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Transitions in cell organization and in expression of contractile and extracellular matrix proteins during development of chicken aortic smooth muscle: evidence for a complex spatial and temporal differentiation program

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Abstract

Whereas the understanding of the mechanisms underlying skeletal and cardiac muscle development has been increased dramatically in recent years, the understanding of smooth muscle development is still in its infancy. This paper summarizes studies on the ontogeny of chicken smooth muscle cells in the wall of the aorta and aortic arch-derived arteries. Employing immunocyto-chemistry with antibodies against smooth muscle contractile and extracellular matrix proteins we trace smooth muscle cell patterning from early development throughout adulthood. Comparing late stage embryos to young and adult chickens we demonstrate, for all the stages analyzed, that the cells in the media of aortic arch-derived arteries and of the thoracic aorta are organized in alternating lamellae. The lamellar cells, but not the interlamellar cells, express smooth muscle specific contractile proteins and are surrounded by basement membrane proteins. This smooth muscle cell organization of lamellar and interlamellar cells is fully acquired by embryonic day 11 (ED11). We further show that, during earlier stages of embryogenesis (ED3 through ED7), cells expressing smooth muscle proteins appear only in the peri-endothelial region of the aortic and aortic arch wall and are organized as a narrow band of cells that does not demonstrate the lamellar-interlamellar pattern. On ED9, infrequent cells organized in lamellar-interlamellar organization can be detected and their frequency increases by ED10. In addition to changes in cell organization, we show that there is a characteristic sequence of contractile and extracellular matrix protein expression during development of the aortic wall. At ED3 the peri-endothelial band of differentiated smooth muscle cells is already positive for smooth muscle alpha actin (α SM-actin) and fibronectin. By the next embryonic day the peri-endothelial cell layer is also positive for smooth muscle myosin light chain kinase (SM-MLCK). Subsequently, by ED5

this peri-endothelial band of differentiated smooth muscle cells is positive for α SM-actin, SM-MLCK, SM-calponin, fibronectin, and collagen type IV. However, laminin and desmin (characteristic basement membrane and contractile proteins of smooth muscle) are first seen only at the onset of the lamellar-interlamellar cell organization (ED9 to ED10). We conclude that the development of chicken aortic smooth muscle involves transitions in cell organization and in expression of smooth muscle proteins until the adult-like phenotype is achieved by mid-embryogenesis. This detailed analysis of the ontogeny of chick aortic smooth muscle should provide a sound basis for future studies on the regulatory mechanisms underlying vascular smooth muscle development.

Keywords

Aortic arch-derived arteries; Vascular system; Chick embryo; Cytoskeletal proteins; Basement membrane

Introduction

The onset of vascular development during early embryogenesis is marked by the appearance of an endothelial tube (Wagner 1980; Pardanaud et al. 1987; Coffin and Poole 1988; Noden 1989; Yablonka-Reuveni 1989; DeRuiter et al. 1993; Wilting et al. 1995; Wilting and Christ 1996). The next step in the histogenesis of the vessel wall is the recruitment of cells forming a layer adjacent to the endothelium. These cells become morphologically distinct from the surrounding cells; presumably marking the early emergence of the smooth muscle cell layer (Hughes 1943). Hiruma and Hirakow (1992) noted that the investment of a morphologically distinct cell layer around the aortic endothelial tube during chicken development begins at stage 12 HH (16 somites; Hamburger and Hamilton 1951). A 'primordial' smooth muscle cell layer surrounding the endothelial layer of the dorsal aorta has been further distinguished, immunohistochemically, in 2.5- to 3-day-old chicken and quail embryos by its expression of smooth muscle alpha actin (α SM-actin; Rosenquist and Beall 1990; Duband et al. 1993; Yablonka-Reuveni et al. 1995; Hungerford et al. 1996). Morphological analysis of chicken embryos has demonstrated that, as development progresses, the aortic wall continues to grow by the addition of concentric cell layers (Huges 1943; Arciniegas et al. 1989). However, by embryonic day 8, only the inner few layers are thought to contain differentiated smooth muscle cells, as determined by morphological criteria (Hiruma and Hirakow 1992). Similarly, only the cell layers nearest to the lumen of the aortic arch-derived arteries in 9-day-old (ED 9) chicken embryos were shown to be positive for α SM-actin (Rosenquist and Beall 1990). These aortic arch arteries are derived from the 3rd, 4th and 6th aortic arches which appear during the early days of development (Romanoff 1960; Le Lièvre and Le Douarin 1975).

Different from the aortic smooth muscle cell organization seen in the earlier stages of development, adult chicken aortic smooth muscle cells display a distinct multi-layer organization. Using morphological criteria, Moss and Benditt (1970) demonstrated that the smooth muscle cells of the aortic arch and of the thoracic aorta are organized in lamellar and interlamellar layers; the lamellar cells show intracellular characteristics of smooth muscle

cells and are surrounded by a basement membrane while the interlamellar cells are lacking these specific characteristics. The morphological studies of Moss and Benditt (1970) further recognized that the lamellarinterlamellar organization becomes less defined in the abdominal aorta. Eventually, in the distal part of the adult abdominal aorta, cells with muscular properties were concentrated in the inner part of the media and were not interspersed with cells lacking smooth muscle characteristics. To date, the majority of studies on the development of chicken aortic smooth muscle have primarily focused on the aortic arch-derived arteries and the thoracic aorta. Conducting preliminary immunohistochemical studies with an antibody against desmin we showed that an organization of interchanging desmin-positive and desmin-negative lamellae is already present in the media of aortic arch-derived arteries of ED13 to ED19 embryos (Yablonka-Reuveni et al. 1995). As previously shown for the adult chicken (Schmid et al. 1982), we further demonstrated that cells in both the desmin-positive and desmin-negative lamellae were positive for vimentin. Staining of these late stage embryos with antibodies against the basement membrane proteins laminin and collagen type IV, or with an antibody against α SM-actin, also revealed alternating positive and negative lamellae (Yablonka-Reuveni et al. 1995). The lamellae stained with the antibodies against the basement membrane proteins and the lamellae stained with the antibodies against the smooth muscle contractile proteins desmin and α SM-actin are presumably the same lamellae. Studies confirming this presumption are included in the present paper.

The source of the cells founding the vascular smooth muscle has not been completely resolved but it is now widely accepted that vascular smooth muscle cells can originate from multiple cell types depending on the location of the embryonic blood vessels. Neural crest cells, locally differentiating mesenchyme from all mesodermal compartments, as well as endothelial cells at the vessel wall have been considered as sources for vascular smooth muscle cells (for summary see Le Lièvre and Le Douarin 1975; Kirby and Waldo 1995; Yablonka-Reuveni et al. 1995; Mikawa and Gourdie 1996; Topouzis and Majesky 1996; DeRuiter et al. 1997). In their study that introduced the quail-chick transplantation approach, Le Lièvre and Le Douarin (1975) showed that the tunica media cells in the proximal great vessels (i.e., the aortic arch-derived arteries) originate from cranial neural crest cells. Tunica media cells of the distal vessels were shown to be derived from mesoderm and the tunica media cells in the interfaces of the great vessels with the distal vessels were shown to be of a mixed origin (Le Lièvre and Le Douarin 1975). More recently, employing quail-chick chimeric embryos and an antibody that recognizes quail cells, we showed that both the lamellar and interlamellar cells in the aortic arch-derived arteries are of neural crest origin (Yablonka-Reuveni et al. 1995).

In the study described in this paper we analyzed the transitions in the organization of the smooth muscle cells in the aortic arch-derived arteries during chicken embryogenesis. This can potentially shed light on the dynamics of vascular smooth muscle development which are poorly understood at present. Employing immunocytochemistry with antibodies against smooth muscle contractile and extracellular matrix proteins, we trace smooth muscle cell patterning from early development throughout adulthood. First, we characterize the lamellar and interlamellar cells in the aortic arch from adult chicken. Second, we demonstrate that this smooth muscle cell organization is fully acquired by mid-embryogenesis; during earlier

stages of vasculogenesis smooth muscle positive cells appear only in the peri-endothelial region of the vessel wall occupying a narrow but continuous region. Third, we show that in addition to changes in cell organization there is a characteristic sequence of contractile and extracellular matrix protein expression. The acquisition of the lamellar-interlamellar cell organization by mid-embryogenesis correlates with the stage by which the vascular smooth muscle acquire the 'adult' complement of smooth muscle characteristic proteins.

Although the temporal expression of some of the above smooth muscle markers have been previously analyzed during chick vascular development (Risau and Lemmon 1988; Duband et al. 1993; Yablonka-Reuveni et al. 1995; Hungerford et al. 1996; Bergwerff et al. 1996), and during vascular development in mammals (Sawtell and Lessard 1989; Frid et al. 1992; Glukhova et al. 1993; Miano and Olson 1996; Samaha et al. 1996; Takahashi et al. 1996), the work described in this paper represents the first comprehensive study analyzing a plethora of both extracellular and contractile proteins throughout a wide range of developmental stages and in reference to the emergence of the lamellar-interlamellar cell organization. Our detailed analysis of the ontogeny of chick aortic smooth muscle should provide a solid foundation for future studies on the complexity of the regulatory mechanisms underlying vascular smooth muscle development.

Materials and methods

Animals

Embryonated chicken eggs (White Leghorn) were the source for all embryonic material used in the study and were purchased from local growers. Young chickens (3-week-old, White Leghorn) were kindly provided by the laboratory of Dr. E. Rubel (Department of Otolaryngology, University of Washington), adult chickens (8-week-old, Hubbard) by Acme Poultry (Seattle, Wash.), and old chickens (2.5-year-old hens, White Leghorn) by a local grower.

Tissue preparation

The following protocols were used to process tissues for immunocytochemistry.

Frozen sections of unfixed tissues – (routinely used for post-hatch and adult chickens and for ED16)

The aortic arch and thoracic aorta were removed from the animal and freed from connective tissue. Aortic segments were then immersed in OCT (Tissue-Tek II; Miles Laboratories, Naperville, Ill.) and frozen in liquid nitrogen. Tissue sections (10 µm) were cut with a cryostat and mounted on slides coated with gelatin. Sections were either processed directly or stored frozen until needed; results were not affected by storage. In the instances when this protocol was used to process the aortic arch arteries from mid stage embryos, the entire tree of arterial arches was isolated from the embryo and immersed in OCT as one unit.

Frozen sections of formaldehyde fixed tissues – (used for ED7-ED13 embryos)

In earlier studies we found that our routine protocol of preparing frozen sections was not suitable for the analysis of isolated aortic arches from mid stage chicken

embryos as the tissue integrity could not be fully maintained under these conditions. On the other hand, routine protocols of tissue fixation followed by paraffin embedding for sectioning and subsequent removal of paraffin reduced or completely eliminated our ability to detect many of the proteins via immunocytochemistry (see description below for individual antibodies). Therefore, for the analysis of the developing aortic tree from ED7-ED13 embryos, we adopted the protocol described by Duband et al (1993). As indicated under Results, isolated aortic arches or whole embryos were fixed at room temperature in 4% formaldehyde in phosphate buffered saline (PBS) for 2–24 h, depending on the size of the tissue. After several extensive washes in PBS, embryos or isolated aortic arch-derived arteries were exposed to a graded series of sucrose solutions (12%, 14%, 16%, 18% wt/vol) and frozen in OCT in liquid nitrogen. Tissue blocks were then sectioned with a cryostat as for frozen sections.

Paraplast embedded sections of Serra fixed tissues – (used for ED2-ED6 embryos)—We used this protocol for the younger age embryos because tissue integrity was better maintained compared to the above protocol where the fixed tissue was subsequently frozen for sectioning. The paraplast embedding reduced or completely eliminated the immunoreactivity of some of the antigens with their corresponding antibodies. Nevertheless, this protocol combined with a step of microwaving of sections (see below) allowed the detection, via immunofluorescence, of many antigens that we could otherwise detect only when using frozen sections of unfixed tissue. Whole, ED2 to ED7 embryos were fixed in Serra's fixative, which is made of 6 parts of 100% ethanol, 3 parts of 40% formaldehyde and 1 part of 100% acetic acid (Serra 1946). Twenty-four hours after fixation the specimens were dehydrated with graded ethanol solutions, embedded in paraplast, and sectioned into 7 μ m sections. Paraplast was removed from the sections by xylene and the sections were rehydrated with graded ethanol solutions and immediately processed for antibody staining as below.

Microwaving of paraplast embedded sections—When sections from ED2–ED6 embryos were analyzed, the sections were submitted to a microwaving protocol that was shown to retrieve many antigens for immunocytochemistry (Cuevas et al. 1994). In the present study the α SM-actin antigen could be visualized without this treatment but reactivity with many other antibodies was weak or completely absent without the microwaving and was enhanced following the procedure. A summary of the conditions under which the different antigens are recognized is provided below with the description of each antibody. Following the processing through xylene and graded ethanol the slides were immersed in 10 mM citrate buffer (pH 6.0) and irradiated for 5 min at the 'high' setting using a domestic microwave oven. Evaporated fluid was replaced by water that brought back the buffer to its original concentration and the microwaving cycle was repeated altogether three times. Slides were allowed to cool in the buffer for 20 min at room temperatures, rinsed with TRIS-buffered saline and further processed as described below.

Indirect immunofluorescence

Sections, prepared by the different ways described above, were first treated with a blocking solution to minimize nonspecific antibody binding, then reacted with the primary and

secondary antibodies. For blocking nonspecific antibody binding, slides were kept for 1–2 h at room temperature in sterile TRIS-buffered saline containing normal goat serum (TBS-NGS; 0.05 M TRIS, 0.15 M NaCl, 1% normal goat serum, pH 7.4). Sections were then reacted with the appropriate primary antibody (listed below) for 1 h at room temperature and the incubation was continued over night at 4°C. Sections were then rinsed with TRIS buffered saline containing Tween 20 (TBS-TW20; 0.05 M TRIS, 0.15 M NaCl, 0.05% Tween 20, pH 7.4) and incubated at room temperature for 1–2 h with fluorescein-conjugated secondary antibodies diluted 1:100 with the blocking buffer TBS-NGS. Sections were rinsed again with TBS-TW20 followed by a final rinse with TRIS-buffered saline. Sections were mounted in VECTASHIELD mounting medium from Vector Laboratories (Burlingame, Calif.). In some instances we analyzed the cross sections via double immunofluorescence using a rabbit polyclonal antibody and a mouse monoclonal antibody. The staining protocol was identical to the one described for single antibody stain with the exception that the sections were exposed simultaneously to both primary antibodies and subsequently reacted simultaneously with both secondary antibodies.

For single antibody staining the secondary antibodies used were fluorescein-conjugated goat anti-rabbit IgG (for the polyclonal primary antibodies) or fluorescein-conjugated goat anti-mouse IgG (for the monoclonal primary antibodies). For double antibody staining the secondary antibody combination was either rhoda-mine-conjugated goat anti-rabbit IgG along with fluorescein-conjugated goat anti-mouse IgG, or fluorescein-conjugated goat anti-rabbit IgG along with rhodamine-conjugated goat anti-mouse IgG. All secondary antibodies were from Organon-Technika Cappel (Downington, Pa.).

In many of the studies, following the removal of the secondary antibodies and prior to the final washes, the sections were exposed for 15 min at room temperature to the fluorescent dye 4,6-diamidino-2-phenylindole (DAPI, 1 µg/ml in PBS). This staining allowed visualization of the nuclei together with immunofluorescence. The DAPI-stained nuclei were traced using Hoechst filters (Yablonka-Reuveni et al. 1995).

Observations were made with a Zeiss microscope equipped for epifluorescence, and Kodak EL 135 film (400 ASA) was used for photography. Photographs were taken with a ×16 objective (lower magnification) or a ×40 objective (higher magnification). Regardless of the magnification used, all photographs within an individual figure were enlarged to the same size.

Primary antibodies

The following primary antibodies diluted in TBS-NGS were used throughout the study:

Mouse anti-smooth muscle alpha actin (αSM-actin)—A mouse monoclonal antibody against αSM-actin which was originally developed in the laboratory of Dr. G. Gabbiani (Skalli et al. 1986) was first provided by Dr. Gabianni and in later stages of the study was purchased from Sigma (clone 1A4). This antibody reacts with αSM-actin from many species; employing Western blotting we demonstrated this specificity in the chicken model (J.M. Benson and Z. Yablonka-Reuveni, unpublished work). In our studies the anti-αSM-actin reacted well with tissues processed by each of the protocols discussed above.

Mouse anti-desmin—Mouse monoclonal antibodies against chicken desmin were obtained from the Developmental Biology Hybridoma Bank (hybridomas D3 and D76). These antibodies were developed in the laboratory of Dr. D. Fischman (Danto and Fischman 1984). Both antibodies produce identical staining patterns of chicken smooth muscle. In our studies these two anti-desmin antibodies provided excellent signals only with frozen sections of unfixed tissues.

Rabbit anti-desmin—Rabbit polyclonal antibody against chicken desmin was prepared in the laboratory of Dr. H. Holtzer (Bennett et al. 1978; Fellini et al. 1978) and was kindly provided by Dr. Holtzer. Additional characterization of this antibody by Western blotting has been described by us for chicken skeletal muscle (Yablonka-Reuveni and Nameroff 1986). Analyzing chicken aorta, this polyclonal antibody produces staining patterns similar to those seen with the monoclonal antibodies against desmin. This polyclonal antibody against desmin produced a strong immunosignal with frozen sections of unfixed and fixed tissues as well as with microwaved sections of fixed/paraplast embedded tissues.

Mouse anti-smooth muscle calponin (SM-calponin)—A mouse monoclonal antibody against chicken gizzard calponin (clone CP-93, originally described by Gimona et al. 1990) was obtained from Sigma. In our studies the anti-calponin antibody reacted well with frozen sections of fixed and unfixed tissues, and with microwaved sections of fixed/paraplast embedded tissues; when analyzing early embryos microwaving allowed a better immuno-signal than that seen with frozen sections of fixed tissue.

Mouse anti-smooth muscle myosin light chain kinase (SM-MLCK)—A mouse monoclonal antibody against MLCK from chicken gizzard was obtained from Sigma (clone K36). In our studies the anti-MLCK antibody reacted well with frozen sections of fixed and unfixed tissues, and with microwaved sections of fixed/paraplast embedded tissues; microwaving resulted in a stronger signal than that seen with frozen sections of fixed tissue.

Rabbit anti-smooth muscle myosin heavy chain (SM-myosin)—A rabbit polyclonal antibody against the 204 kDa isoform of bovine aortic smooth muscle myosin heavy chain (SM-myosin) was produced and provided by Drs. C. Kelley and R. Adelstein (Kelly et al. 1992). This antibody generated an excellent signal only with frozen sections of unfixed tissues. Our studies of cell cultures prepared from aortic arch-derived arteries of ED16 chicken embryos demonstrated that the antibodies against SM-myosin or SMMLCK react with the same cells; only some of the cultured cells were positive for SM-myosin (or SM-MLCK) but all the cells were positive for an antibody against platelet myosin (J.M. Benson and Z. Yablonka-Reuveni, unpublished work). The antibody against the bovine aortic myosin, used throughout the present study, provided patterns of immunostaining and immunoblotting (of chicken aorta and of chick aortic cultures) identical to those obtained in our preliminary studies with a rabbit polyclonal antibody against smooth muscle myosin heavy chain that was produced and provided by Dr. D. Larson. The latter antibody was raised against human uterine muscle (Larson et al. 1984a, b).

Mouse anti-laminin—A mouse monoclonal antibody against chicken laminin was developed and provided by Dr. D. Fambrough (hybridoma 31-2; Bayne et al. 1984). This

antibody is also available through the Developmental Studies Hybridoma Bank. The antibody has been previously used to detect laminin in the myofiber basement membrane of chicken skeletal muscle (Bayne et al. 1984; Yablonka-Reuveni 1995) and it is known to recognize laminin subunits of 200 and 400 kDa (α and β laminin chains, respectively; specific chain type has not been established; data provided by the Developmental Studies Hybridoma Bank). The monoclonal antibody against laminin provided identical results to those obtained with a rabbit polyclonal antibody against laminin from Collaborative Research.

Mouse anti-fibronectin—A mouse monoclonal antibody against chicken fibronectin was obtained from the Developmental Studies Hybridoma Bank (hybridoma B3/D6). This antibody was originally developed by Gardner and Fambrough (1983) and was shown to react with the myofiber basement membrane in chicken skeletal muscle (Bayne et al. 1984). In our studies this anti-fibronectin provided an excellent signal with frozen sections of unfixed and fixed tissues, and with microwaved sections of fixed/paraplast embedded tissues.

Mouse anti-collagen type IV—Mouse monoclonal antibodies against chicken collagen type IV were obtained from Drs. R. Mayne and T. Linsenmayer. The characterization of the different antibodies was described in various publications of these investigators (Fitch et al. 1982; Mayne et al. 1983, 1984). We routinely used clone 1A8, which reacts strongly with the basement membrane of myofibers in chicken skeletal muscle. The use of this anti-collagen type IV resulted in an excellent signal with tissues processed by each of the three protocols discussed above.

For negative controls the following analyses were performed: staining with secondary antibodies only, staining with non-related mouse monoclonal antibodies, and staining with the rabbit preimmune sera and/or with non-related rabbit polyclonal antibodies. No immunostaining was observed with these controls.

Results

Lamellar-interlamellar cell patterning in the media of the aortic arch arteries from post-hatch and adult chickens and from late chicken embryos

Frozen sections of an isolated aortic arch from an adult chicken (8-week-old) were analyzed with antibodies against basement membrane and contractile proteins. Figure 1a shows a representative immunostaining with the antibody against the basement membrane protein collagen type IV. A similar pattern was obtained with the antibody against laminin (data not shown). Figure 1b shows a representative immunostaining with the antibody against α SM-actin. Immunostaining with the antibodies against the contractile proteins SM-calponin, SMMLCK, SM-myosin, and desmin resulted in a similar pattern to that shown for the anti- α SM-actin antibody (data not shown). As shown in Fig. 1, the distribution of the immunosignal was similar for both the anti-collagen IV and the anti- α SM-actin antibodies with the exception that the anti-collagen IV antibody also stained the subendothelial basement membrane (indicated in Fig. 1 by V-shaped arrowheads). Whereas a band of the vessel wall closest to the lumen is negative for the antibodies tested, the majority of the

vessel wall consists of stretches of non-continuous lamellae stained with the antibodies interspersed with stretches negative for the antibodies. The immunopositive material in the region closer to the lumen (i.e., the region bordering the negative band) is organized as multiple patches rather than lamellae.

As shown in Fig. 2, the vessel wall of the aortic arch arteries from 3-week-old chickens and from 16-day-old chicken embryos is also organized in multiple immuno-positive and immunonegative lamellae. Figure 2a,a', c,c', d,d' depict double immunofluorescence with various combinations of polyclonal and monoclonal antibodies against the contractile proteins and show that the different contractile proteins colocalize to the same lamellae. The utilization of double immunofluorescence further showed that the contractile proteins and the basement membrane proteins colocalize to the same lamellar regions as seen in the example shown in Fig. b,b'. Finer analysis of this double immunostaining with the anti-myosin and the anti-collagen IV suggests that the myosin-positive material is surrounded by the collagen-positive material. Staining with the antibody against laminin, which generates a less powerful immunosignal than the anti-collagen, indeed demonstrates a localization at the periphery of the lamellae (data not shown; see Fig. 3 for single antibody staining with the anti-laminin).

Figure 3 summarizes additional immunostaining patterns with antibodies that have not been included in the previous figures. As in the staining with the antibody against collagen IV, the anti-laminin antibody also reacts with the sub-endothelial basement membrane (indicated by V-shaped arrowheads in Fig. 3a). The antibody against the extracellular protein fibronectin reacted with both the lamellar and interlamellar regions, although the lamellar regions stained somewhat stronger than the interlamellar regions (Fig. 3b). This staining pattern may indicate that the fibronectin is more concentrated in the basement membrane associated with the lamellar regions compared to the extracellular matrix present in the inter-lamellar regions. Immunofluorescence analysis of earlier developmental stages further demonstrates that the fibronectin antibody reacts with both the lamellar and interlamellar regions (see results below with ED11). As in the immunostaining with the antibodies against the other contractile proteins, the antibody against SM-MLCK reacts with selective lamellae which are interspersed with negative lamellae (Fig. 3c).

Table 1 summarizes the specificity of the different antibodies discussed above with respect to their staining of the lamellar and/or interlamellar regions in the aortic arch and the thoracic aorta from 3- and 8-week-old chickens and from 2.5-year-old chickens. The vimentin distribution included in the Table is based on our previous study where we demonstrated that this cytoskeletal protein is expressed by both the lamellar and interlamellar cells (Yablonka-Reuveni et al. 1995). This distribution was also confirmed for late stage embryos and post-hatch chickens.

Using post-hatch (3-week-old) and old (2.5-year-old) chickens we examined sections from different regions along the aortic arch, the thoracic aorta and the abdominal aorta. In agreement with the morphological studies of Moss and Benditt (1970), our studies with the multiple immunoreagents demonstrated that the lamellar-interlamellar cell organization becomes less prominent in the abdominal aorta. Closer to the diaphragm we could still

identify both positive and negative lamellae; however, the negative lamellae were narrower than those described above for the aortic arch and the overall area occupied by the lamellae was reduced. At the very end of the abdominal aorta, closer to the bifurcation, cells positive for the different contractile markers are not interspersed with negative cells and appear as a continuous, multi-layered band of cells surrounding the lumen (J.M. Benson and Z. Yablonka-Reuveni, unpublished observations). The differing organization of the smooth muscle cells in the thoracic and abdominal aorta was already apparent in ED16 embryos. The smooth muscle in the thoracic aorta was organized in lamellar and interlamellar regions as discussed above for the aortic arch. However, in the abdominal aorta the cells positive for the smooth muscle contractile proteins occupy only the region closer to the lumen, constituting a continuous band of several cell layers, and the outer region of the abdominal vessel wall is essentially devoid of positive cells (J.M. Benson and Z. Yablonka-Reuveni, unpublished work). While the developmental progression of the smooth muscle cells in the abdominal aorta has not been the focus of the present study, these results indicate that the thoracic and abdominal aorta already differ by ED16 in the organization of the smooth muscle cells.

Smooth muscle cell organization and protein expression in the aortic wall of ED3–ED6 embryos

The early investment of smooth muscle cells at the wall of the dorsal aorta is demarcated by a narrow, peri-endothelial band of cells positive for α SM-actin. This ‘pri mordial’ organization of the aortic smooth muscle which was demonstrated for ED4 embryos (Yablonka-Reuveni et al. 1995) was already present in ED3 embryos but was not detected in ED2 embryos. A similar ‘primordial’ staining pattern was observed in the more rostral or caudal regions of the ED3 embryos but the aortic lumen varied in size and shape depending on its position in the embryo (data not shown). This organization of a peri-endothelial band of cells positive for α SM-actin contrasts profoundly with the lamellar-interlamellar organization seen in the aortic arch-derived arteries and the thoracic aorta from late embryos and from post-hatch and adult chicken. This profound difference led us to question when the transition from the primordial to the adult organization does take place, and whether a specific pattern of smooth muscle protein expression correlates with the transition. Serial sections of ED3, ED4, ED5, and ED6 whole embryos were immunostained with the same antibodies against contractile and extracellular matrix proteins used above for the analysis of late embryos and post-hatch chickens. The results of the analysis, which focused primarily on the aortic arch, are summarized in Table 2. Similar to the younger embryos, the peri-endothelial region of the aortic wall in ED5 and ED6 embryos appeared as an α SM-actin-positive ring. In all four embryonic days analyzed, the ring of α SM-actin-positive cells was found strongly positive for fibronectin. Cells positive for SM-MLCK were very infrequent in ED3 embryos but by ED4 the immunostaining pattern with the anti-MLCK was identical to that seen with the antibody against α SM-actin. Reactivity with the antibodies against SM-calponin and collagen type IV was first detected at ED5, becoming more pronounced in ED6 embryos. Staining with the antibody against laminin did not result in detectable immunostaining of the aortic arch even by ED6, while the basement membrane of various other developing tissues was positive for laminin. Likewise, the aortic wall of ED3 through ED6 embryos was found negative for desmin. Examples of the aortic arch from an ED6

embryo stained with the different antibodies, are shown in Fig. 4. Additional aortic arch-derived arteries, seen as smaller vessels in some of the micro-graphs, appear to follow a similar immunostaining pattern.

The absence of immunoreactivity for laminin by ED6 is in agreement with an earlier study by Risau and Lemmon (1988). However the lack of immunostaining with the antibody against desmin is in disagreement with the studies of Duband et al. (1993). In the latter study desmin was detected in the vascular smooth muscle of chicken embryos from ED2.5 forward. In order to provide further support for the specificity of the anti-desmin antibody used in the current study, we included in Fig. 4 a region containing skeletal muscle which is positive for the antibody against desmin. Similarly, while the dorsal aorta in ED3 was negative for desmin the appropriate myogenic regions in the somites were positive for desmin (data not shown). The studies of Beall and Rosenquist (1990) also noted a discrepancy in desmin expression when staining the aorticopulmonary septum of the embryonic chick with the polyclonal antibody against desmin used by Duband et al. (1993). Whereas the immuno-staining with a monoclonal antibody against desmin resulted in a negative signal, the polyclonal antibody generated a positive immunosignal (Beall and Rosenquist 1990). The study of Bergwerff et al. (1996) additionally suggests that the early desmin expression reported by Duband et al. (1993) might be due to an antigen other than desmin.

Analysis of smooth muscle cell organization and protein expression in the aortic arch arteries from ED7 through ED13 embryos

To study the aortic smooth muscle of ED7 or older embryos we used two different types of histological materials. One preparation, as for ED3 through ED6, consisted of cross sections of whole embryos. The other preparation consisted of sections of the aorta and aortic arches that were first isolated from the embryos prior to fixation. Isolation of aortic arch arteries from earlier stages was not possible technically. Both kinds of histological specimens were prepared from the same batch of embryos, fixed by the same method using formaldehyde, and eventually embedded together in frozen OCT so they could be sectioned and subsequently stained with the antibodies simultaneously while situated side by side. We took this approach in view of our inability to detect lamellar-interlamellar cell organization in the aortic wall even by ED13 when cross sections of whole embryos were analyzed. This was puzzling to us because our preliminary results with frozen sections of isolated aortic arch arteries from ED13 revealed evidence of lamellar-interlamellar cell organization (Yablonka-Reuveni et al. 1995). The finding that the aortic wall does not show the lamellar-interlamellar cell organization, unless the aorta/aortic arch arteries are isolated from the embryo, is demonstrated in Fig. 5. Panels a/a' are micro-graphs showing the organization of the α SM-actin-positive cells in the aortic arch wall and the parallel DAPI counterstain of nuclei in a cross section of a whole ED13 embryo. While the lumen contains many nuclei that are a reflection of red blood cells, only the near lumen region is positive for α SM-actin. The α SM-actin-positive band is wider in the ED13 preparation than in the aortic wall of ED6 shown in Fig. 4. Figure 5b, b' show the results of staining with the antibody against α SM-actin using a cross section of a preparation where the aorta/aortic arch arteries tree was isolated from the embryo prior to fixation. V-shaped arrowheads in panels b, b' mark the

border between the vessel wall and the lumen. This border cannot be readily distinguished by the DAPI stain due to the presence of red blood cells. As in the late embryos and post-hatch chickens, the majority of the vessel wall material is organized in alternating lamellae that are positive/negative for the α SM-actin. Likewise, the region closer to the lumen is devoid of α SM-actin-positive cells. The diameter of the aorta that has not been released from the embryo appears to be considerably narrower than the diameter of the released aorta. This diameter change is probably a reflection of the stretched state of the aorta in vivo. When isolated from the embryo, the released aorta relaxes and appears wider and shorter.

As summarized in Table 3, the lamellar cells of ED13 embryos are positive for all the contractile and extracellular matrix proteins tested. As in the adult, the region closer to the lumen of the ED13 aorta was devoid of cells positive for the smooth muscle markers. With the exception of SM-myosin, expression of all proteins was determined using a fixed tissue. The immunodetection of SM-myosin was performed with frozen sections because the reactivity with the anti-SM-myosin antibody was below detection level when using a fixed tissue. SM-myosin reactivity was not determined for embryos younger than ED13 because the morphology of the younger tissue is poorly preserved without fixation.

Smooth muscle in the ED7 aortic wall does not exhibit the lamellar-interlamellar cell organization and is negative for desmin and laminin

The micrographs in Fig. 6 depict cross sections of aortic arch arteries from ED7 embryos that were reacted with the antibody against α SM-actin. Figure 6a,b,b' shows results with sections of fixed embryos. Figure 6c,c' shows results with an aortic arch that was isolated from the embryo prior to fixation. A summary of the reactivity with the other antibodies at this embryonic stage is included in Table 2. Figure 6a is a lower magnification image at the level where both the ascending and descending portions of the aortic arch were present and appear together as a paired vessel. Two additional aortic arch arteries and the esophagus are also shown stained with the antibody. Figure 6b,b' shows a higher magnification of the region pointed out with an arrow in Fig. 6a. Micrographs of released arteries shown in Fig. 6c,c' were taken only at the higher magnification. A comparison of the antibody and DAPI staining in Fig. 6c,c' reveals that the band of α SM-actin-positive cells is narrower than the entire vessel wall and the positive cells are in the region closer to the lumen. Regardless of the isolation of the arteries, there was no evidence of lamellar-interlamellar organization in ED7 embryos.

Isolated aortic arch arteries from ED9 begin to demonstrate the lamellar-interlamellar cell organization and the expression of laminin

Figure 7a–g shows micrographs of sections of isolated aortic arch arteries prepared from ED9 embryos. Figure 7h shows a section of an isolated aortic arch artery from an ED10 embryo. A summary of the immunoreactivity of ED9 arteries with the different antibodies is included in Table 2. The staining with the antibodies against fibronectin and collagen IV was stronger in the region closer to the lumen but, immunopositive material was detected across the vessel wall. The antibody against SM-MLCK also showed a stronger signal closer to the lumen but as shown in the higher magnification in Fig. 7f, the region of the media

further away from the lumen began showing some SM-MLCK-positive cells. Staining patterns with the antibodies against SM-calponin and α SM-actin were similar to those shown for SM-MLCK (data not shown). Staining with the antibody against laminin revealed an immunopositive signal which was uniform in intensity across the wall. Figure 7g,g' are higher magnification micrographs that demonstrate the cellular organization of the vessel wall at this ED9 stage. We failed to detect cells positive for desmin in the aortic wall of ED9 embryos. The absence of desmin was confirmed for both isolated arteries and sections of whole embryos. However, desmin-positive cells were present in the media of ED10 aortic arch arteries as shown in Fig. 7h. Altogether, the findings illustrated in Fig. 7 indicate that the onset of the transition in smooth muscle cell organization from a uniform band of cells to the lamellar-interlamellar cell organization occurs in ED9 embryos. This transition seems to correlate with the onset of laminin expression. The transition is further progressed in ED10 embryos when desmin-positive cells begin to appear. We noticed that the distribution of the smooth muscle positive cells in ED9 and ED10 embryos is not yet uniform along the aortic arch wall; in some sections these positive cells are not yet detected. It is only in aortic arch arteries from ED11 embryos that the cells positive for the smooth muscle markers were consistently identified throughout the media.

The aortic smooth muscle in ED11 embryos exhibit the lamellar-interlamellar cell organization

Analysis of released aortic trees from ED11 embryos with the antibody against α SM-actin revealed a lamellar-interlamellar organization similar to that shown in Fig. 5 for an isolated aortic arch from an ED13 embryo. α SM-actin-positive lamellae were only present in the region of the media further away from the lumen, whereas the region closer to the lumen consisted entirely of negative cells (data not shown). Figure 8 shows micrographs of additional sections of a released aortic tree from an ED11 embryo, stained with the antibodies against SMMLCK (Fig. 8a–a₄), collagen type IV (Fig. 8b–b₄), laminin (Fig. 8c–c₄), and fibronectin (Fig. 8d–d₄). For each vertical series shown in Fig. 8, the top panel shows a lower magnification micrograph depicting immunofluorescent staining of cross sections of whole embryos at the level of the aortic arch arteries (Fig. 8a–d). The second and third panels shown for each antibody (Fig. 8a₁,a₂, b₁,b₂, c₁,c₂, d₁,d₂) are higher magnification, parallel fluorescent and phase micrographs of one of the arteries shown in the top panels. These micrographs demonstrate that the aortic wall is strongly stained with the different antibodies but does not hint at the presence of the lamellar-interlamellar cell organization. Nevertheless, the lamellar-interlamellar cell organization was detected in the ED11 embryos when the aortic arch arteries were first isolated from the embryo before processing for immunofluorescence. Figure 8a₃, b₃, c₃, d₃ show lower magnification micrographs of immunostaining of cross sections of such isolated arteries. Figure 8a₄, b₄, c₄, d₄ show higher magnification micrographs of the same sections shown in Fig. 8a₃, b₃, c₃, d₃. Section of isolated arteries stained with the antibody against SM-MLCK showed the same distribution of positive cells as discussed above for isolated arteries stained with the antibody against α SM-actin. Immunostaining with the antibodies against collagen IV and laminin also showed a multi-lamellae organization. In contrast, the immuno-staining with the antibody against fibronectin resulted in a uniform signal across the vessel wall and did not show individual lamellae. Studying aortic vessels from different age chickens we

concluded that the immunosignal generated by the antibodies against α SM-actin, SMMLCK, SM-calponin, laminin and collagen IV remains strong throughout adulthood but the immunosignal generated by fibronectin is reduced in intensity in adult chickens.

Discussion

The main focus of this study has been the analysis of aortic smooth muscle cell organization and the temporal expression of contractile and extracellular matrix proteins characteristic of smooth muscle cells throughout development of the aortic arch-derived arteries. The rationale behind this comprehensive investigation of the ontogeny of aortic smooth muscle has been the need to establish a sound base for future investigations into regulatory mechanisms underlying smooth muscle development.

Developmental changes in aortic smooth muscle cell organization

Employing immunocytochemistry with antibodies against smooth muscle contractile and extracellular matrix proteins we traced smooth muscle cell patterning from early development throughout adulthood. Comparing late stage chicken embryos to post-hatch chickens we demonstrated that the cells in the media of aortic arch-derived arteries and of the thoracic aorta are organized in alternating lamellae. The lamellar cells, but not the inter-lamellar cells, express smooth muscle-specific contractile proteins and are surrounded by basement membrane proteins. This smooth muscle cell organization of lamellar and interlamellar cells is acquired in the aortic arch arteries by ED11. We further showed that during earlier stages of embryogenesis ranging from ED3 to ED7, cells expressing smooth muscle proteins appear only in the peri-endothelial region of the aortic and aortic arch wall, organized as a narrow band of cells that does not demonstrate the lamellar-interlamellar pattern. On ED9, infrequent cells organized in lamellar-interlamellar organization can be detected and their frequency increased by ED10. The detection of the lamellar-interlamellar organization by mid development was possible only if the arteries were first isolated from the embryos. This is probably a reflection of the stretched state of the aortic wall by mid stages of embryogenesis. When isolated from the embryos, the arteries become relaxed and demonstrate their multi-lamellae organization. The detection of the lamellar-interlamellar organization upon isolation of the aortic tree from the embryo cannot be due to artifacts of tissue handling. The removal of the aortic arch from the ED9–ED13 embryos is done rapidly by detaching the breast tissue and attached ribs. The aortic tree is then easily removed from the embryo by trimming it at its origin (the heart) and at a mid thorax point. The isolated aortic tree is then rapidly fixed and processed side by side along preparations of whole embryos. The possibility that the multiple lamellae are the result of a smaller number of longitudinal transverse rings that become wider and concentric once they are relaxed, rather than a reflection of multiple alternating lamellae at the same plane, is not supported by our studies. The width of the α SM-actin-positive ring did not demonstrate an obvious change in size or position in relation to the lumen when multiple serial sections along various regions of the aortic arch were analyzed using sections of whole embryos. Furthermore, the lamellar-interlamellar organization can be seen in the aortic arch-derived arteries of later stage embryos even without prior isolation of the vasculature from the embryo (data not shown; for a similar analysis see Bergwerff et al. 1996).

The transition in smooth muscle cell organization from the peri-endothelial band of cells to the more mature smooth muscle that is organized in smooth muscle positive and negative lamellae raises several immediate questions regarding the aortic smooth muscle cells in the early and late embryos:

1. Do the cells in the primordial smooth muscle and in the lamellar-interlamellar smooth muscle belong to different lineages? Preliminary studies of Rosenquist and Beall (1990) with a quail-chick chimera could potentially hint at the possibility that the cells contributing to the primordial smooth muscle organization and cells contributing to the more mature lamellar-interlamellar organization are not of the same origin. However, the observation raising this possibility was only briefly mentioned in the latter study and this issue needs to be investigated in greater detail.
2. Are the lamellar and the interlamellar cells of the same or different origin? Using the quail-chick chimera methodology we showed that the cells comprising the lamellar and interlamellar regions in the aortic arch arteries in ED15 are of the same origin; both are derived from neural crest cells (Yablonka-Reuveni et al. 1995). While the origin of the lamellar and interlamellar cells in the more distal portions of the thoracic aorta has been less studied, it has been suggested that the smooth muscle in the more distal part of the descending aorta is derived from the mesoderm (Le Lièvre and Le Douarin 1975). Hence, it is likely that within a specific site along the aorta the lamellar and interlamellar cells originate from a common lineage; but the specific lineage from which these cells are derived may vary along the aorta, depending on what cells originally surrounded the primordial vessel wall.
3. What type of mechanisms are involved in replacing the primordial organization with the more mature lamellar-interlamellar organization? It is attractive to propose that the cells in the primordial, peri-endothelial smooth muscle are programmed to turn off the expression of smooth muscle proteins and that at the same time the presumptive smooth muscle cells further away from the lumen undergo changes in gene expression that lead to the formation of the lamellar interlamellar organization. It is possible that the primordial smooth muscle is eliminated from the vessel wall by programmed cell death rather than by turning off smooth muscle genes in the sub-endothelial ring. Both alternatives could be supported by comparing the immuno-stain pattern of the aortic arch arteries of ED7 to that of ED9 where the primordial band of cells is significantly diminished while the lamellar-interlamellar organization is initiated. Further experiments are required to determine the actual mechanism underlying the changes from the primordial to the alternating lamellar organization.

A recent study by Bergwerff et al. (1996) concluded that during chicken development the peri-endothelial actin staining is completely lost from the proximal part of the aortic arch arteries and is re-expressed later when elastic cells appear in the media. The study further suggested that arteries that develop a muscular phenotype (such as the abdominal portion of the aorta) do not lose their initially acquired peri-endothelial SM-actin expression. In

contrast, our investigation, which used multiple smooth muscle markers, recognized a transition stage from the peri-endothelial smooth muscle organization to the lamellar-interlamellar smooth muscle organization in the aortic arch arteries. The difference in the antibodies against smooth muscle actin used in the two studies could also lead to the different observations. The anti-actin antibody we used is a well-characterized monoclonal antibody that recognizes specifically α SM-actin. The monoclonal antibody used in the Bergwerff et al. study is a different antibody named HHF35 that recognizes all alpha actins present in cardiac, smooth and skeletal muscle (Tsukada et al. 1987).

The relationship between the temporal expression of contractile and extracellular proteins and the emergence of the lamellar-interlamellar smooth muscle cell organization

In addition to changes in cell organization, we show that there is a temporal program of contractile and extracellular matrix protein expression during development of the smooth muscle in the aortic arch arteries. At ED3 the peri-endothelial band of differentiated smooth muscle cells is already positive for α SM-actin and fibronectin. By the next embryonic day the peri-endothelial cell layer is also positive for SM-MLCK. Subsequently, by ED5 this peri-endothelial band of differentiated smooth muscle cells is positive for α SM-actin, SM-MLCK, SM-calponin, fibronectin, and collagen type IV. However, laminin and desmin (characteristic basement membrane and contractile proteins of smooth muscle) were first seen only at the onset of the lamellar-interlamellar cell organization (ED9–ED10). Like the primordial smooth muscle, the smooth muscle cells organized in the alternating lamella express the contractile proteins α SM-actin, SM-calponin and SM-MLCK (in addition to desmin), and they are surrounded by an extracellular matrix characterized by the expression of collagen type IV (in addition to laminin). Taken together, the data suggest that the development of smooth muscle in the chicken aortic arch arteries involves transitions in cell organization and in expression of smooth muscle proteins until the adult-like phenotype is achieved by mid-embryogenesis.

The finding that the expression of laminin and desmin is initiated at the time that the lamellar-interlamellar cell organization emerges is intriguing, suggesting perhaps a role for laminin in directing the final maturation of smooth muscle in the aortic arch arteries. Interestingly, our studies with cultured cells isolated from the aortic wall of ED16 chicken embryos demonstrated that a sub-stratum of laminin can suppress the expression of the α SM-actin and SM-calponin by the smooth muscle cells without affecting the expression of SM-myosin. On the other hand, smooth muscle cells from ED16 cultured on a substratum of denatured collagen type I were triply positive for the contractile proteins α SM-actin, SM-calponin and SM-myosin (Benson and Yablonka-Reuveni 1993). Hence, it is possible that laminin, via its interaction with surface elements on the vessel wall cells, can affect the emergence of the distinct smooth muscle positive and negative lamellae. As described under Materials and methods, the antibody against laminin used in the present study is known to recognize laminin chains of 200 and 400 kDa (α - and β -chains). Although a further characterization of the specific type of these α and β chains is not available, the antibody is unlikely to recognize the laminin chain β 2. This conclusion stems from our observation that both the lamellar and interlamellar cells in ED16 were positive when reacted with an antibody specific for the laminin chain β 2. Specific integrin chains are thought to act as

receptors for laminin (Timpl and Brown 1994; Giancotti 1996). Studies on human aortic smooth muscle noted the expression of some smooth muscle contractile proteins in the adult but not in the developing embryo (Frid et al. 1992). Likewise, Glukhova et al. (1993) noted developmental changes in the expression of laminin and integrin variants when comparing embryonic and postnatal human arteries. These two human studies support the notion that laminin expression is important for the maturation of the aortic smooth muscle.

Using antibodies against various integrins we attempted to identify a specific integrin expression pattern that may distinguish between the lamellar and interlamellar cells in aortic arch arteries. Focusing on ED16 embryos, we failed to recognize such a possible distinct pattern of integrin expression. Similarly we found both the lamellar and interlamellar cells to be immunopositive when reacted with an antibody against N-CAM. Thiesen et al. (1996) reported on differences in the expression of integrins when comparing cultured chicken smooth muscle cells derived from neural crest cells (i.e., cells isolated from the thoracic aorta) versus smooth muscle cells derived from the mesoderm (i.e., cells isolated from the abdominal aorta). However, in that study the entire cell population was assayed biochemically and it is unclear which specific cells within the culture contribute to the distinctions in integrin expression. Our cell culture studies of aortic smooth muscle from ED16 chicken embryos demonstrated the presence of at least two cell populations; one population expressed characteristic smooth muscle contractile proteins while the other population was negative (Benson and Yablonka-Reuveni 1993). Future studies, identifying molecular markers for the inter-lamellar cells, may shed light on the mechanisms governing the differentiation into lamellar and interlamellar smooth muscle cells.

What might be the physiological basis for the changes in gene expression and cell organization during development of the aortic smooth muscle?

Many studies have provided circumstantial evidence that hemodynamic forces such as shear stress and tangential wall stress may play an important role in the remodeling of the vascular system (Ben-Driss et al. 1997; reviewed by Owens 1995). Hu and Clark (1989) determined that systolic blood pressure increases by 6–7-fold in chicken embryos between stage 12 and stage 29 (stage 29 is equivalent to about ED6 according to Hamburger and Hamilton 1951). A more recent analysis of hemodynamic parameters in chick embryos by Broekhuizen et al. (1993) demonstrated that there is a 17-fold rise in mean dorsal aortic blood flow between stage 20 (ED3) and stage 35 (ED8). The developmental stage at which the dramatic increase in hemodynamic parameters was documented, fits with the developmental stages identified in the present study at which the aortic smooth muscle is still organized in the primordial formation but already expresses the full complement of the primordial markers (i.e., ED6 and ED7). The emergence of the lamellar-interlamellar organization might be linked to additional changes in hemodynamic performance along the aortic arch that are taking place to accommodate the demands of the growing embryo (Hu et al. 1996).

In summary, the present study presents a comprehensive analysis of two developmental processes that take place during embryogenesis of the wall of aortic arch-derived arteries and the thoracic aorta. One process is the transition in smooth muscle cell organization from a primordial, peri-endothelial band of cells to the mature, lamellar-interlamellar

organization. A second process is the temporal expression of smooth muscle contractile and matrix proteins. The physiological cues and the molecular mechanisms underlying these two processes are not yet known. Recent studies on the smooth muscle proteins, SM22 (Li et al. 1996) and telokin (Herring and Smith 1996, 1997), provide initial evidence for distinct transcriptional regulatory programs in vascular smooth muscle. The present study suggests, however, that the development of vascular smooth muscle is 'multidimensional'. Future studies on the development of the vasculature are likely to identify complex regulatory elements that can modulate temporal and spatial processes during smooth muscle development.

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Fig. 1.

a,b Lamellar-interlamellar smooth muscle cell organization in the wall of the aortic arch isolated from an 8-week-old chicken. Frozen sections were reacted with the monoclonal antibody against collagen type IV (**a**) or the monoclonal antibody against α SM-actin (**b**). *V-shaped arrowheads* show the position of the sub-endothelial basement membrane, which is stained with the antibody against collagen IV. *Bar* 110 μ m

Fig. 2.

Double immunofluorescence staining of frozen sections of the aortic arch isolated from 3-week-old embryos (**a,a',a'', b,b'**) and from ED16 embryos (**c,c', d,d'**). The section shown at the top was reacted with the monoclonal antibody against desmin (clone D76) and the polyclonal antibody against myosin along with the DAPI stained nuclei. The section shown in the second row was reacted with the polyclonal antibody against myosin and the monoclonal antibody against collagen type IV. The section shown in the third row was reacted with the monoclonal antibody against SM-calponin and the polyclonal antibody

against desmin. The section shown at the bottom was reacted with the monoclonal antibody against α SM-actin and the polyclonal antibody against myosin. In **b,b'** the secondary antibodies were fluorescein-labeled goat anti-rabbit IgG and rhodamine-labeled goat anti-mouse IgG. For all other combinations the secondary antibodies were fluorescein-labeled goat anti-mouse IgG and rhodamine-labeled goat anti-rabbit IgG. *Bar* 45 μ m

Fig. 3.

Immunofluorescent micrographs of frozen sections of aortic arches isolated from a 3-week-old chicken (**a,a'**, **b,b'**) and from a ED16 embryo (**c,c'**). Sections were reacted with various monoclonal antibodies and counter stained with DAPI, which highlights all nuclei; **a,a'** show reactivity with the antibody against laminin and the corresponding DAPI stain; **b,b'** show reactivity with the antibody against fibronectin and the corresponding DAPI stain; **c,c'** show reactivity with the antibody against SM-MLCK and the corresponding DAPI stain.

Bar 45 μ m

Fig. 4.

a–h Immunofluorescent staining of sections of an ED6 embryo at the area of the aortic arch arteries. Whole embryos were fixed with Serra and embedded in paraplast for sectioning. Sections were microwaved prior to being subjected to the immunostaining protocol. Sections were reacted with the monoclonal antibodies against α SM-actin (a), fibronectin (b), SM-MLCK (c), SM-calponin (d), and collagen type IV (e) and the polyclonal antibody against desmin (f, g, h). In all cases the secondary antibodies were fluorescein-labeled. g, h Higher magnification micrographs of f, included side by side to show that the aortic wall

(*single arrow*) is negative for desmin while the developing skeletal muscle (*double arrow*) is positive, indicating that the absence of desmin expression in the ED6 aortic wall is not a technical artifact. For all sections analyzed, the strong fluorescence of the blood cells in the vessel lumen is non-specific. *Bar* 110 μm for **f**; 45 μm for **a–e, g, h** panels

Fig. 5.

Comparison of the morphology of the smooth muscle in the ED13 aortic arch when the embryo was fixed and then sectioned (**a**) versus first isolating the aortic tree from the embryo prior to fixation and sectioning (**b**). The whole embryo and the isolated aorta were fixed in paraformaldehyde and then embedded together in frozen OCT. The two preparations were then sectioned simultaneously and subsequently reacted together with the antibody against α SM-actin followed by a fluorescein-conjugated goat anti-mouse IgG. **a'**, **b'** show the nuclear organization (as visualized by DAPI stain) of the same sections as in **a** and **b**, respectively. Nuclei of red blood cells in the vessel lumen are stained with DAPI (**a'**) but do not react with the antibody against α SM-actin (**a**). *V-shaped arrowheads* in **b**, **b'** mark the border between the vessel wall and the lumen; vessel wall cells in the region closer to the lumen are negative for α SM-actin. Bar 45 μ m

Fig. 6.

Comparison of the morphology of the smooth muscle in the ED7 aortic arch when the embryo was fixed and then sectioned (**a, b**) versus isolating the aortic tree from the embryo prior to fixation and sectioning (**c**). The whole embryo and the isolated aorta were fixed in paraformaldehyde and then embedded together in frozen OCT. The two preparations were then sectioned simultaneously and subsequently reacted with the antibody against α SM-actin followed by a fluorescein-conjugated goat anti-mouse IgG. **b** Higher magnification micrograph of the aortic arch shown in **a**; the *arrow* in each point to the same location. **b', c'** Show the nuclear organization (as visualized by DAPI stain) of the same sections as in **b** and **c**, respectively. Nuclei of red blood cells in the vessel lumen are stained with DAPI (**a'**); *Es* location of the esophagus, which is also positive for α SM-actin. Bar 110 μ m for **a**; 45 μ m for **b, b', c, c'**

Fig. 7. Immunofluorescent staining of cross sections of aortic arch arteries isolated from an ED9 (a–g') and from an ED10 (h) embryo prior to fixation and sectioning. **b–f** Show micrographs of serial sections reacted with the monoclonal antibodies against fibronectin (b), collagen type IV (c) laminin (d) and SM-MLCK (panel e). **a** Shows the counter stain with DAPI of the same section shown in **b**. **f** Higher magnification image of the area identified with an arrow in **e**. **g, g'** Higher magnification images of corresponding phase and DAPI stain of a section adjacent to that shown in **f**. **h** Higher magnification image of a cross

section from an ED10 embryo stained with the monoclonal antibody against desmin (clone D3). In all cases secondary antibody was fluorescein-labeled goat anti-mouse IgG. *Bar* 110 μm for **a–e**, 45 μm for **f–h**

Fig. 8.

Comparison of smooth muscle morphology in sections of aortic arch arteries from ED11 that were prepared by sectioning whole fixed embryos (**a,a1,a2, b,b1,b2, c,c1,c2, d,d1,d2**) or by sectioning aortic arch arteries that were first isolated from the embryo before fixation (**a3,a4, b3,b4, c3,c4, d3,d4**). **a1, b1, c1, d1** Higher magnification images of **a, b, c, d**. **a2, b2, c2, d2** Corresponding phase micrographs of the fluorescent micrographs shown in **a1, b1, c1, d1**. **a4, b4, c4, d4** Higher magnification images of **a3, b3, c3, d3**. The thoracic portion of a whole embryo and the isolated thoracic portion of the aortic tree were fixed in paraformaldehyde and then embedded together in frozen OCT. The two preparations were then sectioned simultaneously and subsequently reacted together with the different antibodies followed by a flour-escein-conjugated goat anti-mouse IgG. Primary antibodies were anti-SM-MLCK (**a–a4**), anti-collagen type IV (**b–b4**), anti-laminin (**c–c4**), and anti-fibronectin (**d–d4**). Bars 110 μ m for **a,a3, b,b3, c,c3, d,d3**; 45 μ m for **a1,a2,a4, b1,b2,b4, c1,c2,c4, d1,d2,d4**

Table 1

Characteristic antigens expressed by lamellar and interlamellar cells

Antigen	Lamellar cells	Interlamellar cells
α SM-actin	^a +	–
SM-calponin	+	–
SM-MLCK	+	–
SM-myosin	+	–
Desmin	+	–
Vimentin ^b	+	+
Collagen type IV ^c	+	–
Laminin	+	–
Fibronectin	+	+

^a + or – indicates the presence or absence of detectable immunostaining with the appropriate antibody

^b Vimentin distribution was reported in Yablonka-Reuveni et al. (1995)

^c Staining with collagen type IV, laminin and fibronectin reflects the presence of the antigen in the matrix surrounding the cells

Table 2

Temporal expression of cytoskeletal and extracellular proteins in the developing aortic wall (– no detectable immunostain, + rare positive cells by the lumen, ++ strong immunostain but occasional sections show weaker or no stain, +++ strong immunostain)

Antigen	ED3	ED4	ED5	ED6	ED7	ED9	ED11
αSM-actin	+++	+++	+++	+++	+++	+++	+++
SM-calponin	–	–	++	+++	+++	+++	+++
SM-MLCK	+	+++	+++	+++	+++	+++	+++
Desmin	–	–	–	–	–	–	+++
Fibronectin	+++	+++	+++	+++	+++	+++	+++
Collagen type IV	–	–	++	+++	+++	+++	+++
Laminin	–	–	–	–	–	++	+++

Table 3

Temporal appearance of lamellar/interlamellar cell organization in the media of the aortic arch-derived arteries (– positive immunostain but no lamellar/interlamellar organization, + infrequent positive lamellar cells, ++ more frequent positive lamellar cells, +++ complete lamellar/interlamellar cell organization, *ND* not determined, *NIS* no immunostain was detected)

Antigen tested	ED7	ED9	ED10	ED11	ED13	ED15-Adult
α SM-actin	–	+	++	+++	+++	+++
SM-calponin	–	+	ND	+++	+++	+++
SM-MLCK	–	+	ND	+++	+++	+++
SM-myosin	ND	ND	ND	ND	+++	+++
Desmin	NIS	NIS	++	+++	+++	+++
Collagen type IV	–	–	ND	+++	+++	+++
Laminin	NIS	–	ND	+++	+++	+++