Contribution of the Primitive Epicardium to the Subepicardial Mesenchyme in Hamster and Chick Embryos

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ABSTRACT A study about the hypothetical contribution of the epicardial cells to the subepicardial mesenchyme was carried out in Syrian hamster embryos of 9–12 days post coitum (dpc) and chick embryos of 3–5 days of incubation. In the epicardium and subepicardium of these embryos we have immunolocated the proteins cytokeratin (CK), vimentin (VIM), fibronectin (FN), and two antigens related to the transformation of endocardial cells into valvuloseptal mesenchyme, ES/130 and J B3. In the hamster embryos, CK+ subepicardial mesenchymal cells (SEMC) were apparently migrating from the primitive epicardium from 9.5 dpc at the atrioventricular (AV) groove and proximal outflow tract (OFT). The morphological signs of delamination extended by 11 dpc to the epicardium of the interventricular groove and the dorsal part of the ventricle. The relative abundance of the CK+ SEMC decreased in embryos of 12 dpc. VIM colocalized with CK in most SEMC, and in some epicardial mesothelial cells, mainly at the areas of delamination. CK immunoreactivity was also found in some early subepicardial capillaries. Similar observations were made in the chick embryos studied. The immunoreactive patterns obtained at the subepicardium with anti-FN, ES/130, and J B3 antibodies were similar to those reported in the areas of endothelial transformation of the endocardial cushions. We suggest that these observations are compatible with an epithelial-mesenchymal transformation involving the epicardial mesothelium and originating at least a part of the SEMC. Dev. Dyn. 1997;210:96-105. © 1997 Wiley-Liss, Inc.

Key words: epicardium; mesothelium; subepicardial mesenchyme; cytokeratin; vimentin; fibronectin; coronary vessels; chick embryo; Syrian hamster embryo

INTRODUCTION

In the vertebrate embryos, the epicardial investment of the heart is closely followed by the establishment of a space between the primitive epicardium and the myocardium, a space that is initially acellular and filled with an amorphous extracellular matrix. This space, the subepicardium, is rapidly populated by mesenchymal cells whose origin and developmental fate is not yet well known (Choy et al., 1993). Some of them contribute to the cellular component of the atrioventricular valves (Wenink, 1992), others secrete the extracellular matrix elements of the subepicardium (Tidball, 1992), and others might coalesce to form the primary cardiac capillary plexus (Viragh and Challice, 1981; Tokuyasu, 1985; Icardo et al., 1990; Bolender et al., 1990; Viragh et al., 1990).

Our previous morphological studies in a primitive vertebrate, the dogfish (Scyliorhinus canicula), have suggested that, in this species, the early subepicardial mesenchymal cells (SEMC) originate from the epicardial mesothelium. In the dogfish, we could discard the migration of extrinsic mesenchymal cells since the subepicardium is not connected with extracardiac areas until many SEMC and a part of the capillary plexus have already appeared (Muñoz-Chápuli et al., 1996).

We wanted to know if a similar process to that described in the dogfish (i.e., delamination of SEMC from the epicardial mesothelium) actually occurs in the embryos of higher vertebrates. In fact, this possibility has been suggested by some authors on a morphological basis (Viragh et al., 1990, 1993; Icardo et al., 1990).

Our approach, conducted on Syrian hamster and chick embryos, was based on the immunohistochemical localization in the epicardium and SEMC of the proteins cytokeratin (CK) and vimentin (VIM), which constitute the intermediate filaments characteristic of epithelial and mesenchymal cells, respectively. The expression of VIM prior to the transformation of an epithelial cell is probably involved in the migratory shape changes (Hay, 1990). On the other hand, CK immunoreactivity persists for some time in the mesenchymal cells originated from CK+ epithelial cells (Fitchett and Hay, 1989; Hay, 1990). Thus, if epicardial mesothelial cells detach and migrate into the subepicardium, colocalization of CK and VIM should be expected at the migratory as well as at the premigratory stages.

We have also assessed the presence in the subepicardium of three molecules involved in the transformation of endocardial cells into mesenchymal cells, which occurs in the embryonic heart, i.e., fibronectin (FN), the...
fibrillin-like protein recognized by the monoclonal antibody J B3, and the antigen ES/130. Fibronectin shows a characteristic distribution in the subendocardium during the formation of the atrioventricular (AV) and outflow tract (OFT) endocardial cushions (Icardo and Manasek, 1984; Mjaadvedt et al., 1987). On the other hand, only the endocardial cells expressing the J B3 antigen are susceptible to be transformed in mesenchyme cells (Wunsch et al., 1994; Barton et al., 1995; Bouchey et al., 1996). Finally, the ES/130 antigen seems to be involved at a critical step in the initiation of the epithelial-mesenchymal transformation of the cardiac endothelium (Rezaee et al., 1993; Markwald et al., 1995).

These immunohistochemical markers do not provide a direct proof of the SEMC lineage, but they can afford evidence that mesothelial cells from the primitive epicardium are able to transform, migrate, and contribute to the subepicardial mesenchyme.

MATERIALS AND METHODS

The animals used in our research program were handled in compliance with the international guidelines for animal care and welfare. The Syrian hamsters (Mesocricetus auratus) were housed in polypropylene cages in a room with controlled temperature and photoperiod. Commercial mouse food (UAR/Poland, Barcelona, Spain) and water were given ad libitum, starting at weaning. Eggs from chick (Gallus gallus) were obtained by laparotomy and uterotomy, freed from maternal membranes and fixed.

The Syrian hamster sample consisted of 39 embryos. Mature male and female specimens of hamster were mated, and the end of the coitus was considered as the day 0 of the embryonic development. The embryos were collected at the stages 9 (3), 9.5 (5), 10 (9), 10.3 (4), 11 (13), and 12 (5) days post coitum (dpc). Pregnant females were killed by chloroform overdose, and the embryos obtained by laparotomy and uterotomy, freed from fetal membranes and fixed.

The sample of chick embryos consisted of seven specimens of the stages HH17–HH27 of Hamburger and Hamilton (1951) (3–5 days of incubation). The embryos were fixed in 40% methanol, 40% acetic acid, 20% distilled water for 8–12 hr. A few embryos were fixed either in the same fixative including 16% of saturated picric acid solution or in Bouin. After fixation, the embryos were dehydrated in an ethanolic series finishing in butanol, and paraffin-embedded. Serial sections (5 and 10 µm) were obtained with a Leitz (Wetzlar, Germany) microtome and collected on poly-l-lysine coated slides. The sections were dewaxed in xylene, hydrated in an ethanolic series, and washed in Tris-phosphate buffered saline (TPBS, pH = 7.8). The ultrastructural treatment of the slides varied according to the antibody used, as described below.

For the histological study of the subepicardium, two embryos (11 dpc) were fixed by perfusion through the left ventricle with 2% glutaraldehyde and 1% paraformaldehyde in 50 mM cacodylate buffer. The embryos were then immersed in the same fixative for 45 min, washed in cacodylate buffer for 30 min, and postfixed in 1% OsO4 for 90 min. After washing in distilled water (30’) the embryos were dehydrated in an ethanolic series finishing in aceton and embedded in Araldite 502. Semithin sections were obtained in a Reichert UMO-2 ultramicrotome and stained with toluidine blue.

Peroxidase Immunohistochemistry

Endogenous peroxidase activity was quenched by incubation for 30 min with 3% hydrogen peroxide in TPBS. After washing with TPBS, non-specific binding sites were saturated for 30 min with 10% sheep serum, 1% bovine serum albumin, and 0.5% Triton X-100 in TPBS (SBT). The slides were then incubated overnight at 4°C in the primary antibody diluted in SBT. Control slides were incubated in SBT only or with non-immune rabbit serum diluted 1:200.

After incubation, the slides were washed in TPBS, incubated for 1 hr at room temperature in biotin-conjugated anti-mouse or anti-rabbit goat IgG (Sigma, Dorset, UK) diluted 1:100 in SBT, washed again and incubated for 1 hr in avidin-peroxidase complex (Sigma) diluted 1:150 in TPBS. Peroxidase activity was developed with Sigma Fast 3,3’-diaminobenzidine tablets according to the indications of the supplier. In some cases, the staining was intensified with metallic cations or the slide was counterstained with hematoxylin.

Two anti-cytokeratin antibodies were used. Monoclonal anti-pan cytokeratin (C2562, Sigma) is a mix of six monoclonal antibodies, which stains most types of keratin in epithelial cells of several vertebrates, but it shows no cross-reaction with non-epithelial normal human tissues. It was used at 1:200 dilution. Polyclonal anti-cytokeratin (Z622, Dakopatts, Glostrup, Denmark) is used for wide screening of keratins in several tissues. This antibody was diluted at 1:500. No significant differences were found between the results obtained with both antibodies, but the monoclonal one gave less background staining.

Polyclonal rabbit anti-human fibronectin antibody (ICN Pharmaceuticals, Irvine, CA) was used only for hamster embryos at a 1:500 dilution. The monoclonal anti-ES/130 (Rezaee et al., 1993) and the monoclonal J B3 (Wunsch et al., 1994) were a generous gift from Dr. Edward Krug (Medical University of South Carolina). The anti-ES/130 antiserum was diluted 1:500 and used only with hamster embryos. The anti-J B3 supernatant was diluted 1:100 and used only with chick embryos.

Fluorescence Immunohistochemistry and Double Labellings

For VIM immunofluorescence, the dewaxed sections were saturated for 30 min with 10% rabbit serum, 1% bovine serum albumin, and 0.5% Triton X-100 in TPBS (RBT). The slides were then washed and incubated as described above. Polyclonal goat anti-human vimentin (ICN Pharmaceuticals) was used as the primary anti-
body diluted 1:60 in RBT. After washing, the slides were incubated in FITC-conjugated rabbit anti-goat IgG diluted 1:75 in RBT for 1 hr, washed, and mounted in glycerol-TPBS (1:1) containing 0.2% 1,4-diazabicyclo[2.2.2]octane.

The CK/VIM double immunolabelling was performed with a mix of monoclonal and polyclonal primary antibodies. For the hamster embryos, the slides were blocked with SBT for 30 min and incubated for 1 hr with both antibodies, monoclonal anti-human VIM (Boehringer, Mannheim, Germany) diluted 1:50, and polyclonal anti-CK diluted 1:50 in SBT. After washing in TPBS, the slides were first incubated for 45 min in TRITC-conjugated goat anti-mouse IgG (Sigma) diluted 1:50. This solution had been preadsorbed for 1 hr with 10% rabbit serum. After washing, the slides were blocked again in SBT, incubated for 1 hr in biotin-conjugated goat anti-rabbit IgG (Sigma) diluted 1:100 in SBT, washed and incubated in extravidin-FITC conjugate (Sigma) diluted 1:100. For the chick embryos, the procedure was similar, but a monoclonal anti-chick VIM (Developmental Studies Hybridoma Bank, University of Iowa, clone AMF-17b) was used as primary antibody. Controls were incubated only with one primary antibody and then with both secondary antibodies, in order to detect any cross-reaction between the secondary and the primary antibodies.

The sections were observed in a Nikon Microphot FXA equipped with epifluorescence and in a laser confocal microscope Leica TCS-NT (Heidelberg, Germany), using filters specific for the FITC and TRITC fluorochromes. Selected images were captured and printed in a Sony digital color printer.

RESULTS

Development of the Epicardium, Subepicardium, and SEMC in the Syrian Hamster

There are no available data in the literature on the development of the epicardium, subepicardium, and subepicardial capillary vessels in embryos of Syrian hamsters. For this reason, we will start with a brief morphological description of these processes. The chronology of these developmental events was very similar to that of the mouse embryo.

In embryos of 9 dpc, the myocardium was not covered by epicardial cells. Just a few cells, probably precursors of the primitive epicardium, could occasionally be seen adhered to the cardiac wall, mainly in the neighbourhood of the AV junction. Round cells and small vesicles seemed to be released in the coelom from the mesothelial villi of the transverse septum. The vesicles, composed of epithelial cells, occasionally contained mesenchymal cells inside.

The epicardial lining of the heart was relatively fast, as observed in embryos of 9.5 and 10 dpc. The first areas of the heart to be covered by the epicardium, about 9.5 dpc, were the AV canal, the ventral part of the atrium, the ventral part of the sinus venosus, the dorsal part of the ventricle, and a ring around the proximal outflow tract (OFT). By 10 dpc only the distal OFT and some areas of the dorsocephalic part of the atrium were not covered by the epicardium. The epicardial investment was complete in the embryos of 11 dpc, when the mesothelial villi of the transverse septum had already disappeared.

The subepicardial space first appeared around the AV canal and proximal OFT, and it was at its maximum width by 10 dpc (Fig. 1). A subepicardial space subsequently appeared in the sinoatrial (SA) groove, dorsal surface of the ventricle, interventricular (IV) groove, and in the ventral part of the atrium. The cephalic areas of the atrium usually lacked a subepicardium in the embryos studied. In the embryos of 12 dpc, the relative volume of the subepicardium decreased markedly.

Only a few SEMC could be seen in embryos of 9.5 dpc, but they were already abundant by 10 dpc in those areas where the subepicardium was the widest, i.e., the SA, AV, and conoventricular grooves (Fig. 1). SEMC were also present in the ventral part of the sinus venosus and dorsal part of the ventricle, and they appeared by 11 dpc in the IV groove. These cells were frequently in contact with the primitive epicardial cells. A number of epicardial cells, specially those of the intercameral grooves, were large and spheric, they showed rounded nuclei and long basal cytoplasmic processes. Cell overriding also occurred in these areas (Fig. 2). By 11 dpc, the morphological signs of delamination of SEMC from the primitive epicardium had extended to the dorsal part of the ventricle and the IV groove. However, they only persisted at the AV, conoventricular, and IV grooves in the embryos of 12 dpc.

Cytokeratin Immunohistochemistry

In all the embryos studied, the anti-CK antibodies labelled the cells of the primitive epicardium as well as those from the splanchnic mesothelium, the epidermis, and the endodermal epithelium. We will describe now the distribution of the CK+ SEMC in the heart of the embryos studied.

As described above, SEMC were already abundant by 10 dpc in the sinoatrial and AV grooves and also around the proximal OFT. Most of these SEMC were CK+ (Figs. 3, 4). CK+ mesenchymal cells were also abundant in the limit between the liver and the sinus venosus, but a clear discontinuity was usually present between this population and the SEMC of the AV groove. CK immunoreactivity also labelled cells that were forming ring-like structures; sometimes involving several cells. The blood island-like vessels that could be observed in the subepicardium occasionally showed CK+ cells in their endothelial lining (Fig. 3).

In the hamster embryos of 11 dpc, CK+ SEMC have appeared in the dorsal part of the ventricle and in the interventricular (IV) groove. Some of them could be seen infiltrated in inner areas of the myocardial wall, specially at the AV canal and IV septum. Ring-like structures were abundant, and they were usually formed...
by CK⁺ cells (Fig. 5). By 12 dpc the proportion of CK⁺ SEMC had decreased, as well as the intensity of their immunoreactivity. The mesenchymal cells more intensely labelled with the anti-CK antibody were restricted to the AV and IV grooves, the only places where the morphological signs of epicardial delamination remained. Some CK⁺ mesenchymal cells were located in deep areas of the myocardium, as described above. CK⁺ immunoreactivity was detected in the endothelial lining of some subepicardial capillaries (Fig. 6).

In the chick embryos of 3–5 days of incubation (HH stages 17–27), the CK immunoreactive pattern was similar to that described above for the Syrian hamster embryos (Figs. 9, 10). The primitive epicardium of the AV groove and OFT showed signs of delamination, which extended, in the embryos of stages HH21–23, to all the ventricles. CK⁺ cells were very abundant in the subepicardial space as well as inside the proepicardial protrusions located in the sinus venosus wall and in the liver-cardiac limit. There was not a discontinuity be-
tween the CK⁺ cells of the subplanchnic and the subepicardial spaces (Fig. 10). Ring-like structures and blood-containing vessels showed CK⁺ cells in their walls (Fig. 9a,c). Furthermore, some cells lining the hepatic sinusoids were also CK⁺ in these stages (Fig. 9a).

Cytokeratin/Vimentin Colocalization

Vimentin antibodies labelled all the endothelial cells, including the endocardial cells, the subendocardial mesenchyme, and most SEMC. In the 10 and 11 dpc hamster embryos, as well as in the chick embryos studied, colocalization of CK and VIM was relatively frequent in SEMC, either isolated or forming cord-like or ring-like structures (Figs. 7, 8). In both animal models studied, the epicardial mesothelial cells from the areas where delamination was presumably occurring were frequently also CK⁺/VIM⁺. However, the squamous epicardial cells covering other parts of the heart devoid of subepicardial space were CK⁺/VIM⁻.

Fibronectin Immunohistochemistry

In the subepicardium of the Syrian hamster embryos, the anti-fibronectin antibody stained the basal surface of the epicardial cells as well as the SEMC. The mesenchymal cells were stained in a pattern of irregular patches associated to the cell membranes. Filopodial processes were always distinctly stained (Fig. 11). In the areas where the squamous epicardial cells are directly adhered to the myocardial wall, without a subepicardial space, a distinct FN immunostaining was present between the myocardium and the epicardium, probably related to the presence of a basal lamina.

J B3 Immunohistochemistry

In the chick embryos, the fibrillin-like protein detected by the J B3 antibody was present at the subepicardium of the areas where SEMC were presumably delaminating from the primitive epicardium (Figs. 12, 13). In these areas, J B3 stained the cytoplasm of some epicardial cells, their basal cytoplasmic projections, the surface of a number of SEMC, and the subepicardial capillaries. This pattern of immunostaining was similar to that observed in the endocardial cushions (Fig. 12).

ES/130 Immunohistochemistry

In the hamster embryos, the ES/130 antigen labelled the endocardium as well as a part of the epicardial cells that covered the areas of wide subepicardium (Fig. 14), especially those large and with round nuclei. However, the squamous epicardial cells adhered to the myocardium usually were ES/130⁻.

DISCUSSION

We have carried out a study on a poorly known aspect of the cardiac development, the origin of the subepicardial mesenchyme. The hypothesis to be tested was the existence of a contribution of epicardial mesothelial cells to the subepicardial mesenchyme, a process already shown by us in a fish model (Muñoz-Chápuli et al., 1996).

The first evidence that supports our hypothesis is the existence of morphological signs of delamination in specific areas of the epicardium, mainly in those that are not directly adhered to the myocardium and cover the AV and IV grooves as well as the proximal OFT. These evidences, which include cell hypertrophy, basal cytoplasmic processes, and cell overriding, are the same that appear in the endocardium during the formation of the cushion mesenchyme (Bolender and Markwald, 1979; Markwald et al., 1985). The earliest SEMC appear in these areas of wider subepicardium, frequently in contact with the epicardial cells.
Figs. 3–8.
The immunolocation of CK and VIM in the primitive epicardium and SEMC constituted a second test of our hypothesis. CK immunoreactivity is strong in the primitive epicardial cells, and it has been applied to describe the epicardial lining of the heart (Vrancken Peeters et al., 1995). The persistence of the original epithelial-type intermediate filaments, after the transdifferentiation of an epithelium, has been reported both in vivo and in vitro systems (Fitchett and Hay, 1989; Hay, 1990). On the other hand, the expression of VIM prior to the transformation of an epithelial cell is probably involved in the premigratory shape changes (Hay, 1990) and it has been demonstrated in the primitive streak and neural tube (Franke et al., 1982). For these reasons, we expected colocalization of CK and VIM in the mesenchymal cells presumably derived from the epicardial mesothelial cells, as well as in the epicardial cells themselves.

The presence of CK+ mesenchymal cells had been described in the proepicardium and subepicardium of the quail embryo, forming a network of tubular structures morphologically identical to the true capillaries but not expressing the vascular marker QH-1 (Viragh et al., 1993). These authors suggest that the tubular structures form by invagination of the surface mesothelium. In spite of this interesting feature, the significance of the CK+ SEMC has not been hitherto established, and there is no data in the literature about their presence in the mammalian embryos.

In the hamster and chick embryos of the stages studied, CK immunoreactivity was present throughout the epicardial mesothelium, but it also labelled most SEMC cells. The persistence of the epithelial-type intermediate filaments is probably transient. This is suggested by the marked decrease in the relative abundance of CK+ SEMC recorded in the hamster embryo between 10 and 12 dpc. On the other hand, CK localized with VIM in the SEMC and in a number of epicardial mesothelial cells, mainly those covering the cardiac grooves. VIM immunoreactivity was scarce or absent in the squamous epicardial cells directly adhered to the myocardium.

The third evidence of an epicardial contribution to the subepicardial mesenchyme comes from the immunolocalization of the antigens JB3 and ES/130 in a number of epicardial mesothelial cells, the same which were showing the signs of a delamination to the subepicardium. The labelling of these cells was identical to that reported in the endothelial cells which originate mesenchymal cells in the endocardial cushions (Rezaee et al., 1993; Wunsch et al., 1994; Bouche et al., 1996). The JB3 antigen seems to be expressed only in the subset of endocardial cells which are responsive to the inductive signal for a transformation in a migratory mesenchymal cell (Markwald et al., 1995). According to these authors, the JB3+ endocardial cells secrete ES/130 upon induction from the myocardium, before their transformation in mesenchymal cells. We think that the presence of these antigens in a subset of epicardial cells can be significant.

The distribution of FN at the subepicardium was very similar to that reported in the endocardial cushions (Icardo and Manasek, 1984; Mjaadvedt et al., 1987), with a patchy staining of the surface of the mesenchymal cells and the basal filopodial processes of the epithelial cells. Distinctive staining of a continuous basal lamina was only observed beneath the epicardial cells adhered to the myocardium. The presence of FN at the subepicardium had been reported (Choy et al., 1991; Tidball, 1992) but its relation with the migration of epicardial cells to the subepicardium was not suggested.

The mechanism of differentiation of mesenchymal cells from the primitive epicardium, which we herein suggest, seems to be analogous to that occurring at the endocardial cushions and, in fact, there is a relative spatiotemporal colocalization of both phenomena of mesenchymal cell transformation. The epicardium of the AV groove, dorsal part of the ventricle, and proximal OFT showed the larger abundance and persistence of the morphological signs of delamination. Their cells were frequently stained with the anti-VIM, JB3, and anti-ES/130 antibodies. It will be interesting to check if a common inductive mechanism originating from the myocardium might be involved in both processes of mesenchyme formation. The presence of adheron-like particles in the subepicardial space (Bolender et al., 1990), analogous to that reported as playing an inductive role in the endocardial cushions, might be regarded in this context. It is also interesting to quote the report of molecules in the epicardium and subepicardium, which probably play a role in the transformation of epithelial cells, for example, the extracellular matrix proteins tenascin-X (Burch et al., 1995), fibulin-1 and fibulin-2 (Zhang et al., 1995), and the transforming growth factor β1 (Choy et al., 1991). Furthermore, the gene Msx-1 is expressed specifically in the epicardium and subepicardium of the AV canal during the development of the SEMC (Chan-Thomas et al., 1993). These observations, together with those reported in this paper, are consistent with an event of epithelial-mesenchymal transformation involving the epicardial mesothelium.

Sources other than the primitive epicardium probably contribute to the subepicardial mesenchyme. The migration of cells from the liver/transverse septum area has been demonstrated in the avian embryos (Poelmann et al., 1993; Viragh et al., 1993). This contribution might be less significant in the hamster embryos. We have found that the CK immunostaining of many SEMC, specially by 10 dpc, is as intense as that of the neighbour epicardial cells. If CK is progressively disappearing from the SEMC, it is conceivable that mesenchymal cells migrating from extracardiac areas should lose a significant part of their CK immunoreactivity during
the time of their migration. On the other hand, the first appearance of the SEMC in the hamster embryo is virtually simultaneous at the subepicardium of the sinus venosus, AV canal, and ventricle (V). In the epicardial mesothelium (E), there are signs of delamination, such as basal cytoplasmic processes (arrowhead, shown at higher magnification in b). Some vascular structures also involve CK⁺ cells, either forming channels devoid of blood cells (star) or true capillaries (C). This capillary is shown at higher magnification in c. Note the blood cells (arrowheads) and the CK⁺ immunoreactivity of some cells of its wall (arrows). CK⁺ cells (arrow in a) were always seen at these stages in the wall of the liver (L) sinusoids. S: splanchnic mesothelium. Scale bars: a = 26 µm; b = 14 µm, c = 15 µm.

Fig. 9. a: Chick embryo of 4 days of incubation (HH21), sagittal section. Many subepicardial mesenchymal cells are CK⁺ in the wide subepicardium between the sinus venosus (SV), atrium (A), and ventricle (V). In the epicardial mesothelium (E), there are signs of delamination, such as basal cytoplasmic processes (arrowhead, shown at higher magnification in b). Some vascular structures also involve CK⁺ cells, either forming channels devoid of blood cells (star) or true capillaries (C). This capillary is shown at higher magnification in c. Note the blood cells (arrowheads) and the CK⁺ immunoreactivity of some cells of its wall (arrows). CK⁺ cells (arrow in a) were always seen at these stages in the wall of the liver (L) sinusoids. S: splanchnic mesothelium. Scale bars: a = 26 µm; b = 14 µm, c = 15 µm.

Fig. 10. Subepicardium of a chick embryo of 4 days of incubation (HH21). There is a wide communication between the liver (L) mesenchyme and the subepicardium (large arrow), which could be a potential way for the migration of mesenchymal cells. In fact, CK⁺ mesenchymal cells can be seen in all these areas. However, there also are basal cytoplasmic processes (small arrows) in the epicardial mesothelial cells (E). There is a CK⁺ channel (star) similar to that shown in 9a. P: proepicardial villi. Other abbreviations as in Figure 9. Scale bar = 15 µm.

Another possibility that has also been suggested is the migration of mesenchymal cells from the transverse septum through free-floating mesothelial vesicles (Van den Eijnde et al., 1995). We regard this possibility as feasible, but it is difficult to consider it as the main source for the subepicardial mesenchyme. Most of the free mesothelial vesicles observed by us contained no mesenchymal cells inside, and there are CK⁺ SEMC in the AV groove of the hamster embryo by 12 dpc, when the epicardial investment of the heart is complete and the mesothelial villi of the transverse septum have disappeared. In conclusion, we think that the primitive epicardium significantly contributes to the subepicardial mesenchyme, this contribution probably being more substantial in the mammalian than in the avian models.

Two questions have been raised by our findings and they remain open. First, is it possible that the CK⁺ mesenchymal cells of the liver, transverse septum, and mesothelial villi originate from the splanchnic mesothelium in the same way as the SEMC do from the epicardial mesothelium? Second, what is the mechanism involved in the subsequent differentiation of the SEMC either as fibroblasts or as cardiac vessel precursors? Is this mechanism related with a differential origin of the SEMC? Although we have not further investigated these questions at this time, we have
shown that many ring-like and tube-like structures as well as some early cardiac vessels contain \( \text{CK}^+ \) cells, although the intensity of the immunoreactivity was always inversely proportional to the degree of differentiation of the structure. It is also important to remark that a number of cells in the wall of the liver sinusoids in the chick embryos were \( \text{CK}^+ \), although we cannot precise if they were endothelial. An interesting, although highly speculative, hypothesis would be that the liver endothelial cells and the endothelial cells of the primary cardiac plexus share a common mesothelial origin, from the splanchnic mesothelium covering the liver primordium and from the epicardial mesothelium. This hypothesis would be consistent with the

Figs. 11–14. Immunolocalisation in the subepicardium of antigens involved in the epithelial-mesenchymal transition.

Fig. 11. Syrian hamster embryo (10 dpc), posterior part of the atrioventricular groove, fibronectin (FN) immunostaining. The FN antibody labelled the surface of the mesenchymal cells (M) in the subepicardium (SE) and the basal area of the epicardial mesothelial cells (E), especially the cytoplasmic processes (arrow). The basal surface of the endocardium (EN) is also labelled. Scale bar = 8 \( \mu \)m.

Fig. 12. Chick embryo (4 days of incubation, HH21), JB3 immunostaining, counterstained with hematoxylin. The JB3 antigen was present in the endocardial cells (EN) of the atrioventricular cushion as well as in the subendocardium. The subepicardium (SE) showed a similar immunoreactive pattern, including staining of the epicardial cells (arrowhead), fibrous elements of the extracellular matrix, and capillaries (arrows). E: epicardial mesothelium. Scale bar = 12 \( \mu \)m.

Fig. 13. Chick embryo (5 days of incubation, HH27), JB3 immunostaining, counterstained with hematoxylin. This view of the dorsal part of the ventricle shows that the JB3 antigen is present in some epicardial mesothelial cells, presumably in the cytoplasm (arrows). Other abbreviations as in Figure 12. Scale bar = 9 \( \mu \)m.

Fig. 14. Syrian hamster embryo (10 dpc), transverse section of the AV region, ES/130 immunostaining. The ES/130 antigen is present in the endocardial cells (EN), the mesenchyme of the atrioventricular cushion (AVC) as well as in some epicardial mesothelial cells (E), particularly those that are large and rounded. Other squamous epicardial cells, directly adhered to the myocardial wall, were ES/130\(^-\). A, atrium; V, ventricle.
experimental approaches to the study of coronary vessel development. Mikawa and Fischman (1992) demonstrated, through retroviral cell labelling, that the precursors of the chick coronary arteries enter the heart during the epicardial morphogenesis, and that the coronary arteries form by coalescence of discontinuous colonies. Poelmann et al. (1993) obtained quail coronary endothelial cells after transplantation of quail liver to chick. Furthermore, the formation of coronary vessels is severely disturbed or impeded when the epicardium does not develop properly, either by mechanical (Manner, 1993) or genetical (Kwee et al., 1993) manipulations. In order to test this hypothesis, the possibility of a transient colocalization of CK with specific vascular markers in the SEMC will be the subject of our further studies.

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