The G3 Domain of Versican Enhances Cell Proliferation via Epidermal Growth Factor-like Motifs*

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Versican is a member of the large aggregating chondroitin sulfate proteoglycan family. We have expressed in NIH3T3 fibroblasts a recombinant versican mini-gene comprising the G1 and G3 domains and 15% of the CS chains. We observed that expression of the mini-versican gene stimulated cell proliferation as determined by cell counting and cell cycle analysis. Addition of exogenous mini-versican protein to cultured cells produced the same result. The effects of the mini-versican were greatly reduced when the G3 domain was deleted. Expression of the G3 domain alone promotes cell proliferation, and addition of purified G3 gene products to NIH3T3 fibroblasts and cultured chicken fibroblasts enhances cell growth. Further, deletion of the epidermal growth factor (EGF)-like motifs in the versican G3 domain reduced the effects of the mini-versican on cell proliferation. In the presence of the purified mini-versican protein, antisense oligonucleotides to the EGF receptor inhibited proliferation of NIH3T3 fibroblasts, compared with control sense oligonucleotides. Taken together, these results imply that versican enhances cell proliferation, and this effect is mediated, at least in part, by the action of versican EGF-like motifs on endogenous EGF receptor.

Versican (1, 2), or PG-M, is a member of the large aggregating chondroitin sulfate proteoglycan family, which also includes aggrecan, neurocan, and brevican (3). The core proteins of these large proteoglycans have sizes ranging from 200 to 400 kDa. Common features in this family include the presence of N-terminal G1 and C-terminal G3 domains, and a large chondroitin sulfate side chain-bearing sequence localized in the middle region. In versican, 12–15 chondroitin sulfate side chains are covalently attached to this central sequence (2, 4), which also contains two alternatively spliced domains named GAG-α and GAG-β (5, 6). The G1 domain of versican binds hyaluronan (7). The G3 domain consists of a set of lectin- (also called carbohydrate recognition domain or CRD), epidermal growth factor (EGF)1-, and complement-binding protein-like subdomains. In this respect, G3 is similar to the selectin family (8) except that the G3 domain contains only one complement-binding protein-like subdomain while selectins contain at least two. Functionally, selectins play a role in mediating cell adhesion, but no such role has been identified for the G3 domain.

Versican is highly expressed in fast growing tissues and cells, it has been suggested that versican plays a role in cell proliferation. Using molecular biological techniques, we show in this report that the expression of a truncated versican gene, or exogenous addition of a truncated versican gene product, promotes cell proliferation. Expression of the versican G3 domain alone achieves the same effect, but deletion of the EGF-like motifs greatly reduces the ability of versican to stimulate growth. Antisense oligonucleotides to EGF receptor (EGFR) inhibited cell proliferation, and the effect was greater in the recombinant versican-transfected cell line than in the vector-transfected cell line. These results suggest that the EGF-like motifs in versican G3 domain may promote cell proliferation through a direct or indirect interaction with EGFR.

EXPERIMENTAL PROCEDURES

Materials—The reverse transcription-PCR kit was from CLON-TECH. Tag DNA polymerase, T4 DNA ligase, and restriction endonucleases were from Boehringer Mannheim. Bacterial growth medium was from Difco. Prep-A-Gene DNA purification kit, prestained protein markers, and the protein assay kit were from Bio-Rad. DNA mini-prep triltriacetic acid; ELISA, enzyme-linked immunosorbent assay.
kit was from BioCan Scientific. Lipofectin, Genetin G(418), DEMEM growth medium, fetal bovine serum, Hank’s balanced salt solution, trypsin/EDTA, and isopropl-1-tho-β-n-galactosaminopropyl were from Life Technologies, Inc. ECL Western blot detection kit was from Amer-sham Pharmacia Biotech. Goat anti-mouse IgG horseradish peroxidase and anti-rabbit IgG horseradish peroxidase were from Invitrogen and ligated into the recombinant mini-versican from which the full-length G3 domain had been digested by digestion with XhoI and SphI.

The pcDNA1-G1CSG3 construct (mini-versican) was transiently transfected into COS-7 cells (American Type Culture Collection) using Lipofectin (Life Technologies, Inc.) as originally described by Felgner et al. Growth medium and cells were harvested separately after 3 days of transfaction. Expression of genes was analyzed by Western blot, using anti-G3 antibody, as described below. We confirmed that glyco-saminoglycan chains were attached to the expressed core protein, resulting in a characteristic proteoglycan smear on SDS-PAGE and Western blot, and also that gene products were secreted and present in the growth medium.

Once we had verified that the transiently-expressed products were properly processed and secreted, the mini-versican construct was sub-cloned into the pcDNA3 vector (In vitro) and stable transfection of NIH3T3 cells was performed. Three days after transfection, Genetin was incorporated into the growth medium at a concentration of 1 mg/ml, and the cells were maintained in this medium until individual colonies were large enough for cloned cells to be collected. The cells were lysed using 0.5 mg/ml Geneticin or stored in liquid nitrogen. PCR technique was used to confirm that the selected cell lines had incorporated the genes of interest. Briefly, genomic DNA was prepared from cell lystate (25) and used as template in a PCR reaction with primers (5'-GAG CAA GAC ACA GAG ACT and 5'-GGC CCT TGA GTC CCA CCA) which amplify nucleotides 10125–10830 of chicken versican (1).

Western Blot Assays for Proteoglycans—Electrophoresis of recombinant proteoglycans was performed in SDS-PAGE-Western blot assay. Cell lysate and growth medium that contained recombinant gene products were subjected to SDS-PAGE electrophoresis. The stacking gel contained 4% polyacrylamide. A 5% separating gel was used for the mini-versican product and a 12% separating gel for the G3 domain. The buffer system is 1x TG (Tris-glycine buffer, Amresco product) containing 1% SDS. Proteins separated on SDS-PAGE were transblotted onto a nitrocellulose membrane (Bio-Rad) in 1x TG buffer containing 20% methanol. The membrane was blocked in TBST (10 mM Tris-CI, pH 8.0, 150 mM NaCl, 0.05% Tween 20) containing 10% non-fat dry milk powder (TBSTM) for 1 h at room temperature, and then incubated at 4 °C overnight with monoclonal antibody (1:1000 dilution) prepared in 1% SDS. The membranes were washed with TBST (three times for 30 min each) and then incubated for 1 h with goat anti-rabbit antibody conjugated to horseradish peroxidase in TBSTM. After washing as above, the bound antibody was visualized with an ECL kit according to the manufacturer's instructions (Amersham).

Electrophoresis of endogenous versican was performed in agarose gel (Invitrogen) and Western blot as above. The agarose gel (4 cm height containing 1.5% agarose in a barbitul buffer containing 0.124 mM Tris-CI, 27 mM barbituric acid, 1 mM EDTA, pH 8.7) was made on top of a 1-cm conventional 10% polyacrylamide gel. The polyacrylamide gel was used only to seal the bottom of the casting stand and acted as a base for the agarose separating gel. The barbitul buffer was also used as a running buffer and the electrophoresis took place at 40 V for 5 h at room temperature. Molecules, up to 2 million Da in size (blue dextran 2000), are able to enter the agarose gel. Growth media from chicken mesenchymal culture and NIH3T3 fibroblast culture of equal protein concentration were run on 1% agarose in a barbital buffer containing 0.124M Tris-Cl, 27m M glycine and 1x TG agarose separating gel. The barbital buffer was also used as a running buffer and the electrophoresis took place at 40 V for 5 h at room temperature. Molecules, up to 2 million Da in size (blue dextran 2000), are able to enter the agarose gel. Growth media from chicken mesenchymal culture and NIH3T3 fibroblast culture of equal protein concentrations were analyzed in the gel. To allow transfer of such large molecules onto the nitrocellulose membrane, the blotting took place in TBG buffer at 20 V overnight at 4 °C. The rest of the procedure was similar to the Western blot protocol described above.

Digestion of Glycosaminoglycan Chains with Chondroitinase ABC—The conditioned medium collected from the mini-versican-transfected cells was dialyzed overnight against 10 volumes of reaction buffer (100 mM Tris-HCl, pH 8.0, 30 mM sodium acetate). After a buffer change, the mixture was further dialyzed for 1 h. Meanwhile, chondroitinase ABC (Sigma, catalog no. C2909) was dissolved in the reaction buffer at a concentration of 1.33 units/ml. This enzyme was then added to the dialyzed versican solution (1:1), and the mixture was incubated at 37 °C overnight to digest the glycosaminoglycan chains. In some samples, chondroitinase ABC was replaced by hyaluronidase, which was used as a control. The enzyme-digested products were subjected to SDS-PAGE and analyzed on Western blot.

Antigen Preparation and Antibody Generation—Strategy for inser-
tion of the G3 domain into pQE30 (Qiagen Inc., catalog no. 32149) is shown in Fig. 1A. pQE30, a bacterial expression vector, contains an epitope (MRGSHHHHHH) at its amino terminus recognized by the monoclonal antibody anti-

This served a dual purpose; it was followed by addition of 50 μl of purified mini-versican solution at an optimal volume of elution buffer used in each sample was the same as the corresponding medium from COS-7 cells that had been transfected with G3 construct, and cell lysate from G3-expressing bacteria. Briefly, conditioned medium from COS-7 cells that had been transfected with G3 construct was used to prepare affinity column. The antibody specifically recognizes versican and the G3 construct product. Expression of the G3 construct was demonstrated with Western blot assay, using cell growth medium. Briefly, 10 μl of G3-containing mini-versican in the selected cell lines was verified on a Western blot probed with antibody to the G3 domain. Protein bands observed in culture medium corresponded to the expected size of recombinant versican core protein, was observed (Fig. 1A) in pcDNA1 for transient expression and in pcDNA3 for stable expression. To monitor the expression of the recombinant constructs, we raised antibody against the G3 domain. The G3 construct was expressed in E. coli M15. Gene products pro-

G3 constructs, we assigned a value of 100%. Cell Proliferation Affected by Antisense Oligonucleotides—To test the possible role of the EGFR in versican’s effects on cell proliferation, 200 ng/μl of NIH3T3 fibroblast suspension at a concentration of 3 × 10^4 cells/ml were inoculated into each well of 96-well tissue culture plates followed by addition of 50 μl of purified mini-versican solution at an estimated concentration of approximately 0.4 ng/μl. The control solution obtained from the purification was used as a negative control. Human EGF (Sigma, catalog no. E-9644) at a final concentration of 15 ng/ml was used as a positive control. As well, 15 μl of sense (ATG CCG CCA TCA GGG ACC GCG, located in the 5’ end of the coding sequence of EGFR) (29) or antisense (antisense 1, CGG GGT CCC TGA GGG TGC CAT, 5’ end of EGFR; and antisense 2, TGC TCC AAT AAA CTC ACT GCT, 3’ end of EGFR) oligonucleotides were added to the cultures at a final concentration of 3.2 μM. Cells without oligonucleotide treatment served as controls. Cultures were incubated at 37 °C in a tissue culture incubator. After 3 days, the fibroblasts were harvested by incubating the cells with 10 mM EDTA for 10 min. Cell number was determined by cell counting as described above. Each treatment was done in quadruplicate and analyzed statistically.

RESULTS

Expression of a Mini-versican Gene Stimulated Cell Proliferation—The mini-versican gene, containing the G1 domain, a portion of the central sequence, and the G3 domain, was constructed (Fig. 1A) in pcDNA1 for transient expression and in pcDNA3 for stable expression. To monitor the expression of the recombinant constructs, we raised antibody against the G3 domain. The G3 construct was expressed in E. coli M15 using the vector pQE30 (Fig. 1A) and purified on a Ni-NTA affinity column. The antibody specifically recognizes versican and the G3 construct (see below).

We have expressed the recombinant versican in NIH3T3 fibroblasts. For stable expression, cell lines were selected with Geneticin (G418) at a concentration of 4 μg/ml. Expression of the mini-versican in the selected cell lines was verified on a Western blot probed with antibody to the G3 domain. Protein bands observed in culture medium corresponded to the expected size of recombinant glycosylated versican with attached glycosaminoglycan chains (190–250 kDa; Fig. 1A). The G3 construct and the control vector alone in order to produce the G3 gene product. Expression of the G3 construct was demonstrated with Western blot assay, using cell growth medium. Briefly, 10 μl of G3-containing mini-versican in each well of a 96-well plate and incubated at 4 °C overnight. The medium was then removed and the plate was blocked with TBST (200 μl/well) at room temperature for 1 h. Washes and incubation with primary and secondary antibody were similar to Western blot assay. The primary antibody was rabbit anti-G3 antibody, and the secondary antibody was peroxidase-conjugated anti-rabbit antibody. TMB (Amersham code RPN 2718) was used for color development. Optical density was determined using an ELISA reader set to 570 nm according to the manufacturer’s instructions. The optical density value in the medium from the vector-transfected cells was assigned a value of 100%.

Normalization of Gene Products in Conditioned Media—To evaluate the concentration of versican in growth medium, Western blot was not appropriate because versican migrates as a smear in SDS-PAGE, making determination of band density difficult. The ELISA assay provided a useful means of evaluating versican’s concentration. In ELISA, growth medium (100 μl) from vector- and recombinant versican-transfected chicken fibroblasts was coated onto each well of a 96-well plate and incubated at 4 °C overnight. The medium was then removed and the plate was blocked with TBST (200 μl/well) at room temperature for 1 h. Washes and incubation with primary and secondary antibody were similar to Western blot assay. The primary antibody was rabbit anti-G3 antibody, and the secondary antibody was peroxidase-conjugated anti-rabbit antibody. TMB (Amersham code RPN 2718) was used for color development. Optical density was determined using an ELISA reader set to 570 nm according to the manufacturer’s instructions. The optical density value in the medium from the vector-transfected cells was assigned a value of 100%.

Cell Proliferation Affected by Antisense Oligonucleotides—To test the possible role of the EGFR in versican’s effects on cell proliferation, 200 ng/μl of NIH3T3 fibroblast suspension at a concentration of 3 × 10^4 cells/ml were inoculated into each well of 96-well tissue culture plates followed by addition of 50 μl of purified mini-versican solution at an estimated concentration of approximately 0.4 ng/μl. The control solution obtained from the purification was used as a negative control. Human EGF (Sigma, catalog no. E-9644) at a final concentration of 15 ng/ml was used as a positive control. As well, 15 μl of sense (ATG CCG CCA TCA GGG ACC GCG, located in the 5’ end of the coding sequence of EGFR) (29) or antisense (antisense 1, CGG GGT CCC TGA GGG TGC CAT, 5’ end of EGFR; and antisense 2, TGC TCC AAT AAA CTC ACT GCT, 3’ end of EGFR) oligonucleotides were added to the cultures at a final concentration of 3.2 μM. Cells without oligonucleotide treatment served as controls. Cultures were incubated at 37 °C in a tissue culture incubator. After 3 days, the fibroblasts were harvested by incubating the cells with 10 mM EDTA for 10 min. Cell number was determined by cell counting as described above. Each treatment was done in quadruplicate and analyzed statistically.

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We observed that cell lines expressing the recombinant versican had a higher growth rate and reached confluence faster than the vector-transfected cell lines. This observation is extremely important in light of the fact that versican is highly expressed in mesenchymal cells in the early stage of chicken development (16, 17) and restricted to the zone of keratinocyte proliferation in the epidermis (22). To investigate the function of versican in detail, we performed different growth assays on one transfected cell line to confirm this observation. One of the cell lines was monitored by cell counting for three days (Fig. 2).

As well, cell proliferation was confirmed after 3 days of culture in three cell lines transfected with the mini-versican gene and three cell lines transfected with the control vector (Table I). We observed that the mini-versican-transfected cell lines exhibited a 2–3-fold increase in proliferation rate compared with the vector-transfected cell lines (n = 4, p < 0.001).

**TABLE I**

The mini-versican gene enhanced fibroblast proliferation

| Cell line | Cell number |
|-----------|-------------|
| Vector    | ×1000 cells/well |
| Vector 1  | 2.97 ± 0.15 |
| Vector 2  | 2.40 ± 0.40 |
| Vector 3  | 2.60 ± 0.35 |
| Versican 1 | 8.67 ± 0.42 |
| Versican 2 | 10.05 ± 0.04 |
| Versican 3 | 11.23 ± 1.17 |

**TABLE II**

Expression of the mini-versican gene altered the patterns of cell cycles

| Day | G1 % | Versican % | S phase % | Vector | Versican |
|-----|------|------------|----------|--------|---------|
| 1   | 61   | 46         | 9        | 17     |         |
| 2   | 70   | 15         | 11       | 16     |         |
| 3   | 43   | 24         | 15       | 31     |         |

FIG. 2. Expression of the recombinant versican stimulates cell proliferation. In a cell proliferation assay, one recombinant versican-transfected cell line and one vector-transfected cell line were seeded in 96-well tissue culture plates. Each cell line was seeded in four wells at a concentration of 2.5 × 10^3 cells/well. Cell proliferation was evaluated by determining the average number of cells per well at day 1, 2, and 3. The recombinant versican-transfected cell had a higher proliferation rate compared with the vector-transfected cell lines (n = 4, p < 0.001).
To further investigate the enhanced cell proliferative activity, cell cycle patterns were analyzed by flow cytometry. During the first 3 days, nearly twice as many mini-versican-transfected cells were found in S phase compared with the vector-transfected cells (Table II). At all time points tested, fewer cells expressing mini-versican were observed in G1, compared with control. This again suggests that the mini-versican-transfected cells exhibit a higher growth rate than vector-transfected cells.

To determine whether the addition of exogenous mini-versican gene products was sufficient for stimulation of cell proliferation, we collected conditioned medium from the mini-versican- and vector-transfected NIH3T3 cell lines. Media from different sources were added to fibroblasts to assess their effects on cell proliferation. Exogenous addition of the mini-versican product from versican-transfected NIH3T3 fibroblast cell lines (Fig. 3A) significantly stimulated cell proliferation. Similar stimulation was observed using growth media from the mini-versican-transfected COS-7 cells (data not shown). Thus, these results further indicate that versican can stimulate cell growth and plays an important role in cell proliferation.

Because fibroblasts are known to express versican (18), it was necessary to evaluate whether total versican expression in fibroblasts is increased by transient transfection of the mini-versican gene. Growth medium was collected from the mini-versican- and vector-transfected fibroblasts. The ELISA procedure was used to assess versican levels with anti-G3 antibody, as described under “Experimental Procedures.” Recognition of endogenous versican with the anti-G3 antibody was confirmed by agarose-Western blot assay (Fig. 3B). According to our ELISA results, transfection of the mini-versican resulted in a 3-fold increase in total versican expression compared with the fibroblasts transfected with vector alone (Fig. 3C). We also noted that the immunoreactivity of the purified recombinant versican with the anti-G3 antibody was dose-dependent, varying with the concentration of recombinant versican (data not shown).

**Versican G3 Domain Promotes Fibroblast Proliferation—**

Versican consists of a G1 domain, a G3 domain, and a central region for glycosaminoglycan chain attachment. We suspected that the G3 domain is important in stimulating cell proliferation since it has been shown that the EGF motifs in laminin and thrombomodulin are responsible for cell proliferation (30, 31), and versican’s G3 domain contains two EGF-like motifs.

To determine whether recombinant G3 peptide alone was sufficient to stimulate cell proliferation, COS-7 cells were transiently transfected with the G3 construct. Expression of the G3 construct was evaluated using link protein as a standard.
cause both the G3 and link protein contain the same epitope (4B6). The assay showed that a band produced by 10 μl of the growth medium had a density similar to 10 ng of link protein (Fig. 4A), indicating that each milliliter of growth medium contained 1 μg of G3 protein. B, NIH3T3 fibroblasts were seeded in 24-well tissue culture plates at a concentration of 1.25 × 10⁵ cells/well for the cell proliferation assay as described under “Experimental Procedures.” The G3 protein band in 10 μl of growth medium had a density comparable to the 10-ng link protein, indicating that each milliliter of growth medium contained 1 μg of G3 protein.

FIG. 4. The effect of G3 on cell proliferation is dose-dependent. A, COS-7 cells was transiently transfected with the G3 construct. Expression of G3 was analyzed on Western blot. Growth medium (10 μl) was run on SDS-PAGE alongside link protein (10 μl at concentrations of 0.5, 1.0, and 1.5 ng/μl). The resulting blot was probed with monoclonal antibody (4B6) and visualized as described under “Experimental Procedures.” The G3 protein band in 10 μl of growth medium had a density comparable to the 10-ng link protein, indicating that each milliliter of growth medium contained 1 μg of G3 protein. B, NIH3T3 fibroblasts were seeded in 24-well tissue culture plates at a concentration of 1.25 × 10⁵ cells/well for the cell proliferation assay as described under “Experimental Procedures.” The G3 protein band in 10 μl of growth medium had a density comparable to the 10-ng link protein, indicating that each milliliter of growth medium contained 1 μg of G3 protein.
not shown). G3 produced in COS-7 cells had a greater effect on fibroblasts than did the prokaryotic G3 product. Since Western blot had indicated that the COS-7 product was 20-fold more dilute, the difference may indicate that the eukaryotic G3 product is more efficient in promoting proliferation, presumably because it is properly processed and folded.

To further investigate the role of the G3 domain in cell proliferation, we stably expressed the G3 construct in NIH3T3 fibroblasts. Cell lines were selected with Geneticin (G418). Expression of the G3 construct was confirmed by Western blot as above (data not shown). Proliferation of the four cell lines was measured using three vector-transfected cell lines as controls. All G3-transfected cell lines had increased proliferative activity (Fig. 6A). Cell cycle analysis demonstrated that the number of cells in S phase in all G3-transfected cell lines was nearly twice that found in vector-transfected cell lines (Fig. 6B), indicating a high rate of proliferation in the G3-transfected cells.

**Deletion of the G3 Domain Suppresses the Effects of Versican on Cell Proliferation**—We removed the G3 domain from our mini-versican gene making a new construct, G1CS, in pcDNA3. COS-7 cells were transiently transfected with the mini-versican, G3 and G1CS constructs, and the vector, under the same conditions. Growth media from different transfections were examined for their effects on cell proliferation. Mini-versican and G3 alone stimulated cell proliferation to a similar degree. However, the G1CS product showed a significant reduction of cell proliferation when compared with the effects of versican (Fig. 7). These results further demonstrated that the effects of the mini-versican on cell proliferation are mediated, at least in part, by the activity of the G3 domain.

**Deletion of the EGF-like Motifs Greatly Reduced the Ability of Versican to Stimulate Cell Proliferation**—Given the importance of the versican G3 domain in the observed proliferation effects, we sought to characterize the effects of this domain in detail. G3 contains two EGF-like motifs, and to investigate their role in this phenomenon, we deleted them from the mini-versican gene to obtain the versicanΔEGF construct as described under “Experimental Procedures” and shown in Fig. 1A. Fibroblasts were transfected with the versicanΔEGF construct and cell
lines stably expressing the versican\textsubscript{D}EGF construct were isolated. Growth medium collected from these cell lines was analyzed for versican\textsubscript{D}EGF expression and secretion, while growth medium from the mini-versican-transfected cells was used as control. We verified that deletion of the EGF-like motifs resulted in smaller bands which migrated slightly faster than mini-versican on Western blot (Fig. 8A).

In a cell proliferation assay, cell lines stably transfected with versican\textsubscript{D}EGF, the mini-versican, and vector alone were seeded into 96-well tissue culture plates and allowed to grow for 2 days. Cell density was determined. We found that deletion of EGF-like motifs from mini-versican greatly, but not completely, abolished the ability of versican to stimulate cell proliferation (Fig. 8B).

Since the EGF-like motifs are apparently involved in the effects of versican, we next investigated if the effect was specifically mediated by the EGFR. To do so, we used an antisense approach to repress endogenous EGFR expression in our versican-transfected fibroblast cell lines. Fibroblasts were seeded into 96-well tissue culture plates, and the cultures were incubated for 3 days with antisense and sense oligonucleotides of mouse EGFR, in the presence or absence of purified mini-versican products. (Cells not treated with purified mini-versican were incubated in a column elute from vector-transfected cells only.) The antisense oligonucleotides are complementary to sequences located at the 5' and 3' of the gene (29). The growth of both mini-versican-treated and the control NIH3T3 fibroblasts was inhibited in the presence of antisense oligonucleotides, presumably because EGFR is involved in a wide range of cellular processes. However, the extent of inhibition in the mini-versican-treated cells was significantly greater than that of the controls (Table III), consistent with the involvement of EGFR in versican-induced cell proliferation. Cells serving as positive controls were treated with human EGF, and these cells were also subjected to high levels of inhibition in the presence of EGFR antisense oligonucleotides, as compared with the negative control (Table III).

**DISCUSSION**

The large chondroitin sulfate proteoglycan versican is highly expressed in various tissues where the cells are actively metabolizing and proliferating such as in mesenchymal cells. In epidermis, versican is restricted to the proliferating zone. In cell culture, versican is highly expressed in both dermal fibroblasts and keratinocytes while the cells are actively proliferating. Once confluent, the growth rate of these cells slows and the expression of versican is turned off (22). Therefore, it is thought that versican plays a role in cell proliferation.

Chicken mesenchymal cells provide an ideal model for the study of cell proliferation and differentiation. We have previ-
usualy established an in vitro model of chicken chondrogenesis in order to study the role of versican in chicken mesenchymal condensation and chondrogenesis. However, stable transfection of genes into chicken mesenchymal cells is not possible since chicken mesenchymal cells cannot be maintained in culture. We therefore used NIH3T3 fibroblasts as a model to investigate the role of versican. It has been well documented that integration of transfected genes into a genome is a random event. We reported that cell lines transfected with a recombinant mini-versican grew faster, incorporated more [3H]thymidine into DNA, and exhibited faster cell-cycle turnover. Our experiments performed on at least three stably transfected fibroblast cell lines demonstrated that the enhanced cell growth was a result of expression of the mini-versican gene rather than a disruption of genes controlling cell proliferation in the genome. Furthermore, we found that the exogenous addition of recombinant gene product had the same effects on cell proliferation. As versican is a component of extracellular matrix, addition of versican into cell culture may mimic the effect of this molecule when it is secreted by the cells. This indicates that we can study the role of versican in chondrogenesis by introducing gene products generated in NIH3T3 fibroblasts into chicken mesenchymal cells. In other studies, we demonstrated that addition of matrix molecules to the cultures plays a role in mediating cell activities. Such an approach is useful in the investigation of the role of matrix molecules.

Versican is a large molecule and its domains exhibit characteristic properties. For example, the G1 domain, which resides at the N terminus of versican, demonstrates hyaluronan binding activity. The hyaluronan-binding property of the gene may be important in cell-cell and cell-matrix interactions such as those that involve CD44. The G1 domain may also be involved in cell migration, since receptor for hyaluronic acid-mediated motility, another hyaluronan-binding receptor, is known to regulate cell migration. The central sequence is a large region situated between the G1 and G3 domains for the attachment of chondroitin sulfate side chains. It has been reported that the chondroitin sulfate chains are involved in cell adhesion. The lectin-like motif in the G3 domain binds tenascin-R. In this study, we demonstrated that the ability of a mini-versican to stimulate cell proliferation was due to the G3 domain. The G3 domain is composed of a lectin-like subdomain, a complement binding protein-like motif, and one or two EGF-like repeats (depending on splicing). Specifically, we demonstrated that deletion of the G3 domain significantly abolished the effect of the mini-versican on cell proliferation and that the G3 domain alone was as effective as the intact mini-versican.

The G3 domain of versican we studied contains two EGF-like repeats. The EGF-like unit is a sequence of approximately 40 amino acid residues which has a significant homology to epi- dermal growth factor. It contains six conserved cysteine residues which form three disulfide bonds, folding the sequence into its active form. Other amino acids in this motif are also highly conserved. Many proteins contain EGF-like regions in single or multiple copies, including urokinase; laminin B1; low density lipoprotein receptor; tissue plasminogen activator; coagulation factors IV, IX, X, and XII; protein C, S, and Z; fibronectin; thrombomodulin; transforming growth factor; and other proteins. All of these proteins are known or thought to take part in protein-protein or protein-cell interactions. For example, transforming growth factor, fibronectin, laminin, and thrombomodulin are able to stimulate cell proliferation. Fragment containing the inner rodlike segments from the short arm of laminin, which are composed of EGF-like repeats, stimulated thymidine incorporation in cultured cells possessing EGF receptors but had no effect on a cell line lacking this receptor. The recombinant thrombomodulin peptide, which is composed of six EGF-like structures, induced proliferation of Swiss 3T3 cells and accelerated [3H]thymidine uptake into their DNA.

Based on these observations, we hypothesized that the effects of G3 on cell proliferation were mediated by the EGF-like motifs. To investigate this, we deleted the EGF-like motifs from the mini-versican and observed that deletion of the EGF-like motifs greatly, but not completely, reduced the effect of versican on cell proliferation.

It has been reported that NIH3T3 fibroblasts express low levels of functional EGF receptors and respond to EGF stimulation by exhibiting denser growth in monolayer cultures and increased DNA synthesis. Overexpression of EGF conferred on these and other EGF-negative cells heightened the response to EGF stimulation. In our study, deletion of the EGF-like motifs greatly reduced versican’s ability to stimulate NIH3T3 cell proliferation, suggesting that the EGF-like motifs play a role in versican’s enhancement of cell growth, perhaps by interacting with the EGF on the cell surface. Evidence for this was provided by antisense studies in which the growth of NIH3T3 fibroblasts was inhibited by treatment with antisense oligonucleotides to mouse EGF. However, the levels of growth inhibition of cells treated with purified mini-versican were greater as compared with the control. This suggests that EGF is involved in the mini-versican-induced promotion of cell proliferation. The growth of control cells was also reduced after the treatment with antisense oligonucleotides, indicating that antisense treatment also interfered with basal proliferation levels generated by endogenous versican and EGF-binding growth factors. Since deletion of EGF-like motifs did not completely abolish versican’s ability to stimulate cell proliferation, it

| Treatment      | Sense cell no. | Antisense-1 | Antisense-2 |
|----------------|---------------|-------------|-------------|
|                | ×10³ cells    | ×10⁶ cells  | %           | %           |
| Control        | 7.88 ± 1.64   | 6.0 ± 1.41  | 23.9        | 6.38 ± 1.69 | 19.0        |
| Mini-versican  | 11.13 ± 2.7   | 7.75 ± 3.01 | 30.0        | 8.25 ± 1.49 | 26.0        |
| EGF            | 18.0 ± 1.83   | 12.0 ± 1.49 | 33.3        | 13.75 ± 2.0 | 24.0        |

**TABLE III**

Versican Enhances Proliferation

NIH3T3 fibroblasts were cultured in 96-well tissue culture plates at a concentration of 3 × 10⁴ cells/ml. Three different reagents, human EGF, purified mini-versican, and control solution, were added to the fibroblast cultures followed by addition of oligonucleotides as described under “Experimental Procedures” to a final concentration of 3 μM. The cultures were maintained at 37 °C for 3 days, and the cell number was determined. The antisense oligonucleotides were found to inhibit cell proliferation under all conditions. This inhibition was significantly greater in the mini-versican-treated cells and in EGF-treated cells than in the control cells (n = 4, p < 0.01). Effects of antisense oligonucleotides on different treatments (control, mini-versican, and EGF) are expressed as a percentage inhibition, compared to cells treated with sense oligonucleotides, as follows: inhibition = ((number of cells following sense treatment) − (number of cells following antisense treatment))/number of cells following sense treatment) × 100%.
seems that this effect is not mediated entirely by these motifs. Further studies will likely lead to the identification of other elements in the versican molecule which play a role in enhancing cell proliferation.

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