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Influence of growth hormone on the mandibular condylar cartilage of rats

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Summary Growth hormone (GH) stimulates mandibular growth but its effect on the mandibular condylar cartilage is not well understood. *Objective:* This study was designed to understand the influence of GH on mitotic activity and on chondrocytes maturation. The effect of GH on cartilage thickness was also determined. *Design:* An animal model witt differences in GH status was determined by comparing mutant Lewis dwarf rats with reduced pituitary GH synthesis (dwarf), with normal rats and dwarf animals treated with GH. Six dwarf rats were injected with GH for 6 days, while other six normal rats and six dwarf rats composed other two groups. Mandibular condylar tissues were processed and stained for Herovici's stain and immunohistochemistry for proliferating cell nuclear antigen (PCNA) and alkaline phosphatase (ALP). Measurements of cartilage thickness as well as the numbers of immunopositive cells for each antibody were analysed by one-way analysis of variance. *Results:* Cartilage thickness was significantly reduced in the dwarf animals treated with GH. PCNA expression was significant lower in the dwarf rats, but significantly increased when these animals were treated with GH. ALP expression was significant higher in the dwarf animals, while it was significantly reduced in the dwarf animals treated with GH. *Conclusions:* The results from this study showed that GH stimulates mitotic activity and delays cartilage cells maturation in the mandibular condyle. This effect at the cellular level may produce changes in the cartilage thickness.

Introduction

Human craniofacial growth and development depend on complex interactions between genes, hormones, nutrients and epigenetic factors. Growth hormone (GH) is one factor that plays an important role on this process.[1] Pituitary dwarfism is accompanied by smaller jaw with retroposition of the mandible[2] and delayed dental development.[3] Growth of the jaws is affected when patients are treated with GH, particularly in regions where interstitial cartilage is involved such as the mandible.[4,5] In vitro, chondrocytes from the mandibular condylar cartilage respond to GH with cell proliferation, proteoglycan synthesis and possibly enhanced mineralisation.[6] Multiplication of the cells in the germinal layer of the epiphyseal cartilage also occurs in vivo.[7]

The aim of the present study is to determine the effect of GH on the cell division and chondrocyte

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maturation in mandibular condylar cartilage in the Lewis dwarf rat model, to further understand its role in humans. The proposed animal model is a mutant with reduced pituitary GH synthesis from lack of GH-releasing hormone. Concentrations of other anterior pituitary trophic hormones are normal.[8] It is a suitable model for studying the effect of GH on tooth development.[9,10] The growing root tip of the rodent incisor is analogous to the cartilaginous growth plate as both calcifying tissue cell populations pass through different ontogenic phases within well characterised zones.[29] Specifically, the present study investigates the effect of GH on the mitotic activity and on cell maturation of the cartilage cells in the mandibular condyle of GH-treated Lewis dwarf rats, when compared with those from dwarf and normal Lewis rats. In addition, the effect of GH on cartilage thickness is also investigated.

Materials and methods

Six normal Lewis and twelve homozygous Lewis dwarf rats (7 weeks old) were used in the experiment. Rats were divided into three groups (3 males and 3 females in each group). Six normal (controls) and six dwarf rats (dwarfs) composed the first two groups. Six dwarf rats, injected intraperitoneally with 66 μg/100 g body weight of recombinant bovine growth hormone (Monsanto, USA) twice daily for 6 days, composed the third group (dwarfs + GH). This dose has shown an effect on cell proliferation of the odontogenic mesenchyme and epithelia in this animal model.[9,10]

All rats were weighted at the beginning and at 7 days at the end of the experiment. The increase in weight for the different groups was as follows: 2.6% for the controls (170 ± 27 to 175 ± 25 g), 1% for the dwarfs (116 ± 13 to 118 ± 13 g), and 23 for the dwarfs + GH (120 ± 19 to 150 ± 19 g).

At day 7, under general anaesthesia (Xylazil/Ketamine, 0.1 ml/100 g. body weight), the animals were perfused with 2% paraformaldehyde and 1% glutaraldehyde in 0.1 M phosphate buffer for tissue fixation, and then sacrificed by exsanguination. The right condyles were harvested and postfixed for four hours in the same fixative. After decalcification with EDTA (4.13%), tissues were individually embedded in paraffin with the dorsal face of the condyle against the bottom of the embedding cage. Serial sagittal sections (5 μm) from each condyle in the different groups were used to perform the measurements. The maximum antero-posterior condylar axis was determined prior to decalcification (2.8 ± 0.3) and only those even sections with a length between 2.5 and 3 mm were used for this study.[12] The tissues were processed and stained for Herovici's stain to measure cartilage thickness, and immunohistochemistry for proliferating cell nuclear antigen (PCNA), also known as cyclin,[11] and alkaline phosphatase (ALP) following the methodology described previously.[12] Mitotic activity was measured by counting immunopositive nuclei for PCNA (DAKO clone PC10). This intracellular polypeptide antigen increases its expression during the G1 phase of the cell cycle, reaches its maximum expression in the S-phase, and declines during the G2/M phase.[13] Cartilage cell maturation was measured by counts of ALP-immunoreactive chondrocytes. ALP is a bone marker commonly expressed by the chondrocytes of the mature and hypertrophic zones. In the mandibular condylar cartilage, ALP activity has been related with the onset of chondrocyte maturation and to mineralisation of the cartilage matrix.[14,15] The anti-bone ALP antibody was a gift from Dr. Greg Welowski, Merck Research Laboratories, West Point, USA.

Although PCNA has been employed by other authors in similar experiments[16] and it reacts with the PCNA molecule from all vertebrate species, positive and negative controls were performed during the experiment. Sections of the developing rat incisors were used as positive controls for dividing cells. For the negative controls, the procedure was performed in the rat mandibular condylar cartilage using an equivalent dilution of anti-mouse IgG, instead of the primary antibody. In addition, the optimal dilution for the antibody against PCNA was assessed in cartilage by several dilutions and different time frames. A dilution of 1:50 for 90 min at room temperature (RT) was determined as the optimal dilution for PCNA in chondroblasts. Thus, Immunohistochemical staining was performed using mouse monoclonal antibody anti-PCNA as stated earlier and anti-bone ALP at a dilution of 1:300.

In this study, both cartilage thickness measurement and cell counting were performed with no knowledge of treatment by two observers. The numbers of immunoreactive cells to each antibody present in a specified area of the condylar cartilage were counted.[12] Briefly, an area of 0.122 mm^2 covering the total thickness of the proliferative and the mature layers, as well as part of the articular and the hypertrophic layers was determined on digitised pictures from the immunostained sections. Total thickness of the cartilage was determined as previously reported[12] measuring the distance from the superior border of the mandibular cartilage to boundary with the zone of endochondral ossification (Scion image Beta 4.0.2, Scion Corp, MD, USA).

Statistical analysis

The data from each observer were analysed by Spearman correlation test to determine the level of agreement between the examiners. A high agreement ($r > 0.95$) was observed between the examiners for all the cases. A final average from the results of both examiners was used for statistical analysis.

Once the data was obtained, paired *t*-test was used to determine if any significant differences existed regarding gender. It was determined that no significant difference existed between males and females within the different groups, and thus the data were pooled in each group.

The pooled data obtained were compared statistically by one-way analysis of variance (one-way ANOVA). Newman–Keuls post-test was computed when a significant difference at the 95% level of significance was observed, discriminating where the significant difference was present. Statistical analysis was performed using Prism 2.1 software (GraphPad Prism Software Inc., San Diego, CA, USA).

Results

Sections for the primary antibodies tested in the developmental tip of the rat incisors showed positive staining for PCNA on the odontogenic mesenchymal cells. ALP-immunoreactivity in the cells of the bone surrounding the incisor was also observed. Control sections stained with anti-mouse IgG showed no immunoreactivity under the staining conditions used.

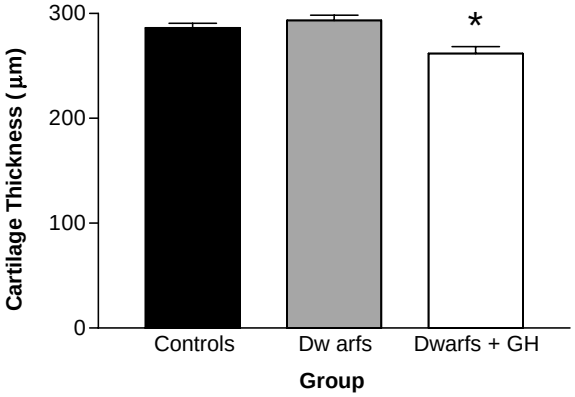

Figure 1 Cartilage thickness is significantly reduced ($P < 0.01$) when the dwarf rats were treated with GH during 6 days. A slight but not significant increase in cartilage thickness is observed in the GH-deficient animals (dwarfs) compared with the non GH deficient rats (controls). Mean and S.E.M. are shown.

Cartilage thickness

Cartilage thickness measured on sections stained with Herovici's was significantly decreased in the dwarf rats treated with GH, compared with the other two groups ($P < 0.01$). No significant difference in cartilage thickness was observed between the dwarfs and the controls ($P > 0.05$). However, a slight increase in cartilage thickness was noted in the dwarfs compared with controls, but was not significant (Fig. 1).

Immunohistochemistry

PCNA, as a marker of cell division, showed significant differences when the numbers of immunopositive

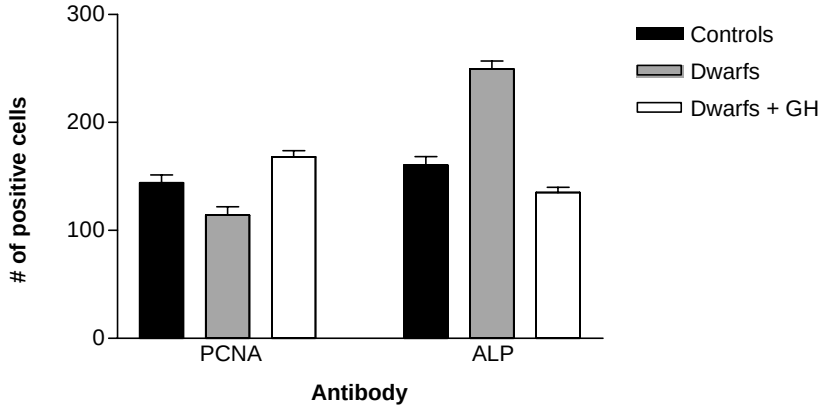

Figure 2 Mitotic activity measured by PCNA expression is significantly reduced in the dwarf animals ($P < 0.01$), whereas is significantly increased in the dwarfs treated with GH (dwarfs + GH) for 6 days ($P < 0.001$). ALP expression is significant higher in the GH deficient animals (dwarfs), and significantly reduced when the dwarf animals are treated with GH ($P < 0.001$). Mean and S.E.M. are shown.

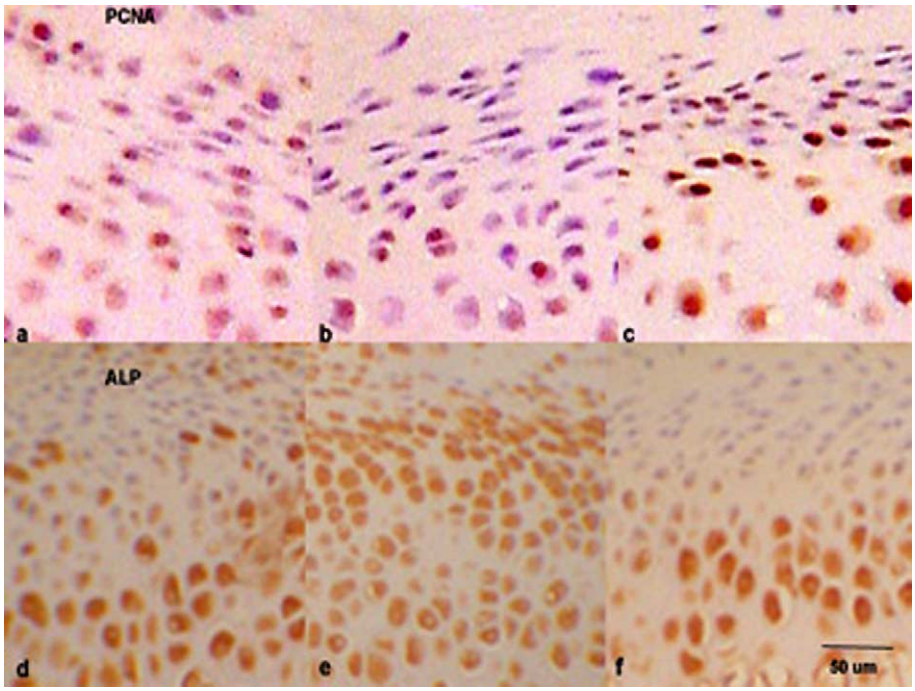

Figure 3 Immunohistochemical stain of cartilage sections for PCNA and ALP in the non GH-deficient animals (a, d), the GH-deficient rats (b, e) and the GH-deficient rats treated with GH for 6 days (c, f). Magnification 400×.

cells on the condylar cartilage where compared between the three groups. Significantly higher numbers of cells entered the mitotic cycle on the cartilages of the dwarfs + GH compared with the dwarfs ($P < 0.001$) and with the controls ($P < 0.05$). The dwarfs showed a significant lower numbers of PCNA-immunopositive cells than those observed in the controls ($P < 0.01$) (Figs. 2 and 3).

The numbers of ALP-immunopositive cells were significantly different between the three groups. The dwarfs + GH showed significant lower numbers of immunopositive cells for ALP than the dwarfs ($P < 0.001$) and the controls ($P < 0.05$). Comparing the controls with the dwarfs, the numbers of ALP-immunopositive cells in the dwarfs were significantly higher than in the controls ($p < 0.001$) (Figs. 2 and 3).

Discussion

In the present study, effects of GH on cartilage thickness, on mitotic activity, and on chondrocytes maturation were found in the mandibular condylar cartilage of rats. Differences were appreciated between non GH deficient animals (controls), GH deficient rats (dwarfs), and GH deficient animals treated with GH (dwarfs + GH).

The data shows that mitotic activity as determined by the expression of PCNA, is significantly reduced in GH deficient rats, whereas it is significantly increased in the dwarf rats treated with GH, reaching numbers of PCNA-immunopositive cells even higher than those observed in the normal animals. Therefore, this study confirms that GH stimulates mitotic activity in the mandibular condylar cartilage in vivo, as it does in vitro in mandibular condylar explants,[17] and in vivo in tibial cartilage.[7] In humans, GH treatment has been shown to increase mandibular growth.[5] Stimulation in mitotic activity results in more cartilage cells being available to differentiate into chondrocytes from the mesenchymal cells. Thus, a higher number of cells will be depositing extracellular matrix that later will turnover into bone.[18] In this way, GH would be responsible, at least in part, for increases in length of the mandible.

More cells expressing ALP are observed in the GH deficient rats. However, these animals treated with GH for a week show significant reductions of the numbers of cartilage cells expressing ALP. The numbers of ALP-immunopositive cells in the normal rats are in between the values for the other two groups. Cartilage cells of the proliferative zone normally show low reactivity for ALP, with higher expression in the chondrocytes of the mature and hypertrophic zones.[19–21] So, ALP expression relates to the onset of the maturation process of cartilage cells, and with the mineralisation process of the cartilage matrix.[14,22–25] In the dwarfs' condyles, a

higher number of cells of the proliferative and mature layers expressing ALP is an indication of their maturity. However, this differentiation (ALP expression) is not a feature of the proliferative zone of the controls or of the dwarfs + GH. It seems that GH treatment produces a delay in the maturation process of the cartilage cells.

Petrovic has proposed that an increase in the numbers of mature cartilage cells produces a negative feedback on the numbers of cartilage cells dividing.[26] Conversely, the present study shows that increased mitotic activity is correlated with decreased cartilage cell maturation. GH stimulus to cell division maintain the cartilage cells in proliferation for a longer period of time and the number of cells differentiating into chondroblasts and later maturing as chondrocytes is diminished. Therefore, the delay caused by GH in chondrocytes maturation may be a consequence of that effect on cartilage cell proliferation.

Interestingly, the cartilage thickness is significantly reduced in the dwarf animals treated with GH. An increase in the mitotic activity accompanied with a delay in the chondrocytes maturation would imply an increase in the thickness of the mandibular cartilage, but this did not occur in this study. Increased cell volume and higher rates of matrix synthesis in the hypertrophic layer contribute to cartilage thickness three or four times more than the proliferative and the mature layers.[18] In the dwarf rats treated with GH, cell proliferation is stimulated, increasing the number of cells in the proliferative layer and therefore, increasing the thickness of this layer. However, these are flattened cells and an increase in the proliferative layer does not markedly change the total cartilage thickness. Chondrocytes maturation is delayed in these animals, which means lower numbers of cells are going through the mature layer into the hypertrophic layer. There are diminished numbers of cells of high volume secreting cartilage matrix. Thus, the reduction in the cartilage thickness observed in the dwarf rats treated with GH is explained by a reduction in the numbers of cells differentiating, maturing and reaching the hypertrophic layer. A reverse effect may also explain the slight but not significant increase noted in the cartilage thickness of the GH deficient rats. In these animals, higher numbers of cartilage cells are passing through the mature layer into the hypertrophic zone producing a thicker cartilage. However, lower numbers of cells multiplying in the proliferative layer reduce the availability of cells reaching the hypertrophic layer, which affects the significance in the increase of cartilage thickness noted in the dwarf animals not treated with GH.

We have used the Lewis dwarf rat as a model to understand the effect of GH in the mandibular condylar cartilage. Nevertheless, the animals are treated with a single dose of GH for 6 days. This scheme of treatment has shown to affect tooth development.[9,10] In the mandibular condyle, the same scheme stimulates stronger responses in the cartilage cells compared to that normal behaviour observed in those cells of the non-GH-deficient rats. Although, a clear effect of GH on mitotic activity and on maturation of the mandibular condylar cartilage cells are determined with this scheme, a dose-related effect of GH on costal cartilage have been reported previously.[27] Also, variations in schedule of GH injections produce different responses in tibial cartilage of hypophysectomized animals.[28] Therefore, further studies are necessary to determine a dose- and time-related effect of GH on the mandibular condylar cartilage.

Conclusions

This study tested the effect of GH treatment on the mandibular condylar cartilage of rats over 6 days. GH treatment stimulates mitotic activity and delays cell maturation in the mandibular condylar cartilage. Thus, GH seems to regulate an inverse relationship between proliferation and chondrocytes maturation. This effect of GH on cartilage cell division and maturation may produce changes in the cartilage thickness.

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