

Anatomy and Formation of Congenital Bicuspid and Quadricuspid Pulmonary Valves in Syrian Hamsters

Borja Fernández, M. Carmen Fernández, Ana C. Durán, David López, Alessandra Martire, and Valentín Sans-Coma

Department of Animal Biology, Faculty of Science, University of Málaga, Málaga, Spain

ABSTRACT

Background: Congenital bicuspid and quadricuspid pulmonary valves have received little attention because of their limited clinical relevance. However, knowledge of the mechanisms by which these anomalous valves develop is essential to obtain a more accurate survey of the etiological factors implicated in the malformations of the cardiac outflow tract in mammals. The present study was designed to assess the anatomical features of bicuspid and quadricuspid pulmonary valves in Syrian hamsters as well as to elucidate the mechanisms involved in the formation of these defective valves.

Methods: The sample examined consisted of 206 adults and 28 embryos belonging to a laboratory-inbred family of Syrian hamsters with a high incidence of congenital anomalies of the pulmonary and aortic valves. The study was carried out using histological techniques for light microscopy, semithin sections, and scanning electron microscopy.

Results: The pulmonary valve was tricuspid in 140 of the 206 adult hamsters, and in 124 of these tricuspid valves the dorsal commissure was more or less extensively fused. Another 45 hamsters possessed a bicuspid pulmonary valve with the sinuses oriented ventrodorsally. In 43 of these bicuspid valves, a raphe was located in the dorsal pulmonary sinus. The pulmonary valve was quadricuspid in a further nine specimens. The remaining 12 hamsters had a tricuspid pulmonary valve with a raphe-like ridge located in the right pulmonary sinus. In seven of these valves, the dorsal commissure showed a more or less extensive fusion. The embryos examined, aged between 11 days, 3 hours and 12 days, 6 hours postcoitum, were at the beginning of the valvulogenesis. In five of the 28 embryos, the pulmonary valve consisted of three mesenchymal valve cushions, right, left, and dorsal. In a further 17 embryos, the right and left valve cushions were more or less fused toward the lumen of the pulmonary artery. In the remaining six embryos, the left and dorsal valve cushions were normal, whereas the right cushion was divided into two lobes.

Conclusions: The present findings suggest that in the Syrian hamster: (1) bicuspid pulmonary valves result from the extensive fusion of the right and left pulmonary valve cushions at the beginning of the valvulogenesis, (2) the partial fusion of the right and left pulmonary valve cushions leads to the formation of tricuspid pulmonary valves with a more or less extensive fusion of the dorsal commissure, (3) quadricuspid pulmonary valves result from the partition of one of the three valve cushions at a very early stage of the valvulogenesis, and (4) the partial division of the right pulmonary valve cushion may lead to the development of tricuspid pulmonary valves with a raphe-like ridge located in the right pulmonary sinus. In addition, the present findings, together with previous observations in Syrian hamsters, indicate that in this

species the mechanisms by which bicuspid and quadricuspid pulmonary valves develop are similar to those by which bicuspid and quadricuspid aortic valves form, respectively. However, the primary factor or factors that induce the malformations of the pulmonary valve operate independently from those inducing the malformations of the aortic valve.

Key words: Bicuspid pulmonary valve; quadricuspid pulmonary valve; congenital anomalies; heart; anatomy; embryology; Syrian hamster.

INTRODUCTION

Most of the previous work on congenital malformations of the cardiac semilunar valves is concerned with the aortic valve, and especially with the bicuspid aortic valve, which, in humans, significantly predisposes to aortic stenosis by dystrophic calcification, incompetence, and infective endocarditis (Fenoglio et al., 1977; Cheitlin et al., 1978; Roberts, 1987, 1989; Giusti et al., 1991). In contrast, little attention has been paid to the congenital anomalies of the pulmonary valve, except for the congenital pulmonary stenosis, a disease that is very often associated with a dome or funnel-shape pulmonary valve (Roberts, 1993). Bicuspid and quadricuspid pulmonary valves are usually considered to be minor cardiac defects because of their limited, if any, clinical relevance (Koletsky, 1941; Lewis and Cammarosano, 1984; Roberts, 1993). However, knowledge of the mechanisms by which these anomalous valves develop is essential to obtain a more accurate survey of the etiological factors implicated in the malformations of the cardiac outflow tract in mammals.

As noted in previous reports, relevant information on the morphogenesis of bicuspid (Sans-Coma et al., 1996) and quadricuspid (Fernández et al., 1994) aortic valves was obtained from Syrian hamsters belonging to a single laboratory-inbred family. It was demonstrated through statistical methods that, in this rodent species, the concurrence of a bicuspid aortic valve and a bicuspid pulmonary valve in an individual is a random event (Sans-Coma et al., 1992). This result already suggested that the conditions of both cardiac semilunar valves are independent traits, thus supporting the hypothesis of Koletsky (1941) that defective aortic and pulmonary valves might have different morphogenetic origin.

Bearing this in mind, we studied a series of pulmonary valves from both adults and embryos belonging to the inbred family of Syrian hamsters mentioned above, thereby obtaining data on the anatomy and formation of tricuspid, bicuspid, and quadricuspid pulmonary valves. The aim here is to report our findings.

MATERIALS AND METHODS

Animals

The sample consisted of 206 adults and 28 embryos belonging to a single Syrian hamster family subjected to systematic inbreeding by crossing siblings or, occasionally, the offspring of siblings. As reported elsewhere (Sans-Coma et al., 1992, 1993, 1996), the incidence of anomalous cardiac semilunar valves is relatively high in this family, which originated from an unrelated pair with tricuspid aortic and pulmonary valves. In the family, 27 inbred generations with > 1,700 animals were produced. The animals examined were taken at random among those belonging to generations 15-23. Most of the present embryos were used in a preceding study on the formation of bicuspid aortic valves (Sans-Coma et al., 1996).

The hamsters used in our research program are handled in compliance with the international guidelines for animal care and welfare. They are housed in polypropylene cages in a room in which both the temperature and photoperiod are controlled. Commercial mouse food (UAR/Panlab s.l. A.04) and water are given as required, starting at weaning. There is no known exposure of the animals to teratogenic agents.

The adult hamsters, ages 49-724 days, were examined by means of a stereomicroscope, histological techniques for light microscopy, or scanning electron microscopy. All of the animals were killed by overdose with chloroform. The heart was exposed by means of a thoracotomy at the level of the fifth intercostal space.

The embryos examined were aged between 11 days, 3 hours and 12 days, 6 hours postcoitum, and their total length (TL) ranged between 8.1 and 12.0 mm. Pregnant females were killed by overdose with chloroform. The embryos were obtained by laparotomy and uterotomy, freed from fetal membranes, and examined using histological techniques for light microscopy or semithin sections of the heart.

Stereoscopy

In 167 of the 206 adult hamsters, the heart was removed after perfusion through the ventricles with physiological saline and transferred to the same solution for dissection. Pulmonary and aortic valves were removed and then preserved in 10% neutral formalin buffered with magnesium carbonate. The condition of each valve was assessed by means of a stereomicroscope.

Light Microscopy

In a further 22 adult hamsters, the heart was perfused through the ventricles with heparinized 0.9% physiological saline, fixed in 10% neutral formalin buffered with magnesium carbonate (ratio of fixative to tissue volume = 80:1), and embedded in paraffin. Transverse sections at the level of the pulmonary valve, serially cut at 10 μ m, were stained with haematoxylin-eosin, alcian blue, orcein-picricfuchsin, or Mallory's trichrome stains for a general assessment of the histological components of the valves. Twenty-six of the 28 embryos were fixed in 10% neutral formalin buffered with magnesium carbonate (ratio of fixative to tissue volume = 80:1), dehydrated in increasing concentrations of ethanol, cleared with xylene, and embedded in paraffin. Serial sections of the heart, sagittally or transversely cut at 5 μ m, were stained with haematoxylin-eosin.

Scanning Electron Microscopy

The hearts of the remaining 17 adult hamsters were perfused through the ventricles with 0.1 M phosphatebuffered saline (pH 7.3). Hearts were then removed, transferred to the same solution, and dissected to expose both semilunar valves. Having assessed the condition of each valve, the pulmonary valve was removed and fixed by immersion in 1% paraformaldehyde and 2% glutaraldehyde in 0.05 M sodium cacodylate buffer (pH 7.3), with osmolarity adjusted to 330 milliosmol/liter, for 3 hours (ratio of fixative to tissue volume = 80:1). Finally, the specimen was dehydrated in increasing concentrations of ethanol, dried by the critical point method, and gold sputter coated. Observations were made using a Jeol JSM-840 scanning electron microscope, operated at 10, 20, or 25 kV.

Semithin Sections

The remaining two embryos were perfusion-fixed with 1% paraformaldehyde and 2% glutaraldehyde in 0.05 M sodium cacodylate buffer (pH 7.3), with osmolarity adjusted to 330 milliosmol/liter. After subsequent immersion in the same fixative for 1 hour (ratio of fixative to tissue volume = 80:1), the embryos were rinsed with 0.05 M sodium cacodylate buffer (pH 7.3), postfixed by immersion in 1% osmium tetroxide in sodium cacodylate buffer for 1 1/2 hours, and rinsed with the same buffer. Thereafter, the heart was removed, dehydrated in acetone, and embedded in araldite. Sagittal sections, serially cut at 1 μ m with an ultramicrotome Reichert UMO-2, were stained with 1% toluidine blue in borax. Observations were made using a light microscope Nikon Microphot FXA.

Nomenclature

The nomenclature used for cardiac semilunar valve components was that of Angelini et al. (1989), McKay et al. (1992), and Sans-Coma et al. (1992). It should be noted, however, that we use the terms “tricuspid,” “bicuspid,” and “quadricuspid” instead of “trifoliate,” “bifoliate,” or “tetrafoliate,” considering that the former are of much more common usage.

RESULTS

Figure 1 shows the pulmonary valve morphologies observed in the adult hamsters as well as the different arrangements of the pulmonary valve cushions at the beginning of the valvulogenesis. We refer to this diagram throughout in both the description and discussion of our findings.

Pulmonary Valve Morphology in Adult Syrian Hamsters

In 140 of the 206 adult hamsters examined, the pulmonary valve was tricuspid. In 16 of these 140 tricuspid valves, there were three pulmonary sinuses, right, left, and ventral, three leaflets, and a triangular space between each adjacent leaflet, so that overall three interleaflet triangles of similar size were present in the subvalvular outflow tract (Figs. 1A,2). The triangles were located in the right-ventral, left-ventral, and dorsal positions, respectively. In the other 124 cases, the pulmonary valve also had three leaflets and three sinuses, but the dorsal commissure, between the right and left leaflets, was more or less fused, and the dorsal interleaflet triangle was decreased in size, according to the degree of the fusion (Fig. 1B). When the commissure was completely fused, the dorsal triangle was lacking.

Forty-five hamsters possessed a bicuspid pulmonary valve. In all of these valves, there were two pulmonary sinuses, a ventral and a dorsal, each supporting one leaflet, and two interleaflet triangles, a right and a left. In 43 cases, a raphe was located in the dorsal pulmonary sinus (Fig. 1C). The raphe varied in size, ranging from a raphe that widely encroached toward the leaflet to a raphe confined to the pulmonary artery wall. The remaining two bicuspid pulmonary valves were devoid of any raphe (Figs. 1D,3).

The fused commissures of the tricuspid pulmonary valves and the raphes reaching the leaflet (Fig. 4) of the bicuspid pulmonary valves displayed similar histological conditions. Both valvar structures consisted of a proximal and a distal portion, with regard to the pulmonary artery wall. The proximal portion was mainly composed of collagenous and elastic fibers that merged with the collagenous and elastic fibers of the pulmonary artery media, and contained sparse connective tissue cells. The distal portion displayed a structure like that of the leaflet. It consisted of an arterial zone similar to the fibrosa layer of the leaflet, and a ventricular zone,

rich in glycosaminoglycans, similar to the spongiosa layer of the leaflet. The distal portion was absent in the raphe confined to the pulmonary artery wall.

It is often difficult to differentiate a tricuspid pulmonary valve with a complete fusion of the dorsal commissure from a bicuspid pulmonary valve with a greatly developed raphe located in the dorsal pulmonary sinus. The histological conditions of the fused commissures and raphes are not helpful in making this distinction. In addition, there are only two interleaflet triangles in both pulmonary valve variants. Therefore, the only characteristic that may define the boundary between a tricuspid pulmonary valve with a complete fusion of the dorsal commissure and a bicuspid pulmonary valve with a greatly developed raphe in the dorsal pulmonary sinus is the cephalad level reached by each of these two valve elements. The most cephalad margin of a completely fused commissure is on the same level as the cephalad margins of the other two commissures, whereas the raphe does not reach this level.

In a further five hamsters, the pulmonary valve was basically tricuspid, with three sinuses, three leaflets, and three interleaflet triangles of similar size. However, a raphe-like ridge was present in the right pulmonary sinus (Figs. 1E,5A). In three cases, the ridge reached the leaflet and divided the sinus into two subequal cavities. Histologically, the ridge consisted of two portions, a proximal and a distal, with regard to the pulmonary artery wall. The proximal portion was rich in collagen and elastic fibers, whereas the distal portion, attached to the leaflet, was mainly composed of glycosaminoglycans and contained sparse connective tissue cells and collagen fibers (Fig. 5B). In the other two cases, the raphe-like ridge was confined to the wall of the pulmonary artery and consisted only of the proximal histological portion.

In nine other hamsters, the pulmonary valve was quadricuspid (Fig. 1F). It consisted of four pulmonary sinuses of variable size, two dorsal and two ventral, four leaflets, and four interleaflet triangles located in the dorsal, right, left, and ventral positions, respectively.

In the remaining seven hamsters, the pulmonary valve had three sinuses and three leaflets. However, the dorsal commissure displayed a more or less extensive fusion, and the dorsal interleaflet triangle was decreased in size, according to the degree of the fusion. In addition, a more or less developed raphe-like ridge reaching the leaflet was located in the right pulmonary sinus (Fig. 1G).

Pulmonary Valve vs. Aortic Valve Morphology in Adult Syrian Hamsters

In 136 of the 206 hamsters examined, the aortic valve was tricuspid, whereas it was bicuspid in the remaining 70. In all bicuspid aortic valves, the aortic sinuses were arranged in ventrodorsal orientation.

The distribution of pulmonary and aortic valve morphologies in the sample studied is given in Table 1: observed values. In addition, the table shows the results of a χ^2 -test, which was performed to determine if there was any association between the pulmonary and aortic valve conditions. The null hypothesis was that both traits are independent. First, the frequency of each valve type in the sample studied was calculated. The results obtained were 0.680 for tricuspid pulmonary valves, 0.218 for bicuspid pulmonary valves, 0.024 for tricuspid pulmonary valves with a raphe-like ridge in the right pulmonary sinus and no commissural fusion, 0.044 for quadricuspid pulmonary valves, 0.034 for tricuspid pulmonary valves with a raphe-like ridge in the right pulmonary sinus and a more or less extensive fusion of the dorsal commissure, 0.660 for tricuspid aortic valves, and 0.340 for bicuspid aortic valves. Thereafter, the expected value (Table 1) of each pair of valve morphotypes was obtained by the product

of their respective frequencies multiplied by the total number of specimens ($n = 206$). The computed value of the χ^2 statistic is 1.268, with nine degrees of freedom. Therefore, the null hypothesis is accepted at $P > 0.99$.

Embryologic Findings

The embryos examined were at the beginning of the valvulogenesis. The septation of the conotruncus had just taken place at the level of the cardiac semilunar valve primordia. The valve cushions showed no sign of excavation.

In five of the 28 embryos, the pulmonary valve consisted of three valve cushions, right, left, and dorsal (Figs. 1a,6A). Each cushion was composed of a core of mesenchymal tissue, covered by the endocardial endothelium, and appeared as a semiconical swelling with the apex pointing toward the ventricle.

In 17 other embryos, the right and left pulmonary valve cushions were fused from their most cephalic to their most caudal margins. The extension of the fusion toward the lumen of the pulmonary artery was variable, ranging from cases in which the cushions were slightly fused, sharing a small amount of mesenchymal tissue, to cases in which the cushions were extensively fused, giving rise to a single dorsal cushion (Figs. 1b,6B). In all of these cases, the ventral valve cushion was well defined and clearly separated from the more or less extensively fused right and left cushions.

In a further six embryos, the left and ventral pulmonary valve cushions were normal, whereas the right valve cushion appeared as a more or less divided structure. The degree of division of the cushion varied among specimens, ranging from a slight division into two lobes (Figs. 1c,6C) to a complete division into two true cushions, a right-dorsal and a right-ventral (Fig. 6D).

DISCUSSION

In the Syrian hamster as in other mammals (Barone, 1972; Lawson, 1979), the normal condition of the pulmonary valve is characterized by the existence of three pulmonary sinuses, three leaflets, and three interleaflet triangles (Sans-Coma et al., 1992). Bicuspid and quadricuspid pulmonary valves can be regarded as anomalous valves. The high incidence of congenital anomalous pulmonary and aortic valves in the present laboratory family of Syrian hamsters is thought to be the product of systematic inbreeding (Sans-Coma et al., 1992).

Our anatomical and histological observations concerning tricuspid and bicuspid pulmonary valves in adult Syrian hamsters suggest the existence of a continuous spectrum of pulmonary valve anatomy (Fig. 1A-D), ranging from a tricuspid pulmonary valve with no fusion of the dorsal commissure to a bicuspid pulmonary valve devoid of any raphe. The intermediate stages are represented by tricuspid pulmonary valves with a more or less extensive fusion of the dorsal commissure and bicuspid pulmonary valves with a more or less developed raphe. This morphologic spectrum is similar to that described for the aortic valve of the Syrian hamster (Sans-Coma et al., 1992, 1996).

In addition, our findings suggest the existence of another continuum of pulmonary valve anatomy, which ranges from a tricuspid pulmonary valve devoid of any raphe-like ridge to a quadricuspid pulmonary valve (Fig. 1A,E,F). The intermediate stages are represented by tricuspid pulmonary valves with a more or less developed raphe-like ridge located in the right pulmonary sinus. To our knowledge, the present report is the first to describe a continuous

phenotypic spectrum of valve morphology between the tricuspid and quadricuspid conditions of the cardiac semilunar valves. In this context, it should be emphasized that, histologically, the raphe-like ridges do not differ from the raphes of the bicuspid pulmonary valves.

In a previous work (Sans-Coma et al., 1996), it was demonstrated that, in the Syrian hamster, the fusion of the right and left aortic valve cushions at the beginning of the valvulogenesis is a key factor in the formation of both tricuspid aortic valves with fusion of the ventral commissure and bicuspid aortic valves with the aortic sinuses oriented ventrodorsally. The present embryologic findings strongly suggest that this is also the case for the pulmonary valve. Indeed, at the beginning of the valvulogenesis, the right and left pulmonary valve cushions, originating from the conotruncal ridges, may fuse in a variable degree toward the lumen of the pulmonary artery. When they are totally fused, only two valve cushions, a ventral and a dorsal, are present in the pulmonary outflow tract. This indicates that the more or less extensive fusion of the right and left pulmonary valve cushions (Fig. 1b) leads to the formation of tricuspid pulmonary valves with fusion of the dorsal commissure (Fig. 1B) and bicuspid pulmonary valves with the pulmonary sinuses in dorsoventral orientation (Fig. 1C,D).

Fernández et al. (1994) described the case of a Syrian hamster embryo with four aortic valve cushions, concluding that the supernumerary valve cushion resulted from the division of one of the three normal aortic valve cushions at a very early stage of the valvulogenesis. The present findings suggest that a similar mechanism (Fig. 1c) may account for the formation of quadricuspid pulmonary valves (Fig. 1F). These anomalous valves seem to be the result of the total partition of one of the three valve cushions that normally give rise to a tricuspid pulmonary valve. In addition, the present findings indicate that the partial division of one of the pulmonary valve cushions at the beginning of the valvulogenesis (Fig. 1C) leads to the formation of tricuspid pulmonary valves with a raphe-like ridge located in one of the sinuses (Fig. 1E). This statement relies on two facts: (1) in all tricuspid pulmonary valves having a raphe-like ridge, this structure was located in the right pulmonary sinus, and (2) in all embryos displaying a partially divided valve cushion, the affected cushion was the right.

The formation of tricuspid pulmonary valves with a raphe-like ridge in the right pulmonary sinus and a more or less extensive fusion of the dorsal commissure (Fig. 1G) might be explained by the concurrence of two mechanisms, namely, the partial division of the right pulmonary valve cushion and the partial fusion of the resulting right-dorsal lobe of the right cushion with the left pulmonary valve cushion (Fig. 1d). However, further embryologic evidence is needed to verify this hypothesis.

We found no evidence of maldevelopment of the conotruncal ridges, anomalous septation of the conotruncus, or abnormal mesenchymal proliferations in the common trunk of the embryos examined. Therefore, our findings disagree with the hypotheses, already proposed in classic studies, that the bicuspid condition of the pulmonary valve may result from the improper initial development of the conotruncal ridges (De Vries, 1918), from the erroneous septation of the conotruncus (De Vries, 1918; Simonds, 1923; Wauchope, 1928; Koletsky, 1941), or from the absence of one of the three valve cushions (De Vries, 1918; Simonds, 1923; Shaner, 1963). Moreover, our findings contradict the hypotheses that the quadricuspid condition of the pulmonary valve might be due to the interpolation of an extra swelling or pad before the conotruncal septation, or to alterations in the formation of the aorticopulmonary septum (Simonds, 1923).

In summary, the present findings, together with those reported by Fernández et al. (1994) and Sans-Coma et al. (1996), strongly suggest that, in the Syrian hamster: (1) bicuspid aortic and pulmonary valves result from the extensive fusion of the respective right and left valve cushions, derived from the conotruncal ridges, at the beginning of the valvulogenesis, (2) the partial fusion of these valve cushions leads to the formation of tricuspid aortic valves with fusion of the ventral commissure and tricuspid pulmonary valves with fusion of the dorsal commissure, (3) the quadricuspid condition of both cardiac semilunar valves is due to the division of one of the three valve cushions at a very early stage of the valvulogenesis, and (4) the partial division of the right pulmonary valve cushion may play a fundamental role in the formation of tricuspid pulmonary valves with a raphe-like ridge located in the right pulmonary sinus.

Therefore, at least in the Syrian hamster, the anomalies in the number and arrangement of the leaflets and sinuses of both cardiac semilunar valves seem to rely on two principal morphogenetic mechanisms, namely, the fusion and the partition of valve cushions at the beginning of the valvulogenesis. It should be noted, however, that, as already suggested by Sans-Coma et al. (1992) and substantiated herein by the results of the χ^2 -test, the conditions of the aortic and pulmonary valves are independent traits. This means that the factor or factors that induce the fusion and/or partition of the pulmonary valve cushions are different, or at least operate independently from the factor or factors that induce the fusion and/or partition of the aortic valve cushions. However, the fact that in some cases both cardiac semilunar valves exhibit the same anomalous condition, i.e., the bicuspid condition, indicates that the morphogenetic factors operating at the aortic and pulmonary sides are not mutually exclusive.

To our knowledge, no morphogenetic factor has been currently adduced to explain the partition of a semilunar valve cushion at the beginning of the valvulogenesis. Hence, the primary cause of quadricuspid semilunar valve development remains a question.

With regard to the formation of bicuspid cardiac semilunar valves, two main factors have been proposed as primary causes of maldevelopment: (1) alterations of the fetal cardiac hemodynamics, and (2) anomalous behaviour of the cells from the cardiac neural crest.

The classical theory that altered flow relationships might account for the development of bicuspid aortic and pulmonary valves (Shaner, 1963; Moore et al., 1980; Clark, 1986) relies on the study of congenitally malformed cardiac semilunar valves from autopsied human hearts and is mainly supported by the fact that, in humans, bicuspid aortic and pulmonary valves are significantly associated with congenital cardiac malformations with outflow tract obstruction, such as aortic coarctation (Becker et al., 1970; Hutchins, 1981; Moore et al., 1980) and tetralogy of Fallot (Winn and Hutchins, 1973; Moore et al., 1980; Anderson et al., 1981), respectively. Disproportionate flow reduction of the right or left side of the heart has been adduced as a factor in the development of malformations in the respective semilunar valve (Moore et al., 1980). According to these authors, the majority of congenital anomalies of the cardiac semilunar valves are due to lesions acquired during fetal life after a normal valvulogenesis. This would rule out the early fusion of the valve cushions as a key factor in the formation of bicuspid aortic and pulmonary valves, a suggestion that disagrees with our observations in the Syrian hamster.

More recently, Hogers et al. (1997) have reported that in the chick embryo, the ligation of the right lateral vitelline vein alters the intracardiac blood flow pattern, resulting in intracardiac

and pharyngeal arch artery malformations comparable to defects observed in chicken embryos subjected to neural crest ablation, cervical flexure experiments, and excessive retinoic acid treatment. Among these malformed embryos, there was a remarkable proportion of cases of semilunar valve abnormalities in association with ventricular septal defects, a combination that has not been found in our hamsters. Yet, the malformed embryonic hearts obtained experimentally by Hogers et al. (1997) displayed a malalignment of the septal components due to the impaired septation of the outflow tract. This is not the case in our hamster model, where the septation of the outflow tract takes place normally, and the embryonic septal components of the outflow tract show a normal alignment (see also Sans-Coma et al., 1996).

The hypothesis that the formation of bicuspid cardiac semilunar valves may be due to the anomalous behaviour of the cells from the cardiac neural crest bases on the following facts: (1) the cardiac neural crest gives rise to ectomesenchyme, which supports the development of the aortic arch arteries and the aortopulmonary septum that divides the cardiac outflow tract into the aortic and pulmonary tracts (Fukiishi and Morris-Kay, 1992; Kirby et al., 1983), (2) in quail-chick chimera embryos, ectomesenchymal cells from the cardiac neural crest have been found to colonize the aortic and pulmonary valve primordia, indicating that they are involved in the formation of the cardiac semilunar valves (Sumida et al., 1989; Takamura et al., 1990), and (3) in humans, the bicuspid aortic valve is significantly associated with congenital malformations of the aortic arch and other neural crest-derived systems (Van Mierop and Kutsche, 1986; Kappetein et al., 1991; Miyabara et al., 1993a,b; Durán et al., 1995). These data, together with the embryologic observations in the Syrian hamster, suggest that the fusions of the aortic and pulmonary valve cushions, which lead to the formation of bicuspid aortic and pulmonary valves, may be the outcome of anomalous behaviour of the cells from the cardiac neural crest. Further support for this suggestion comes from the fact that in the Syrian hamster, bicuspid aortic valves are significantly associated with several congenital anomalies in the origin of the coronary arteries, which are thought to be due to defective behaviour of neural crest cells (Sans-Coma et al., 1991; Cardo et al., 1995). Indeed, as substantiated in chick embryos, anomalies in the cells from the cardiac neural crest may cause spatial disorder on the development of the proximal coronary arteries, thus resulting in a wide spectrum of coronary artery abnormalities (Hood and Rosenquist, 1992).

In this context, it should be emphasized that the conditions of the aortic and pulmonary valves are independent traits. This already indicates that the neural crest cells involved in the fusion of the right and left pulmonary valve cushions must diverge from the neural crest cells implicated in the fusion of the right and left aortic valve cushions. This suggestion agrees with current knowledge on neural crest behaviour. Indeed, grafting experiments have demonstrated that the cranial neural crest becomes segmentally committed prior to migration. This means that regional diversity in neural crest-derived structures is the consequence of early differences in the morphogenetic specification of individual neural crest populations (Noden, 1983; Kirby, 1989). Further support for the specificity of the cells from the cranial neural crest comes from the recent finding that cranial muscle connective tissues derived from a specific neural crest rhombomeric level are always specifically attached to skeletal regions of the same origin (Köntges and Lumsden, 1996). Therefore, we hypothesize that the formation of the cardiac semilunar valves may be mediated by specific subpopulations of cardiac neural crest cells, acting separately at the pulmonary and aortic sides of the embryonic cardiac outflow tract.

ACKNOWLEDGMENTS

The authors express deep gratitude to G. Martín and J.J. Canca, Málaga, for their assistance in operating the scanning electron microscope, and to L. Vida, Málaga, for his technical assistance.

LITERATURE CITED

- Anderson, R.H., S.P. Allwork, S.Y. Ho, C.C. Lenox, and J.R. Zuberbuhler 1981 Surgical anatomy of tetralogy of Fallot. *J. Thorac. Cardiovasc. Surg.*, 81:887-896.
- Angelini, A., S.Y. Ho, R.H. Anderson, W.A. Devine, J.R. Zuberbuhler, A.E. Becker, and M.J. Davies 1989 The morphology of the normal aortic valve as compared with the aortic valve having two leaflets. *J. Thorac. Cardiovasc. Surg.*, 98:362-367.
- Barone, R. 1972 Appareil circulatoire. In: *Traité de Zoologie*, t. 15, fasc 4. P.-P. Grassé, ed. Masson et Cie, Paris, pp. 702-921.
- Becker, A.E., M.J. Becker, and J.E. Edwards 1970 Anomalies associated with coarctation of the aorta: Particular reference to infancy. *Circulation*, 41:1067-1075.
- Cardo, M., B. Fernández, A.C. Durán, M.C. Fernández, J.M. Arqué, and V. Sans-Coma 1995 Anomalous origin of the left coronary artery from the dorsal aortic sinus and its relationship with aortic valve morphology in Syrian hamsters. *J. Comp. Pathol.*, 112:373-380.
- Cheitlin, M.D., J.J. Fenoglio, H.A. McAllister, J.E. Davia, and C.M. DeCastro 1978 Congenital aortic stenosis secondary to dysplasia of congenital bicuspid aortic valves without commissural fusion. *Am. J. Cardiol.*, 42:102-107.
- Clark, E.B. 1986 Mechanisms in the pathogenesis of congenital cardiac malformations. In: *Genetics of Cardiovascular Disease*. M.E.M. Pierpont and J.H. Moller, eds. Martinus Nijhoff, Boston, pp. 3-11.
- De Vries, W.M. 1918 Ueber Abweichungen in der Zahl der Semilunarklappen. *Beitr. Pathol. Anat.*, 64:39-54.
- Durán, A.C., C. Frescura, V. Sans-Coma, A. Angelini, C. Basso, and G. Thiene 1995 Bicuspid aortic valves in hearts with other congenital heart disease. *J. Heart Valve Dis.*, 4:581-590.
- Fenoglio, J.J., H.A. McAllister, C.M. DeCastro, J.E. Davia, and M.D. Cheitlin 1977 Congenital bicuspid aortic valve after age 20. *Am. J. Cardiol.*, 39:164-169.
- Fernández, B., A.C. Durán, G. Thiene, M. Cardo, J.M. Arqué, and V. Sans-Coma 1994 Embryological evidence for the formation of a quadricuspid aortic valve in the Syrian hamster. *Cardiovasc. Pathol.*, 3:287-291.
- Fukiishi, Y., and G.M. Morris-Kay 1992 Migration of cranial neural crest cells to the pharyngeal arches and heart in rat embryos. *Cell Tissue Res.*, 268:1-8.
- Giusti, S., P. Cocco, and G. Thiene 1991 Valvola aortica bicuspidale: Una cardiopatia congenita "minore" a rischio di gravi complicanze. *G. Ital. Cardiol.*, 21:189-201.

- Hogers, B., M.C. DeRuiter, A.C. Gittenberger-de Groot, and R.E. Poelmann 1997 Unilateral vitelline vein ligation alters intracardiac blood flow patterns and morphogenesis in the chick embryo. *Circ. Res.*, 80:473-481.
- Hood, L.C., and T.H. Rosenquist 1992 Coronary artery development in the chick: origin and deployment of smooth muscle cells, and the effects of neural crest ablation. *Anat. Rec.*, 234:291-300.
- Hutchins, G.M. 1981 Coarctation of the aorta explained as a branchpoint of the ductus arteriosus. *Am. J. Pathol.*, 63:203-214.
- Kappetein, A.P., A.C. Gittenberger-de Groot, A.H. Zwinderman, J. Rohmer, R.E. Poelmann, and H.A. Huisman 1991 The neural crest as a possible pathogenetic factor in coarctation of the aorta and bicuspid aortic valve. *J. Thorac. Cardiovasc. Surg.*, 102:830- 836.
- Kirby, M.L. 1989 Plasticity and predetermination of mesencephalic and trunk neural crest transplanted into the region of the cardiac neural crest. *Dev. Biol.*, 134:402-412.
- Kirby, M.L., T.F. Gale, and D.E. Stewart 1983 Neural crest cells contribute to normal aorticopulmonary septation. *Science*, 220: 1059-1061.
- Koletsky, S. 1941 Congenital bicuspid pulmonary valves. *Arch. Pathol.*,31:338-353
- Köntges, G., and A. Lumsden 1996 Rhombencephalic neural crest segmentation is preserved throughout craniofacial ontogeny. *Development*, 122:3229–3242.
- Lawson, R. 1979 The comparative anatomy of the circulatory system. In: Hyman's Comparative Vertebrate Anatomy. M.H. Wake, ed. University of Chicago Press, Chicago, pp. 448–554.
- Lewis, W., and C. Cammarosano 1984 Quadricuspid pulmonic valve. *Am. J. Cardiol.*, 53:971–972.
- McKay, R., A. Smith, M.P. Leung, R. Arnold, and R.H. Anderson 1992 Morphology of the ventriculoaortic junction in critical aortic stenosis: Implications for hemodynamic function and clinical management. *J. Thorac. Cardiovasc. Surg.*, 104:434–442.
- Miyabara, S., K. Suzumori, Y. Uemura, and M. Tokunga 1993a Fetal cystic hygroma in sibs: Developmental analysis of coexisting cardiovascular malformations relevant to pathogenesis. *Birth Defects; Original Article Series*, 29:303–315.
- Miyabara, S., K. Suzumori, H. Winking, and M. Nakayama 1993b Structural defects of the vascular system in 45,X fetuses: Integrated interpretation of the pathogenesis. In: *Basic and Clinical Approach to Turner Syndrome*. I. Hibi and K. Takano, eds. Elsevier Science, New York, pp. 61–68.
- Moore, G.W., G.M. Hutchins, J.C. Brito, and H. Kang 1980 Congenital malformations of the semilunar valves. *Human Pathol.*, 11:367– 372.
- Noden, D.M. 1983 The role of the neural crest in patterning of avian cranial skeletal connective, and muscle tissues. *Dev. Biol.*, 96:144– 165.
- Roberts, W.C. 1987 Congenital cardiovascular abnormalities usually silent until adulthood. In: *Adult Congenital Heart Disease*. W.C. Roberts, ed. F.A. Davis Philadelphia, pp. 631–691.
- Roberts, W.C. 1989 Living with a congenitally bicuspid aortic valve. *Am. J. Cardiol.*, 64:1408–1409.

- Roberts, W.C. 1993 Valvular heart disease of congenital origin. In: Valvular Heart Disease. Comprehensive Evaluation and Treatment. W.S. Frankl and A.N. Brest, eds. F.A. Davis, Philadelphia, Cardiovasc. Clin., 23:25–52.
- Sans-Coma, V., J.M. Arqué, A.C. Durán, M. Cardo, and B. Fernández 1991 Coronary artery anomalies and bicuspid aortic valves in the Syrian hamster. *Basic Res. Cardiol.*, 86:148–153.
- Sans-Coma, V., M. Cardo, A.C. Durán, D. Franco, B. Fernández, and J.M. Arqué 1993 Evidence for a quantitative genetic influence on the formation of aortic valves with two leaflets in the Syrian hamster. *Cardiol. Young*, 3:132–140.
- Sans-Coma, V., M. Cardo, G. Thiene, B. Fernández, J.M. Arqué, and A.C. Durán 1992 Bicuspid aortic and pulmonary valves in the Syrian hamster. *Int. J. Cardiol.*, 34:249–254.
- Sans-Coma, V., B. Fernández, A.C. Durán, G. Thiene, J.M. Arqué, R. Muñoz-Chápuli, and M. Cardo 1996 Fusion of valve cushions as a key factor in the formation of congenital bicuspid aortic valves in Syrian hamsters. *Anat. Rec.*, 244:490–498.
- Shaner, R.F. 1963 Abnormal pulmonary and aortic semilunar valves in embryos. *Anat. Rec.*, 143:5–13.
- Simonds, J.P. 1923 Congenital malformations of the aortic and pulmonary valves. *Am. J. Med. Sci.*, 166:585–595.
- Sumida, H., N. Akimoto, and H. Nakamura 1989 Distribution of the neural crest cells in the heart of birds: A three dimensional analysis. *Anat. Embryol.*, 180:29–35.
- Takamura, K., T. Okishima, S. Ohdo, and K. Hayakawa 1990 Association of cephalic neural crest cells with cardiovascular development, particularly that of the semilunar valves. *Anat. Embryol.*, 182:263–272.
- Van Mierop, L.H.S., and L.M. Kutsche 1986 Cardiovascular anomalies in DiGeorge syndrome and importance of neural crest as possible pathogenetic factor. *Am. J. Cardiol.*, 58:133–137.
- Wauchope, G.M. 1928 The clinical importance of variations in the number of cusps forming the aortic and pulmonary valves. *Quart. J. Med.*, 21:383–399.
- Winn, K.J., and G.M. Hutchins 1973 The pathogenesis of the tetralogy of Fallot. *Am. J. Pathol.*, 73:157–172.

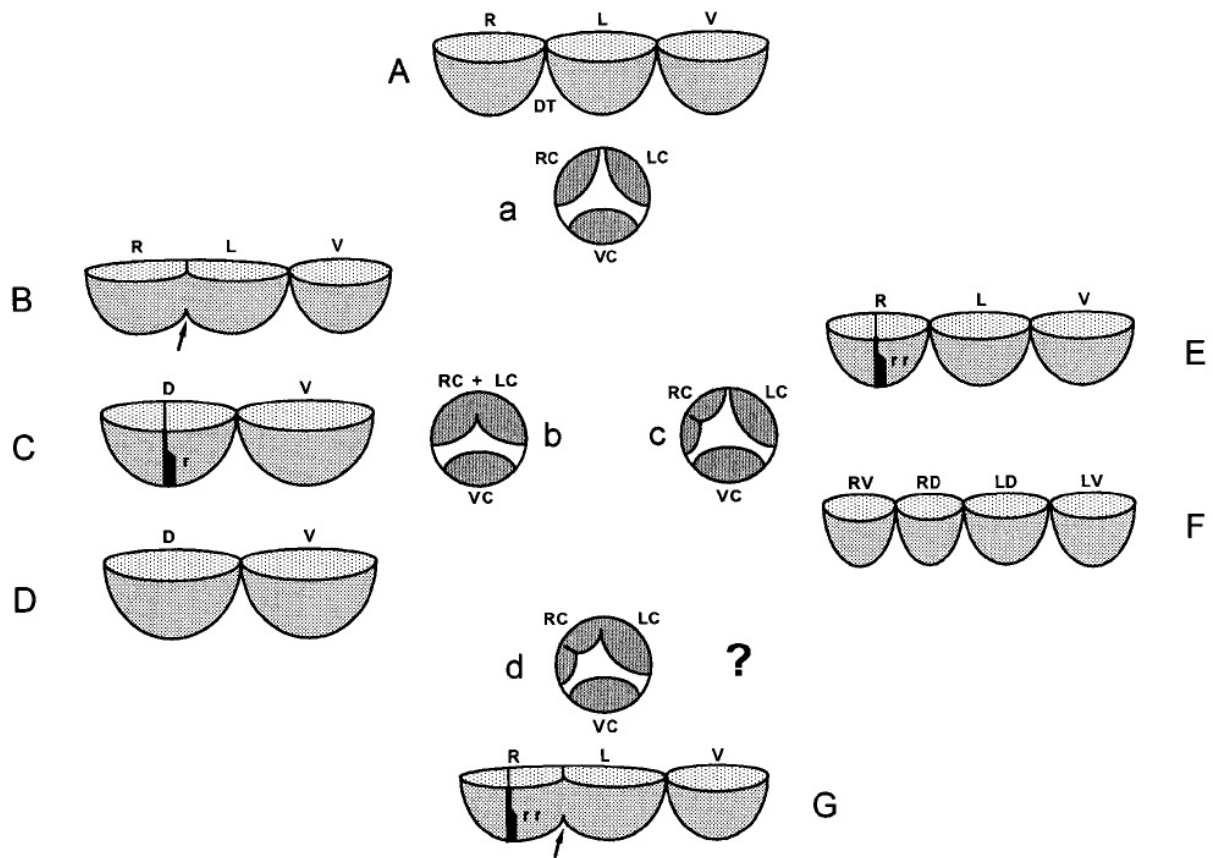


Fig. 1. Pulmonary valve morphologies in the adult hamsters (A–G) and arrangements of the pulmonary valve cushions at the beginning of the valvulogenesis (a–d). A: Tricuspid pulmonary valve with no fusion of the dorsal commissure and a well-developed dorsal interleaflet triangle (DT). B: Tricuspid pulmonary valve with partial fusion of the dorsal commissure. The dorsal interleaflet triangle (arrow) is reduced in size. C: Bicuspid pulmonary valve with a raphe (r) located in the dorsal pulmonary sinus. D: Bicuspid pulmonary valve devoid of any raphe. E: Tricuspid pulmonary valve with no fusion of the dorsal commissure and a raphe-like ridge (rr) located in the right pulmonary sinus. F: Quadricuspid pulmonary valve. G: Tricuspid pulmonary valve with partial fusion of the dorsal commissure and a raphe-like ridge (rr) located in the right pulmonary sinus. The dorsal interleaflet triangle (arrow) is reduced in size. (a) Arrangement of the right (RC), left (LC) and ventral (VC) pulmonary valve cushions giving rise to tricuspid pulmonary valves with no fusion of the dorsal commissure. (b) Right and left pulmonary valve cushions (RC + LC) can fuse in a variable degree, resulting in either tricuspid pulmonary valves with fusion of the dorsal commissure or bicuspid pulmonary valves. (c) Right (RC) pulmonary valve cushion divides into two lobes, giving rise to tricuspid pulmonary valves with partial fusion of the dorsal commissure and a raphe-like ridge located in the right pulmonary sinus. The total division of that or another valve cushion into two completely separated lobes or true cushions is a key factor for the formation of quadricuspid pulmonary valves. (d) Hypothetical (?) arrangement of the valve cushions leading to the formation of tricuspid pulmonary valves with partial fusion of the dorsal commissure and a raphe-like ridge located in the right pulmonary sinus. The right pulmonary cushion (RC) divides into two lobes, one of which fuses with the left pulmonary valve cushion (LC). D, dorsal pulmonary sinus; L, left pulmonary sinus; LD, left-dorsal pulmonary sinus; LV, left-ventral pulmonary

sinus; RD, right-dorsal pulmonary sinus; RV, right-ventral pulmonary sinus; V, ventral pulmonary sinus.

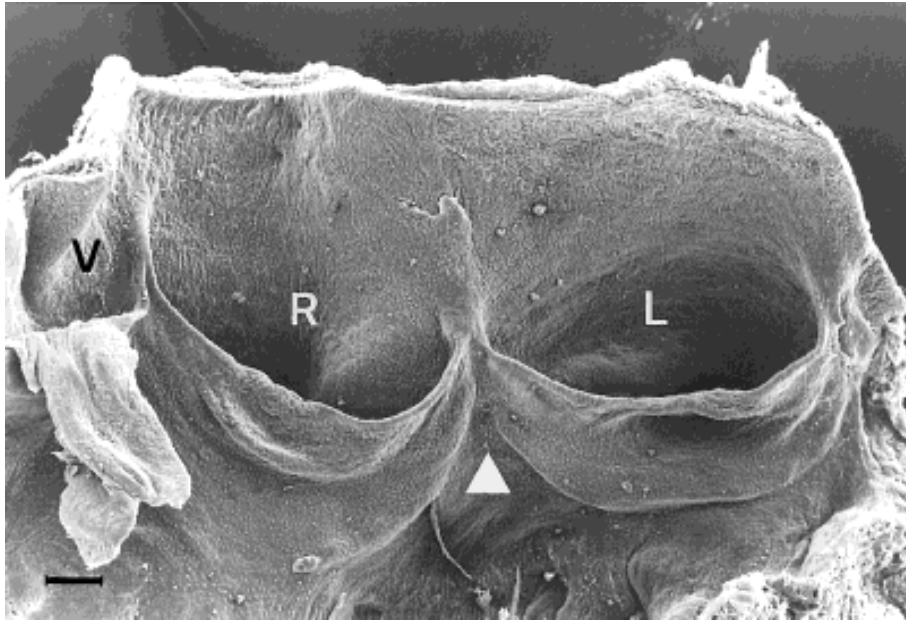


Fig. 2. Scanning electron micrograph of a tricuspid pulmonary valve with no fusion of the ventral commissure and a well-developed dorsal interleaflet triangle (triangle) from an adult Syrian hamster. The specimen was opened through the ventral pulmonary sinus (V) to expose the dorsal aspect of the valve. L, left pulmonary sinus; R, right pulmonary sinus. Scale bar = 140 μm .

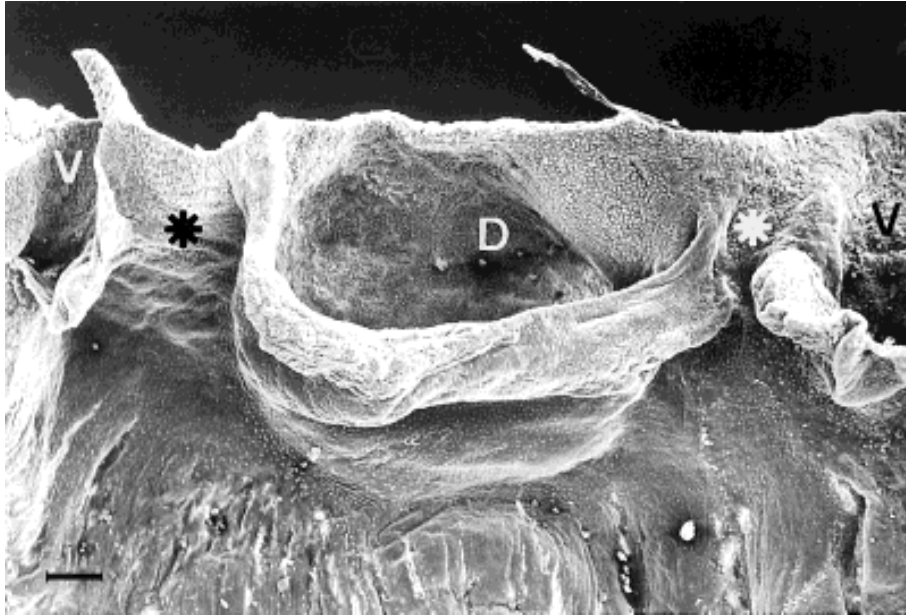


Fig. 3. Scanning electron micrograph of a bicuspid pulmonary valve devoid of any raphe from an adult Syrian hamster. The specimen was opened through the ventral pulmonary sinus (V) to expose the dorsal aspect of the valve. In this specimen, the adjacent leaflets did not join along their attachments to the sinus wall, so that the spaces between leaflets (asterisks) had a trapezoidal, and not a triangular contour. D, dorsal pulmonary sinus. Scale bar = 125 μ m.

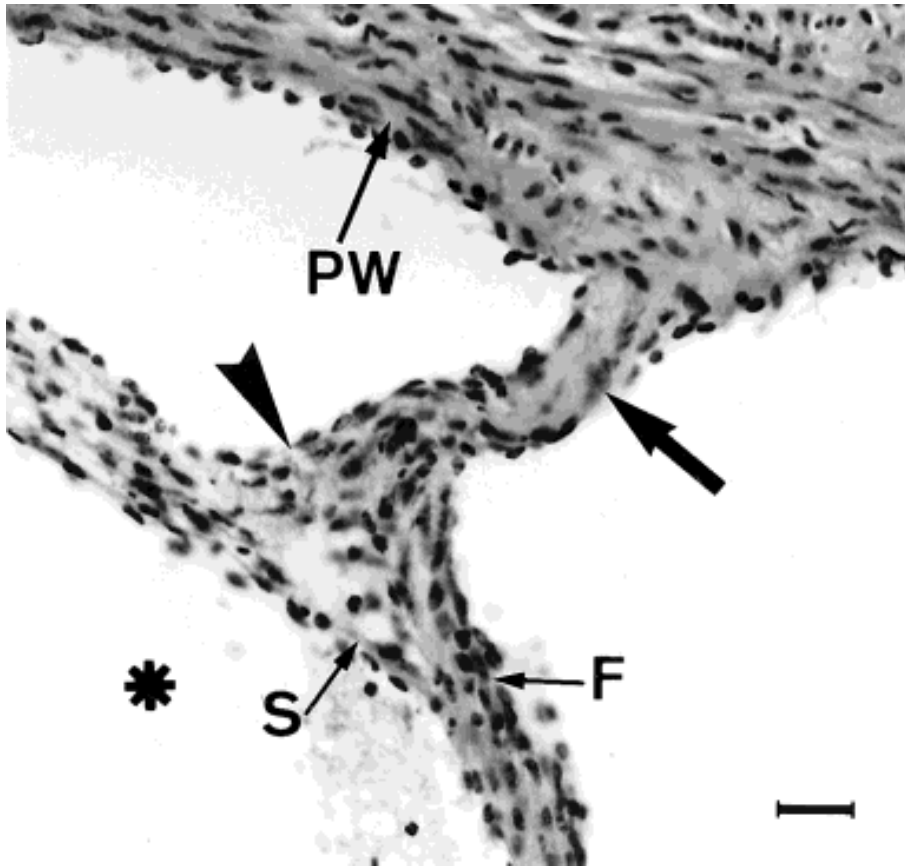


Fig. 4. Transverse section of a bicuspid pulmonary valve with a raphe located in the dorsal pulmonary sinus from an adult Syrian hamster. At this level, the raphe widely encroaches toward the leaflet and consists of two histological portions, a proximal (arrow) and a distal (arrowhead), with regard to the pulmonary artery wall (PW). The asterisk indicates the lumen of the pulmonary artery. F, fibrosa layer of the dorsal leaflet; S, spongiosa layer of the dorsal leaflet. Haematoxylin-eosin. Scale bar = 25 μ m.

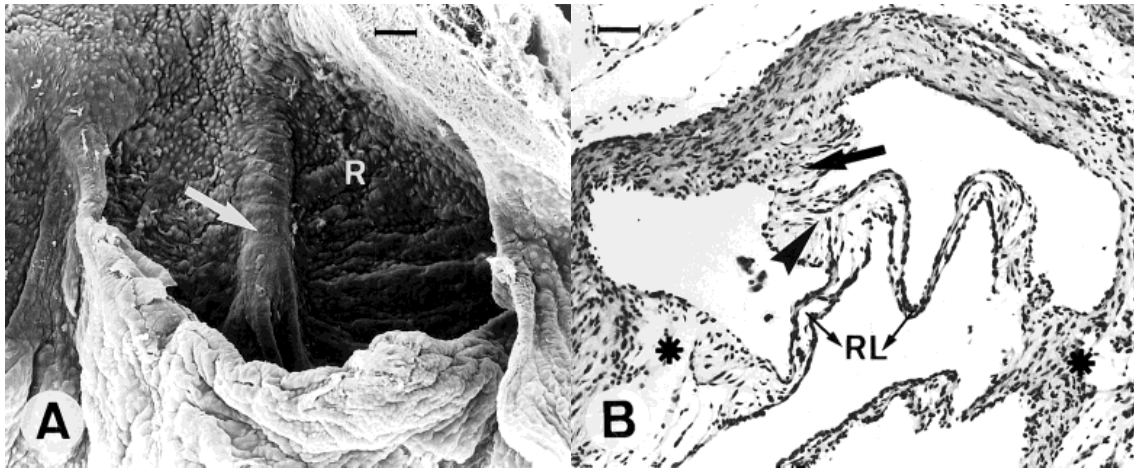


Fig. 5. Tricuspid pulmonary valves with a raphe-like ridge located in the right pulmonary sinus from adult Syrian hamsters. A: Scanning electron micrograph of the right pulmonary sinus (R). The raphe-like ridge (arrow), which divides the sinus into two cavities, enters into contact with the leaflet at the caudad level of the valve. B: Transverse section of the pulmonary valve. The raphe-like ridge, which consists of two histological portions, a proximal (arrow) and a distal (arrowhead), with regard to the pulmonary artery wall, widely encroaches toward the right leaflet (RL). Asterisks indicate the true commissures of the leaflet. Haematoxylin-eosin. Scale bars = 60 μ m.

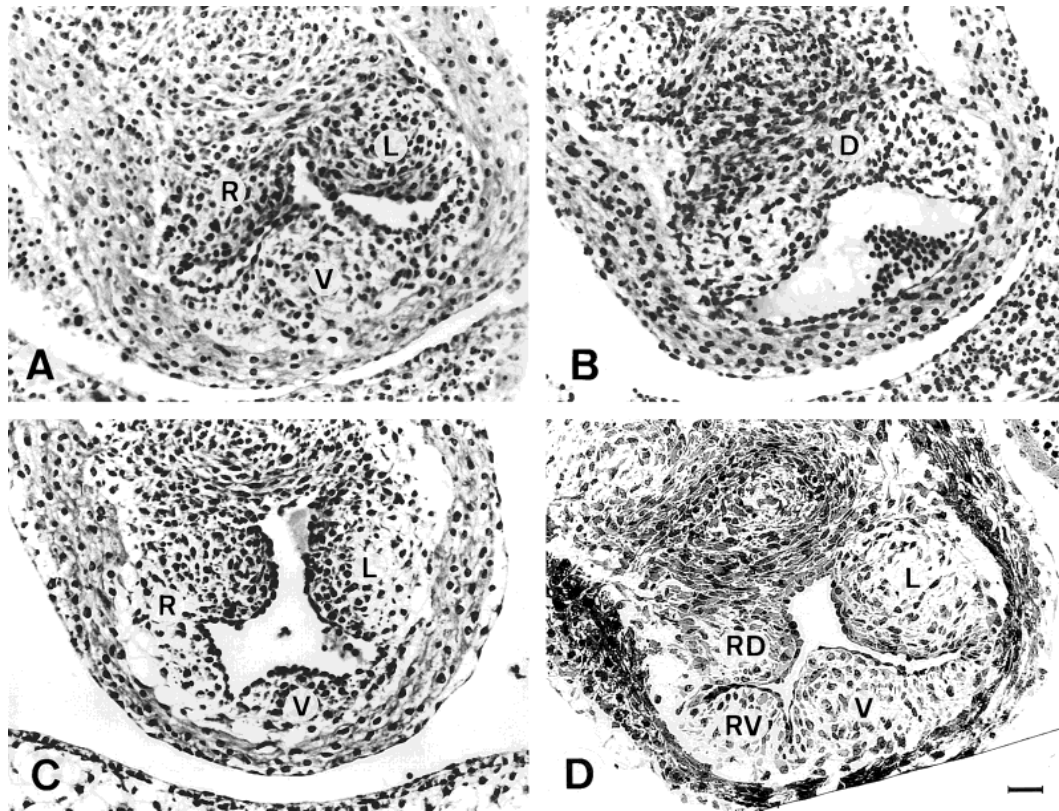


Fig. 6. Transverse sections of the pulmonary artery of Syrian hamsters at the beginning of the valvulogenesis, just before the excavation of the pulmonary sinuses takes place. The sections correspond to the cephalad level of the cushions. In panel A, three cushions, right (R), left (L), and ventral (V) can be identified, each of them growing as an independent structure. In panel B, the right and left cushions are totally fused, giving rise to a single dorsal cushion (D). The ventral cushion is not visible because of the orientation of the section. In panel C, the right cushion is partially divided into two lobes. In panel D, the right cushion is totally divided into two cushions, a right-dorsal (RD) and a right-ventral (RV). Age (postcoitum) and total length of the embryos: A: 11 days, 3 hours, TL = 9.1 mm; B: 11 days, 3 hours, TL = 9.5 mm; C: 11 days, 4 hours, TL = 9.1 mm; D: 11 days, 4 hours, TL = 8.9 mm. A–C: Haematoxylin-eosin; D: Semithin section stained with toluidine blue. Scale bar = 30 μ m.

Table 1. Pulmonary and Aortic Valve Conditions in Adult Hamsters an results of the Y² test

Valve conditions

	TPV ² TAV ⁷	TPV ² BAV ⁸	BPV ³ TAV ⁷	BPV ³ BAV ⁸	TPVr ⁴ TAV ⁷	TPVr ⁴ BAV ⁸	QPV ⁵ TAV ⁷	QPV BAV ⁸	TPVrf ⁶ TAV ⁷	TPVrf ⁶ BAV ⁸	TN1	OX ²
O9	91	49	32	13	3	2	5	4	5	2	206	
E ¹⁰	92.4	47.6	29.7	15.3	3.3	1.7	6.0	3.0	4.6	2.4	206	
x2	0.021	0.041	0.178	0.346	0.027	0.053	0.167	0.333	0.035	0.067		1.268

¹Total number of specimens.

²Tricuspid pulmonary valve.

³Bicuspid pulmonary valve.

⁴Tricuspid pulmonary valve with raphe-like ridge in the right pulmonary sinus.

⁵Quadricuspid pulmonary valve.

⁶Tricuspid pulmonary valve with raphe-like ridge in the right pulmonary sinus and partial fusion of the dorsal commissure.

⁷Tricuspid aortic valve.

⁸Bicuspid aortic valve.

⁹Observed values.

¹⁰Expected values.