ORIGINAL ARTICLE

Hyaluronic acid-binding insulin-like growth factor-1: Creation of a gene encoding a bifunctional fusion protein

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Abstract

Chondrogenic growth factors are promising therapeutic agents for articular cartilage repair. A persistent impediment to fulfilling this promise is a limited ability to apply and retain the growth factors within the region of cartilage damage that is in need of repair. Current therapies successfully deliver cells and/or matrices, but growth factors are subject to diffusion into the joint space and then loss from the joint. To address this problem, we created a novel gene that encodes a bifunctional fusion protein comprised by a matrix binding domain and a growth factor. The gene encodes the hyaluronic acid binding region of the cartilage matrix molecule, versican, and the chondrogenic growth factor, insulin-like growth factor-1 (IGF-1). We delivered the gene in an adeno-associated virus-based plasmid vector to articular chondrocytes. The cells synthesized and secreted the fusion protein gene product. The fusion protein bound to hyaluronic acid and retained the anabolic and mitogenic actions of IGF-1 on the chondrocytes. This proof-of-concept study suggests that the bifunctional fusion protein, in concert with chondrocytes and a hyaluronic acid-based delivery vehicle, may serve as an intra-articular therapy to help achieve articular cartilage repair.

Keywords Genetic engineering · Articular chondrocyte · Insulin like growth factor-1 · Versican · Hyaluronic acid

Abbreviations

AAV	Adeno-associated virus
AG1	Aggrecan G1 domain
AU	Arbitrary unit
CM	Conditioned medium
DMB	Dimethylmethlyene blue
DMEM	Dulbecco's minimum essential medium
EDTA	Ethylenediaminetetraacetic acid
F	Forward primer
FBS	Fetal bovine serum
G1	Globular domain 1
GAG	Glycosaminoglycan
HA	Hyaluronic acid
HB-EGF	Heparin-binding epidermal-like growth factor
HEK-293	Human embryonic kidney 293 cells
HRP	Horseradish peroxidase
IGF-1	Insulin-like growth factor-1
Ka	Equilibrium association constant
PBS	Phosphate buffered saline
R	Reverse primer
SDS	Sodium dodecyl sulfate/sulphate
TBST	Tris-buffered saline/Tween
VG1	Versican G1 domain

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Introduction

Articular cartilage provides a gliding surface that enables pain-free motion of diarthrodial joints. Articular cartilage formation, maintenance, and repair depend on the chondrocytes embedded in the cartilage extracellular matrix. These

cells have a poor intrinsic healing capability after cartilage damage [1]. As a result, the articular cartilage loss that occurs in osteoarthritis is often progressive and is currently a leading cause of disability among adults [2]. Currently available treatments for osteoarthritis are effective for the symptoms of the disease, but treatments are needed that correct the cartilage damage.

Polypeptide growth factors play a central role in articular cartilage function [3]. Prominent among these is insulin-like growth factor-1 (IGF-1). IGF-1 stimulates both chondrocyte proliferation and synthesis of articular cartilage matrix [4–9]. In addition, IGF-1 reduces chondrocyte catabolic activity and inhibits the action of catabolic cytokines [10, 11].

Articular cartilage matrix is composed largely of a high molecular weight ($\geq 10^8$ daltons) aggregate molecules formed by a hyaluronic acid (HA) backbone decorated with covalently bound proteoglycans [12]. Two of these proteoglycans, versican and aggrecan, bind to HA through a peptide sequence designated the globular 1 (G1) domain at their N terminal end [13]. Though closely related, the G1 domain of versican has a higher binding affinity for HA than that of aggrecan [14]. These HA-proteoglycan aggregates help enable cartilage to support the compressive loads that are required for joint function. These cell–matrix interactions participate in the maintenance of cartilage homeostasis and in promoting cartilage tissue development and repair [15]. Due to the central role of HA in the assembly, organization, function and regulation of articular cartilage, HA has played a prominent role in efforts to treat articular cartilage damage [16] and has been successfully employed as a carrier or scaffold for chondrogenesis in vitro [17–19] and in vivo [20], and in clinical applications [21–24].

A major and pervasive problem in the therapeutic application of chondrogenic growth factors to articular cartilage repair is the inability to retain the growth factors at the repair site. To be effective, the growth factors must remain at the repair site long enough to interact with their receptors on their target cells before being lost by diffusion out of the joint space. Because growth factor proteins are relatively small molecules, this loss by diffusion can occur in a matter of hours [25]. To improve the effectiveness of growth factors in cartilage repair and regeneration, several delivery systems have been developed to localize and retain them where they are needed. IGF-1 has been genetically modified by adding a heparin binding domain from heparin binding epidermal growth factor (HB-EGF) to enable binding to heparin [26] by chondrocytes, an α_2 Plasmin Inhibitor domain to enable binding to plasmin by smooth muscle cells [27], and vitronectin sequences to enable binding to the vitronectin receptor on MCF-7 human breast cancer cells [28]. Matrix modifications include grafting of a short peptide sequence from IGF binding protein-5 onto alginate to promote chondrocyte matrix synthesis by retaining IGF-1 in the hydrogel [29].

In the present study, we created a novel bifunctional fusion protein designed to improve the function of IGF-1 in an HA-based, chondrocyte-bearing hydrogel. Specifically, we combined the HA-binding domain of versican (VG1) and full-length IGF-1 to create the fusion protein VG1–IGF-1 (Fig. 1). We hypothesized that VG1–IGF-1 binding via VG1 to the HA surrounding the cells will serve to retain the fusion proteins in the HA hydrogel, and that binding of the growth factor domain to cell surface receptors will stimulate reparative cellular functions.

Results

Synthesis of fusion proteins by articular chondrocytes

Chondrocytes were transfected with adeno-associated virus-based vectors pAAV-IGF-1, pAAV-VG1–IGF-1, or pAAV-MCS (empty vector) control, cultured for 48 h, and conditioned medium (CM) was harvested for IGF-1 and VG1–IGF-1 measurement. Chondrocytes transfected with empty vector produced no detectable IGF-1. Those transfected with pAAV–IGF-1 or pAAV–VG1–IGF-1 produced IGF-1 and VG1–IGF-1, respectively (Fig. 2). The VG1–IGF-1 in CM from chondrocytes transfected with pAAV–VG1–IGF-1 also possessed HA binding activity

Fig. 1 Schematic illustration of the proteins encoded by the expression constructs. *SP* signal peptide, *G1* globular domain 1, *CS* chondroitin sulfate domain, *G3* globular domain 3, *his* his tag. Light gray (blue) denotes the versican domains included in the constructs. Dark grey (tan) denotes the IGF-1 segments included in the constructs. (Color figure online)
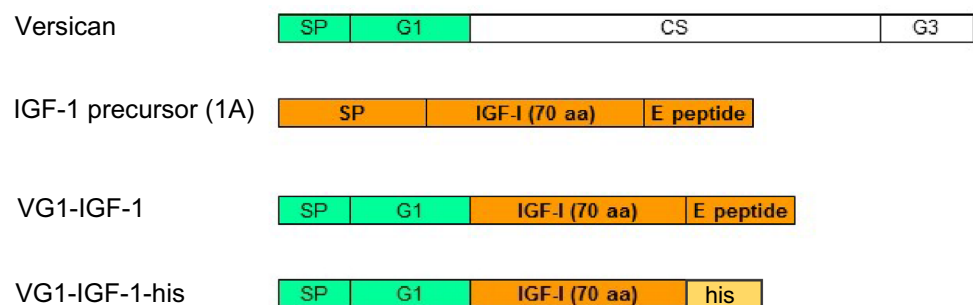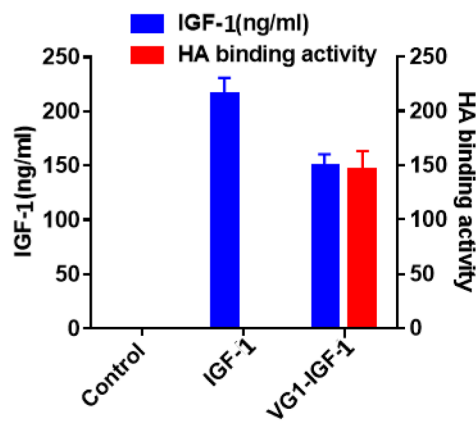
Fig. 2 IGF-1 and its fusion protein expression and HA binding activity. Chondrocytes were transfected by empty vector (control) or pAAV vector expressing IGF-1, or VG1–IGF-1. On day 2 after transfection, conditioned medium was collected for analyses. Data are presented as mean ± SD of triplicate wells from a representative experiment. (Color figure online)

(Fig. 2). No HA binding activity was detected in CM from cells transfected with empty vector or pAAV–IGF-1.

To determine whether the VG1 and IGF-1 components of the protein product were successfully combined in the same molecule, the expressed protein was assessed by western blot using antibodies against VG1 (anti-VG1) and IGF-1 (anti-IGF-1), respectively. No cleavage between the VG1 domain and the IGF-1 component was detected (Fig. 3). These data indicate that the pAAV–VG1–IGF-1 gene product secreted by these chondrocytes contains both IGF-1 and VG1.

Biological activity of VG1–IGF-1 fusion protein

To determine whether the IGF-1 component of the fusion protein retains its ability to stimulate chondrocytes, we measured the glycosaminoglycan (GAG) in the conditioned medium and the cell layer of the transfected chondrocytes as an index of matrix synthesis, and measured the DNA content in the cell layer as an index of cell proliferation. We found that delivery of pAAV–VG1–IGF-1 stimulated both chondrocyte GAG production and proliferation, and that the magnitude of this stimulation was comparable to that generated by pAAV–IGF-1 (Fig. 4). There was no difference in GAG production or cell proliferation between cells transfected with pAAV–VG1 and empty vector control. These data indicate that the IGF-1 component in VG1–IGF-1 retains the ability to regulate chondrocyte biosynthesis.

Production and purification of VG1–IGF-1 fusion protein

To purify VG1–IGF-1 fusion protein from the conditioned medium of transfected cells, we created a cDNA encoding VG1–IGF-1–his by replacing the E peptide of IGF-1 with a his tag (Fig. 1). This enabled VG1–IGF-1–his purification using Ni-affinity chromatography. To determine whether this substitution affects the function of the fusion protein, we transfected chondrocytes with pAAV–VG1–IGF-1–his and measured the expression, HA binding, and biological activity of the purified his-tagged fusion protein. The results
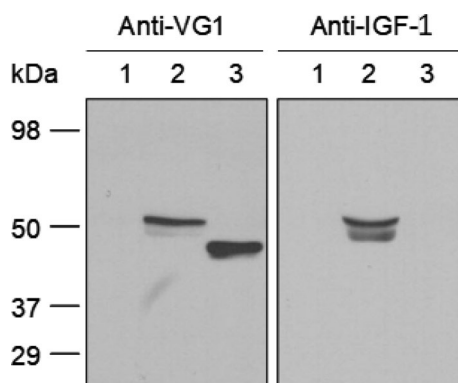

Fig. 3 Western blot of fusion proteins in conditioned medium probed with anti-VG1 (left panel) or anti-IGF-1 (right panel) antibodies. Chondrocytes were transfected by empty vector control (lanes 1), pAAV vector expressing VG1–IGF-1 (lanes 2), or pAAV vector expressing VG1 (lanes 3). On day 2 after transfection, conditioned medium was collected for analyses
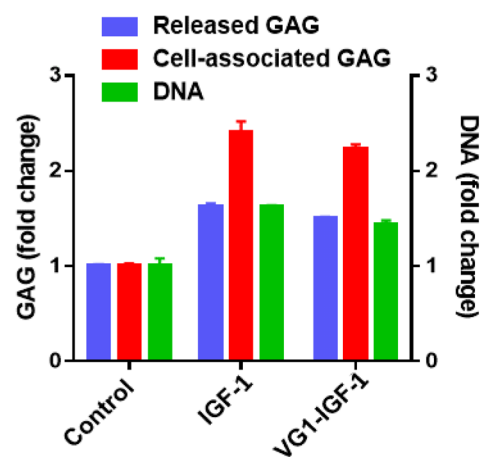

Fig. 4 Effects of IGF-1 and its fusion protein on chondrocyte GAG and DNA content. Chondrocytes were transfected by empty vector (control) or pAAV vector expressing IGF-1, or VG1–IGF-1. The cells were cultured for 6 days after transfection. GAG released into the medium and retained in cell layer were measured separately. DNA content was measured in the cell layer. Data are normalized to control and presented as mean ± SD of triplicate wells from a representative experiment. (Color figure online)

showed that the substitution of the E peptide by the his tag did not alter any of these parameters (Fig. 5).

To characterize VG1–IGF-1–his, human embryonic kidney (HEK)-293 cells were selected for VG1–IGF-1–his overexpression because these cells generate more VG1–IGF-1–his fusion protein than do chondrocytes. Following transfection of HEK-293 cells with pAAV–VG1–IGF-1–his, the cells secreted 3020 ng/ml of VG1–IGF-1–his protein into the conditioned medium. The HA binding activity of the fusion protein was 2864 AU/ml. Purification yielded ~2 mg of recombinant VG1–IGF-1–his protein per liter of conditioned medium. The purity of the recombinant protein was assessed by SDS-PAGE and silver staining and appeared as a single band (Supplementary Fig. 1).

Discussion

IGF-1 was chosen for the present study due to its ability to stimulate the dual cartilage repair functions of chondrocyte proliferation and of cartilage matrix synthesis, as well as to reduce chondrocyte catabolic activity and promote articular cartilage repair, in both in vitro and in vivo cartilage repair models [30–33]. Versican was selected as the source of the hyaluronic acid binding domain due to its presence as a constituent of normal articular cartilage matrix, and its higher affinity for HA compared to the related HA binding molecule, aggrecan. We previously compared the interaction of the aggrecan G1 domain (AG1) and versican G1 domain (VG1) with HA by surface plasmon resonance analysis. The data demonstrated that the equilibrium association constant (Ka) of VG1 with HA was 5.1-fold greater than that of AG1 (0.1×10^8 M^{-1} and 0.6×10^8 M^{-1} respectively [14].

The therapeutic application of chondrogenic growth factors, such as IGF-1, has been hindered by difficulties in retaining the growth factor within the joint at the site of articular cartilage damage. We have developed a system that may help resolve this problem. The system includes the triad of tissue engineering elements: cells, biological stimulus, and carrier, and also adds a novel bifunctional fusion protein that physically and functionally integrates these elements. The results of this study indicate that the fusion protein comprised by VG1 and IGF-1 possesses both the ability to bind to HA and to stimulate biosynthesis by chondrocytes. Interestingly, even though the VG1 component of the fusion protein is much bigger than that of the IGF-1 component (343 aa vs. 70 aa), the VG1 does not appear to interfere with the ability of the IGF-1 to interact with its receptor so as to stimulate its target cells. Additionally, the transfected chondrocytes produced similar molar amounts of VG1–IGF-1 and of IGF-1, suggesting that the post-translational processing of the fusion protein is not substantially limited in comparison to IGF-1.

The results of this study suggest that this strategy can be applied to either cell-based growth factor gene therapy or to exogenous protein therapy for cartilage repair. Each of these approaches has advantages and disadvantages.
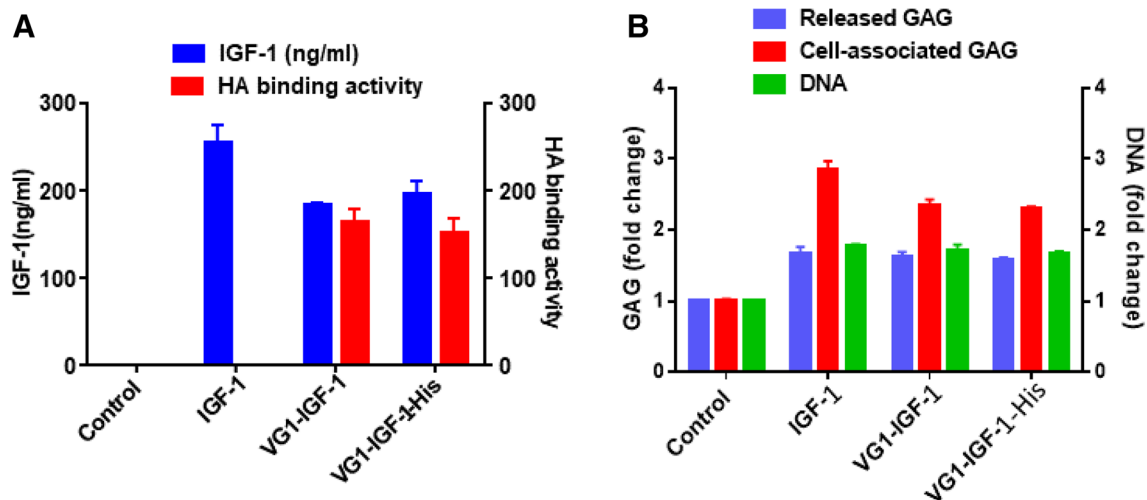

Fig. 5 Comparison of VG1–IGF-1–His with VG1–IGF-1. Chondrocytes were transfected by empty vector (control) or pAAV vector expressing IGF-1, VG1–IGF-1 or VG1–IGF-1–His. **a** Expression of IGF-1, VG1–IGF-1 and VG1–IGF-1–His (blue), and HA binding activity of VG1–IGF-1 and VG1–IGF-1–His (red). On day 2 after transfection, conditioned medium was collected for analyses. Data are presented as mean±SD of triplicate wells from a representative experiment. **b** Effects of IGF-1, VG1–IGF-1 and VG1–IGF-1–His on chondrocyte GAG and DNA content. The cells were cultured for 6 days after transfection. GAG released into medium (blue) and retained in cell layer (red) were measured separately. DNA content (green) was measured in the cell layer. Data are normalized to control and presented as mean±SD of triplicate wells from a representative experiment. (Color figure online)

Gene therapy has the advantage of providing an ongoing source of locally concentrated fusion protein. Potential disadvantages include the requirements that the synthesized VG1–IGF-1 fusion protein be produced in biologically relevant amounts, bind to the HA-based vehicle containing the transfected cells, and act on the cells through paracrine and/or autocrine mechanisms. However, the data demonstrate that all of these requirements were met. Protein therapy has the potential disadvantage of requiring a method of timed release. However, this is a rapidly advancing field with many that is likely to provide suitable options. Advantages include the ability to produce much larger amounts of fusion protein and to more precisely adjust its dose. This study suggests that either approach could be effective. An additional advantage of protein therapy over gene therapy is its substantially less complex regulatory approval process. Going forward the simplest next steps in the translational pathway may be to focus on the protein as the therapeutic agent and on gene transfer as the tool with which to produce it.

In this study, articular chondrocytes were selected for their ability to produce and maintain articular cartilage matrix. Other cell types can also be used. Various progenitor or stem cells have chondrogenic potential. Many of these cells have the advantage of being more easily obtained than articular chondrocytes, but all have the disadvantage of needing to be converted to mature chondrocytes.

HA is found in variable amounts in most tissues. It is used as vehicle for drug delivery and also to create various HA-based carriers for the regeneration of multiple tissue types. IGF-1 regulates many cells in other tissues in addition to chondrocytes in cartilage. For these reasons, VG1–IGF-1 may be applicable to tissues other than cartilage.

Compared to other forms of controlled release, the system tested here offers the advantage that it does not require the development of additional biomaterials to store and release the growth factors. The approach is also tunable. In addition to varying doses, the affinity for HA of the fusion proteins can be varied by the choice of HA binding domain and the proportion of fusion proteins containing different growth factors. Further, the time course of fusion protein release timing could, in future iterations, be controlled by inserting cleavable linkers between the growth factors and HA binding domains.

The translational pathways for this approach to cartilage repair involve established and developing methods. A well-established method for the treatment of damaged articular cartilage are autologous chondrocyte transplantation (ACI) [34] and its successor, matrix-induced ACI (MACI) [35–37]. Substitution of the currently employed, untransfected chondrocytes in suspension by transfected chondrocytes in HA (gene therapy), or the addition of VG1–IGF-1 fusion protein in HA to the currently employed untransfected cells (protein therapy) are straightforward potential pathways.

The experimental design for randomized controlled trials testing the safety and efficacy of autologous cell therapies for knee cartilage defects has been published and implemented [38], and would be appropriate for such studies. A similar pathway has been employed in evaluating cartilage regeneration in osteoarthritis patients using a combination of stem cells and hyaluronic acid as an addition to subchondral drilling [39]. In addition to the established translational pathways noted above, developing methods relevant to the translation of the present combination of chondrocytes, fusion protein and HA include advances in gene vector and controlled protein release technologies as well as evolving surgical techniques. Progress in these fields may be expected to open additional translational pathways for this approach to cartilage repair.

A limitation in this study is that, as a proof-of-concept study, it covers the relatively early stage of development and investigation of this class of molecules. Further studies will be needed to determine the release kinetics and test whether the biological effects of the fusion protein are superior to those of the native growth factor in a composite of chondrocytes and an HA-based delivery vehicle. An additional limitation is that these data are specific to IGF-1, the G1 domain of versican, and a hyaluronic acid-based vehicle. However, this approach, including these methods of fusion protein construction, may be applicable to other growth factors, other binding domains, and other carriers.

Conclusion

These data support the concept that a tripartite construct, composed of a bifunctional fusion protein containing IGF-1 and the HA-binding versican G1 domain, an HA-based vehicle, and articular chondrocytes, may have potential value for articular cartilage repair. If confirmed by further studies, these data would serve as a basis for future in vitro and in vivo studies to assess the effectiveness of this approach.

Materials and methods

Expression vector construction

Vector pAAV-IGF-1 was prepared as previously described [40]. The cDNA encoding human versican G1 domain (VG1) (Fig. 1), the N-terminal 363 amino acid sequence of human versican (Accession #NM_001164097), was generated by PCR using pFastBac-VG1 [14], as a template and, Forward primer VG1–F1 and Reverse primer VG1–R1 (Supplementary Table 1). The PCR product was cloned into pCR II-TOPO (Invitrogen). After confirming the sequences, the

cDNA was sub-cloned into pAAV-MCS (Stratagene, La Jolla, CA) to obtain pAAV-VG1.

The cDNA encoding VG1–IGF-1 fusion protein (Fig. 1) was generated by initially cloning two separate DNA fragments and then assembling them. The first DNA fragment, encoding the N-terminal 361 amino acid sequence of human versican (Accession #NM_001164097), was generated by PCR using pAAV-VG1 as a template, Forward primer VG1-F1 and Reverse primer VG1-R2 (Supplementary Table 1). The second DNA fragment encoding the Ser^{362}Glu^{363} of human versican (Accession #NM_001164097) and the C-terminal 105 amino acid sequence of human IGF-1 (Accession #NM_000618), was generated by PCR using pAAV-IGF-1 as a template, Forward primer IGF-1-F1 and Reverse primer IGF-1-R1 (Supplementary Table 1). The Ser^{362}Glu^{363} of human versican was introduced by Forward primer IGF-1-F1 during PCR. The PCR products were separately cloned into pCR II-TOPO (Invitrogen). After confirming the sequences, the fragments were sequentially sub-cloned into pAAV-MCS at EcoRI and XhoI sites, and assembled through a Mfe I restriction enzyme site to obtain pAAV–VG1–IGF-1. The Mfe I site (CAATTG) in 5′-CCAATTGAT-3′, was generated from the sequence 5′-CCAATAGAT-3′ of Genebank accession number NM_001164097 from 1431 to 1439 encoding Pro^{359}Ile^{360}Asp^{361} in human versican (Fig. 1).

The cDNA encoding VG1–IGF-1 with a his tag (VG1–IGF-1–his), in which the E peptide of IGF-1 was replaced by a his tag, was generated by PCR using pAAV–VG1–IGF-1 as a template, Forward primer VG1-F1 and Reverse primer His-R1 (Supplementary Table 1). The PCR product was cloned into pCR II-TOPO (Invitrogen). After confirming the sequences, the cDNA was sub-cloned into pAAV-MCS (Stratagene) to obtain pAAV–VG1–IGF-1–his.

Chondrocyte isolation, monolayer culture and transfection

Dulbecco's minimum essential medium (DMEM), fetal bovine serum (FBS), penicillin, streptomycin, glutamine and proteinase k were from Life Technologies. Ascorbic acid was from Sigma. Basal medium contained DMEM, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine and 50 μg/ml ascorbic acid. Complete medium contained basal medium supplemented with 10% FBS. Bovine articular chondrocytes were isolated as previously described [40]. Briefly, chondrocytes were isolated from the carpal articular cartilage of ~ 1 year old bovids and placed in monolayer culture in 6-well plates at a density of 3×10^5 cells/well in 4 ml of complete medium. After 3 days, cells were transfected using FuGENE 6 (Roche Applied Science) and 2 μg of each plasmid DNA per well. The ratio of plasmid DNA (μg) to

FuGENE 6 reagent (μl) was 1:3. After 16 h, transfection was stopped by replacing the medium with 4 ml of fresh complete medium. On day 2 and day 4 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 2 ml proteinase k solution (0.5 mg/ml proteinase k in 10 mM Tris, pH 8.2, and 5 mM EDTA) at 65 ℃ for 2 h. CM was stored at − 20 ℃ for analyses of IGF-1 and proteoglycan in medium. The cell digest was stored at − 20 ℃ for analyses of proteoglycan and DNA in the cell layer. This study did not include the use of animals. We used only bovine articular cartilage obtained from leg joints that had been discarded by a local abattoir. The bovine articular cartilage was used for chondrocyte isolation.

Analysis of VG1–IGF-1 fusion protein by ELISA and Western blot

IGF-1 in conditioned medium was measured by human IGF-1 ELISA (DuoSet, R&D Systems) according to the manufacturer's protocol. Specifically, phosphate buffered saline (PBS, 11.9 mM phosphate, 137 mM sodium chloride, 2.7 mM potassium chloride, pH 7.4) was used to dilute capture antibody for plate coating. PBS with 0.05% Tween 20 was used as wash buffer. PBS with 5% Tween 20 was used as block buffer, and also as a reagent diluent to dilute samples, IGF-1 standard, detection antibody and streptavidin-HRP.

HA binding activity was measured by a functional ELISA using HA in place of the anti-IGF-1 capture antibody in the human IGF-1 ELISA. Specifically, HA purchased from Sigma at 0.5 mg/ml in PBS was used instead of anti-IGF-1 capture antibody to coat plates. HA binding activity was calculated based on the IGF-1 standard curve of the human IGF-1 ELISA conducted on the same ELISA plate. The resulting value, expressed in arbitrary unit (AU), reflects the fusion protein concentration, the HA amount relative to the amount of IGF-1 capture antibody coated on the ELISA plates and the affinity of fusion protein with HA relative to that of IGF-1 with IGF-1 capture antibody. This assay measures HA binding only of molecules that include IGF-1.

VG1–IGF-1 fusion protein in conditioned medium was analyzed by western blotting. One part of 6X SDS-Sample Buffer (375 mM Tris–HCl pH 6.8, 6% SDS, 48% glycerol, 9% 2-Mercaptoethanol, and 0.03% bromophenol blue) was mixed with 5 parts of sample, and heated at 95 ℃ for 10 min. The prepared samples were electrophoresed on a 10% SDS–polyacrylamide gel, and the proteins were transferred to nitrocellulose membranes. The membranes were blocked with 5% fat-free milk (Bio-Rad) in Tris-buffered saline/Tween (TBST) for 1 h at room temperature and were then separately probed with primary antibodies against to IGF-1 (Epitomics, Cat.#:5217-1) or versican (12C5, Developmental Studies Hybridoma Bank) overnight at 4 ℃.

Proteoglycan and DNA analysis

Glycosaminoglycan (GAG) released into the medium (released GAG) and retained with the chondrocytes (cell-associated GAG) were separately measured by dimethylmethlyene blue (DMB) assay using chondroitin sulfate A (Sigma) as the standard. Cell proliferation was assessed by DNA analysis of the cell digest by Picogreen dsDNA assay (Molecular Probes) according to the manufacturer's instructions using pure phage λ DNA as the standard.

Expression and purification of VG1–IGF-1 fusion protein with a his tag

HEK-293 cells (ATCC) were cultured in complete medium without ascorbic acid and transfected by pAAV–VG1–IGF-1–his using FuGENE 6 (Roche Applied Science). After 12 h, transfection was stopped by replacing the medium with serum free 293 expression medium (Gibco). On day 2 after transfection, conditioned medium (CM) was harvested and centrifuged to remove cell debris. Recombinant protein VG1–IGF-1–his was purified from the conditioned medium by Sephadex G-25 and nickel-nitrilotriacetic acid-agarose (Qiagen, Mississauga, ON) column chromatography as described previously [14]. The purity of purified VG1–IGF-1–his recombinant protein was analyzed by 10% SDS-PAGE under reducing conditions and silver staining.

Compliance with ethical standards

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