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Gene therapy: an alternative strategy for correction of severe craniofacial deformities

ABSTRACT

Objective. Gene therapy is a novel strategy for correction of severe deformities. To test whether such therapy could be used to control condylar growth, an experimental model entailing Sprague-Dawley rats was developed. **Methods.** Either recombinant adeno-associated virus (rAAV)–mediated angiogenesis inhibitor (experimental group) or rAAV-mediated reporter expressing green fluorescent protein (control group) was transduced into the temporomandibular joint of Sprague-Dawley rats for 3 weeks. Rats in both groups were sacrificed and samples were collected for image analysis, immunostaining, periodic acid-Schiff staining, and tartrate-resistant acid phosphatase staining. Vascular formations, bone formation, and osteoclast activities of the two groups were compared. **Results.** Virus vector–mediated angiogenesis inhibitor expression in condylar cartilage inhibited vascular formation, bone formation, osteoclast activity, and condylar growth. **Conclusion.** Gene therapy can be considered an alternative strategy for the correction of severe craniofacial growth deformities.

Key words: Angiogenesis inhibitors; Craniofacial dysostosis; Gene therapy/methods; Rats, Sprague-Dawley

Introduction

Condylar overgrowth is one of the major symptoms of many congenital and acquired craniofacial deformities [1-4]. However, restraint of this growth is considered to be one of the most difficult targets to achieve clinically [5-7]. Treatment options like orthognathic surgery have been widely used and efficiently conducted. Nevertheless they have limitations, in that they give rise to temporomandibular disorders, condylar resorption, neurosensory disturbance, infection, and relapse [8-10]. Therefore, exploration of novel strategies could provide alternative treatment options for affected patients.

In the past few years, important progress has been reported with therapeutic strategies to overcome craniofacial defects, including matrix-based, cell-based, and factor-based therapies [11]. Matrix-based therapies introduce biocompatible materials to provide bio-environments for proper bone remodeling, but their role as vehicles only seems to weaken their importance. Cell-based therapies aim to transfer cells with therapeutic potential into the target sites and achieve positive effects, but are limited by the number of donor cells that can be obtained. Factor-based therapies

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have boomed due to the development of recombinant DNA technology, but need to be applied repeatedly; the short half-life of the relevant factors being one possible reason [11]. Gene therapy using recombinant adeno-associated virus (rAAV) vectors combines the advantage of facilitating therapeutic gene delivery and mediating the expression of any therapeutic gene at a given site, which makes it a better strategy to develop for treating craniofacial defects [12].

Our earlier study showed that gene therapy using rAAV-mediated vascular endothelial growth factor enhanced condylar growth in an animal model [13]. Recombinant AAV type 2 (rAAV2) vector was also reported to be safe and capable of mediating long-term transgene expression, without inducing overt systemic side-effects [12,13]. As an extension of our interest in gene therapy, in this study we used rAAV2 as the vector to inhibit condylar growth. It was reported that angiogenesis is critical for condylar growth [14], and ultimately facilitates conversion of cartilage to bone [15]. In this study, we therefore used an angiogenesis inhibitor known as vastatin [16] as the therapeutic gene to inhibit condylar growth.

The aim of this study was to examine whether such gene therapy could be used to retard condylar growth in a Sprague-Dawley (SD) rat model.

Methods

Virus construction

Recombinant AAV vectors were generated by a two-plasmid packaging system with minor modifications [17]. Briefly, the expression plasmid and the helper plasmid were co-transfected into human embryonic kidney 293FT cells (American Type Culture Collection, USA) by calcium phosphate precipitation. Cell pellets were collected and processed for heparin column (Sigma-Aldrich, USA) purification after culture for 60 to 72 hours. Purified virus particles were quantified by real-time polymerase chain reaction and stored at -80°C. The schematic illustration of expression plasmids (pAAV-vastatin, pAAV expressing green fluorescent protein [pAAV-eGFP]) is shown in Figure 1.

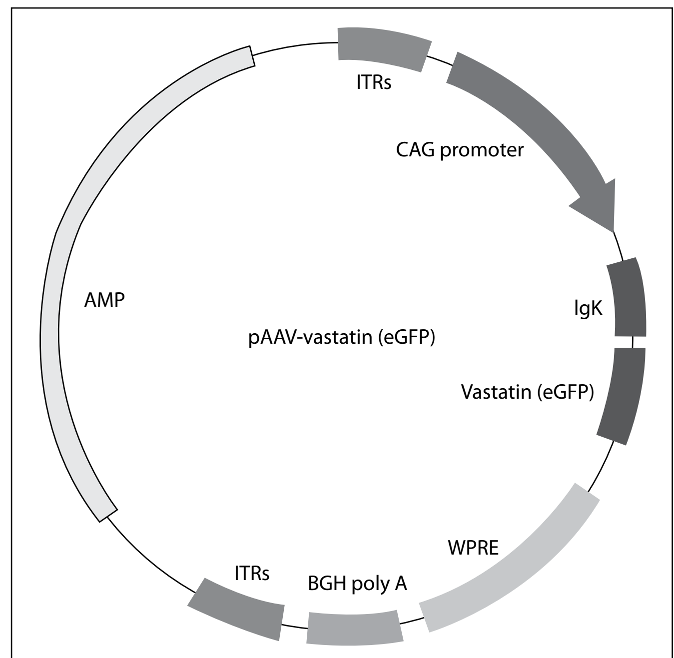

Figure 1 The schematic diagram of expression plasmids (pAAV-vastatin, pAAV-eGFP)*

* AAV=adeno-associated virus; Vastatin=vastatin sequence cloned from the human collagen type VIII; IgK=IgK leader cloned from pSecTag2A; eGFP=enhanced green fluorescent protein; WPRE=woodchuck hepatitis B virus postregulatory element; BGH poly A=bovine growth hormone polyadenylation signal; ITRs=inverted terminal repeats; AMP=ampicillin; CAG=cytomegalovirus enhancer plus chicken beta-actin

Experimental animal

Animal work in this study was approved by the committee on the use of live animals in teaching and research of the University of Hong Kong. Surgical procedures and animal care were carried out according to institutional regulations. Sixteen 30-day-old female SD rats weighing 100±5 grams were randomly divided into experimental and control groups. They were injected with either rAAV-vastatin (producing the experimental group) or rAAV-eGFP (producing the control group) into both temporomandibular joints (TMJs) at a dosage of 2 x 10^{11} vg. The method of injection was as described in earlier reports [13,18]. The rats were sacrificed after 3 weeks by intraperitoneal overdosing with dorminal.

Sample collection

The right mandibles of rats were immediately fixed with 4% paraformaldehyde. Digital images of the mandibles were

obtained for later measurements as previously described [13]. The condylar process of each mandible was collected and decalcified for about 3 weeks, and then double-embedded in paraffin blocks, and continuous sections were prepared as explained before [19].

Staining methods

A standard avidin-biotin complex method of immunostaining was used to examine the formation of blood vessels in condylar cartilage [11]. Primary antibody polyclonal anti-rabbit von Willebrand factor VIII (Blood vessel staining kit ECM 590; Chemicon International, California, USA) and secondary antibody biotinylated polyclonal goat anti-rabbit immunoglobulins (Dako A/S, Denmark) were used. Signals were visualized using DAB chromogen substrate (Chemicon International). Periodic acid-Schiff (PAS) [reagent] staining [20] and tartrate-resistant acid phosphatase (TRAP) staining (Leukocyte Acid Phosphatase Kit; Sigma, St. Louis, USA) were carried out as per previous descriptions [21].

Image analyses

A computer-assisted image analyzing system (Leica DC300 V2.0; Leica, Wetzlar, Germany) installed with software (QWin V2.4; Leica, Cambridge, UK) was connected with a three-channel red-green-blue color video camera to capture images from stained slides. The QWin V2.4 software was used for measurements and obtaining counts from captured images [22].

Quantitative analyses

Slides obtained from both groups were stained simultaneously in the same staining system in order to balance deviations. For each slide, three independent image analyses were carried out and the mean values recorded. At least five stained slides from each group were chosen for statistical analysis.

Statistics

Data were analyzed using the Statistical Package for the Social Sciences Windows version 15.0. The data were evaluated for normality by the Kolmogorov-Smirnov test. Independent sample *t* tests were used to analyze differences between means for the results from PAS staining, TRAP staining, and immunostaining. A P value of smaller than 0.05 was considered statistically significant.

Results

The expression of therapeutic gene in condylar cartilage

Our earlier study examined the expression pattern of transgene mediated by rAAV2 in condylar cartilage after 3 weeks of injection, in which it was clearly shown that at this time-point the expression of vastatin is located in the pre-hypertrophic chondrocyte layer and hypertrophic chondrocyte layer [19]. Based on the results of immunostaining, the extent of expressed vastatin in the cartilage layers mentioned above could be derived [19].

Neovascularization of condylar cartilage

The quantitative analyses of von Willebrand factor VIII immunostaining revealed that on average the numbers of newly formed blood vessels in the erosive layer of condylar cartilage of the experimental group were reduced by 21.9% compared with the controls (Table 1). This indicated that angiogenesis of condylar cartilage was retarded.

Bone formation

The high gray area of Figure 2 represents the newly formed bone by PAS staining. The percentage of bone formation in a given frame (500 μm x 300 μm) in the subchondral area of the condyles were compared, and revealed that on average bone formation in the experimental group was reduced by 32.6% when compared with the controls (Table 2). The data showed that bone formation was delayed in the experimental group.

Table 1 New blood vessel formation in condylar cartilage after 3 weeks of treatment *

	Control group	Experimental group	Mean difference	P value
Mean (±standard deviation) No. of small capillaries	42.1±2.3	32.9±1.7	9.2†	0.001

* At least five stained slides from each group were chosen for comparison
† Compared with the controls, the mean value for the numbers of small capillaries was 21.9% smaller in the experimental group

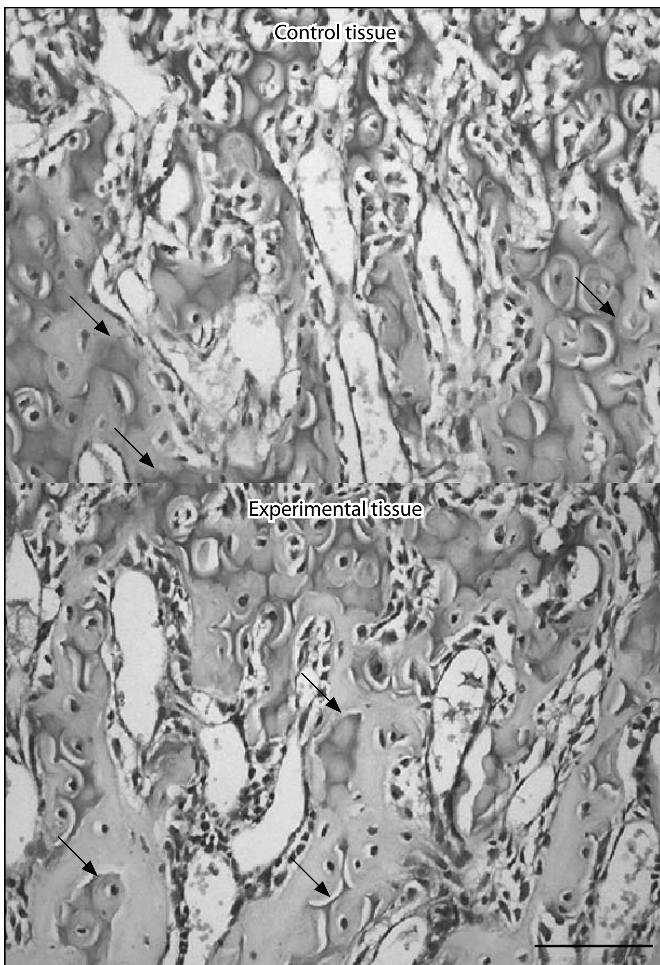

Figure 2 New bone formation examined by periodic acid-Schiff (PAS) staining. The high gray area represents the sites where new bone was formed. The black arrows indicate the area of new bone formation. New bone formation in the experimental group was less evident than that in the control group. Error bar: 100 µm

Table 2	**New bone formation in condylar cartilage after 3 weeks of treatment** *			
	Control group	**Experimental group**	**Mean difference**	**P value**
Mean (±standard deviation) area of new bone formation (%)	11.2±1.4	7.5±1.0	3.6†	0.001

* At least five stained slides from each group were chosen for comparison
† Compared with the controls, the mean value for new bone formation was 32.6% smaller in the experimental group

Number of osteoclasts

The numbers of TRAP-positive osteoclasts in a given frame (500 µm x 300 µm) in the subchondral area of the condyles were counted and compared. On average, TRAP-positive osteoclasts in the experimental group were reduced by 13.4% (Table 3). This demonstrated that there were fewer osteoclasts in the condylar cartilage of the experimental group.

Table 3	**Number of osteoclasts in condylar cartilage after 3 weeks of treatment** *			
	Control group	**Experimental group**	**Mean difference**	**P value**
Mean (±standard deviation) No. of osteoclasts	17.7±1.7	15.3±2.0	2.4†	0.034

* At least five stained slides from each group were chosen for comparison
† Compared with the controls, the mean value for the numbers of osteoclasts was 13.4% smaller in the experimental group

Condylar growth

The length of the condylar process (gray line in Fig 3a) was used to reflect the growth of condyle. From the scanned images of mandibles, the average length of the condylar process in the experimental group was shorter (Fig 3b), indicating inhibition of condylar growth in the experimental group after angiogenesis inhibitor treatment.

Discussion

This work demonstrated that gene therapy using rAAV2 as the therapeutic vector can limit condylar growth in an animal model of SD rats.

Recombinant AAV2 is a non-pathogenic, replication-defective small DNA virus, which can infect both dividing and non-dividing cells [23]. Its ability to mediate long-term transgene expression without triggering a cellular response makes it an efficient and safe vector to select in preference to other current available vectors [24]. Our earlier work showed that for different periods of time, rAAV2 can mediate transgene expression in condylar cartilage, the glenoid fossa, and TMJ discs. Moreover, the location of transgene expression changed depending on the time-points at which transfected chondrocytes underwent their pre-hypertrophic and hypertrophic phases [19]. The tissue structure and histology of condylar cartilage appeared to be normal after gene delivery as observed from the immunostaining results [19]. Therefore, rAAV2 can be considered a very promising vector for the treatment of TMJ growth deformities.

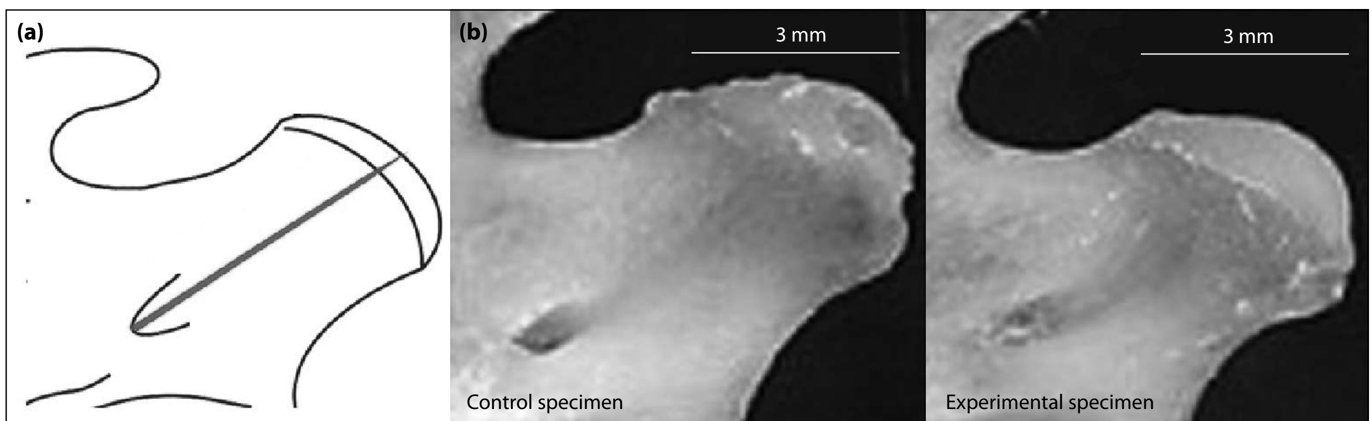

Figure 3 Condylar growth examined by scanned images of the peeled samples of mandibles from Sprague-Dawley rats. (a) Schematic diagram explaining the growth of condyle. The thick gray line indicates the length of the condylar process (representing the amount of growth). (b) The length of the condylar process in the experimental specimen was shorter than the control specimen, which demonstrates that condylar growth was inhibited after 3 weeks of active treatment

Neovascularization is essential for cartilage transition to bone, and as such, is important for bone and condylar growth [14]. Targeting angiogenesis to inhibit neovascularization of condylar cartilage is therefore a promising strategy to control condylar growth. In this study, the local delivery of the angiogenesis inhibitor vastatin inhibited the process (Table 1) and delayed bone formation (Table 2). In the experimental group, the numbers of osteoclasts were also reduced (Table 3). According to the literature, the circulation is the major source of osteoblasts and osteoclast precursors [25]. Thus, it is possible that limiting neovascularization could reduce the source of these precursor cells, and the consequential reduction of osteoblasts and osteoclasts may contribute to delayed bone formation and cartilage turnover. In which case, growth retardation of condyles (Fig 3) after treatment could well be a cumulative effect of delayed bone formation (Table 2).

The application of gene therapy as an interventional strategy in dentistry, though still in its infancy, reveals an alternative option for the management of patients with severe craniofacial growth deformities. Regarding the technical aspects of gene therapy, although there is still a need to improve specificity and achieve better control of gene-mediated expression, this approach deserves further exploration as a potential therapeutic vector. With the development of molecular biology and the emergence of novel techniques, delivery of a therapeutic gene into a specific organ (the condyle) may be possible, as a means of overcoming condylar overgrowth in patients with severe deformities.

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