The G3 Domain of Versican Inhibits Mesenchymal Chondrogenesis via the Epidermal Growth Factor-like Motifs*

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Yao Zhang‡, Liu Cao, Chris Gholam Kiani, Bing L. Yang, and Burton B. Yang§
From the Sunnybrook Health Science Centre and Department of Laboratory Medicine and Pathobiology, University of Toronto, Toronto M4N 3M5, Canada

Versican is a highly expressed proteoglycan in zones of developing tissues. To investigate whether versican plays a role in cell differentiation, we studied its role in mesenchymal condensation and chondrogenesis. Here we report that a mini-versican gene product inhibits mesenchymal chondrogenesis but not condensation. The mini-versican-treated mesenchymal cultures formed fewer, smaller cartilaginous nodules and produced lower levels of link protein and type II collagen. The versican G3 domain alone, but not G1, was sufficient to inhibit mesenchymal chondrogenesis. Deletion of two epidermal growth factor (EGF)-like motifs in the G3 domain abolished the effect of versican. The G3 domain of aggrecan, which does not contain an EGF-like motif, did not inhibit mesenchymal chondrogenesis. We also generated a chimera construct containing the two EGF-like motifs of versican and the G3 domain of aggrecan, and we observed that this chimera construct inhibited chondrogenesis to a lesser extent than did the full-length versican G3 construct. Direct transfection of mesenchymal cells with different constructs produced similar results. Furthermore, treatment with versican antisense oligonucleotides and transfection with a versican antisense construct promoted chondrogenesis. Taken together, our results strongly suggest that versican inhibits mesenchymal chondrogenesis via its EGF-like motifs.

Versican is expressed in a variety of tissues. High concentrations are found in embryonic tissues, such as human embryonic lung (1), the mesenchymal cell condensation area of limb buds (2, 3), the perinotochordal mesenchyme between the notochord and neural tube, and basement membranes facing the neuroepithelial cells of chicks (4). It is also present in embryonic aorta, lung, cornea, and skeletal muscle (4). In adult tissues, versican is detected in the loose connective tissue of various organs including most smooth muscles, the central and peripheral nervous systems, the luminal surface of glandular epithelia (5), blood vessels (6), vessels of brain tumors (7), dermis, and in the proliferative zone of the epidermis (8).

Versican (1, 9) is a member of the large aggregating chondroitin sulfate proteoglycan family (10), which also includes aggrecan, neurocan, and brevican. All contain G1 domains at their amino termini and G3 domains at their carboxyl termini. A varying number of chondroitin sulfate side chains are attached to a central chondroitin sulfate bearing sequence; in versican, 12–15 chondroitin sulfate side chains are covalently attached to this sequence (1, 11). This central sequence contains two alternatively spliced domains, glycosaminoglycan-α and glycosaminoglycan-β (12–14).

The functions of these proteoglycans remain elusive. Different domains may be involved in different cellular processes. The G1 domain of versican, as well as that of other members of that family, binds hyaluronan and may participate in matrix assembly and cell-cell and cell-matrix interactions. The G3 domain, consisting of lectin-like (also called carbohydrate regulatory domain), epidermal growth factor-like (EGF†-like), and complement binding protein motifs, is structurally similar to the selectin family (15). But whereas selectins play a role in mediating cell adhesion, no such function has been identified for the G3 domain of versican. It is possible that the EGF-like and lectin-like motifs are involved in intercellular signaling or cell recognition processes. The chondroitin sulfate chains of versican are reported to inhibit cell adhesion (16, 17).

Thus, versican is suspected to play a role in cellular attachment, migration, and proliferation by interacting with cell surfaces and extracellular matrix molecules (1, 3). It has been reported that versican interferes with the attachment of cells to various extracellular matrix components such as type I collagen, fibronectin, and laminin (16) and acts to inhibit cell adhesion (17, 18). It may also play a role in the formation of tissues that act as barriers to migratory neural crest cells and outgrowing axons during embryonic development (19). The expression of versican appears to be developmentally regulated in some cases. For example, during hair development, versican is not expressed until the hair follicle starts to grow (20). Versican expression intensifies with follicle maturation, reaches a maximum at the height of the growth phase, diminishes at the end of the phase, and disappears as the follicle enters the next hair cycle. Thus, versican expression is turned on and off in each cycle, and it appears that its expression correlates with cell differentiation. Furthermore, versican is transiently expressed during mesenchymal condensation. Versican is highly expressed in condensing mesenchyme, but during the transition from mesenchyme to cartilage, the expression of versican is down-regulated and finally is restricted to the periphery of the newly formed cartilage.

To study the role of versican in tissue development, we introduced a mini-versican gene product into an in vitro mesenchymal condensation and chondrogenesis model, and we observed that it inhibited mesenchymal differentiation. This was further confirmed using antisense and transfection studies.

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‡ Postdoctoral Fellow of Sunnybrook Trust.
§ Scholar of the Arthritis Society of Canada. To whom correspondence should be addressed: Research Bldg., Sunnybrook Health Science Centre, 2075 Bayview Ave., Toronto M4N 3M5, Canada. Tel.: 416-480-5874; Fax: 416-480-5737; E-mail: byang@srlc.sunnybrook.utoronto.ca.

† The abbreviations used are: EGF, epidermal growth factor; RT, reverse transcriptase; PCR, polymerase chain reaction; DMEM, Dulbecco’s modified Eagle’s medium; PAGE, polyacrylamide gel electrophoresis; NTA, nitrilotriacetic acid; PNA, peanut agglutinin.
Studies using versican constructs lacking the EGF-like motif indicated that this motif is responsible for the inhibiting effect.

**EXPERIMENTAL PROCEDURES**

**Materials**—Chicken eggs (products of Hamburger and Hamilton strains) were purchased from Brampton Chick Hatchery Co., Ltd., Brampton, Ontario, Canada. Lipofectin, Genetecin (G418), DMEM growth medium, fetal bovine serum, Hanks’ balanced salt solution (HBSS), trypsin/EDTA, and RT-PCR kit were from Life Technologies, Inc. (Canadian Life Technologies, Inc., Burlington, Ontario, Canada). Taq DNA polymerase, T4 DNA ligase, and restriction endonucleases were from Boehringer Mannheim. The Prep-A-Gene DNA purification kit were from Bio-Rad. The DNA mini-prep kit was from Bio/Ca Scientific. The ECL Western blot detection kit was from Amersham Pharmacia Biotech. The goat anti-mouse IgG horseradish peroxidase and goat anti-rabbit horseradish peroxidase were from Sigma. The DNA Mid-prep kit, Ni-NTA agarose and mRNA purification kit were from Qiagen Inc. A monoclonal antibody (4B6) was from Dr. Paul F. Goetinck’s laboratory. The 6-, 24-, and 96-well tissue culture plates were from Nunc Inc. Cell Strainers were from Falcon. All chemicals were from Sigma. Monoclonal antibody to type II collagen was purchased from Developmental Studies Hybridoma Bank (University of Iowa). Oligonucleotides were synthesized by ACGT Corp. (Toronto, Ontario, Canada).

**Construction and Expression of Recombinant Genes**—Construction of a mini-versican has been described by us previously (21). To allow sufficient secretion of the mini-versican product, the leading peptide of link protein was added to this construct. This also allowed the versican mini-gene products to be detected by a monoclonal antibody (4B6) that recognizes an epitope in the leading peptide (22). The leading peptide of link protein was added to this construct. This also allowed the versican mini-gene products to be detected by a monoclonal antibody (4B6) that recognizes an epitope in the leading peptide (22). The leading peptide of link protein was then ligated into pcDNA1.

The aG3 construct was generated by linking the leading peptide of link protein and the G3 domain of aggrecan together. The leading peptide contained an EcoRI and BamHI site at its 5’ end and a BamHI site at its 3’ end, whereas the G3 domain contains an internal BamHI site at its 5’ end (nucleotides 244–249) (9). The leading peptide and the mini-versican (vG3His) fragment were ligated into EcoRI- and SpHII-digested pcDNA1. The recombinant versican gene was 3.2 kilobase pairs and was expected to yield a core protein of 150 kDa. With the attachment of glycosaminoglycan chains, we expected that the recombinant proteoglycan would migrate on SDS-PAGE gel as a smear, at around 200 kDa or more.

The G1 construct (vG1) was generated with two primers, 5’-AAA AAG CTT GCC GCC ACC ATG TTG TTA AAC ATA AAA AGC-3’ and 5’-AAA GCA TGC TTC GTA GCA GTA GGC ATC-3’, in a PCR reaction. The PCR product was digested with BamHI (restriction site present at nucleotides 244–249) (9) and SpHII. This fragment and the leading peptide of link protein were then ligated into pcDNA1.

The aG3 construct was generated by linking the leading peptide of link protein and the G3 domain of aggrecan together. The leading peptide contained an EcoRI and an Xhol site at the 5’ and 3’ terminus of the fragment. The G3 domain of aggrecan was obtained by restriction digestion with Xhol and SpHII from a mini-aggrecan previously constructed (24).

The chimeric construct vEGFaG3 contained the link protein leading peptide, the two EGF-like motifs of versican, and the G3 domain of aggrecan. The leading peptide contained an EcoRI at the 5’ end and an Xhol at the 3’ end of the cDNA fragment. The EGF-like motifs were synthesized in a PCR reaction with two primers, 5’-AAA GAA TTC GCC -3’ (located in nucleotides 10129–10143 of versican sequence) and 5’-AAA GCA TGC ACC ATG CTA CTC TTT CTG G3’ (complementary to nucleotides 10810–10830) as primers in a PCR reaction using the mini-versican as a template. The product, a G3 fragment lacking the EGF-like motifs (G3EGF), was digested with Xhol and SpHII and ligated into the recombinant mini-versican from which the full-length G3 domain had been removed by digestion with Xhol and SpHII.

**FIG. 1. Strategy for generation of different constructs.** Strategy for construction of a mini-versican gene that contains the G1 domain (nucleotides 145–1182 of mature versican), 15% of the entire chondroitin sulfate domain (nucleotides 1183–2424), and the G3 domain (nucleotides 9904–10830) is shown. Also shown are mini-versican lacking EGF-like motifs (ΔEGF), vG1 and vG3 constructs obtained from the mini-versican gene, and aG3 construct obtained from aggrecan G3 domain. vEGFaG3 is a chimeric construct consisting of retaining the two EGF-like motifs of versican and the G3 domain of aggrecan. The leading peptide added to each construct was obtained from link protein. IgG, immunoglobulin domain; TR, tandem repeat; EGF, epithelial growth factor-like motif; CBP, complement-binding protein; His, MRGSHHHHHH. Numbers above schematic correspond to nucleotides in the sequence of full-length versican.
The amplified cDNA was digested with HindIII and SacI. The above three cDNA fragments were ligated together into EcoRI- and SacI-digested pCDNA1 to obtain the construct vEGFaG3.

The antisense construct was generated with two primers, 5'-AAA AAG CTT GAC CTG GGT GGG TCT GTG CAC-3' and 5'-AAA GCC TCC ATG GGT GGG TCT GTG CAC-3'. The amplified cDNA was digested with HindIII and SacI. The above three cDNA fragments were ligated together into EcoRI- and SacI-digested pCDNA1 to obtain the construct vEGFaG3.

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taining 5% fetal bovine serum) and cell lysate from COS-7 cells transiently transfected with the His-tagged mini-versican were harvested and purified with Ni-NTA beads according to manufacturer’s instructions. The eluted product was dialyzed extensively against PBS and analyzed in Western blot and silver staining to confirm that the mini-versican had been purified.

**RT-PCR Assay**—Standard protocol was used for mRNA purification and RT-PCR assay with kits from Life Technologies, Inc. Briefly, 5 µg of mRNA were used to synthesize cDNA, a portion of which (equal to 1 µg mRNA) was used in a PCR reaction. The PCR products were analyzed in agarose gel electrophoresis and stained with ethidium bromide for cDNA detection.

**Western Blot Assay**—Cell lysate or growth medium containing recombinant gene products was subjected to SDS-PAGE and stained with primary antibody (4B6 or monoclonal antibody to type II collagen) in a buffer containing 10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% Tween 20, 5% milk (TBSTM). The membranes were washed with TBST (3 times, 30 min each) and then incubated for 1 h in secondary antibody conjugated to horseradish peroxidase in TBSTM. After washing as above, the membranes were developed with an ECL kit according to the manufacturer’s instructions (Amer sham Pharmacia Biotech).

**Isolation and Growth of Chicken Mesenchymal Cells**—Fertilized chicken eggs were incubated at 37°C for 4 days. Limb buds were isolated from chicken embryos (stages 22–24) under a dissection microscope. Buds were rinsed with HBSS and then dissociated with 0.5% trypsin in 10 ml of HBSS at 37°C for 30 min. The digested limb buds were washed twice with DMEM and resuspended in 2 ml of DMEM supplemented with 5% fetal bovine serum. To obtain single cell suspensions, the limb buds were vortexed thoroughly and filtered through Cell Strainers (40 µm nylon) to obtain single mesenchymal cells. The cells were used for micromass cultures.

**Micromass Cultures**— Micromass cultures were grown on either 24- or 96-well tissue culture plates. Mesenchymal cell suspension was incubated with a micropipette tip on the middle of each well (approximately 2–3 × 10⁶ cells/ml) for the 24-well plates and 10 µl (3 × 10⁶ cells/ml) for the 96-well plates. The cells were incubated at 37°C in a tissue culture incubator for 2 h to allow cellular attachment. After cells had attached, growth medium was gently introduced (1 ml for the 24-well plates and 200 µl for the 96-well plates). To enhance mesenchymal condensation and chondrogenesis, the micromass cultures were incubated in a medium containing 50% standard growth medium and 50% enriched growth medium that had been preincubated with subconfluent fibroblasts such as NIH3T3 fibroblasts for 2 days. The enriched medium contained different kinds of growth factors that enhanced the growth and differentiation of the micromass cultures. Growth medium was changed every other day, and cultures were examined under light microscopy every day.

**Alcian Blue Staining**—Alcian blue is a dye that binds to the glycosaminoglycan chains of proteoglycans. Because aggrecan, a major product of chondrocytes, contains many more glycosaminoglycan chains than other proteoglycans, Alcian blue can be used to monitor chondrogenesis (26). Staining of the glycosaminoglycan chains was examined and photographed under an inverted light microscope.

**Transient Expression of Genes in Mesenchymal Cells**— Plasmid DNA (15 µg) was incubated with Lipofectin (10 µl) for 15 min in 100 µl of DMEM. DMEM was added to a final volume of 2 ml. Meanwhile, mesenchymal cells freshly isolated from chicken embryos were rinsed with DMEM, pelleted by centrifugation at 1000 rpm, and suspended in 2 ml of DMEM supplemented with 5% fetal bovine serum. To obtain single cell suspension at a concentration of 3 × 10⁶ cells/ml, the cell suspensions were placed in 25 cm² tissue culture flasks and incubated in a tissue culture incubator. After 3–5 days, the growth medium was changed every other day. Cells were transfected with Lipofectin, resuspended in 2 ml of DMEM supplemented with 5% fetal bovine serum, and the mixture was introduced into each well. The final concentration of each oligonucleotide was 4.2 µM. The mesenchymal cultures were incubated in a tissue culture incubator, and levels of condensation and chondrogenesis were determined using light microscopy and PNA (peanut agglutinin) or Alcian blue staining. Staining of cultures with PNA, which binds to extracellular aggregates in condensed mesenchymal cultures, was performed according to the methods described earlier (27). The stained cultures were examined under light microscopy.

**RESULTS**

**Versican Inhibits Mesenchymal Chondrogenesis**—It has been reported that versican is highly expressed in chicken mesenchymal cells in the early developmental stages (2, 3) and is restricted to the zone of keratinocyte proliferation in the epidermis (8). These results imply that versican may play an important role in cell differentiation and tissue development. To test this, we chose to use an in vitro model as follows: we used chicken mesenchymal cells at developmental stages 22–24, which are able to undergo condensation and chondrogenesis in culture.

To study the functions of versican, we generated a mini-
versican gene (Fig. 1, versican) and transfected it transiently into COS-7 cells and stably into NIH3T3 fibroblasts. Expression of the mini-versican and its secretion in the growth medium was confirmed by Western blot. The band observed was diffuse and migrated slower than the size predicted by the amino acid sequence, indicating that the product is glycosylated. Fig. 2A (COS) shows a typical sample, obtained from the growth medium of COS-7 cells transfected with mini-versican. This mini-versican-containing growth medium was introduced into mesenchymal cultures, and growth medium from the vector-transfected COS-7 cells was used as a control. Chondrogenesis was monitored using light microscopy and Alcian blue staining. We observed that mini-versican treatment enhanced cell proliferation but inhibited chondrogenesis by producing fewer cartilaginous nodules (Fig. 2B). Undifferentiated cells died quickly, and proliferation was inhibited at high cell densities, causing the total cell number in the mini-versican-treated culture to be slightly higher than that of the control culture (Fig. 2B). A higher cell density normally favors condensation and chondrogenesis with the formation of cartilaginous nodules; however, the more confluent mini-versican-treated cultures formed significantly fewer cartilaginous nodules (Fig. 2B). Growth media from NIH3T3 fibroblasts transfected with the mini-versican also inhibited chondrogenesis (data not shown).

To rule out the possibility of nonspecific effects arising from factors present in the growth medium, we purified the mini-versican from growth medium and cell lysate of COS-7 cells transfected with the mini-versican construct. Vector-transfected COS-7 cells were used as controls in the purification. Purified mini-versican was analyzed by Western blot to ensure that the mini-versican product was isolated by the Ni-NTA column (Fig. 2A). Since the concentrations of purified mini-versican were low (0.05 ng/μl), we were not able to detect the product with silver staining (partially due to the smear of the product). Nevertheless, treatment of cultured mesenchymal cells with the purified mini-versican inhibited the formation of cartilaginous nodules, in a dose-dependent manner, compared with treatment with elution from the control (Fig. 2C). As the column was extensively washed during product purification, the yield was low but no contaminant was detected with silver staining (data not shown).

Finally, freshly isolated mesenchymal cells were transfected with the mini-versican construct or the control vector. The transfected mesenchymal cells were incubated at 37 °C for 3–5 days before analysis. Expression of mini-versican was found to

**FIG. 4. The effect of versican antisense on chondrogenesis.** Mesenchymal cells were cultured in 96-well tissue culture plates and incubated in 4.2 μM antisense oligonucleotides (A) or sense oligonucleotides (B). As well, mesenchymal cells were directly transfected with plasmids containing an antisense construct to versican (C) or the control vector (D) as described under “Experimental Procedures.” Both antisense oligonucleotides (A) and expression of antisense construct (C) enhanced chondrogenesis.
inhibit chondrogenesis in each of the 20 transfections performed. A typical result is shown in Fig. 2D.

The effects of mini-versican on differentiation was monitored by examining the expression of link protein and type II collagen. Growth media from the control- and the mini-versican-treated cultures were collected and analyzed with Western blots. The cultures treated with the mini-versican expressed only negligible amounts of secreted link protein, even after 5 days (Fig. 3A). Medium from controls, however, exhibited significant immunoreactivity to link protein and type II collagen. Link protein was not detected in cultures at day 1. Since link protein expression is characteristic of cartilaginous nodule formation, low levels of link protein expression corroborate the observed inhibitory effects of versican on chondrogenesis. Expression of type II collagen was similar to link protein; type II collagen expression was not detected at day 1, and treatment with the mini-versican inhibited type II collagen expression (Fig. 3B).

Versican Antisense Enhanced Mesenchymal Chondrogenesis but Not Condensation—To confirm the effect of the mini-versican on mesenchymal chondrogenesis, an antisense approach was employed. Mesenchymal cells freshly isolated from chicken mesenchyme were seeded into 96-well tissue culture plates, and the cultures were incubated with one of three antisense oligonucleotides of versican for 5 days. For each antisense oligonucleotide, one complementary sequence (sense) and one oligonucleotides of versican for 5 days. For each antisense oligonucleotide, one complementary sequence (sense) and one oligonucleotide and sense construct. The level of reduction in antisense construct did reduce levels of free versican mRNA, we performed RT-PCR. Mesenchymal cells treated with antisense oligonucleotides and cells transfected with antisense construct had reduced amounts of RT-PCR product, analyzed in agarose gel electrophoresis (Fig. 5).

The above experiments demonstrate that the mini-versican has a negative effect on chondrogenesis. A prerequisite of chondrogenesis is mesenchymal condensation, and it has been reported that versican is expressed in areas of mesenchymal condensation (2, 3). To investigate whether versican inhibits chondrogenesis by preventing mesenchymal condensation, cultures were analyzed for condensation with or without exogenous addition of versican. Growth medium containing the mini-versican was introduced into mesenchymal cultures, with growth medium from vector-transfected cells used as a control. Cultures were fixed, treated with PNA, a marker of condensation, and examined under light microscopy. Areas of condensation appeared as characteristically discrete circular regions of brown staining. At 18 to 20 h after versican treatment, similar levels of condensation were observed in both the mini-versican- and vector-treated cultures (Fig. 6). When the areas of condensation were counted, no significant difference between the vector- and versican-treated mesenchymal cultures was found (Table III). We also used antisense oligonucleotides to study the effects of endogenous versican on mesenchymal condensation. Antisense oligonucleotides to versican had no effect on mesenchymal condensation (Fig. 6). With respect to the number of areas of condensation, cultures treated with sense or antisense oligonucleotides showed no significant difference (Table III), indicating that versican does not inhibit mesenchymal condensation.

The Versican G3 Domain, Especially the EGF-like Motifs, Is

| Table II |

| Treatment | Well Score | Score Score/well |
|-----------|------------|------------------|
| Vector    | 14 | 21 | 28 | 15 | 64 | 4.57 |
| Versican  | 14 | 15 | 10 | 1 | 26 | 1.86* |
| vG1       | 7  | 18 | 8  | 2 | 28 | 4.0  |
| vG3       | 8  | 3  | 6  | 1 | 10 | 1.25* |
| ΔEGF      | 8  | 18 | 10 | 3 | 31 | 3.9  |
| aG3       | 8  | 18 | 14 | 0 | 32 | 4.9  |
| vEGFaG3   | 4  | 4  | 3  | 2 | 9  | 2.25* |
| Antiversican | 8 | 30 | 20 | 4 | 54 | 6.75* |

* p < 0.05.
the Critical Element in Inhibiting Mesenchymal Chondrogenesis—Given the negative effect of versican on chondrogenesis, we sought to determine which specific domains were involved in the inhibition. Constructs encoding the versican G1 and G3 domains were generated, and the constructs (see Fig. 1) were expressed in COS-7 cells. Expression of both constructs was confirmed (Fig. 7A). Growth medium was collected from cells transfected with the vG1 or vG3 construct and introduced into mesenchymal cultures as described under “Experimental Procedures.” Growth medium from vector-transfected cells was used as a control. After chondrogenesis, growth medium from all cultures was collected and analyzed by Western blot; the vG3 construct-treated cultures were found to secrete much less link protein than did the vG1- or control-treated cultures (Fig. 7B). The cultures were also stained with Alcian blue and examined under a light microscope. The vG3 construct alone, but not the vG1 construct, was sufficient to inhibit chondrogenesis (Fig. 8), since cultures incubated with growth medium from G3-transfected cells formed significantly fewer nodules. In a quantitative assay, the effect of the vG3 construct was similar to that of the mini-versican and both inhibited chondrogenesis significantly. The cultures treated with growth medium containing vG3 formed fewer cartilaginous nodules compared with vG1 treatment (Table IV).

It has been reported that EGF plays a role in cell differentiation (28–31). Because the G3 domain of versican contains two EGF-like motifs, we sought to investigate the potential role of the EGF-like motifs in mesenchymal chondrogenesis. After deleting the EGF-like motifs from the mini-versican as shown

**Fig. 6. The effects of versican and versican antisense on condensation.** Growth medium collected from vector- or versican-transfected cell lines was introduced into mesenchymal cultures, and the cultures were stained with PNA after 20 h incubation. Mesenchymal condensation was indicated by the appearance of 3,3′-diaminobenzidine (DAB) label foci. The mini-versican had no effect on mesenchymal condensation. In antisense oligonucleotide assays, mesenchymal cells were cultured in 96-well tissue culture plates, and 4.2 μM oligonucleotides were introduced into each well. The mesenchymal cultures were allowed to condense and stained with PNA after 24 h. The antisense oligonucleotides to versican had no effect on mesenchymal condensation.

**Table III**

| Treatment  | Wells | Nodules (per well) |
|------------|------|--------------------|
| Vector     | 8    | 2.38 ± 1.69        |
| Versican   | 8    | 2.13 ± 1.55*       |
| Sense      | 8    | 2.0 ± 1.31         |
| Antisense  | 8    | 2.5 ± 0.93*        |

* p > 0.05.
in Fig. 1, the versican-ΔEGF construct was expressed in COS-7 cells and NIH3T3 fibroblasts. The G3 domain of chicken aggrecan (aG3), which does not contain an EGF-like motif, was also constructed and expressed. Finally, a chimera construct (vEGFaG3) containing the two versican EGF-like motifs and the G3 domain of aggrecan was generated as described under “Experimental Procedures.” Expression of these constructs by COS-7 cells was confirmed on Western blot (Fig. 7A). Growth media containing versican-ΔEGF, aggrecan G3 construct (aG3), or the chimera construct vEGFaG3 were introduced into mesenchymal cultures. Growth medium from the vector- and the mini-versican-transfected cells was used as controls. Link protein expression was analyzed on Western blot after chondrogenesis. Cultures treated with aG3, versican-ΔEGF, or vector were found to express high levels of link protein (Fig. 7B), further suggesting that the EGF-like motifs are critical for inhibition of chondrogenesis. Growth medium from mesenchymal cultures treated with the chimera construct contained moderate amounts of link protein (Fig. 7B). Cartilaginous nodules were detected by Alcian blue staining. The ability of versican to inhibit chondrogenesis was substantially reduced when its EGF-like motifs were removed, whereas the aG3 construct had no effect on chondrogenesis (Fig. 8). The chimera construct inhibited chondrogenesis to a greater extent than the aG3 construct, but to a lesser extent than did the vG3 construct (Fig. 8).

FIG. 7. The EGF-like motifs of versican inhibited link protein expression. A, a number of different domains were individually sub-cloned into expression vectors, as shown in Fig. 1: a mini-versican lacking EGF-like motifs, vG1 and vG3 constructs derived from the G1 and G3 domains of versican, aG3 construct derived from the G3 domain of aggrecan, and a chimera construct vEGFaG3. These constructs were transiently expressed in COS-7 cells. The mini-versican and the vector were used as controls. Expression of these constructs was analyzed in Western blot probed with the monoclonal antibody 4B6. All constructs had the expected sizes. The mini-versican product migrated as a smear similar to that seen in Fig. 2A. The versican-ΔEGF construct also migrated as a smear but was slightly faster than the mini-versican product. The products of vG3 and vEGFaG3 constructs migrated as a single band with size of 58 kDa, vG1 construct, 54 kDa and aG3, 50 kDa. B, growth media were collected from cultures described B to assay link protein expression. vG3- and mini-versican-treated cultures showed a significant reduction in link protein expression, but cells treated with aG3 and versican-ΔEGF products exhibited no change in expression of this protein, as compared with the control. The product of chimeric construct had some inhibitory effect on link protein expression, but its effects were smaller than those of the vG3 product.


discussion

Versican is found in significant concentrations in tissues where the cells are actively proliferating, for example, embryonic tissues and the epidermal proliferative zone (3, 8, 19). This indicates that versican may be involved in cell growth, and our previous work has indeed confirmed that versican stimulates cell proliferation (21). Since cell growth and differentiation are usually antagonistic, we hypothesized that versican would affect cell differentiation. In this study, we report that versican does inhibit mesenchymal chondrogenesis but not condensation. The inhibition is apparently mediated, at least in part, by the G3 domain of versican and specifically by the EGF-like motifs.

To determine the effects of versican, we devised an in vitro assay system using chick mesenchymal cells in culture. Our findings show that such assays are sensitive and require careful calibration of mesenchymal age and cell plating density to detect the effects of versican. After extensive experimentation, we determined that limb buds harvested in stages 22–24 were optimal for our work.

In the in vitro assay, cell differentiation was monitored using three separate methods. Morphological changes provided one measure of cell differentiation: as cultures differentiate, they produce characteristic “swirls” (high densities of cells and matrix) which later develop into cartilaginous nodules and increase in size over time. The second method of assessing differentiation involved histochemical techniques. Two major products of chondrocytes are aggrecan and link protein (32, 33). Alcian blue, a dye that nonspecifically stains glycosaminoglycan chains, easily detects aggrecan that possesses 100–150 chondroitin sulfate and keratin sulfate side chains. Thus, staining cultures with Alcian blue allowed detection of chondrogenesis even at early stages. Third, although aggrecan is a poor choice for monitoring the onset of chondrogenesis by immunoblot analysis as it appears on SDS-PAGE as a large smear over 500 kDa, link protein is a convenient marker for chondrogenesis. Anti-link protein antibody recognizes a discrete 52-kDa protein on an immunoblot, and although link protein appears in most tissues, it is found at high concentrations only in mature cartilage (33). We did not detect link protein in areas of cell condensation. Since type II collagen is also a major component of cartilage, we have confirmed our results by collagen staining. Results obtained using the above techniques (morphologic analysis, Alcian blue staining, and expression of
link protein) were all consistent with the inhibition of chondrogenesis by the mini-versican.

In our first studies of the effects of versican on chondrogenesis, we used versican gene product, manufactured by transfected COS-7 or NIH3T3 cells and secreted into their growth media (21). By using this system, however, we could not exclude the possibility that inhibitory effects of versican on differentiation were indirect. The gene product may have interacted with other proteins to produce unidentified factors that may have been secreted into the medium, causing the observed effects. To investigate if versican acts directly, we purified the mini-versican product and demonstrated that the purified product also inhibited chondrogenesis. We used a Ni-NTA affinity column to purify the mini-versican. With extensive washing, we were always able to obtain purified products with undetectable contamination stained by silver. However, the purified products were always diluted during dialysis. The products were not concentrated in order to maintain the products in its active forms. What is important in our assay is that the purified mini-versican, although at a much lower concentration compared with those expressed by COS-7 and mesenchymal cells, inhibited mesenchymal chondrogenesis. We also used transfection and antisense approaches. Results obtained from mesenchymal cell cultures transfected with versican constructs confirmed our hypothesis that the mini-versican itself inhibits differentiation. Moreover, all versican antisense oligonucleotides and versican antisense constructs also inhibited the effect of versican on chondrogenesis. We hypothesize that at the concentration of oligonucleotides used or in the presence of antisense construct, translation of versican mRNA was inhibited.

Before cells can differentiate, they must first undergo condensation. The question that arises is whether versican interferes with the differentiation process by preventing condensation. To monitor condensation, PNA, which binds to the extracellular aggregates in condensed mesenchymal cultures (27), was used. Treatment with versican or versican antisense

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**TABLE IV**

**Chondrogenesis was affected by products of different constructs**

Mesenchymal cells were seeded to tissue culture plates and incubated with conditioned medium from NIH3T3 fibroblasts transfected stably with different constructs, for 5 days. Cartilaginous nodules were stained with Alcian blue and examined under a light microscope. Mini-versican and versican G3 domain (vG3) significantly inhibited chondrogenesis compared with control vector, whereas versican G1 domain (vG1) and aggrecan G3 domain (aG3) had no effect. A versican construct lacking two EGF-like motifs (ΔEGF) also had no inhibitory effect. The chimeric construct containing two EGF-like motifs and the G3 domain of aggrecan inhibited nodule formation to a limited extent.

| Treatment | Well | Score | Total | Score/well |
|-----------|------|-------|-------|------------|
|           |      | Large¹ | Small² | Swirl² |       |
| Vector    | 13   | 27    | 6     | 17      | 50    | 3.85  |
| Versican  | 9    | 3     | 2     | 3       | 8     | 0.89* |
| vG1       | 12   | 27    | 0     | 10      | 38    | 3.16  |
| vG3       | 11   | 3     | 6     | 2       | 11    | 1.0*  |
| ΔEGF      | 8    | 15    | 6     | 7       | 28    | 3.50  |
| aG3       | 8    | 15    | 4     | 9       | 28    | 3.50  |
| vEGFaG3   | 8    | 9     | 4     | 8       | 21    | 2.63* |

* p < 0.01.
oligonucleotides had no obvious effect on the number of PNA-stained foci observed during early stages of treatment. However, we cannot rule out the possibility that mesenchymal cells isolated from stage 22–24 embryos already contain endogenous condensation signals nor that condensation was an automatic response to high culture density. Neither exogenous addition of versican gene product nor versican antisense oligonucleotides affected condensation; however, whereas the gene product also inhibited subsequent differentiation in the culture, treatment with antisense oligonucleotides and antisense construct actually seemed to enhance chondrogenesis. Given that versican stimulates cell proliferation, and versican expression must be down-regulated before chondrogenesis can proceed, it may be that exposure to the versican gene product prevented the initiation of chondrogenesis, whereas blocking expression of versican with antisense oligonucleotides actually triggered a differentiation pathway.

Given, then, that versican does not function by blocking events prerequisite to differentiation, such as condensation, but seems to inhibit the differentiation process itself, we were interested in characterizing the mechanism of its activity. By treating mesenchymal cultures with the gene products of constructs expressing various domains of the versican molecule, we found in the present study that the G3 domain of versican alone can inhibit differentiation. This domain comprises three separate sections as follows: a set of epidermal growth factor-like motifs, a carbohydrate recognition sequence, and a complement-binding protein-like sequence. When cells were treated with the products of a mini-versican construct lacking the EGF-like motifs or with aggrecan G3 domain (which does not contain EGF-like motif), the culture underwent chondrogenesis, implying that the EGF-like motifs were involved in the inhibition. These results were confirmed by direct transfection of mesenchymal cells with the above constructs. Consistent with our results, a protein containing EGF-like repeats has also been reported to prevent differentiation in adipocyte precursors (29). Nor is this inhibition directly related to the ability of EGF to stimulate proliferation: EGF’s inhibition of differentiation appears to operate via a separate mechanism (34). In our experiments too, the inhibitory effects of versican are distinct and not directly related to its stimulatory effects, since the amount of versican gene product required to prevent differentiation was less than the amount required to stimulate mesenchymal proliferation (data not shown). It should be mentioned that the EGF-like motifs of versican are necessary for the inhibition of chondrogenesis but are by themselves not sufficient for this function since a chimeric construct containing the EGF-like motifs of versican and the G3 domain of aggrecan only partially inhibited chondrogenesis as compared with the vG3 domain construct. These results suggest that other motifs in the G3 domain are involved in the effect of versican on chondrogenesis. We are currently studying the roles of other motifs.

Evidence from this study and from other studies suggests that the proteoglycan versican may play an important and complex role in cell growth and differentiation. It simulates cell proliferation, both directly, through its G1 and EGF-like subdomains, and indirectly; and it inhibits cell differentiation, an effect mediated by its EGF-like subdomains. The physiological significance of these findings remains to be determined.

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