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Endogenous versus Exogenous Growth Factor Regulation of Articular Chondrocytes

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Abstract

Anabolic growth factors that regulate the function of articular chondrocytes are candidates for articular cartilage repair. Such factors may be delivered by pharmacotherapy in the form of exogenous proteins, or by gene therapy as endogenous proteins. It is unknown whether delivery method influences growth factor effectiveness in regulating articular chondrocyte reparative functions. We treated adult bovine articular chondrocytes with exogenous recombinant insulin-like growth factor-I (IGF-I) and transforming growth factor-beta1 (TGF-β1), or with the genes encoding these growth factors for endogenous production. Treatment effects were measured as change in chondrocyte DNA content, glycosaminoglycan production, and aggrecan gene expression. We found that IGF-I stimulated chondrocyte biosynthesis similarly when delivered by either exogenous or endogenous means. In contrast, exogenous TGF-ß1 stimulated these reparative functions, while endogenous TGF-ß1 had little effect. Endogenous TGF-ß1 became more bioactive following activation of the transgene protein product. These data indicate that effective mechanisms of growth factor delivery for articular cartilage repair may differ for different growth factors. In the case of IGF-I, gene therapy or protein therapy appear to be viable options. In contrast, TGF-ß1 gene therapy may be constrained by a limited ability of chondrocytes to convert latent complexes to an active form.

Keywords

articular chondrocyte; insulin-like growth factor-I; transforming growth factor-beta1; gene transfer; gene expression

INTRODUCTION

Trauma or impaired articular chondrocyte homeostasis can result in loss of articular cartilage and disability. Anabolic growth factors are candidates for augmenting articular cartilage repair. One potential method of growth factor therapy is the administration of exogenous growth factor in a delivery vehicle. A second potential approach is the administration of cells that produce endogenous growth factors. These two approaches differ in clinically important respects. The use of exogenous growth factors requires that the

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recombinant growth factor protein be correctly folded and purified by the manufacturer. The use of endogenous growth factors requires that the growth factor transgene be correctly processed by the cells' transcriptional, translational and post-translational machinery. Exogenous growth factors function in an endocrine fashion, while endogenous growth factors produced by the chondrocytes act on their target cells in an autocrine/paracrine fashion. For these reasons, it is possible that growth factors delivered by these different methods will not produce the same effects on their target cells. To our knowledge, direct comparison of these two approaches to growth factor regulation of articular chondrocytes is lacking.

Multiple growth factors have been identified that promote articular chondrocyte anabolic activity. Prominent among these are insulin-like growth factor I (IGF-I) and transforming growth factor beta1 (TGF-β1)[1]. IGF-I is a mitogenic and anabolic factor that stimulates chondrocyte proliferation and aggrecan synthesis[2-9] and reduces chondrocyte catabolic activity[8]. TGF-β1 is multifunctional growth factor that has pleiotrophic effects on chondrocytes, including the stimulation of chondrocyte aggrecan and collagen synthesis[10-13]. We used these growth factors to test the null hypothesis that endogenous and exogenous approaches to the regulation of articular chondrocyte mitogenic and anabolic activity are not different.

MATERIALS AND METHODS

Chondrocyte cell culture and vector construction

Basal medium was prepared with DMEM, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine (Invitrogen) and 50 μg/ml ascorbic acid (Sigma). Complete medium was prepared by supplementing basal medium with 10% FBS (Invitrogen). Adult bovine articular chondrocytes were isolated as previously described[14] and placed in monolayer culture in 6-well plates at a density of 3×10^5 cells/well in complete medium. The vectors pAAV-IGF-I, and pAAV-TGF-β1 were generated as previously described[15] by inserting the human growth factor cDNA coding regions, following sequence confirmation, into pAAV-MCS (Stratagene).

Delivery of endogenous growth factors

Chondrocytes were transfected in complete medium with pAAV-IGF-I or pAAV-TGF-β1 plasmids and FuGENE 6 (Roche Applied Science) in a ratio of 1:3. Mock-transfected chondrocytes were transfected with pAAV-MCS (empty vector). At 2 and 4 days after transfection, conditioned medium (CM) was collected and replaced by basal medium. On day 6, CM was collected and the cell layer was digested in proteinase k solution (0.5 mg/ml proteinase k in 10 mM Tris, pH 8.2, and 5 mM EDTA) at 65°C for 2 h. CM was stored at -20°C for analyses of glycosaminoglycan (GAG) released into the CM. The cell digest was stored at −20°C for analyses of cell-associated GAG, and DNA in the cell layer. In additional experiments, the cell layer was harvested for analysis of aggrecan gene expression by real time PCR as previously described[15].

Delivery of exogenous growth factors

Chondrocytes used for treatment with exogenous IGF-I or TGF-β1 were transfected with 2 μg pAAV-MCS plasmid DNA (single mock-transfected). Chondrocytes used for treatment with IGF-I plus TGF-β1 were transfected with 4 μg pAAV-MCS plasmid DNA (double mock-transfected). The exogenous IGF-I (PeproTech) was recombinant mature human IGF-I (70 amino acids) produced in *E.coli*. The exogenous TGF- β1 (R&D Systems) was recombinant mature human TGF- β1 (112 amino acids) produced in CHO cells. Following transfection, medium was replaced with fresh complete medium supplemented with

exogenous recombinant IGF-I (200 ng/ml), TGF-β1 (50 ng/ml) or the combination of IGF-I (200 ng/ml) plus TGF-β1 (50 ng/ml) for 2 days. Conditioned medium (2 day CM) was harvested and replaced with basal medium supplemented with 0.1% BSA, exogenous recombinant IGF-I (100 ng/ml), TGF-β1 (35 ng/ml) or the combination of IGF-I (100 ng/ml) plus TGF-β1 (35 ng/ml) for 2 days. Conditioned medium (4 day CM) was harvested and replaced with basal medium supplemented with exogenous IGF-I (10 ng/ml), TGF-β1 (10 ng/ml) or the combination of IGF-I (10 ng/ml) plus TGF-β1 (10 ng/ml) for 2 days. Conditioned medium (6 day CM) was harvested and cultures were terminated. The designated exogenous growth factor concentrations were approximated from prior studies that measured growth factor production following gene transfer[16].

DNA, glycosaminoglycan, growth factor, and aggrecan expression analysis

DNA, GAG, growth factor protein and aggrecan gene expression were assessed as previously described.[15,17] Briefly, cell proliferation was assessed by DNA analysis of the cell digest by Picogreen dsDNA assay (Molecular Probes). GAG released into the medium (released GAG) and retained with the chondrocytes (cell-associated GAG) were separately measured by dimethylmethlyene blue assay. Data for cells and medium from individual culture wells were normalized to well. IGF-I and TGF-β1 protein in the conditioned medium were analyzed by ELISA using DuoSet ELISA kits (R&D Systems). In other studies employing the same treatments, less than 5% of the growth factor transgene product was retained in the cell layer (STable 1); therefore the cells were not assayed for growth factor content. To assess free mature TGF-β1 peptide, CM was assayed directly. To additionally assess latent TGF-β1, CM was assayed following pretreatment with HCl (1 N HCl, 1/5 volume of CM) and neutralization with NaOH and HEPES (1.2 N NaCl/0.5 N HEPES, 1/5 volume of CM). Aggrecan gene (ACAN) expression was measured by real time PCR of reverse transcribed RNA from chondrocyte lysates. Data were normalized to 18S ribosomal RNA.

Statistical analysis

Three independent experiments using chondrocytes from three bovids were performed. Within each experiment, all treatment conditions were tested in triplicate. Statistical analyses of DNA, cell-associated GAG, GAG/DNA, released GAG (at 2, 4, 6 days and total), cell-associated + total released GAG, and the ratio of cell-associated GAG to total released GAG were performed on the ratios to controls. Group comparisons were evaluated using mixed-model analysis of variance (ANOVA), which included a random effect for experimental run. The analyses of cell-associated GAG were performed after a natural log transformation of the data to satisfy the normality assumption for the ANOVA. Statistical analyses of aggrecan mRNA fold change vs control at 1, 2, 3, 4, 5, and 6 days were performed using repeated measures analysis of variance (ANOVA), which included a random effect for experimental run and an unstructured variance/covariance matrix for the repeated measurements over time. The analyses were performed after a natural log transformation of the data to satisfy the normality assumption for the ANOVA. Using the ANOVAs, tests were performed to determine whether simultaneous growth factor treatment generated synergistic or inhibitory effects compared to treatment by individual growth factors. Synergistic effects were defined as those for which the value of the combined growth factors was significantly greater than the sum of the effects of the individual growth factors. Inhibitory effects were defined as those for which the value of the combined growth factors was significantly less than the sum of the effects of the individual growth factors. p values less than 0.05 were considered statistically significant. To improve readability, p values in the text are used sparingly. A complete set of p values is provided in the Supplementary Material. Non-significant comparisons are designated NS, nanograms per milliliter as ng/ml, and standard deviation as SD.

RESULTS

Chondrocyte proliferation

Exogenous and endogenous IGF-I stimulated cell proliferation 1.8- and 1.9-fold respectively (NS), as measured by increases in DNA content. Exogenous and endogenous TGF-β1 increased DNA content 2.6- and 1.4-fold respectively ($p<0.0001$). Treatment with exogenous or endogenous [IGF-I + TGF-β1] generated 2.6- and 1.9-fold increases respectively ($p=0.0005$). The effect of the combined exogenous growth factors, [IGF-I + TGF-β1], was not different than the effect of exogenous TGF-β1 alone, while the effect of the combined endogenous factors was not different than that of either endogenous or exogenous IGF-I alone (Figure 1. STable 2). These data indicate that the effect of these growth factors on cell proliferation is not additive, and that the respective role of each growth factor differs according to the method of delivery.

Glycosaminoglycan production

Exogenous and endogenous IGF-I stimulated total (cell-associated and released) GAG production 2.0- and 2.1-fold respectively (NS). Exogenous and endogenous TGF-β1 increased GAG production 4.9- and 1.2-fold respectively ($p<0.0001$). Treatment with exogenous or endogenous [IGF-I + TGF-β1] generated increases of 7.4- and 2.5-fold ($p<0.0001$) respectively. Both the exogenous and endogenous growth factor combinations were synergistic (C/S=1.31 and 1.23, respectively) (Figure 2, STable 2).

Cell-associated vs released glycosaminoglycan

To distinguish between GAG that contributes to the structure of new matrix and non-structural GAG, cell-associated and released GAG were measured separately. Exogenous and endogenous IGF-I stimulated the production of cell-associated GAG 2.6- and 2.5-fold respectively (NS) and of released GAG 1.9-fold each. In contrast, exogenous and endogenous TGF-β1 stimulated cell-associated GAG 5.6- and 1.4-fold respectively ($p<0.0001$), and released GAG 4.7- and 1.1-fold respectively ($p<0.0001$). Exogenous and endogenous [IGF-I + TGF-β1] stimulated cell-associated GAG 10.0- and 2.9-fold respectively ($p<0.0001$). The combined exogenous factors were synergistic (C/S=1.62), but the effect of the combined endogenous factors was only additive. Exogenous and endogenous [IGF-I + TGF-β1] stimulated released GAG 6.1- and 2.4-fold respectively ($p<0.0001$). The combined exogenous factors and the combined endogenous factors were synergistic (C/S=1.12 and C/S=1.31 respectively) (Figure 2, STable 2). Similar relationships were observed when data were expressed as the ratio of cell-associated to released GAG, an index of the structural component of chondrocyte GAG production (SFigure 1).

Chondrocyte anabolic activity

To separate the effect of these growth factors on changes in GAG production due to stimulation of chondrocyte biosynthesis from changes due to the increase in cell number over the course of the experiment, GAG production was normalized to DNA content. The effects of exogenous and endogenous IGF-I on GAG/DNA were not significantly different. In contrast, exogenous TGF-β1 increased GAG/DNA 2.2-fold while endogenous TGF-β1 had no effect ($p<0.0001$). Exogenous and endogenous [IGF-I + TGF-β1] stimulated GAG/DNA 4.1- and 1.5-fold respectively ($p<0.0001$). The exogenous, but not the endogenous combined factors were synergistic (C/S=2.01)(Figure 3, STable 2).

Time course of action of IGF-I and TGF-β1 on released glycosaminoglycan

Exogenous and endogenous IGF-I stimulated released GAG production in a progressive, time-dependent fashion to peaks of 3.5- and 3.9-fold respectively at 6 days (NS). At no time

point did the magnitude of stimulation differ significantly for the methods of IGF-I delivery. Exogenous and endogenous TGF-β1 stimulated the production of released GAG in a progressive, time-dependent fashion to a peak of 20.6- and 1.8-fold respectively at 6 days ($p<0.0001$). The magnitude of stimulation by endogenous TGF-β1 never exceeded 6% of the effect generated by exogenous TGF-β1. Exogenous and endogenous [IGF-I + TGF-β1] stimulated released GAG in a time-dependent fashion to maxima of 32.6- and 7.6-fold respectively at 6 days ($p<0.0001$). Both combinations were synergistic (C/S=1.42 and C/S=1.79 respectively). The time course patterns of exogenous and endogenous [IGF-I + TGF-β1] action were identical, but stimulation by the endogenous growth factors was of a markedly lower magnitude (Figure 4, STable 2).

Aggrecan gene expression

Exogenous and endogenous IGF-I regulated ACAN expression similarly during their early time courses, both becoming stimulatory at 4d. Through 6 days, endogenous IGF-I maintained a constant 2-fold stimulation of ACAN expression, while the effect of exogenous IGF-I declined to 1.5-fold ($p=0.0018$). This modest difference represents the only significant difference in the effect of exogenous and endogenous IGF-I observed in these studies. Exogenous and endogenous TGF-β1 progressively stimulated ACAN expression to maxima of 7.7- and 2.0-fold respectively at 6d ($p<0.0001$). Exogenous [IGF-I + TGF-β1] synergistically increased ACAN expression from 3d to 5d (maximum C/S=2.24, STable 2), but became inhibitory at 6d. Compared to TGF-β1 alone, the addition of IGF-I shifted the time course of stimulation to the left by approximately 2 days. Endogenous [IGF-I + TGF-β1] progressively stimulated ACAN expression to a plateau at ~3.7-fold after 3 days, reflecting an additive effect of the two endogenous growth factors. (Figure 5, STable 4).

Characterization of endogenous growth factors

Following transfection by pAAV-IGF-I, chondrocytes produced physiological concentrations of IGF-I in the conditioned medium (CM) (Table 1). To control for the effect of time in culture, CM from pAAV-MCS mock-transfected chondrocytes treated with exogenous growth factor(s) was also harvested and measured for IGF-I content (Table 1). Following transfection by pAAV-TGF-β1, no TGF-β1 was detected in the CM at any time point. Following acid treatment of the CM to activate any latent TGF-β1, re-assay detected TGF-β1 at physiological concentrations at all time points. To control for the effect of acid treatment, medium containing exogenous TGF-β1 was subjected to acid treatment and then re-assayed. Acid treatment increased the assayed concentrations of TGF-β1, though to a much smaller degree than for endogenous TGF-β1. (Table 2) Acid treatment of CM from chondrocytes that received exogenous [IGF-I + TGF-β1] yielded results similar to those for exogenous TGF-β1 alone (Table 2).

Detailed data and comparisons are provided in Supplementary Data (STables 1-8 and SFigures 2-5).

DISCUSSION

Growth factors may be delivered as therapeutic agents by two general approaches. The first employs pharmacologic methods, the second is accomplished by gene transfer techniques. These two approaches lead to quite different lines of research toward a therapy for articular cartilage repair. Our data suggest that the differences between modes of delivery can make the difference between efficacy and lack of efficacy of growth factors as regulators of articular chondrocyte function. The results further indicate that this difference does not occur for all growth factors.

Our data support the null hypothesis for IGF-I. It was equally effective whether delivered in endogenous or exogenous form. Endogenous IGF-I is synthesized as a precursor protein consisting of three distinct domains: a signal peptide, the mature IGF-I, and the C-terminal propeptide (E peptide). Mature, active IGF-I is generated by sequential proteolytic removal of the signal peptide and C-terminal propeptide regions of the precursor protein.[18,19] Our observation that the IGF-I produced by the chondrocytes is as effective as the exogenous peptide suggests that these cells are able to generate active IGF-I.

The data reject the null hypothesis for TGF-β1. The endogenously produced growth factor lacked most of the biological activity of the exogenous protein. TGF-β1 is synthesized as a precursor protein consisting of a signal peptide, a latency-associated peptide (LAP), and the mature peptide. For endogenous TGF-β1, during post-translational processing the LAP is cleaved from the precursor protein but remains non-covalently associated with the mature TGF-β1 to form a small latent complex (SLC). The SLC complex can become associated with a latent TGF-β1 binding protein (LTBP) to form a large latent complex (LLC) by one disulfide bond between LAP and LTBP. The binding sites of the mature TGF-β1 in SLC or LLC are shielded, preventing interaction with the TGF-β1 receptor[20]. An additional activation process releases mature TGF-β1 from LLC or SLC, enabling the mature TGF-β1 to bind to its receptors. Previous studies have shown that acid treatment activates latent TGF-β1[21] and enables its detection by the ELISA assay employed in this study[16]. These results suggest that the chondrocytes produced correct TGF- β1 in a latent complex and lack an efficient mechanism for activating TGF-β1.

Despite the failure of chondrocytes carrying the TGF-β1 transgene to produce detectable levels of growth factor, the gene product did not entirely lack a biological effect. Although endogenous TGF-β1 achieved a relatively small fraction of the stimulation by the exogenous growth factor, it did significantly increase all tested cell functions. In the case of released GAG, endogenous TGF-β1 interacted with endogenous IGF-I to regulate production in a synergistic fashion. The observation that endogenous TGF-β1 regulates cell proliferation, GAG production and ACAN gene expression without detectable levels of the growth factor in the culture medium may reflect a limitation of the ELISA in distinguishing between active and inactive forms of TGF-β1, an ability of chondrocytes to respond to TGF-β1 at sub-threshold assay levels, or higher concentrations of TGF-β1 in the pericellular region of the chondrocytes than in the conditioned medium.

Limitations of this work include limitations intrinsic to cell culture models. For the present studies, we chose a 2D culture system rather than a 3D system. This model permits comparison to other studies of these factors in this and other cell types. It also enables a focus on growth factor action by removing multiple variables that characterize 3D culture systems, such as differences in matrix structure and hydration, growth factor diffusion and binding, and cell-matrix interactions. Thus, if the observed mechanisms are sensitive to experimental conditions, it is possible that the results would differ in different cultures and from in vivo conditions. A further limitation of this study is the imperfect match between the concentration of exogenous growth factor delivered and the amount of endogenous growth factor measured at the given time points. This resulted from the need to estimate in advance the concentrations of exogenous factor to be delivered. For this reason, we have not attempted to compare the magnitude of the response to the growth factors delivered by exogenous and endogenous means. It is important to note the presence of endogenous growth factors, amounting to approximately 1-10% of that measured, that is produced from their native genes, rather than from the inserted transgenes. Any functional effect of these factors would represent an additional contribution to the observed data. Finally, the current data do not investigate the specific protein structure of these growth factors. Further studies

will be required to elucidate the mechanisms of growth factor protein processing by these cells.

These results demonstrate that the growth factors and the delivery methods for those factors are interdependent. In the case of TGF-β1, but not IGF-I, endogenous delivery appears to restrict the action of the growth factor compared to exogenous delivery. However, the relationships between growth factor and delivery method also offer another level of control of growth factor action. In the case of TGF-β1, this could take the form of regulated activation of the precursor complex. The data further indicate that the effect of delivery on growth factor function is much less for IGF-I than for TGF-β1, suggesting a trade-off between flexibility and complexity in regulating these cells. Additional studies will be needed to determine the respective actions of the endogenous and exogenous forms of other chondrotrophic growth factors. Such data will be important in selecting growth factors for cell-based articular cartilage repair.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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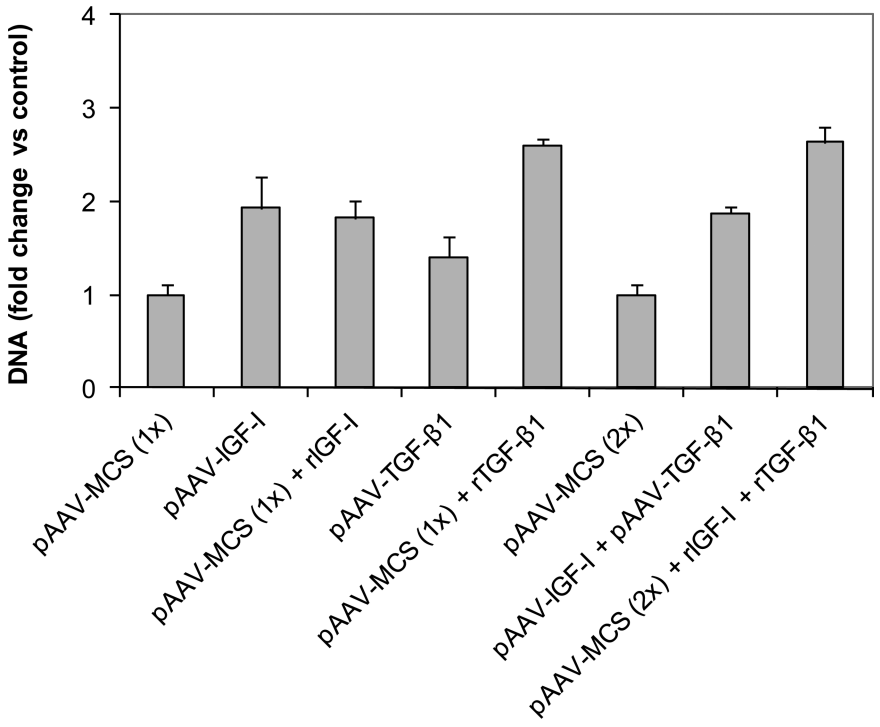

Figure 1.

Change in DNA content of articular chondrocytes in response to the designated treatments. Chondrocytes were transfected with control vectors containing 2 ug (1x) or 4 ug (2x) empty vector DNA (pAAV-MCS 1x or pAAV-MCS 2x), or vectors encoding IGF-I (pAAV-IGF-I), TGF-β1 (pAAV- TGF-β1), or both IGF-I and TGF-β1 (pAAV-IGF-I + pAAV- TGF-β1). Additional chondrocytes were transfected with control vectors and also exposed to recombinant IGF-I (pAAV-MCS 1x + rIGF-I), recombinant TGF-β1 (pAAV-MCS 1x + rTGF-β1), or both recombinant growth factors (pAAV-MCS 2x + rIGF-I + rTGF-β1). pAAV-MCS 1x was used as control for single transgenes. pAAV-MCS 2x was used as control for two transgenes. Cultures were terminated at 6 days. Data are expressed as DNA normalized to the respective control ± SD for 3 independent experiments.

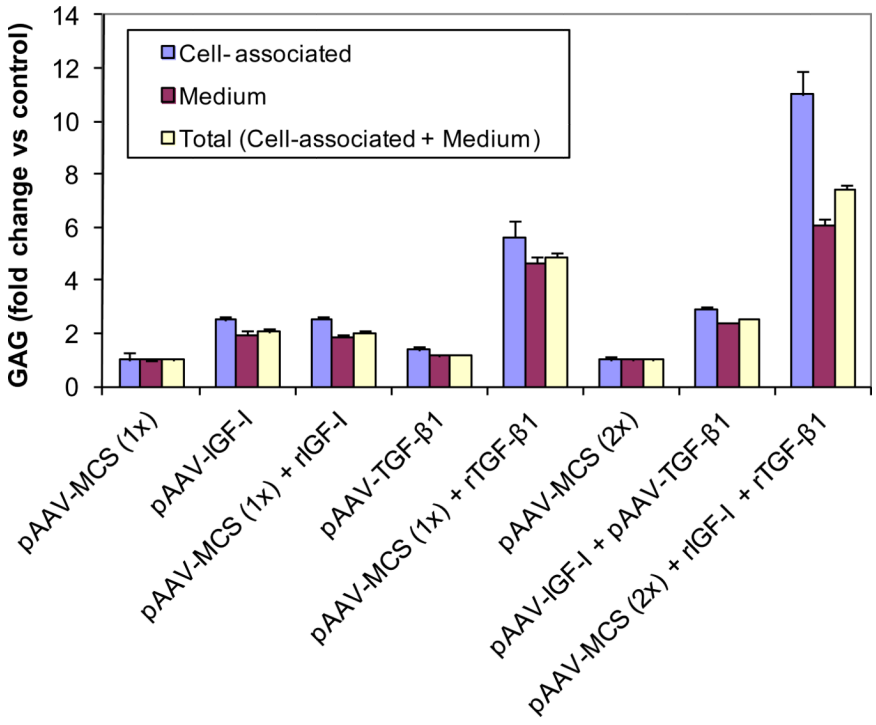

Figure 2.

Changes in glycosaminoglycan (GAG) production by articular chondrocytes in response to the designated treatments. Chondrocytes were treated with recombinant growth factor(s) and/or transfected as described above. Cell-associated and released GAG were analyzed separately. Cultures were terminated at 6 days. Data are expressed as cell associated, released, and total GAG normalized to the respective control ± SD for 3 independent experiments.

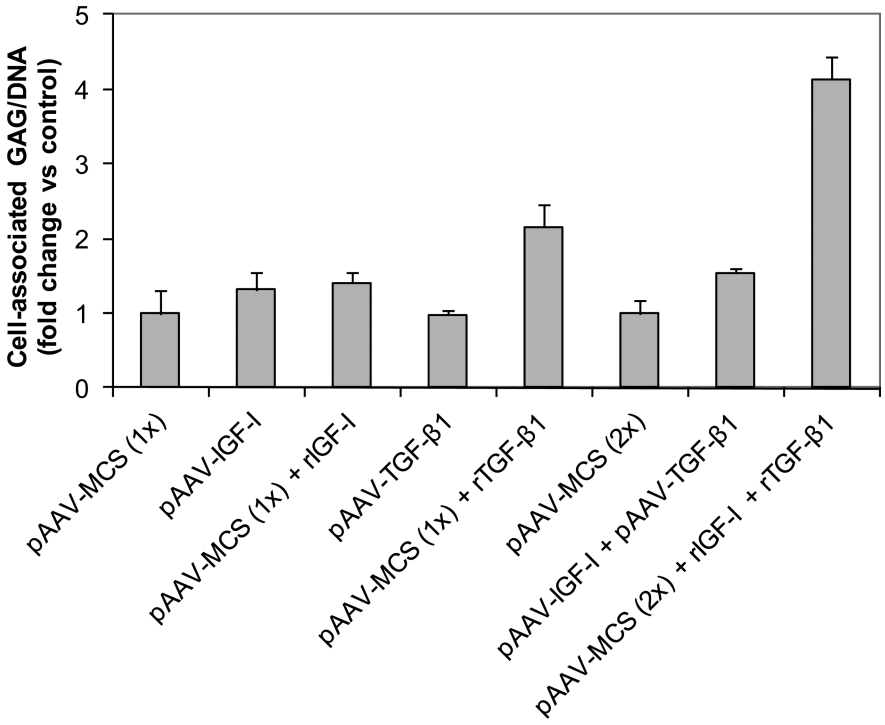

Figure 3.

Changes in cell-associated GAG normalized to DNA content. Chondrocytes were treated as described above. Chondrocytes were treated with recombinant growth factor(s) and/or transfected as described above. Cultures were terminated at 6 days. Data are expressed as the ratio of cell-associated GAG to DNA content normalized to the respective control ± SD for 3 independent experiments.

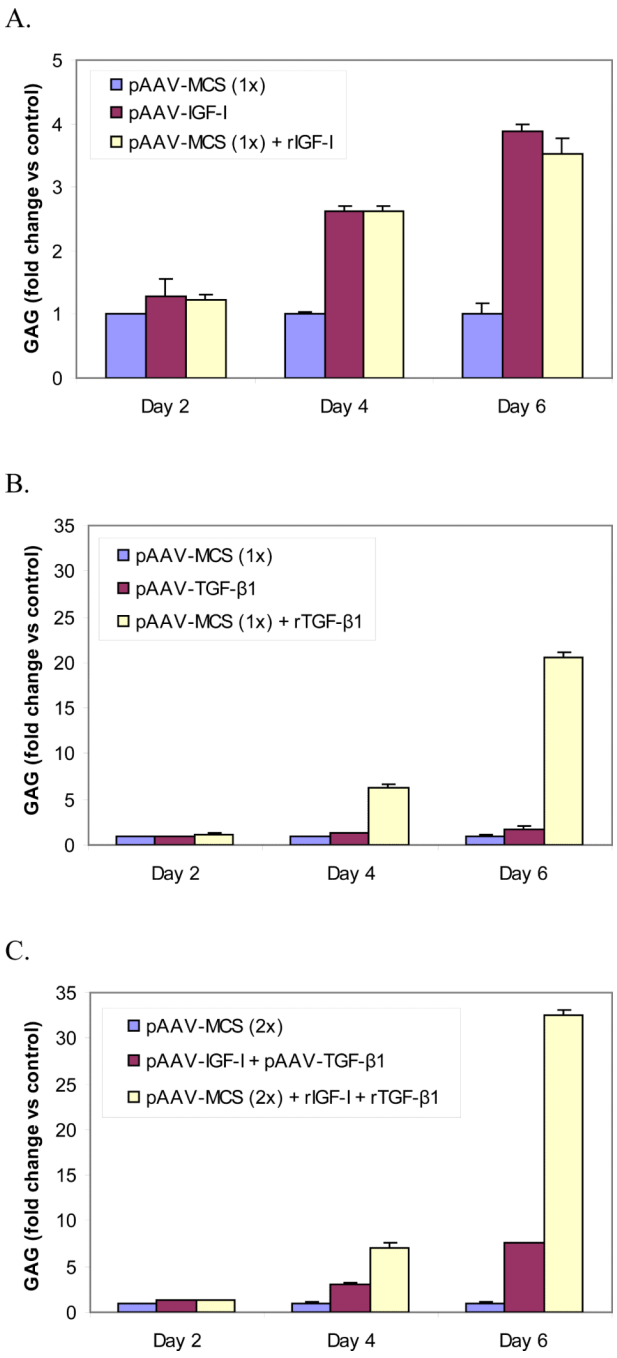

Figure 4.

Time-dependent changes in articular chondrocyte released GAG production in response to the designated treatments. Chondrocytes were treated with recombinant growth factor(s) and/or transfected as described above. GAG released into the culture medium was collected at 2-day intervals. Data are expressed as released GAG normalized to the respective control ± SD for each time point for 3 independent experiments.

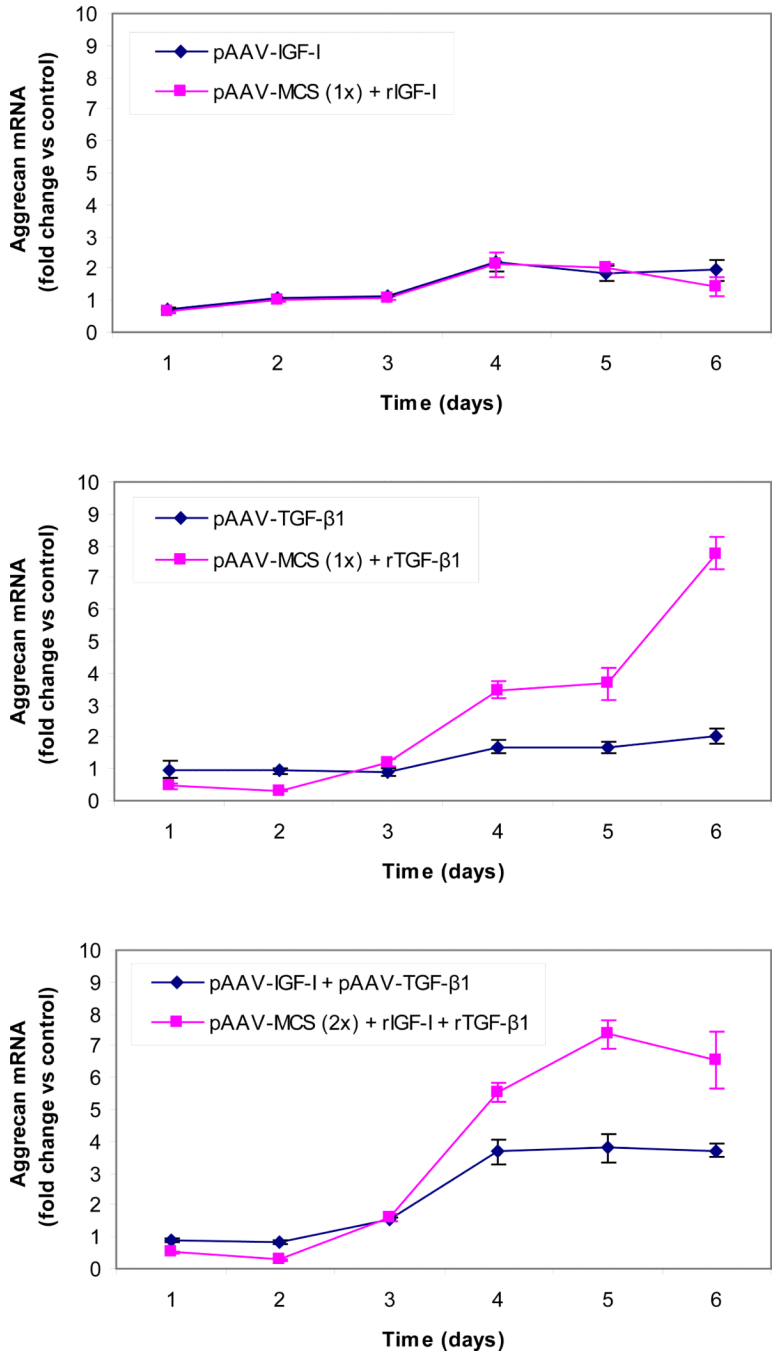

Figure 5.

Time course of aggrecan gene expression in response to the designated treatments. Chondrocytes were treated with recombinant growth factor(s) and/or transfected as described above. Aggrecan gene expression was measured by real-time PCR at daily intervals and normalized to 18S RNA. Data are expressed as aggrecan mRNA normalized to the respective control for each time point ± SD for 3 independent experiments.

Table 1

IGF-I produced by articular chondrocytes following the designated treatments. pAAV-MCS(1x): Empty control vector (single dose). pAAV-MCS (2x): empty control vector (double dose). pAAV-IGF-I: vector encoding IGF-I (endogenous IGF-I). pAAV-TGF-β1: vector encoding TGF-β1 (endogenous TGF-β1). rIGF-I: recombinant insulin-like growth factor I (exogenous IGF-I). rTGF-β1: recombinant transforming growth factor β1 (exogenous rTGF-β1).

Treatment	IGF-I (ng/ml±SD)		
	Day 2	Day 4	Day 6
pAAV-MCS (1x)	1.41±0.09	ND	ND
pAAV-IGF-I	289.89±20.11	115.81±1.10	12.03±0.49
pAAV-MCS (1x) + rIGF-I	192.36±22.23	63.73±0.67	3.48±0.14
pAAV-MCS (2x)	1.36±0.10	ND	ND
pAAV-IGF-I + pAAV-TGF-β1	137.22±19.44	62.23±7.00	7.00±0.40
pAAV-MCS (2x) + rIGF-I + rTGF-β1	174.99±32.70	53.77±6.56	2.42±0.27

ND: not detectable

Table 2

TGF-β1 produced by articular chondrocytes following the designated treatments. pAAV-MCS(1x): Empty control vector (single dose). pAAV-MCS (2x): empty control vector (double dose). pAAV-IGF-I: vector encoding IGF-I (endogenous IGF-I). pAAV-TGF-β1: vector encoding TGF-β1 (endogenous TGF-β1). rIGF-I: recombinant insulin-like growth factor I (exogenous IGF-I). rTGF-β1: recombinant transforming growth factor β1 (exogenous rTGF-β1).

Treatment	TGF-β1 (ng/ml±SD)					
	Day 2		Day 4		Day 6	
	HCl	No HCl	HCl	No HCl	HCl	No HCl
pAAV-MCS (1x)	3.38±0.08	ND	1.54±0.16	ND	1.13±0.05	ND
pAAV-TGF-β1	33.39±1.14	ND	26.26±1.21	ND	6.27±0.37	ND
pAAV-MCS (1x) + rTGF-β1	52.89±4.21	27.81±2.24	27.92±1.73	3.68±0.54	8.15±0.27	0.24±0.06
pAAV-MCS (2x)	3.09±0.34	ND	1.54±0.13	ND	1.16±0.03	ND
pAAV-IGF-I+pAAV-TGF-β1	30.69±1.39	ND	27.58±1.45	ND	7.19±0.12	ND
pAAV-MCS (2x) + rIGF-I + rTGF-β1	54.20±3.37	27.47±3.73	30.88±0.74	2.81±0.36	8.79±0.10	0.24±0.03

ND: not detectable