MARKWALD RR (1985). Protein extracts from early embryonic hearts initiate cardiac endothelial cytodifferentiation. *Dev Biol*, 112: 414-426.

LINASK KK and LASH JW (1993). Early heart development: dynamics of endocardial cell sorting suggests a common origin with cardiomyocytes. *Dev Dyn*, 195: 62-69.

LUIDER TM, BRAVENBOER N, MEIJERS C, VAN DER KAMP AWM, TIBBOEL D and POELMANN RE (1993). The distribution and characterization of HNK-1 antigens in the developing avian heart. *Anat Embryol*, 188: 307-316.

MARKWALD RR, FITZHARRIS TP and ADAMS SMITH WN (1975). Structural analysis of endocardial cytodifferentiation. *Dev Biol*, 42: 160-180.

MARKWALD RR, MJAATVEDT CH and KRUG EL (1990). Induction of endocardial cushion tissue formation by adheron-like, molecular complexes derived from the myocardial basement membrane. In: Clark EB and Takao A, (eds). *Developmental Cardiology: Morphogenesis and Function*. Mount Kisco, NY, Futura Publishing Co., Inc. pp. 191-204.

MARTÍN-RODRÍGUEZ JC, PÉREZ-MIGUELSANZ J, MAESTRO C, MURILLO J and PUERTA J (1993). Study of the first cells seen in the cardiac jelly using monoclonal antibody 13F4. *Cardiol Young*, 3, Suppl.: 125.

MJAATVEDT CH, KRUG EL and MARKWALD RR (1991). An antiserum (ES1) against a particulate form of extracellular matrix blocks the transition of cardiac endothelium into mesenchyme in culture. *Dev Biol*, 145: 219-230.

NODEN DM (1991). Origins and patterning of avian outflow tract endocardium. *Development*, 111: 867-876.

NODEN DM, POELMANN RE and GITTENBERGER-DE GROOT AC (1995). Cell origins and tissue boundaries during outflow tract development. *Trends Cardiovasc Med*, 5: 69-75.

PARDANAUD L, ALTMAN C, KITOS P, DIETERLEN-LIEVRE F and BUCK C (1987). Vasculogenesis in the early quail blastodisc as studied with a monoclonal antibody recognizing endothelial cells. *Development*, 100: 339-349.

REZAEE M, ISOKAWA K, HALLIGAN N, MARKWALD RR and KRUG EL (1993). Identification of an extracellular 130-kDa protein involved in early cardiac morphogenesis. *J Biol Chem*, 268: 14404-14411.

SIEBER-BLUM M and ITO K (1995). In vitro clonal analysis of cardiac outflow tract mesenchyme. *Ann N Y Acad Sci*, 27. 752:92-100.

SINNING AR, HEWITT CC and MARKWALD RR (1995). A subset of sba lectin-binding proteins isolated from myocardial-conditioned media transforms cardiac endothelium into mesenchyme. *Acta Anat*, 154: 111-119.

SINNING AR and HEWITT CC (1996). Identification of a 283-kDa protein component of the particulate matrix associated with cardiac mesenchyme formation. *Acta Anat*, 155: 219-230.

SINNING AR, KRUG EL and MARKWALD RR (1992). Multiple glycoproteins localize to a particulate form of extracellular matrix in regions of the embryonic heart where endothelial cells transform into mesenchyme. *Anat Rec*, 232: 285-292.

SUGI Y and MARKWALD RR (1996). Formation and early morphogenesis of endocardial endothelial precursor cells and the role of endoderm. *Dev Biol*, 175: 66-83.

WUNSCH AM, LITTLE CD and MARKWALD RR (1994). Cardiac endothelial heterogeneity defines valvular development as demonstrated by the diverse expression of JB3, an antigen of the endocardial cushion tissue. *Dev Biol*, 165: 585-601.

ZACCHEI AM (1960). Lo sviluppo embrionale della quaglie giapponese. *Archivo di Anatomia*, 66: 36-61.

ABBREVIATION LIST

A: Primitive Auricle
B: Bulbus Cordis
BV: Bulbo-ventricle
CAV: Atrioventricular Canal
CJ: Cardiac Jelly
E: Endocardium
F: Foregut
M: Myocardium
N: Notochord
OFT: Outflow Tract
TN: Neural Tube
V: Primitive Ventricle