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Comparison of Efficacy of Endogenous and Exogenous IGF-I in Stimulating Matrix Production in Neonatal and Mature Chondrocytes

Izath N. Aguilar[1], Stephen B. Trippel[2,3], Shuiliang Shi[2], and Lawrence J. Bonassar[1,4]

Abstract

Objective. The goal of this study was to compare the efficacy of endogenous upregulation of IGF-I by gene therapy and exogenous addition of insulin-like growth factor I (IGF-I) in enhancing proteoglycan synthesis by skeletally mature and neonatal chondrocytes. Chondrocyte transplantation therapy is a common treatment for focal cartilage lesions, with both mature and neonatal chondrocytes used as a cell source. Additionally, gene therapy strategies to upregulate growth factors such as IGF-I have been proposed to augment chondrocyte transplantation therapies. *Methods*. Both skeletally mature and neonatal chondrocytes were exposed to either an adeno-associated virus-based plasmid containing the IGF-I gene or exogenous IGF-I. *Results*. Analysis of IGF-I and glycosaminoglycan production using a 4-parameter dose-response model established a clear connection between the amount of IGF-I produced by cells and their biosynthetic response. Both neonatal and mature chondrocytes showed this relationship, but the sensitivities were quite different, with EC50 of 0.57 ng/mL for neonatal chondrocytes and ED_{50} of 8.70 ng/mL IGF-I for skeletally mature chondrocytes. *Conclusions*. These data suggest that IGF-I gene therapy may be more effective with younger cell sources. Both cell types were less sensitive to exogenous IGF-I than endogenous IGF-I.

Keywords

Gene Therapy, Osteoarthritis, Cartilage Development, Growth Factor

Introduction

Traumatic joint injuries resulting in damage to articular cartilage are common in young adults, leading to osteoarthritis later in life.[1] Among the more promising treatments for focal cartilage lesions is chondrocyte transplantation therapy, which can generate tissue resembling hyaline cartilage after 6 to 8 months.[2] While this technique is often successful, frequently the tissue formed at the implant site contains type I collagen that can progressively degenerate to a fibrocartilaginanous scar tissue and ultimately fail.[2] As such, effort has been focused on enhancing chondrocyte matrix production for cartilage repair. A common approach to enhance matrix production is through growth factors. Specifically insulin-like growth factor I (IGF-I), a potent anabolic regulator of metabolism, has been widely studied as a means to enhance chondrocyte matrix synthesis.

Insulin-like growth factor I is a 70 amino acid polypeptide that stimulates proliferation and matrix synthesis by chondrocytes.[3] The receptor engagement and subsequent activation are determined by the availability of free IGF-I.[4] Unfortunately, IGF-I delivered to joints is rapidly cleared, so direct addition of IGF-I peptide requires multiple large doses. Additionally, chondrocytes are surrounded by a dense matrix and their supply of IGF-I is limited by transport of this protein through the dense tissue. This transport through cartilage is further limited by its binding to IGF-I binding proteins,[5] limiting its availability to chondrocytes. For these reasons, direct *in vivo* administration of IGF-I protein for cartilage repair is likely to be highly inefficient.

Gene therapy is a valuable tool to control local protein production to articular tissues that be used to improve approaches to the treatment of injured cartilage.[6-12] Although recombinant proteins, such as IGF-I, are difficult to apply

[1]Department of Biomedical Engineering, Cornell University, Ithaca, NY, USA
[2]Department of Orthopaedic Surgery, Indiana University School of Medicine, Indianapolis, IN, USA
[3]Department of Anatomy and Cell Biology, Indiana University School of Medicine, Indianapolis, IN, USA
[4]Sibley School of Mechanical and Aerospace Engineering, Cornell University, Ithaca, NY, USA

Corresponding Author:
Lawrence J. Bonassar, Department of Biomedical Engineering, Cornell University, 149 Weill Hall, Ithaca, NY 14853, USA.
Email: lb244@cornell.edu

effectively *in vivo*, gene delivery provides a mechanism to enable sustained protein production by targeted cells. The most frequent delivery vectors used for IGF-I are viruses and nonviral vectors. Adeno-associated virus (AAV) is a nonpathologic human parvovirus and persists in nondividing cells for extended periods of time and can transduce normal and osteoarthritic human articular cartilage *in vitro*.[13] Saxer *et al*.[6] used adenoviral vectors (Ad.IGF-I) to express IGF-I in synovial tissue of horses for 8 days, with peak expression on day 4. Also, adenovirus vectors have been used to transfect chondrocytes harvested from human cartilage for up to 21 days, with considerable vector leakage from the gel used as a carrier.[11] Although Ad.IGF-I and rAAV vectors have high transduction efficiency, both have shortcomings such as the potential of significant inflammatory immune responses for Ad.IGF-I and the difficulty to produce high enough titer of rAAV viral vectors to efficiently transduce chondrocytes.[10] Such, developing nonviral gene delivery remains an important goal.

Several studies have investigated transfection of chondrocytes with plasmid DNA for IGF-I gene therapy. Such methods require lipid-based reagents such as FuGENE or Lipofetamine to enhance DNA uptake. Using such methods, plasmid-based gene therapy has enhanced IGF-I production and matrix synthesis in monolayers and 3-dimensional alginate cultures *in vitro* and *in vivo*.[14] Additionally, transplantation of chondrocytes transfected with IGF-I plasmid DNA enhanced cartilage formation and defect repair *in vivo* for 32 days.[8] A more recently developed plasmid (pAAV/IGF-I) has been shown to have higher transfection efficiency than pcDNA containing IGF-I.[15] Both skeletally mature[16] and neonatal[17] chondrocytes are used clinically for cartilage repair. The mechanisms by which specific growth factors such as IGF-I regulate cartilage metabolism are age dependent.[18] However, it is unclear whether adult or juvenile chondrocytes are the best targets for IGF-I gene therapy. Available data indicates that there is a differential response to exogenous IGF-I between adult and juvenile chondrocytes. In articular cartilage explant culture, exogenous IGF-I increased collagen synthesis by both adult and juvenile chondrocytes, with a more marked reponse in adult than juvenile cartilage[18] In isolated chondrocytes in monolayer and 3D culture exogenous IGF-I increased proteoglycan and protein synthesis with a greater response by juvenile than adult cells[19], and it was hypothesized that juvenile chondrocytes are more responsive to exogenous IGF-I than adult chondrocytes. More recently, studies with human articular cartilage indicated that chondrocyte responsiveness to IGF-I progressively diminished with age[20]. To our knowledge, the application of IGF-I gene therapy to mature and neonatal chondrocytes has not been compared.

The relative ability of these cells to be transfected with IGF-I, and the ability of the transfected cells to respond to this treatment, are unknown. The goals of this study were to determine the efficiency of pAAV/IGF-I transfection of skeletally mature and neonatal chondrocytes, to compare the effects of this transfection on stimulation of proteoglycan synthesis and to compare the response of these cells to endogenously produced and exogenously added IGF-I.

Methods

Endogenous IGF-I

Articular chondrocytes were harvested from carpal joints of four skeletally mature (growth plate closed) and from the stifle (knee) condyles of five 1- 3-day-old bovids via collagenase digestion overnight. Isolated chondrocytes (9×10^5 cells/well) were placed in 6-well plates with 4 mL of DMEM (Dulbecco modified Eagle's medium) containing 100 U/mL penicillin, 100 μg/mL streptomycin, and 10% fetal bovine serum (FBS) at 37°C in 5% CO_2. After 48 hours, cells were transfected in DMEM using a complex of FuGENE 6 (Roche Applied Science, Indianapolis, IN) and pAAV plasmids carrying IGF-I or multiple cloning site (MCS) referred as the empty vector at a ratio of 3:1,[15] made 30 minutes before transfection. After 16 hours, the transfection was stopped by replacing 2 mL of fresh media without FBS, and 100 U/mL penicillin, 100 μg/mL streptomycin. There were six transfected (pAAV/IGF-I) and untransfected wells maintained in culture for 6 days. pAAV/empty neonatal chondrocytes were used as a secondary control. For each of the four animals of each age, the media was collected from both transfected and untransfected cells on days 2, 4, and 6. The IGF-I content in the media was measured with the DueSet ELISA kits from R&D Systems (Minneapolis, MN). GAG produced was normalized to the amount of GAG produced by untransfected chondrocytes at day 2 in each experiment for Fig.1B and 2B. All data were analyzed using 2-way and 3-way ANOVA analysis with Tukey Test for post-hoc analysis($p<0.05$ for significance).

Exogenous IGF-I

Articular chondrocytes were harvested from 2 skeletally mature (growth plate closed) and two 1- to 3-day old bovids. Cartilage samples were cut away from the periosteum with a thickness of 1 to 2 mm and placed in collagenase digestion over night. Both populations of chondrocytes were placed in 6 well plates at the same density as above. Cells were maintained in media containing IGF-I (Peprotech, Rocky Hill, NJ) at concentrations 0.1, 0.3, 1, 3, 10, 30, and 100 ng/mL,to have comparable range of concentrations with endogenous IGF-I. For both experiments cultures were performed in triplicate at all IGF-I concentrations, with media collected on days 2, 4 and 6. For both experiments, endogenous and exogenous, glycosaminoglycan (GAG) content

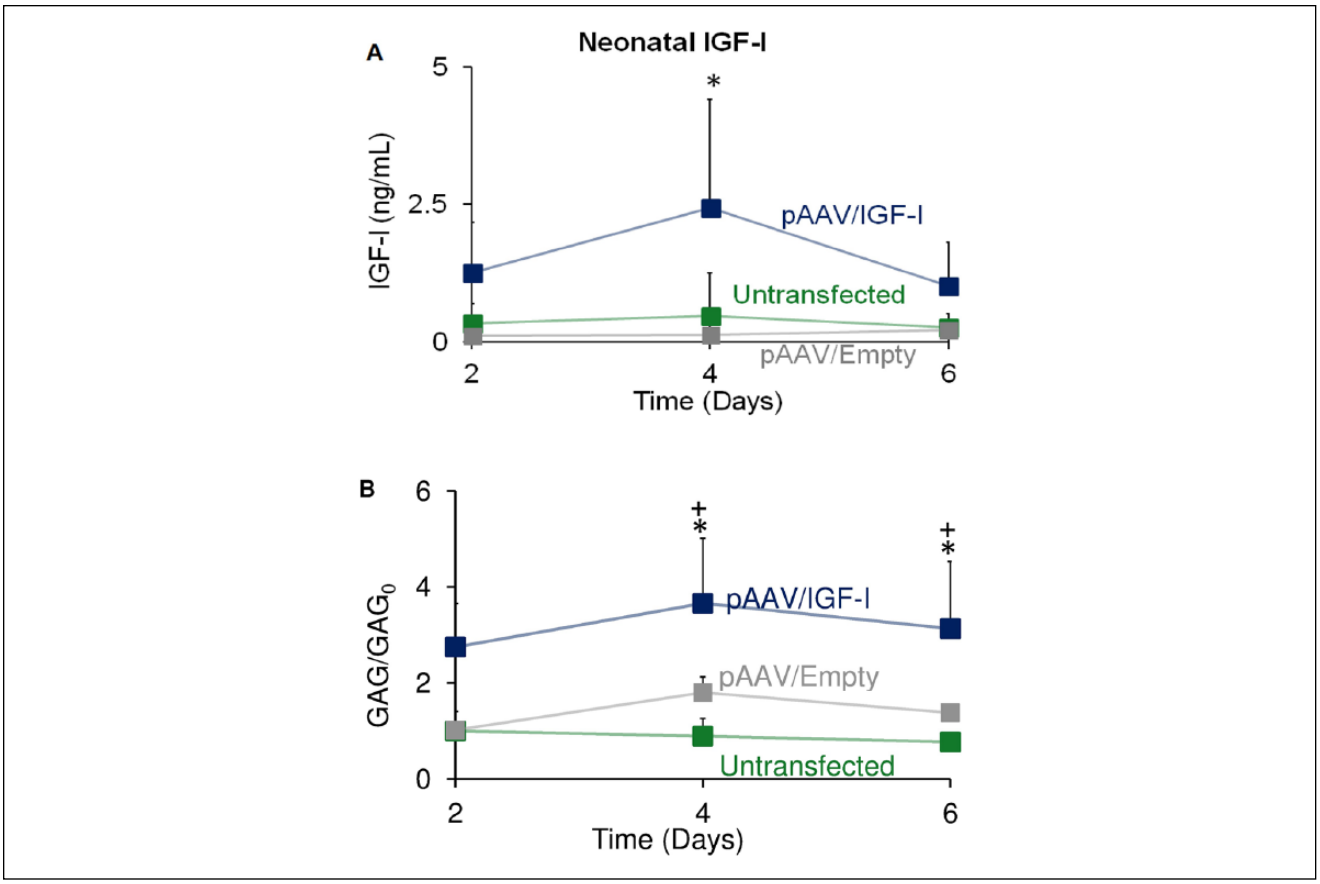

Figure 1. Neonatal chondrocytes. $N = 8$ experiments with 3 wells per experiments. Data are normalized to day 2 values for untransfected chondrocytes. (**A**) insulin-like growth factor I (IGF-I) and (**B**) glycosaminoglycan (GAG) produced in media at the designated time periods. $^{*}P < 0.05$ with respect to untransfected chondrocytes. $^{+}P < 0.01$ with respect to transfected skeletally mature chondrocytes.

was measured using DMMB (1,9-dimethylmethylene blue) dye[21] on both the cell layer and media. Cell proliferation was assessed by DNA and was measured using Hoechst 33258 dye[22] on the cell layer previously digested with cell lysis buffer from BD PharmLyse. IGF-I released at all time points was normalized to the minimal amount of IGF-I released by untransfected chondrocytes at day 2 in each experiment. Similarly, GAG produced was normalized to the amount of GAG produced by untransfected chondrocytes at day 2 in each experiment. All data were analyzed using analysis of variance on ranks with Kruskal-Willis test for *post hoc* analysis ($P < 0.05$ for significance).

Concentration-Response Model

In endogenous stimulation studies, a concentration-response model was used to assess the extent to which the amount of IGF-I produced in each experiment influenced GAG production. For each transfection experiment (i.e. mature or neonatal chondrocytes), the total amount of GAG released over 6 days in culture in three wells was plotted against the average of concentration of IGF-I released in the same three wells over the course of culture. For exogenous stimulation studies, the average of the total of the GAG released in three wells was plotted against the total IGF-I added to the media, with exogenous IGF-I concentrations chosen to span the range of those resulting from transfection studies.

Data from each condition (mature vs. neonatal and endogenous vs. exogenous) was fitted to a generalized variable slope concentration response (VSCR) model to determine if a first-order binding mechanism explained the differences in GAG production and IGF-I release (Equation 1).

$$\mathrm{GAG} = A + \frac{B - A}{1 + \left(\frac{\mathrm{IGF\text{-}I}}{\mathrm{EC}_{50}}\right)^{-D}}. \quad (1)$$

The 4-parameter model included the baseline stimulation (A) and maximal stimulation (B) parameters, EC_{50}, which is the concentration required to provoke a response of half-maximal stimulation, and the Hillslope (D), which characterizes the steepness of the curve. The VSCR model is a

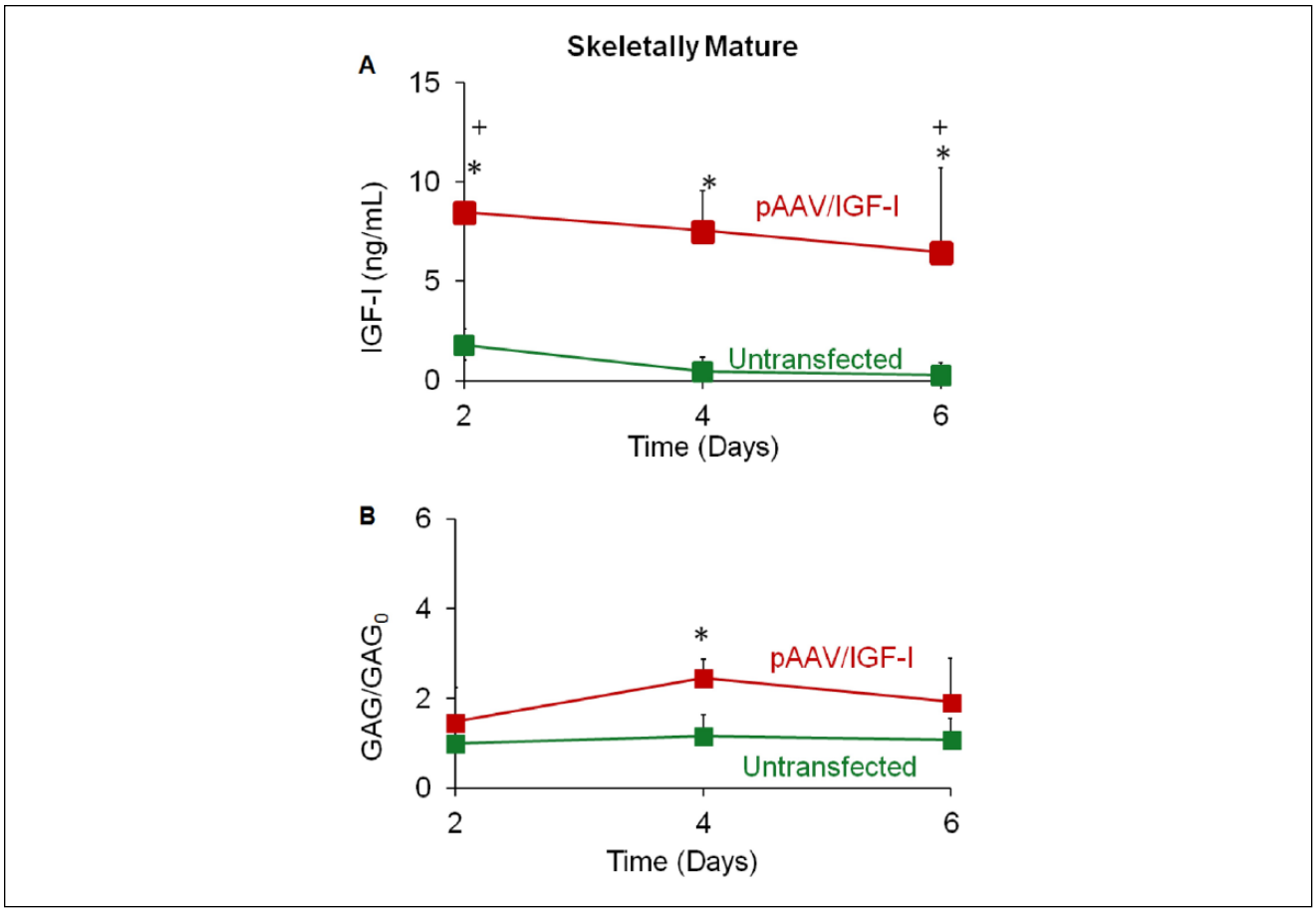

Figure 2. Skeletally mature chondrocytes. *N* = 7 experiments with 3 wells per experiment. Data are normalized to day 2 values for untransfected chondrocytes (**A**) insulin-like growth factor I (IGF-I) and (**B**) glycosaminoglycan (GAG) produced in media at the designated time periods. **P* < 0.01 with respect to untransfected chondrocytes. ^{+}P < 0.01 with respect to transfected neonatal chondrocytes.

general form of an agonist binding model that does not assume that the observed effect is linearly proportional to the agonist binding ratio. If the effect is first order, then the value for Hillslope is 1 and the equation reduces to the classic Langmuir binding/chemisorptions isotherm.[23,24] Because each condition had different baseline amounts of GAG production, the GAG production of each group was normalized to the baseline stimulation (i.e. GAG production at 0 ng/ml IGF-I predicted by the model) to calculate the relative increase in GAG production at maximal stimulation. Thus for all model fits, the parameter A is set to 1 and B represents the level of maximal stimulation.

Results

IGF-I Release and GAG Production

The production of IGF-I and GAG were compared between pAAV/IGF-I and pAAV/Empty vectors and untransfected controls for neonatal chondrocytes. The empty vector had no effect in either IGF-I or GAG production (Fig. 1A &1B). Data presented are averages of 3 (pAAV/Empty) or 8 (pAAV/IGF-I and untrasnfected) experiments, with each condition cultured in triplicate. pAAV/IGF-I increased IGF-I production by both mature and neonatal chondrocytes when compared to unstransfected controls or pAAV/Empty. On day 2, transfected neonatal chondrocytes increased IGF-I production from 0.34±0.4 ng/mL (unstransfected) to 1.3±1 ng/mL (transfected)) (p= 0.095) (Fig. 1A). On day 2, pAAV/IGF-I increased IGF-I production by mature chondrocytes from an average of 1.8±0.80 ng/mL (unstransfected) to 8.5±1.0 ng/mL (transfected) (p=0.013) (Fig.2A). On day 4, neonatal chondrocytes' increment was from 0.50 ± 0.79 ng/mL (unstransfected) to 2.5±2 ng/mL (transfected) (p<0.001), while mature chondrocytes had an increment from 0.50±0.80ng/mL (unstransfected) to 7.6±2ng/mL (transfected) (p=0.009). (Fig.1A&2A). On day 6 transfection increased neonatal chondrocytes IGF-I production from 0.26±0.25 ng/mL to 1±0.8 ng/mL (p=0.055) (Fig. 1A). On day 6 mature chondrocytes had an increment

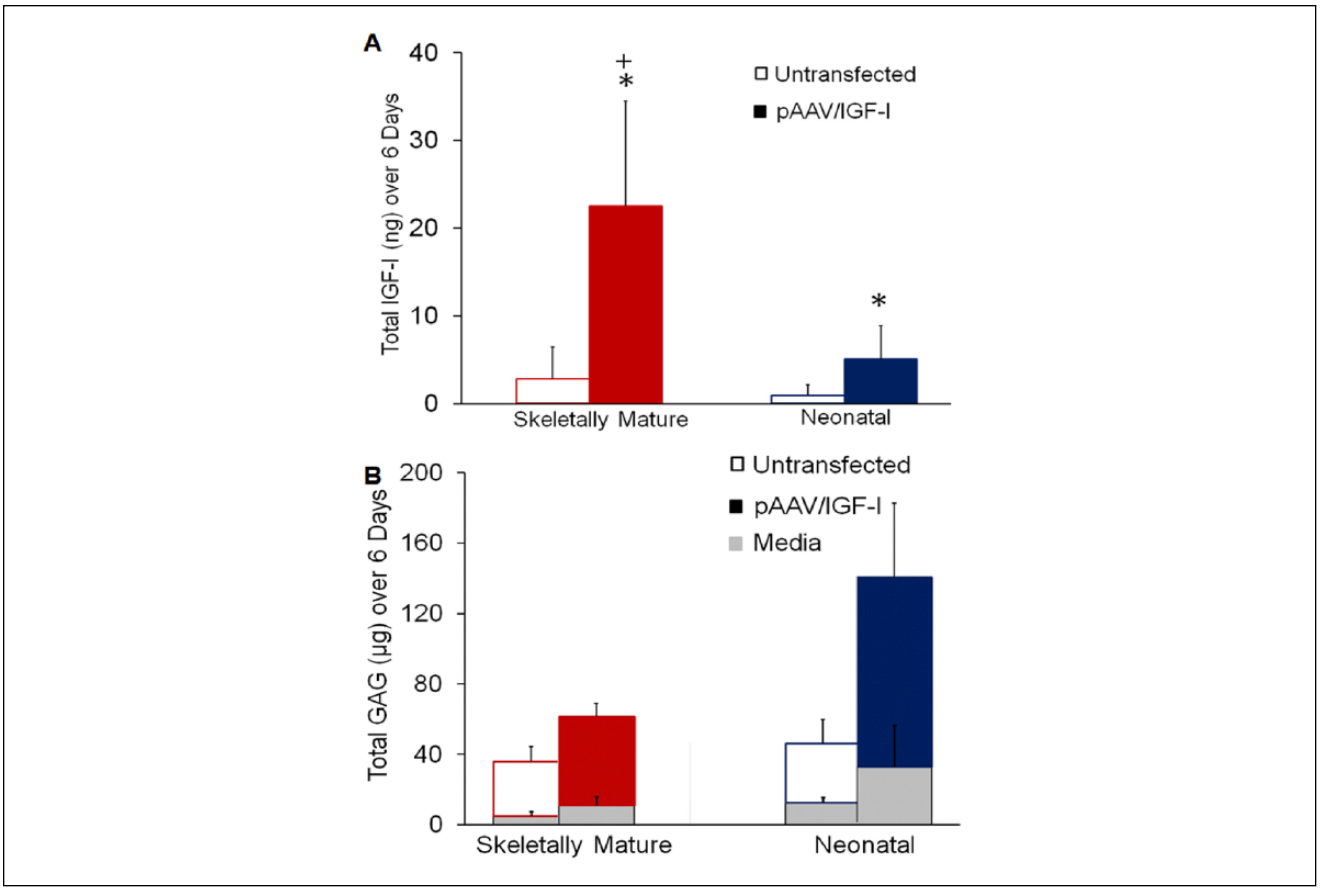

Figure 3. (**A**) Total insulin-like growth factor I (IGF-I) and (**B**) total glycosaminoglycan (GAG) production over 6 days in culture by skeletally mature and neonatal chondrocytes. * is p<0.05 with respect to untransfected.+ is P<0.01 with respect to transfected skeletally mature chondrocytes.

from 0.27 ± 0.70 ng/mL (unstransfected) to 6.5 ±4.20 ng/mL (transfected) (p=0.021). The amount of IGF-I released was slightly greater for untransfected mature chondrocytes than untransfected neonatal chondrocytes, but without any significance.

More GAG was lost to media from transfected neonatal chondrocytes (3-fold) than from transfected mature chondrocytes (2-fold) (*$P < 0.05$) when compared with untransfected (**Fig. 1B** and **2B**) chondrocytes. The amount of GAG produced by untransfected mature chondrocytes was slightly, but not significantly, more than by untransfected neonatal chondrocytes. However, pAAV/IGF-I increased GAG levels more by transfected neonatal chondrocytes ($^{+}P < 0.01$) than by transfected mature chondrocytes (**Fig. 1B** and **2B**).

Total IGF-I and GAG in Cell Layer and Media

We compared the effect of IGF-I transfection on total IGF-I and GAG production over 6 days in culture between skeletally mature and neonatal chondrocytes. pAAV/IGF-I increased the total IGF-I synthesis in both transfected mature and neonatal chondrocytes when compared with untransfected (**Fig. 3A**). However, even though transfected mature chondrocytes produced more IGF-I than transfected neonatal chondrocytes, transfected neonatal chondrocytes produced more GAG than transfected mature chondrocytes (**Fig. 3B**). IGF-I increased the total GAG production in both the cell layer and the media in both mature and neonatal chondrocytes. pAAV/IGF-I increased GAG by 60% in transfected mature (from 31µg±8 to 50±7µg) and ~3 fold in transfected neonatal chondrocytes (from 33±14 µg to 107±42 µg) in media when compared to untransfected mature and neonatal chondrocytes respectively.

Also, GAG production in the cell layer was higher in both transfected mature and transfected neonatal chondrocytes when compared to untransfected chondrocytes (~2-fold for mature and ~3-fold for neonatal). At the same time, transfected neonatal chondrocytes produced more GAG in the cell layer (~3-fold) than transfected skeletally mature chondrocytes (**Fig. 3**).

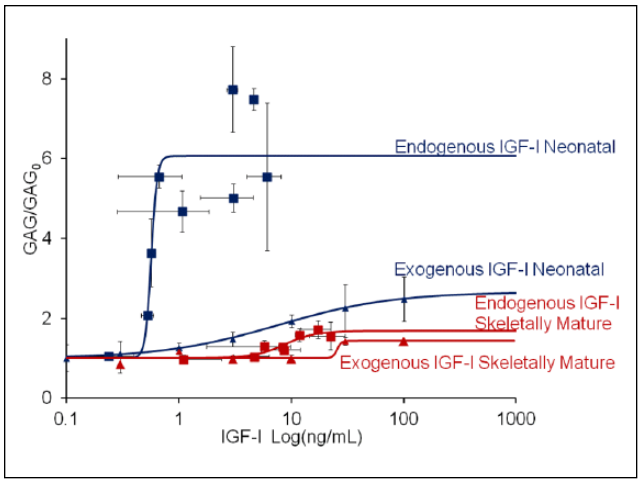

Figure 4. Dose response. Total insulin-like growth factor I (IGF-I) versus total glycosaminoglycan (GAG) production from each experiment fitted to a VSCR model. Blue represents neonatal chondrocytes. Red represents mature chondrocytes. ■ Endogenous IGF-I neonatal (pAAV/IGF-I). ▲ Exogenous IGF-I neonatal. ■ Endogenous IGF-I skeletally mature (pAAV/IGF-I). ▲ Exogenous IGF-I skeletally mature. VSCR, variable slope concentration response; pAAV, adeno-associated virus–based plasmid.

Dose-Response Model

Combining data on average concentrations of IGF-I in the media and normalized GAG production from each experiment and fitting this data set to a four parameter model revealed a clear relationship between the IGF-I production and GAG released. Using this technique, we compared the dose-response relationships for neonatal and adult cell sources and endogenous and exogenous sources of IGF-I. The correlation between IGF-I production and GAG released in both neonatal and skeletally mature chondrocytes was strong, correlation coefficients (R2)greater than 0.80 for all the fits. The sensitivity to IGF-I was quantified by the maximal amount of GAG released and by the EC_{50}, which is the concentration of IGF-I producing half the maximum amount of GAG. Therefore, transfected neonatal chondrocytes were 15 times more sensitive to IGF-I (EC_{50} of 0.57 ng/mL) than transfected mature chondrocytes (EC50 of 8.70 ng/mL), and produced approximately 4 times more GAG when compared with transfected mature chondrocytes (**Fig. 4** and **Table 1**). Similarly, the stimulation by exogenous IGF-I was greater in neonatal chondrocytes than in mature chondrocytes with an EC_{50} of 7.56 and 25.55 ng/mL, respectively (**Table 1** and **Fig. 4**). Such exogenous stimulation produced almost 2 times more GAG when comparing neonatal and mature chondrocytes (**Fig. 4** and **Table 1**). As a result, neonatal chondrocytes were more responsive to both endogenous and exogenous IGF-I when compared with mature chondrocytes. Significant differences were also noted in the response of chondrocytes (GAG production) to the source of IGF-I(endogenous vsexogenous). Transfected neonatal chondrocytes reached steady state of GAG levels at lower concentrations of IGF-I than neonatal chondrocytes exposed to exogenous IGF-I (**Fig. 4**). Only 0.57 ng/mL of IGF-I was required to reach the half-maximum matrix production (**Table 1**) when compared with neonatal chondrocytes exposed to exogenous IGF-I (EC_{50} of 7.56 ng/mL) with a difference of 13 times the sensitivity to endogenous IGF-I than exogenous IGF-I. The matrix production of transfected neonatal chondrocytes was 3 times higher than neonatal chondrocytes exposed to exogenous IGF-I (**Fig. 4** and **Table 1**). The differences in sensitivity between endogenous IGF-I and exogenous IGF-I were less in mature chondrocytes. Mature chondrocytes were only 3 times more sensitive to endogenous IGF-I than exogenous IGF-I with an EC_{50} of 8.70 and 25.55 ng/mL, respectively (**Fig. 4** and **Table 1**). At the same time, transfected mature chondrocytes produced only 85% more matrix than mature chondrocytes exposed to exogenous IGF-I (**Table 1**).

Table 1. Dose-Response Summary: Parameters EC_{50}, Maximal Stimulation over Minimal Stimulation (Max/Min), and R^2 of the VSCR Model for Neonatal and Mature Chondrocytes.

	Neonatal		Skeletally Mature	
	Endogenous IGF-I	Exogenous IGF-I	Endogenous IGF-I	Exogenous IGF-I
EC_{50} (ng/mL)	0.57	7.56	8.70	25.55
Max/Min	6.25	2.65	1.69	1.44
R^2	0.80	0.99	0.85	0.85

EC_{50} = half-maximal effective concentration; IGF-I, insulin-like growth factor I; VSCR, variable slope concentration response.

Discussion

The current study compared the efficacy of endogenous and exogenous IGF-I in stimulating proteoglycan synthesis by skeletally mature and neonatal articular chondrocytes. We first analyzed the ability of pAAV/IGF-I to transfect skeletally mature and neonatal chondrocytes. The empty vector had no effect on either the IGF-I or GAG production (**Fig. 1**). pAAV/IGF-I upregulated IGF-I synthesis in both cell types, but skeletally mature chondrocytes released more IGF-I to the media than did neonatal chondrocytes (**Fig. 2**). However, the matrix synthesis was upregulated to a greater degree in neonatal cells (**Figs. 1B** and **3B**). For all experiments there was a clear dose-dependent relationship between IGF-I exposure and GAG production. The differential effects of pAAV/IGF-I on IGF-I production and matrix synthesis were highlighted by comparing these parameters on a sample-by-sample basis (**Fig. 4**). Neonatal

chondrocytes were very sensitive to stimulation by endogenous IGF-I (**Fig. 4**). GAG production increased 6-fold at steady state and required only 0.57 ng/mL of IGF-I in media for half-maximal stimulation (Table 1). Skeletally mature chondrocytes had lower sensitivity to endogenous IGF-I (**Fig. 4**) increasing GAG production 1.69-fold, with half-maximal stimulation at 8.70 ng/mL (**Table 1**). We also investigated the stimulation of chondrocytes by exogenous IGF-I added to the media. GAG production was related to IGF-I concentration in a clearly dose-dependent manner (R^2 fits are 0.80-0.99) (**Table 1**). For both cell types exogenous IGF-I added to media was less effective at stimulating GAG production than endogenously produced IGF-I, where the differences between maximum and minimum GAG stimulation were 2.7- versus 6.1-fold increase in neonatal and 1.4- versus 1.7-fold for skeletally mature. Furthermore, both cell types were less sensitive to exogenous IGF-I than endogenous IGF-I, with EC_{50} values that were 13 and 3 times lower than those from pAAV/IGF-I studies.

There is an extensive amount of research using gene therapy to try to deliver IGF-I to chondrocytes to treat injured cartilage. These studies vary greatly in the amount of IGF-I produced and resultant matrix synthesis. Viral methods using adenovirus (Ad.IGF-I) in chondrocytes from 3- to 6-month-old foals released a maximum of 66 ng/mL of IGF-I and a minimum of 10 ng/mL. This treatment stimulated proteoglycan production to 6.25 μg/mL on day 14, which dropped which dropped to 3.2 μg/mL on day 28.[25] Nonviral methods[9] with cDNA/IGF-I to transfect rabbit chondrocytes augmented IGF-I release to 3.6 ng on day 4 and 0.72 ng on day 36. Shi *et al.*[15] used pAAV/IGF-I, a nonviral method, to transfect skeletally mature bovine chondrocytes. IGF-I released was up to 300 ng/mL on day 2 and a minimum of 50 ng/mL on day 6. The proteoglycan in the media ranged from 20 to 130 μg/mL.[15] The amount of IGF-I produced by chondrocytes in the current study is less than seen in previous work in young equine[25] and adult bovine[15] samples, but similar to that seen in skeletally mature rabbits.[9] However, proteoglycan production rates in the current study were comparable to those noted previously. A previous comparison of endogenous and exogenous IGF-I found similar effects, but employed only skeletally mature chondrocytes.[26] Despite the extensive previous work, to our knowledge, no study has shown a dose-dependent response for the amount of IGF-I released by cells and the amount of GAG produced, and no study has compared skeletally mature and neonatal articular chondrocytes as potential targets for gene therapy.

To our knowledge, these studies, for the first time, established a clear connection between the amount of IGF-I produced by transfected chondrocytes and their biosynthetic response. The current study demonstrated a saturation effect by 20 ng/mL IGF-I in adults and 1 ng/mL IGF-I in neonatal chondrocytes, above which excess IGF-I does not enhance matrix production. This observation suggests that studies aimed at growth factor therapy via gene transfection should be calibrated to produce the maximal biosynthetic effect with the minimum amount of growth factor necessary. The data further suggest that this calibration will likely differ for chondrocytes from donors of different age.

The VSCR models[23,24] used to describe the dose-dependent effects of IGF-I on GAG production describe the data well. However, the mechanism responsible for the increased sensitivity of neonatal cells to endogenous IGF-I is unclear and may involve differences in IGF-I receptor or IGF-I binding protein concentration. A number of studies describe age-related changes in chondrocytes related to IGF-I action. For example sensitivity to exogenous IGF-I in adult chondrocytes declined because of fewer IGF-I receptors,[19] defective signal transduction pathways, or production of other factors necessary for full response to IGF-I.[27] Another possibility previously analyzed was the presence of insulin-like growth factor proteins (IGFBPs) as modulators for IGF-I. IGFBPs regulate the access of native tissue cells to IGF-I, specifically, IGFBP-1 to IGFBP-6.[28] Previously, it has been shown that IGFBP-3 and 4 have been identified in human articular chondrocyte cultures with arthritic diseases.[29] In contrast, IGFBP-5 has shown to be beneficial for enhancing IGF-I signal and therefore proliferation of matrix production.[30] The amount of IGFBPs changes throughout the life span of the chondrocytes. IGFBP-5 is more active in neonatal chondrocytes and activity decreases with age.[31]

Even though the protein and the gene structure of IGF-I have been highly conserved throughout evolution in vertebrates,[32] the differences in biosynthetic response to endogenous and exogenous IGF-I may be due to possible differences in tertiary or quaternary structure. Exogenous IGF-I came from recombinant human IGF-I which was commercially obtained and the endogenous IGF-I source was the chondrocytes that were transfected. Furthermore, the availability of the IGF-I to the IGF-I receptor could vary between exogenous addition and endogenous production. Theoretical models describing the transport of endogenously produced epidermal growth factor (EGF) show significant local gradients in concentration because of the binding to cell surface receptors.[27] Such may be the case for case for IGF-I as well, causing the concentration in the bulk of the media to be different from that near the cell surface. This could explain the low levels of EC_{50} (0.57 ng/mL) for IGF-I from transfected neonatal chondrocytes.

Taken as a whole, this study shows distinct differences in the upregulation of proteoglycan synthesis by skeletally mature and neonatal chondrocytes when exposed to endogenously produced or exogenously added IGF-I. Nevertheless, there are clear limitations to this study. Previous studies have established that pAAV/Empty vectors have no effect on IGF-I synthesis or GAG production in skeletally mature chondrocytes thus, these controls were

omitted from the current study.[15] However, pAAV/Empty vector had no effect in either the production of IGF-I or GAG in neonatal chondrocytes when compared to transfected neonatal chondrocytes. Another factor that could contribute to differences in IGF-I production between neonatal and mature chondrocytes is differential transfection efficiency between the two cell types. To assess this directly, we measured transfection efficiency using two related vectors (pAAV/hrGFP and pAAV/IRES/hrGFP) (See Supplemental Data). These studies showed that transfection efficiency was ~20% higher in mature chondrocytes than in neonatal; however, this level of effect did not achieve statistical significance and does not account for the 450% difference in IGF-I production noted in Figure 3A.In addition, GAG production is not the only indicator of matrix production for chondrocytes. Indeed previous studies show differential dose responses of neonatal and adult cartilage explants with regard to upregulation of collagen and GAG synthesis.[18] As such it is possible that collagen synthesis patterns due to IGF-I transfection may be different than those seen for GAG production. Last, it is important to note that the current study was conducted with chondrocytes in monolayer culture. These studies used primary chondrocytes, at short times where there should be minimal de-differentiation. Nevertheless, future studies on this topic using appropriate 3-dimensional culture system would be of great interest.

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Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Supplementary Material

Supplementary material for this article is available on the Cartilage website at http://cart.sagepub.com/supplemental.

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