Insulin-like growth factor-1 boosts the developing process of condylar hyperplasia by stimulating chondrocytes proliferation

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Objective: The etiology of Condylar hyperplasia (CH) of human temporomandibular joint (TMJ) remains largely unknown. Our previous study has demonstrated that enriched insulin-like growth factor-1 (IGF-1) was expressed in the proliferation and hypertrophic layers of CH cartilage. Accordingly, this study was aimed to investigate whether IGF-1 regulates CH chondrocytes proliferation in condylar cartilage overgrowth and explore the molecular mechanism of IGF-1 involved in.

Methods: Chondrocytes were isolated from 6 CH and 3 normal cartilage (NC) specimens and cultured in alginate beads or monolayer, treated with IGF-1 or specific inhibitors such as 7-[(trans-3-[(azetidin-1-yl)-methyl]cyclobutyl]-5-(3-benzylxoyphenyl)-7H-pyrrolo[2,3-d]pyrimidin-4-amine (NVP-AEW541), U0126, and LY294002. Thereafter, cellular proliferation capacity was evaluated by Cell Viability Analyzer (alginate beads culture) or 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (monolayer culture). Gene expression levels of IGF-1, IGF-1 receptor (IGF-1R), collagen type II, type X and those genes associated with proliferation were evaluated by realtime PCR. Protein levels of IGF-1 and IGF-1R synthesized by CH chondrocytes were accessed by enzyme-linked immunosorbent assay (ELISA) and western blotting.

Results: CH chondrocytes enhanced cellular proliferation capacity and expressed significantly higher levels of messenger RNA (mRNA) and protein expressions of IGF-1 and IGF-1R, as compared with NC chondrocytes. Furthermore, enriched IGF-1 enhanced CH chondrocytes proliferation, up-regulated the expressions of specific genes associated with cellular proliferation and elevated the gene expression of collagen type II A1 (COL2A1). Besides, IGF-1-mediated CH chondrocytes proliferation mainly depended on the mitogen-activated protein kinase (MAPK)–ERK pathway.

Conclusions: IGF-1 promotes human TMJ cartilage overgrowth in the developing process of CH by enhancing chondrocytes proliferation via MAPK–ERK pathway.

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Introduction

Condylar hyperplasia (CH) in human temporomandibular joint (TMJ) is defined as a postnatal and self-limited growth abnormality which occurs unilaterally and leads to facial asymmetry and occlusal disturbance. It usually develops in the teens, early 20s and sometimes after the opposite condyle has stopped growing with equal frequency in males and females. It is widely accepted that an accelerated or prolonged growth after the end of general skeletal growth of a condyle is the main cause of such deformity in human TMJ. Single-photon emission computed tomography (SPECT) bone scintigraphy, the golden standard for the clinical diagnosis of CH, is frequently used to the growth activity of the TMJ condyle. The typical histological features of CH include the presence of an uninterrupted layer of undifferentiated germinating mesenchymal cells, hypertrophic cartilage, and islands of chondrocytes in the subchondral trabecular bone. Insulin-like growth factors (IGF-1) exert important roles in human growth and development. IGF-1 regulates the metabolism like increasing lipid, glycogen, and protein synthesis. IGF-1 has effects on muscle and bone by stimulating proliferation and promoting differentiation of myoblastic or osteoblastic tissues. In the growth plate of longitudinal bone, IGF-1 is a key growth factor exerting its actions to stimulate proliferation and differentiation of chondrocytes by endocrine and paracrine/autocrine mechanisms. The IGF-1 receptor (IGF-1R), acting as a docking protein for the
downstream signaling transduction, is overexpressed in many tumors and have vastly different functions like mediates cancer cells proliferation, motility and apoptosis protection both in vitro and in vivo.

Concerning the unique role of the IGF-1 in somatic development, recent studies have started focusing on the relationship between IGF-1 and TMJ condyle cartilage growth. IGF-1 regulated adaptive remodeling of mandibular condylar cartilage by an onset or enhanced proliferation of chondrocytes. Elevated expressions of IGF-1 and IGF-1R were found in cartilage and bone of active CH. Also, our previous study showed that strong immunostaining of IGF-1 was in the proliferative chondrocyte layer and the hypertrophic chondrocyte layer, indicating that the abnormality of the cartilage growth in CH is probably attributed to IGF-1 overexpression.

The aim of this study was to investigate whether IGF-1 regulates CH chondrocytes proliferation, thereby resulting in condylar cartilage overgrowth. The molecular mechanism of IGF-1 mediated CH chondrocytes proliferation was also investigated.

Materials and methods

Samples and chondrocyte culture

The condyle cartilage samples of CH were obtained from six patients in Table I following surgeries of condylectomy in the stomatology hospital of Wuhan university. All patients were diagnosed by SPECT and confirmed growth activity in the affected lateral condyle. Normal cartilage (NC) samples as the control group were harvested from condyle fractured patients in Table II, and the specimens were confirmed as NC tissues by histological examination. The experimental protocol was approved by the Human Research Ethics Committee, School & Hospital of Stomatology, Wuhan University and written consent was obtained from all patients included in this study.

Chondrocytes were isolated by collagenase B digestion of cartilage, as described previously, and grown in DMEM (Hyclone, USA) supplemented with 10% fetal bovine serum (FBS) at 37°C, in a humidified atmosphere containing 5% CO2 in air. After chondrocytes reached 80–90% confluence, they were encapsulated in alginate beads as described previously. Normal cartilage (NC) samples as the control group were harvested from condyle fractured patients (Table II), and the specimens were confirmed as NC tissues by histological examination. The experimental protocol was approved by the Human Research Ethics Committee, School & Hospital of Stomatology, Wuhan University and written consent was obtained from all patients included in this study.

Table I

<table>
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Enzyme-linked immunosorbent assay (ELISA) for IGF-1

Culture supernatants of alginate beads were collected from three wells of NC, CH and CH + NVP-AEW541 group at day 7, 14, and 21. Monolayer culture supernatants were also collected at 24 h, 48 h, and 72 h. IGF-1 concentrations were measured by ELISA using the commercial Human IGF-1 Quantikine Kit (R&D Systems, dg100), according to the manufacturers’ protocol. Absorbance was measured at 450 nm using a microplate reader (Nanodrop, Thermo). The parameters were normalized by the cellular quantity.

RNA extraction and real-time polymerase chain reaction (real-time PCR)

Chondrocytes in alginate beads culture were incubated with or without IGF-1 and U0126 for 12 h at day 14 and 21. Next, cellular pellets were harvested after dissolving alginate beads. Total RNA were extracted from cells using trizol (TAKARA, D9108A) and reverse transcribed in cDNA using oligo (dT)15 as a reverse primer according to the manufacturer’s protocol (TAKARA, DRR037A). Equivalent amounts of cDNA were used for real-time PCR in a 20ul reaction mixture with 10ul of 2 × SYBR Green PCR Mastermix and 1 μl of specific primer pair. Reaction was run in triplicate with 40 cycles of amplification on an ABI Prism 7500 real-time PCR (Applied Biosystems, USA). The sequences of primers were shown in Table III. The expression levels of target genes were normalized by the expression of 18S gene measured in parallel samples.

Western blot analysis for IGF-1R

At day 21, six beads from each sample were dissolved and cellular lysis was collected. Lysis supernatant containing 50 μg proteins from each sample was collected as we previously described. Samples were loaded and analyzed by SDS-PAGE followed by Western blot analysis.
underwent electrophoresis in 10% sodium dodecyl sulfate polyacrylamide gels, and were transferred to polyvinylidene difluoride membranes. After the membrane was blocked in 5% non-fat milk, the membranes were washed, and specific bands were visualized on X-ray films using enhanced chemiluminescence kit (Roche, Germany). The gray value of bands of IGF-1R were read by the In-vivo multispectral imaging system (KODAK, USA) and normalized by that of GAPDH.

Statistics analysis

Statistical analyses were done with the SPSS 18 statistical software program. Data are expressed as mean ± 95% confidence interval (95% CI), and were considered statistically significant for P < 0.05. Gaussian distribution and homogeneous variance were tested in all original data. For comparisons among normally distributed data, two-sample t-test and one-way analyses of variance (ANOVA) followed by pairwise multiple comparisons were used, when a significant F-ratio was found, significant differences among treatment groups were identified by the post hoc Scheffé test. For non-normally distributed data, Mann–Whitney non-parametric analyses and non-parametric Kruskal–Wallis test followed by pairwise multiple comparisons were used.

Results

CH chondrocytes possess higher proliferation capacity than NC chondrocytes

The morphology of primary CH chondrocytes cultured in monolayer was round or polygonal [Fig. 1(A,B)], while NC chondrocytes displayed a homogeneous small fibroblast-like appearance [Fig. 1(C, D)]. Chondrocytes proliferation capacity was compared between NC and CH group when cells were cultured in alginate beads within 21 days. As illustrated in Fig. 1(E), cell amount in CH group was significantly higher than that in NC group at each time point (day 7, P = 0.042; day 14, P = 0.036; day 21, P = 0.002), although a continuous increase of cellular quantity from day 7 was presented in these two groups. To exclude the potential effects of three-dimensional culture on cell proliferation, cells cultured in monolayer were also investigated by MTT assay. As shown in Fig. 1(F), the cellular quantity in CH group chondrocytes was higher at day 5 and 7 (day 5, P = 0.002; day 7, P = 0.001).

Enriched expressions of IGF-1 and IGF-1R in CH chondrocytes

A time course study was performed to compare IGF-1 protein production in alginate beads culture between CH and NC group. The levels of IGF-1 concentration in CH group were significantly higher than those in NC group from day 7 to day 21 [P < 0.0001 Fig. 2(A)]. At day 7, an average IGF-1 concentration over than 281.1 pg/ml (95% CI = 238.3–323.89, N = 3) was detected in CH cells. Thereafter, a peak level occurred at day 14. On the contrary, the level of IGF-1 concentration in NC group (mean = 57.6 pg/ml, 95% CI = 44.75–70.56, N = 3) was relatively constant during the whole observing period. Addition of NVP-AEW541, a specific inhibitor against IGF-1R, significantly suppressed the level of IGF-1 concentration of CH chondrocytes at day 7 (P < 0.0001), day 14 (P = 0.002) and day 21 (P = 0.01) [Fig. 2(B)]. Again, our monolayer culture experiment demonstrated that the level of IGF-1 expression in CH chondrocytes was also significantly higher than that in NC cells at day 21 [P < 0.0001, Fig. 2(C)].

Subsequently, we perform real-time PCR to compare gene expression alterations of IGF-1 and IGF-1R from between CH and NC chondrocytes. Compared with NC cells, significantly higher expression levels of IGF-1 ( ~52-fold at day 14, P = 0.007; ~5.8-fold at day 21, P = 0.035) and IGF-1R genes (P = 0.022) were presented in CH cells (Fig. 3). Likewise, the expression level of IGF-1R protein from CH cells detected by western blotting at day 21 was as about 15.7-fold higher as that of NC cells (P = 0.037) (Fig. 4).

IGF-1 enhanced CH chondrocyte proliferation

We next examined the effect of IGF-1 on chondrocytes proliferation by addition of IGF-1 or NVP-AEW541 [Fig. 5]. The data showed that proliferation capacity of CH chondrocytes significantly declined with the supplement of NVP-AEW541 at day 14 (P = 0.002) and day 21 (P < 0.0001), while NC cells proliferated rapidly with the exposure of IGF-1 at day 14 (P = 0.005) and day 21 (P < 0.0001). Cellular viability of CH chondrocytes was more than 90% after the treatment.

Furthermore, specific genes associated with cellular proliferation control and cell cycling was investigated by real-time PCR. As shown in Fig. 6(A), treatment of NVP-AEW541 significantly suppressed proliferating cell nuclear antigen (PCNA) (P = 0.002), G2/mitotic-specific cyclin B1 (P = 0.046) and M-phase inducer phosphatase (P = 0.002) gene expressions. On the other hand, IGF-1 significantly enhanced gene expressions of PCNA (P = 0.049), but it was difference in gene expressions of G2/mitotic-specific cyclin B1 (P = 0.05) and M-phase inducer phosphatase (P = 0.084) in NC chondrocytes.

CH chondrocytes retain higher matrix synthesis activity than NC chondrocytes

To compare matrix synthesis activity of chondrocytes between NC and CH group, we investigated gene expressions of collagen type II A1 (COL2A1) and collagen type X A1 (COL10A1) from cells cultured in alginate beads within 21 days of both groups. As shown in Fig. 6(D), gene expression levels of COL2A1 was 4.63-fold higher in CH cells than those in NC group (P = 0.018), while there was no significant difference for COL10A1 between these two groups (P = 0.531). Besides, treatment of IGF-1 significantly enhanced COL2A1 expression (P = 0.027) in NC chondrocytes. When treated with NVP-AEW541, CH chondrocytes did not exhibit significant
expression alteration of both COL2A1 \((P = 0.845)\) and COL10A1 \((P = 0.998)\) genes [Fig. 6(E, F)].

**IGF-1-induced CH chondrocytes proliferation is mediated through the mitogen-activated protein kinase (MAPK)–ERK pathway**

We further explored the signaling pathways involved in IGF-1-induced CH chondrocytes proliferation. The addition of U0126, a specific inhibitor of MAPK–ERK pathway, at each time point suppressed the proliferation capacity of CH chondrocytes (Fig. 7, \(P = 0.029\)). Whereas, the treatment of LY294002, a specific inhibitor of phosphatidylinositol-3 kinase (PI3K) pathway, exhibited no effects on the capacity of CH cells proliferation during the observing period (Fig. 7, \(P = 0.45\)). Taken together, these data indicated that IGF-1-stimulated CH chondrocytes proliferation depends on MAPK–ERK pathway.

In addition, specific genes of MAPK–ERK pathway downstream molecules, which regulate cell-cycle to start the cellular
proliferation process, were tested by real-time PCR. As shown in Fig. 8, there were higher gene expression levels in CH cells than in NC cells for Cyclin D1 ($P = 0.002$) and cyclin-dependent kinase2 (CDK2) ($P = 0.001$), while there was a marked decline in CH cells under the incubation of U0126 for Cyclin D1 ($P = 0.03$) and CDK2 ($P = 0.042$).

Discussion

In the former studies, chondrocytes were proved to have a tendency to dedifferentiate if they grew in monolayer culture\textsuperscript{15}. We also found the round CH chondrocytes rapidly transformed into flattened fibroblast-like cells in the monolayer culture, and lost their high proliferation capacity as the same as NC chondrocytes did at passage 2 (data not shown). The classic experiments on chondrocyte culture suggested three-dimensional culture offers a scaffolding material maintaining the original chondrocyte phenotype as it facilitates chondrocytes aggregate and mimic vivo environment\textsuperscript{15}. Therefore, alginate beads culture was preferred in the present study as such method avoids passage culture and enables cells continue to proliferate, maintain their phenotypes\textsuperscript{22}.

In this study, amount of IGF-1 protein in CH showed a peak at day 7, decreased at day 21 in alginate culture, meanwhile constantly increased in monolayer culture. This is due to the different culture condition.

The initial stage of condylar growth and remodeling undergoes chondrogenesis, which results from the chondrocytes proliferation. Our results showed that CH chondrocytes possess higher proliferation capacity than NC chondrocytes, which is in agreement with the histopathological results that the actively growing type of CH condyles was characterized by a broad proliferation zone or mesenchyme layer\textsuperscript{2,23}. Our previous study from immunohistochemistry analysis had found that the expressions of IGF-1 were strong in the active CH condyle and presented mainly in the proliferative chondrocyte layer\textsuperscript{12}. In this study, ELISA and western blotting results demonstrated that CH chondrocytes significantly increased IGF-1 and IGF-1R protein production levels than NC chondrocytes. Moreover, gene expressions of IGF-1 and IGF-1R
were also remarkably elevated in CH cells. IGF-1 is a key regulator in many tissues during their postnatal development through promoting mitogenesis, cell migration, survival and protein synthesis by endocrine and autocrine mechanisms. However, aberrant IGF-1 expression or signaling would cause various human diseases, such as certain growth disorder or cancer. In human melanoma, Molhoek suggested that IGF-1 is a growth factor for autocrine driven proliferation in vitro. Melanoma cells elevated IGF-1 synthesis. In turn, IGF-1 enhanced proliferation of melanoma cell. Other investigators had proven that IGF-1 also promotes the proliferative activity of colon cancer cells, breast cancer cells, and tendon cell. Accordingly, it is reasonable for us to examine the effect of IGF-1 on CH chondrocytes proliferation by addition of IGF-1 or NVP-AEW541, which is a specific inhibitor against IGF-1R.

The proliferation capacity of CH chondrocytes was significantly declined by the supplement of NVP-AEW541, while NC cells proliferated rapidly with the exposure of IGF-1. Furthermore, treatment of NVP-AEW541 significantly suppressed the expression levels of specific genes associated with cellular proliferation control and cell cycling. Thus, these findings demonstrated that the enriched IGF-1 in CH cartilage enhanced CH chondrocytes proliferation. Besides, using NVP-AEW541, we demonstrated that IGF-1 induced its own synthesis in chondrocytes. The elevated IGF-1 in CH cartilage is the production of local chondrocytes suggesting that the secreted IGF-1 from CH chondrocytes is utilized by themselves to enhanced chondrocytes proliferation. Subsequently, increased chondrocytes in CH cartilage continue to produce IGF-1, which keeps an aberrant high concentration level in local cartilage. This positive feedback loop indicated that IGF-1 is a potent growth factor in the CH pathological development process. Collectively, the abnormality of cartilage growth in CH is attributed to IGF-1 over-expression for autocrine driven proliferation.

Another important factor in the cartilage growth pattern is matrix synthesis. Our findings showed that an enhanced gene expression level of COL2A1 in CH chondrocytes, and IGF-1 promoted the expression of COL2A1 in chondrocytes, suggesting IGF-1 participates in the CH pathological development partially through augmenting matrix synthesis capacity of chondrocytes. When treated with NVP-AEW541, CH chondrocytes did not remarkably alter the expression levels of COL2A1, indicating that IGF-1 may not be the only critical factor affecting the matrix synthesis of CH chondrocytes. Cellular hypertrophy is an important factor for cartilage interstitial growth. Collagen Type X is known to be a marker for hypertrophic chondrocytes. In the present study, CH chondrocytes exhibited a low expression of COL10A1 gene and no significant alteration of such gene expression in chondrocytes when treated with IGF-1 or NVP-AEW541, which suggested that IGF-1 would not mediate chondrocytes toward hypertrophic differentiation in the process of CH development.

Some studies have shown that IGF-1 modulates the proliferation of somatic cells, such as tendon cells and costal chondrocytes, by activating two major intracellular signaling pathways, MAPK and PI3K pathway. It is notable that different cell types comprise the specific signaling pathways involved in IGF-1-induced proliferation. For example, MAPK pathway is exclusively involved in the IGF-1-induced cellular proliferation of myoblasts and adipocytes, while stimulation of MCF-7 mammary tumors and brain capillary

![Fig. 4. IGF-1R protein expression at CH and NC chondrocytes. GAPDH was used as a control. The bar represents mean ± 95% CI, results shown represent three independent experiments. Comparisons of IGF-1R protein expressions from between CH and NC chondrocytes were statistics analyzed by Mann-Whitney non-parametric test. The mean relative gray value of IGF-1R of CH group was 1.574-fold (1.35, 1.797) to that of NC group (*represents P = 0.037, N = 3).](image-url)

![Fig. 5. Effects of IGF-1 and NVP-AEW541 on CH and NC chondrocytes proliferation. Encapsulated beads were categorized and treated as described in Materials and methods. The bar represents mean ± 95% CI, results shown represent three independent experiments. Comparison of cell amount in CH and NC groups were statistics analyzed by ANOVA (N = 3). The initial cellular density was 1.66 × 10⁵ cells/2 beads/well in each group. At day 7, visible cell amount in CH group was significantly higher than that of other three groups (*represents P = 0.024). The data showed that proliferation capacity of CH chondrocytes markedly declined with the supplement of NVP-AEW541 at day 14 (*represents P = 0.002) and day 21 (*represents P < 0.0001), while NC cells proliferated rapidly with the exposure of IGF-1 at day 14 (*represents P = 0.005) and day 21 (*represents P < 0.0001).](image-url)
cellular proliferation by IGF-1 is mediated via PI3K pathway. In addition, in mammary epithelial cells, IGF-1 induce cellular proliferation via both two pathways. To determine pathways responding to IGF-1-induced CH chondrocytes proliferation, we used two specific pharmacological inhibitors, U0126 and LY294002. As a result, U0126 but not LY294002, significantly inhibited cellular proliferation indicating MAPK–ERK pathway is mainly responsible for IGF-1 induced CH chondrocytes proliferation. IGF-1 induces the sequential activation of MAPK, which then activates ‘classical’ MAPK family members like extracellular-signal-regulated kinase 1/2 (ERK1/2), ERK5. Activated ERK1/2 phosphorylates transcription factors and protein kinases, thereby transmitting extracellular signals to the nucleus and inducing immediate early genes associated with cell-cycle. It is widely accepted that chondrocytes proliferation is initiated by cell-cycle entry, which is mainly promoted by transcription factors and protein kinases, such as Cyclin D1 and CDK2. The present study demonstrated that such two gene expression levels in CH cells were significantly higher than those in NC cells, which suggests that increased cell-cycle entry signals transmit in CH cell nucleus to start cellular proliferation. Besides, when ERK1/2 was blocked by U0126, Cyclin D1 and CDK2 messenger RNA (mRNA) down-regulated to similar levels of those in NC cells, which indicated that MAPK–ERK pathway plays important roles to mediate such signals transmitting from extracellular into CH cellular nucleus.

Interestingly, even in the long-term culture, CH chondrocytes proliferation started to stagnated since from the fourth week. By telomeric repeat amplification protocol assay, we found no detectable telomerase activity in CH chondrocytes (data not shown), which suggested CH chondrocyte is not a kind of immortalized cell. Furthermore, silver staining combined light microscopic examination experiment showed no cytologic and nuclear atypia in CH chondrocytes and no difference in mitotic counts between CH and NC cells (data not shown). These findings implied that CH chondrocyte is not a kind of immortalized or tumor-like cell. To further clarify the characteristics of CH chondrocyte is a major objective in our ongoing study.

In conclusion, IGF-1 promotes human TMJ cartilage overgrowth in the CH developing process by enhancing chondrocytes proliferation via MAPK–ERK pathway. These results will be helpful to
explore a novel therapeutic method for CH in its actively growing stage.

**Author contributions**

Xing LONG designed the research and wrote the paper; Yuxiang CHEN and Jin KE performed the research and contributed new reagents/analytic tools; Qinggong MENG and Mohong DENG critically revised the draft; Jian LI and Wei FANG analyzed data; Hengxing CAI and Si CHEN collected experimental data; Xing Long had full access to all the data in this study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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**Conflict of interest**

The authors report no conflicts of interest.

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