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Formation of Cartilage in Congenital Bicuspid Aortic Valves of Syrian Hamsters (*Mesocricetus auratus*)

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SUMMARY

The formation, topographical location and calcification of cartilage in congenital bicuspid aortic valves of 235 Syrian hamsters aged 0–173 days were studied by histological, histochemical and immunohistochemical techniques. In all animals the aortic valve was bicuspid; it had two leaflets, ventral and dorsal, each supported by its own aortic sinus. In 141 valves, a more or less developed raphe was located in the ventral sinus. The remaining 94 valves were devoid of any raphe. The first sign of valvular chondrogenesis was the presence of small groups of cells embedded in a type II collagen-positive extracellular matrix. These cellular groups, which appeared as early as 2 days after birth, became converted into hyaline cartilage or fibrocartilage. A considerable proportion (67%) of the valvular cartilages developed within the first 6 weeks of life. The cartilaginous tissue was capable of forming anywhere along the attachments of the valve leaflets to their supporting sinuses. However, statistical analyses substantiated the observation that the bases of the sinuses and raphes were the valvular regions particularly prone to the development of cartilage. At these sites, the cartilage was usually hyaline and often became calcified. The findings were consistent with the assumption that intense mechanical stimulation plays an important role in the formation of the valvular cartilage. Moreover, these findings supplied new evidence that in the cardiac semilunar valves of Syrian hamsters, cartilage formation does not involve the aggregation of large numbers of cells before their differentiation into chondrocytes. The valvular hyaline cartilages appear to act as competent pivots, resisting mechanical tensions generated during the cardiac cycle. Deposition of calcium in the matrix can be regarded as a reinforcement process of the cartilaginous tissue. Finally, it is hypothesized that the formation of cartilage in the aortic valves of hamsters prevents dystrophic calcification of the valve, a pathological change that causes aortic stenosis in man, especially in patients with a bicuspid aortic valve.

Key Words: bicuspid aortic valve, cartilage, heart, Syrian hamster.

INTRODUCTION

Cartilaginous foci are regularly present in the aortic valve of the Syrian hamster (Kelsall and Visci, 1970; Sans-Coma et al., 1994; López et al., 2004). They appear along the fibrous attachments of the valve leaflets (cusps) to their respective sinuses and in front of the valvular commissures (López et al., 2004). Cartilage occurs significantly more frequently in the ventral region of the valve and in the dorsal aortic sinus than in other valvular regions (Sans-Coma et al., 1994; López et al., 2004). Locally intense, mechanical stimulation has been adduced as a possible cause in the development of the valvular cartilaginous tissue (Sans-Coma et al., 1994; López et al., 2004).

Congenital bicuspid aortic valves (BAVs) similar to those occurring in man have been reported in Syrian hamsters (Sans-Coma et al., 1992, 1993, 1996; Fernández et al., 1999). The presence of cartilaginous foci has been described in BAVs from adult hamsters (Sans-Coma et al., 1994). However, the mechanism by which such foci are formed remains unknown.

The aims of this study were (1) to gather information on the appearance, differentiation and structural features of cartilaginous foci in the BAVs of Syrian hamsters, and (2) to gain new insight into the factors implicated in the formation of the valvular cartilage. In addition, an attempt was made to assess whether the valvular cartilage becomes calcified. The study was carried out in young and adult hamsters examined by histological, histochemical and immunohistochemical techniques.

MATERIAL AND METHODS

Animals

The Syrian hamsters belonged to a family subjected to systematic inbreeding by crossing siblings. As described elsewhere (Sans-Coma et al., 1994; Fernández et al., 1999), the incidence of BAVs is relatively high in this inbred family. The present study was based on 235 hamsters (111 male; 124 female), aged 0–731 days, possessing a BAV. The animals belonged to a single substrain in which the incidence of cartilaginous tissue in aortic valves of adult specimens was 100%.

All hamsters were handled in accordance with the Spanish Regulations for the Protection of Experimental Animals (Real Decreto 223/1988; B.O.E. 18.03.1988). They were housed in polypropylene cages in a room in which both the temperature and photoperiod were controlled. Commercial mouse food (A.04; UAR/Panlab, Barcelona, Spain) and water were given ad libitum from the time of weaning. There was no known exposure of the animals to teratogenic agents.

The hamsters were killed by overdosing with chloroform or with carbon dioxide at a concentration of 75%. Seventy-six hearts were examined by histological, histochemical and immunohistochemical techniques. A further 55 specimens were examined by a whole-mount immunolabelling technique to demonstrate type II collagen. The remaining 104 hearts were examined for the detection of calcific deposits.

Histological and Histochemical Techniques (Light Microscopy)

The hearts were perfused with 0.02 M phosphate-buffered saline (PBS), pH 7.3, fixed by immersion in Bouin's solution (ratio of fixative to tissue volume, 80:1), and embedded in Paraplast (Sigma Chemical Co., Poole, England). Transverse, longitudinal or sagittal sections, cut serially at 10 mm for light microscopy, were stained with haematoxylin and eosin (HE) for a general assessment of the tissue structure, with Mallory's trichrome or Masson-Goldner's trichrome for connective tissue, with Weigert-van Gieson stain for the detection of elastin, or with 0.05% Alcian blue 8GX in 0.05 M acetate buffer (pH 5.8) plus 0.65 M magnesium to identify sulphated glycosaminoglycans (Scott and Dorling, 1965).

The Alizarin red S in-toto staining technique was used for the specific detection of calcium in the aortic valve tissues. The heart was removed, transferred to Ringer's solution, and dissected to expose the cardiac outflow tract. The specimens were fixed by immersion in 10% neutral formalin buffered with magnesium carbonate (ratio of fixative to tissue volume, 80:1) and stained with Alizarin red S, according to the procedure described by Richmond and Bennet (1938). Whenever calcium deposition was detected, the specimen was embedded in Paraplast and sectioned transversely for examination with a light microscope.

Immunohistochemical Techniques for the Detection of Type II Collagen

The two techniques used were as follows.

Detection in tissue sections (light microscopy). The hearts were washed in PBS and fixed by immersion in Bouin's solution (ratio of fixative to tissue volume, 80:1). The specimens were embedded in Paraplast, and sectioned transversely at 10 mm. Sections were dewaxed in xylene, dehydrated in an ethanolic series, and washed in Tris-phosphate buffered saline (TPBS), pH 7.8. Thereafter, the tissues were digested for 30–60 min at 37 8C with papain 0.5% in phosphate buffer (pH 4.7). Endogenous peroxidase activity was quenched by incubation with hydrogen peroxide 3% in TPBS for 30 min. After washing in TPBS, nonspecific binding sites were saturated for 30 min with SB (sheep serum 10% and bovine serum 1% albumin in TPBS). Sections were then incubated overnight at 4 8C with the monoclonal antibody CIIC1 (Developmental Studies Hybridoma Bank, University of Iowa, USA), diluted in SB; this antibody is specific for type II collagen. Control slides were incubated with SB or with nonimmune rabbit serum diluted 1 in 200.

After incubation, the sections were washed in TPBS (3!5 min), incubated for 1 h at room temperature with biotin-conjugated anti-mouse IgG (Sigma) diluted 1 in 100 in TPBS, washed again, and incubated for 1 h in ExtrAvidin© peroxidase conjugate (Sigma) diluted 1 in 150 in TPBS. Peroxidase activity was developed with Sigma Fast© 3,30-diaminobenzidine tablets, according to the instructions of the supplier. In some cases, the sections were counterstained with haematoxylin.

Detection by the whole mount immunolabelling technique. The aortic valves were removed and transferred to Cornwelle™ centrifuge tubes, fixed by immersion in

Bouin's solution, washed with TPBS, permeated in acetone, digested with proteinase K and papain, and then processed by the method used for the detection of type II collagen in tissue sections, starting from the incubation with the primary antibody (see above). A more detailed description of this procedure is given in López et al. (2001).

Statistical Analysis

The Student t-test and the $\chi 2$-test were used. In both cases, a probability of 0.05 or less (P%0.05) was required as evidence of a significant difference.

Nomenclature

The nomenclature employed for aortic valve components was that of Angelini et al. (1989), McKay et al. (1992), Sans-Coma et al. (1996), and Hokken et al. (1997). The terms proximal and distal are used to describe the location of such components with regard to the ventricles.

RESULTS

Cartilaginous Deposits in Aortic Valves of Young and Adult Hamsters

In all hamsters examined, the aortic valve was bicuspid; it had two aortic sinuses, ventral and dorsal, two leaflets, and a fibrous interleaflet triangle between each adjacent leaflet. Overall, therefore, two interleaflet triangles, right and left, were present in the subaortic outflow tract. In 141 (60%) of the 235 specimens, a fibrous raphe was located in the ventral aortic sinus. The raphe varied in size, ranging from a raphe that reached the free margin of the leaflet to a raphe that contacted the leaflet only at its most proximal level. The remaining 94 (40%) BAVs were devoid of any raphe.

Cartilaginous foci occurred at different sites of the fibrous attachments of the valve leaflets to their supporting aortic sinuses. These findings are represented by means of two diagrams (Fig. 1), one for BAVs with a raphe (BAVRs) and the other for BAVs with no raphe (BAVNRs). In each diagram eight topographical regions are considered. The following description of these topographical regions refers to the adult condition of the aortic valve.

Regions RC and LC are the two collagenous condensations of the aortic wall which constitute the most distal extensions of the valvular commissures and which, more proximally, where the leaflets emerge from the arterial wall, bulge into the arterial lumen. RC corresponds to the collagenous condensation of the right commissure, and LC to that of the left commissure.

Regions V1, V2, D1 and D2 are the lateral fibrous attachments of the leaflets to the aortic sinuses. V1 and V2 correspond, respectively, to the left and right lateral attachments of the ventral leaflet, and D1 and D2 to the right and left lateral attachments of the dorsal leaflet.

Region D3 corresponds to the proximal attachment of the dorsal leaflet; it consists of fibrous tissue which is continuous with that of the right fibrous trigone.

In BAVRs (Fig. 1a), region R corresponds to the raphe and the proximal attachment of the ventral leaflet to its sinus. In BAVNRs, it is the proximal attachment of the ventral leaflet to its sinus. In both cases, the proximal attachment of the ventral leaflet is composed of fibrous tissue inserted into the myocardium of the left ventricle free wall.

In this study, differences related to sex were not observed with regard to the occurrence of cartilage in the aortic valve. Therefore, male and female data were pooled. The following observations were based on the 131 specimens examined by histological, histochemical or immunohistochemical techniques (light microscopy) or by means of the whole mount immunolabelling technique for type II collagen.

The first event that could be related to cartilage formation was the appearance of small groups of mesenchymal cells embedded in a type II collagen-positive, extracellular matrix. This occurred in one of seven hamsters, aged 2 days, with BAVRs, and in one of two hamsters, aged 2 days, with BAVNRs (Table 1). In each specimen there was a single type II collagen-positive cellular group. It was situated in front of the raphe (region R) in the BAVR, and in the proximal attachment of the dorsal leaflet to its sinus (region D3) in the BAVNR (Fig. 2a).

None of the animals aged 3 days displayed type II collagen-positive cellular groups in the aortic valve.

Most of the hamsters aged 4 to 40 days had cartilaginous foci in the aortic valve: 24 (96%) of 25 having a BAVR and all (100%) of nine having a BAVNR (Table 1). In the BAVRs, the cartilaginous tissue appeared in the lateral or proximal attachments (or both) of the dorsal leaflet to its sinus (regions D1, D2, D3), in the left lateral attachment of the ventral leaflet (region V1), and, more frequently, either in front of the raphe or in the proximal attachment of the ventral leaflet (region R). In the BAVNRs, the cartilaginous foci occurred in the proximal attachment of the dorsal leaflet to its sinus (region D3) and in the left lateral or proximal attachments of the ventral leaflet (regions V1, V3).

In the animals aged 4 to 10 days, the developing cartilaginous foci could be assessed only by means of immunohistochemical techniques (Figs 2b, c). The type II collagen-positive cellular groups were embedded in a markedly developed fibrous cellular matrix, which hindered their detection by conventional histological procedures. In the animals aged 11 to 40 days, the cartilaginous foci varied in size and histological features. Some were composed of a small group of cells surrounded by a type II collagen-positive matrix. Other foci consisted of a large number of chondrocytes embedded in an extracellular matrix containing a considerable amount of glycosaminoglycans (Fig. 2d). The remaining foci were made up of small rows of cells surrounded by a type II collagen-positive matrix and were contained within a meshwork of collagen fibres.

All of the 72 hamsters aged 41 days and older had cartilaginous foci in the aortic valve. The distribution of such foci, according to the aortic valve morphology and topographical regions, is given in Table 1. In 55 specimens the cartilage was detected

by means of the whole mount immunolabelling technique for type II collagen. In the remaining 17, it was observed in tissue sections.

Histologically, two types of cartilaginous foci were identified, namely, hyaline and fibrocartilaginous. The hyaline cartilaginous foci were composed of chondrocytes embedded in a type II collagen-positive matrix that stained metachromatically with haematoxylin. In some cases, the chondrocytes were of similar size; in other cases, the central part of the hyaline cartilage was occupied by hypertrophic chondrocytes (Fig. 2e). The foci displayed a thin perichondrium consisting of collagen fibres that ran circumferentially and contained one to three layers of flattened cells (Fig. 2e). Sometimes, however, the perichondrium was incomplete. At the sites devoid of perichondrium, the cartilaginous tissue gradually merged with the adjacent fibrous tissue. In the BAVRs, the hyaline cartilages were mainly located in front of the raphe (region R), sometimes penetrating the raphe, and in the proximal attachment of the dorsal leaflet to its sinus (region D3). In the BAVNRs, they usually occurred in the proximal attachments of the ventral and dorsal leaflets (regions V3 and D3). The presence of hyaline cartilage in any other topographical region must be regarded as an uncommon event. Most hyaline cartilages displayed a nodular shape, varying in size. Those located in the proximal part of the ventral and dorsal sinus walls (regions V3 and D3) often appeared as C-shaped bars.

The fibrocartilaginous foci were composed of a few rows of cells embedded in a type II collagen-positive matrix and were contained within a meshwork of collagen fibres and a few elastic fibres. The arrangement of these foci varied widely, ranging from an isolated, small focus to a series of chain-like foci that extended along a variable number of adjacent topographical regions. In the latter case, the foci were regarded as a single focus for statistical purposes. The fibrocartilaginous foci were prevalent in the valvular commissures (regions RC and LC) and in the lateral attachments of the leaflets to their respective sinuses (regions V1, V2, D1 and D2); they rarely appeared in other valvular regions.

Calcification of the Valvular Cartilages

The presence of calcium deposits in the aortic valve was examined in 63 hamsters, aged 56 to 714 days, with a BAVR and in 41 hamsters, aged 24 to 708 days, with a BAVNR. Valvular calcification (Fig. 2f) was detected in 30 specimens with a BAVR and nine with a BAVNR. It should be noted that the presence of calcium in Alizarin red S-positive cartilages had been confirmed previously in normal aortic valves of Syrian hamsters by means of X-ray diffraction (unpublished data). As revealed by the tissue sections, the mineral was deposited in the extracellular matrix of cartilaginous tissue (Fig. 2g).

In the 30 BAVRs there was a single calcified cartilaginous focus. In 27 cases, the focus was located in front of the raphe (position R). In another case it was situated in the right commissure (position RC), and in the remaining two cases it was found in the proximal attachment of the dorsal leaflet to its supporting sinus (position D3). Seven of

the nine BAVNRs had a focus of calcified cartilage in the proximal attachment of the ventral leaflet to the sinus wall (position V3). In the remaining two BAVNRs, two calcified cartilaginous foci, one in position V3 and the other in position D3, were found. These findings are shown diagrammatically in Figs 3a and b.

The youngest animal with a calcific deposit was aged 37 days; it had a BAVNR. The remaining specimens with calcified cartilaginous tissue in the aortic valve were aged 79 days and older. As shown in Table 2, the incidence of cartilage calcification was higher in BAVRs than in BAVNRs. In addition, calcification increased in prevalence with advancing age in both valvular morphotypes.

Statistical Analysis

With regard to the number of cartilaginous foci occurring in the valve, no statistical differences relating to the presence versus absence of a raphe were observed. Therefore, data concerning BAVRs and BAVNRs were pooled for the following statistical analysis.

In the animals aged 4 to 40 days, the number of cartilaginous foci occurring in the valve ranged between one and three, while in those aged 41 days or older, it ranged from one to five. The mean values and standard deviations were 1.73±0.63 for the first group of specimens and 2.58±1.18 for the second group. This difference between means was statistically significant at $P<0.001$ (Student *t*-test). From these computed mean values (1.73 versus 2.58) it can be concluded that about 67% of the valvular cartilages formed within the first 40 days of life.

To test for any association between the incidence of cartilaginous foci and their location in the aortic valve, a χ^2 contingency test was performed under the null hypothesis that they were independent events. The test was carried out for specimens aged 41 days and older, and separately for those with BAVRs and those with BAVNRs (Table 3). The computed value of the $\chi 2$ statistic was 63.88 for hamsters with BAVRs and 52.17 for hamsters with BAVNRs, with 7 degrees of freedom in each series. Therefore the null hypothesis was rejected at $P<0.001$ in both cases. As is also shown in Table 3, the major departure from homogeneity in specimens with BAVRs was the relatively large proportion of animals with cartilaginous foci in front of the raphe (region R) and in the proximal attachment of the dorsal leaflet to its sinus (region D3). In the specimens with BAVNRs, the major departure from homogeneity was the relatively high proportion of animals with cartilaginous tissue in the proximal attachments of the ventral and dorsal leaflets (regions V3 and D3). These results are shown diagrammatically in Figs 3c and d.

DISCUSSION

In the present study, immunohistochemical techniques for detection of type II collagen were used, bearing in mind that the synthesis of type II collagen is considered to be a cartilage-characteristic event (Miller and Matukas, 1969; Miller, 1976; Kosher, 1983; Hall and Miyake, 1992, 1995). It should be noted, however, that type II collagen

is also produced by several nonchondrogenic cell types (see Kosher (1983) and Swiderski et al. (1994) for extensive reviews).

Previous work indicates that the formation of cartilaginous foci in normal (tricuspid) aortic valves of Syrian hamsters can start from the first day of life (López et al., 2004). In the present BAVRs and BAVNRs, the earliest sign of cartilage formation was recorded in the second day of life. This observation contradicts our former suggestion, based on the study of adult Syrian hamsters, that chondrogenesis takes place earlier in BAVRs and BAVNRs than in tricuspid aortic valves (Sans-Coma et al., 1994).

As in normal aortic (López et al., 2004) and pulmonary (López et al., 2001) valves, the first evidence of cartilage formation in BAVs of Syrian hamsters is the appearance of small groups of mesenchymal cells, embedded in a type II collagen-positive extracellular matrix. This contrasts with reptiles (López et al., 2003) and birds (López et al., 2000), in which the development of cardiac cartilaginous tissue begins with the formation of the so-called prechondrogenic condensations, composed of a considerable number of loosely packed, mesenchymal cells embedded in a type II collagen-positive extracellular matrix. Synthesis of type II collagen begins in the core of such cell condensations, which are readily recognized in tissue sections, even with conventional histological techniques (López et al., 2000, 2003). These observations in reptiles and birds are consistent with the common assumption that acquisition of the ability of the chondrocyte precursors to aggregate, establishing cell to cell contact, constitutes the first step of the cascade of events that regulates the early stages of the cartilage differentiation (Thorgood and Hinchcliffe, 1975; Ede, 1983; Tachetti et al., 1992; Cancedda et al., 1995; De Crombrugghe et al., 2000). In contrast, we detected no conspicuous prechondrogenic condensation in the BAVs; this supports a previous statement (López et al., 2001, 2004) that, at least in rodents, the morphogenesis of valvular cartilage does not involve the aggregation of large numbers of cells before their differentiation into chondrocytes. This points to a possible induction of differentiation in prechondrogenic cells independent of cell condensation, a morphogenetic mechanism already adduced to explain the results of experimental studies carried out in cell cultures (Benya and Schaffer, 1982; Glowacki et al., 1983; Zanetti and Solursh, 1984; Newman and Watt, 1988). Nonetheless, further investigations by more appropriate methods should be carried out to decide whether or not precartilage condensation represents an essential step in the formation of the valvular cartilage.

In normal aortic valves of Syrian hamsters, most cartilaginous foci begin to develop within the first 40 days of life (López et al., 2004), when the histogenesis of the valve takes place (Sans-Coma et al., 1994). The present findings indicate that most (67%) of the cartilaginous foci occurring in the BAVs of this rodent species also form during this period. Nonetheless, the number of cartilaginous foci was significantly higher in the hamsters with BAVs aged 41 days or older than in those aged 4 to 40 days. This denotes that, as in normal aortic valves (López et al., 2004), the formation of new cartilage in BAVs continues after the sixth week of life. Indeed, small groups of cells

surrounded by an extracellular matrix containing type II collagen occurred in the BAVs of both young and adult hamsters.

The number of hamsters aged 0 to 40 days in the present study was too small to seek any statistically significant association between the occurrence of cartilaginous foci and their location in the anomalous valves. Nevertheless, the data given in Table 1 indicate that chondrogenesis takes place relatively early in front of the raphe in BVARs, in the proximal attachment of the ventral leaflet in BAVNRs, and in the proximal attachment of the dorsal leaflet in both BAV morphotypes. In addition, they prove that these topographical regions are those which show a particular predisposition to the development of cartilaginous tissue during the first 6 weeks of life.

The results of the $\chi 2$ contingency tests (Table 3) indicate that in the BAVRs and BAVNRs of hamsters aged 41 days and older, the occurrence of cartilaginous foci is not a random event. It was significantly increased in (1) the region consisting of the raphe and the proximal attachment of the ventral leaflet to the aortic root in BAVRs, (2) the proximal attachment of the ventral leaflet to its sinus in BAVNRs, and (3) the fibrous, proximal attachment of the dorsal leaflet to its sinus in both BAVRs and BAVNRs (Fig. 3). Interestingly, the cartilaginous foci are regularly hyaline in these topographical regions, whereas in both the lateral attachments of the leaflets to the sinuses and valvular commissures, the foci are usually of a fibrocartilaginous nature.

It is well known that the differentiation of an individual cell is influenced by the extracellular environment (Hunter and Caplan, 1983). In this context, mechanical action has been adduced as a factor that may induce the transformation of mesenchymal cells into cardiac chondrocytes (Hueper, 1939; Hollander, 1968; Sans-Coma et al., 1994; Egerbacher et al., 2000; López et al., 2000, 2004). In normal (tricuspid) aortic valves of Syrian hamsters, cartilage may develop in any part of the sinus boundaries (Lo´pez et al., 2004), i.e., the valvular portions that bear the brunt of the stress generated by the valve leaflets during the cardiac cycle (Broom, 1998). Specifically, the hyaline cartilaginous foci develop in the valvular commissures and sinus bottoms (López et al., 2004), which are subject to particularly intense mechanical stimulation during ventricular systole (Thubrikar, 1990). In the BAVs of hamsters, the cartilaginous tissue also develops along the attachments of the leaflets to their supporting sinuses. Nonetheless, the hyaline cartilage usually appears in the sinus bottoms and in front of the raphe, which is a fibrous structure that increases flexion stresses locally (Thubrikar et al., 1986). The sinus bottoms, and especially the raphe, are also the valvular sites in which the hyaline cartilaginous tissue shows greater predisposition to become calcified with age (compare Figs 3a, b with Figs 3c, d).

In man, the bicuspid condition of the aortic valve is the congenital cardiac anomaly seen most frequently in adults, with an incidence of 0.5%–2% in autopsy surveys (see Giusti et al. [1991] and Fedak et al. [2002] for extensive reviews). The defect may be inapparent clinically and compatible with normal functions (Roberts, 1989). However, it is associated with the risk of multiple complications, the most frequent of which is aortic stenosis, with or without coexistent regurgitation, due to dystrophic calcification

of the valve (Roberts, 1970, 1987; Fenoglio et al., 1977; Giusti et al., 1991; Sabet et al., 1999; Fedak et al., 2002). Calcification tends to be greatest along the raphes and at the base of the valve pockets (Fenoglio et al., 1977; Subramanian et al., 1984; Thubrikar et al., 1986; Sabet et al., 1999; Fedak et al., 2002), probably as a result of locally intense mechanical stimulation (Thubrikar et al., 1986; Robiseck et al., 2004). There is now a growing awareness that vascular calcification is a well-regulated biological process, and not a passive mineral precipitation. It has many similarities to embryonic bone formation and bone repair (Bostro¨m, 2000, 2001; Bostro¨m and Demer, 2000; Rajamannan et al., 2003). Indeed, cell differentiation proceeds along osteogenic and chondrogenic lineages, resulting in calcified tissues (Boström, 2000, 2001; Boström and Demer, 2000).

The present observations and those reported by Sans-Coma et al. (1994) are the only ones that illustrate the natural history of congenital BAVs in a nonhuman mammalian species. They show that, at least in the Syrian hamster, BAVs seem not to calcify as human BAVs do. Instead, they form cartilaginous tissue, a fact with no pathological consequences. Fibrocartilage forms in many valvular regions subject to mechanical stresses, and conspicuous hyaline cartilages appear where these stresses are particularly intense. Their occurrence in specific valvular regions suggests that they play a role in valve performance, acting presumably as competent pivots. Accordingly, deposition of calcium in the extracellular matrix of intact cartilage, containing viable cells, can be regarded as a reinforcement of the cartilaginous tissue. This accords with the view of Egerbacher et al. (2000) that calcification of cartilage matrix material located in the heart skeleton of the otter, *Lutra lutra*, should be interpreted as a process of mechanical reinforcement rather than the result of a degenerative process. Thus, the formation of cartilage in the aortic valve of Syrian hamsters probably represents a protection mechanism against the strong mechanical tensions generated during the cardiac cycle. Possibly it prevents damage of valvular tissues, which has been adduced as an important factor in the development of the initial nuclei (i.e., products of cellular degradation) of valvular calcification (Kim, 1976; Valente et al., 1985).

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Table 1. Cartilaginous foci in bicuspid aortic valves with raphes (BAVRs) or without raphes (BAVNRs).

Ages of Animals (days)	n	nC^+	%	BAVRs: number of specimens with foci in the stated topographical regions							
				RC	LC	V1	V2	R	D1	D2	D3
0	4	0	0	0	0	0	0	0	0	0	0
1	5	0	0	0	0	0	0	0	0	0	0
2	7	1	14.3	0	0	0	0	1	0	0	0
3	1	0	0	0	0	0	0	0	0	0	0
4–40	25	24	96.0	0	0	1	0	24	1	2	15
>40	36	36	100.0	5	11	2	12	36	15	17	33
Ages of Animals (days)	n	nC^+	%	BAVNRs: number of specimens with foci in the stated topographical regions							
				RC	LC	V1	V2	V3	D1	D2	D3
0	5	0	0	0	0	0	0	0	0	0	0
1	1	0	0	0	0	0	0	0	0	0	0
2	2	1	50.0	0	0	0	0	0	0	0	1
3	0	0	0	0	0	0	0	0	0	0	0
4–40	9	9	100.0	0	0	1	0	9	0	0	6
>40	36	36	100.0	6	11	8	14	36	11	20	33

N=Number of specimens examined; nC^+=number of specimens with cartilaginous foci in the aortic valve. See the text and Fig. 1 for definitions of the topographical regions of the aortic valve.

Table 2. Calcified cartilaginous foci in bicuspid aortic valves of Syrian hamsters, according to the age of the animals.

Ages of animals (days)	BAVR			BAVNR		
	n	n calcif.	%	n	n calcif.	%
<78	6	0	0	7	1	14.3
79–179	13	5	38.5	3	0	0
180–359	18	9	50.0	12	3	25.0
360–539	21	11	52.4	8	2	25.0
>539	5	5	100	11	3	27.3

BAVNR= bicuspid aortic valve with no raphe; BAVR= bicuspid aortic valve with raphe; n= number of specimens examined; n calcif.=number of specimens with calcified cartilaginous foci in the aortic valve.

Table 3. Contingency table of location of the cartilaginous foci in bicuspid aortic valves of hamsters aged 41 days or older, and results of the χ^2 contingency tests.

BAVR										
Values	RC	LC	V1	V2	R	D1	D2	D3	n	χ^2
O	5	11	2	12	36	15	17	33	131	
E	16.4	16.4	16.4	16.4	16.4	16.4	16.4	16.4	131	
χ^2	7.92†	1.78	12.64†	1.18	23.42*	0.12	0.02	16.80*		63.88*
BAVNR										
Values	RC	LC	V1	V2	V3	D1	D2	D3	n	χ^2
O	6	11	8	14	36	11	20	33	139	
E	17.4	17.4	17.4	17.4	17.4	17.4	17.4	17.4	139	
χ^2	7.47†	12.35	5.08†	0.66	19.88*	2.35	0.39	13.99*		52.17*

BAVNR= bicuspid aortic valve with no raphe; BAVR= bicuspid aortic valve with raphe; E= expected values; O= observed values; n= total number of cartilaginous foci; *P< 0.001; †P< 0.05. See the text and Fig. 1 for definitions of the topographical regions of the aortic valve.

Fig. 1. Location of the topographical regions established for (a) bicuspid aortic valves with a raphe (arrows) located in the ventral aortic sinus (BAVR), and (b) bicuspid aortic valves with no raphe (BAVNR). See the text for the definition of each region. DS, dorsal aortic sinus; VS, ventral aortic sinus.

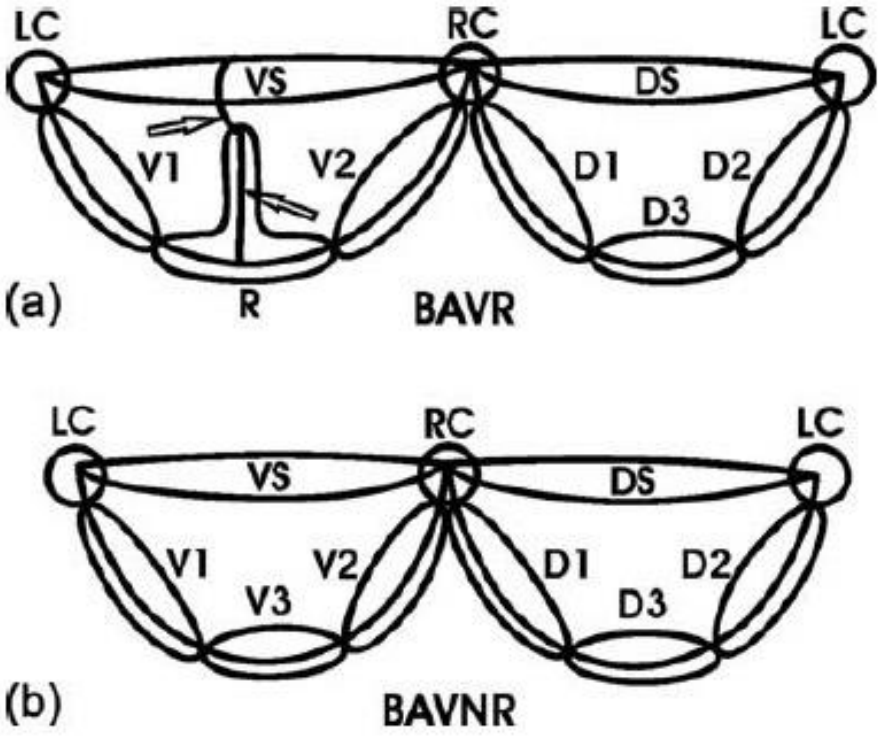

Fig. 2a–g. Cartilaginous foci in congenital bicuspid aortic valves of Syrian hamsters. (a) Transverse section of a bicuspid aortic valve devoid of any raphe from a hamster aged 2 days. Type II collagen immunolabelling, counterstained with haematoxylin. The arrowheads indicate a small, C-shaped group of cells, surrounded by a type II collagen-positive extracellular matrix, located in the dorsal aortic sinus (DS) (region D3). (b) Transverse section of a bicuspid aortic valve with a raphe (star) located in the ventral aortic sinus from a hamster aged 10 days. Type II collagen immunolabelling, counterstained with haematoxylin. A group of cells surrounded by a type II collagen-positive extracellular matrix is located in front of the raphe (region R). (c) Transverse section of a bicuspid valve with no raphe from a hamster aged 10 days. Type II collagen immunolabelling, counterstained with haematoxylin. The arrows point to two small groups of cells surrounded by a type II collagen-positive extracellular matrix, placed in the proximal portion of the ventral aortic sinus (VS) (region V3). DS, dorsal aortic sinus. (d) Transverse section of a bicuspid valve with no raphe from a hamster aged 25 days. Alcian blue stain. A cartilaginous focus is located in the proximal portion of the dorsal aortic sinus (region D3). DS, dorsal aortic sinus. (e) Sagittal section of a bicuspid aortic valve with no raphe from a hamster aged 525 days. A focus of hyaline cartilage is located in the proximal portion of the dorsal aortic sinus (region D3). Masson-Goldner's trichrome. Note the presence of hypertrophic chondrocytes in the core of the focus, which is surrounded by a thin perichondrium (arrowheads) composed of collagen fibres and two or three layers of flattened cells. Ao, aorta. (f) Occlusal view of the ventral sinus and leaflet of a bicuspid aortic valve with a well-developed raphe (arrow) from a hamster aged 305 days. Alizarin Red S stain. The arrowheads point to calcium deposits located in the base of the raphe and proximal attachment of the ventral leaflet to the sinus wall (region R). (g) Histological section of an Alizarin Red S-positive focus located in front of the raphe of a bicuspid aortic valve from a hamster aged 668 days. The calcium is deposited in the extracellular matrix of cartilaginous tissue. Scale bars: a, d, e, g, 50 μm; b, 25 μm; c, 150 μm; f, 250 μm.

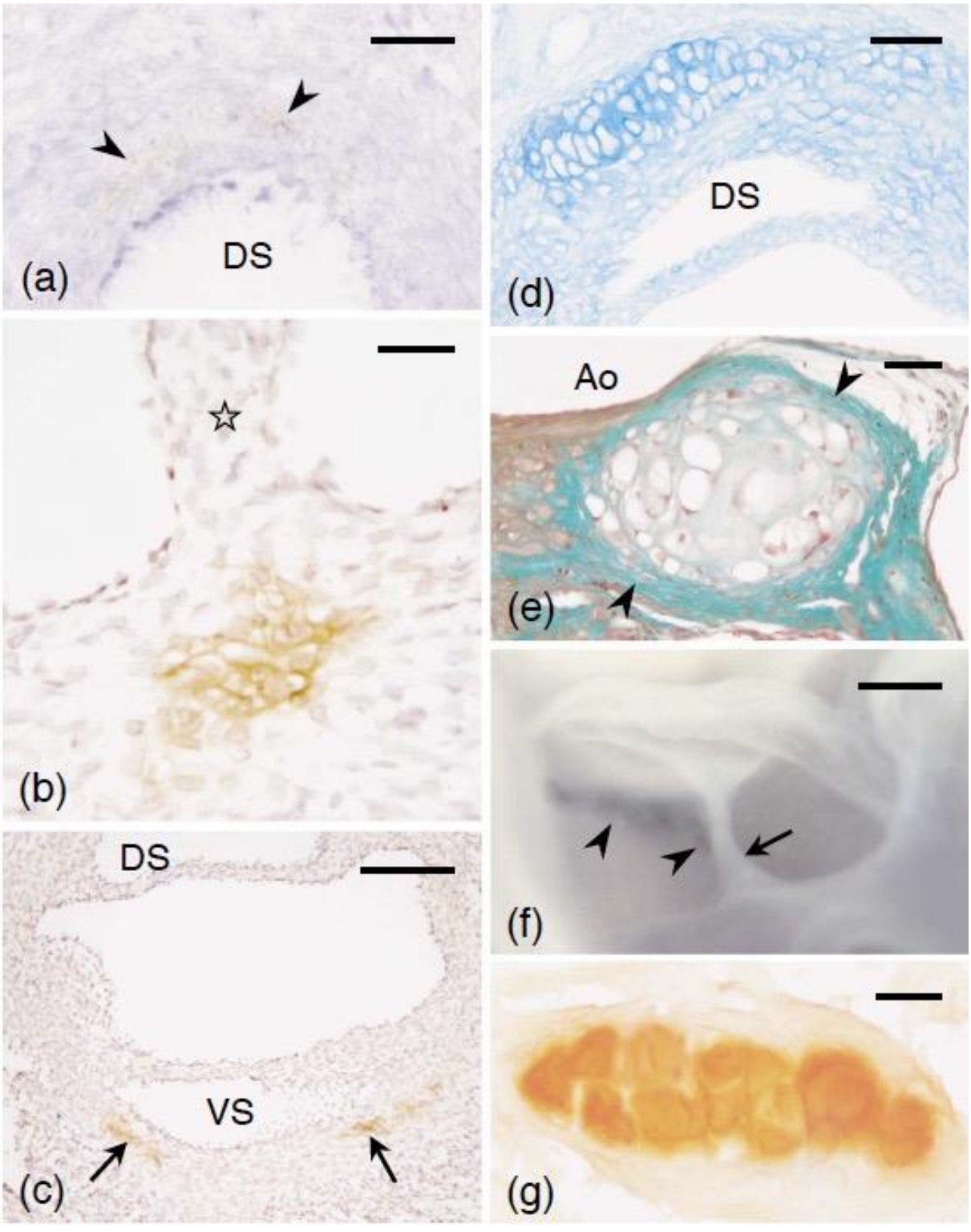
(a)
DS
(b)
DS
(c)
VS
(d)
DS
Ao
(e)
(f)
(g)

Fig. 3a–d. Open cut views of bicuspid aortic valves showing (a, b) the location of calcified cartilaginous foci (black areas) and (c, d) the topographical regions with significantly higher incidence of cartilaginous foci (grey areas). Note the similarity between the valvular regions more prone to develop cartilaginous foci and those in which the cartilaginous tissue becomes calcified. BAVR, bicuspid aortic valve with a raphe located in the ventral aortic sinus. BAVNR, bicuspid aortic valve with no raphe. DS, dorsal aortic sinus; VS, ventral aortic sinus.