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Elastic Extracellular Matrix of the Embryonic Chick Heart: An Immunohistological Study Using Laser Confocal Microscopy

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ABSTRACT **The "elastic matrix" constitutes a specialized component of the extracellular matrix which confers resiliency to tissues and organs subjected to repeated deformations. The role of the elastic matrix in living organisms appears to be of key importance since diseases characterized by expression of defective inherited genes which encode components of the elastic matrix lead to premature death. While the elastic matrix of adult organs has received a great deal of attention, little is known about when it first appears in embryonic tissues or its possible role in developing organs. In the present study we have performed an immunohistochemical study of the distribution of elastin and three additional components often associated with elastic matrices in adult tissues (i.e., fibrillin, emilin, and type VI collagen) during the development of the chicken embryonic heart. The three-dimensional arrangement of these components was established through the observation of whole-mount specimens with scanning laser confocal microscopy. Our results revealed three different periods of heart development regarding the composition of the elastic matrix. Prior to stage 21 the embryonic heart lacks elastin but exhibits a matrix scaffold of fibrillin and emilin associated with the endocardium and the developing cardiac jelly. Between stages 22 and 29 the heart shows a transient elastic scaffold in the outflow tract which contains elastin, fibrillin, and emilin. Elastin-positive fibrillar material is also observed during these stages in the base of the atrioventricular cushion adjacent to the myocardial wall. In addition, emilin-positive material appears to be associated with the zones of formation of ventricular trabeculae. Collagen type VI was not detected during these early stages. From stage 30 to stage 40 a progressive modification of the pattern of distribution of elastin, fibrillin, emilin, and collagen type VI is observed in association with the formation of the definitive four-chambered heart. The distribution of the elastic scaffold in the outflow tract appears to be rearranged and becomes restricted to the roots of the main arteries. Each of the components studied here is also deposited at increasing levels in the developing valvular apparatus including the valve leaflets and the chordae tendinea. The components are also present in the subendocardial space where they form aligned fibrillar tracts, an arrangement suggestive of a role in ventricular contractile function. The epicardium constitutes an additional region of elastic matrix deposition during these later stages and contains elastic, fibrillin, and collagen type VI. Finally, during the later stages the intramyocardial matrix ("myocardial interstitium") is formed and characterized by an abundance of collagen type VI, emilin, and fibrillin but lacks elastin-positive material. This study suggests that during cardiac development there is not a fixed composition of the so-called "elastic matrix." Rather, combinations of the different components of the elastic matrices appear to characterize the matrix associated with specific regions of the embryonic heart and may reflect the different tensile properties required in these regions during development. Possible roles for these specific elastic matrices during heart morphogenesis are discussed.**

Key words: Heart development, Embryo, Extracellular matrix, Elastin, Fibrillin, Emilin, Collagen type VI, Myocardium, Immunohistochemistry

INTRODUCTION

Elastic matrices are a specialized kind of extracellular matrix in living organisms and play a crucial role in the adaptation of tissues to mechanical stress (for reviews, see Mecham and Heuser, 1991; Pasquali-Ronchetti et al., 1993; Rosenbloom et al., 1993). At the ultrastructural level elastic matrices consist of large tracts of amorphous material containing peripherally associated microfibrils 10–12 nm in diameter (Ross

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and Bornstein, 1969; Sear et al., 1978; Rosenbloom et al., 1993). In most vertebrates elastin is the basic component of the amorphous material in elastic matrices (Sage and Gray, 1979). Although there are considerable differences in amino acid composition of elastin between species, in all cases the protein is characterized by an abundance of hydrophobic amino acids and extensive polypeptide cross-linking (Rosenbloom, 1993). Because these features provide elastin with rubber-like elastic properties (Gray et al., 1973), its function has been primarily correlated with gross and continuous tissue deformation (Parks et al., 1988; Pasquali-Ronchetti et al., 1993). Most studies of elastic matrices have focused on adult tissues and organs which are subjected to periodic mechanical deformations such as skin, pulsatile blood vessels, lung, and some ligaments where elastin comprises a very high percentage of their dry weight (Sage and Gray, 1979). Studies of elastin in the developing chick embryo using in situ hybridization techniques have indicated that elastin mRNA is expressed early during development (Selmin et al., 1991; Holzenberger et al., 1993). However, these studies depended on the relative abundance of mRNA and did not establish the distribution pattern of elastin itself, or the distribution of the other proteins often associated with elastic matrices.

While elastin has been found to be the main constituent of the amorphous component of the elastic fibers, the composition of the associated microfibrils appears to consist of a heterogeneous group of glycoproteins, including fibrillin (Sakai et al., 1986), MAGP (microfibril-associated glycoprotein; Gibson et al., 1990), and emilin (elastic microfibril interface located protein; Bressan et al., 1993). Collagen type VI has also been proposed to be a constituent of the elastic matrices (Knight et al., 1984) although this feature has been questioned and may be restricted to specific elastic matrices (Sakai et al., 1986). The importance of the association between the fibrillar and amorphous proteins in the formation of elastic matrices remains to be clarified (Mecham and Heuser, 1991; Pasquali-Ronchetti et al., 1993). In general, little is known about the function of the elastin-associated glycoproteins, and all of them have been shown to have a wider distribution than that of elastin. However, the identification of genetic diseases affecting the elastic properties of tissues based on alteration of the genes for the elastin-associated glycoproteins (Dietz et al., 1991; Lee et al., 1991) suggests an important role for these components in elastic functions. The study of the distribution of elastin and associated glycoproteins in elastogenic models should be of great interest and should help to clarify their functions.

The developing heart constitutes a unique organ model which is subjected to continuous cycles of mechanical stress due to the onset of myocardial contraction/relaxation and the pumping of blood shortly after its initial formation. The intensity and the pattern of mechanical stress associated with heart function undergoes changes during the course of development. In the chick embryo hemodynamic changes are particularly evident between stages 17 and 27 (Clark and Hu, 1982; Cuneo et al., 1993). Moreover, the septation of the embryonic tubular heart into a four-chambered organ causes extensive redistribution of stress, and structures such as the valve leaflets and pericardium serve a key role in supporting mechanical tension during heart maturation (Tidball, 1992). Comparative studies in vertebrates have found that increasing blood pressure is accompanied by modifications in elastin composition which increase its elastic properties (Sage and Gray, 1979). Further, at least in the aortic wall, mechanical stress has been identified as a factor stimulating elastogenesis (Keeley and Alatawi, 1991; Sutcliffe and Davidson, 1990). In view of these facts, it has been assumed that the embryonic heart elastic matrix undergoes significant changes during the course of development. Elastin and fibrillin have been found to be present during the embryonic stages of heart development (Rosenquist et al., 1988; Gallagher et al., 1993). In addition, since elastin has been found to interact with neighboring cells by means of specific receptors (Hornebeck et al., 1986; Hinek et al., 1988), it may provide a structural link required for the generation of mechanical forces which are important during the morphogenesis of the heart.

In this work we have studied the temporal and spatial distribution of elastin and several of the proteins proposed to be associated with elastic matrices during the development of the chick heart. Our purpose was twofold. First, we wanted to obtain information concerning the composition of an elastic matrix in a developing model. Such information should improve our understanding of the genesis of elastic matrices. In addition, we wanted to assess a possible role of these matrix components during heart development on the basis of their distribution.

RESULTS

Elastin in the Embryonic Heart

Prior to stage 21 the embryonic heart was negative for elastic labeling. In stage 22 embryos, elastin-positive material was observed at the level of the junction between the outflow tract of the heart and the body of the embryo (Fig. 1). In this region elastin was distributed in a fibrillar pattern in the matrix surrounding the origin of the aortic arches. From this area a well-defined tract of elastin positive fibers extended deep into the dorsal truncal cushion of the outflow tract of the heart. In the following stages the number of elastin fibers in this region increased and by stage 24 two bundles of filamentous material were detected extending between the aortic arches and the primitive ventricle (Fig. 2). Each of these tracts appeared to be associated with one of the conotruncal ridges. Although the predominant direction of the fibers was parallel to the lumen of the outflow tract, fibers were also observed radiating into the surrounding mesenchyme (Fig. 1B).

Low-magnification views with the confocal microscope gave the impression of a fibrillar system anchoring the heart to the embryonic body.

Between stage 25 and stage 29 the number of elastin-labeled fibers in the outflow tract of the heart was at a maximum (Fig. 3). As can be seen in Figure 3A–C, elastin formed a continuous layer of circumferential fibers located at the interface between the cushion tissue and the myocardial layer of the outflow tract. At the upper limit of the outflow tract, fibers extending from the outflow tract to the mesenchyme surrounding the origin of the aortic arches were still detected.

Elastin was also localized in the atrioventricular cushion tissue in the heart between stages 26 and 29. In this region elastin exhibited a striking and well-defined fibrillar pattern, extending from the ventricular myocardium toward the lower part of the atrioventricular cushions (Fig. 4). At first, this fibrillar system gave the impression of a valvular-like system. However, careful observations of several hearts revealed that these fibrils were located adjacent to the ventricular walls at the bases of the dorsal and ventral atrioventricular cushions.

During the following stages changes in the structure and shape of the heart were prominent and accompanied by changes in elastin distribution. The truncal and conal portions of the outflow tract of the embryonic heart from the roots of the great arteries (truncus) and the outflow portion of the right ventricle (conus). Immunolabeling of elastin appeared positive in the root of the great arteries and extended along the aortic wall (Fig. 5), but disappeared from the conus region which becomes incorporated into the ventricle. Elastin-positive matrix was also observed in the area of the atrioventricular cushions which had fused to form the continuous septum separating the two atrioventricular orifices. In the subsequent stages of development elastin in this area became restricted to the developing valve leaflets of both the arterial (semilunar valves) and atrioventricular orifices. In addition to these changes, from day 6 of development an increased amount of elastin was detected in the ventricular epicardium, forming a gradient of immunopositive material which extended from the atrioventricular sulcus toward the apex of the ventricle (Fig. 6).

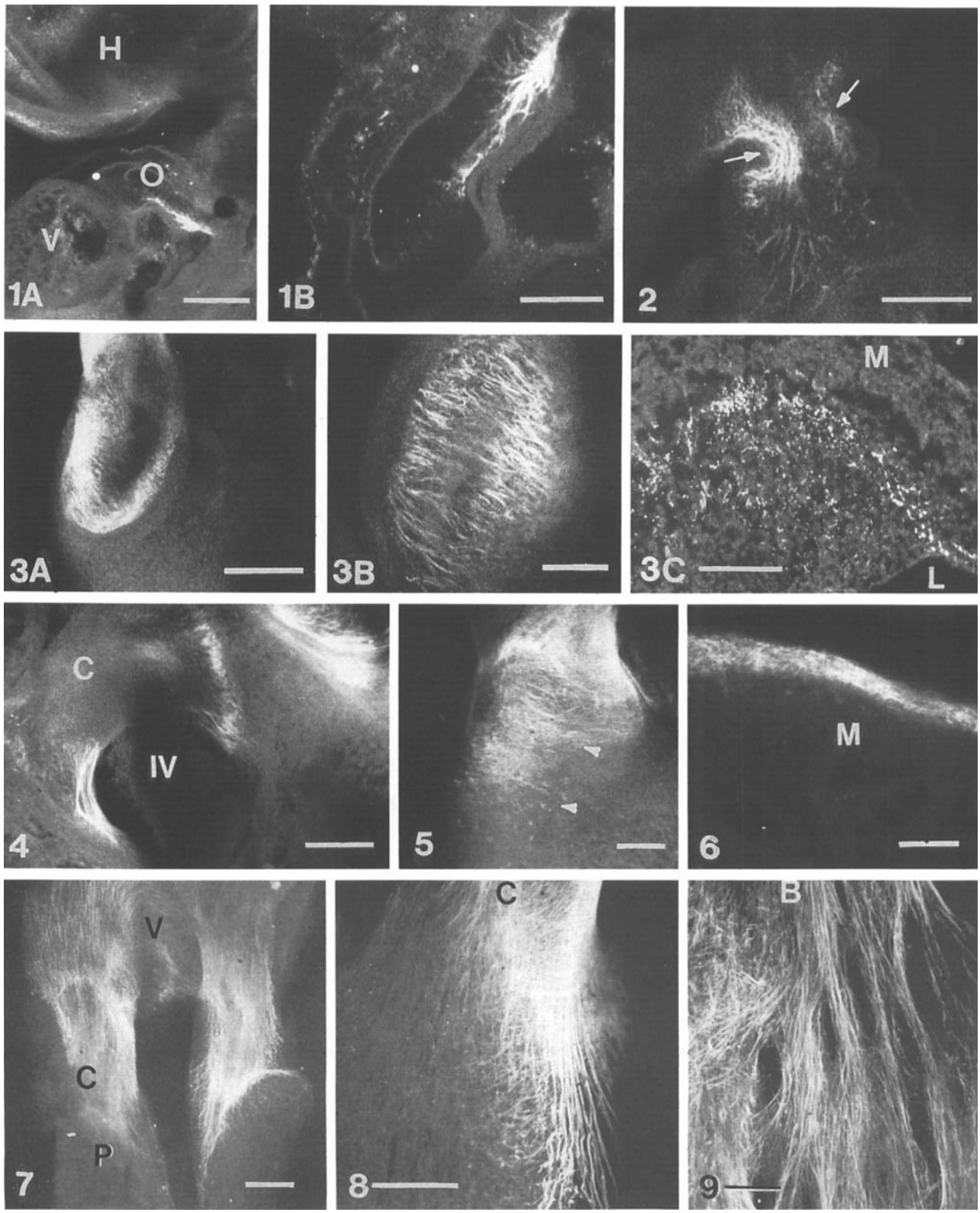
In the oldest stage studied here (stage 40, day 14), elastin displayed a prominent localization in the epicardium and subendocardial space of the atria. In the ventricles elastin was present in the walls of the coronary arteries and appeared to be very abundant in the epicardium and valvular apparatus. As shown in Figure 7, a pattern of parallel elastin-positive fibers extending from the base of the valve leaflets toward their free margins was the prominent feature in the atrioventricular valves. These fibers continued into the tendinous cords and a considerable number of them extended into the papillary muscles of the ventricle where they became intertwined with a plexus of elastin-positive fibers under the subendocardial surface (Fig. 8). Many of these fibers were present in large bundles which branched and covered most the subendocardial surface of the ventricle from the bases of the papillary muscles to the apex of the ventricle (Fig. 9).

Elastin-Associated Glycoproteins and Collagen VI Distribution in the Embryonic Heart

Although the components studied here (i.e., fibrillin, emilin, and collagen VI) were expected to be associated with elastin, they showed patterns of distribution which were not always related to elastin, or to each other, up to stage 30. By stage 40, a point at which the heart begins to exhibit a definitive more mature pattern of elastic matrix, all the components shared a common distribution in the valvular apparatus. However, differences in other zones of localization such as the epicardium and the intramyocardial connective tissue were still present in the older stage embryos.

Fibrillin

Fibrillin was consistently detected in association with the basal surface of the endocardial layer at all stages, including the earliest stage studied (i.e., stage 11). Prior to the formation of the cushion tissue mesenchyme, fibrillin appeared to be concentrated under the endocardial surface (Fig. 10). As endocardial cells delaminated to form the cushion tissue mesenchyme (stages 17–20), fine fibrillar tracts positive for fibrillin were also detected in association with these cells, possibly contributing to the expansion of the matrix in cushion tissue regions (Fig. 11). At stages 23–25 fibrillin localization was restricted to the cushion tissue forming regions in the outflow tract and atrioventricular canal with the most intense labeling still associated with the basal surface of the endothelium in these regions (Fig. 12). From stage 28 onward fibrillin labeling in the outflow tract region became more intense in the same cushion tissue mesenchymal layers in which elastin had been found to be abundant (compare Fig. 13 and Fig. 3). In the atrioventricular cushions fibrillin labeling remained concentrated in the subendocardial region, particularly in the areas of future leaflet formation surrounding the atrioventricular orifices. After stage 32 fibrillin was detected in the arterial walls (Fig. 14), atrioventricular valve leaflets, tendinous cords, and epicardium. As observed for elastin, a subendocardial network of fibrillin-positive fibers was also detected (Fig. 15). In the epicardium maximum labeling was located in the proximity of the atrioventricular sulcus. At stage 40 (day 14) fibrillin showed intense labeling in the valvular apparatus and epicardium (Figs. 16, 17). As can be observed in Figure 16, there appeared to be a parallel arrangement of fibrillin-positive fibers in the valve leaflets. In these older embryonic hearts fibrillin was also localized in the myocardial perimysium, a region in which elastin was not detected (Fig. 17).



Figs. 1–9.

Emilin

As for fibrillin, emilin was detected on the basal surface of the endocardium in the heart from the earliest stages studied. It was also detected as scattered fluorescent clumps in the myocardial layer. By stage 19 emilin was present in tracts extending through the cardiac jelly (Fig. 18). In sections of stage 20 hearts emilin-positive fibrillar material in the cardiac jelly was associated with the migrating cushion mesenchymal cells (Fig. 19). In the stage 20–21 ventricle intramyocardial deposits positive for emilin appeared to be associated with the invaginating endocardium in the zones of myocardial trabeculation (Fig. 20). By stages 28–29, myocardial labeling in the ventricle was widespread but faint and appeared to correspond to the developing perimysial connective tissue matrix. During these stages, as cardiac septation is being completed, emilin showed a significant amount of labeling in the external walls of the atria which was not present at the level of the atrioventricular orifices and interatrial septum (Fig. 21). In the outflow tract region a pattern of emilin labeling, similar to that seen for elastin and fibrillin, remained detectable in the cushion tissue mesenchyme at the level of origin of the great arteries (Fig. 22). At stage 34 (day 8) emilin labeling was observed in the subepicardial and subendocardial epimysium and the intramyocardial perimysium of the myocardial wall of the ventricle (Fig. 23). At stage 40 emilin was detected in the valvular apparatus, ventricular perimysium, and in the wall of the coronary vessels (Fig. 24). Emilin was very abundant in the valve leaflets and formed fibrils which were continuous with the tendinous cords (Fig. 25) and extended through the papillary muscles to the subendocardial surface as observed for elastin and fibrillin.

Collagen Type VI

Collagen type VI was not detected in the embryonic heart prior to stage 26–27. Beginning at stage 28 collagen type VI was detected in the atrioventricular cushion tissue and the epicardium (Fig. 26). In the atrioventricular cushion tissue collagen type VI immunolabeling was intense and showed a wide area of distribution which included the primordia of the atrioventricular valve leaflets. In the central region of the atrioventricular septum the zone of collagen type VI labeling established sharp boundary with the origin of the interatrial septum which was negative (Fig. 27). In the following stages the atrioventricular valve leaflets were positive for collagen type VI (Fig. 28) and remained positive through stage 40. In the epicardial region collagen type VI labeling initially appeared restricted to the atrioventricular sulcus and adjacent ventricular and atrial surfaces (Fig. 26). However, from stage 31 the epicardial distribution of collagen type VI extended in the direction of the ventricular apex. A faint labeling of the outer layers of the aortic and pulmonary roots was also detected by day 8 (stage 34). At stage 40, in a pattern comparable with the components of the elastic matrix described above, collagen type VI labeling was prominent in the valvular apparatus including the valve leaflets, the tendinous cords (Fig. 29) and subendocardial fibrillar tracts (Fig. 30). Collagen type VI labeling also showed an intense dis-

Fig. 1–9. Chick embryo heart specimens after elastin immunolabeling.

Fig. 1. **A:** Stage 23 embryonic chick showing the appearance of elastin positive material in the heart as a tract of fibrillar material originating at the junction with the embryonic body and running longitudinally in the cushion tissue of the dorsal wall of the outflow tract (O). V, ventricle; H, embryonic head. Bar = 500 μm. **B:** Detailed view of the outflow tract in A, showing the presence of fibers branching radially from the longitudinal fibrillar elastin positive tract toward the adjacent cushion tissue. Bar = 200 μm.

Fig. 2. Lateral view of the embryonic heart at stage 24 showing the presence of two elastin-positive bundles (arrows) running through the dorsal and ventral cushion tissue regions of the outflow tract. The dorsal bundle appears earlier in development and is richer in fibrillar material. Bar = 200 μm.

Fig. 3. Illustration showing the elastin-labeled matrix in the outflow tract of the heart between stages 27 and 28. **A:** Low-magnification anterior view of the heart at stage 27 showing the restricted distribution of the labeled material in the outflow tract region. Bar = 500 μm. **B:** Detailed view of A showing the circular arrangement of the elastin positive fibrillar material in the outflow region. Bar = 200 μm. **C:** A transverse section through the outflow tract at stage 28 showing the elastin-positive matrix in the cushion tissue mesenchyme. M, myocardial layer; L, heart lumen. Bar = 100 μm.

Fig. 4. Lateral view of the heart at stage 28 displaying the arrangement of elastin-positive fibrillar material in relation to the atrioventricular cushions (C). Note the precise arrangement of elastin-positive fibrillar material in the zones of junction between the atrioventricular cushion and the anterior and posterior ventricular walls. The interventricular foramen (IV) appears partially filled by a clot of blood. Bar = 200 μm.

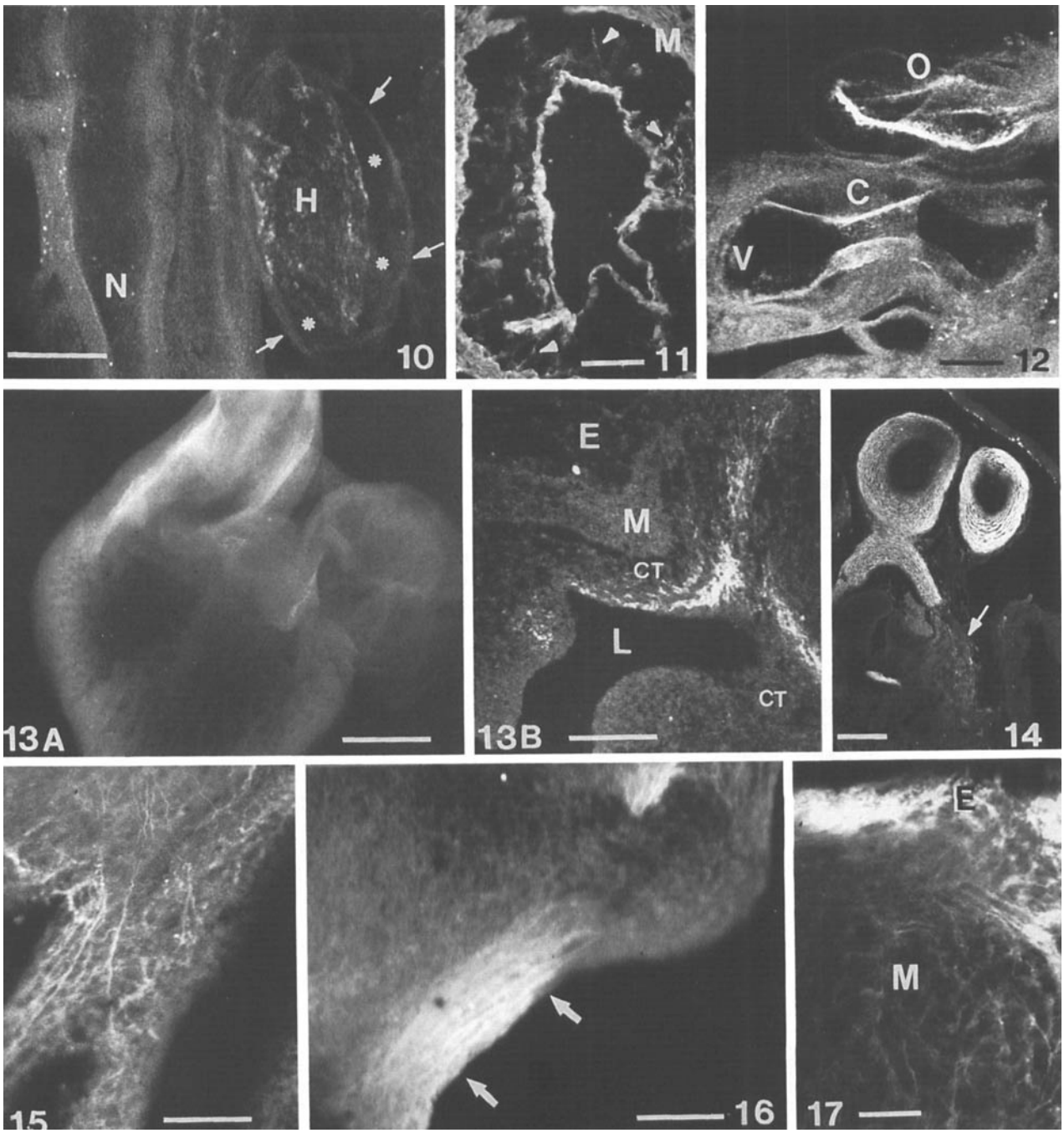
Fig. 5. Low-magnification ventral view of the outflow tract region of the heart at stage 30. Note the loose arrangement of the elastic fibrillar material in the former conal region (arrowheads), while labeling remained intense in the upper region of the outflow tract (former truncus) which now forms the roots of the aortic and pulmonary arteries. Bar = 200 μm.

Fig. 6. Detailed view of the pericardial surface of the ventricle at stage 31. Note the intense labeling of the epicardium. M, myocardium. Bar = 100 μm.

Fig. 7. Low-magnification micrograph showing the left atrio-ventricular valvular apparatus at stage 40. The valve leaflet (V) and the tendinous cords (C) are very rich in elastin-positive fibrillar matrix. Note the arrangement of the elastin-positive fibers extending from the leaflet into the papillary muscles (P). Bar = 200 μm.

Fig. 8. Detailed view of a papillary muscle showing the elastin fibrillar material extending from the tendinous cords (C) and spreading through the endocardial surface of the ventricle. Bar = 200 μm.

Fig. 9. Low-magnification micrograph displaying the abundance of elastin-positive fibers running from the base of papillary muscle (B) toward the apex of the ventricle under the endocardial surface of the left ventricle at stage 40. Bar = 200 μm.



Figs. 10–17. Chicken embryonic heart specimens after fibrillin immunolabeling.

Fig. 10. Dorsal view of a stage 11 chick embryo showing fibrillin-positive material associated with the endocardial layer of the heart loop (H). The optical section of this specimen is tangential to the endocardial tube and shows that the "cardiac jelly" filling the space between the endocardium and the myocardial layer (arrows) lacks labeling (*). N, neural tube. Bar = 200 μm.

Fig. 11. Transverse histological section of the outflow tract at stage 20. The endocardial layer shows a continuous labeling on its basal surface. Positive fibrils extend from the endocardium toward the myocardial layer (M) in areas associated with the developing cushion mesenchyme (arrowheads). Bar = 100 μm.

Fig. 12. Lateral view of the heart at stage 23 showing intense labeling of the endocardial basal surface in the outflow tract (O) and atrioventricular cushions (C). V, ventricle. Bar = 200 μm.

Fig. 13. **A:** Low-magnification ventral view of the heart at stage 29 showing the intense labeling in the outflow region. Bar = 500 μm. **B:** Oblique histological section through the upper part of the outflow region at stage 28 showing the distribution of the labeled material within the cushion tissue mesenchyme (CT). The upper limit of the myocardial layer extending into the outflow tract is observed in the section (M). L, lumen; E, epicardium. Bar = 100 μm.

Fig. 14. Frontal section of the heart at stage 32 displaying intense labeling in the roots of the aortic and pulmonary arteries. Faint labeling is also detected in the epicardial region (arrow). Bar = 200 μm.

Fig. 15. Detailed view with the confocal microscope of the subendocardial surface of a ventricular trabeculum at stage 36 displaying fine fibrils positive for fibrillin immunolabeling. Bar = 100 μm.

Fig. 16. Detailed view of an atrioventricular valve leaflet at stage 40 showing faint homogeneous labeling and a fibrillar bundle very positive for fibrillin running under the free margin of the leaflet (arrows). Bar = 100 μm.

Fig. 17. Detailed view of the ventricular wall of a stage 40 heart. Note the intense labeling of the epicardium (E) and the presence of a network of fine fibrils extending into the underlying myocardium (M). Bar = 100 μm.

tribution in the epicardium forming a complex network of fibers which were continuous with the intramyocardial extracellular matrix (Fig. 31). In the latter region collagen type VI was abundant and showed a coiled fibrillar pattern which was arranged in a direction parallel to the muscle fibers.

DISCUSSION

First Period

Our observations have allowed us to distinguish three distinct periods of heart development regarding the components of the elastic matrix. The first period extends up to stage 22 of chick embryo development and is characterized by the presence of fibrillin and emilin and the absence of elastin in the heart. Fibrillin and emilin exhibit a common distribution in association with the endocardial layer and the cushion tissue mesenchymal cells which originate from it. The formation of the cushion tissue mesenchyme constitutes as critical event in heart development and has been the subject of many investigations. This process constitutes a typical example of epithelial-mesenchymal transformation which appears to be triggered in part by the local production of transforming growth factor β (see Runyan et al., 1992). The components of the extracellular matrix adjacent to the endocardium at these stages are critical for these events since they constitute the substrate for cushion mesenchymal cell migration. They may also provide a mechanism by which growth factors are retained in the area where mesenchymal cells are forming. Numerous extracellular matrix components are detected in the interface of myocardium and endocardium, often referred to as "cardiac jelly," including type I, III, and IV collagens, fibronectin, hyaluronic acid, chondroitin sulfate, laminin (Markwald et al., 1984; Kitten et al., 1987; Little et al., 1989), fibulin (Spence et al., 1992), tenascin (Hurle et al., 1990), vitronectin (Sumida et al., 1992), and collagen type II (Swiderski et al., unpublished data). There is abundant evidence suggesting that most of these individual components may play a significant role as specific substrata for cushion mesenchyme migration and may themselves be produced by the cushion mesenchyme. Since our study shows that fibrillin and emilin are two additional glycoproteins present in the cardiac jelly associated with cushion mesenchyme formation, they should be considered as additional candidates which may play a role in cushion morphogenesis.

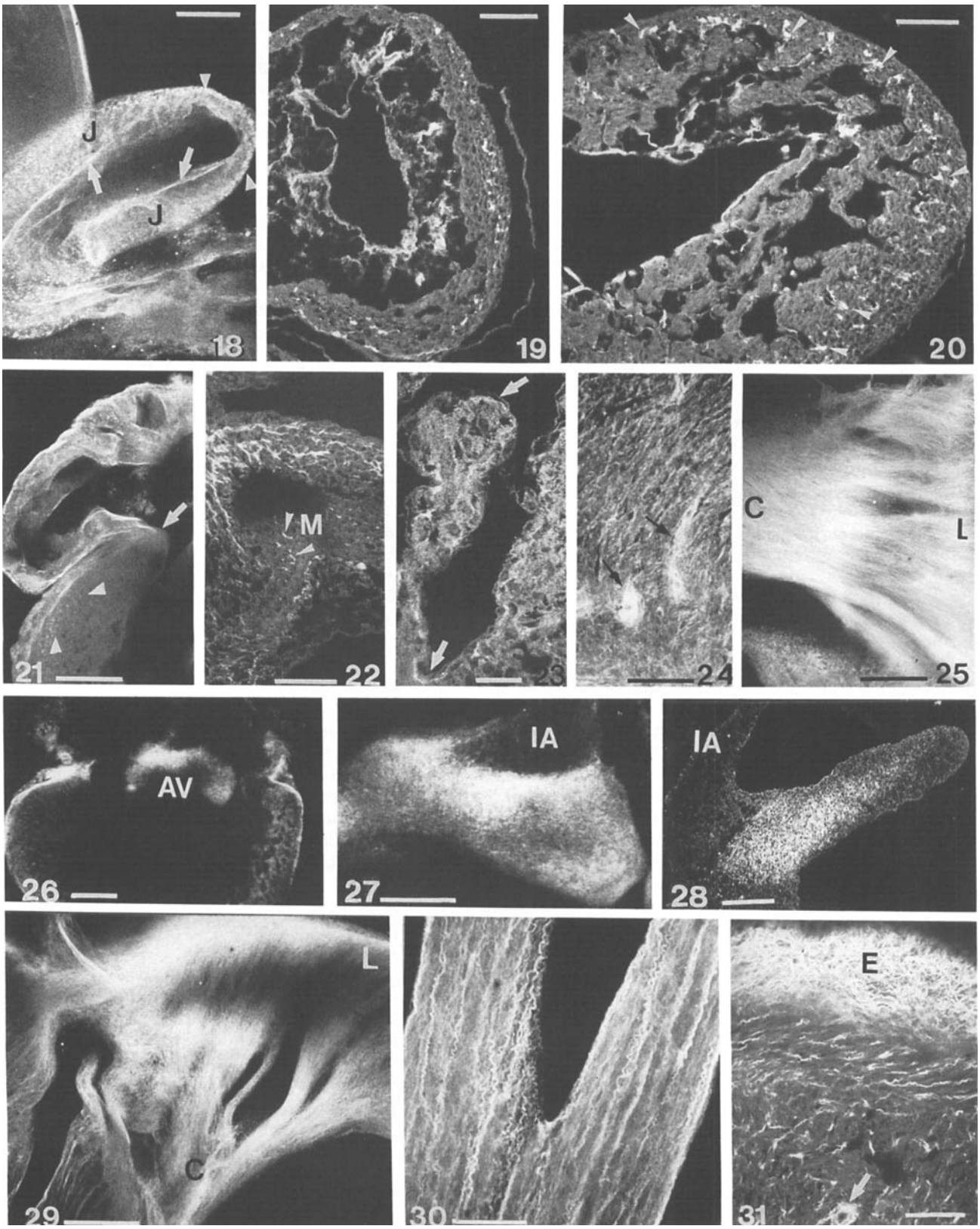
Alternatively, due to the characteristic presence of both emilin and fibrillin in elastic matrices and the proposed importance of fibrillin in conferring elastic properties to the connective tissue as may be deduced by the alterations present in the Marfan syndrome which is caused by mutations in the fibrillin gene (Dietz et al., 1991), it is tempting to suggest that fibrillin and emilin constitute a rudimentary elastic scaffold in the heart in the stages prior to elastin production. It has been clearly established that all animal species have specific extracellular matrix components which provide their tissues with the tensile properties which are required to meet the demands of mechanical stress (Sage and Gray, 1979). Gallagher et al., (1993) have previously suggested a mechanical function for fibrillin in the process of gastrulation. The heart, even during the earliest stages of development, undergoes significant mechanical stress (Clark and Hu, 1982) and therefore most probably requires a specialized elastic matrix. Our results indicate that some of the endocardial cells and the cushion tissue mesenchyme derived from these cells account for the production of the first elastic matrix components of the heart.

In addition to the distribution of emilin and fibrillin in the cardiac jelly it is also worth noting the distribution of emilin among the myocardial cells and on the basal surface of the endocardium in the zones of ventricular trabeculation. This localization is suggestive of a role for this matrix component in the trabeculation of the myocardial wall during heart development.

Second Period

The second period of heart development in relation to elastogenesis commences at stage 22 (day 3.5) and extends to stages 29–30 (day 6.5) when the four-chambered heart is established. The most characteristic feature of this period is the formation of a prominent elastic layer in the wall of the outflow tract of the heart. This elastic matrix contains primarily elastin, and is associated with fibrillin and emilin as observed in several fetal and adult elastic matrices (Sakai et al., 1986; Bressan et al., 1993), but appears to lack collagen type VI. The presence of elastin in the heart at these stages has been previously reported by Rosenquist et al (1988). In the present work, the use of the confocal microscope to study whole-mount embryonic heart specimens has revealed that elastin exhibits a precise fibrillar arrangement in one case extending between the outflow tract and the embryonic body and in another as a circular array surrounding the whole length of the outflow tract. These observations are in agreement with the longitudinal and radial gradient patterns observed with in situ hybridization studies of the distribution of tropoelastin mRNA (Selmin et al., 1991; Holzenberger et al., 1993). It is also interesting to note that this arrangement of elastin in the outflow tract of the heart is reminiscent of the prominent conus arteriosus characteristic of the heart of the elasmobranch fish (Sanchez-Quintana and Hurle, 1987) which, as in the embryonic chick heart at these stages, consists of a single ventricle with an outflow chamber containing myocardium and elastic fibers. This feature suggests that the pattern of elastogenesis observed in the course of phylogeny is conserved during ontogeny.

The mechanisms controlling elastin fibrogenesis constitute a complex process. It has been classically proposed that the microfibrillar components of the elastic fibers constitute a template which precedes elastin deposition (see Clearly et al., 1981; Pasquali-Ronchetti et al., 1993). In this study, although fibrillin and emilin



Figs. 18–31.

are present in the cushion tissue prior to detectable elastin deposition, their initial pattern of distribution as radial fibers diverging from the endocardium does not fit with such a possible template role in elastin fibrillogenesis. Furthermore, at slightly later stages when elastin fibers are prominent in the bases of the atrioventricular cushions, possibly involved in anchoring the cushions to the ventricular walls, no fibrillin is associated with the elastin in these areas. As previously shown by Rosenquist et al. (1988, 1990) it is likely that the presence of some of the elastin in the heart is related to the arrival of neural crest cells which could constitute an elastogenic cell population. However, we also observed elastin in areas not known to contain neural crest cells, suggesting that other cells in the developing heart are also capable of producing elastin.

A striking feature of this early cardiac elastic matrix is its apparent transitory nature which is in contrast with the stability of elastin in postnatal tissues (Davis, 1993). The outflow tract by stages 29–30 becomes in part (conus cordis) incorporated into the ventricles and loses the elastic matrix, while its distal segment (truncus arteriosus) forms the roots of the great arteries where elastin becomes structured in concentric lamellae intercalated between smooth muscle cell layers (Davis, 1993). The degradation of elastin during these events might well be related with the presence of macrophages and the prominent cell death processes occurring both in the mesenchymal and in the myocardial layers at these stages (Hurle and Ojeda, 1979), since macrophages constitute a major source of elastase in tissues (see Rosenbloom, 1984).

Third Period

The last period of heart development in relation to elastogenesis extends from stages 30–31 up to the last stage studied here (stage 40, day 14), although it is very likely that elastogenesis continues through hatching and into the posthatching period as can be deduced from previous histological and in situ hybridization studies of the developing heart (Hurle, 1979; Rosenquist et al., 1988; Selmin et al., 1991; Holzenberger et al., 1993).

During this third period, the heart has already acquired its four-chambered structure and changes are now related to the maturation of the heart tissues and the establishment of the definitive functional heart. Two major events are characteristic of this period: the formation of the mature valvular apparatus and the establishment of the typical compact myocardium. In terms of elastogenesis, this period appears to be very

Figs. 18–25. Embryonic heart specimens after emilin immunolabeling.

Fig. 18. Lateral view of the stage 19 heart displaying emilin labeling at the endocardial basal surface (arrows), in the cardiac jelly (J) and in the myocardial layer (arrowheads). Bar = 200 μm.

Fig. 19. Transverse section through the outflow tract (stage 20) showing positive tracts in the cardiac jelly associated with the basal surface of the endocardium and cushion mesenchymal cells. Note clumps positive for emilin immunolabeling along the zone dividing the myocardium into outer and inner layers. Bar = 100 μm.

Fig. 20. Section through the ventricle during the process of trabeculation (stage 20) showing the association of the intramyocardial clumps positive for emilin with the invading endothelial sprouts (arrowheads) which also exhibit labeling. Bar = 100 μm.

Fig. 21. Low-magnification view of the heart at stage 29 displaying emilin labeling in the myocardial and endocardial layers of the auricle. Note the interruption of the endocardial labeling at the level of the atrioventricular orifice (arrow). At this magnification only a faint labeling is detected in the ventricular myocardium (arrowheads). Bar = 200 μm.

Fig. 22. Section through the upper limit of the outflow tract at stage 28 showing emilin labeling in the cushion mesenchymal layer. The myocardial layer (M) also shows small clumps of labeled material (arrowheads). See Figure 13, which shows an adjacent section after fibrillin immunolabeling, and note the similar pattern of distribution of both antigens at this level. Bar = 100 μm.

Fig. 23. Detailed view of a myocardial trabeculum at stage 34 showing emilin labeling in the subendocardial space (arrows) and among the myocardial cells. Bar = 20 μm.

Fig. 24. Low-magnification micrograph of the myocardial wall of the ventricle at stage 40 showing emilin labeling in "myocardial interstitium" and in the arterial walls (arrows). Bar = 100 μm.

Fig. 25. Lateral view of a leaflet of the left atrioventricular valve (L) at stage 40 showing emilin-positive fibrils which extend into the tendinous cord (C). Bar = 100 μm.

Figs. 26–31. Embryonic heart specimens after collagen type VI immunolabeling.

Fig. 26. Low-magnification view of the heart at stage 28 showing the presence of labeling in the atrioventricular cushion (AV) and in the epicardial layer. Note the decreasing labeling of the epicardium from the atrio-ventricular sulcus toward the apex of the heart. Bar = 300 μm.

Fig. 27. Detailed view of the atrioventricular septum at stage 28 displaying intense labeling. Note the sharp limit of the labeling at the level of the junction with the interatrial septum (IA). Bar = 200 μm.

Fig. 28. Micrograph showing the defined distribution of collagen type VI in a developing atrioventricular leaflet at stage 33. Interatrial septum (IA). Bar = 100 μm.

Fig. 29. Panoramic view of a leaflet (L) of the left atrioventricular valve at stage 40. Note the abundance of positive fibrillar material in the leaflet which extends into the tendinous cord (C). Figures 7 and 25 show a similar view of the atrioventricular valve, after elastin and emilin labeling, respectively. Bar = 200 μm.

Fig. 30. Detailed view of the subendothelial space of a ventricular trabeculae at stage 40 showing the presence of coiled fibrils positive for collagen type VI oriented parallel to the myocardial fibers. Bar = 200 μm.

Fig. 31. Section through the ventricular wall at stage 40. Note the abundance of collagen type VI in the epicardial layer (E) and its extension into the "myocardial interstitium." Arrow shows labeling in the wall of an arterial vessel. Bar = 200 μm.

active and involves the formation of the definitive elastic matrix of the cardiac valves, the maturation of the epicardial connective tissue, and the formation of the intramyocardial extracellular matrix. By combining immunolabeling of specific elastic components with the use of whole-mount specimens we were able to discern important features undetected in previous studies of the heart extracellular matrix. We found that the valvular leaflets contain all the elastic components described in studies of other typical elastic fetal or adult tissues (Sakai et al., 1986; Bressan et al., 1993) including elastin, fibrillin, emilin, and collagen type VI, a component not always associated with elastic matrices (Knight et al., 1984; Sakai et al., 1986). Each of these components exhibited a fibrillar pattern in the atrioventricular valve leaflets oriented in the direction of the blood flow. The fibers were continuous with the subendocardial extracellular matrix of the ventricles through the tendinous cords and papillary muscles. This pattern of distribution suggests a role for the whole ventricular myocardium in valve dynamics rather than a restricted valvular function for the myocardium of the papillary muscles.

The epicardium constitutes another zone of intense elastogenesis during the later period. In this case elastin is associated with fibrillin and collagen type IV throughout the epicardial layer, while emilin is only present at the surface of the myocardial cells. The importance of the epicardium for myocardial function in advanced stages of heart development has been previously suggested on the basis of the abundance of collagen type I and collagen type III fibers which are present in a precise spatial arrangement (Tidball, 1992). The results obtained here show the presence of additional extracellular matrix molecules in the epicardium and support the idea that the epicardium has a biomechanical function. Further, our study shows that the fibers positive for collagen type VI are continuous with the intramyocardial extracellular matrix as previously observed for collagen type III fibers (Tidball, 1992). This feature suggests that the epicardium constitutes a continuous elastic scaffold with the intramyocardial extracellular matrix and consequently both structures may be involved in a common biomechanical function.

The connective tissue extracellular matrix of the ventricular myocardium functions as a stress-tolerant network which dissipates mechanical forces throughout the walls of the heart. It has been shown that myocardial cells and the surrounding extracellular matrix (myocardial interstitium) are closely associated and constitute a single functional unit (Borg and Caufield, 1981; Robinson et al., 1983; Carver et al., 1993). The extracellular matrix appears to be responsible for translating tension generated by contraction of each myocardial fiber into coordinated patterns of stress through the ventricular wall and, by its elastic properties, returns the ventricle to diastolic parameters when myocardial cells relax. Several myocardial pathological conditions are characterized by alterations in the composition of the intramyocardial extracellular matrix (Weber, 1989). Among the extracellular matrix components identified in the adult myocardium are type I, III, and IV collagens, laminin, fibronectin, and emilin (see Colombatti et al., 1985; Bashey et al., 1992). In addition, Bashey et al. (1992) identified collagen type VI in the myocardium and suggested that this component may play a key role in the interconnections between the myocardial basement membrane and the myocardial interstitium. However, there are few studies addressing the formation of the intramyocardial extracellular matrix (Sanchez-Quintana et al., 1991; Carver et al., 1993). The present study shows that collagen type VI appears in the embryonic heart during the period when the myocardial wall is completing compaction. Further, collagen type VI-positive fibers exhibit the typical coiled morphology characteristic of the extracellular matrices subjected to mechanical stress, and as we have mentioned above, collagen type VI-positive extracellular matrix appears to constitute a fibrillar system continuous with the epicardial connective tissue. All these features emphasize the possible importance of collagen type VI in the ventricular dynamics.

In addition to collagen type VI, our study shows that emilin and fibrillin, two glycoproteins characteristic of elastic matrices, are early constituents of the myocardial extracellular matrix. Further histological and ultrastructural studies will help to identify the precise spatial relationships of these components and their relationships with the myocardial cells.

This study suggests that there is not a unique or fixed composition of the so-called "elastic matrix" during the development of the embryonic heart. It is interesting to note that while emilin and fibrillin are the first elastic components detected in the developing heart, elastin appears in the following stages and collagen type IV is the last to appear. In the heart the combination of the different components of the elastic matrix appears to change in the different regions in relation to the elastic requirements needed during the course of development.

EXPERIMENTAL PROCEDURES

White Leghorn chick embryos ranging from 2 to 14 days of incubation (stages 11–40: Hamburger and Hamilton, 1951) were used in the present study. Between stages 11 and 31 the embryos were studied at 12 hr intervals. After stage 31 the embryos were studied at 24 or 48 hr intervals.

Elastic components of the ECM were detected with monoclonal antibodies against chick elastin (mAb 10B8: Hurle et al., 1994), fibrillin (mAb 201: Sakai et al., 1986), and emilin (mAb 147H11: Bressan et al., 1993). Collagen type VI was analyzed using a monoclonal antibody obtained from the Developmental Studies Hybridoma Bank (mAb 39: D.M. Fambrough).

Depending on the stage, the whole embryo, the

heart, or small heart fragments were dissected free and fixed at 4°C for up to 8 hr in methanol plus 20% dimethyl sulfoxide (DMSO). The specimens were then washed in Tris-buffered saline (TBS) and digested for 30 min in 1,600 U/ml of testicular hyaluronidase (Sigma). After a prolonged rinse in TBS they were immersed for 30 min in 10% goat serum in 10 mM glycine and then transferred to the primary antibody for 6 hr (undiluted hybridoma supernatant). The specimens were washed again for 3–5 hr in several changes of TBS, and incubated overnight in fluroescein-conjugated goat anti-mouse IgG (Cappel) at a 1:300 dilution. The specimens were then washed thoroughly in TBS and attached in the appropriate orientation on depression slides using Cell-Tak (Becton Dickinson Labware, Bedford, MA). After dehydration in methanol the cavity of the slide was filled with a 2:1 solution of benzyl benzoate:benzyl alcohol and sealed with a cover glass attached to the slide by means of nail polish. For each of the stages studied, at least one specimen was embedded in paraffin, sectioned, and then immunostaining was performed on 10 μm thick sections. In all cases controls omitting the first antibody were made. The specimens were examined in a Bio-Rad MRC-600 scanning confocal microscope equipped with a krypton-argon laser (Bio-Rad Laboratories, Richmond, CA). Immunolabeled paraffin sections were examined either with the confocal or with a standard fluorescence microscope.

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