Primary and secondary cartilages of the neonatal rat: the femoral head and the mandibular condyle


Primary and secondary cartilages differ in embryonic origin and in histological organization, and are generally considered to have a different mode of growth. However, few studies have directly compared the two types of cartilage of the same animal at the same age. Therefore, we analysed several histological and biochemical differences between secondary cartilage of the mandibular condyle and primary cartilage of the femoral head of 4-d-old rats. We evaluated the tissue organization, the level of DNA and glycosaminoglycan (GAG) synthesis, and the GAG and collagen content. The expression of collagen types I, II and III and of receptors for insulin-like growth factor (IGF)-I, fibroblast growth factor (FGF), and transforming growth factor (TGF)-β were investigated by immunohistochemistry. The ex vivo DNA and GAG synthesis as well as the GAG content of femoral heads were much higher than that of mandibular condyles. Mandibular condyles expressed both collagen types I and II, while femoral heads expressed only type II collagen. In the mandibular condyles, receptors for IGF-I, FGF, and TGF-β were observed mainly in the superficial layers, whereas they were found throughout the entire femoral head. In conclusion, major differences were found between both types of cartilage, which might be related to their specific functional demands.

The mandibular condyle is a major growth site and plays a significant role during mandibular development. Cartilage of the mandibular condyle provides regional adaptive growth, endochondral bone growth and a movable articulation (1). This type of cartilage, often referred to as secondary, is thought to differ from the major type of cartilage, usually described as primary (2). Primary cartilages are considered to be part of the primary cartilaginous skeleton, which acts as a scaffold for the ossification process. In the craniofacial region, the basicranial synchondroses and the cartilages in the nasal and otic capsules represent primary cartilages (3). Secondary cartilages appear later in the embryonic development and are independent of the chondroskeleton (4). They appear at the margins of membranous bones during prenatal and early postnatal development, and are generally transient in nature (2). In contrast, the secondary cartilage of the mandibular condyle develops into permanent articular cartilage.

The two types of cartilage show differences in histological organization and in the pattern of cell proliferation. In primary cartilage, interstitial cell proliferation occurs in chondrocytes, while in secondary cartilage undifferentiated prechondroblasts show appositional proliferation (5). However, it was recently shown that a portion of the proliferative cells in the mandibular condyle of young rats are chondroblasts (6). Further, secondary cartilages contain not only type II collagen, which is characteristic for all cartilages, but also type I collagen which is absent from all primary cartilages (7).

The regulation of growth of primary and secondary cartilages by growth factors may also be different. Growth factors such as insulin-like growth factor (IGF)-I, transforming growth factor (TGF)-β1, and fibroblast growth factor (FGF)-2 are all expressed within the epiphysial growth plate, where they regulate chondrocyte proliferation, matrix synthesis, and terminal differentiation (8). More recently, growth of the mandibular condyles was also reported to be regulated by these growth factors (9–11). Furthermore, primary and secondary cartilages were recently shown to differ in the synthesis of some growth factors (12, 13), and in the expression of their receptors (14).

In summary, many reports describe the properties of primary or secondary cartilages from all kinds of animals at different ages under varying experimental conditions. However, none have directly compared several characteristics of the mandibular condyle and the femoral head of the same animal at the same age. The aim of our study, therefore, was to analyse several histological and biochemical differences between secondary cartilage from the mandibular condyle and primary cartilage from the femoral head of 4-d-old rats. We investigated the following parameters: the localization and rate of DNA
and glycosaminoglycan (GAG) synthesis, the GAG and collagen content, as well as the distribution of collagen types I, II and III. Furthermore, the expression of receptors for IGF-I, FGF, and TGF-β was investigated.

Material and methods

Explants

Eighteen 4-d-old Wistar rats (Harlan, Zeist, The Netherlands) were decapitated and the cartilage explants (36 mandibular condyles and 36 proximal femoral heads) were dissected under stereomicroscopic observation. The adhering soft tissue and muscles were removed. The experiments were approved by the Board for Animal Experiments of the University Medical Center Nijmegen. During dissection, the tissue was bathed in IMDM medium (Life Technologies, Breda, The Netherlands) supplemented with 100 U ml⁻¹ penicillin and 100 µg ml⁻¹ streptomycin (Life Technologies). The mandibular condyles and the femoral heads were cut to a length of approximately 1.5 mm. Mandibular condyles contained the cartilaginous tissue of the organ and a small amount of calcified cartilage. Femoral heads contained the cartilaginous tissue of the proximal head and a small part of the epiphyseal growth plate.

In total, 40 explants (mandibular condyles plus femoral heads) were processed for histology, autoradiography and biochemical analyses, and 32 explants were used for enzyme histochemistry and immunohistochemistry. Half of the latter group was embedded in paraffin for the immunohistochemistry and immunohistochemistry. Half of the latter group was embedded in paraffin for the immunohistochemistry of collagen types I and III, and of receptors for IGF-I, FGFs, and TGF/β. The other half was used to make frozen sections to examine the expression of collagen type II and alkaline phosphatase.

Ex vivo labeling

Mandibular condyles and femoral heads were placed in a 24-well culture plate (Nunc, Roskilde, Denmark) in 0.5 ml incubation medium per well. The incubation medium consisted of IMDM supplemented with penicillin (100 U ml⁻¹) and streptomycin (100 µg ml⁻¹), ascorbic acid (150 µg ml⁻¹; Sigma, St Louis, MO, USA), β-glycerophosphate (5 mM, Sigma) and 0.1% bovine serum albumin (BSA; Sigma). To analyse the GAG synthesis of both cartilages, 10 mandibular condyles and 10 femoral heads were labeled with 10 µCi/ml [³⁵S]sulfate (Amersham, Little Chalfont, UK) for 17 h at 37°C in a humidified incubator in an atmosphere of 5% CO₂ in air. Two other groups were labeled with 5 µCi/ml [methyl-³¹H]thymidine (Amersham) to analyze DNA synthesis. Seven explants of each group were harvested for biochemical analyses (GAG content, hydroxyproline (Hyp) content and either GAG or DNA synthesis) and their wet weight was determined. Three explants of each group were processed for histology and autoradiography.

Histology and autoradiography

Explants were fixed in 4% neutral formaldehyde solution, dehydrated and subsequently embedded in paraffin. Serial sections of 7 µm were cut in the sagittal plane of the mandibular condyles and along the long axis of the femoral heads. Central sections were stained with hematoxylin and eosin, and the von Kossa method to detect calcification sites. To visualize cell proliferation and GAG production, sections of explants labeled with [methyl-³¹H]thymidine or with [³⁵S]sulfate were processed for autoradiography. Briefly, sections were coated with Ilford Nuclear Emulsion K5 (Ilford Scientific Product, Mobberley, UK), stored at 4°C, developed, and stained with hematoxylin and eosin.

Immunohistochemistry

Paraffin sections were collected on superfrost plus slides (Menzel-Gläser, Brauwischweig, Germany), deparaffinized and rehydrated. Before staining, the slides were rinsed in phosphate-buffered saline (PBS) for 10 min.

Collagen type I staining: Sections were subsequently pretreated with 1 mg ml⁻¹ hyaluronidase and 1 mg ml⁻¹ trypsin in PBS for 5 min at 37°C. After washing in PBS, they were treated with 3% H₂O₂ in PBS for 10 min to block endogenous peroxidase activity and rinsed in PBS. The sections were subsequently preincubated with 5% BSA in PBS. After pretreatment, the sections were incubated with goat anti-type I collagen (Harlan Sera-Laboratory, Belton, UK) 1: 80 for 90 min. After washing with PBS, the sections were incubated with rabbit anti-goat peroxidase (DAKO, Glostrup, Denmark) 1: 50 for 45 min. Peroxidase activity was visualized by incubation with Sigma Fast (Sigma). The staining was enhanced with 0.5% CuSO₄ in a 0.9% NaCl solution for 5 min. The sections were counterstained with hematoxylin.

Collagen type III staining: The pretreatment of the sections was similar as for collagen type I. After pretreatment, the sections were incubated with rabbit anti-type III collagen (Chemicon, Temecula, CA, USA) 1: 5000 for 45 min. After washing with PBS, the sections were incubated with biotinylated goat antirabbit (Chemicon) 1: 400 for 45 min. After washing with PBS, the sections were treated with ABC-peroxidase (Vector Laboratory, Burlingame, CA, USA). Peroxidase activity was visualized as for collagen type I.

Growth factor receptors: Sections were pretreated as described for collagen type I. After pretreatment, sections were incubated either with rabbit anti-IGF-I receptor (IGF-IR) (H-60), anti-β subunit; Santa Cruz Biotechnology, Santa Cruz, CA, USA), or with rabbit anti-FGF receptor 2 (Bek (C-17); Santa Cruz Biotechnology) or with rabbit anti-TGF-β receptor II (TGF-β RII (L-21); Santa Cruz Biotechnology) overnight at dilutions of 1: 100, 1: 200 and 1: 100, respectively. Afterwards, sections were incubated with biotinylated goat antirabbit (Chemicon) 1: 400 for 45 min, and were finally incubated with ABC-peroxidase. The visualization was the same as that described for collagen type I.

Collagen type II staining: This staining was performed on frozen sections. Explants were decalcified in ethylenediaminetetraacetic acid (EDTA) for 2 d at 4°C, snap-frozen in liquid nitrogen, and stored at −80°C. Serial sections of 7 µm were cut. The pretreatment of the sections was the same as for collagen type I, only the trypsin treatment was omitted. Next, sections were incubated with mouse antitype II collagen (DSHB, Department of Biological Sciences, University of Iowa, Iowa City, IA, USA) 1: 200 for 1 h at room temperature. After washing, the sections were incubated with biotinylated donkey antimouse (Jackson Laboratories, West Grove, PA, USA) 1: 500, and then incubated with ABC-peroxidase. Peroxidase activity was visualized as for collagen type I. All collagen antibodies had a cross-reactivity against
the other collagen types of less than 10%. For the receptor antibodies no cross-reactivity was observed. The negative controls, in which the first antibodies were omitted, showed no background staining.

Alkaline phosphatase staining: Frozen sections were incubated with Naphthol AS-TR phosphate (Sigma) as a substrate in the presence of the chromogen Fast Bleu B (Gurr, BDH Chemicals, Poole, England) for 30 min at 37°C. After washing in PBS, the sections were counterstained with hematoxylin. Alkaline phosphatase activity was indicated by a violet-coloured precipitate.

Biochemical analyses

Explants were washed three times with PBS and digested overnight in 550 µl 1 mg ml⁻¹ papain (Merck, Darmstadt, Germany) in 0.2 M NaCl, 0.1 M sodium acetate, 0.01 M L-cysteine and 0.05 M EDTA, pH 6, at 60°C. Aliquots of the digests were analysed for total sulfated GAG with a spectrophotometric assay using dimethylmethylene blue (15). The hydroxyproline (Hyp) content, a measure for the amount of collagen, was determined after alkaline hydrolysis (16). For both assays, all samples were analysed in one batch and were well within the range of the standard curves. The intra-assay coefficients of variation were below 5%. The Hyp and GAG contents were expressed per mg wet weight. To analyse GAG and DNA synthesis, samples from the digests and the label media were diluted in Aqua Luma (Lumac-LSC, Groningen, The Netherlands) and counted in a liquid scintillation counter (LKB-Wallac, Turku, Finland). The rate of incorporation was expressed as cpm mg⁻¹ wet weight in 17 h.

Statistics

The data were expressed as means ± standard deviation (SD). Differences in the biochemical parameters were evaluated by *t*-tests. A *P*-value of less than 0.05 was considered to be significant.

Results

Histology, autoradiography and enzyme histochemistry

The mandibular condyles of 4-d-old rats showed a fibrous layer, a layer of chondroprogenitor cells, and a layer of chondrocytes in the upper half of the condyle, while the lower half consisted of hypertrophied chondrocytes (Fig. 1A). The femoral heads consisted mainly

![Fig. 1. Central sections of mandibular condyles (A,C) and femoral heads (B,D) of 4-d-old rats ex vivo (bar, 260 µm). (A) Hematoxylin and eosin staining shows the zones of the mandibular condyle: a, fibrous layer; b, chondroprogenitor layer; c, differentiated chondrocytes; d, hypertrophied chondrocytes. (B) Hematoxylin and eosin stained section of a femoral head shows mainly differentiated chondrocytes: a, differentiated chondrocytes; b, groups of dividing cells; c, hypertrophied layer. [³H]thyidine incorporation takes place only in the non-differentiated layers of the mandibular condyle (C) and in the entire femoral head, except in the hypertrophied chondrocytes (D).](image-url)
of differentiated chondrocytes covered by a thin fibrous layer (Fig. 1B). Small groups of dividing cells and some hypertrophied chondrocytes were observed in the neck of the femoral heads.

Autoradiography of mandibular condyles showed that DNA synthesis took place only in the non-differentiated superficial layers (Fig. 1C). In contrast, in femoral heads cell proliferation was observed in all of the non-hypertrophied cartilage (Fig. 1D). Autoradiography of [35S]sulfate-labeled explants showed a very diffuse signal only in the differentiated cartilage of both organs (not shown).

Frozen sections of the mandibular condyles showed staining for alkaline phosphatase in hypertrophied chondrocytes and in the perichondrium near the hypertrophied zone (Fig. 2A). A weaker staining extended into the outer margins of the mature chondrocyte layer. In the femoral heads, staining was very weak and was restricted to the hypertrophied chondrocytes and at the outer margins of this zone (Fig. 2B).

In sections of the mandibular condyles, the von Kossa staining demonstrated calcification sites in the extracellular matrix (ECM) of hypertrophied chondrocytes (Fig. 2C). No staining was observed in the femoral heads (Fig. 2D).

**Immunohistochemistry**

**Collagen type I:** In the mandibular condyles, collagen type I was observed in the extracellular matrix of all layers (Fig. 3A). Staining was most prominent in the fibrous layer, the chondroprogenitor layer, and the chondrocyte layer, but was much weaker in the hypertrophied layer. In the femoral heads, clear staining was observed in the matrix of the fibrous layer and in the ligament attachments on the surface (Fig. 3B). No staining was found in the deeper layers of the femoral heads.

**Collagen type II:** There was no staining in the superficial layers of the mandibular condyles (Fig. 3C). Collagen type II staining was apparent in the ECM of the differentiated layers. In contrast, an intense staining was observed throughout the ECM of the entire femoral head (Fig. 3D). The ligament attachments did not show any collagen type II staining.

**Collagen type III:** Weak staining was found in the most superficial layers of the mandibular condyles. Collagen type II staining was apparent in the ECM of the differentiated layers. In contrast, an intense staining was observed throughout the ECM of the entire femoral head (Fig. 3D). The ligament attachments did not show any collagen type II staining.

**IGF-1R receptor:** Staining was prominent in cells of the fibrous and chondroprogenitor zones of the mandibular condyles (Fig. 4A). A less intense staining was found in the other layers, decreasing down to the hypertrophied layer. All cells of the femoral heads showed staining for the IGF-I receptor except the hypertrophied chondrocytes (Fig. 4B).

**TGF-βRII receptor:** Staining for the TGF-β receptor in the mandibular condyles was found in the fibrous layer, the chondroprogenitor layer and in the chondrocyte layer, while the hypertrophied chondrocytes were only weakly stained (Fig. 4C). In the femoral heads, all cells were equally well stained, except for the hypertrophied chondrocytes (Fig. 4D).

**FGFR2 receptor:** Cells in all layers of the mandibular condyles showed clear staining for the FGF receptor with a higher intensity in the fibrous and chondroprogenitor zone (Fig. 4E). In the femoral heads, staining for the FGF receptor was observed throughout the organ, except in the hypertrophied zone (Fig. 4F). The intensity of the staining was increased in columns of cells in the neck of the femoral heads.

**Biochemical analyses**

The ex vivo GAG and DNA synthesis rates of the mandibular condyles and the femoral heads from 4-d-old rats were analysed. The synthesis rates were expressed as incorporation of label per mg wet weight in 17 h (Table 1). The incorporation of both [35S]sulfate (GAG-synthesis) and [3H]thymidine (DNA synthesis) in the femoral heads was about twice that in the mandibular condyles. The ex vivo GAG and Hyp contents were expressed as µg mg⁻¹ wet weight (Table 1). The GAG content was about three times higher in the femoral heads than in the mandibular condyles. The Hyp content was only a little higher in the femoral heads.
We have compared several (immuno)histochemical and biochemical characteristics of the mandibular condyle and the femoral head from 4-d-old rats, which are secondary and primary cartilages, respectively. The mandibular condyle contained several distinct layers, which was also shown earlier (5, 17). In contrast, the femoral head was a more homogenous tissue. About half of the mandibular condyle consisted of hypertrophied cartilage, which was partly mineralized. The perichondrium in this region showed some alkaline phosphatase activity, as was also shown by others (18, 19). In the femoral heads, mineralization was practically absent and

**Fig. 3.** Immunohistochemical staining for collagen type I (A,B), type II (C,D) and type III (E,F) in the mandibular condyles (A,C,E) and femoral heads (B,D,F) of 4-d-old rats ex vivo (bar, 260 μm). In the mandibular condyle, clear staining for collagen type I is observed in the extracellular matrix of all layers (A) while in the femoral head no staining is found in the deeper layers (B). Staining for collagen type II is present in the differentiated layers of the mandibular condyle (C) and in the entire femoral head (D). Staining for collagen type III is very weak (E,F).

**Fig. 4.** Immunohistochemical staining for receptors for insulin-like growth factor (IGF)-I (A,B), transforming growth factor (TGF)-β (C,D) and fibroblast growth factor (FGF) (E,F) in the mandibular condyles (A,C,E) and femoral heads (B,D,F) of 4-d-old rats ex vivo (bar, 260 μm). In the mandibular condyles, clear staining for the three receptors types (IGF-IRβ, TGF-βRII, and FGFR2) is observed in cells of the fibrous and chondroprogenitor zones and decreases in the deeper layers (A,C,E). In the femoral heads, all chondrocytes are stained for IGF-IRβ, TGF-βRII, and FGFR2 except for hypertrophied chondrocytes (B,D,F). The staining intensity for FGFR2 receptors is strongest in the neck of the femoral head (F).
Table 1

Biochemical analyses of mandibular condyles and femoral heads of 4-d-old rats ex vivo

<table>
<thead>
<tr>
<th></th>
<th>GAG synthesis</th>
<th>DNA synthesis</th>
<th>GAG content</th>
<th>Hydroxyproline content</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mandibular condyles</td>
<td>6.0 ± 0.8</td>
<td>17.7 ± 2.4</td>
<td>6.4 ± 0.6</td>
<td>2.2 ± 0.3</td>
</tr>
<tr>
<td>Femoral heads</td>
<td>10.5 ± 1.0*</td>
<td>30.2 ± 2.4*</td>
<td>20.6 ± 1.9*</td>
<td>2.6 ± 0.1*</td>
</tr>
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</table>

Glycosaminoglycan (GAG) and DNA synthesis are expressed as cpm (x 10^6) per mg wet weight; mean values ± SD of 14 explants are given. The GAG and hydroxyproline contents are expressed as chondroitin sulfate and hydroxyproline in µg mg⁻¹ wet weight; mean values ± SD of seven explants are given.

*P < 0.001, significant difference between mandibular condyles and femoral heads (Student’s t-tests).

is probably restricted to the epiphyseal growth plate at this age.

The distribution of collagen types I and II was completely different in the mandibular condyles and the femoral heads. The femoral heads only expressed collagen type I in the fibrous layer, while type II collagen was shown throughout the cartilage. In general, mesenchymal cells synthesize only type I collagen, but when they differentiate into chondrocytes, they start to synthesize type II collagen (20). In the mandibular condyles, however, type I collagen was observed in the non-differentiated layers but also around differentiated chondrocytes. In addition, the expression of type II collagen was weaker in the femoral cartilage. These findings are in agreement with other investigations on young (21) and neonatal rats (7) and on fetal mice (22, 23). This indicates that these specific features are established at an early developmental stage. In the mandibular condyle of young rats, the local distribution of type I and type II collagens was shown to depend on the local type of functional loading (24). In that study, type I collagen was shown to be mainly expressed in regions subjected to tensile forces, while type II collagen was mainly expressed in regions subjected to compressive forces. The higher content of collagen type II in the femoral heads suggests that resistance to compressive forces is more important for this cartilage than for the mandibular condyle. Indeed, femoral cartilage is meant for weight-bearing, and therefore subject to high compressive forces. In contrast, cartilage of the mandibular condyle, which is mainly involved in regional adaptive growth, is subjected to multidirectional forces and may therefore require both types of collagen (21). Furthermore, the deposition of type I and type II collagens in the mandibular condyles of rats was found to be sensitive to alterations in loading (25). Some type III collagen was found in the superficial layers of the mandibular condyles and in the ligament attachment sites of the femoral heads. Further analyses are required to determine if collagen type III is limited to the fibrous layer or extends deeper into the mandibular condyle.

The distribution of IGF-IRβ, TGF-βRII and FGFR2 was similar in the mandibular condyles. Expression of the three growth factor receptors was more intense in the fibrous and chondroprogenitor layers and decreased in the deeper layers. The abundance of the receptors on the chondroprogenitor cells might indicate that IGF-I, TGF-β and FGF all play a role in the regulation of cell proliferation. Indeed, it was shown in vitro that these growth factors stimulate cell proliferation in the mandibular condyle (10, 26, 27). A similar distribution of IGF-I and FGF receptors was shown by others in mandibular condyles from young mice and rats (27–30). We also found a high number of FGF receptors on the hypertrophied chondrocytes. Fibroblast growth factors might therefore be associated with the regulation of chondrocyte differentiation, as was suggested for rat rib growth plate (31). A recent study further showed expression of IGF-I receptors mainly in the superior and posterosuperior regions of young rat mandibular condyles, which might be explained by regional variations in functional loading (14). It was also noted that the distribution of FGF receptors changes in the rat mandibular condyle during growth, probably in response to functional conditions (29, 30). In contrast to the mandibular condyle, the three growth factor receptors were found to be distributed throughout the entire femoral head. The expression of FGF receptors was more intense in small columns of cells in the neck of the femoral head. This might indicate a regulatory function of FGFs in cell division, as was shown for isolated growth plate chondrocytes (31). Hence, IGF-I, TGF-β, and FGFs all seem to play a role in the growth regulation of both mandibular condyles and femoral heads.

The ex vivo DNA and GAG synthesis rates of the femoral heads were about two times higher than that of the mandibular condyles. The difference in the rate of cell proliferation might be explained by the different mode of growth of both cartilages. Femoral heads grow by interstitial cell division in the entire organ, while mandibular condyles grow by appositional cell division mainly in the chondroprogenitor zone (17). The higher GAG synthesis rate in femoral heads is probably caused by the higher fraction of matrix-synthesizing chondrocytes in femoral cartilage. In addition, the GAG content was three times higher in the femoral heads than in the mandibular condyles. Sulfated GAGs are bound to a core protein to form large proteoglycans with a high water-binding capacity (32). The proteoglycans provide the swelling pressure and resilience of the cartilage. The higher amount of glycosaminoglycans shown in femoral heads probably leads to a higher resilience. This supports the above-mentioned relation between the weight-bearing function of the femoral head and its matrix composition. In addition, it is suggested that the lower amount of proteoglycans in the mandibular condyle is associated with its lower independent growth potential (33, 34).

In conclusion, the matrix composition of both cartilages appears to be related to their specific biomechanical
requirements. Structural strength and resilience have to be higher in the femoral head than in the mandibular condyle because it grows under high pressure and is mainly weight-bearing. In contrast, the mandibular condyle is less weight-bearing, but provides mainly regional adaptive growth. Similar growth factors appear to be involved in the regulation of cell metabolism.

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References