Formation of cartilage in cardiac semilunar valves of chick and quail

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Summary. The development of cartilage in the aortic and pulmonary valves of chick and quail was studied using histological, histochemical and immunohistochemical techniques. In both species, the earliest evidence of chondrogenesis is the formation of smooth muscle α-actin-negative prechondrogenic (type II collagen-negative) cellular condensations in the tunica media of the proximal aorta and pulmonary trunk, in front of or slightly distal to the valvular commissures. Such condensations are present as early as stage 37 of Hamburger-Hamilton in the aortic and pulmonary valves of the chick. In quail embryos, they form somewhat later, namely, at stage 38 in the aortic valves and stage 39 in the pulmonary valves. In the chick, synthesis of type II collagen starts in the central core of the aortic cellular condensations at stage 38. In the pulmonary valves of chick and aortic and pulmonary valves of quail, production of type II collagen does not begin until stage 40. This production then gradually increases toward the periphery of the condensations, which remain devoid of perichondrium prior to hatching. After birth, the condensations become transformed into hyaline cartilaginous foci. In the aortic valves of some chickens and quails, more or less extensive deposits of hyaline cartilage or fibrocartilage form along the attachments of the leaflets to their supporting sinuses. They develop later than the commissural cartilages. The present findings, together with previous data from the literature, suggest that the aortic and pulmonary valve cartilages differentiate from neural crest-derived nonmuscular cells.

Key words: Aortic valve – Pulmonary valve – Cartilage – Chick – Quail – Embryo

Introduction

Stiefel (1926) and Kern (1926) were the first authors who mentioned the occurrence of cartilaginous foci in the aortic and pulmonary valves of the chick. In addition, Stiefel (1926) described the main steps in the development of these foci by using histological techniques for light microscopy. Matumoto (1938) and Tsusaki et al. (1956) also obtained further data concerning the morphology and development of cardiac cartilage in birds by means of histological methods. More recently, Sumida et al. (1989) transplanted quail neural folds and neural crest between the level of the myelencephalon and the fourth somite into chick embryos. They demonstrated thereby that the cartilage tissue located between the aorta and pulmonary trunk differentiates from neural crest cells.

The aim of the present paper is to report the results of a comparative study of cartilage formation in the cardiac semilunar valves of the chick and quail which was carried out using histological, histochemical and immunohistochemical techniques.

Materials and methods

Animals. Fertilized chick and quail eggs were kept in a rocking incubator at 38°C. Thirty-nine chick embryos were killed between 8 and 21 days (stages of chick development 34–46 of Hamburger and Hamilton 1951), and 31 quail embryos between 8 and 16 days (stages 34–42). The number of embryos examined from each developmental stage is given in Table 1. In addition, the following specimens were studied: 6 chickens and 8 quails aged 1 day, 3 chickens and 4 quails aged 7 days, 28 adult chickens and 10 adult quails.

Thirty-nine chick embryos, 31 quail embryos, 9 young chickens, 6 young quails, 6 adult chickens, and 4 adult quails were examined by means of histological, histochemical and immunohisto-
Histological and histochemical techniques for light microscopy. Whole embryos or removed hearts were washed in Ringer’s solution and fixed by immersion in Bouin (ratio of fixative to tissue volume = 80:1), and embedded in Paraplast (Sigma Chemical Co., England). Serial sections, transversely, longitudinally, or sagittally cut at 10 μm, were stained with haematoxylin-eosin or Mallory’s trichrome stain for a general assessment of the histological components of the heart, and with resorcin-fuchsin for the detection of elastic fibres. Other procedures applied were picrosirius for the specific detection of collagen by polarization microscopy (Junqueira et al. 1979), and the differential staining of sulphated glycosaminoglycans with alcian blue (Scott and Dorling 1965).

Immunohistochemical techniques for light microscopy. Whole embryos or removed hearts were washed in Ringer’s solution and fixed by immersion in a mixture of methanol, acetone and distilled water (2:2:1) or in Bouin (ratio of fixative to tissue volume = 80:1). The specimens were embedded in Paraplast, and transversely, longitudinally, or sagittally cut at 10 μm. Sections were stained with monoclonal antibody CIIC1 (Developmental Studies Hybridoma Bank, University of Iowa) recognizing type II collagen, or with monoclonal anti-smooth muscle α-actin (α-SMα-actin; Sigma, clone 1A4).

The sections were dewaxed in xylene, dehydrated in an ethanol series, and washed in Tris-phosphate buffered saline (TBPS, pH 7.8). For the detection of type II collagen, the tissues were then digested for 30 min at 37 °C 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 with TBPS, nonspecific binding sites were saturated for 30 min with 10% sheep serum and 1% bovine serum albumin in TPBS (SB) for staining with CIIC1, or with the same solution plus 0.5% Triton X-100 (SBT) for staining with anti-α-SMα-actin. Sections were then incubated overnight at 4 °C in the primary antibody diluted in SB when staining with CIIC1 or in SBT when staining with anti-α-SMα-actin. Control slides were incubated in SB or SBT only.

After incubation, the sections were washed in TPBS (3×5 min), incubated for 1 h at room temperature in biotin conjugated anti-mouse IgG (Sigma) diluted 1:100 in TPBS, washed again, and incubated for 1 h in ExtrAvidin® conjugate (Sigma) diluted 1:150 in TPBS. 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 or haematoxylin-cosin.

Alcian blue in toto staining technique. The heart was removed, transferred to Ringer’s solution, and dissected to expose the cardiac semilunar valves. Removed valves were fixed by immersion in 5% trichloroacetic acid (ratio of fixative to tissue volume = 80:1) and stained with alcian blue 8 GX (Gurr), according to the technique described by Ojeda et al. (1970), then rinsed with 70% aqueous ethanol.

Type II collagen whole-mount immunostaining technique. Cardiac semilunar valves were transferred to Cornwall™ centrifuge tubes, and fixed by immersion in Bouin. The valves were then washed in TPBS and permeated for 15 min in acetone at -20 °C. After washing in TPBS, the specimens were immersed for 30 min in a 3% Triton X-100 solution 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 °C. 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.

Nomenclature. We use the terms proximal and distal to describe the location of the cardiac outflow tract components with regard to the ventricles.

Bearing in mind that chick and quail are commonly used in biomedical research, we employ the terms aortic and pulmonary valves to designate the valvular structures located at the base of the aorta and pulmonary trunk respectively, each of which is normally composed of three semilunar pocket valves. Therefore, as in humans (Angelini et al. 1989; McKay et al. 1992), both the aortic and pulmonary valves have to be regarded as complex structures made up of several components, namely the leaflets (or cusps), the sinuses, and the fibrous interleaflet triangles of the subaortic outflow tract. To prevent potential confusion, we will define these terms briefly.

The leaflets are the most mobile components of the valve that open and close during cardiac cycle. The sinuses are the supporting structures of the leaflets; they can be defined as the hollow portions of the aortic and pulmonary roots, the borders of which support the leaflets in a semilunar fashion. Each leaflet is attached laterally and proximally to its supporting sinus. The adjacent leaflets join distally along their attachments to the sinus wall. These junctions between leaflets are the commissures. The attachments of the leaflets diverge from the commissures toward the ventricle. As a result of this divergence, there is a triangular space between each two adjacent sinuses called the interleaflet triangle.

The sinuses and leaflets are named according to both their location in the heart and the normal origin of the coronary arteries. Hence, in the aortic valve there are a right, a left, and a dorsal sinus, each one supporting its own leaflet. The right and left aortic sinuses are the sites from which the right and left coronary arteries arise respectively. The dorsal aortic sinus is that located in the posterodorsal position with regard to the right and left aortic sinuses. The pulmonary sinuses and leaflets are termed right, left and ventral. The right and left pulmonary sinuses face the right and left aortic sinuses respectively. The ventral pulmonary sinus is placed anteroventrally with regard to the right and left pulmonary sinuses.

The commissures of the aortic valve are the ventral commissure, between the right and left sinuses, the dextrodorsal commissure, between the right and dorsal sinuses, and the sinistrodorsal commissure, between the left and dorsal sinuses. The commissures of the pulmonary valve are the dorsal commissure, between the right and left sinuses, the dextroventral commissure, between the right and ventral sinuses, and the sinistroventral commissure, between the left and ventral sinuses.

Results

Embryological findings. The first event in both the aortic and pulmonary valves which could be related to cartilage formation was the pre-
sence of cellular condensations in the tunica media of the proximal aorta and pulmonary trunk (Fig. 1A), one in front of or slightly distal to each valve commissure. We will refer to these condensations by using the term commissural condensations. They consisted of a limited number of loosely packed mesenchymal cells embedded in a type II collagen-negative extracellular matrix (Fig. 1A). The cells were SMα-actin-negative, whereas the surrounding medial tissue of the aorta and pulmonary trunk displayed an organization of smooth muscle α-actin-positive lamellar cells and smooth muscle α-actin-negative interlamellar cells (Fig. 1B).

Such commissural condensations were present in aortic and pulmonary valves of chick embryos as early as stage 37. In quail embryos, they appeared somewhat later, namely, at stage 38 in the aortic valve and stage 39 in the pulmonary valve.

The second event which could be related to chondrogenesis in the cardiac semilunar valves was the presence of cellular condensations in the pulmonary trunk of an HH 37 chick embryo. A. Type II collagen immunostaining, counterstained with haematoxylin-eosin. A type II collagen-negative cellular condensation (arrow) is located slightly distal to the dorsal pulmonary commissure. B. Smooth muscle α-actin immunostaining. The tissue of the pulmonary media displays an organization of smooth muscle α-actin-positive lamellar cells and smooth muscle α-actin-negative interlamellar cells. The location (arrowhead) of the cellular condensation shown in panel A can be well recognized because of its smooth muscle α-actin-negative nature. Ao = aorta. Bars = 100 μm

Fig. 1. Transverse sections of the pulmonary trunk of an HH 37 chick embryo. A. Type II collagen immunostaining, counterstained with haematoxylin-eosin. A type II collagen-negative cellular condensation (arrow) is located slightly distal to the dorsal pulmonary commissure. B. Smooth muscle α-actin immunostaining. The tissue of the pulmonary media displays an organization of smooth muscle α-actin-positive lamellar cells and smooth muscle α-actin-negative interlamellar cells. The location (arrowhead) of the cellular condensation shown in panel A can be well recognized because of its smooth muscle α-actin-negative nature. Ao = aorta. Bars = 100 μm

Fig. 2. Transverse section of the aorta of an HH 39 chick embryo immunostained with an antibody against type II collagen and counterstained with haematoxylin. A cellular condensation is present in the aortic media, slightly distal to the sinistrodorsal aortic commissure. The arrowhead points to a small spot of immunoreactivity located in the central core of the condensation. The asterisk indicates the aortic adventitia. Bar = 50 μm
Fig. 3. Oblique longitudinal sections of the pulmonary trunk of an HH 41 chick embryo. A: Smooth muscle α-actin immunostaining. B: Type II collagen immunostaining, counterstained with haematoxylin. The smooth muscle α-actin-negative region indicated by the arrow in panel A corresponds to the type II collagen-positive cellular condensation delimited by the arrowheads in panel B. Type II collagen is expressed throughout the whole cellular condensation, which is located in front of the dorsal pulmonary commissure (asterisk). Ao = aorta. Bars: A = 200 µm; B = 50 µm

Fig. 4. Transverse section at the level of the cardiac outflow tract of an HH 44 chick embryo. Type II collagen immunostaining, counterstained with haematoxylin. The aortic valve is sectioned obliquely. A group of cells embedded in a type II collagen-positive extracellular matrix (arrow) is located in the proximal part of the wall of the left aortic sinus. The asterisk indicates a type II collagen positive cellular condensation placed in front of the dextrodorsal aortic commissure; the arrowhead points to a similar condensation located in front of the ventral aortic commissure. LD = dorsal aortic valve leaflet; LL = left aortic valve leaflet; LR = right aortic valve leaflet; P = pulmonary trunk; SD = wall of the dorsal aortic sinus; SL = wall of the left aortic sinus; SR = wall of the right aortic sinus. Bar = 200 µm

Fig. 5. Transverse section of the aorta of a chick aged 7 days, stained with 0.05% alcian blue 8GX in 0.05 M acetate buffer (pH 5.8) plus 0.65 M magnesium chloride. A cartilaginous deposit is located in front of the ventral aortic commissure. Note the high amount of sulphated glycosaminoglycans in the cartilaginous tissue. Bar = 100 µm
Table 1. Commissural cell condensations in cardiac semilunar valves of chick and quail embryos

| HH | n  | Chick Aortic valve | | | | | | Pulmonary valve | | | | | | | | | | |
|----|----|--------------------|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
|    |    | nC⁻ | nC⁺ | V | DD | SD | nC⁻ | nC⁺ | D | DD | DV | SV |
| 34 | 2  | 0   | 0   | 0   | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 36 | 4  | 0   | 0   | 0   | 0 | 1 | 0 | 3 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 37 | 4  | 3   | 0   | 2   | 0 | 1 | 0 | 3 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 38 | 6  | 4   | 1   | 2   | 0 | 1 | 2 | 0 | 4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 39 | 4  | 4   | 2   | 2   | 3 | 1 | 1 | 2 | 3 | 2 | 3 | 2 | 3 | 2 | 3 | 2 | 3 | 2 |
| 40 | 5  | 5   | 0   | 5   | 3 | 2 | 3 | 2 | 3 | 2 | 3 | 2 | 3 | 2 | 3 | 2 | 3 | 2 |
| 41 | 5  | 4   | 4   | 2   | 3 | 4 | 1 | 3 | 2 | 3 | 2 | 3 | 2 | 3 | 2 | 3 | 2 | 3 |
| 42 | 4  | 4   | 3   | 1   | 3 | 2 | 2 | 1 | 3 | 0 | 3 | 0 | 2 | 0 | 2 | 0 | 2 | 0 |
| 43-46 | 5  | 5 | 5 | 0 | 5 | 0 | 5 | 0 | 5 | 0 | 5 | 0 | 5 | 0 | 5 | 0 | 5 | 0 |

| Quail Aortic valve | | | | | | | | Pulmonary valve | | | | | | | | | | |
| HH | n  | nC⁻ | nC⁺ | V | DD | SD | nC⁻ | nC⁺ | D | DD | DV | SV |
| 34 | 3  | 0   | 0   | 0   | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 36 | 4  | 0   | 0   | 0   | 0 | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 37 | 5  | 1   | 0   | 1   | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 38 | 4  | 2   | 0   | 0   | 0 | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 39 | 5  | 3   | 1   | 1   | 1 | 1 | 1 | 2 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 40 | 2  | 2   | 1   | 2   | 0 | 2 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 41 | 3  | 3   | 0   | 3   | 1 | 2 | 1 | 2 | 1 | 2 | 1 | 2 | 1 | 2 | 1 | 2 | 1 | 2 |
| 42 | 3  | 3   | 0   | 3   | 1 | 2 | 1 | 2 | 1 | 2 | 1 | 2 | 1 | 2 | 1 | 2 | 1 | 2 |

Abbreviations: HH = developmental stages of Hamburger and Hamilton (1951); D = dorsal commissure; DD = dextrodorsal commissure; DV = dextroventral commissure; SD = sinistrodorsal commissure; SV = sinistroventral commissure; V = ventral commissure; n = number of specimens examined; nC⁻ = number of specimens with type II collagen-negative commissural cell condensations; nC⁺ = number of specimens with type II collagen-positive commissural cell condensations; C⁻ = number of type II collagen-negative commissural cell condensations; C⁺ = number of type II collagen-positive commissural cell condensations.

of type II collagen in the central core of the commissural condensations (Fig. 2). This was observed at the ventral aortic commissure of a chick embryo as early as stage 38. Type II collagen was not detected until stage 40 in the commissural condensations of both the pulmonary valve of the chick and aortic and pulmonary valves of the quail.

Thereafter, the type II collagen-positive condensations gradually increased in size. The cells became rounded and production of type II collagen proceeded toward the periphery of the condensations (Figs. 3 A, B).

The embryonic developmental stage at which type II collagen-negative condensations appeared at the valvular commissures and the stage at which synthesis of type II collagen started in each condensation varied from specimen to specimen. Type II collagen-negative and positive commissural condensations often coexisted in the aortic and pulmonary valves of an individual. Table 1 shows the distribution of both types of condensations in chick and quail embryos, according to the developmental stages and aortic and pulmonary commissures.

A group of rounded cells, embedded in a type II collagen-positive extracellular matrix, occurred in the wall of the left aortic sinus of an HH 42 chick embryo. The cells were located in the proximal portion of the attachment of the leaflet to the sinus. Similar type II collagen-positive groups of cells were seen in the proximal portions of 2 right aortic sinuses and 3 left aortic sinuses of HH 43-46 chick embryos (Fig. 4).

Another HH 44 chick embryo displayed 2 type II collagen-positive noncommissural cell condensations. One of them extended along the proximal and left portions of the attachment of the right aortic leaflet to its sinus; the other occupied the proximal and right portions of the attachment of the left aortic leaflet to its sinus. Both condensations converged at the level of the ventral aortic commissure, where a type II collagen-positive commissural condensation existed.

**Cartilage in the cardiac semilunar valves of young animals**

In all specimens aged 1 day (6 chickens, 8 quails) there was a cellular condensation in front of each aortic and pulmonary commissure. Tissue sections revealed that the condensations were type II collagen-positive and devoid of perichondrium.

In the 3 chickens and 4 quails aged 7 days, a type II col-
lagen-positive cellular condensation was present in front of each aortic commissure. All of these condensations exhibited a considerable amount of glycosaminoglycans (Fig. 5), and in most cases there was a developing perichondrium (Fig. 6): 5 of 9 chick condensations and 10 of 12 quail condensations. A cellular condensation also occurred in front of each pulmonary commissure of these chickens and quails. Four of the 9 chick pulmonary condensations were type II collagen-positive, but none of them displayed a perichondrium. In contrast, all quail pulmonary condensations (n = 12) were type II collagen-positive, and 2 of them showed a developing perichondrium.

Two type II collagen-positive, noncommissural groups of cells were present in the aortic valve of a chick aged 1 day. The cells were located in the proximal portions of the attachments of the right and left leaflets to their supporting sinuses. Similar cellular groups were present in the proximal attachments of the right, left, and dorsal aortic leaflets of 2 chickens aged 7 days, and in the proximal attachment of the dorsal aortic leaflet of a quail aged 7 days.

**Cartilage in the cardiac semilunar valves of adult animals**

In all the adult chickens and quails, a cartilaginous focus was located in front of each aortic and pulmonary commissure (Fig. 7). Tissue sections revealed that the foci were of hyaline nature. They were surrounded by a more or less developed perichondrium, composed of collagen fibres that ran in a circumferential direction and contained two or three layers of flattened cells (Fig. 8).

The foci located in front of the aortic commissures were usually ellipsoidal. In some cases, however, they displayed an inverted-Y shape. The perichondrium was incomplete. It partially surrounded the focus and was lacking at its proximal part, where the cartilaginous tissue gradually merged with the fibrous tissue of the adjacent interleaflet triangle. Around the perichondrium there was a collagenous capsule to which collagen fibres from the aortic media were attached.

The pulmonary commissural foci were ellipsoidal and displayed a less developed perichondrium, surrounded by a collagenous capsule. Collagen fibres from the pulmonary media and the adjacent interleaflet triangle were attached to each capsule (Fig. 9).

In 20 (71%) of the 28 adult chickens and in 4 (40%) of the 10 adult quails, deposits of cartilaginous tissue were present along the attachments of the aortic leaflets to their supporting sinuses. Presence of such deposits according to each aortic sinus is given in Table 2. Histologically, two types of deposits could be recognized; namely, hyaline cartilages and fibrocartilages. The hyaline cartilages occurred as small foci located near the commissural cartilages; they were composed of a few chondrocytes embedded in an homogeneous extracellular matrix that was surrounded by a thin, often incomplete perichondrium. The fibrocartilages consisted of a few rows of cells embedded in a type II collagen-positive matrix (Fig. 10A) and were contained within a meshwork of thick collagen-
Fig. 9. Transverse section of a cartilaginous focus located in front of the sinistroventral pulmonary commissure of an adult quail. The focus is surrounded by a collagenous capsule to which collagen fibres from the pulmonary media are attached. Picrosirius staining and polarization microscopy. Bar = 100 μm

Table 2. Deposits of hyaline cartilage or fibrocartilage along the attachments of the aortic valve leaflets to their supporting sinuses in adult chickens and quails

<table>
<thead>
<tr>
<th>Aortic sinuses</th>
<th>Chick (28) n</th>
<th>Quail (10) n</th>
</tr>
</thead>
<tbody>
<tr>
<td>R + L + D</td>
<td>6</td>
<td>1</td>
</tr>
<tr>
<td>R + L</td>
<td>2</td>
<td>1</td>
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<tr>
<td>R + D</td>
<td>5</td>
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<td>L + D</td>
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<td>tn</td>
<td>20</td>
<td>4</td>
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Abbreviations: D = dorsal aortic sinus; L = left aortic sinus; R = right aortic sinus; n = number of specimens with cartilaginous deposits; tn = total number of specimens with cartilaginous deposits; in parenthesis = total number of specimens examined.

Fig. 10. Transverse sections of the aorta of an adult chick. A: Type II collagen immunostaining, counterstained with haematoxylin. B: Resorcin-fuchsin stain. A fibrocartilage is present in the proximal part of the wall of the dorsal aortic sinus, close to the attachment of the corresponding valve leaflet. The cartilaginous tissue consists of a few rows of chondrocytes embedded in a type II collagen-positive matrix (panel A) and contained within a meshwork of thick collagenous fibres and a few elastic fibres (panel B). M = myocardium. Bars = 100 μm

Discussion

Formation of cartilage in the chick. Our findings indicate that the earliest evidence of chondrogenesis in the cardiac semilunar valves of chick embryos is the presence of SMα-actin-negative prechondrogenic (type II collagen-negative) condensations in the tunica media of the aorta and
pulmonary trunk, in front of or slightly distal to the valve commissures. These cellular aggregations increase in size, while cell differentiation into chondrocytes begins at their central core. This is well-documented by the synthesis of type II collagen at this site, an event which is considered cartilage-characteristic (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 nonchondrogenic cell types (see Kosher 1983 and Swiderski et al. 1994 for extensive reviews of the literature). In the chick, for example, type II collagen is transiently expressed during cardiac morphogenesis in the interface between the epimyocardium and endocardium at stage 11 (Kosher and Solursh 1989), and in the endocardial cushion cells which populate the developing valve regions at stage 18 (Swiderski et al. 1994).

In the commissural condensations, cell differentiation into chondrocytes seems to proceed from the central core of the condensation to its periphery. This suggestion relies on the fact that, in the specimens examined, expression of type II collagen increased radially in successive developmental stages. The commissural condensations remain devoid of perichondrium prior to birth.

Stiefel (1926) described a possible precartilaginous cellular aggregation in the zone of attachment of two cardiac semilunar valves to the aortic root in an embryo aged 8 days (stage 34). However, at this developmental stage, we were unable to identify any mesenchymal condensation at the level of the cardiac semilunar valves other than that of the aortopulmonary septum. Moreover, Stiefel (1926) reported the presence of a group of rounded cells, embedded in an amorphous extracellular matrix, in the attachment of one of the cardiac semilunar valves to the aorta of a chick embryo aged 10 (stage 36). According to this author, the arrangement of these cells was similar to that of the chondroblasts in the developing ribs. On this basis, he concluded that there are evident signs of cardiac cartilaginous tissue in chick embryos aged 10 days. Our observations do not support this statement; indeed, they indicate that the prechondrogenic condensations, devoid of type II collagen, are not discernible until stage 37 (11 days).

Matumoto (1938) stated that cartilaginous tissue does not appear in the aortic valves of the chick until embryonic day 14 (stage 40), whereas Tsusaki et al. (1956) reported its presence from embryonic day 15 (stage 41). Our observations indicate that synthesis of type II collagen starts earlier in the aortic commissural condensations, namely at stage 38. In the aortic valves of embryos belonging to stages 40 and 41 there were several well-developed commissural condensations, expressing type II collagen from their central region as far as their periphery. In most of these specimens, the type II collagen-positive condensations coexisted with prechondrogenic commissural condensations.

There is a significant disagreement between authors when deciding the time at which commissural cartilage forms in the pulmonary valves. Tsusaki et al. (1956) reported that this occurs from embryonic day 17 (stage 43). Stiefel (1926) considered that the pulmonary cartilage develops just before hatching, whereas Matumoto (1938) claimed that it appears after birth. Our findings contradict these statements. Indeed, they demonstrate that prechondrogenic commissural condensations are already present in the pulmonary valves of HH 37 embryos, and that production of type II collagen begins at stage 40.

Our findings agree with those of Tsusaki et al. (1956) in indicating that more or less extensive deposits of cartilage form along the attachments of the aortic leaflets to their supporting sinuses in some individuals. These deposits develop later than the commissural cartilages. Tsusaki et al. (1956) found them at stages 45 and 46. In the present specimens, we observed such noncommisural deposits earlier, namely, from stage 42. In this context, it should be noted that we detected no sign of cartilaginous tissue along the attachments of the pulmonary leaflets to their corresponding sinuses.

**Formation of cartilage in the quail.** To our knowledge, the present report is the first to describe the formation of cartilage in the cardiac semilunar valves of the quail. At the level of the valvular commissures, this morphogenetic process is similar to that which takes place in the chick. However, there are some differences concerning the timing of events.

The prechondrogenic condensations form later in the quail than in the chick. They do not appear until stage 38 in the aortic valves and stage 39 in the pulmonary valves. Synthesis of type II collagen also begins later in the quail; namely, at stage 40 in both the aortic and pulmonary valves.

In HH42 quail embryos, there are both prechondrogenic and type II collagen-positive commissural condensations in the aortic valves just before birth. In contrast, all aortic commissural condensations express type II collagen when hatching takes place in the chick, at stage 46 (embryonic day 21).

In the present quail embryos there was no evidence of cartilaginous deposits along the attachments of the aortic and pulmonary valve leaflets to their corresponding sinuses.

**Cartilage in young and adult chickens and quails.** In both the chick and quail, the aortic commissural condensations are type II collagen-positive 1 day after birth. From that date, they become gradually converted into hyaline cartilage. A developing perichondrium can be already seen 1 week after birth.

The transformation of the commissural condensations into hyaline cartilaginous foci takes place later in the pulmonary than in the aortic valves. This statement relies on two facts: (1) there were prechondrogenic commissural condensations in the pulmonary valves of several chicks aged 1 week, and (2) most type II collagen-positive pulmonary commissural condensations displayed no developing perichondrium at this age.

Our findings in adult chickens and quails agree with those of Stiefel (1926), Matumoto (1938), and Tsusaki et al. (1956) in indicating that more or less extensive deposits of cartilage form along the attachments of the aortic leaflets to their supporting sinuses in some individuals. These deposits develop later than the commissural cartilages. Tsusaki et al. (1956) found them at stages 45 and 46. In the present specimens, we observed such noncommisural deposits earlier, namely, from stage 42. In this context, it should be noted that we detected no sign of cartilaginous tissue along the attachments of the pulmonary leaflets to their corresponding sinuses.
al. (1956) in suggesting that in both species, presence of a hyaline cartilaginous focus at the level of each aortic valvular commissure can be regarded as a common event. In contrast, occurrence of commissural cartilaginous foci in the pulmonary valves seems to be not such a regular episode. We did indeed find a hyaline cartilaginous focus at the level of each pulmonary commissure in all of the 28 adult chickens and 10 adult quails, but Stiefel (1926) reported their presence in only 2 of 7 adult chickens and Matumoto (1938) detected no pulmonary commissural focus in 2 adult chickens and only 2 pulmonary commissural foci in a single adult quail.

According to Tsusaki et al. (1956), the cartilaginous deposits located in the attachments of the aortic leaflets to their sinuses disappear shortly after their formation. Our observations disagree with this statement. We found this kind of cartilages in 71% of the adult chickens and 40% of the adult quails; hence, their presence in the aortic valves cannot be viewed as an uncommon event. In the present specimens, several of these deposits were of fibrocartilage. This should be stressed, since both Matumoto (1938) and Tsusaki et al. (1956) stated that the cardiac cartilaginous foci in birds are exclusively of a hyaline nature.

**Origin and significance of the valvular cartilaginous deposits.** In quail-chick chimeras embryos HH 45, Sumida et al. (1989) found a cluster of neural crest-derived cells between the proximal aorta and the pulmonary trunk which had differentiated into cartilage and surrounding connective tissue. This finding is relevant because it reveals the identity of potential precursors of chondroblasts in the cardiac outflow tracts. However, it does not explain the usual distribution of the cartilaginous foci around the cardiac semilunar valves. Actually, Sumida et al. (1989) detected the neural crest-derived cartilaginous cells approximately at the level of the ventral aortic and dorsal pulmonary commissures, but not at the level of the remaining commissures. However, we observed type II collagen-positive condensations at all aortic commissures and prechondrogenic condensations or type II collagen-positive condensations at most pulmonary commissures of HH 43–46 chick embryos.

In the chick embryos, the earliest commissural prechondrogenic condensations were seen at stage 37. As in quails, such condensations could be unequivocally distinguished by their SMα-actin-negative condition. They stood out against the surrounding medial arterial tissue which, in HH 37 chick embryos, has fully acquired its organization of SMα-actin-positive lamellar cells and SMα-actin-negative interlamellar cells (Yablonka-Reuveni et al. 1995, 1998; Bergwerff et al. 1996, 1998). This strongly suggests that the commissural prechondrogenic condensations consist of interlamellar cells, which are also known as nonmuscular cells because of the lack of most of the characteristics used to describe the smooth muscle cells (Hughes 1943; Hiruma and Hirakow 1992).

In the aortic and pulmonary trunks of HH 35 chick embryos and older specimens, the lamellar and interlamellar cells located downstream from the distal level of the aortic and pulmonary sinuses are of neural crest origin (Yablonka-Reuveni et al. 1995, 1998; Bergwerff et al. 1998). In contrast, from this topographical level upstream, mesodermal cells clearly outnumber neural crest cells in both arterial roots; the walls of the aortic and pulmonary sinuses consist of mesodermal cells (Bergwerff et al. 1998; Waldo et al. 1998). The commissural prechondrogenic condensations develop at the boundary between these two regions, so that the possibility of a mesodermal origin of chondrocytes cannot be ruled out conclusively. Nonetheless, the finding of Sumida et al. (1989) concerning the differentiation of cartilage from neural crest cells, together with similar experimental results reported by Bachnou et al. (1996), suggest that the nonmuscular interlamellar cells that we believe to be precursors of chondroblasts are indeed neural crest-derived. The deposits of hyaline cartilage or fibrocartilage that form along the attachments of the leaflets to their supporting sinuses in the aortic valves of the chick are probably of neural crest origin as well. This assumption relies on the following two observations indicating that cells from the neural crest are actually present where noncommissural cartilages develop: (1) Takamura et al. (1990) found neural crest cells in all aortic valve cushions of quail-chick chimeras at embryonic day 10 (stage 36), and (2) Poelmann et al. (1998) detected neural crest cells in the bases of some of the semilunar valve leaflets of the chick at stages 38–40, when using both quail-chick chimeras and retroviral infection.

The functional significance of cartilaginous deposits in the cardiac semilunar valves is still an open question. The following facts suggest that valvular cartilage might form as a response to mechanical stimulation: (1) in the adult chickens and quails, the deposits are located in the sinus boundaries of the valves, that is at the sites to which the large stresses, generated in the leaflets during the cardiac cycle, are distributed (Broom 1988), and (2) the topographic distribution of the cartilaginous tissue is very similar to that of tenascin during valvular morphogenesis (García Martínez et al. 1990), and the distribution of tenascin during valvulogenesis is associated with zones specialized in bearing mechanical loads (García Martínez et al. 1990). However, it is well known that presence of valvular cartilage is not a requisite for normal function of the cardiac semilunar valves in birds (Matumoto 1938; Tsusaki et al. 1956). This is also the case in reptiles (Matumoto 1938; Kashyap 1959; Young 1994) and mammals (Matumoto 1938; Huerper 1939; Hollander 1968; Wexler 1964; Kelsall and Vischi 1970; Sans-Coma et al. 1994), where the incidence of cartilage in aortic and pulmonary valves varies widely between species and between members of the same species.

The presumed precursors of chondrocytes, i.e., interlamellar neural crest-derived cells are regularly present where semilunar valve cartilages develop, at least in birds. However, the reason why some of these precursors differentiate into chondrocytes, while others do not, remains...
unknown. Chondrogenesis is a complex morphogenetic process, in which multiple factors such as the expression of specific genes, cell interactions, and synthesis of specific gene products are involved (Hall and Newman 1991; Adolphe M 1992; Hall and Miyake 1992, 1995; De Crombrugge 1996; Lefebvre et al. 1998). Knowledge about the morphogenesis of skeletal cartilage has strikingly increased in the last three decades. In contrast, information on the developmental biology of spontaneous extraskeletal cartilages, and especially cardiac cartilages is still scarce. Hence, the factor or factors that may induce or hinder the formation of cartilage in the cardiac semilunar valves remain unknown. So far, occurrence of cartilaginous tissue in these valves can only be regarded as an outcome of the chondrogenetic potential of specific cells existing in the embryonic cardiac outflow tract (see Young, 1994); yet, the expression of this potential is subject to wide individual variation.

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