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Immunohistochemical evidence for a mesothelial contribution to the ventral wall of the avian aorta

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Summary

We report morphological and immunohistochemical evidence for a translocation of cells from the coelomic mesothelium to the aortic wall between the developmental stages HH16 and HH22 of the quail embryos. The coelomic mesothelial cells closest to the aorta showed, at these stages, increased mitotic activity, reduced intercellular adhesion, loss of tight junctions, and long basal cytoplasmic processes. Coinciding with these morphological traits, cytokeratin immunoreactivity was found in the mesothelium, in cells of the aortic wall and throughout the ventral periaortic mesenchyme (but not in the lateral and dorsal aortic regions). Vimentin immunoreactivity colocalized with cytokeratin in the mesothelial cells adjacent to the aorta. In the ventral aortic wall, cytokeratin colocalized with smooth muscle α-actin and with the 1E12 antigen (a smooth muscle-specific α-actinin isoform). We think that the morphological and immunolocalization data observed are compatible with an epithelial–mesenchymal transition by which mesothelial-derived cells contribute to the splanchnic mesoderm and aortic wall. The precise coincidence between the mesothelial contribution and the emergence of the aortic smooth muscle cells progenitors, as well as the immunolocalization data, suggest a potential relationship of the mesothelial-derived cells with this cell lineage. This may explain the observed ventrodorsal asymmetry in the distribution of smooth muscle cells progenitors in the aortic wall.

Introduction

The dorsal aorta, in avian embryos of 3–4 days of incubation, is composed of (1) mesenchymal cells, a number of which express smooth muscle cell-specific α-actin, (2) endothelial cells, and (3) clusters of hemopoietic stem cells which will colonize the rudiments of the hemopoietic organs giving rise to the definitive blood cell system (Dieterlen-Liévre & Martin 1981, Cormier & Dieterlen-Liévre 1988, Lee *et al.* 1997). Mesenchymal cells and hemopoietic stem cells show, in these embryonic stages, an asymmetrical distribution in the aortic wall, being more abundant in the ventral half (Pardanaud *et al.* 1996, Lee *et al.* 1997). In fact, hemopoietic stem cells have not been reported in the dorsal half of the aorta.

The transient asymmetry of the embryonic dorsal aorta remains basically unexplained. Regarding the smooth muscle progenitor cells, an asymmetrical expression of factors responsible for the recruitment of mesenchymal progenitors, or a ventrally-located source for these progenitors, has been suggested (Hungerford *et al.* 1996, Lee *et al.* 1997). Based on their studies of the aortic endothelium and the intraaortic clusters of hemopoietic stem cells, Pardanaud *et al.* (1996) proposed a mosaic origin for the dorsal aorta. One source would be precursors of paraxial origin (in the dorsal part) and the other being precursors arising from the splanchnopleural mesoderm. Such cells, it is proposed, will give rise to the ventral aortic endothelium and, presumably, to the hemopoietic stem cells (Pardanaud *et al.* 1996).

Therefore, the origin of the aortic smooth muscle cells and the intraaortic clusters of hemopoietic stem cells have been traced back to the aortic wall and neighbouring areas of chick embryos of 3 days of incubation. However, the progenitors of these cells have not been followed back towards earlier developmental stages, previous to the expression of specific markers of these cell types.

On a morphological basis, Olah *et al.* (1988) suggested that the intraaortic hemopoietic stem cells originate from cells migrating from the adjacent coelomic mesothelium in chick embryos. This proposal has been surprisingly overlooked in most reviews about the origin of the intraembryonic progenitors of the hemopoietic stem cells. However, the hypothesis put forward by Olah *et al.* (1988) may be related to recent findings on the developmental fate of mesothelial-derived cells in the subepicardium of avian and mammal embryos (Markwald *et al.* 1996, Pérez-Pomares *et al.* 1997, 1998, Dettman *et al.* 1998). We have proposed elsewhere that most, if not all, subepicardial mesenchymal cells derive from the surface mesothelium by a process of epithelial–mesenchymal transformation. These cells may be involved in the formation of vessels, blood island-like structures and coronary vessel smooth muscle cells (Pérez-Pomares *et al.* 1997, 1998, Dettman *et al.* 1998).

We have performed a histomorphological and immunolocalization study of the embryonic dorsal aorta in order to test the hypothesis that mesothelia contribute to the mesenchyme which surrounds the embryonic aortic wall. The study was

done on quail embryos (*Coturnix coturnix*). We aimed to correlate the morphological information with an immunolocalization of the cytoskeletal proteins cytokeratin, vimentin, smooth muscle cell α-actin and the 1E12 antigen. We also immunolocalized the QH1 antigen as a marker of hemopoietic and endothelial cells (Pardanaud *et al.* 1987). Cytokeratin is present in the coelomic mesothelium (Vrancken-Peeters *et al.* 1995). During the epithelial–mesenchymal transition, cytokeratin transiently persists in the mesenchymal cells (Fitchett & Hay 1989, Hay 1990, Pérez- Pomares *et al.* 1997). Vimentin is the typical intermediate filament of the mesenchymal and endothelial cell, but it has also been reported in premigratory epithelial cells (Franke *et al.* 1982, Hay 1990). The smooth muscle α-actin is specific of the smooth muscle cells, although this actin isoform is also expressed in the cardiac endothelium during its transformation to valvuloseptal mesenchyme, and may be related to the phenotypic changes associated with the acquisition of a migratory phenotype (Nakajima *et al.* 1997). Finally, the 1E12 antigen is most probably a member of the α-actinin family of cytoskeletal proteins. This antigen is specifically expressed in cells restricted to the smooth muscle lineage (Hungerford *et al.* 1996, 1997).

We think that the immunoreactive pattern of these antigens and their eventual colocalization in the embryonic aortic wall can provide new information about the hypothetical contribution of mesothelial-derived cells and the differentation of the components of the embryonic dorsal aorta.

Materials and methods

The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health (NIH publication No. 8523, revised 1985). Quail eggs were kept in a rocking incubator at 38 °C. The embryos were staged according to the Hamburger and Hamilton (1951) stages of chick development.

Semithin sections and transmission electron microscopy

Twenty-two embryos ranging between Hamburger and Hamilton (1951) stages HH15 and HH24 were fixed in 1% paraformaldehyde, 1% glutaraldehyde in phosphate-buffered saline (PBS), washed and postfixed in 1% OsO_4 for 90 min. After washing, the embryos were dehydrated in an ethanol series finishing in acetone and embedded in Araldite 502. Semithin (0.5–1μm) and ultrathin sections were cut on a Reichert UMO-2 ultramicrotome. Semithin sections were stained with toluidine blue and observed under a light microscope. Ultrathin sections were contrasted with lead citrate and uranyl acetate and were observed and photographed in a Philips 300 transmission electron microscope.

Immunolabeling

The immunolocalization study was performed on 16 quail embryos ranging from HH17 to HH24. The embryos were excised and fixed in 40% methanol, 40% acetone and 20% distilled water for 8–12 h. After fixation, the embryos were dehydrated in an ethanol series finishing in butanol and paraffin-embedded. Ten μm serial sections were obtained with a Leitz microtome and collected on poly-L-lysine coated slides.

Selected sections were dewaxed in xylene, hydrated in an ethanol series, washed in Tris–phosphate buffered saline (TPBS, pH 7.8) and blocked for 1 h with 16% sheep serum, 1% bovine serum albumin and 0.5% Triton X-100 in TPBS (SBT). Then, the slides were blocked with the avidin–biotin blocking kit (Vector, Burlingame, CA) according to the instructions of the supplier. For the immunoperoxidase technique, the sections, which have been incubated in 3% hydrogen peroxide for 30 min in TPBS, were incubated overnight at 4 °C with the primary antibody diluted in SBT. After washing in TPBS, they were incubated for 1 h at room temperature in biotin-conjugated anti-mouse or anti-rabbit goat IgG (Sigma) diluted 1 : 100 in SBT, washed again and incubated for 1 h in avidin–peroxidase complex (Sigma) diluted 1 : 150 in TPBS. Peroxidase activity was developed with Sigma Fast 3,3′-diaminobenzidine (DAB) tablets according to the directions of the supplier.

For the double immunofluorescence study, the sections were incubated overnight at 4 °C with the primary polyclonal antibody diluted in SBT. After washing in TPBS, they were then incubated for 1 h in biotin-conjugated goat anti-rabbit IgG (Sigma) diluted 1 : 100 in SBT, washed and incubated for 1 h in extravidin–FITC or TRITC conjugate (Sigma) diluted 1 : 150. The sections were blocked again in SBT for 30 min, incubated for 1 h in the primary monoclonal antibody diluted in SBT, washed and incubated for 1 h in TRITC or FITC-conjugated goat anti-mouse IgG (or FITC-conjugated anti-mouse IgM in the case of the 1E12 antibody) diluted 1 : 50 in SBT. After washing, the sections were observed in a Nikon Microphot FXA equipped with epifluorescence and in a laser confocal microscope Leica TCS-NT, using filters specific for the FITC and TRITC fluorochromes. Selected images were captured and printed in a Sony digital colour printer.

The QH1 monoclonal antibody, which stains quail endothelial and hemopoietic cells, was supplied by the Developmental Studies Hybridoma Bank, University of Iowa. It was used at a 1 : 200 or 1 : 500 dilution of the supernatant for immunofluorescence and immunoperoxidase, respectively. Polyclonal anti-bovine epidermal cytokeratin (Z622, Dakopatts, Denmark) is used for wide screening of keratins in several epithelial tissues. It has been used to describe the epicardial covering of the heart of quail embryos (Vrancken-Peeters *et al.* 1995). This antibody was diluted at 1 : 1000 for immunoperoxidase and 1 : 100 for immunofluorescence. The monoclonal anti-chick vimentin (clone AMF-17b) was also from the Hybridoma Bank, and it was used at a 1 : 200 and 1 : 100 dilution in immunoperoxidase and immunofluorescence techniques, respectively. The monoclonal anti-smooth muscle α-actin (clone 1A4, Sigma) is specific for this actin isoform and shows a wide reactivity in vertebrates. It was used at a 1 : 200 and 1 : 100 dilution in immunoperoxidase and

immunofluorescence, respectively. The monoclonal 1E12, developed at the laboratory of Dr. Little (Medical University of South Carolina) is an IgM isotype (Hungeford *et al.* 1996, 1997). The supernatant was used undiluted.

Results

Light and transmission electron microscopy

The dorsal aorta was studied in transverse serial sections at the level of the heart and liver primordium.

Stage HH15

In this stage, the mesothelial cells located in the corner between the dorsal mesentery and the lateral body wall showed a characteristic bottle-like shape, with long basal cytoplasmic processes and wide extracellular spaces between them. These morphological traits were more evident throughout the dorsal mesentery, and around the endodermal tissue, where mesenchymal cells were very abundant. However, mesenchymal cells were relatively scarce in the area ventral to the dorsal aorta, a site distant from the coelomic mesothelium (Figure 1A).

The aortic endothelium was squamous, without noticeable differences between the dorsal and the ventral areas (Figure 1A). The endothelial nuclei were ovoid or elliptical, and the lateral cytoplasmic projections were long and thin.

Stages HH16–HH17

At the HH16 and HH17 stages, the morphological traits of mesothelial cell migration towards the lateroventral areas of the aorta were more conspicuous than in the previous stage. At the ultrastructural level, tight junctions displaying a well-developed zonula occludens were observed at the HH16 stage between the mesothelial cells located close to the lateroventral aortic wall (Figure 2A). However, well-developed tight junctions were not observed in this area at the stage HH17, when large spaces were conspicuous between the mesothelial cells. Instead, mesothelial cells were connected by small junctions. Sometimes, only a narrow process of the cell was connected to the mesothelial lining by these junctions, while most of the cell projected towards the submesothelial space (Figure 2B).

Mesenchymal cells, showing the same ultrastructural features as the neighbouring mesothelial cells, were loosely arranged in the periaortic areas, with their longest axis oriented towards the aorta (Figures 1B and 2B). In the lateroventral areas of the aorta, the mesenchymal cells were more closely packed and arranged in layers under the endothelium.

The aortic endothelial cells displayed a clear heterogeneity between the dorsal and the ventral areas (Figure 1B). The dorsal endothelial cells remained flattened and squamous. In contrast, the ventral and ventrolateral endothelial cells showed more rounded nuclei, protruding frequently into the aortic lumen. Ventral cells were more closely arranged than in the dorsal area, and the lateral cytoplasmic projections were consequently shorter and thicker. The presence of vacuoles, which were sometimes very large, was noticeable in the ventral endothelial cells. These vacuoles were frequently located in the ventral part of the cells, causing the protrusion of the nucleus and the cytoplasm towards the aortic lumen. These ventral aortic endothelial cells formed a continuous lining.

Stage HH18–HH19

The signs of cell migration from the mesothelium to the lateroventral areas of the aorta continued to be conspicuous (Figure 1C). These signs were more distinct in the area closer to the aortic wall. Mitotic images were relatively frequent in the mesothelial and submesothelial areas close to the aorta.

Mesenchymal cells aggregated in the ventral aortic wall. Only a few of them, located in the innermost layer, showed a flattened morphology with elongated nuclei.

The endothelium of the dorsal half of the dorsal aorta retained the squamous morphology, while the distinctive features of the lateroventral areas were more manifest, with large luminal clusters of rounded cells. By these stages, vacuolized endothelial cells were scarce and they were mainly located in the midventral region of the aorta.

Stage HH20–HH21

At HH20–HH21, the morphological traits of migration in the mesothelium adjacent to the aorta persist. Most of the ventral aortic endothelial cells have clearly diminished their protruding aspect and seem to be returning to the endothelial phenotype described for the HH15 stage; however, a clear difference can still be detected between the dorsal and the ventral areas of the aortic endothelium.

Stage HH22–HH24

Between stages HH22 and HH24 there is a progressive decrease in the number of rounded cells in the lateroventral aortic wall as well as in the signs of migration from the neighbouring mesothelium. Instead, a typical, squamous endothelium invests the lateroventral regions of the aorta. At the same time, the number of layers of flattened cells around the aorta increases, and cells showing features of apoptosis become abundant in the areas located ventrally to the dorsal aorta (Figure 1D). By HH24, a continuous tunica media seems to be organized within the aortic wall at the thoracic level.

Immunohistochemistry and immunofluorescence

Cytokeratin immunoreactivity

Cytokeratin immunostaining was prominent in the coelomic mesothelium and also in a number of mesenchymal cells distributed throughout the splanchnic mesoderm (Figure 3A,C–E). The number of cytokeratin-positive mesenchymal cells increased from HH15 to HH22, although they were progressively disappearing in older embryos. A sharp dorsal boundary of the area occupied by these cells coincided with the floor of the dorsal aorta. Cytokeratin-positive mesenchymal cells were not observed dorsally to this level (Figure 3C,E). In the aortic wall, cytokeratin-positive cells were

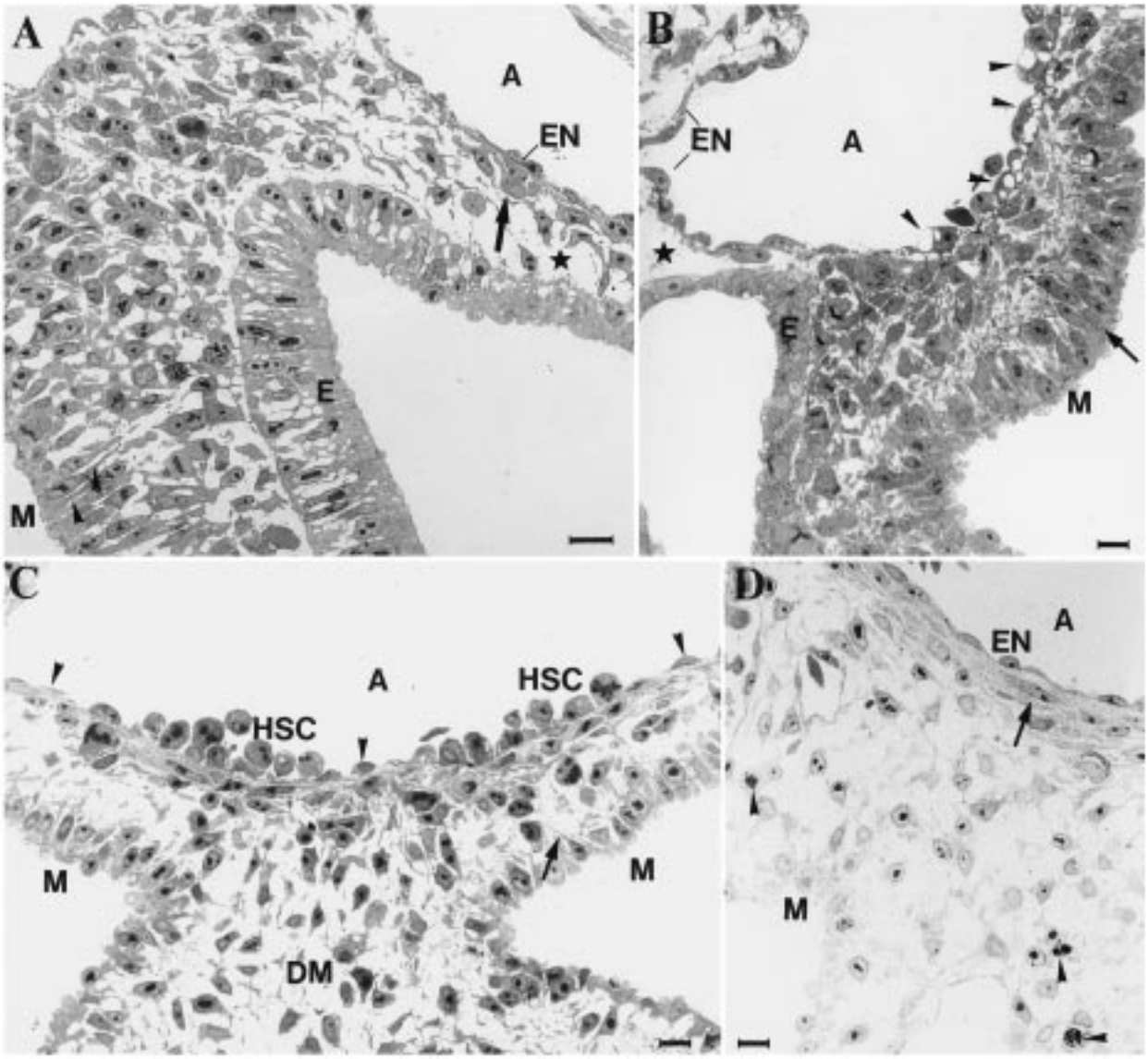

Figure 1. Transverse semithin sections of the aorta (A) and periaortic areas in quail embryos. A: Stage HH15. The mesothelial cells (M) close to the endoderm (E) show signs of migration to the splanchnic mesoderm, which is filled by mesenchymal cells. However, mesenchymal cells are scarce in the periaortic areas located away from the mesothelium (star). Only a few cells (arrow) contact the ventral aortic endothelium (EN) which is squamous. Scale bar: 9 μm. B: Stage HH16. In this region, the mesothelium (M) is located closer to the aortic wall. The mesothelial cells show long basal cytoplasmic processes and remarkable overriding (arrow). Note the differences in cell density between the submesothelial region and the area between the endoderm (E) and the aorta (star). The dorsal and medial aortic endothelium (EN) is squamous, but the lateroventral endothelium (arrowheads) show large vacuoles and signs of releasing into the aortic lumen. The zone of the modified endothelium coincides with the proximity of the mesothelium. Scale bar: 10 μm. C: Stage HH18. The modified endothelium has been replaced by clusters of hemopoietic stem cells in the lateroventral areas of the aorta, coinciding with the proximity of the mesothelium (M). In such regions the cells continue to show long basal cytoplasmic processes (arrow). However, the ventral and lateral aortic lumen is lined by squamous cells (arrowheads). DM: dorsal mesentery. Scale bar: 10 μm. D: Stage HH22. The clusters of hemopoietic stem cells have been replaced by a squamous endothelial lining (EN), and the tunica media is developing in the aortic wall (arrow). The signs of migration (cell overriding, basal processes) have decreased in the mesothelium (M). Apoptotic cells are frequent in the periaortic areas and dorsal mesentery (arrowheads). Scale bar: 9 μm.

found in the ventral and lateroventral areas, sometimes in contact, apparently, with the aortic lumen (Figure 3A).

Vimentin immunoreactivity

Vimentin staining was extensive in the mesenchymal and endothelial cells. However, a number of mesothelial cells, particularly those from the areas located close to the aorta, were also strongly vimentin immunoreactive, especially between HH17 and HH22 (Figure 3B). Colocalization of vimentin and cytokeratin was confirmed in these cells as well as in many splanchnic mesodermal cells (Figure 3C).

Smooth muscle cell α-actin/α-actinin immunoreactivity

The smooth muscle cell-specific α-actin was detected in a number of cells surrounding the dorsal aorta and in the cells which were apparently migrating towards the aorta from the

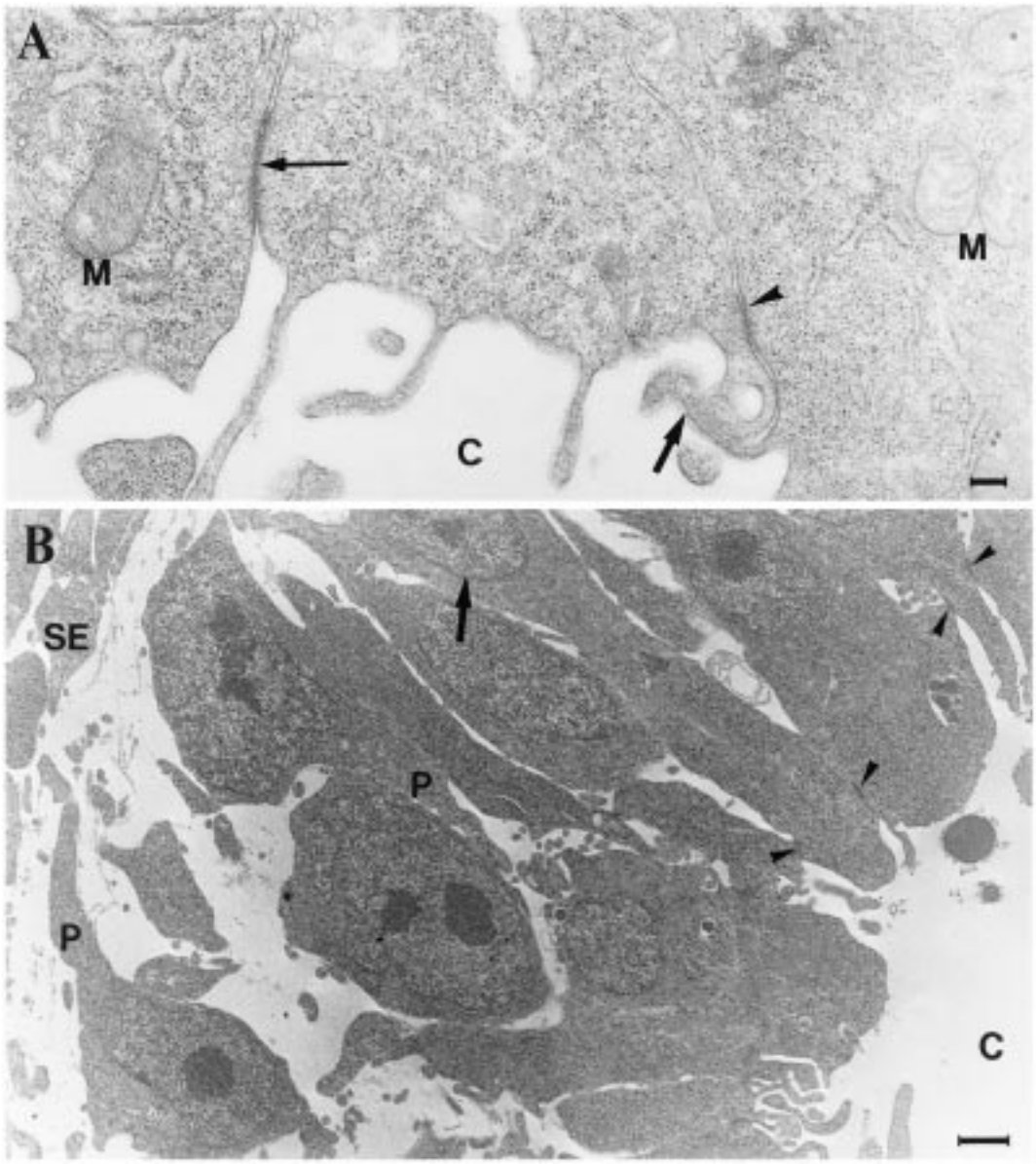

Figure 2. Transmission electron microscopy of the mesothelium adjacent to the aortic wall in quail embryos. A: Stage HH16. Apical surface of the mesothelial cells. The junctional complex of the left (long arrow) shows a well-developed zonula occludens which seems to be absent in the dot-like junction of the right (arrowhead). The contact between the finger-like cytoplasmic projections of two adjacent cells (short arrow) might be related with the model described by Viebahn (1995) on the remodelling in apical junctions during mesoderm ingression. C: coelom; M: mitochondria. Scale bar: 0.2 μm. B: Stage HH17. Mesothelial and submesothelial areas adjacent to the dorsal aorta. Large spaces between the mesothelial cells have appeared. The contact between the mesothelial cells is reduced to small junctions which sometimes connect only a narrow cytoplasmic process to the mesothelial lining (arrowheads). Note the projection of some mesothelial cells into the submesothelial space (arrow). Mesenchymal cells can be seen in this space, displaying leading or trailing pseudopodia (P). C: coelom; SE: subendothelial layer of cells in the aortic wall. Scale bar: 1.3 μm.

ventral areas (Figure 3D). The immunoreactive cells were arranged loosely in the early stages, and more abundant in the ventral than in the dorsal aortic wall. In older embryos, the aortic smooth muscle α-actin positive cells showed a flattened shape, and were arranged in concentric layers around the aorta. A gradient of increasing smooth muscle α-actin immunoreactivity was evident between the outer and the inner cells of the aorta (Figure 3D). However, the 1E12 immunoreactivity (a smooth muscle specific α-actinin) was confined to the juxtaluminal areas (Figure 3E).

Colocalization of cytokeratin with both the smooth muscle cell α-actin and the 1E12 antigen was frequent in the aortic wall by HH17 (Figure 3D,E).

QH1 immunoreactivity

The QH1 antibody labelled the endothelium of the aorta and other vessels, the aortic clusters of hemopoietic stem cells and a number of cells scattered throughout the tissues, including the dorsal mesentery and the aortic wall. In the embryos of the stages HH17– HH18, these aortic QH1+ mesenchymal cells

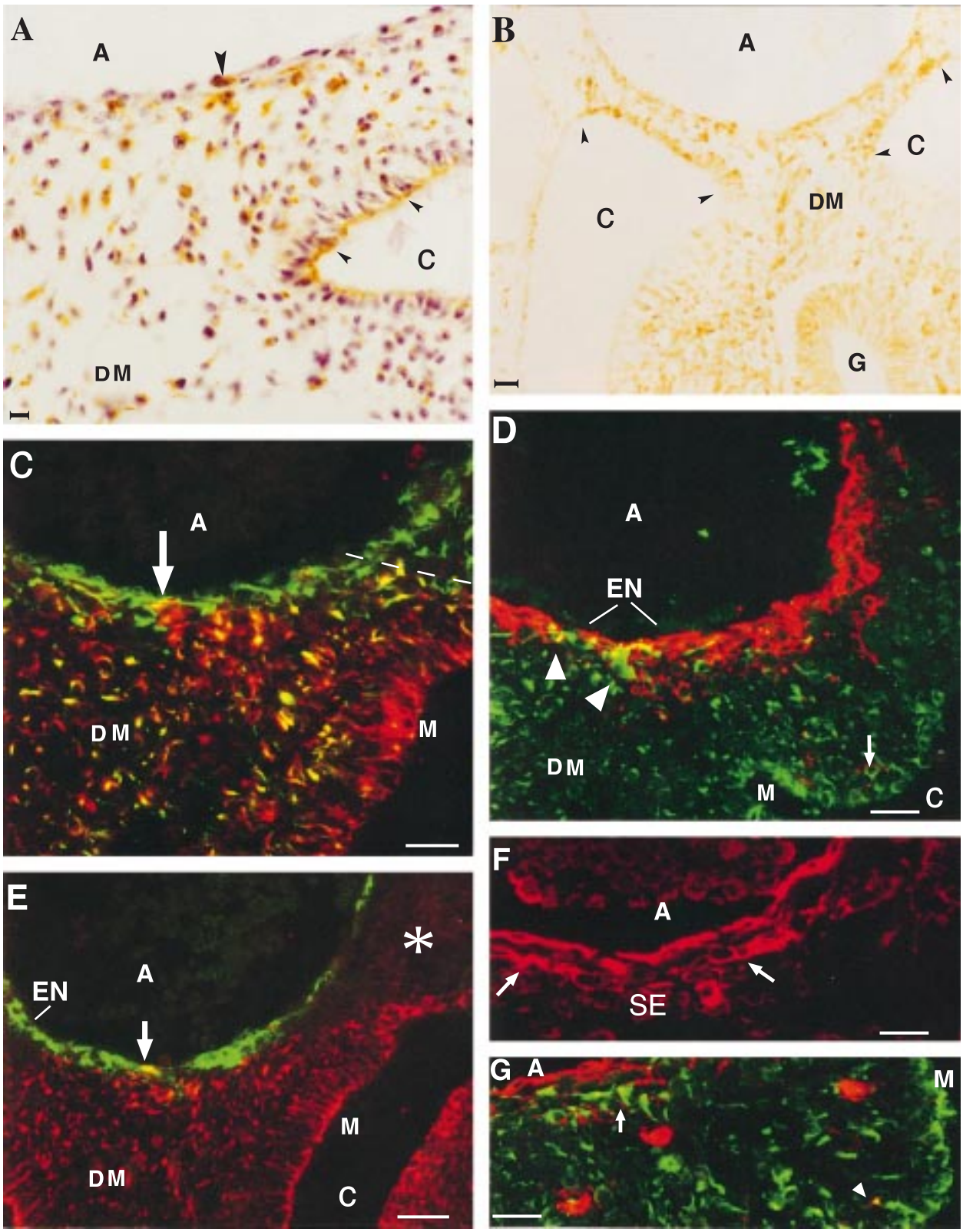

Figure 3. Immunohistochemical features of the aortic wall, periaortic region and adjacent coelomic mesothelium in quail embryos. A: Stage HH17. Cytokeratin immunostaining. Cytokeratin-positive cells are present in the mesothelium lining the coelom (C). Note the signs of ingression (arrowheads) of the mesothelial cells. Cytokeratin-positive cells are abundant in the dorsal mesentery (DM), the periaortic region and the aortic wall, even at adluminal locations (large arrowhead). However, cytokeratin-positive mesenchymal cells were not observed dorsally to the level of the aortic floor. A: aorta. Scale bar: 5 μm. B: Stage HH18. Vimentin immunostaining. Positive mesenchymal cells are abundant in the splanchnic mesoderm surrounding the gut (G) and in the dorsal mesentery (DM). The mesothelium adjacent to the dorsal aorta (A) shows an intense immunostaining (between arrowheads).

showed a very complex arrangement, displaying an increasing gradient of immunoreactivity from the outer to the inner aortic wall (Figure 3F,G). In embryos of stage HH19, two adluminal layers of QH1 immunoreactive cells were sometimes observed in the lateroventral areas (Figure 3F). However, in later embryos, only an endothelial QH1+ layer was observed, neatly separated from the developing media.

QH1-positive and cytokeratin-positive cells were located very close in the aortic wall. The contact between these cells made unequivocal evidence for colocalization of these antigens difficult (Figure 3F). However, some cytokeratin-positive cells seemed to show small areas of QH1 immunoreactivity on their outer surface (Figure 3G).

Discussion

The main purpose of this paper is to test, by means of a histomorphological, ultrastructural and immunolocalization study, the hypothesis that mesothelial cells contribute to the ventral aortic wall (Olah *et al.* 1988). We hoped to establish the developmental timing of this event, and the significance that this observation may have for the origin of the embryonic aortic wall.

Our results show evidence for a substantial translocation of mesothelial cells to the submesothelial space and subsequent migration to the ventrolateral areas of the aorta. This event coincides with the recruitment of the smooth muscle cell precursors and the formation of the intraaortic cell clusters. The existence of this translocation, in chick embryos at 3 days of incubation, was first suggested by Olah *et al.* (1988), based on morphology. According to our observations in the quail embryo, the phenotypic shift of mesothelial cells into mesenchyme seems to be a generalized event in the developmental stages studied, starting in the domains closest to the endoderm, and progressing later to the dorsal mesentery and the mesothelium neighbouring the dorsal aorta; the process there reaches its maximum by HH18. This translocation, which probably supplies abundant mesenchymal cells to the splanchnic mesoderm, can be described in terms of an epithelial–mesenchymal transition (Hay, 1995).

The hypothesis that mesothelial-derived cells contribute to the dorsal aorta was supported by histomorphological information, such as (i) the signs of decreased cell adhesion in the mesothelium, (ii) the disappearance of the epithelial-type junctional complexes, and (iii) the development of long basal migratory appendages. These results correlated precisely with the immunolocalization evidence. Coinciding with the emergence of the morphological traits of mesothelial–mesenchymal transformation in the areas close to the aorta, by HH16, the underlying splanchnic mesoderm was progressively populated by cytokeratin-immunoreactive mesenchymal cells. In later stages, between HH17 and HH22, cytokeratin immunoreactive cells were observed in the aortic wall, including cells that were virtually adjacent to the aortic lumen. However, cytokeratin immunoreactive mesenchymal cells were not observed adjacent to the dorsal aspect of the aorta. The strikingly sharp limit between the areas with and without cytokeratin immunoreactive cells may be related with the proposed mosaic origin of the dorsal aortic wall which we described in the introduction (Pardanaud *et al.* 1996).

The presence of cytokeratin immunoreactive mesenchymal cells in the paraaortic region was transient, disappearing progresively beyond HH22. In order to assess these observations, it is important to remark that cytokeratin is a main component of the cytoskeleton of diverse embryonic and adult epithelia, including the coelomic mesothelium (Vrancken-Peeters *et al.* 1995). Cytokeratin transiently persists after the transdifferentiation of an epithelium into mesenchyme, as it has been reported both *in vivo* and *in vitro* systems (Fitchett & Hay 1989, Hay 1990, Pérez-Pomares *et al.* 1997).

On the other hand, strong vimentin immunoreactivity was conspicuous within the mesothelial cells located adjacent to the aorta at the time of the presumed epithelial–mesenchymal transition. In this area, vimentin colocalized with cytokeratin in the mesothelial as well as in the mesenchymal cells. The expression of vimentin in an epithelial cell may be an early indication of its transformation into a migratory, mesenchymal cell, as it has been demonstrated in the primitive streak and neural tube (Franke *et al.* 1982). Vimentin expression is probably involved in the premigratory shape changes (Page 1989, Hay 1990). We think that the simplest way to explain the cytokeratin and vimentin immunoreactive pattern observed in the paraaortic areas is to assume that cells delaminating from the coelomic mesothelium undergo a cytoskeletal shift from an epithelial type (containing cytokeratin) to a mesenchymal type.

Thus, our results suggest a significant mesothelial-derived cell contribution to the wall of the dorsal aorta coinciding with critical stages of its differentiation. It is tempting to suggest

Figure 3. (*Continued*) C: coelom. Scale bar: 15 μm. C: Stage HH20. Double immunostaining for cytokeratin (red) and vimentin (green). Colocalization is frequent in the coelomic mesothelium (M), mesenchyme of the dorsal mesentery (DM) and aortic wall, even at adluminal levels (arrow). Note the sharp limit of the cytokeratin-positive cells (dashed line). Scale bar: 8 μm. D: Stage HH17. Double immunostaining for smooth muscle cell-specific α-actin (red) and cytokeratin (green). Smooth muscle α-actin immunoreactive cells are abundant around the aorta, especially in the ventral region, where smooth muscle α-actin colocalizes with cytokeratin (arrowheads). The endothelium (EN) and the hemopoietic cells are negative. Migratory cells display a weak smooth muscle α-actin immunoreactivity (small arrow). Scale bar: 20 μm. E: Stage HH20. Double immunostaining for cytokeratin (red) and the 1E12 antigen (a smooth muscle-specific α-actinin) (green). The cells 1E12 immunoreactive are located around the aortic lumen, and more abundant in the ventral region, where 1E12 colocalizes with cytokeratin (arrow). The cytokeratin-positive cells are excluded from areas dorsal to the level of the aortic floor (asterisk). Scale bar: 16 μm. F: Stage HH19. QH1 immunostaining. A subendothelial layer of QH1+ cells can be seen in the lateroventral region of the aortic wall. Scale bar: 9 μm. G: Stage HH17. Double immunostaining for QH1 (red) and cytokeratin (green). Some cases of colocalization occur in the splanchnic mesoderm (arrow) and perhaps in the ventrolateral aortic wall (arrow), where the cells show a complex pattern of alternate QH1 and cytokeratin immunoreactivity. Scale bar: 9 μm.

that both processes are causally related. The colocalization of cytokeratin with both smooth muscle cell α-actin and α-actinin in cells of the aortic wall clearly supports an origin of vascular smooth muscle cells from mesothelial-derived cells. It does not preclude alternative hypotheses proposed for the origin of the smooth muscle cells, such as transdifferentiation of embryonic endothelial cells, as suggested by experimental studies (De Ruiter *et al.* 1997).

There is some indirect support for the possibility of a mesothelial contribution to the progenitors of the aortic smooth muscle cells. By means of an experimental approach, Dettman *et al.* (1988) have shown that the smooth muscle cells of the coronary arteries, as well as perivascular and intermyocardial fibroblasts, arise from the epicardial mesothelium through an epithelial–mesenchymal transition.

The evidence of cytokeratin/QH1 colocalization was not conclusive, perhaps due to the different subcellular distribution of these antigens, intracytoplasmic and cell surface, respectively. In any case, we have shown a precise temporal and spatial coincidence of the hemopoietic stem cells clusters with the areas populated by mesothelial-derived cells. Therefore, we cannot rule out a possible relationship between the migration of mesothelial cells and the intraluminal hemopoietic stem cells clusters. This relationship could be direct or indirect, considering the proposal about an origin of these clusters by sprouting and dissociation of the aortic endothelium (Jaffredo *et al.* 1998).

We think that there are reasons to believe that the cells arising from the splanchnic mesothelium might be pluripotent. Our working hypothesis, published elsewhere (Muñoz-Chápuli *et al.* 1999), is that the embryonic coelomic mesothelium can be regarded as a pluripotent mesodermal tissue, which would supply fibroblasts, smooth muscle cells and perhaps hemangioblasts to all the splanchnic areas in response to local signals. This proposal is highly speculative, but we think that it is experimentally testable and it has explanatory potential. Studies involving direct cell labelling and the culture of embryonic coelomic mesothelium may provide support to the hypothesis. We are currently working in our laboratory in these directions.

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