THE JOURNAL OF EXPERIMENTAL ZOOLOGY 275:95–111 (1996)

Development of the Subepicardial Mesenchyme and the Early Cardiac Vessels in the Dogfish (*Scyliorhinus canicula*)

RAMÓN MUÑOZ-CHÁPULI, DAVID MACÍAS, CASTO RAMOS, ALEJANDRO GALLEGO, AND VICTORIA DE ANDRÉS
Department of Animal Biology, Faculty of Science, University of Málaga, 29071 Málaga, Spain

ABSTRACT A study was designed to assess the development of the subepicardial mesenchyme and the early cardiac vessels in the elasmobranch dogfish (*Scyliorhinus canicula*). The findings showed that the subepicardial mesenchymal cells originated, at least in part, from the primitive epicardium. This process seemed to be more intense where the subepicardium was the widest, namely, at the atrioventricular and conoventricular grooves as well as at the lateral margins of the ventricle. At these sites, the epicardial cells displayed morphological features usually related to epithelial-mesenchymal transitions, i.e., cell hypertrophy, motility-like basal appendages, cell overlapping, intercellular gaps, and acquisition of a secretory phenotype. The epicardial cells which covered other parts of the heart were flattened and showed smaller nuclei; their basal surface was fibronectin-immunoreactive, unlike that of the hypertrophied epicardial cells of the atrioventricular groove. Fibronectin immunoreactivity also developed in the subepicardial space as the mesenchymal cell population increased.

In the dogfish, a subepicardial network of capillaries developed subsequent to the epicardial covering of the heart. Before this network was established, numerous capillary-like structures were present in the subepicardial space. These capillary-like structures appeared as single cells with a large vacuole or as connections of cytoplasmic processes of one or several cells by junctional complexes. The cells that formed the capillary-like structures probably originated from the subepicardial mesenchymal cells. The main ultrastructural difference between the mesenchymal cells and the capillary-like structures was the presence, in the latter, of membrane-bound, electron-dense cytoplasmic inclusions 0.2–1.0 μm in diameter. Morphological evidence suggested that both the subepicardial capillary plexus and the endothelial precursors of the adult cardiac veins resulted from the coalescence of capillary-like structures.

In the embryos of higher vertebrates whose cardiac development is known, the epicardial investment of the heart is closely followed by the successive appearance of a subepicardial acellular space between the primitive epicardium and the myocardium, numerous subepicardial mesenchyme cells and a network of subepicardial capillaries.

Little is known regarding the origin of the embryonic subepicardial mesenchyme. Some accounts claim that a mesenchyme "appears in" or "invades" the subepicardial space shortly after the epicardial investment (Hiruma and Hirakow, '89; Hirakow, '92; Tidball, '92). Others suggest that it is formed by delamination from the epicardium (Icardo et al., '90). A recent study has shown that the subepicardial mesenchyme in the quail has a double origin, from the epicardium and from extracardiac proepicardial tissue (Viragh et al., '93).

Regarding the fate of the subepicardial mesenchyme, the involvement of mesenchymal cells in the formation of capillaries has been proposed (Viragh and Challice, '81; Tokuyasu, '85; Viragh et al., '90; Bolender et al., '90; Icardo et al., '90). However, other authors have suggested that the subepicardial capillary plexus originates from endocardial sprouts, blood island structures or extracardiac angioblasts (reviewed in the discussion).

The main animal models for the study of these cardiogenetic processes have been the chick and quail embryos among the birds, and the mouse embryo among the mammals. There are only a few papers dealing with these matters in lower vertebrates. Only a few descriptions of the main

Address reprint requests to Ramón Muñoz-Chápuli, Department of Animal Biology, Faculty of Science, University of Málaga, 29071 Málaga, Spain.

features of the coronariogenesis in fishes are available (Lewis, '04; Nair, '70; De Andrés et al., '93). Furthermore, fish models currently used by developmental biologists, such as the zebrafish (*Brachydanio rerio*) or the ricefish (*Oryzias latipes*), lack a well-developed coronary system.

For these reasons, we have chosen an elasmobranch, the dogfish (*Scyliorhinus canicula*), as a fish model for the study of cardiogenetic processes. Some features of dogfish embryos are advantageous for the study of the development of heart vascularization, for example, the large size of the embryos, their slow development, and its well-developed coronary system (De Andrés et al., '93; Muñoz-Chápuli et al., '94).

The specific aims of this paper are: (1) to describe the development and morphological features of the subepicardial mesenchyme. To further analyse the process of the development of this mesenchyme, an immunohistochemical survey of the distribution of fibronectin in the embryonic subepicardium was carried out. (2) To study the possible involvement of the subepicardial mesenchymal cells in the development of the early cardiac vessels.

MATERIALS AND METHODS

Fifteen hearts from dogfish embryos were used for morphological studies. Their sizes were 17, 19 (n=2), 21, 22, 23, 27 (n=2), 28, 31, 33, 34, 40, 55, and 69 mm in total length (TL). Fibronectin immunohistochemistry was assessed in six embryos of 16, 24, 29, 31, 35, and 65 mm TL. The size of the embryos has proved to be well-correlated with the successive developmental stages of the dogfish (De Andrés et al., '93).

Fertilized eggs were obtained from adult females collected in the Bay of Malaga (western Mediterranean) by commercial trawl vessels. The eggs were kept in indoor tanks of well-aerated sea water which was monitored for nitrite, specific gravity and pH. Egg capsules were opened at intervals, and the embryos carefully removed, anaesthetised in 0.04% tricaine methanesulphonate (MS-222, Sigma, U.K.) in sea water and measured. Whole embryos were fixed for 2–3 h in freshly prepared 1.25% glutaraldehyde and 1% paraformaldehyde diluted in elasmobranch buffer (16.38 g/l NaCl, 0.89 g/l KCl, 1.11 g/l $CaCl_2$, 0.38 g/l $NaHCO_3$, 0.06 g/l NaH_2PO_4, 21.6 g/l urea, pH 7.2). The fixation was by immersion. The embryos of 28, 31 and 33 mm in total length were previously perfused with the fixative through the vitelline vein. The fixed embryos were washed in elasmobranch buffer, postfixed in 1% OsO_4 in the same buffer for 2 hours at 4°C, washed, dehydrated and embedded in Araldite 502. Thick sections 0.5 μm were obtained with an Ultracut E Reichert-Jung ultramicrotome and stained with toluidine blue. The embryos of 17, 23 and 34 mm TL were sagittally sectioned, while the others were sectioned in a transverse plane. Ultrathin sections were obtained from embryos of 27, 28 and 31 mm TL. These sections were contrasted with lead citrate and uranyl acetate, observed and photographed in a Jeol JEM-100CX transmission electron microscope.

Fibronectin immunoreactivity (FNIR) was assessed with an immunoperoxidase technique. The embryos were fixed for two hours in 4% paraformaldehyde, 0.08% glutaraldehyde and 15% picric acid in elasmobranch buffer. Then, the embryos were placed overnight in the same fixative without glutaraldehyde, dehydrated and embedded in paraffin wax. Transverse sections 10 μm in thickness were obtained at the level of the atrioventricular (AV) groove. Sections were dewaxed in xylene, hydrated and preincubated for 15 min with 3% H_2O_2 in TRIS-phosphate-buffered saline (TPBS; 10 mM TRIS-HCl, 8.3 mM Na_2HPO_4, 3.5 mM KH_2PO_4, 0.12 M NaCl, pH 7.8). The sections were then blocked for 90 min in GBT (2% goat serum, 1.5% bovine serum albumin in TPBS) and incubated for 16 h with the primary antiserum, anti-human plasma fibronectin diluted 1:200 in GBT. Controls were incubated with GBT instead of the primary antibody. The sections were washed three times in TPBS and incubated with the secondary antiserum, goat anti-rabbit IgG (1:20) for 45 min, washed, incubated with the peroxidase-antiperoxidase (PAP) complex (1:50) for 45 min, washed again and developed with 0.03% hydrogen peroxide and 0.2% 3,3′ diaminobenzidine. The reaction was stopped by washing and the sections were mounted. All the antisera were provided by Sigma (UK).

RESULTS

Development and structure of the subepicardial mesenchyme

A narrow subepicardial space developed in the embryo of 17 mm TL subsequent to the establishment of the first patches of epicardium at the atrioventricular (AV) canal and surrounding areas. This space, the subepicardium, was wide in the embryos of 19 mm TL around the AV canal, in the dorsal surface of the ventricle and in the ventral surface of the atrium (Fig. 1). In one em-

bryo of 19 mm TL, a contact existed between the subepicardial extracellular matrix and the cardiac jelly through a discontinuity of the ventral myocardial wall of the atrium (Fig. 1A).

A few mesenchymal cells were seen in the subepicardium of both embryos of 19 mm TL. Most of these cells were very close or in contact with the primitive epicardium of the AV junction or the lateral margins of the ventricle (Fig. 1A). Some epicardial cells from these areas were larger than the squamous epicardial cells which coated other parts of the heart, and they showed rounded nuclei and cytoplasmic processes towards the subepicardium (Fig. 1B).

Subepicardial mesenchymal cells were more numerous in the embryos of 21–23 mm TL, especially in the wide subepicardium existing around the AV and the conoventricular (CV) grooves (Fig. 2A) and, to a lesser extent, along the lateral ventricular margins. As described above, some epicardial cells from these areas displayed a characteristic appearance. They were large, with rounded nuclei, and bearing long basal cytoplasmic processes (Fig. 2A–C). Some images showed overlapping cells and others suggested that a translocation of epicardial cells to the subepicardium was occurring. A number of epicardial and mesenchymal cells of these areas were in mitosis (Fig. 2C). All these features were also exhibited by the epicardial cells of the embryos of 27–34 mm TL in the AV and CV grooves, and, to a lesser degree, along the lateral and ventral ventricular margins and the lateral margins of the conus arteriosus.

In contrast, the epicardial cells which covered other parts of the ventricle and atrium where the subepicardium was thin or nonexistent, were flat, with small, elongated nuclei and no cytoplasmic processes. Images of mitosis were infrequent in the primitive epicardium of these areas.

A number of myocardial discontinuities or gaps put the subepicardial matrix in contact with the cardiac jelly, especially in the ventral areas of the atrium and sinus venosus closer to the AV junction (Fig. 2D). Myocardial discontinuities were observed in all embryos between 19 and 33 mm TL.

The mesenchymal cells and the adjacent epicardial cells showed similar ultrastructural features (Fig. 3), namely, abundance of polyribosomes and rough endoplasmic reticulum, rounded and elongated mitochondria, clear vesicles and long cytoplasmic processes. Large intercellular spaces were present between the mesenchymal and the

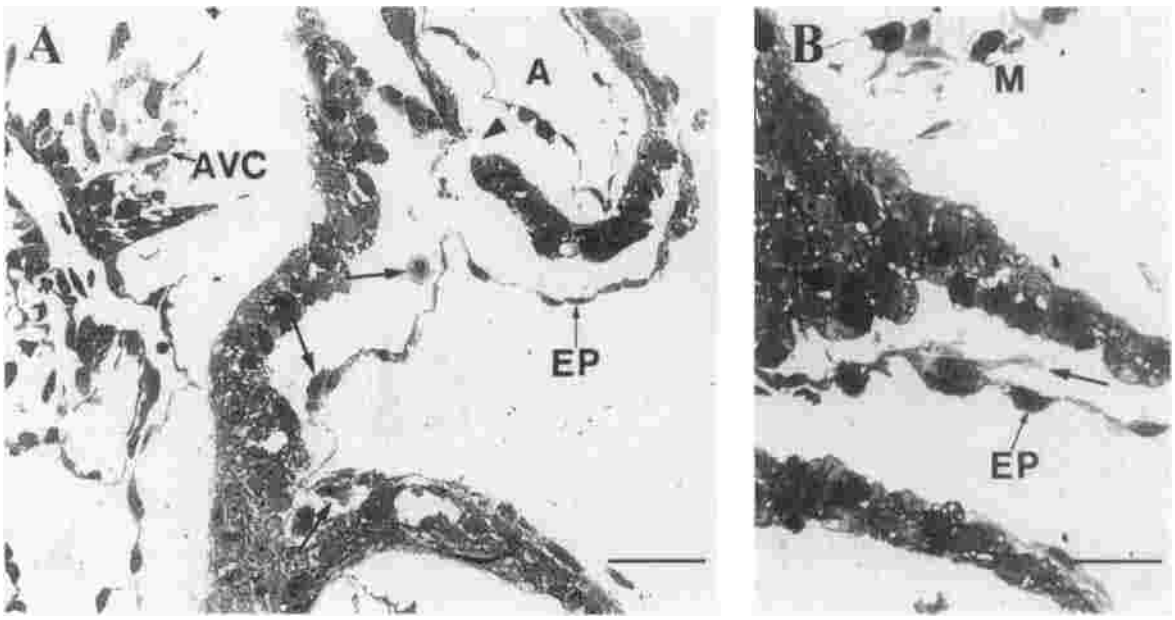

Fig. 1. Early stages of the development of the subepicardial mesenchyme. Transverse sections of the embryo of 19 mm total length (TL). **A:** A few mesenchymal cells (arrows) are present in the subepicardium of the right part of the atrioventricular groove, either free or adhered to the primitive epicardium (EP). Note the connection between the cardiac jelly and the subepicardium through a myocardial discontinuity of the ventral part of the atrium (A) (arrowhead). AVC, atrioventricular endocardial cushion. **B:** A basal appendage (arrow) in a cell of the ventral part of the atrium, cephalic to the atrioventricular junction. This cell is overlayed by the primitive epicardium (EP). M, mesenchymal cells of the anterior atrioventricular endocardial cushion. Scale bars = 50 μm for A; 25 μm for B.

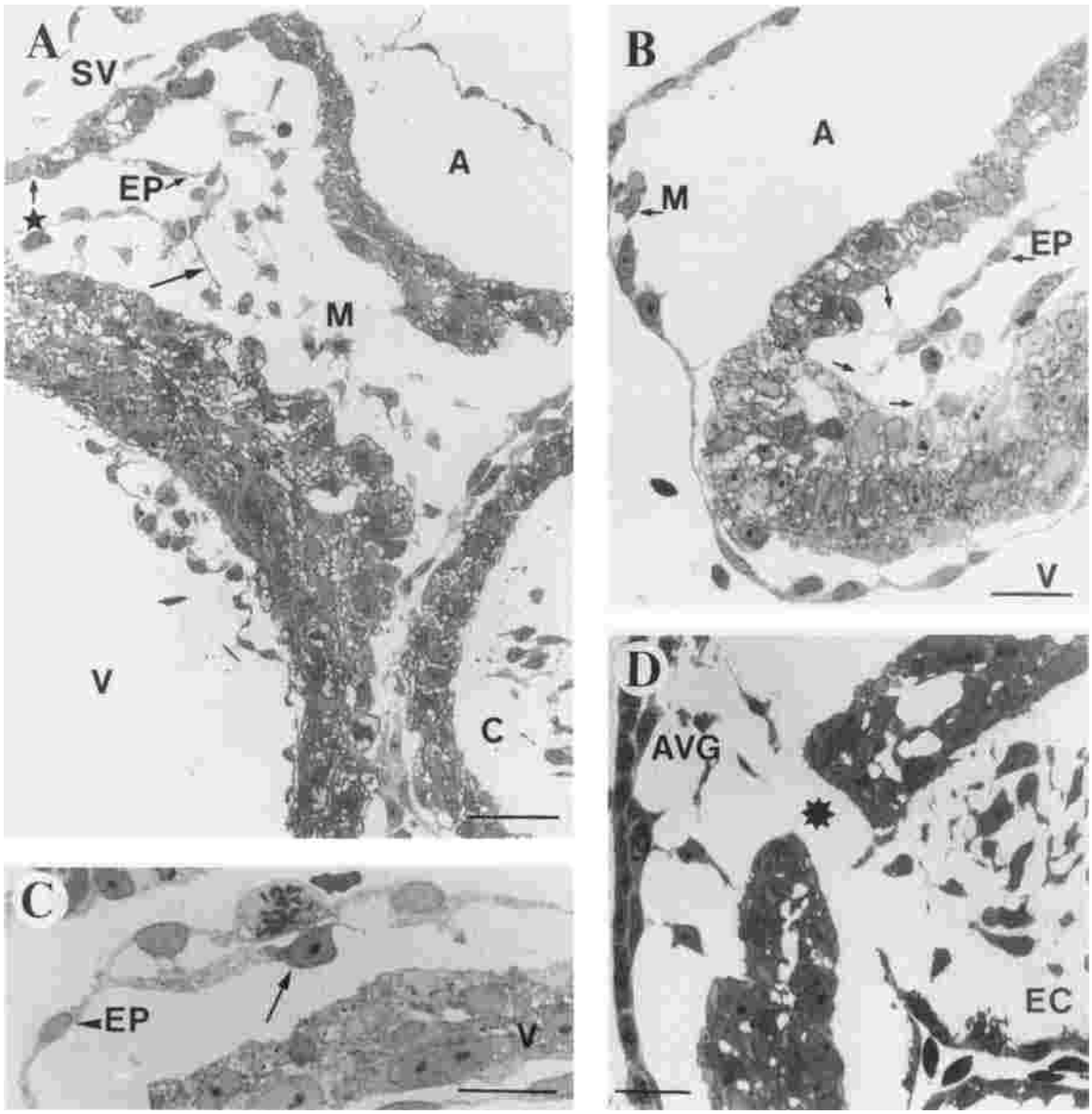

Fig. 2. Development of the subepicardial mesenchyme in embryos of 21–23 mm TL. **A:** Parasagital section at the level of the conus arteriosus (right part of the heart) in the embryo of 23 mm TL. The wide subepicardium existing around the atrioventricular and conoventricular grooves is populated by mesenchymal cells (M). A long basal appendage (arrow) arises from the primitive epicardium (EP) of the atrioventricular groove. The posterior boundary of the primitive epicardium on the sinus venosus (SV) is indicated by a star. A, atrium; V, ventricle; C, conus arteriosus. **B:** Transverse section at the level of the right part of the atrioventricular groove in an embryo of 22 mm TL. Some cells of the primitive epicardium (EP) are hypertrophied and show basal appendages (arrows). Some mesenchymal cells (M) are differentiating in the atrioventricular endocardial cushion. A, atrium; V, ventricle. **C:** Transverse section. Right ventricular margin of the 22 mm TL embryo. There is an apparent translocation of an epicardial cell (arrow) towards the subepicardial space, close to a mitotic cell in the primitive epicardium (EP). V, ventricular myocardium. **D:** Embryo of 21 mm TL. Transverse section. There is a large discontinuity in the left myocardial wall of the atrium (star), close to the atrioventricular junction. Mesenchymal cells are present in the subepicardium of the left atrioventricular groove (AVG) as well as in the posterior atrioventricular endocardial cushion (EC). Scale bars = 50 μm for A; 25 μm for B–D.

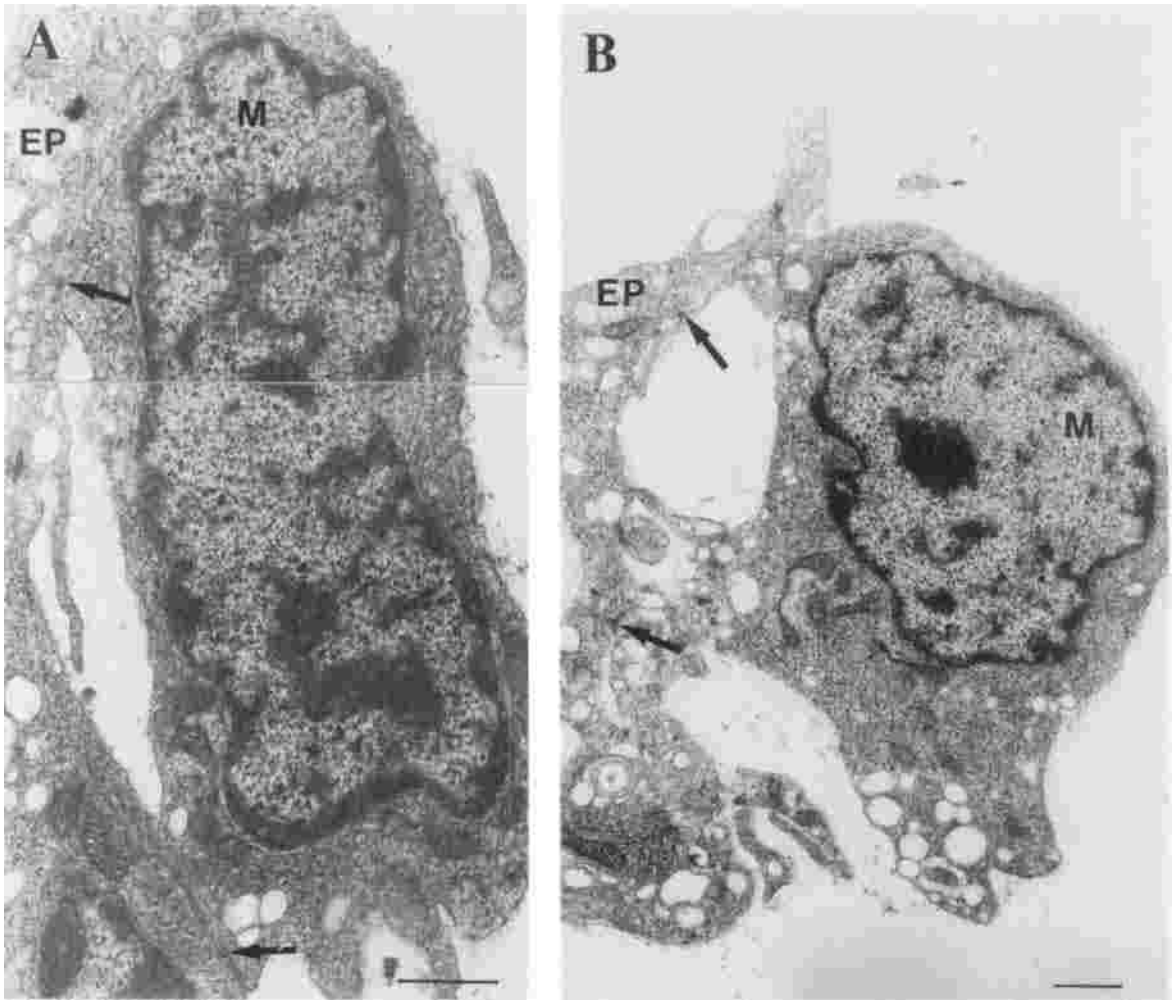

Fig. 3. Ultrastructure of the early mesenchymal cells in the embryos of 31 (**A**) and 28 (**B**) mm TL. Both mesenchymal cells (M) are joined to the primitive epicardium (EP) through small intercellular junctions (arrows). Mesenchymal and adjacent epicardial cells show similar ultrastructural features, including extensive rough endoplasmic reticulum and abundant polyribosomes, mitochondria and clear vesicles. Large intercellular spaces are present between the mesenchymal and the epicardial cells. Scale bars = 1 μm.

epicardial cells, which were connected through small intercellular junctions. Although mesenchymal cells were sometimes adjacent to the myocardial cells, no junctional complexes were noticed between them.

The primitive epicardium progressively lost its areas of rounded cells in the larger embryos studied. Only a few cells with these traits were present in the ventral ventricular margin of the 55 mm TL embryo. The epicardial cells of the 69 mm TL embryo showed a flattened, squamous appearance.

Development of capillary-like structures

Some capillary-like structures were present in the subepicardium of the AV and CV groove and along the lateral ventricular margins of the embryos of 21–23 mm TL (Fig. 4A). In the embryos of 27–31 mm TL, the capillary-like structures were numerous throughout the subepicardium (Figs. 4B–D and 5–7). In some cases, these structures consisted of cells containing a single large vacuole (Figs. 4A, 5, 6A and 7). These vacuoles were usually placed at one end of the cell and were covered by a more or less thin layer of cytoplasm. Some images suggested that the large vacuoles of two or more cells could merge in a common cavity. Figure 6B shows a ring formed by four cells, one of them apparently bearing a large vacuole not covered by cytoplasm in its luminal boundary. The structure shown in Figure 7 might also

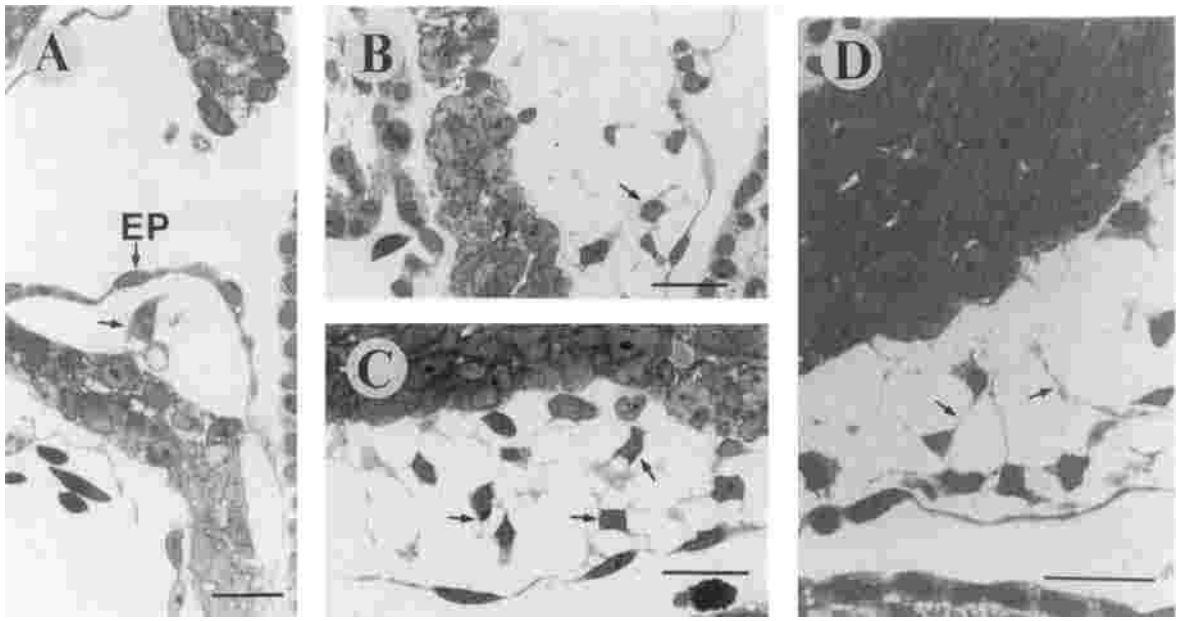

Fig. 4. Capillary-like structures. Transverse sections. **A:** An early capillary-like structure (arrow) at the right ventricular margin of the embryo of 22 mm TL. This structure is formed by two contacting vacuolated cells. The photomicrograph shows the vacuoles and the bilobed nucleus of one of the cells. EP, primitive epicardium. **B–D:** Capillary-like structures (arrows) in the subepicardium of the left part of the atrioventricular groove (B) and the ventral part of the conoventricular groove (C, D) of an embryo of 27 mm TL. The structures shown in B and C are formed by one or two cells, while the large rings displayed in D are formed by the connection of cytoplasmic processes of several cells. Scale bars = 25 μm.

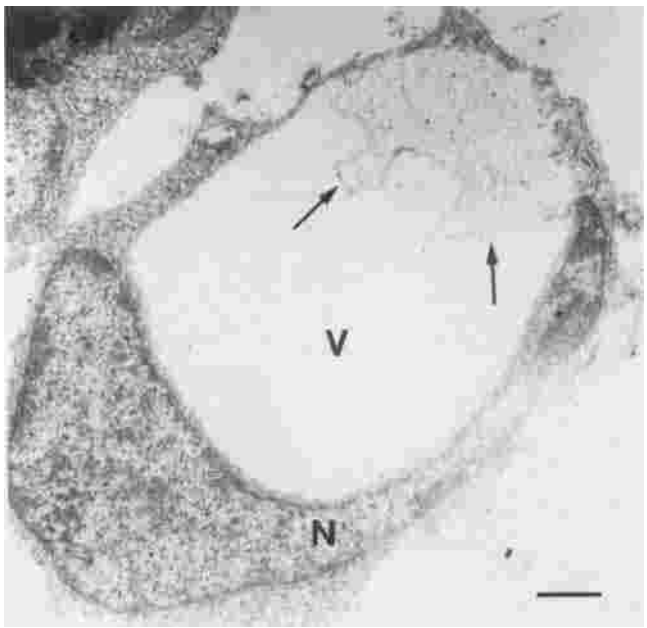

Fig. 5. Capillary-like structure in the embryo of 31 mm TL. This cell shows a large vacuole (V) and a nucleus (N) peripherally located. The vacuolar membrane (arrows) seems to be partially collapsed in an area where the cytoplasm is very thin. Scale bar = 1 μm.

be interpreted as a large vacuole partially encircled by another cell.

A second kind of capillary-like structure was formed by the assembling of the cytoplasmic processes from two or more cells (Fig. 4B–D). These structures, more frequent in the AV and CV grooves, showed intercellular junctions (Fig. 6C).

The cells which constituted the capillary-like structures and the subepicardium mesenchymal cells displayed only a few ultrastructural differences. Most remarkable was the presence, in the capillary-like cells, of membrane-bound, electron-dense vesicles with heterogeneous content and circular, elliptical or irregular shape (Figs. 6 and 7C). The largest diameter of these vesicles ranged between 0.2 and 1.0 μm. The survey of consecutive semithin sections demonstrated that the capillary-like structures formed no continuous vessels.

The sinus venosus, as well as the conus arteriosus, was already covered by epicardial cells in the embryos of 27–28 mm TL. However, some areas of the most posterior part of the sinus venosus were only composed of myocardial and endocardial cells, with no mesothelial lining. Except for the sinoatrial junction, there was no conspicuous space between epicardium and

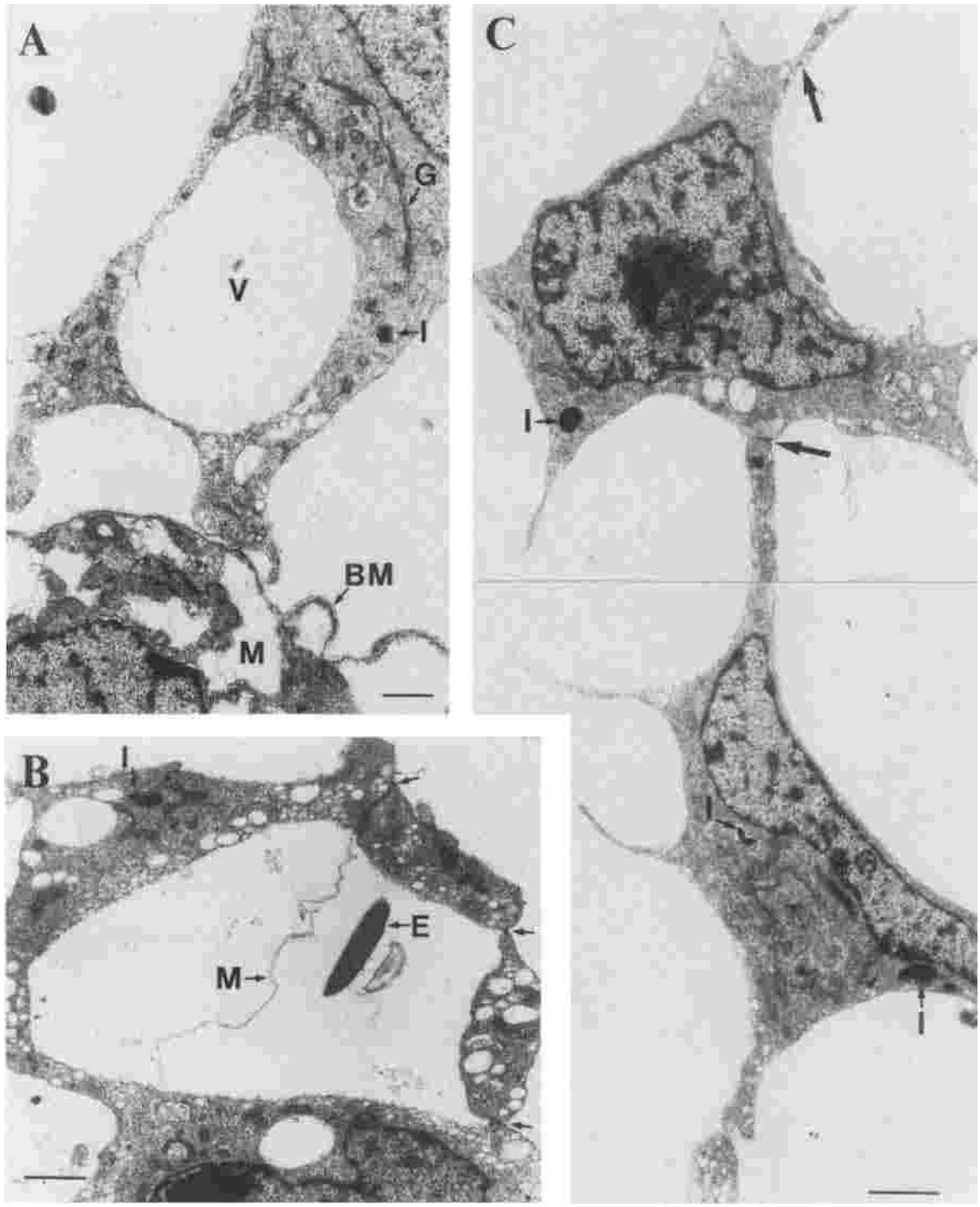

Fig. 6. Capillary-like structures in the embryo of 28 mm TL. **A:** A single cell with a large vacuole (V) close to the myocardial cell layer (M). The vacuolated cell shows abundant mitochondria, rough endoplasmic reticulum, clear vesicles, a well-developed Golgi apparatus (G) and some membrane-bound, electron-dense cytoplasmic inclusions (I). Note the basement membrane (BM) of the myocardial cell layer. **B:** Capillary-like structure formed by the coalescence of four cells. Junctional complexes are indicated by arrows. The membrane observed inside the structure (M) seems to be that of a large vacuole from the cell in the upper left corner. A membrane-bound, electron-dense cytoplasmic inclusion (I) is conspicuous in this cell. Note the presence of a probable erythrocyte (E) in the lumen of a capillary-like structure. **C:** Part of a capillary-like structure similar to the rings shown in Figure 4D. Several cells are connected by intercellular junctions (arrows). Note the presence of membrane-bound, dense inclusions (I) with heterogeneous content. Scale bars = 1 μm for A; 0.5 μm for B; 2 μm for C.

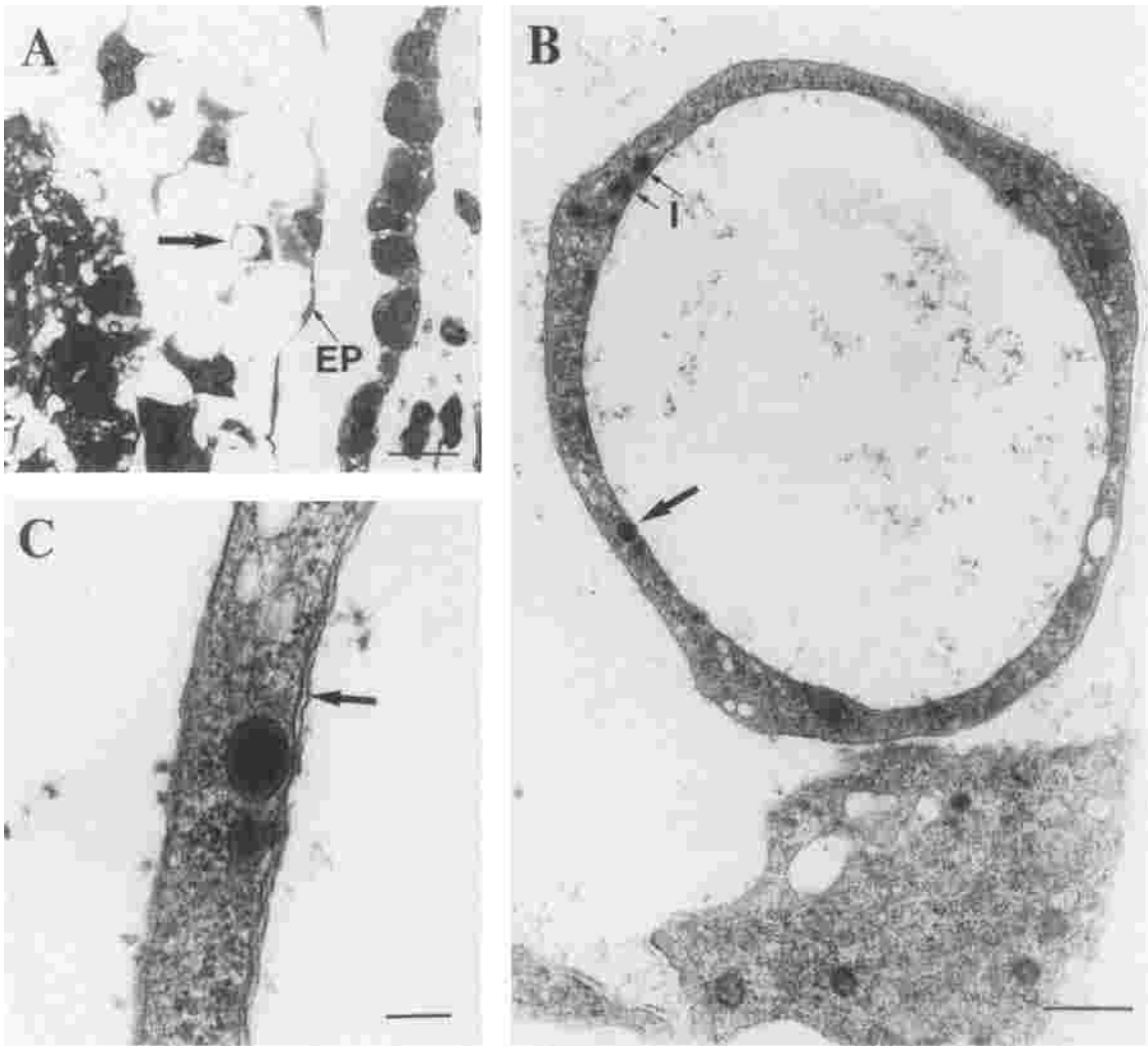

Fig. 7. Capillary-like structure in the 28 mm TL embryo. The arrow in **A** shows the location of this structure at the subepicardium of the atrioventricular groove. EP, primitive epicardium. **B:** This structure resembles a true capillary, but it is probably formed by a large vacuole of the cell labeled with a star wholly encircled by another cell which shows membrane-bound, dense inclusions (I and large arrow). **C:** Higher magnification of the cytoplasmic inclusion labelled with the large arrow in **B**. The vacuolar membrane of the inner cell (arrow) is conspicuous and distinguishable from the cytoplasmic membrane of the outer cell. Scale bars = 10 μm for A; 1 μm for B; 0.2 μm for C.

myocardium in the sinus venosus of these and other larger embryos.

Development of subepicardial capillaries and cardiac veins

In one of the embryos of 27 mm TL, a true vessel was present in the subepicardium of the most posterior part of the AV groove. This vessel was open to the lumen of the ventral part of the sinus venosus through a myocardial discontinuity (Fig. 8A). The vessel split in two dead-ended branches which were located under the left sinoatrial junction and at the right part of the AV groove, respectively (Fig. 8B). The end of the branches was apparently not formed by endocardial cells. Instead, their most terminal cells displayed morphological features similar to those of the subepicardial mesenchymal cells and the capillary-like structures (Fig. 8C). Another vessel was placed at the anterior and right part of the AV groove, near the CV junction. This vessel contained a few red blood cells, but it was not apparently connected to the above-mentioned vessel nor to the cardiac lumen (Fig. 8D).

Examination of the other embryo of 27 mm TL and the embryo of 31 mm TL revealed some vas-

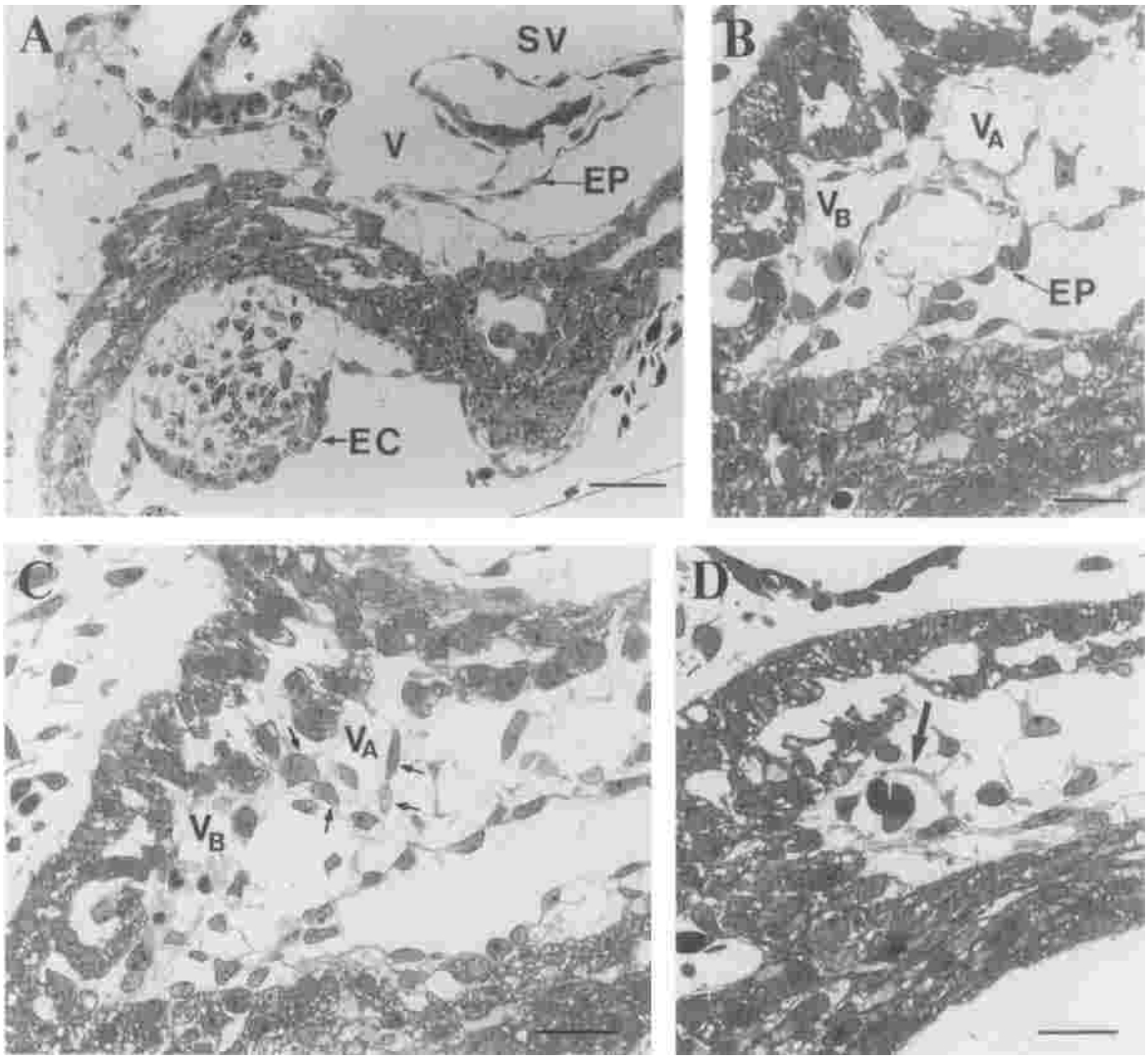

Fig. 8. Putative precursors of the cardiac veins in an embryo of 27 mm TL. Transverse sections. **A:** A vessel (V) located in the subepicardium of the right and posterior part of the atrioventricular groove opens to the sinus venosus (SV) through a myocardial discontinuity. EC, posterior atrioventricular endocardial cushion; EP, primitive epicardium. **B:** The vessel shown in A has split in two vascular structures (V_A and V_B) at the right part of the atrioventricular groove. EP, primitive epicardium. **C:** The anterior rim of V_A is apparently formed by mesenchymal cells (arrows). V_B also disappears some sections beyond this. **D:** Another vascular structure (arrow), containing red blood cells, is present at the right anterior part of the atrioventricular groove. Scale bars = 50 μm for A; 25 μm for B–D.

cular structures located at the subepicardium of the ventral sinoatrial junction, AV and CV grooves. The lack of material precluded checking whether these vessels were connected or not to the cardiac lumen. No differentiated vessels were present in the subepicardium of the embryo of 28 mm TL, although capillary-like structures were abundant.

Embryos larger than 33 mm TL showed a clear decrease of the subepicardial volume in relation to the whole cardiac volume, especially at the intercameral grooves. The subepicardium was always populated by mesenchymal cells, capillary-like structures and apparently true capillaries containing red blood cells (Fig. 9A).

Two subepicardial annular vessels were present, respectively, around the AV and CV grooves of the 33 mm TL embryo (Fig. 9B–D). These vascular rings contacted each other in the subepicardium existing between the AV and the CV grooves. They were easily distinguishable from the capillaries by their wider lumen. Their walls were formed by a single layer of endothelial-like cells, although

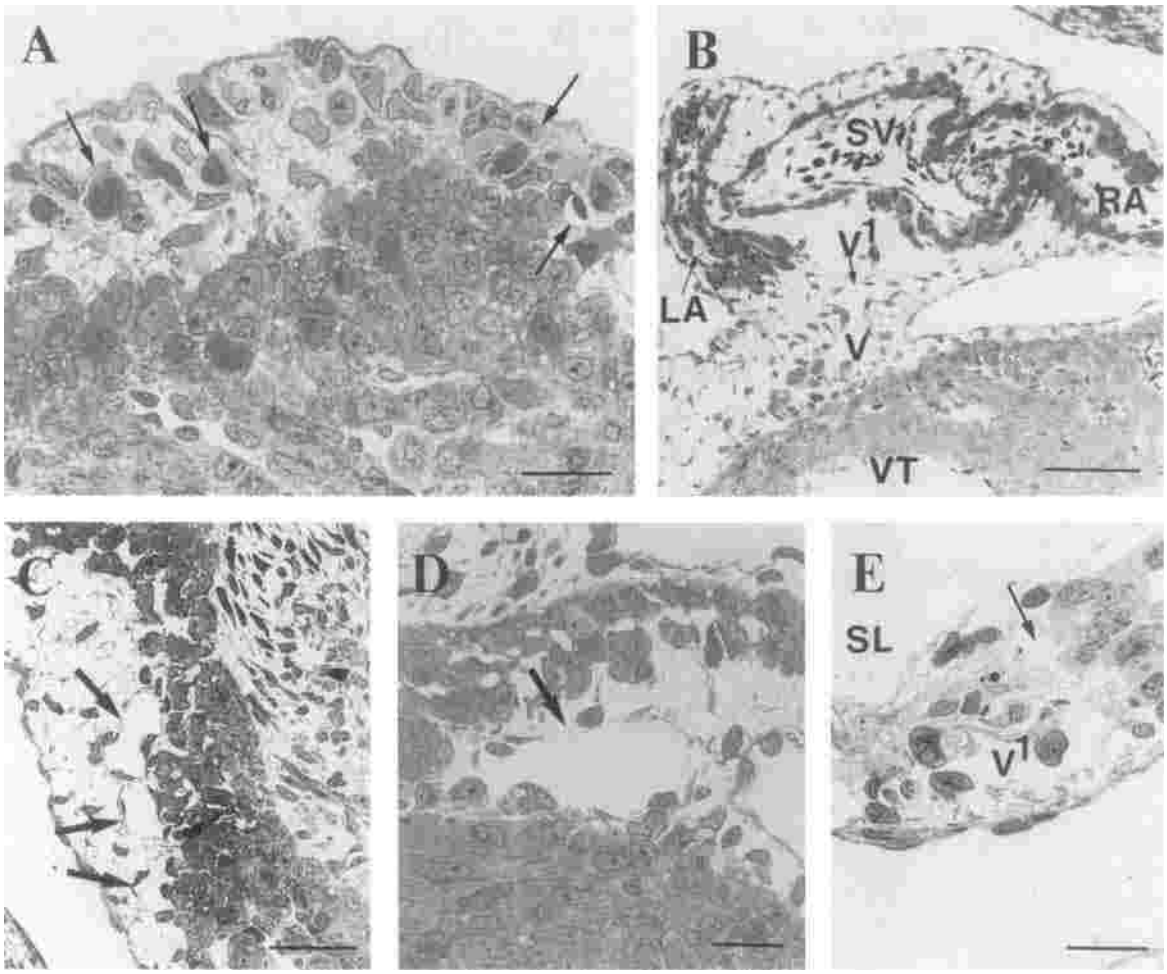

Fig. 9. Early subepicardial vessels in an embryo of 33 mm TL. Transverse sections: **A:** Putative subepicardial capillaries (arrows) in the posterodorsal area of the ventricle. Blood cells are frequent inside these vessels. **B:** A subepicardial annular vessel (V) surrounds the subepicardium of the atrioventricular groove, and it is connected with another vessel (V^1) in the posterior and right part of the atrioventricular junction. SV, sinus venosus; LA, RA, left and right posterior horns of the atrium, respectively; VT, ventricle. **C,D:** The annular vessel which surrounds the atrioventricular groove split in three branches in the right part (arrows in C) and its wall shows some discontinuities in the left part (arrow in D). **E:** The vessel V^1 shown in B ends a few sections beyond this, at the subepicardium of the sinoatrial junction, close to a myocardial discontinuity (arrow). Thus, no connection was recorded between these putative precursors of the cardiac veins and the sinus venosus lumen (SL). Scale bars = 25 μm for A, D, E; 100 μm for B; 50 μm for C.

other cells were occasionally adhered to their outer side. At the left part of the AV groove, the vascular ring split into two or three smaller branches (Fig. 9C). The vessel wall showed discontinuities in some points (Fig. 9D). Blood cells were rare in the lumen. These vascular rings, located around the intercameral grooves, are probably the precursors of the adult main cardiac veins, as discussed below.

A vessel located at the right and posterior part of the AV subepicardium contacted twice, at two different levels, with the AV vascular ring (Fig. 9B). This vessel ran backwards from these anastomoses to the ventral subepicardium of the sinoatrial groove, where it ended near to a myocardial discontinuity (Fig. 9E). Thus, no contact was recorded in this embryo between the system of developing cardiac veins and the cardiac lumen.

In the embryos of 34–69 mm TL, the developing cardiac veins drained into the sinus venosus lumen through an opening located at the ventral wall of this cardiac chamber, close to the base of the left sinoatrial valve (Fig. 10). Other veins were present at the lateral and ventral margins of the ventricle as well as running along the lateral margins of the conus arteriosus. All these veins

Fig. 10. The star shows the opening of the atrioventricular cardiac vein (AV) to the sinus venosus (SV) in the embryo of 55 mm TL. Transverse section. SA, base of the left sinoatrial valve; A, left posterior horn of the atrium; V, ventricle. Scale bar = 100 μm.

drained either into the CV or the AV annular veins. This anatomical arrangement of the cardiac veins conformed to that of the adult dogfish (unpublished observations).

Fibronectin immunoreactivity

Fibronectin-like immunoreactivity was assessed in transverse sections obtained at the level of the AV canal (Fig. 11). No significant labelling was found in the cardiac tube of the 16 mm TL embryo, when the primitive epicardium was just beginning to cover the heart. In the embryo of 24 mm TL, when the atrium and ventricle are covered by the epicardium, FNIR was present at the basal level of the epicardium, as well as at the subendocardium and throughout the myocardial

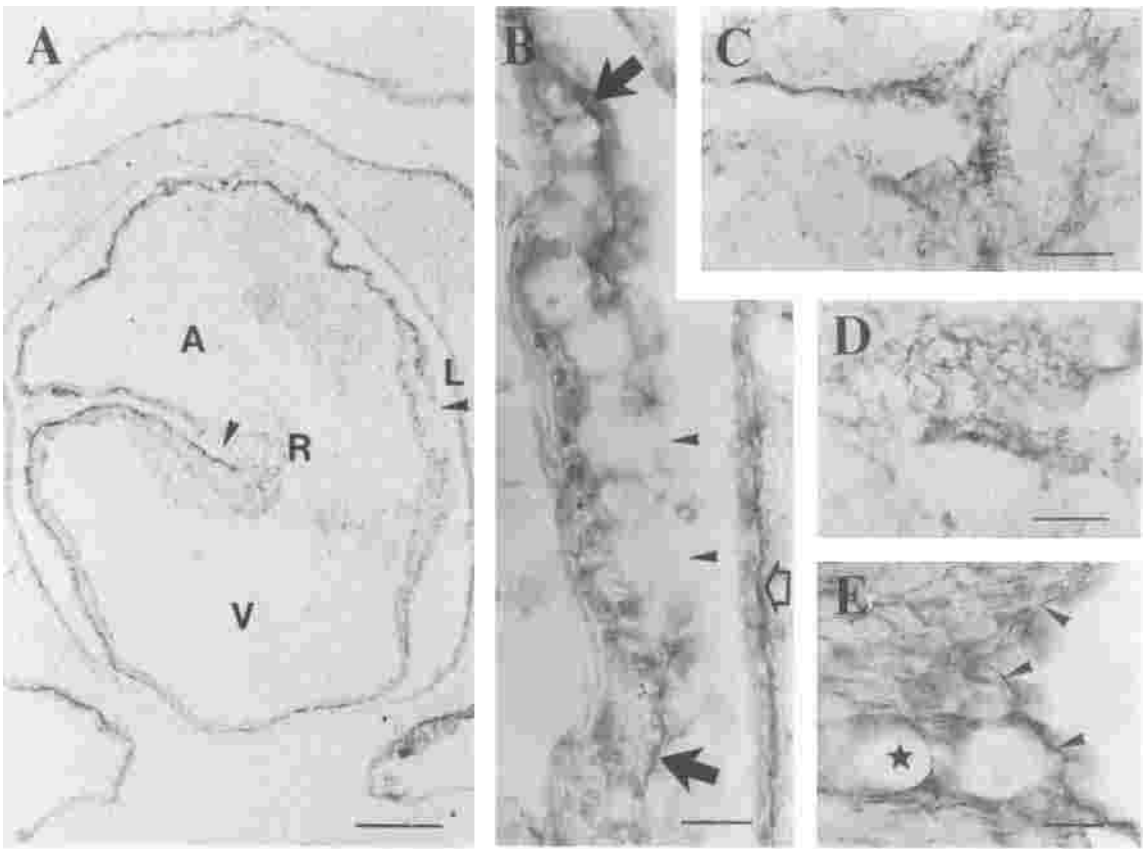

Fig. 11. Fibronectin immunoreactivity (FNIR) in the subepicardium of the dogfish embryos. Transverse sections. **A:** Embryo of 24 mm TL. A distinct FNIR is detected throughout the basal surface of the primitive epicardium except for the left (L) and right (R) parts of the atrioventricular groove (arrowheads). A, atrium; V, ventricle. **B:** Higher magnification of the left part of the atrioventricular groove shown in A. The subepicardium is clearly devoid of FNIR (arrowheads). Large arrows show the labelled base of the epicardium in the atrium and ventricle. Note the labelling of the basal surface of the parietal pericardium (open arrow). **C, D:** Right and left atrioventricular grooves, respectively, of an embryo of 35 mm TL. A conspicuous FNIR has developed in the subepicardium, associated with a network of thin filaments. **E:** FNIR is present at the basal surface of the primitive epicardium of the left atrioventricular groove of the embryo of 65 mm TL (arrowheads). Note the filamentous FNIR in the subepicardium as well as the labelling of the atrioventricular cardiac vein wall (star). Scale bars = 100 μm in A; 20 μm in B–E.

wall (Fig. 11A). However, the base of the epicardium was not labelled at the AV junction, that is, in the place where the apparent differentiation of mesenchymal cells is occurring at this stage of development (Fig. 11A,B). Only a faint FNIR in the basal surface of the epicardium of the AV groove became visible in embryos of 29, 31, and 35 mm. However, a distinct FNIR progressively increased in the AV subepicardium of these embryos as it was being populated with mesenchymal cells. This FNIR was associated with a complex network of thin filaments and, probably, with the surface of the mesenchymal cells (Fig. 9C,D).

A basal FNIR was conspicuous throughout the basal surface of the epicardium of the embryo of 65 mm TL, including that of the AV junction (Fig. 9E). In this embryo, the seeding of subepicardial mesenchymal cells had apparently ceased. A distinct FNIR persists into the AV subepicardium, especially at the wall of the developing cardiac veins. Sections incubated with GBT instead of the primary antibody were unlabelled.

DISCUSSION

Origin of the subepicardial mesenchyme

Little attention has been paid to the origin and fate of the subepicardial mesenchyme in the vertebrate embryo. Most accounts state that a mesenchyme "infiltrates," "appears in" or "invades" the subepicardium shortly after the development of the epicardium, but no specification is provided regarding its origin (Hiruma and Hirakow, '89; Hirakow, '92; Tidball, '92). The latter author remarked that little is known of the development, organization and composition of the subepicardium in late cardiac morphogenesis.

Other accounts, however, provide some hints to clarify the origin of the subepicardial mesenchyme. Fransen and Lemanski ('90) suggested that the pericardial stalk which attaches the pericardium to the ventricle of the axolotl embryo is a potential conduit for other cell types, as fibroblasts, to migrate to the subepicardial space. Icardo et al. ('90) stated that the subepicardial mesenchyme of the chick embryo originates most probably by delamination from the epicardium. Viragh et al. ('90) described how some cells of the primary epicardium of higher vertebrates can detach from the outer layer and migrate into the subepicardium. Tidball ('92) remarked on the ultrastructural similarities between the subepicardial mesenchyme and the epicardial cells of the chick embryo. Both cell types show extensive rough endoplasmic reticulum and villous processes that extend to the subepicardial space. However, this author did not infer an epicardial origin of the subepicardial mesenchyme, and he maintained that these similarities suggest that both epicardial and mesenchymal cells secrete the subepicardial extracellular components. Manner ('93) listed the possible sources of the subepicardial mesenchyme, as pericardial mesenchyme, epicardium, myocardium or endocardium. He noted that when the adhesion of the proepicardium to the heart was surgically prevented in chick embryos, the epicardial ensheathing of the heart was delayed, but the subepicardial mesenchyme failed to develop. It would suggest a relationship between the processes of epicardial covering of the heart and the formation of the subepicardial mesenchyme. Our previous report on dogfish embryo cardiogenesis (Muñoz-Chápuli et al., '94) also suggested that at least a part of the subepicardial mesenchyme in this species originates from the epicardium.

A comprehensive account on the origin and characteristics of the subepicardial mesenchyme in the quail embryo concluded that the subepicardial cells are derived from two sources, the perihepatic mesenchyme and the surface mesothelial layer of the early epicardium (Viragh et al., '93). The perihepatic mesenchyme can freely migrate to the subepicardium via the dorsal mesocardium and the proepicardial organ, a structure derived from the extracardiac proepicardium and located in the sulci of the U-shaped heart tube. Other cells were observed detaching or invaginating from the superficial epicardial layer to form strands in the proepicardial matrix.

The morphological evidence obtained in the dogfish embryos indicated that the subepicardial mesenchymal cells originate, at least in part, from the epicardium. An origin from the migration of perihepatic mesenchymal cells seems questionable since, unlike the chick and quail embryos, the dorsal mesocardium disappears early (in embryos smaller than 10 mm TL), and the epicardial development progresses from the AV groove to the sinus venosus and the conus arteriosus (Muñoz-Chápuli et al., in press). Thus, the sinus venosus-liver limit is not reached by the epicardium until the embryo measures about 27 mm TL, that is, when a subepicardial mesenchyme is already abundant around the AV and CV grooves (see Fig. 2A). Even when the sinus venosus epicardium has joined caudally to the mesothelial lining of the septum transversum, no subepicardial

space develops in the posterior part of the sinus venosus which can be a potential path for the migration of extracardiac mesenchymal cells (Muñoz-Chápuli et al., in press). Furthermore, no proepicardial organ similar to that described by Viragh et al. ('93) can be recognized in the dogfish heart.

Thus, only the myocardium and the primitive epicardium could be the sources of the subepicardial mesenchyme in the dogfish. The endocardium can be ruled out; when the earliest mesenchyme first appears in embryos of 19–21 mm TL, the endocardium is still widely separated from the cardiac wall by a thick layer of cardiac jelly. Regarding the myocardium, evidence of myocardiocyte differentiation to connective tissue cells in the chick embryo has been described (Argüello et al., '78), but the cardiac mesenchymal cells have also shown their potential for myocardial differentiation (Morris, '76). The intercellular junctions observed between epicardial and mesenchymal cells, but not between mesenchymal and myocardial cells, also seem to suggest a relationship between these cell types. Furthermore, the ultrastructural features of the mesenchymal cells were virtually identical to those of the hypertrophied epicardial cells. This was previously noted in the chick embryo by Tidball ('92), as commented on above. Thus, although it cannot be excluded that some late subepicardial mesenchymal cells of the dogfish embryo can originate from alternative sources, the images obtained clearly suggest that most mesenchymal cells originate by delamination from the primitive epicardium.

The process of differentiation of the subepicardial mesenchymal cells seemed to be more intense at the places where the subepicardial space was the widest, that is, at the AV and CV grooves, and later at the lateral ventricular and conal margins. Some epicardial cells of these areas were remarkably different than the squamous epicardial cells from other parts of the heart. This fact brought us to conjecture that the formation of the subepicardial mesenchyme in the dogfish might be described as a process of localized epithelial-mesenchymal transition analogous to that occurring at the endocardial cushions.

In fact, the epicardial cells of the areas where the subepicardial mesenchyme develops showed the morphological features usually associated with epithelial-mesenchymal transitions summarized by Bolender and Markwald ('79) and Markwald et al. ('85). Namely, the epicardial cells showed hypertrophy, motility-like appendages, cell overlapping, intercellular gaps and acquisition of a secretory phenotype. Morphological traits like these allowed Arrechedera et al. ('87) to explain the origin of the mesenchymal tissue in the septum primum of the chick embryo.

A study of the distribution of fibronectin in the heart of the dogfish embryos was carried out in order to test the hypothesis of an epithelial-mesenchymal transition in specific areas of the primitive epicardium. One of the first steps in the epithelial transformation is the extension of small cell processes from the basal surface followed by digestion of the basement membrane (Fitchett and Hay, '89; Hay, '90). Fibronectin is known to be a main component of the epicardial basement membrane, secreted early by both the epicardial (Fransen and Lemanski, '88) and the myocardial cells (Icardo and Manasek, '84). The disappearance of basal FNIR has been reported in the endocardium of chick embryos just before the formation of the endocardial cushion tissue (Icardo and Manasek, '84). In addition, fibronectin distribution in the developing heart seems to be associated with cellular movements or rearrangements prior to expression of their final phenotypic characteristics (Manasek et al., '86).

After these antecedents, basal FNIR should not be present in those basal areas of the epicardium where an epithelial-mesenchymal transition is presumably occurring. The results obtained in the dogfish embryos agreed with our expectations. A conspicuous FNIR was present throughout the basal surface of the epicardium, but not in the AV groove of the embryos of 24–35 mm TL, where subepicardial mesenchymal cells were presumptively differentiating from the epicardium. Furthermore, a strong FNIR progressively developed in the subepicardium of the AV groove of these embryos as the mesenchymal cell population increased. This FNIR was associated with a complex network of thin filaments and to the mesenchymal cell surface. A similar pattern of FNIR has also been observed during the formation of the endocardial cushion mesenchyme (Icardo and Manasek, '84).

Basal FNIR was already distinct at the AV epicardium of an embryo of 65 mm TL, when the epicardial transformation had ceased. The FNIR persisted at the AV subepicardium of this embryo, associated with the developing cardiac veins.

Morphological evidence and the pattern of FNIR agreed with the hypothesis of a localized epithelial-mesenchymal transition as a source of subepicardial mesenchymal cells. Additionally, it is interesting to remark on the similarities between

the subepicardium and the subendocardium during the formation of the subepicardial and subendocardial mesenchymal tissues. These similarities refer to the overall appearance (Viragh and Challice, '73), the mechanical properties (Magovern et al., '86), the protein composition (Kitten et al., '87; Tidball, '92), and the presence of adheron-like complexes (Bolender et al., '90). The latter authors remarked that the adheron-like particles observed in the subepicardium of the quail embryo are similar to those reported within inductively active areas of the developing heart.

Similarities between the proepicardial organ and the endocardial cushions of the quail embryo have also been noted by Viragh et al. ('93). These authors consider as probable that the myocardiocytes of the AV canal wall secrete similar components into the subendocardial and the subepicardial spaces.

The resemblance between the subendocardial and subepicardial environments of the dogfish embryos was further stressed by our observation of transient myocardial discontinuities in the ventral wall of the atrium and sinus venosus. These discontinuities put the cardiac jelly in contact with the subepicardial extracellular matrix. The possibility of a flow of some cardiac jelly components into the subepicardial space cannot be ruled out.

In summary, evidence of a localized epithelial-mesenchymal transition as a source for the subepicardial mesenchyme of the dogfish has been provided, and it would be interesting to assess if a similar process takes place in higher vertebrates. As Hay ('90) has pointed out, "...nature has been ingenious in creating epithelial-mesenchymal transitions to solve many different morphogenetic problems, thereby modifying specific cell behaviours in the various cases to suit each developmental situation."

Origin of the subepicardial capillaries

The development of a subepicardial network of capillary vessels closely follows the epicardial covering of the heart (Viragh and Challice, '81). The origin of this subepicardial network and its involvement in the establishment of the definitive coronary system has been subject to wide debate. The extensive revision of the literature made by Waldo et al. ('90) concluded that sprouting of the sinus venosus endocardium forms a network of capillary vessels which either forms or joins networks of capillaries in the subepicardial space whose origin is unclear.

A number of reports have mentioned the possibility that the subepicardial capillary plexus arises, at least in part, from the subepicardial mesenchymal cells. This was proposed by Viragh and Challice ('81) in their study on the vascularization of the mouse embryo heart. Tokuyasu ('85) demonstrated that the subepicardial mesenchymal cells of the chick embryo form capillary-like structures, but he was not certain whether these structures were nascent blood capillaries. Icardo et al. ('90) asserted that groups of mesenchymal cells organize in the subepicardium of the chick embryo to form vessels of small diameter. Noden ('90) demonstrated that most mesodermal populations (including lateral splanchnic mesothelium) contain cells committed to the endothelial lineage. Viragh et al. ('90) stated that subepicardial mesenchymal cells have the ability to transform into blood capillaries and blood cells. However, Viragh et al. ('93) have described, in quail embryos, the formation of thin-walled tubular strands histologically similar to the capillaries and formed by infolding of epicardial cells. They suggested that these strands are glandular structures and they are probably not involved in the differentiation of the subepicardial capillary network since they lack expression of the QH-1, a quail endothelial cell marker. Finally, Rongish et al. ('94) have shown that endothelial cell precursors form coronary vessels in embryonic rat hearts cultured in oculo.

The hypothesis of differentiation of epicardial-derived mesenchymal cells into capillary vessels has received varied support as it can be inferred from the above-mentioned references. However, it is becoming clear that the forerunners of the coronary vessels have an extracardiac origin. Some recent experimental approaches have favoured this tenet. Bolender et al. ('90), working with explanted quail embryo hearts onto collagen, showed that during the first day in culture an outgrowth of epithelial cells extends from the cut ends of the explants onto the surface of the collagen lattice. These epithelial cells generate a population of mesenchymal cells that migrate into the collagen lattice and interconnect into primitive vascular networks which react positively with QH-1. Significantly, only limited mesenchyme formation was observed when hearts were explanted prior to the epicardial covering. Mikawa and Fischman ('92) demonstrated, through retroviral cell labelling, that the precursors of the chick coronary arteries enter the heart during epicardial morphogenesis, and that the coronary arteries form by coalescence of discontinuous colonies, that is, by in situ vasculogenesis. Poelmann et al. ('93), studying chicken-quail chimaeras,

showed that the coronary endothelial cells reach the heart from the liver region. Further support for this hypothesis was provided by Viragh et al. ('93); angioblasts, blood cells and capillaries were observed migrating to the quail embryonic heart from the perihepatic area via the proepicardial organ.

The present findings have shown that capillary-like structures developed in the subepicardial space of the dogfish embryos studied from 21 mm TL onwards. Morphological evidence suggests that these capillary-like structures arise from the subepicardial mesenchymal cells which previously originated from the epicardium. In fact, when these structures first appear, the endocardium is still well-separated from the myocardium by the cardiac jelly. Hence, no endocardial outpocketings can reach the subepicardium. Migration of vascular precursors from extracardiac areas seems improbable, as discussed above. The dorsal mesocardium disappears in earlier stages. When the epicardium reaches the mesothelial lining of the septum transversum, a potential connection is established between the subepicardium and the perihepatic areas, but at that time numerous capillaries and even developing cardiac veins are already present in the subepicardium. Thus, epicardial-derived, subepicardial mesenchymal cells seem to be the only apparent source of the capillary-like structures and the early capillaries of the dogfish.

There are remarkable similarities between the capillary-like structures observed in the dogfish heart and the capillary tubes formed when human endothelial cells are cultured in tumour-conditioned medium (Folkman and Haudenschild, '80). In accordance with these authors, when the endothelial cells are in sparse culture, they form "rings" by the movement of cytoplasmic extensions along the bottom of the culture dish, until the tips of these extensions join to form a flat, two-dimensional ring. In confluent cultures, the first evidence of tube formation is the appearance of cylindrical vacuoles within an endothelial cell. Later, contiguous cells develop similar vacuoles which form a long tube connecting one cell to another. This connection is apparently formed when the initially intracellular vacuole opens to form two cytoplasmic sheets which are capable of joining with similar cytoplasmic extensions of a neighbouring cell through a structure which resembled a junctional complex. All these steps in the formation of capillaries in vitro can probably be recognized in the subepicardium of the dogfish embryo (see Figs. 5–7).

The presence of membrane-bound, electron-dense vesicles with heterogeneous content, between 0.2 and 1.0 μm in diameter, is a constant feature of the fish endothelial cells. They have been called "dense bodies," "moderately dense bodies" or "endothelial specific granules" (Berge, '79; Leknes, '81; Santer, '85). We have also observed dense bodies in the cells of the endocardial endothelium of the adult dogfish. The presence of these vesicles in the capillary-like structures of the dogfish embryo brings further support to our hypothesis of a differentiation of cardiac vessels from the capillary-like structures. If this hypothesis can be experimentally demonstrated in the future, this system might become an interesting model for the study of in situ vasculogenesis as defined by Risau et al. ('88) and Risau ('91). If a similar process also occurs in the heart of higher vertebrates, it may have been obscured by concomitant processes of heart vascularization, such as endocardial sprouting, formation of blood island-like structures or migration of vascular precursors from extracardiac areas.

Origin of the cardiac veins

Wide subepicardial vessels were placed around the AV and CV grooves of all dogfish embryos larger than 33 mm TL. The anatomical arrangement of these vessels and their drainage to the sinus venosus lumen in all the embryos larger than 34 mm TL, are reasons to consider them as the forerunners of the adult cardiac veins.

Cardiac veins in higher vertebrates have been traditionally considered as sprouts of the sinus venosus endocardium (references in Poelmann et al., '93). However, these authors, working with chick-quail chimaeras, provided the first experimental evidence that subepicardial vessels can actually grow into the sinus venosus.

The study of our embryos has shown an apparent discrepancy regarding the relationship of the cardiac vein precursors with the endocardium. A vessel placed at the caudal part of the AV groove was widely open to the sinus venosus lumen in one of the embryos of 27 mm TL. This could suggest that a nascent vein was being formed by an outgrowth of the sinus endocardium. However, a survey of the serial sections showed that the wall of the vessel, at its anterior ends, was apparently composed of mesenchymal instead of endocardial cells. Furthermore, an isolated vein-like structure placed at the AV subepicardium of the same em-

bryo, close to the CV groove, substantiated the possibility that the subepicardial venous precursors of this embryo would have developed in situ.

The embryo of 33 mm TL showed wide vessels arranged in a circular fashion around the AV and CV grooves. Unlike the adult cardiac veins, they were occasionally split into separate branches and their walls were discontinuous in some points. No contact of these vessels with the cardiac lumen was recorded.

These findings, as a whole, suggest that the cardiac veins form in situ, maybe through coalescence of capillary-like structures, without involvement of the sinus venosus endocardium. Developing cardiac veins probably connect with the cardiac lumen at the level of the sinus venosus when the embryo is about 30 mm TL. This connection with the cardiac lumen might occur whether the veins have already differentiated all around the AV and CV junctions (as in the embryo of 33 mm TL) or whether they are only partially organized in the caudal part of the AV groove. This may explain why De Andrés et al. ('93) have shown that the first evidence of cardiac vein development in the dogfish is the appearance of a diverticulum of the sinus venosus in embryos about 30 mm TL. The thin walls of the developing cardiac veins cannot be easily discerned in the paraffin sections used by these authors, and they only become apparent when the connection with the sinus venosus occurs. This may also be the reason that most of the authors have regarded the vertebrate cardiac veins as sprouts of the endocardium.

Future directions

The dogfish embryo seems to be an interesting model for the study of some basic processes of the cardiogenesis in vertebrates, but it is difficult to directly extrapolate our results to higher vertebrates. In this paper, we have advanced several hypotheses which should be empirically tested in the future. These hypotheses include the origin of the subepicardial mesenchyme through an epithelial-mesenchymal transition localized in specific areas of the epicardium or the differentiation of mesenchymal cells in capillary-like structures which can coalesce to form true capillaries and the precursors of the cardiac veins.

The origin of the coronary arteries in the dogfish remains unclear. A preliminary study showed that coronary arteries were present in the ventricle when the embryo has about 50 mm TL (De Andrés et al., '93). However, it is necessary to determine if arterial branches arising from the hypobranchial artery invest the heart or if some of the capillary vessels observed at the lateral margins of the conus arteriosus connect to the hypobranchial artery. According to the recent theory of angiogenesis, new blood vessels can only originate from small venules and capillaries (Waldo and Kirby, '90). If this postulate applies to the dogfish embryo, it should be impossible for the hypobranchial artery to give vascular sprouts towards the heart. Instead, if we assume that the conal capillaries developed in situ join the hypobranchial artery, these capillaries would probably become arterialized in response to the sudden change in blood pressure. If this is the case, the ingrowth hypothesis of the coronary arteries (Bogers et al., '89) could be extended to the lower vertebrates. Further experimental work should address the question in the future.

ACKNOWLEDGMENTS

We thank Dr. Lourdes Vidal for her kind help with the transmission electron microscopy, and Drs. Valentín Sans Coma, Salvador Guirado, José Carlos Dávila and Borja Fernández for their valuable suggestions. Paule Alen reviewed the English translation. This study was supported by grant PB92-0413 from the DGICYT (Ministerio de Educación y Ciencia, Spain). Alejandro Gallego and David Macías are the recipients of fellowships from the Ministerio de Educación y Ciencia, Spain and from the Junta de Andalucía, Spain, respectively.

LITERATURE CITED

Argüello, C., M.V. de la Cruz, and C. Sánchez (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., M. Alvarez, M. Strauss, and C. Aycsta (1987) Origin of mesenchymal tissue in the septum primum: A structural and ultrastructural study. J. Mol. Cell. Cardiol., *19*:641–651.

Berge, P.I. (1979) The cardiac ultrastructure of *Chimaera monstrosa* L. (Elasmobranchii: Holocephali). Cell Tissue Res., *201*:181–195.

Bogers, A.J.J.C., A.C. Gittenberger-de Groot, R.E. Poelmann, B.M. Peault, and H.A. Huysmans (1989) Development of the origin of the coronary arteries, a matter of ingrowth or outgrowth? Anat. Embryol., *180*:437–441.

Bolender, D., and R. Markwald (1979) Epithelial-mesenchymal transformation in chick atrioventricular cushion morphogenesis. Scan. Electr. Micr., *3*:313–322.

Bolender, D., L.M. Olson, and R.R. Markwald (1990) Coronary vessel vasculogenesis. In: Embryonic Origins of Defective Heart Development. D.E. Bockman and M.L. Kirby, eds. Ann. N.Y. Acad. Sci., *588*:340–344.

De Andrés, A.V., R. Muñoz-Chápuli, and V. Sans-Coma

(1993) Development of the coronary arteries and cardiac veins in the dogfish (*Scyliorhinus canicula*). Anat. Rec., *235*:436–442.

Fitchett, J.E., and E. Hay (1989) Medial edge epithelium transform to mesenchyme after embryonic palatal shelves fuse. Dev. Biol., *131*:455–474.

Folkman, J., and C. Haudenschild (1980) Angiogenesis *in vitro*. Nature, *288*:551–556.

Fransen, M.E., and L.F. Lemanski (1988) Fibronectin and laminin in the developing heart of the axolotl, *Ambystoma mexicanum*. J. Cell Biol., *107*:600.

Fransen M.E., and L.F. Lemanski (1990) Epicardial development in the axolotl, *Ambystoma mexicanum*. Anat. Rec., *226*:228–236.

Hay, E. (1990) Epithelial-mesenchymal transitions. Sem. Dev. Biol., *1*:347–356.

Hirakow, R. (1992) Epicardial formation in staged human embryos. Acta. Anat. Nippon., *67*:616–622.

Hiruma, T., and R. Hirakow (1989) Epicardial formation in embryonic chick heart: Computer-aided reconstruction, scanning and transmission electron microscopic studies. Am. J. Anat., *184*:129–138.

Icardo, J.M., and F.J. Manasek (1984) An indirect immunofluorescence study of the distribution of fibronectin during the formation of the cushion tissue mesenchyme in the embryonic heart. Dev. Biol., *101*:336–345.

Icardo, J.M., M.A. Fernández-Terán, and J.L. Ojeda (1990) Late heart embryology. The making of an organ. In: Handbook of Human Growth and Developmental Biology. Vol. III(B). E. Meisami and P.S. Timiras, eds. CRC Press, Boca Raton, pp. 25–49.

Kitten, G.T., R.R. Markwald, and D.L. Bolender (1987) Distribution of basement membrane antigens in cryopreserved early embryonic hearts. Anat. Rec., *217*:379–390.

Leknes, I.L. (1981) Ultrahistochemical studies on the moderately electron dense bodies in teleostean endocardial cells. Histochemistry, *72*:211–214.

Lewis, F.T. (1904) The question of sinusoids. Anat. Anz., *25*:261–279.

Magovern, J.H., G.W. Moore, and G.M. Hutchins (1986) Development of the atrioventricular valve region in the human embryo. Anat. Rec., *216*:167–181.

Manasek, F.J., J. Icardo, A. Nakamura, and L. Sweeney (1986) Cardiogenesis: Developmental mechanisms and embryology. In: The Heart and Cardiovascular System. H.A. Fozzard et al., eds. Raven Press, New York, pp. 965–985.

Manner, J. (1993) Experimental study on the formation of the epicardium in chick embryos. Anat. Embryol., *187*:281–289.

Markwald, R.R., E.L. Krug, R.B. Runyan, and G.T. Kitten (1985) Proteins in cardiac jelly which induce mesenchyme formation. In: Cardiac Morphogenesis. V.J. Ferrans, G. Rosenquist, and C. Weinstein, eds. Elsevier, New York, pp. 60–69.

Mikawa, T., and D.A. Fischman (1992) Retroviral analysis of cardiac morphogenesis: discontinuous formation of coronary vessels. Proc. Natl. Acad. Sci. USA, *89*:9504–9508.

Morris, E.W.T. (1976) Observations on the source of embryonic myocardioblasts. J. Anat., *121*:47–64

Muñoz-Chápuli, R., D. Macías, C. Ramos, A.V. De Andrés, A. Gallego, and P. Navarro (1994) Heart development in the dogfish (*Scyliorhinus canicula*): A model for the study of the basic processes of vertebrate cardiogenesis. Cardioscience, *5*:245–253.

Muñoz-Chápuli, R., D. Macías, C. Ramos, B. Fernández, and V. Sans-Coma (in press) Development of the epicardium in the dogfish (*Scyliorhinus canicula*). Acta. Zool., (Stockholm).

Nair, M.G.K. (1970) The anatomy and embryology of the heart and its conducting system of the dogfish *Carcharias sorrah* Müller and Henle. Zool. Anz., *185*:265–274.

Noden, D.M. (1990) Origin and assembly of avian embryonic blood vessels. In: Embryonic Origins of Defective Heart Development. D.E. Bockman and M.L. Kirby, eds. Ann. N.Y. Acad. Sci., *588*:236–249.

Poelmann, R.E., A.C. Gittenberger-de Groot, M.M.T. Mentink, R. Bökenkamp, and B. Hogers (1993) Development of the cardiac coronary vascular endothelium, studied with anti-endothelial antibodies, in chicken-quail chimeras. Circ. Res., *73*:559–568.

Risau, W. (1991) Vasculogenesis, angiogenesis and endothelial cell differentiation during embryonic development. In: The Development of the Vascular System. R.N. Feinberg, G.K. Sherer, and R. Auerbach, eds. Karger, Basel, pp. 58–68.

Risau, W., H. Sariola, H.G. Zerwes, J. Sasse, P. Ekblom, R. Kemler, and T. Doetschman (1988) Vasculogenesis and angiogenesis in embryonic-stem-cell-derived embryoid bodies. Development, *102*:471–478.

Rongish, B.J., R.J. Torry, D.C. Tucker, and R.J. Tomanek (1994) Neovascularization of embryonic rat hearts cultured in oculo closely mimics in utero coronary vessel development. J. Vasc. Res., *31*:205–215.

Santer, R.M. (1985) Morphology and innervation of the fish heart. Adv. Anat. Embryol. Cell. Biol., *89*:1–102.

Tidball, J.G. (1992) Distribution of collagens and fibronectin in the subepicardium during avian cardiac development. Anat. Embryol., *185*:155–162.

Tokuyasu, K.T. (1985) Development of myocardial circulation. In: Cardiac Morphogenesis. V.J. Ferrans, G. Rosenquist, and C. Weinstein, eds. Elsevier, New York, pp. 226–237.

Viragh, S., and C.E. Challice (1973) Origin and differentiation of cardiac muscle cells in the mouse. J. Ultrastruct. Res., *42*:1–24.

Viragh, S., and C.E. Challice (1981) The origin of the epicardium and the embryonic myocardial circulation in the mouse. Anat. Rec., *201*:157–168.

Viragh, S., A.C. Gittenberger-De Groot, R.E. Poelmann, and F. Kalman (1993) Early development of quail heart epicardium and associated vascular and glandular structures. Anat. Embryol., *188*:381–393.

Viragh, S., F. Kalman, A.C. Gittenberger-De Groot, R.E. Poelmann, and A.F.M. Moorman (1990) Angiogenesis and hematopoiesis in the epicardium of the vertebrate embryo heart. In: Embryonic Origins of Defective Heart Development. D.E. Bockman and M.L. Kirby, eds. Ann. N.Y. Acad. Sci., *588*:455–458.

Waldo, K., and M.L. Kirby (1990) A new perspective on the development of the coronary arteries in the chick embryo. In: Embryonic Origins of Defective Heart Development. D.E. Bockman and M.L. Kirby, eds. Ann. N.Y. Acad. Sci., *588*:459–460.

Waldo, K., W. Willner, and M. Kirby (1990) Origin of the proximal coronary artery stems and a review of ventricular vascularization in the chick embryo. Am. J. Anat., *188*:109–120.