Original Article

Gene Therapy to Enhance Condylar Growth Using rAAV-VEGF

Juan Dai[a]; A. Bakr M. Rabie[b]

ABSTRACT

Objective: To test the hypothesis that the introduction of specific vascular growth inducting genes would favorably affect mandibular condylar growth in Sprague-Dawley (SD) rats over a limited experimental period. Therefore, the aim of this study is to examine the effect of gene therapy on condylar growth by means of a morphological assessment.

Materials and Methods: Ninety 35-day-old female SD rats were randomly divided into three groups, which received any of the injections of recombinant adeno-associated virus mediated vascular endothelial growth factor (rAAV-VEGF), rAAV mediated enhanced green fluorescence protein (rAAV-eGFP), or phosphate-buffered saline (PBS) into both mandibular condyles. Each group of rats was sacrificed on the following experimental days: 7, 14, 21, 30, and 60. Left halves of the mandibles were isolated and digital pictures were obtained in a standardized manner.

Results: The length of condylar process (B-F) as well as mandibular length (A-F) significantly increased on day 30 and continued to increase until the end of the experiment. Moreover, the width of condyle (Q-R) had increased significantly from day 30 and lasted to day 60. Condylar length (C-D) was found to be significantly longer on day 60.

Conclusions: Gene therapy with VEGF stimulates condylar growth at will. The rAAV-VEGF is an excellent candidate for future gene therapy to induce mandibular growth.

KEY WORDS: Gene therapy; rAAV; VEGF; Mandibular condylar growth

INTRODUCTION

Scientific advances in the 21st century have helped us to understand molecular factors that regulate condylar growth.[1] Genetic disorders in the craniofacial region lead to a number of craniofacial anomalies. For example, micrognathia and hemifacial microsomia are such conditions where impaired condylar growth requires comprehensive surgical intervention. Currently, the available approaches include distraction osteogenesis, orthognathic surgery, bone grafting using autogenous bone, allogenic bone grafts, and others. Osteoinductive matrices are limited by their disadvantages such as bone graft resorption, their association with lack of volume, donor site morbidity, potential for antigenicity, and disease transmission. Most would agree that the current patient care regimen is inefficient, expensive, and painful, and therefore needs to be improved.[2–5]

The rapid development of recombinant DNA technology has led to the development of growth factor-based approaches. Using the specific gene encoding the proteins, we are now able to synthesize large quantities of the therapeutic proteins for treatment purposes.[6,7] Local administration of insulin-like growth factor (IGF-I) in the mandibular condyle of rats has been shown to induce actual bone formation.[8,9]

Although these findings are promising, the possible applications of these growth factors are limited by their short biological half-life which requires repeated administrations and expensive dosages.[10] Recent advances in molecular biology have led to fast progress in the development of gene therapy and the most promising novel approaches to maximally stimulate bone formation in animals as well as in humans.[11] Since Baum and O'Connell[12] first described the potential impact of gene therapy on dentistry, gene therapy has forged its own position as a novel strategy to induce bone formation. Earlier, we reviewed advances of gene therapy for the repair of craniofacial bone defects.[3] By means of this technique, genes can be

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therapeutically used to produce osteoinductive growth factors in the local environment to heal experimental craniofacial defects.[13–15] This has direct and profound implications for the treatment of craniofacial deformities. Most recently, we were able to construct a delivery vehicle where potential therapeutic genes could be delivered to the condyle.[16]

Vascular endothelial growth factor (VEGF) has been shown to play an important role in mandibular condylar growth.[1,17–19] VEGF is a potent regulator of neovascularization expressed during endochondral ossification of the condyle.[1,17] Chondrocytes of the mandibular condyle express VEGF which stimulates neovascularization and marks the onset of endochondral ossification.

Some successful gain of function studies with recombinant VEGF proteins or gene therapy has yielded significant increases in vascularization and bone regeneration in a defects model.[20,21] Local administration of recombinant human vascular endothelial growth factor (rhVEGF) was found to enhance the amount of tooth movement.[22,23] Moreover, rhVEGF administration leads to enhanced blood vessel formation and ossification in bone defects.[20] In vivo gene therapy with adenovirus mediated VEGF proved to modify bone defect healing.[21,24] Thus, VEGF has possible clinical applications for inducing bone formation. More recently, Rabie et al[25] successfully established recombinant adeno-associated virus (rAAV) mediated VEGF delivery system and identified transgene distribution in the condylar cartilage and significant increase in the expression of chondrogenic and osteogenic markers. Therefore, the aim of this study is to further investigate the morphological changes in the mandibular condyles treated by VEGF gene therapy.

MATERIALS AND METHODS

Local Injection of rAAV-VEGF and rAAV-eGFP Into Rat Condyle

The animal experiments were approved by the Committee on the Use of Live Animals in Teaching and Research (CULATR 897-04) of the University of Hong Kong. Ninety 35-day-old female Sprague-Dawley (SD) rats were obtained from the Animal Units of the University of Hong Kong. These SD rats were randomly allotted into one experimental group with rAAV-VEGF injection and two control groups with rAAV-eGFP (enhanced green fluorescence protein) or PBS (phosphate-buffered saline) injection. The body weight of each rat was measured both at the beginning and at the end of the experiment.

After anesthetization of the rats, an anterior-posterior sterile incision, 1.5 cm in length, was made in the skin between the posterior end of the zygomatic arch and the ear cartilage, followed by a blunt dissection to expose the zygomatic arch and the posterior margin of the articular eminence.[16] The 50 μL volume of rAAV-VEGF$_{164}$ (2×10^{11}, genome copies), rAAV-eGFP (2×10^{11}, genome copies) or PBS was slowly injected directly into the posterior attachment of rat condyle through a 30-gauge needle connected with a microsyringe in a downward and backward direction. The needle was allowed to remain in place for another 5 minutes before being slowly retracted at the end of each injection. Care was taken to avoid injecting the blood vessels around the condyle. One interrupted 4-0 silk suture was used to close the incision. Six rats from each subgroup were sacrificed at 7, 14, 21, 30, and 60 days postinjection by an intraperitoneal injection of Dorminal. After death, the heads of those SD rats were first skinned and then dissected into two halves. The left halves were immediately delivered to do the morphological analysis.

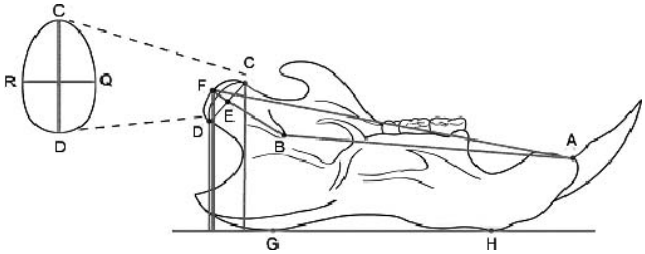

Figure 1. Schematic illustration of method for condylar measurements.[26] A: The most anterior point of the lingual alveolar bone; B: The midpoint of mandibular foramen; C: The most anterior point of condyle; D: The most inferior (posterior) point of condyle; E: The middle of point C and D; F: Intersection point of B-E extension line and outer contour of condyle; G: Posterior–inferior point of attachment of digastric muscle; H: The most inferior point of lower border of angular process; Q: The outermost point of ventral contour of condyle; R: The outermost point of dorsal contour of condyle; BF: Condylar process axis; GH: Mandibular plane.

Morphological Measurement

Gross morphological analysis was carried out as described in detail previously.[26] Digital photos of the lateral view of the left mandibles were taken using a true color video camera (JVC TK-C1380, Tokyo, Japan) to allow for the angular and linear measurements.[26] The condylar head in particular was separated from the mandible and inserted into a homemade columniform abutment for photo taking.[27] The photos were taken through the digital picture capture system with a ruler to standardize the amplification. To increase the accuracy of the small morphological measurement changes, the images were magnified two times the original size with the known scale and were traced with selected landmarks and distance (Figure 1, Table 1). Measurement was blind and evaluated by two inde-

Table 1. Definition of Linear and Angular Measurements[26]

Variables	Definition
B-F	The length of condylar process
A-F	Mandibular length
A-B	Length of mandibular base
C-D	Length of condyle
Q-R	Width of condyle
C-GH	The distance from point C to mandibular plane
F-GH	The distance from point F to mandibular plane
D-GH	The distance from point D to mandibular plane
BF/GH	Angle of condylar process axis to mandibular plane

pendent tracings, which were carried out at an interval of at least 2 weeks. Ten species were randomly selected for the evaluation of method error, which was calculated with Dahlberg's formula $Me = \sqrt{\Sigma d^2/2n}$, where d represents the difference between two registrations and n is the number of duplicate registrations. The method error determined was 0.13 mm for linear measurement and 1.4° for angular measurement, which were both statistically insignificant ($P > .05$).

Statistical Analysis

The statistical analysis was processed with SPSS for Windows (SPSS Inc, Chicago, IL) for one-way analysis of variance (ANOVA) with a Bonferroni multiple comparisons test to compare the mean difference among the experimental group and two control groups at each time point. P values were considered to be statistically significant when less than .05.

RESULTS

Changes in Body Weight During the Experimental Period

A gradual increase in body weight was recognized during the experimental period in both experimental and control groups (Figure 2). However, no significant difference in body weight was detected among the one experimental group and two control groups ($P > .05$).

Morphological Analysis

The length (C-D) and width (Q-R) of the condylar head increased significantly from day 30 to day 60. Moreover, the length of the condylar process (B-F) together with the dependent mandibular length (A-F) significantly increased from day 30 to day 60. The distance from the posterior part of the condylar head (D) to the mandibular plane (GH plane) increased on day 60 followed by the increased distance between the midpoint of the condylar surface (F) to the GH plane. However, the change of position of the point F did not affect the angle of BF/GH. The length of mandibular

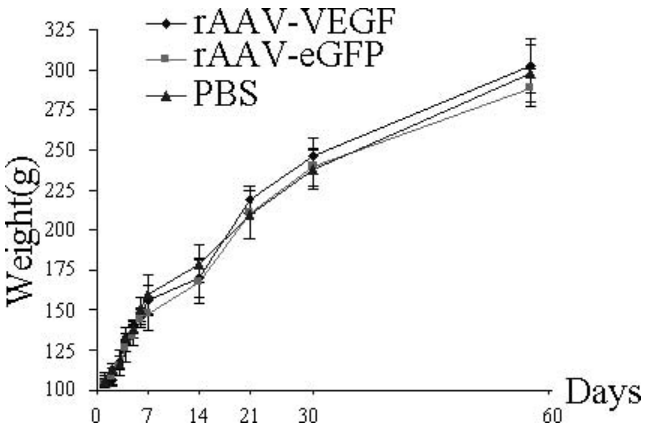

Figure 2. Body weight during the study period. There were no significant differences in body weight among the three groups.

base (A-B) and the distance between the reference point on the most anterior surface of the condyle (C) and the GH plane remained unchanged in both groups throughout the whole period (Table 2).

DISCUSSION

In the present study, we injected the mandibular condyle with rAAV mediated VEGF and demonstrated significant condylar growth. Such growth lasted for the duration of the experiment, which was 60 days after injection. This work should be considered as "proof of principle" that a key regulatory growth factor can stimulate mandibular growth at will.

Growth of mandibular condylar cartilage is partly genetically determined, but is also influenced by exogenic factors including mechanical factors and numerous systemic and local mediators.[28] Furthermore, the effects of mechanical and systemic factors is, in part, mediated by growth factors, which play a central role in the local regulation of cartilage growth and metabolism.[1,29] This gives gene therapy a critical role in the future for management of anomalies related to growth. Gene therapy offers new possibilities to either correct a defective gene in a given tissue or organs or to deliver the lacking growth factors in a given tissue to stimulate a function.

Osteogenesis (the formation of new bone) and angiogenesis (the invasion of new blood vessels) are closely correlated.[30] VEGF, the best-characterized angiogenic factor, has been shown to play an important role in long bone and mandibular condylar growth.[1,31] When VEGF was inactivated in mice through the systemic administration of a soluble receptor chimeric protein (sFlt-1), blood vessel invasion was almost completely suppressed, concomitant with impaired trabecular bone formation and expansion of hypertrophic chondrocyte zone.[31] Some reports demonstrated a successful effect of recombinant VEGF and in vivo

Table 2. Values of Linear and Angular Measurements of Mandibular Morphology in Experimental and Control Groups at Different Time Points[a]

	7 days			14 days			21 days		
	VEGF	eGFP	PBS	VEGF	eGFP	PBS	VEGF	eGFP	PBS
B-F	5.25 ± 0.25	5.30 ± 0.26	5.18 ± 0.26	5.78 ± 0.24	5.69 ± 0.28	5.67 ± 0.30	6.02 ± 0.34	5.73 ± 0.36	5.83 ± 0.43
A-F	22.7 ± 0.41	22.55 ± 0.57	22.35 ± 0.76	22.34 ± 0.51	22.61 ± 0.68	22.54 ± 0.51	24.28 ± 0.47	24.10 ± 0.58	24.19 ± 0.32
A-B	18.00 ± 0.26	18.15 ± 0.49	18.23 ± 0.34	18.18 ± 0.52	18.54 ± 0.16	18.46 ± 0.51	18.56 ± 0.49	18.61 ± 0.41	18.81 ± 0.23
C-D	3.26 ± 0.21	3.21 ± 0.42	3.12 ± 0.12	3.32 ± 0.19	3.19 ± 0.17	3.25 ± 0.19	3.38 ± 0.27	3.31 ± 0.19	3.27 ± 0.28
Q-R	1.24 ± 0.15	1.27 ± 0.12	1.26 ± 0.15	1.38 ± 0.11	1.35 ± 0.18	1.34 ± 0.13	1.54 ± 0.12	1.41 ± 0.13	1.43 ± 0.13
C-GH	8.83 ± 0.25	8.64 ± 0.16	8.73 ± 0.18	9.26 ± 0.17	9.11 ± 0.12	9.27 ± 0.16	10.11 ± 0.26	10.18 ± 0.12	10.26 ± 0.23
F-GH	8.54 ± 0.18	8.36 ± 0.15	8.47 ± 0.19	9.17 ± 0.31	8.91 ± 0.21	9.02 ± 0.16	9.67 ± 0.35	9.53 ± 0.38	9.87 ± 0.39
D-GH	7.27 ± 0.13	7.14 ± 0.12	7.21 ± 0.17	7.75 ± 0.32	7.77 ± 0.24	7.82 ± 0.21	8.30 ± 0.39	8.15 ± 0.22	8.51 ± 0.25
BF/GH	42.51 ± 2.60	42.33 ± 1.50	42.17 ± 1.72	42.75 ± 3.44	43.25 ± 1.95	43.17 ± 2.92	41.28 ± 2.46	42.08 ± 3.18	43.64 ± 2.54

[a] VEGF indicates vascular endothelial growth factor; eGFP, enhanced green fluorescence protein; PBS, phosphate-buffered saline.
* $P < .05$; ** $P < .01$.

gene therapy on bone formation.[20,21] The advance of virus research has brought rAAV into the limelight of viral gene therapy. The major advantages of rAAV include the potential for long-term transgene expression, less pathogenicity, low immunogenicity, and the ability to infect a broad range of host tissues including both dividing and nondividing cells.[3] Most recently, we first reported that transgene can be transferred to the mandibular condyle in vivo by rAAV delivery.[16]

The reporter gene of eGFP, an exogenous intracellular molecule, was used for in situ identification of delivered gene[32] and to eliminate the role of delivery vehicle. The local delivery of rAAV-VEGF in the present study resulted in elongated mandibular condylar growth in the posterior part of the condyle. This was due to the fact that VEGF was locally delivered into the mandibular condyle through the posterior attachment. The rAAV-VEGF vector (22–25 nm in size) was small enough to diffuse from the injected joint space to the cell surface layers, and then deeper to infect chondrocytes and hypertrophic chondrocytes and to make them secrete the growth factor, VEGF, to regulate the microenvironment in the condyle.[25] Moreover, in the previous study, we extracted RNA from the remote organs of liver, kidney, heart, and spleen of rAAV-VEGF injected animals for reverse transcription polymerase chain reaction (RT-PCR) analysis. The exogenous VEGF was not detected, thereby reducing the prospects of systemic adverse effects.[25]

Treated by VEGF gene therapy, the growth of the condyle ultimately resulted in the increase in mandibular length (A-F); no increase was identified in the length of mandibular base (A-B); and the distances of point F as well as point D to mandibular plane demonstrated an increase, but the position of point C was kept stable. Moreover, the changed position of point F did not affect the angle of condylar process to the mandibular plane (BF/GH). Thus, it pointed out that mandibular condylar growth occurred in a backward and upward direction.

After continuous bite-jumping treatment, the most prominent cellular response was documented in posterior surface of the condyle, but not in the anterior surface. The condyle expressed its adaptability in terms of directional changes in mandibular growth,[1] the distance from point F and D to GH was reduced, together with the reduction of the angle of the condylar process to the mandibular plane, which displaced the condylar process backwards but downwards.[26] Hence, the directional remodeling of mandibular condyle by gene therapy and bite-jumping treatment was different in the vertical direction, but similar in the sagittal direction. Gene therapy is still in its infancy stage for experimental growth of the mandibular condyle. We hope that in the near future we can target cells in the superior layer of the condyle that could result in a more upward growth of the condyle and thus help in regulating direction of growth.

One interesting finding in this study was the delayed effect on morphological change. Five time points, 7, 14, 21, 30, and 60 days, were selected because each of these time points demonstrate the peak expression of one of the growth factors expressed during induced growth of the mandible.[1,18] For example, VEGF expression was evident on day 14, while chondrogenesis peaked on day 21, and osteogenesis on day 30 during mandibular advancement. Day 60 was added to determine whether the gene expression was still evident after the injection for a longer time. On day 7, 14, and 21, all the measured morphological parameters of the rats' mandibles showed no significant difference among the rAAV-VEGF delivery, rAAV-eGFP, and PBS injected group. From day 30, parameters of condylar width and length demonstrated a significant increase. Up to day 60, more parameters showed significant change. Since it was known that the rAAV vector exists as a single stranded DNA virus, while the viral genome is transported to the nucleus within minutes after infection,[33] in vivo transduction takes days to weeks as a consequence of lagging second-strand

Table 2. Extended

30 days			60 days		
VEGF	eGFP	PBS	VEGF	eGFP	PBS
6.55 ± 0.44	5.85 ± 0.23**	6.06 ± 0.22*	6.58 ± 0.46	6.07 ± 0.19*	6.05 ± 0.18*
26.21 ± 0.42	25.33 ± 0.23*	25.44 ± 0.60*	27.13 ± 0.61	26.35 ± 0.40*	26.37 ± 0.34*
19.66 ± 0.37	19.83 ± 0.31	19.74 ± 0.28	21.28 ± 0.37	20.97 ± 0.39	20.88 ± 0.32
3.63 ± 0.17	3.39 ± 0.20*	3.41 ± 0.15*	3.68 ± 0.21	3.27 ± 0.19**	3.25 ± 0.17**
1.64 ± 0.12	1.46 ± 0.10*	1.45 ± 0.11*	1.72 ± 0.11	1.56 ± 0.07*	1.52 ± 0.05**
10.38 ± 0.3	10.40 ± 0.11	10.31 ± 0.14	10.77 ± 0.50	10.54 ± 0.14	10.55 ± 0.24
9.96 ± 0.26	9.77 ± 0.19	9.84 ± 0.29	10.36 ± 0.20	9.87 ± 0.24**	9.93 ± 0.21**
8.35 ± 0.33	8.46 ± 0.33	8.44 ± 0.21	9.28 ± 0.34	8.58 ± 0.33**	8.69 ± 0.36**
40.24 ± 4.02	42.31 ± 3.66	41.83 ± 3.28	38.54 ± 4.10	41.22 ± 3.03	42.80 ± 2.39

synthesis.[34] On the other hand, VEGF expressed on the basis of extractable protein from mandibular condyle was significantly increased from day 14, and the biochemical analysis showed that osteogenic markers of alkaline phosphatase activity and osteocalcin content significantly increased from day 30.[25] Taken together, it was consistent with the earlier reports that VEGF expression was closely correlated to mandibular condylar growth, which preceded the peak of bone formation.[1,19,27] In the near future, it is necessary to know whether these genetically treated rats need reinjection or inhibition to avoid relapse or overgrowth. Therefore, the long-term effect on mandibular condylar growth by VEGF gene therapy needs to be investigated further.

CONCLUSIONS

- The present study provides further evidence that local rAAV mediated VEGF gene transfer enhances the size of mandibular condyle leading to mandibular condylar growth. It provides the basis to regulate mandibular condylar growth for future clinical practice.

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