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Promotion of Chondrocyte Proliferation by Versican Mediated by G1 Domain and EGF-like Motifs

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Abstract We have previously demonstrated that versican stimulated NIH3T3 fibroblast proliferation. Since versican is expressed in cartilage, we investigated whether versican plays a role in chondrocyte proliferation. We developed a technique to stably express a recombinant versican mini-gene in chicken chondrocytes, and its effect on chondrocyte proliferation was analyzed by the increase in cell number. The effect of cell adhesion on cell proliferation was tested. Finally, the versican mini-gene was truncated to assess the role of EGF-like motifs in cell proliferation. Expression of the recombinant versican mini-gene stimulated chondrocyte proliferation. Antisense oligonucleotides complementary to versican inhibited chondrocyte proliferation. The G1 domain of versican stimulated chondrocyte proliferation by destabilizing chondrocyte adhesion. Furthermore, deletion of the two EGF-like motifs from the G3 domain also reduced the function of versican in stimulating cell proliferation. Versican enhances chondrocyte proliferation through a mechanism involving its G1 and G3 domains. This finding may have implications for our understanding of the pathogenesis of various joint diseases. J. Cell. Biochem. 73:445–457, 1999.

Key words: G1 domain; G3 domain; EGF; adhesion; proliferation

Versican was originally isolated from human fibroblasts [Zimmermann and Ruoslahti, 1989; Naso et al., 1994] and chicken developing limb buds [Kimata et al., 1986]. Sequence analysis indicated that versican is a member of the large aggregating chondroitin sulfate proteoglycan family [Zimmermann and Ruoslahti, 1989; Shinomura et al., 1993]. All members of the group contain a G1 domain and a G3 domain (also called a selectin-like domain) at their N- and C-terminal ends, respectively. A large chondroitin sulfate side chain-bearing sequence (CS sequence), to which 12–15 chondroitin sulfate side chains are covalently attached, is localized in the middle region to versican. The N-terminal of versican binds hyaluronan [Lebaron et al., 1992]. The G3 domain, consisting of two set of epidermal growth factor-like (EGF-like) motifs—one lectin-like (or carbohydrate recognition domain [CRD]-like) motif, and one complement-binding protein-like (or CBP-like) motif [Krusius et al., 1987]—has been shown to bind tenascin [Aspberg et al., 1995, 1997]. With alternative splicing, the versican gene has many isoforms, which may express different functions.

Versican is expressed in a variety of tissues, especially in fast-growing cells [Zimmermann et al., 1994; Bode-Lesniewska et al., 1996]. For example, versican is transiently and highly expressed during mesenchymal condensation process in developing chicken limb buds and disappears in mature cartilage. It has been suggested that versican plays a role in cell proliferation and mesenchymal chondrogenesis. Using a very sensitive approach, Grover and Roughley [1993] recently demonstrated that versican is expressed in human articular cartilage at all ages, albeit at very low concentrations. The proliferative activity of normal chondrocytes is also low. In osteoarthritic cartilage, versican expression is predominantly detected in the pericellular matrix of chondrocytes [Nishida et al., 1994]. The fact that the increased versican protein level is much higher than its mRNA level suggests accumulation or enhanced versican synthesis in osteoarthritis (OA) [CS-Szabó et al., 1997]. The effects of increased versican in OA

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are unclear. It is believed that the increased versican is synthesized by the osteoarthritic chondrocytes [Nishida et al., 1994]. Because chondrocytes usually display increased proliferation during OA, it is likely that chondrocytes produce more versican to stimulate chondrocyte production for cartilage repair.

To investigate the effects of versican in chondrocyte activities, we chose to mimic the increased expression and accumulation of versican in osteoarthritic cartilage using gene expression in primary chondrocyte cultures. In this article, we report that the expression of a mini-versican gene, or exogenous addition of the mini-versican gene product, stimulates chondrocyte proliferation. Versican enhanced chondrocyte proliferation through its G1 domain and G3 domain. The G1 domain reduced chondrocyte adhesion and thus enhanced chondrocyte proliferation. Expression of the versican G3 domain alone achieves the same effect on enhancing chondrocyte proliferation, but deletion of the EGF-like motifs from G3 greatly reduces its effect. This work thus provides the first evidence that versican is able to stimulate chondrocyte growth. This effect appears to be mediated through the G1 domain and, at least partly, through the EGF-like motifs of the G3 domain.

MATERIALS AND METHODS

Materials

The chicken eggs (products of Hamburger and Hamilton strains) were purchased from Brampton Chick Hatchery Co. Ltd. (Brampton, Ontario, Canada). Bacterial growth medium was from Difco. Lipofectin, Geneticin (G418), Dulbecco's modified Eagle's medium (DMEM) growth medium, fetal bovine serum (FBS), Hank's balanced salt solution (HBSS), and trypsin/EDTA were from GIBCO-BRL (Canadian Life Technologies, Burlington, ON, Canada). The ECL Western blot detection kit was from Amersham. Taq DNA polymerase, T4 DNA ligase, and restriction endonucleases were from Boehringer-Mannheim. The Prep-A-Gene DNA purification kit, prestained protein markers, were from Bio-Rad. The DNA mini-prep kit was from Bio/Can Scientific. The DNA Midi-prep kit was from Qiagen, Inc. The goat anti-mouse IgG (horseradish peroxidase) (HRP) and goat anti-rabbit HRP were from Sigma Chemical Co. (St. Louis, MO). The 24-well and 96-well tissue culture plates were from Nunc, Inc. Oligonucleotides were from BioBasic, Inc. (Scarborough, ON). All chemicals were from Sigma.

Chondrocyte Isolation and Growth

Chicken sterna from 16-day embryos were removed and rinsed with phosphate-buffered saline (PBS). Each sterna was cut into pieces sagitally and incubated at 37°C for 30 min in dissociation medium containing 0.1% trypsin and 0.3% collagenase (Sigma product, Cat. No. C-6885) in HBSS. The dissociation medium was changed and the tissue was incubated for a further 1 h. An equal volume of growth medium (DMEM supplementary with 10% FBS) was then added to stop the enzyme reaction. Cells were collected by passing the digested mixture through a filter (40 μm nylon) and centrifuging the filtrate at 1,100 rpm. The chondrocytes were then resuspended in growth medium, plated in tissue culture dishes, and grown as a suspension culture in the growth medium. Within 1 week, some cells gradually attach to the tissue culture plates. The chondrocytes could then be cultured as two pools: a pool of adherent chondrocytes and a pool of chondrocytes in suspension. During growth, a small proportion of suspended chondrocytes tended to lose the suspension property and attach to the tissue culture plates. Nevertheless, the pool of suspension chondrocytes could be maintained by passing only the suspended chondrocytes to a new culture every time. The adherent chondrocytes developed a fibroblast-like morphology and could be maintained for many months. Initially, both pools of cultures expressed sufficient concentrations of aggrecan, link protein and type II collagen, characteristics of chondrocytes. This confirmed that the fibroblast-like cultures were chondrocytes, which obtained a changed morphology.

Expression of Recombinant Gene

To construct a recombinant versican minigene, we cloned the G1 domain, a part of the CS domain and the G3 domain into the pUC19 vector individually. The sequence of versican has been published by Shinomura et al. [1993]. The protocol to generate a mini-versican construct has been described by us previously [Zhang et al., 1998]. Briefly, the G1 domain was produced with two primers G1N*Hin*d III (AAA AAG CTT GCC GCC ACC ATG GTG TTA AAC ATA AAA AGC ATC [Shinomura et al., 1993] and G1C*MluI* (AAA ACG CGT TTC GTA GCA

GTA GGC ATC AAA). The CS sequence was synthesized with CSN*MluI* (5′ AAA ACG CGT CGT AAA AAA ATT GTA TCA GAG) and CNC*XhoI* (5′ AAA CTC GAG ACT CAT TTC TGG CTC CTT TGT) as primers. The G3 fragment was prepared using primers G3N*Xho*I (5′ AAA CTC GAG GGA CAG GAT CCA TGC AAA AGT) and G3C*SphI* (5′ AAA GCA TGC GCG CCT TGA GTC CTG CCA CGT). These three DNA fragments were ligated together into pcDNA1 (Invitrogen), producing a G1CSG3 construct. Constructs obtained by PCR were sequenced, and domains were confirmed to be identical as that reported previously [Shinomura et al., 1993].

In generating the G3 construct, the leading peptide of link protein [Deak et al., 1986] was joined with the versican G3 domain in order to allow secretion of the G3 gene product. The leading peptide of link protein was amplified with the primers 5′ AAA GAA TTC GCC GCC ACC ATG GCA AGT CTA CTC TTT CTG and 5′ AAA CTC GAG AGG CAG TGT GAC GTT GCC in a PCR reaction. The PCR product was purified and digested with *Eco*RI and *Xho*I. The G3 domain was derived from the G1CSG3 construct using restriction enzymes *Xho*I and *Sph*I. The G3 fragment and link protein leading peptide were ligated into the pcDNA1 vector digested with *Eco*RI and *Sph*I. The leading peptide of link protein contains an epitope recognized by a monoclonal antibody 4B6 [Binette et al., 1994]. We have successfully used this antibody in detection of recombinant products in previously studies [Yang et al., 1997, 1998; Zhang et al., 1998; Cao et al., 1998].

The G1CS construct shown in Figure 1A was generated by insertion of the link protein leading peptide and G1CS sequence into between the *Eco*RI and *Xho*I sites of pcDNA1. The leading peptide contains an *Eco*RI site at its 5′ end and a *Bam*HI site at its 3′ end, while the G1CS sequence contains a *Bam*HI site at its 5′ end (nucleotides 244–249) [Shinomura et al., 1993] and a *Xho*I site at its 3′ end created as described above. Using the same strategy, the signal peptide of link protein was manipulated in the same reading frame into the G1 domain to generate the G1 construct, as well as to the CSG3 sequence, to obtain the CSG3 construct.

To delete the EGF-like motifs from the G3 domain of versican, a site for the restriction enzyme *Xho*I was engineered at the 5′ end of the carbohydrate recognition domain (CRD). This was performed by using CRDN*Xho*I (AAA CTC GAG C(10129)AA GAC ACA GAG ACT) and G3C*SphI* (5′ AAA GCA TGC G(10830)CG CCT TGA GTC CTG CCA CGT) as primers in a PCR reaction. The product was digested with *Xho*I and *Sph*I and ligated into the recombinant mini-versican from which the full-length G3 domain had been removed by digestion with *Xho*I and *Sph*I.

The constructs made in pcDNA1 (Invitrogen) were transiently transfected into COS-7 cells (American Type Culture Collection) using Lipofectin [Felgner et al., 1987] according to the manufacturer's instructions (GIBCO) to analyze the gene product. Briefly, COS-7 cells were seeded to a six-well plate with 1.5×10^5 cells per well in DMEM supplemented with 5% FBS (GIBCO). The next day, when the cells had reached 70% confluence, plasmid DNA (5 μg) was incubated with Lipofectin (10 μl) for 15 min in 200 μl DMEM; then, 800 μl DMEM was added. The COS-7 cell culture was washed briefly, and the Lipofectin–DNA mixture was applied. After 5 h, the DNA–Lipofectin mixture was replaced with 2 ml of DMEM supplemented with 5% FBS. The growth medium and cells were harvested separately after 3 days and samples were frozen until analysis.

The constructs were also subcloned into pcDNA3 and transfected into NIH3T3 fibroblasts as described above. The day after transfection, Geneticin (0.5 mg/ml) was added to the growth medium and the cells were maintained in this medium until individual colonies were large enough for cloning. Chemically selected cell lines were maintained in medium containing 0.5 mg/ml of Geneticin or stored in liquid nitrogen.

Isolation of Individual and Pooled Chondrocyte Cell Lines

The newly isolated chondrocytes were initially grown as a suspension culture. The cells gradually attached to the tissue culture plates and exhibited a fibroblast-like morphology. The adherent cells were transfected with the versican mini-gene in expression vector pcDNA3, using Lipofectin; however, as the efficiency of chondrocyte transfection is much lower than that of fibroblasts, the amount of Lipofectin and DNA used for transfection was increased by 50%. The length of incubation was increased to 10 h, in some cases. Transfected cells were selected in DMEM containing 10% FBS and 0.5 mg/ml Geneticin. To accelerate the prolifera-

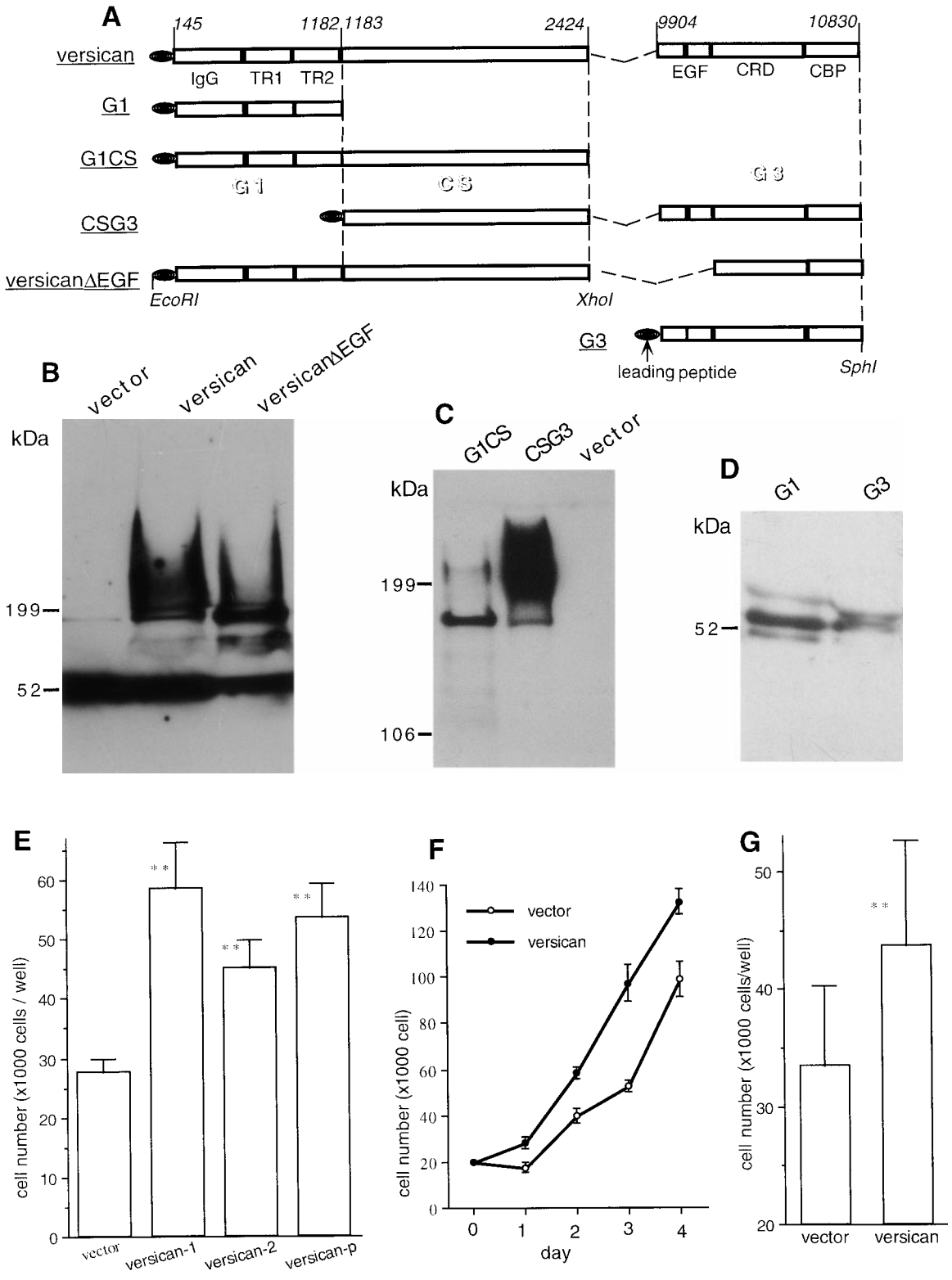
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Figure 1.

tion of transfected chondrocytes, culture medium, which had been incubated with 60% confluent chondrocytes for one day, and contained growth factors secreted by the chondrocytes, was added to the transfected cultures. This medium was also used to enhance proliferation of transfected chondrocytes after colony isolation. With this technique, transgenic chondrocyte cell lines could be obtained within one month. The selected cell lines were stored in liquid nitrogen or maintained in growth medium containing 0.5 mg/ml Geneticin for subsequent gene expression assays and functional studies. Incorporation of interesting constructs into the selected cell lines was verified by PCR as described earlier [Yang et al., 1997]. Cell lines were monitored to ensure expression of the transgene for the duration of functional studies.

For isolation of pooled cell lines, the individual colonies, which harbored the transgene and were resistant to Geneticin, were pooled together and grown as a pooled cell line. In this way, a pooled cell line contained 5–10 individual cell lines.

Chondrocyte Proliferation Assays

Chondrocyte proliferation was measured using a cell counting assay. In chondrocyte cell lines stably transfected with different constructs, cells were seeded at a concentration of 2×10^4 cells/ml into each well of 24-well plates and cultured with standard medium (DMEM supplemented with 5% FBS, unless otherwise stated in the legends) at 37°C in an incubator. Vector-transfected cell line was used as a control. Each cell line was grown in four wells. Cell numbers were determined in each well using a cytometer, either after 3 days or each day for 4 days. The results were analyzed statistically using Student's *t*-test with $P < 0.05$ (*) or $P < 0.01$ (**). To test the effects on chondrocyte proliferation of growth media collected from transfected COS-7 cells, the media were mixed with low serum media (DMEM containing 2.5% FBS) in a 1:1 ratio and added to the chondrocyte cultures. Cell proliferation was evaluated after 3 days.

Studies Using Versican Antisense Oligonucleotides

Chondrocytes were cultured in 96-well tissue culture plates in DMEM containing 5% FBS. 200 µl of chondrocyte culture suspension at a concentration of 1.5×10^4 cells/ml was inoculated in each well; 20 µl of oligonucleotides (complementary to the 5′ of versican, ATG TTG TTA AAC ATA AAA AGC) was then added to obtain a final concentration of 4.2 µM. Cultures were incubated at 37°C in a tissue culture incu-

Fig. 1. Chondrocyte proliferation stimulated by expression of a recombinant versican. **A:** Constructs used in this study. The leading peptide added to the constructs was obtained from link protein. The G1 domain includes nucleotides 145–1182 of mature versican; the truncated CS region, nucleotides 1183–2424; and the G3 domain, nucleotides 9904–10830. SP, signal peptide or leading peptide; IgG, immunoglobular domain; TR, tandem repeat; EGF, epithelial growth factor-like motif, CRD, carbohydrate recognition domain; CBP, complementary binding protein. Numbers above schematic correspond to nucleotides in the sequence of full-length versican. B–D: Gene expression was analyzed by Western blot analysis stained with a monoclonal antibody against an epitope engineered into the constructs. A vector-transfected chondrocyte line was used as a control. All products were collected from different transfection assays mentioned in different sections of this paper. All proteins had expected sizes measured in the blots, and all CS sequence-containing constructs migrated as smears because of the attachment of chondroitin sulfate chain. **B:** A two-gel system containing a 12% gel in the lower portion and 5% gel on the higher portion was used. Link protein (52 kDa) migrated into the 12% gel, while the recombinant versican and versicanΔEGF (recombinant versican lacking EGF-like motifs) stayed in the 5% gel. To observe the bands of proteoglycans, the amount of samples loaded in each well had to be double (40 µl), and the exposure time was longer. Gel concentration: 5% (**C**) and 12% (**D**). Link protein in Panel D migrated as 52-kDa bands, while G3 as 54-kDa bands and G1 as 56-kDa band. **E:** Chondrocyte proliferation was assayed on two individual and one pooled recombinant versican-transfected chondrocyte cell lines controlled with a vector-transfected cell line. Chondrocytes from each line were seeded into 24-well tissue culture plates at a concentration of 2×10^4 cells/well. The cultures were incubated at 37°C in an incubator 5% CO_2 humidified air for three days. Cell number was determined. The versican-transfected cell lines, including individual cell lines and pooled cell line, showed significantly greater proliferation as compared to the vector-transfected cell line (**, $P < 0.01$). **F:** The pooled recombinant versican-transfected chondrocyte cell line and a vector-transfected cell line were seeded into 24-well tissue culture plates at a concentration of 2×10^4 cells/well. To calculate growth rates, the cell number was determined at the indicated times. Proliferation was increased in the pooled versican-transfected chondrocyte cell line as compared to the vector-transfected chondrocyte cell line. **G:** Chondrocytes were seeded into 24-well tissue culture plates. Growth medium collected from a recombinant versican- and the control vector-transfected COS-7 cells was added into the chondrocyte cultures and incubated for 3 days. Growth media from the versican-transfected cells promoted chondrocyte proliferation significantly, as compared with that from the vector-transfected cells (n = 4; **, $P < 0.01$).

bator and chondrocytes were harvested after 3 days by incubating the cells in 10 mM EDTA for 10 min. Cell number was determined by cell counting as described above.

Chondrocyte–Plate Interaction Assay

Cells were seeded in 24-well plates at a concentration of 2×10^4 cells/well in 0.5 ml DMEM containing 5% FBS and allowed to grow to 90% confluence. To test cell adhesion, growth medium was removed and cells were then incubated with 1 ml of trypsin/EDTA solution at a concentration of 0.025× of the original product (0.05% trypsin and 0.53 mM EDTA, GIBCO Cat. No. 15405-012) for 30 min with shaking at 50 rpm on a platform shaker at room temperature. The trypsin/EDTA solution was removed and cells were washed with PBS. Cells remaining adherent to the plates were washed gently with PBS once more. The highly adherent cells that remained on the plates were incubated with 10 mM EDTA (0.5 ml) for 10 min for complete cell detachment. Cells in each well were agitated with a micropipette to obtain a single-cell suspension. The cell number in each well were determined using a cytometer. Cell adhesion was expressed as

$$\text{Cell adhesion (\%)} = T/C$$

where T is the number of cells that remained adherent to the plates in each well, and C is the total cell number without trypsin/EDTA treatment (control). Each treatment had one control and was done in quadruplicate. In this way, the error that may occur during cell counting can be corrected and cell adhesion in different treatments is comparable.

Western Blot Analysis of Proteoglycans

Cell lysate and growth medium from transfected cells was collected and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The stacking gel contained 4% polyacrylamide. A 5% separating gel was used for the products of recombinant versican, the G1CS, CSG3 and versicanΔEGF constructs, and a 12% separating gel for the products of G1 and G3 constructs. The buffer system is 1×TG (Amresco) containing 1% SDS. Proteins separated on SDS-PAGE were transblotted onto a nitrocellulose membrane (Bio-Rad) in 1× TG buffer (Tris-glycine buffer, Amresco) containing 20% methanol. The membrane was blocked in TBST (10 mM Tris-Cl, pH 8.0, 150 mM NaCl, 0.05% Tween 20) containing 10% nonfat dry milk powder (Carnation) (TBSTM) for 1 h at room temperature, and then incubated at 4°C overnight with appropriate primary antibodies in TBSTM. The membranes were washed with TBST (3 × 30 min) and then incubated for 1 h with goat antirabbit (or antimouse) antibody conjugated to HRP in TBSTM. After washing as above, the bound antibody was visualized with an ECL kit according to the manufacturer's instructions (Amersham).

RESULTS

Overexpression of Versican Stimulated Chondrocyte Proliferation

Versican is known to be highly expressed in actively developing tissues [Margolis and Margolis, 1994; Shinomura et al., 1990; Naso et al., 1995; Landolt et al., 1995], and it has been suggested that versican plays a role in cell proliferation. In transfection studies, we have observed that a recombinant versican stimulated NIH3T3 fibroblast proliferation [Zhang et al., 1998]. Although versican is downregulated during mesenchymal chondrogenesis, in joint diseases, its expression is upregulated [Nishida et al., 1994; CS-Szabo et al., 1997]. We sought to investigate if versican is expressed in cartilage and plays a role in enhancing chondrocyte proliferation during wound repair. We isolated the G1 domain, the G3 domain and a part of the CS sequence of versican using RT-PCR and linked them together to form a recombinant versican mini-gene (Fig. 1A).

To study the effect of the recombinant versican on chondrocyte proliferation, chondrocytes were isolated from chicken embryos and maintained as an adherent culture. The adherent chondrocytes were stably transfected with the recombinant versican mini-gene. Expression of the recombinant versican was analyzed on western blot stained with a monoclonal antibody 4B6 [Binette et al., 1994] against an epitope at the leading peptide of link protein, which had been engineered into the construct. The recombinant versican was expressed as an extracellular proteoglycan; it was secreted into the growth medium and had glycosaminoglycan chains attached to its core protein, producing a characteristic proteoglycan smear in the blot (Fig. 1B). Other constructs, comprising deletion mutants for various domains, are shown in Figure 1A. Their expression is presented in Figure 1B–D.

We have previously shown that treatment with chondroitinase ABC reduced the recombinant versican to the expected size of the core protein [Zhang et al., 1998].

Recombinant versican-transfected individual and pooled chondrocyte cell lines were assayed for cell proliferation. We observed that versican-transfected individual and pooled cell lines exhibited increased cell proliferation after 3 days as compared to the vector-transfected cell line (Fig. 1E).

To obtain a time course of the versican effect, chondrocyte proliferation was monitored for four days. Chondrocyte cell lines stably transfected with vector or versican were cultured in tissue culture plates. Chondrocyte proliferation was assayed by determining the cell density of each well. The recombinant versican-transfected chondrocytes had higher proliferative activity at each time point tested, as compared with the vector-transfected chondrocytes (Fig. 1F). Since the expression of endogenous versican is low, the 20–50% increase in chondrocyte proliferation stimulated by versican transfection suggests that the mechanism by which versican acts is saturable, or limited in some way.

Versican is an extracellular matrix molecule. To confirm that versican can enhance chondrocyte proliferation under in vitro conditions, it was necessary to employ secreted versican in the assay. Growth media containing expressed recombinant versican from COS-7 cells was added to chondrocyte cultures. This exogenous addition of the recombinant versican gene product was also found to enhance chondrocyte proliferation significantly, compared with growth media from vector-transfected cells (Fig. 1G).

To investigate whether endogenous versican is sufficient to stimulate chondrocyte proliferation, antisense oligonucleotides complementary to versican gene were added to normal chondrocyte cultures to block the expression of endogenous versican. After 3 days, we observed that chondrocyte proliferation was inhibited in cultures treated with antisense oligonucleotides, as compared to those treated with sense oligonucleotides (Fig. 2).

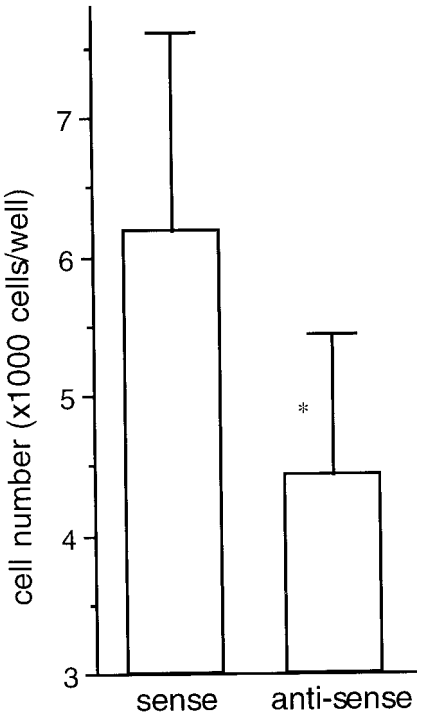

Fig. 2. Versican antisense oligonucleotides inhibited chondrocyte proliferation. Chondrocytes were seeded into 96-well tissue culture plates at a concentration of 3×10^3 cells/well. Oligonucleotide was introduced into each well of the chondrocyte cultures at a concentration of 4.2 μM. After 3 days, cell number was determined. Cells treated with antisense oligonucleotide showed reduced proliferation ($n = 4$; *, $P < 0.05$).

The G1 Domain Stimulates Cell Proliferation by Destabilizing Cell–Substratum Interaction

The versican molecule is comprised of a G1 domain, a G3 domain and a CS region for glycosaminoglycan chain attachment. As a consequence of alternative splicing, CS sequences of different lengths are observed [Dours-Zimmerman and Zimmermann, 1994; Paulus et al., 1996; Ito et al., 1995]. In an extreme case, a G1G3 isoform completely lacking CS sequence was detected in mouse brain tissues [Zako et al., 1995]. In all cases, the presence of the G1 and G3 domains is conserved, implying that these domains may be essential for versican function. To investigate this, we tested the effects of G1 and G3 individually.

Recently, we demonstrated that the G1 domain of aggrecan is essential for cell adhesion mediated by aggrecan–hyaluronan–link protein ternary complex [Yang et al., 1998]. Expression of the G1 domain of aggrecan inhibited chondrocyte adhesion [Cao et al., 1998], and the G1 domain of versican is homologous to the G1 domain of aggrecan. We thus tested chondrocyte adhesion in cell lines stably transfected with the G1 construct and control vector (pcDNA3) alone. Three G1-transfected individual chondrocyte cell lines (Fig. 3A, G1–1, G1–2, and G1–7) and one pooled cell line (G1-p) showed decreased adhesion as compared to the

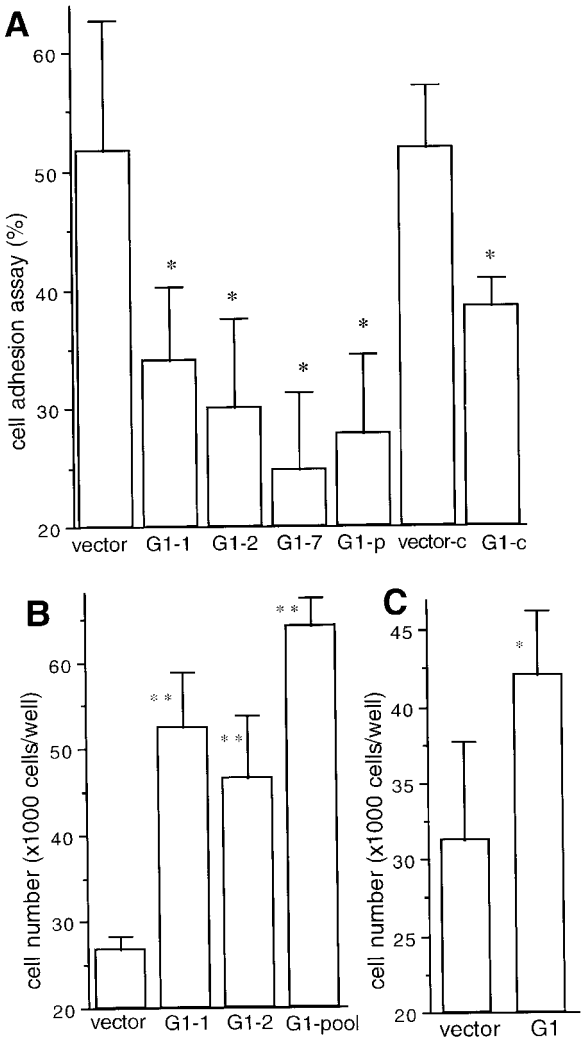

Fig. 3. The G1 domain of versican stimulated chondrocyte proliferation through destabilizing chondrocyte–substratum interaction. **A:** Chondrocyte-substratum interaction was tested on three individual G1-transfected cell lines (G1-1, G1-2, and G1-7) and one pooled G1-transfected (G1-p) cell line controlled with a vector-transfected cell line (vector). As well, growth medium collected from the G1 (G1-c) and vector-transfected (vector-c) COS-7 cells were introduced into chondrocyte cultures to test their effect on chondrocyte–substratum interaction. Expression of G1 and growth media from the G1-transfected cells reduced chondrocyte adhesion ($n = 4$; *, $P < 0.05$). **B:** Chondrocyte proliferation was assayed on two individual (G1-1 and G1-2) and one pooled G1-transfected (G1-pool) chondrocyte cell lines. A vector-transfected cell line (vector) was used as a control. Expression of the G1 construct enhanced chondrocyte proliferation ($n = 4$; **, $P < 0.01$). **C:** Growth medium from COS-7 cells transfected with the G1 construct and the control vector was collected and introduced into chondrocyte cultures. The cultures were incubated at 37°C in a humidified tissue culture incubator filled with 5% CO_2 for 3 days. Chondrocytes in each well were collected and the cell number determined. Chondrocyte proliferation was promoted by growth medium containing the G1 gene product ($n = 4$; *, $P < 0.05$).

chondrocyte cell line transfected with vector alone (vector). Growth media was also collected from COS-7 cells transiently transfected with the G1 construct or vector, and introduced into the normal chondrocyte cultures. Growth media from the G1-transfected COS-7 cells (Fig. 3A, G1-c) reduced the adhesion of primary chondrocyte cultures to tissue culture plates as compared with that from vector-transfected COS-7 cells (vector-c). Expression of the G1 construct is shown in Figure 1D.

Chondrocyte proliferation was assayed in two individual chondrocyte cell lines (Fig. 3B, G1-1 and G1-2) and one pooled chondrocyte cell line (G-pool) transfected with the G1 construct, and one cell line transfected with the control vector alone (vector). G1 expression in individual cell line and pooled cell line enhanced chondrocyte proliferation as compared with the vector-transfected cell line. Finally, growth media from G1-transfected COS-7 cells also enhanced chondrocyte proliferation (Fig. 3C).

We then generated a CSG3 construct, a versican mutant lacking a G1 domain, and expressed this construct in COS-7 cells. Growth medium containing the CSG3 product was introduced into chondrocyte cultures. We observed that the CSG3 product enhanced chondrocyte proliferation significantly compared to medium from vector-transfected cells, but showed less activity than that from the recombinant versican-transfected cells (Fig. 4A). Chondrocyte adhesion was also tested using the above media. Chondrocytes incubated with medium from vector-transfected cells exhibited the highest levels of adherence, followed by the cells incubated with medium from the CSG3-transfected cells and then the cells incubated with medium from the mini-versican-transfected cells (Fig. 4B). This finding suggests that the G1 domain plays a role in versican's ability to enhance chondrocyte proliferation and reduce chondrocyte adhesion.

The G3 Domain Stimulated Chondrocyte Proliferation through the EGF-like Motifs

To study the role of the G3 domain of versican in chondrocyte proliferation, we generated chondrocyte cell lines stably transfected with the G3 construct. Two individual cell lines (Fig. 5A, G3-1 and G3-2) stably expressing the G3 construct were selected, and all other viable colonies were mixed and maintained as a pooled chondrocyte cell line (Fig. 5A, G3-pool). G3 ex-

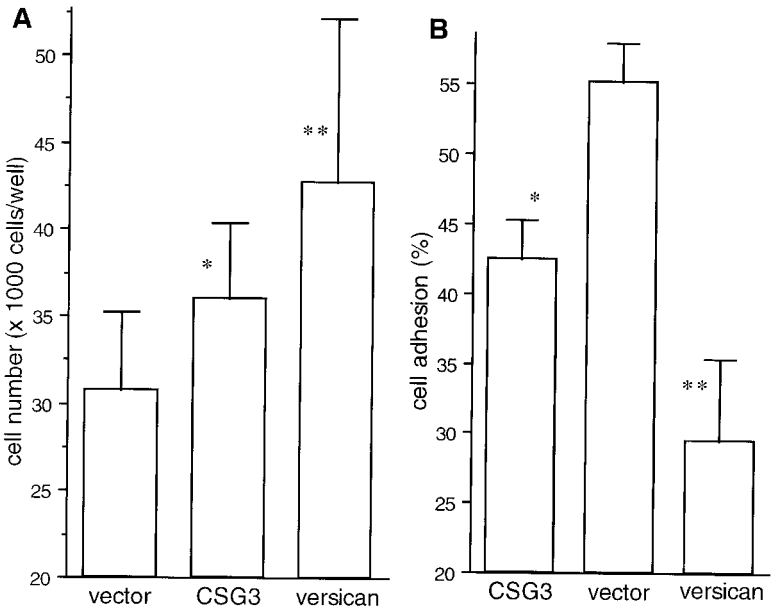

Fig. 4. Deletion of the G1 domain reduced the effect of the recombinant versican on chondrocyte proliferation and adhesion. **A:** The G1 domain was deleted from the recombinant versican and the resultant CSG3 construct was transfected transiently into COS-7 cells. Growth medium containing the CSG3 gene product was collected and introduced into chondrocyte cultures that had been seeded in 24-well tissue culture plates at a concentration of 2×10^4 cells/well. The cultures were incubated for 3 days as above. The cell number was determined (n = 4; *, $P < 0.05$; **, $P < 0.01$). **B:** Growth medium collected from the CSG3 and vector-transfected COS-7 cells was introduced into chondrocyte cultures to test its effect on chondrocyte–substratum interaction. The cultures were incubated for 1 day. Chondrocyte–substratum interaction was tested as described under Materials and Methods. The CSG3 gene product had moderate ability to destabilize chondrocyte–substratum interaction.

pression was analyzed in the two individual and one pooled cell lines with Western blot to ensure the maintenance of the G3 construct in the selected cell lines (Fig. 5A). A vector-transfected cell line was used as a control. These cell lines were assayed for chondrocyte proliferation, and all G3-transfected cell lines exhibited a high level of proliferation, compared with the vector-transfected cell line (Fig. 5B). Growth media collected from vector- and G3-transfected COS-7 cells were introduced into untransfected chondrocyte cultures to test their effect on chondrocyte proliferation. We found that the growth media from G3-transfected COS-7 cells enhanced chondrocyte proliferation, as compared with that obtained from vector-transfected COS-7 cells (Fig. 5C). To corroborate this observation, COS-7 cells were transiently transfected with a versican mini-gene and from which the G3 domain had been removed (the G1CS construct). Growth media was collected and added to chondrocyte cultures. Analysis of chondrocyte proliferation demonstrated that the proliferation was greatly reduced when the G3 domain was deleted (Fig. 5D, G1CS compared to versican).

It has been shown that the EGF motif is responsible for cell proliferation, and the EGF-like motifs in laminin have the ability to stimulate cell proliferation [Panayotou et al., 1989; Maruo et al., 1995]. The G3 domain of versican contains two EGF-like motifs, which may contribute significantly to chondrocyte proliferation. Chondrocyte cell lines stably expressing the versicanΔEGF construct, which lacked an EGF-like motif, were generated. The cell lines were pooled into two groups (versicanΔEGF1 and versicanΔEGF2), and chondrocyte proliferation was tested in these two groups. One pooled chondrocyte cell line transfected with the recombinant versican was used as a control. Deletion of the EGF-like motifs reduced the effect of versican on chondrocyte proliferation (Fig. 6A).

Growth media from COS-7 cells transiently transfected with recombinant versican and the versicanΔEGF construct was introduced into normal chondrocyte cultures to test their effect on chondrocyte proliferation. We found that growth media from COS-7 cells transfected with the versicanΔEGF construct had significantly impaired ability to enhance chondrocyte proliferation when compared to versican-transfected COS-7 cells (Fig. 6B).

DISCUSSION

The large chondroitin sulfate proteoglycan versican was initially described in human fibroblasts [Zimmermann and Ruoslahti, 1989] and was then found in a variety of tissues [Yamagata et al., 1993b; Yao et al., 1994; Nara et al., 1997; Stigson et al., 1997; du Cros et al., 1995]. The tissues that contain versican include embryonic tissues such as embryonic lung fibroblasts [Zimmerman and Ruoslahti, 1989]; embryonic aorta, cornea, and skeletal muscle [Yamagata et al., 1993b]; the mesenchymal cell condensation area of limb buds [Kimata et al., 1986; Shinomura et al., 1990]; the perinotochordal

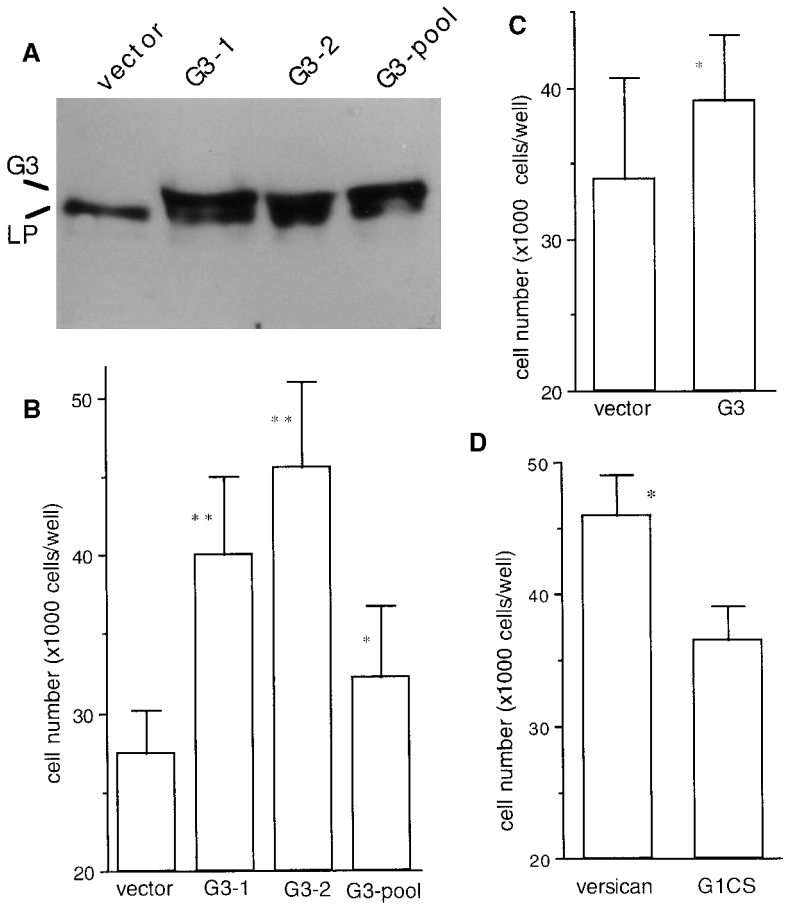

Fig. 5. The G3 domain of versican-stimulated chondrocyte proliferation. **A:** The G3 construct was stably expressed in chondrocytes. Expression of the G3 construct (55 kDa) was analyzed by Western blot in two individual cell lines (G3-1 and G3-2) and one pooled cell line (G3-pool) using the monoclonal antibody 4B6. A vector-transfected cell line was used as a control. The 4B6 antibody also recognized endogenous link protein (LP, 52 kDa). **B:** Chondrocyte proliferation was assayed in the two individual and one pooled G3-transfected chondrocyte cell lines. Expression of the G3 construct enhanced chondrocyte proliferation as compared with the vector-transfected cell line (n = 4; *, $P < 0.05$; **, $P < 0.01$). **C:** Growth medium from COS-7 cells transfected with the G3 construct and the control vector was collected and introduced into chondrocyte cultures. The cultures were incubated at 37°C in a humidified tissue culture incubator filled with 5% CO_2 for 3 days. Chondrocytes in each well were collected and the cell number determined. Growth medium containing the G3 gene product promoted chondrocyte proliferation (n = 4; *, $P < 0.05$). **D:** The G3 domain was deleted from the recombinant versican, and the resultant G1CS construct and the versican mini-gene were transiently expressed in COS-7 cells. Growth medium was collected and introduced into chondrocyte cultures that had been seeded into 24-well tissue culture plates at a concentration of 2×10^4 cells/well. The cultures were incubated as described above for 3 days. Cell number was determined. Deletion of the G3 domain greatly reduced the effect of versican on chondrocyte proliferation (n = 4; *, $P < 0.05$).

mesenchyme; and basement membranes [Yamagata et al., 1993b]. In adult tissues, versican is detected in the loose connective tissue of various organs [Bode-Lesniewska et al., 1996], blood vessels [Yao et al., 1994], vessels of brain tumors [Paulus et al., 1996], human breast tumors [Nara et al., 1997], dermis, and the proliferative zone of the epidermis [Zimmermann et al., 1994]. It is highly expressed in tissues in which cells are actively metabolizing and proliferating. Therefore, it has been suspected that versican plays a role in cell proliferation. Versican is also suspected to play a role in cellular attachment, migration, and proliferation by interacting with cell surfaces and extracellular matrix molecules [Zimmermann et al., 1994; Yamagata and Kimata, 1994; Yamagata et al., 1989]. It has been reported that versican interferes with the attachment of cells to various extracellular matrix components such as collagen I, fibronectin, and laminin [Yamagata et al., 1989] and acts to inhibit cell adhesion [Yamagata and Kimata, 1994; Yamagata et al., 1993a]. It may also play a role in the formation of tissues that act as barriers to migratory neural crest cells and outgrowing axons during

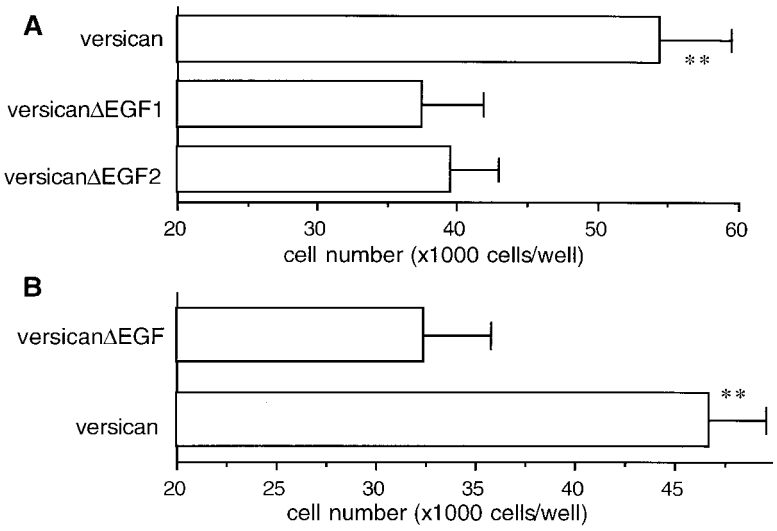

Fig. 6. Role of the EGF motifs in chondrocyte proliferation. **A:** The EGF-like motifs were deleted from versican and the resultant versicanΔEGF construct was stably expressed in chondrocytes. Chondrocyte proliferation was tested in two pooled cell lines expressing the versicanΔEGF construct (versicanΔEGF1 and versicanΔEGF2) and one pooled cell line expressing the recombinant versican (versican). Deletion of the EGF-like motifs greatly reduced chondrocyte proliferation, compared with recombinant versican (n = 4; **, $P < 0.01$). **B:** Growth medium collected from versicanΔEGF and versican-transfected COS-7 cells was introduced into chondrocyte cultures. The cultures were incubated at 37°C as described above for 3 days. Chondrocyte number was determined. Chondrocyte proliferation was lower in the presence of the versicanΔEGF gene product (n = 4; **, $P < 0.01$).

embryonic development [Landilt et al., 1995]. To investigate the function of versican, in previous studies, we expressed a recombinant versican in NIH3T3 fibroblasts in cultures. We demonstrated that overexpression of versican stimulated fibroblast proliferation [Zhang et al., 1998]. In particular, the EGF-like motifs in the G3 domain of versican are involved in the promotion of cell growth. As well, versican enhances NIH3T3 fibroblast proliferation through the G1 domain that also reduces cell adhesion [Yang et al., 1998]. These abilities may enable versican to enhance cell growth in tissue development, tumor formation, and wound repair. Indeed, we have demonstrated that versican could reduce the adhesion of a brain tumor cell line U98. As a result, the locomotion of these glioma cells was stimulated [Ang et al., in press]. Versican is also involved in the growth of the glioma cells (unpublished data). It should be noted that versican does not play such functional roles in all cell types. At least, we did not observe these functions (i.e. promotion of cell proliferation and reduction of cell adhesion) of versican in a squamous carcinoma cell line A431 (data not shown).

Chicken chondrocytes in culture are a useful model to study the role of certain proteins. Chondrocytes are easily isolated from chicken embryos and maintained as an adherent culture for many weeks. They can be transfected, either transiently or stably, and the effect of the transfected gene can be monitored. In this study, the effects of the transfected genes were moderate, compared with effects observed in a transfected fibroblast cell line (unpublished data). For example, chondrocytes transfected with the recombinant versican mini-gene or with versican G3 domain construct sustained a small but significant increase in proliferative activity. However, it is likely that this result is more indicative of the in vivo situation. In order to investigate which versican domains are responsible for its effects, we performed transfection studies, added exogenous gene products to chondrocyte cultures and inhibited the endogenous expression of versican using antisense oligonucleotides. Our studies demonstrated that versican enhanced chondrocyte proliferation by means of two mechanisms: the G1 domain enhanced chondrocyte proliferation by destabilizing chondrocyte adhesion; the EGF-like motifs are involved in G3 stimulating proliferation. However, the increase of chondrocyte proliferation was only around or even under one fold. The limited effects of increased versican expression are perhaps related to the fact that versican is normally expressed at very low levels. Chondrocytes may be limited in their capacity to respond to increased versican expression, perhaps because of the presence of a saturable intracellular pathway mediating the effect. This explanation may be supported by data from OA cartilage, in which chondrocyte proliferation is limited and cartilage repair is arrested in spite of an observed 8- to 9-fold increase in versican expression [CS-Szabo et al., 1997]. Since, under normal conditions, rapid chondrocyte proliferation would not be required, it is conceivable that factors involved in the versican effect are limited. Only in cases of injury or diseases would the tissue need greater numbers of these

factors to mediate repair. Thus, studies of the details of the pathway by which versican stimulates fibroblast and chondrocyte proliferation may shed light on the mechanism of joint diseases such as OA.

The fact that cartilage repair in disease and injury states is always poor and often results in loss of function suggests that the newly synthesized cells do not produce sufficient extracellular matrix molecules, which are critical in maintaining a functional joint. Indeed, chondrocytes do not distribute evenly in cartilage in the late stages of joint diseases; some areas contain few chondrocytes, whereas the others contain groups of chondrocytes. Perhaps, the groups of chondrocytes are the result of versican stimulating chondrocyte proliferation and do not produce sufficient matrix. This suggests that the expression of versican can affect the production of other matrix molecules. We are currently undertaking these studies in our laboratory.

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