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Cartilage in pulmonary valves of Syrian Hamsters

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Summary

The presence of cartilage in the pulmonary valve has been reported in birds, but not in mammals. We describe here the occurrence of cartilaginous tissue in the pulmonary valves of 40 (11.4%) of 351 Syrian hamsters examined using histological, histochemical and/or immunohistochemical techniques. The cartilaginous deposits were located along the fibrous attachments of the valve leaflets to the wall of the pulmonary artery trunk. Our findings indicate that the proximal attachments of the leaflets to their respective sinuses, and especially that of the ventral leaflet, are the most prone valvular regions to develop cartilaginous foci. Nonetheless, the possible function of these foci remains an open question. Formation of cartilage in the pulmonary valve starts within the first month of life, that is during the period in which the valve reaches histological maturation. The earliest evidence of chondrogenesis is the presence of small groups of cells embedded in a type II collagen-positive extracellular matrix. These groups of cells, which can appear as early as one day after birth, increase moderately in size and differentiate into hyaline cartilaginous tissue. The precursors of the cartilaginous cells are presumed to be neural crest-derived elements. However, the factor or factors involved in the differentiation of these precursors into chondrocytes are still unknown. In this regard, our observations cast doubt on the hypothesis that the formation of cardiac cartilages is primarily due to locally intense mechanical stimulation.

Key words: Heart, Pulmonary valve, Cartilage, Syrian hamster, Mammals

Introduction

It is well known that cartilage and/or bone develop in the cardiac skeleton of several mammalian species (see Matumoto (1938), Kelsall and Visci (1970) and Sans-Coma et al. (1994) for reviews of the literature). Cartilaginous deposits have been observed in the aortic valve, fibrous trigones and interventricular septum. Up to now, the presence of cartilage in the pulmonary valve of mammals has not been reported.

In a study of the cardiac semilunar valves of Syrian hamsters, *Mesocricetus auratus*, several animals were found to have cartilaginous tissue in the pulmonary valve, thereby furnishing information on the formation, location and structure of this cardiac

cartilage. The study was carried out using histological, histochemical and immunohistochemical techniques. The aim here is to report our observations.

Material and methods

Animals. The sample examined consisted of 351 Syrian hamsters (158 male, 193 female), aged 0---422 days, belonging to a complex of breeding colonies subjected to systematic inbreeding by mating siblings. The number of animals according to age is given in Table l.

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

The animals were sacrificed by overdosing with chloroform or with carbon dioxide at a concentration of 75% delivered into a chamber. One hundred ninety-six animals were examined by means of histological, histochemical and immunohistochemical techniques for light microscopy. In the remaining 155, aged 42 days or older, a whole-mount immunostaining technique was applied for the specific detection of type II collagen.

Histological and histochemical techniques for light microscopy. Hearts were removed after perfusion with 0.02 M phosphate buffered saline (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., England). Serial sections, transversely cut at 10 µm, were stained with haematoxylin-eosin or Mallory's trichrome stain for a general assessment of the histological components of the valves, and with resorcin-fuchsin for the detection of elastic fibres. In addition, the differential staining of sulphated glycosaminoglycans with alcian blue (Scott and Dorling 1965) was applied as an alternative procedure.

Immunohistachemical technique for the detectian af type II collagen. The use of this technique relied on the fact that synthesis of type II collagen is considered to be of characteristic cartilage (Miller and Matukas 1969; Miller 1976; Kosher 1983; Hall and Miyake 1992, 1995), even though type II collagen is also produced by a limited number of non-chondrogenic cell types (see Kosher (1983) and Swiderski et al. (1994) for extensive reviews of the literature).

The removed hearts were washed in phosphate buffered saline and fixed by immersion in Bouin’s solution (ratio of fixative to tissue volume = 80:1). The specimens were then embedded in Paraplast, and transversely cut at 10 µm. Sections were dewaxed in xylene, hydrated in an ethanol series, and washed in Tris-phosphate buffered saline (TPES, pH 7.8). Thereafter, the tissues were digested for 30 min - 1 h at 37 ºC with 0.5% papain in phosphate buffer (pH 4.7). Endogenous peroxidase activity was quenched by incubation with 3% hydrogen peroxide in TPBS for 30 min. After washing in TPBS, nonspecific binding sites were saturated for 30 min with 10%

sheep serum and 1% bovine serum albumin in TPBS (SB). Sections were then incubated overnight at 4ºC in the monoclonal antibody CIIC1 (Developmental Studies Hybridoma Eank, University of Iowa), which recognizes type II collagen, diluted in SB. Control slides were incubated in SB or in nonimmune rabbit serum at a dilution of 1:200.

After incubation, the sections were washed in TPBS (3x5 min), incubated for 1 h at room temperature in biotin-conjugated anti-mouse IgG (Sigma) at a dilution of 1: 100 inTPBS, washed again, and incubated for 1 h in ExtrAvidin® conjugate (Sigma) at a dilution of 1:150 inTPBS. Peroxidase activity was developed with Sigma Fast® 3,3' - diaminobenzidine tablets, according to the instructions of the supplier. In several cases, the sections were counterstained with haematoxylin.

Type II callagen whale-maunt immunastaining technique. Removed aortic valves were transferred to Cornwell™ centrifuge tubes, and fixed by immersion in Bouin's solution for 12 h. The valves were then washed in TPBS and permeated for 15 min in acetone at -20 ºC. After washing in TPBS, the specimens were immersed for 30 min in 3% Triton X-100 in TPBS, and washed again in TPBS. Thereafter, the tissues were digested with 10 µg/ml proteinase K for 15 min, washed in TPBS and digested with 0.5% papain in phosphate buffer (pH 4.7) for 4 h at 37 ºC. Endogenous peroxidase activity was quenched for 1 h by incubation in 3% hydrogen peroxide in TPBS. After washing with TPBS, nonspecific binding sites were saturated for 2 h with SB. Finally, the specimens were processed following the protocol used for the detection of type II collagen in tissue sections, starting from the incubation with the primary antibody.

Statistical methods. The x2-test was used. A probability of 0.05 or less was required as evidence for a significant difference.

Nomenclature. The nomenclature used for pulmonary valve components is that of Hokken et al. (1997) and Fernández et al. (1998). The terms proximal and distal are used to describe the location of these components with regard to the ventricles.

Results

In all hamsters examined, the pulmonary valve displayed a tricuspid structure. It had three pulmonary sinuses, right, left, and ventral, three leaflets, and a triangular space between each adjacent leaflet, so that overall three fibrous interleaflet triangles were present in the subpulmonary outflow tract. In 173 (49.3%) of the 351 specimens, the dorsal commissure, between the right and left leaflets, was slightly fused. In the Syrian hamster, this arrangement of the pulmonary valve can be considered to be within the spectrum of anatomical normality (Fernández et al. 1998).

Cartilaginous tissue was present in the pulmonary valves of 40 (11.4%) of the 351 specimens. The cartilaginous deposits occurred at different sites of the fibrous attachments of the valve leaflets to their respective pulmonary sinuses. For a clear presentation of these findings, we made a diagram, in which 12 topographic regions (A-L) of the pulmonary valve are considered (Fig. 1). The following description of these

topographic regions refers to adult hamsters, in which the pulmonary valve has completed its histogenesis.

Regions A-C are the three collagenous condensations of the pulmonary artery 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. A corresponds to the collagenous condensation of the dorsal commissure, B to that of the right-ventral commissure, and C to that of the left-ventral commissure.

Regions D-I are the lateral fibrous attachments of the leaflets to the pulmonary sinuses. E and F correspond, respectively, to the dorsal and ventral lateral attachments of the right leaflet, G and H to the right and left lateral attachments of the ventral leaflet, and D and I to the dorsal and ventral lateral attachments of the left leaflet.

Regions J-L are the proximal attachments of the leaflets to their respective sinuses. These attachments consist of collagenous tissue extending in a fingerlike fashion into the underlying myocardium of the right ventricle. J corresponds to the proximal attachment of the left leaflet, K to that of the right leaflet, and L to that of the ventral leaflet. In the present specimens, no differences related to sex were observed with regard to the occurrence of cartilaginous tissue in the pulmonary valve. Therefore, male and female data have been pooled.

The first event which could be related to cartilage formation in the pulmonary valve was the appearance of small groups of cells embedded in a type II collagen-positive extracellular matrix (Fig. 2). This occurred in 2 (6.9%) of the 29 hamsters aged 1 day (Table 1). In each specimen there was a single type II collagen-positive cellular group in the proximal attachment of the right leaflet to its supporting sinus (region K).

Similar groups of cells were present in different topographic regions of 7 (20.0%) of the 35 animals aged 2days, and 5 (17.9%) of the 28 animals aged 3 days (Fig. 3). There was only one group of such cells found in each specimen (Table 1).

In all of the preceding hamsters, aged 1 to 3 days, the wall of the pulmonary artery had already become stratified in intima, media and adventitia. The leaflets of the pulmonary valve displayed a very cellular condition; they consisted of a mesenchymal core covered by the endothelium. Both the most distal extensions of the commissures (regions A-C) and attachments of the leaflets to the pulmonary sinuses (regions D-L) were composed of a mesenchymal tissue, containing few scattered collagenous fibres. Among the 14 animals aged 4 to 9 days, 2 (14.2%) had a single cartilaginous deposit in the pulmonary valve (Table 1); they were 6 and 9 days old, respectively. Each deposit consisted of a small group of chondrocytes embedded in a type II collagen-positive matrix. In both cases, the histogenesis of the pulmonary valve had progressed markedly, and the cartilaginous deposits were surrounded by a more developed fibrous tissue than in the hamsters aged 1 to 3 days.

In the age range between 10 and 42 days, only 1 (2.4%) of the 42 hamsters had a cartilaginous deposit in the pulmonary valve (Table 1). The animal was 17 days old; its pulmonary valve displayed a considerable degree of histological maturation. The cartilage, which was of small size (Fig. 4), was located in the well-developed fibrous

tissue of the proximal attachment of the ventral leaflet to its corresponding sinus (region L).

Cartilaginous tissue occurred in the pulmonary valves of 23 (13.8%) of the 167 hamsters aged 43 days and older (Table 1). In 19 specimens there was a single deposit in the valve; in the remaining 4 there were 2 deposits. The 27 deposits were relatively small, either nodular or somewhat ellipsoidal. Twenty-four of them were detected by means of the type II collagen whole-mount immunostaining technique; the other 3 were found in tissue sections. As far as the sections revealed, the cartilaginous foci were of hyaline nature. They were composed of chondrocytes embedded in a type II collagen-positive matrix (Fig. 5 A) that stained metachromatically with haematoxylin. The loci displayed a thin perichondrium composed of a few collagen fibres that ran in a circumferential direction and contained a single layer of flattened cells (Fig. 5 B).

Finally, it should be mentioned that 90 (48.9%) of the 184 hamsters aged between 0 and 42 days, and all of the 167 hamsters aged 43 or older displayed cartilaginous tissue in the aortic valve.

Discussion

To the best of our knowledge, this is the first report to describe the presence of cartilage in pulmonary valves of mammals. This occurred in a relatively low percentage (11.4%) of the hearts examined. In 1938, Matumoto emphasized the absence of cartilage in a large series of specimens, belonging to 40mammalian species, a considerable number of which had cartilaginous deposits in the aortic valve and/or fibrous trigones. Since than, no other mention of the pulmonary valve has been made in papers reporting the presence of cardiac cartilage in mammals. Cartilaginous tissue has been observed in the pulmonary valves of 66 bird species (Stiefel 1926; Matumoto 1938; Tsusaki et al. 1956; López et al. 2000). However, this is not regarded to be a regular event, in contrast to the formation of cartilage in the aortic valve, which is indeed a common occurrence in birds.

Knowledge on the formation of cartilage in the mammalian heart is scarce (Sans-Coma et al. 1994). In the rat, the cartilaginous foci occurring in the aortic valve were thought to be related to cardiac aging (Wexler 1964), until Hollander (1968) showed their presence from the second week of life. Sans-Coma et al. (1994) detected no cartilage in the aortic valves of Syrian hamsters aged less than 40 days, using histological methods for light microscopy. Nonetheless, these authors could rule out an aging effect in the formation of cartilaginous tissue at this site, by comparing their findings in young and adult animals.

None of the 36 neonates reported herein displayed type II collagen in the pulmonary valve. This indicates that in the Syrian hamster, formation of cartilage in this valve starts after birth, and not during embryonic life as is the case in birds (López et al. 2000).

The earliest sign of chondrogenesis in the pulmonary valves of the Syrian hamster is the formation of small groups of cells embedded in a type II collagen-positive

extracellular matrix. Thereafter, such groups of cells increase moderately in size and differentiate into cartilaginous tissue of hyaline nature.

In the cardiac semilunar valves of chick and quail, the first evidence of cartilage development is the formation of the so-called prechondrogenic condensations (López et al. 2000). They consist of loosely packed mesenchymal cells embedded in a type II collagen-negative extracellular matrix, and can be well recognized in tissue sections using histological techniques for light microscopy. The synthesis of type II collagen starts in the central core of the condensations and gradually increases toward their periphery. We were unable to detect any prechondrogenic condensation in the pulmonary valves of the present Syrian hamsters. This might be due to the fact that in this rodent species, as in the mouse (Hurle et al. 1980; Colvée and Hurle 1981), the cardiac semilunar valves display a very cellular condition at birth, so that presence of type II collagen-negative condensations might be undiscernible at that time using conventional histological techniques. Another possibility is that in the pulmonary valve of the Syrian hamster, formation of cartilage does not involve the aggregation of a large number of cells prior to their differentiation into chondrocytes. Further studies are needed to elucidate this question.

Our findings indicate that in the Syrian hamster, chondrogenesis in the pulmonary valve can start from the first day after birth. Moreover, they suggest that most cartilaginous deposits begin to form within the first month of life, that is during the period in which the valve reaches its histological maturation. This suggestion relies on the following facts: (1) in the sample studied, the percentage (9.2%) of hamsters aged 42 days or younger, possessing cartilage in the pulmonary valve, did not significantly differ from that (13.8%) of the animals aged 43 days or older (χ^2-test, $p > 0.20$); and (2) most of the cartilaginous deposits detected in the pulmonary valves of this latter group of animals were more developed in size and displayed a greater degree of histological maturation.

The number of cartilaginous deposits observed in the present specimens was too small to apply any statistical analysis in order to seek any association between the occurrence of the cartilaginous tissue and its location in the pulmonary valve. Yet, the data given in Table 1 already indicate that the proximal attachments of the leaflets to their respective sinuses, and especially that of the ventral leaflet (region L), are the regions most prone to develop cartilaginous tissue. In this regard, it should be noted that none of the animals aged less than 10 days displayed a cartilaginous deposit in region L. However, this seems to be a mere effect of sampling.

The function of cartilaginous tissue in the cardiac semilunar valves of birds and mammals remains unclear; in fact, its presence is not a prerequisite for normal valve performance (Matumoto 1938; Kelsall and Visci 1970; Sans-Coma et al. 1994; López et al. 2000). However, it has been suggested that its formation may be the result of locally intense mechanical stimulation (Hueper 1939; Hollander 1968; Sans-Coma et al. 1994). The present findings cast doubt on this hypothesis in showing that the

cartilage usually forms early after birth, and not as a response to mechanical tensions that operate over a long period of time. In addition, the small size of the foci observed in the pulmonary valves of the adult hamsters rather indicate that the cartilage plays no substantial role in the performance of these valves.

The cartilage occurring in the aortic and pulmonary valves of birds is believed to differentiate from neural crest-derived cells (Sumida et al. 1989; Bachnou et al. 1996) of nonmuscular nature (López et al. 2000) which populate the cardiac outflow tract during its septation (Hiruma and Hirakow 1992; Yablonka-Reuveni et al. 1995, 1998; Bergwerff et al. 1996, 1998; Waldo et al. 1998, 1999). In the aortic (Kelsall and Visci 1970; Sans-Coma et al. 1994) and pulmonary valves (present data) of the Syrian hamster, the cartilaginous tissue forms at the same sites at which it develops in the cardiac semilunar valves of birds, namely along the attachments of the valve leaflets to their corresponding sinuses. Recent work has shown that in the mouse, neural crest cells are present in the presumptive fibrous skeleton of the cardiac semilunar valves during both the embryonic (Waldo et al. 1999; Jiang et al. 2000) and postnatal life (Jiang et al. 2000). From these observations it can be inferred that in mammals, the cartilaginous tissue located in both aortic and pulmonary valves probably originates from neural crest-derived cells. In this regard, it should be noted that these neural crest cells are regularly present where cartilaginous loci develop. However, as in birds (López et al. 2000), the reason why some of them differentiate into chondrocytes, while others do not, is still an open question.

Finally, another aspect which should be emphasized is that in both birds (Stiefel 1926; Matumoto 1938; Tsusaki et al. 1956; López et al. 2000) and mammals (present observations), formation of cartilage in the aortic and pulmonary valves are independent events. This fits in with the hypothesis of Fernández et al. (1998) that the morphogenesis of the cardiac semilunar valves may be mediated by specific subpopulations of cardiac neural crest cells, acting separately on the pulmonary and aortic sides of the embryonic cardiac outflow tract.

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Table 1. Cartilage in pulmonary valves of Syrian hamsters

Age in days	n	nC^+	%	A	B	C	D	E	F	G	H	I	J	K	L
0	36	0	0	0	0	0	0	0	0	0	0	0	0	0	0
1	29	2	6.9	0	0	0	0	0	0	0	0	0	0	2	0
2	35	7	20.0	1	0	0	3	0	0	0	0	0	1	2	0
3	28	5	17.9	1	0	0	2	0	0	0	0	0	1	1	0
4-9	14	2	14.3	0	0	0	1	0	0	0	0	0	0	1	0
10-42	42	1	2.4	0	0	0	0	0	0	0	0	0	0	0	1
>43	167	23	13.8	0	1	2	1	0	0	3	0	0	1	4	15
Total	351	40	11.4	2	1	2	7	0	0	3	0	0	3	10	16

Abbreviations: A-L = topographic regions of the pulmonary valve (see text for definitions); n = number of specimens examined; nC+ = number of specimens with cartilaginous deposits in the pulmonary valve.

Fig. 1. Location of the topographic regions A-L of the pulmonary valve. See the text for the definition of each region. VS = ventral pulmonary sinus; LS = left pulmonary sinus; RS = right pulmonary sinus.

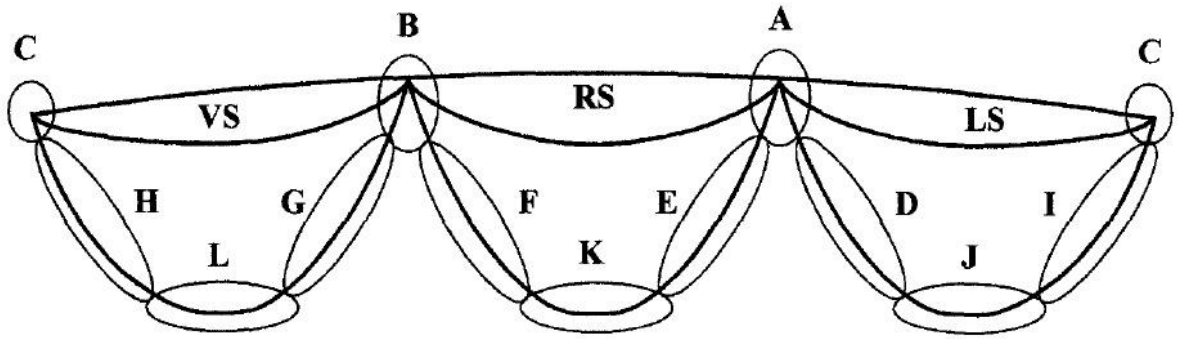

Fig. 2. Transverse section of the pulmonary valve of a Syrian hamster aged 1 day. Type II collagen immunostaining, counterstained with haematoxylin-eosin. The arrow points to a small spot of immunoreactivity that surrounds a single cell located at the proximal attachment of the right leaflet to its pulmonary sinus (topographic region K). Bar = 30 μm

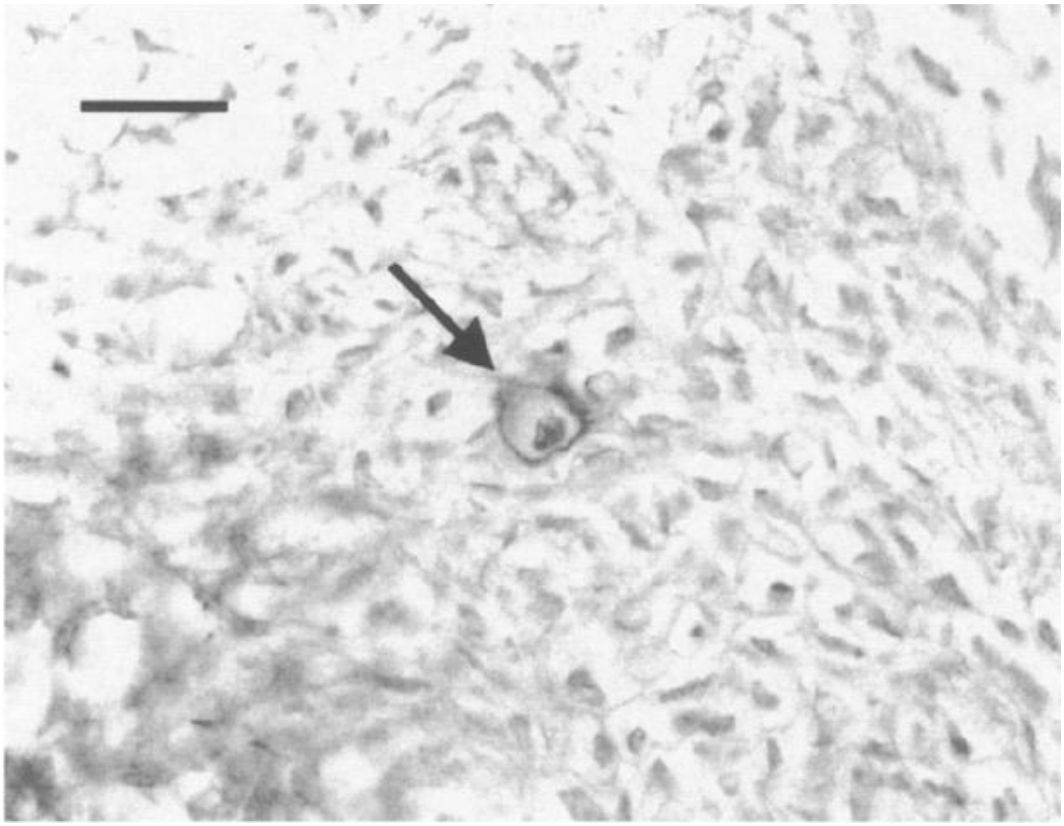

Fig. 3. Transverse section of the pulmonary valve of a Syrian hamster aged 3 days. Type II collagen immunostaining. A small group of cells embedded in a type II collagen-positive extracellular matrix (arrow) is located at the proximal attachment of the right leaflet to its pulmonary sinus (topographic region K). RS = right pulmonary sinus. Bar = 30 μm

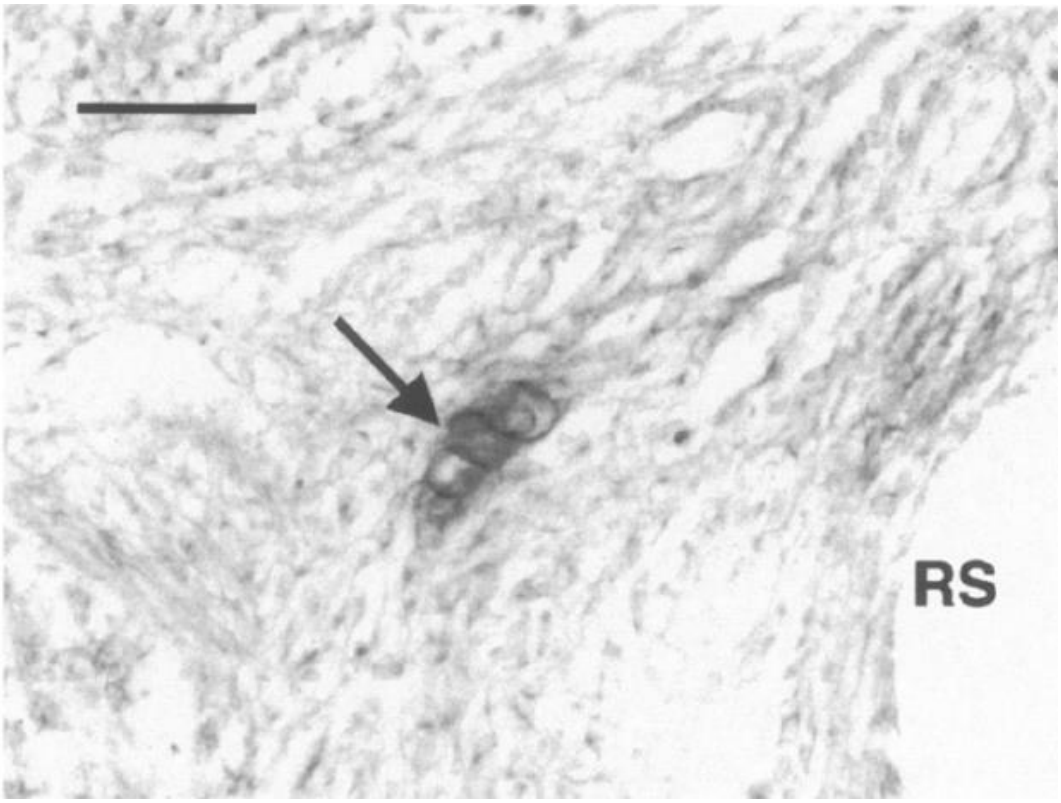

Fig. 4. Transverse section of the pulmonary valve of a Syrian hamster aged 17 days, stained with 0.05% alcian blue 8GX in 0.05 M acetate buffer (pH 5.8) plus 0.65 M magnesium chloride. A small cartilaginous deposit is located at the proximal attachment of the ventral leaflet (VL) to its pulmonary sinus (topographic region L). Bar = 25 μm

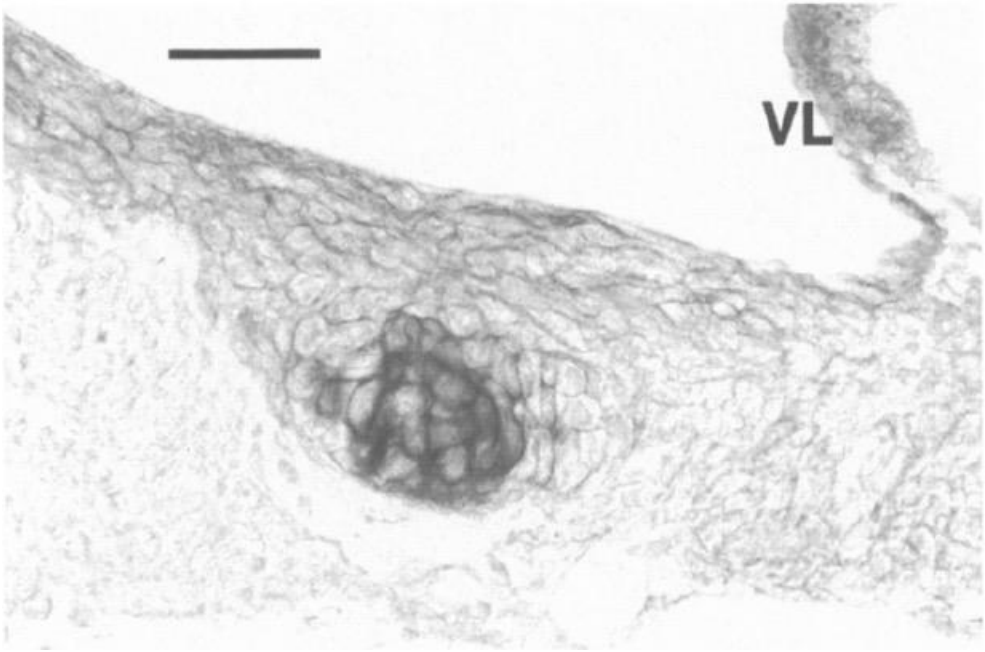

Fig. 5. Transverse sections of the pulmonary valve of a Syrian hamster aged 152days. A: Type II collagen immunostaining, counterstained with haematoxylin. B: Mallory's trichrome stain. A cartilaginous deposit is located at the proximal attachment of the ventral leaflet to its pulmonary sinus (topographic region L). Note the presence of a thin perichondrium in panel B. Bars = 20 μm