Versican V2 isoform enhances angiogenesis by regulating endothelial cell activities and fibronectin expression

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\textbf{A B S T R A C T}

Versican is a proteoglycan expressed in the extracellular matrix, where it regulates a variety of cell activities and affects tumor development. With alternative splicing, there are four versican isoforms, denoted V0, V1, V2 and V3. The V2 isoform is highly expressed in the mature brain but its function in the mature brain has not yet been elucidated. Since brain tumors are among the most angiogenic of human tumors, we investigated whether or not the V2 isoform plays a role in angiogenesis and found that the glioblastoma cell line U87 stably transfected with V2 formed tumors containing extensive vasculature. Although the V2-expressing cells grew slowly, they survived well in serum-free medium. They also displayed high adhesive ability to endothelial cells and facilitated tube-like structure formation. Importantly, fibronectin was up-regulated by V2 and mediated V2 function. Thus, versican V2 could be a potential target for intervention of brain tumor angiogenesis.

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1. Introduction

Glioblastomas are among the most life-threatening brain tumors due to their invasive and infiltrative character. Through a process known as angiogenesis, glioblastomas stimulate the formation of new blood vessels, which are structurally and functionally abnormal. Glioblastomas are among the most angiogenic human tumors. High-grade glioblastomas are characterized by more extensive vascularization than low-grade glioblastomas. The degrees of neovascularization in high-grade glioblastomas are a histological indicator of malignancy and patient prognosis. As a consequence of extensive angiogenesis, a hostile microenvironment containing low oxygen tension and high interstitial fluid pressure leads to a more malignant phenotype associated with increased morbidity and mortality. We hypothesize that malignant tumor cells are capable of controlling endothelial cell activities by modulating the expression of extracellular matrix molecules within the tumor microenvironment.

Versican is a hyaluronan-binding protein that belongs to a class of the large aggregating chondroitin sulfate proteoglycans located primarily in the extracellular matrix [1]. By alternative splicing of two exons encoding chondroitin sulfate attachment sequences (CS\textsubscript{a} and CS\textsubscript{b} domains), there exist four versican isoforms [2–4]. These four isoforms include the largest isoform (versican V0), which is not subject to alternative splicing and contains both the CS\textsubscript{a} and CS\textsubscript{b} domains; versican V1, the larger of the alternatively spliced forms, which has the β domain for chondroitin sulfate attachment [5]; versican V2, the smaller of the alternatively spliced forms, which contains the β domain; and versican V3, which contains neither of the CS sequences. Versican V0 has a core protein of 3650 amino acids [3]. Versican V0 is synthesized by prechondrogenic mesenchymal cells and plays a role in cell migration [3,6]. Versican V1 is synthesized by embryo and endothelial cells, contributing to cell proliferation and tumor growth [7,8]. Versican V2 is expressed in mature tissues and can induce cell apoptosis and repress tumor growth [2,7]. Versican V3 is expressed in various mouse and human tissues and can inhibit tumor growth [9–11]. While the functions of versican have been reported in different cell types and tissues [12–15], different isoforms also play different functions [7,16,17].

Versican is highly expressed in the early stages of development, but becomes downregulated after tissue maturation [5]. In the adult human central nervous system, versican is expressed mainly in the white matter of the frontal lobe, cerebellum, brain stem, and spinal cord, in close association with astrocytes and oligodendrocytes [18–21]. Interestingly, during wound repair or tumor formation, versican expression is again upregulated [22–26].
Additionally, increases in versican expression are involved in tumor formation [27–30]. Versican is detected in interstitial tissues at the invasive margins of tumors, and in the perivascular elastic tissues associated with tumor invasion [26]. Increased versican immunostaining has been detected during tumor blood vessel formation [29]. It appears that V0 is the key isoform in the early stages of tissue development. The V1 isoform plays central roles in cell proliferation and tumor growth, while the V2 and V3 isoforms inhibit cell proliferation and tumor growth. Since the V2 isoform is the major isoform expressed in brain tissues, and brain tumors have been shown to be greatly enriched in vascularization, we hypothesized that the V2 isoform may play a role in brain tumor angiogenesis. By in vitro and in vivo approaches, this study was designed to test the role of the versican V2 isoform in angiogenesis.

2. Methods

2.1. Cell proliferation assay

Glioblastoma cell line U87 cells stably transfected with versican V2 isoform or a control vector (1 × 10^5 cells) were seeded onto 6-well tissue culture plates in 10% FBS/DMEM medium and maintained at 37°C overnight. Cells were harvested daily and cell number was determined by a Coulter Counter.

2.2. Cell survival assay

Cells (1.5 × 10^5 cells per well or 2 × 10^5 cells per well) were seeded on 6-well tissue culture plates in 10% FBS/DMEM. Next day, the medium was removed and the culture was washed with PBS and replaced with serum-free DMEM. The cultures were incubated for different time periods. The cell numbers were counted by using trypan blue staining as described [31,32]. The experiments were repeated three times.

2.3. Cell cycle analysis

U87 cells stably transfected with V2 or the control vector were cultured overnight. The cells were then harvested, washed, and resuspended in cold PBS and incubated in ice-cold 70% ethanol for 3 h. The cells were then centrifuged at 1,500 rpm for 10 min and resuspended in propidium iodide (PI) master mix (40 mg/ml PI and 100 mg/ml RNase in PBS) at a density of 5 × 10^5 cells/ml and incubated at 37°C for 30 min before analysis with flow cytometry.

2.4. Tube formation assay

Vector- or V2-transfected U87 cells were grown to ~80% confluency and then harvested from the plates by 0.05% trypsin with 0.53 mM EDTA. The cells were recovered by centrifugation, washed three times with DMEM, suspended in this medium at a density of 4 × 10^4 cells/ml, and mixed with endothelial cells EOMA or YPEN. The mixture was inoculated to Matrigel-coated 8-chamber culture slides. Formation of tube-like structure was examined under a light microscope and photographed.

2.5. Co-culture experiments

The V2- or vector-transfected cells were mixed with YPEN or EOMA cells (1.5 × 10^5 cells/ml for each) and seeded on 3.5-cm culture dishes in DMEM supplemented with 10% FBS (2 ml). The cultures were maintained for 5 days. The interaction of both types of cells were examined with light and fluorescent microscopy.

2.6. Western blotting

Cell lysates were prepared from V2- or vector-transfected cells seeded on 6-well plates at 10^6 cells/well by lysing the cells in each well with 100 µl lysis buffer containing Triton X-100, SDS, and protease inhibitors. Protein concentrations were measured by Bio-Rad Protein Assay kit. Lysates containing 50 µg protein were subjected to SDS-PAGE. The separated proteins were transferred to a nitrocellulose membrane followed by immunostaining with a primary antibody against fibronectin (BD Transduction Laboratories 610078) or versican (US Biological L1350) overnight at 4°C. Next day, the membrane was washed and incubated with HRP-conjugated goat-anti-mouse secondary antibody for 2 h at room temperature followed by ECL detection. After detection of protein bands, the blot was re-probed with anti-β-actin antibody to confirm equal loading of samples.

2.7. Tumorigenicity assays in nude mice and immunohistochemistry analysis

Five-week-old CD1 strain nude mice were injected subcutaneously with V2- or vector-transfected U87 cells (5 × 10^4 cells per mouse) using the methods described previously [33]. When the sizes of the tumors reached the limited size set by the Animal Care Committee of Sunnybrook Research Institute, the mice were sacrificed for tumor harvest. Organs were freshly excised and fixed in formalin overnight, immersed in 70% ethanol, embedded in paraffin, and sectioned by a microtome (Leica RM2255). The sections were de-paraffinized with xylene and ethanol and then boiled in a pressure cooker. After washing with Tris-Buffered-Saline (TBS) containing 0.025% Triton X-100, the sections were blocked with 10% goat serum and incubated with primary antibodies against CD34, versican or fibronectin in TBS containing 1% bovine serum albumin (BSA) overnight. The sections were washed and labeled with biotinylated secondary antibody, followed by avidin conjugated horse–radish peroxidase provided by the Vectastain ABC kit (Vector, PK-4000). The staining was developed by DAB kit (Vector, SK-4100). The slides were subsequently stained with Mayer’s Hematoxylin for counter staining followed by slide mounting.

2.8. Statistical analysis

The results (mean values ± SD) of all the experiments were subject to statistical analyses by two-tailed t-test. The level of significance was set at P < 0.05, and P < 0.01.

3. Results and discussion

3.1. Versican V2 enhances angiogenesis

U87 glioblastoma cells stably transfected with a versican V2 expression construct or a control vector, were subcutaneously injected into nude mice. Six weeks after injection, the mice were sacrificed. The tumors formed by the V2-transfected cells were visibly enriched in vascularisation, whereas the tumors formed by the vector-transfected cells did not exhibit this phenotype (Fig. 1A). During retrieval of the tumors, we found that the V2 tumors were very adhesive to mouse tissues and a high level of vascularity was observed at the site of tumor growth. By H & E staining of tumor sections, it was observed that a large number of red blood cells were observed at the site of tumor growth. By H & E staining of tumor sections, it was observed that a large number of red blood cells were observed at the site of tumor growth.
tumor section was counted in 6 randomly selected image fields and analyzed statistically. Significantly more vascular structures were detected in the V2 tumors than in the control tumors (Fig. 1C). Careful examination of the structures of each blood vessel did not reveal obvious structural differences between the V2 and control tumors. Typical blood vessel structures from each of the tumors are shown (Fig. 1D). We confirmed expression of the V2 construct within tumors formed by the V2-transfected cells using Western blotting (Fig. 1E) and immunohistochemistry (Fig. 1F).

3.2. Versican V2 reduces tumor proliferation but enhances survival

To test how the versican V2 isoform affected tumor cell activities, we initially determined proliferation rates of U87 cells stably transfected with versican V2 isoform or a control vector were injected subcutaneously into CD-1 nude mice. Tumors were removed from the mice 4 weeks after the injection. Tumors formed by the V2-transfected cells showed increased vasculature compared to those formed by the control cells; (B) tumors formed by cells transfected with V2 isoform (V2 tumors) or a control vector were subjected to H & E staining. Extensive bleeding was detected in the V2 tumors, but not in the control tumors; (C) the tumor sections were subject to immunohistochemistry probed with anti-CD34 antibody to detect blood vessel formation. The number of blood vessels was counted in 10 randomly selected imaging fields and statistically analyzed. Expression of V2 promoted blood vessel formation as compared with the control. \( n = 5 \); (D) typical images of blood vessels from each mouse are shown; (E) tumor lysates were analyzed on Western blot to confirm that the tumor cells were transfected with the versican expression construct; (F) tumor sections were also subject to immunohistochemistry to confirm expression of the versican expression construct.
Expression versican V2 slows down cell proliferation but enhances survival. (A) U87 cells stably transfected with versican V2 isoform or a control vector were seeded on tissue cultures plates containing 10% FBS for six days for proliferation assays. Expression of V2 slowed cell proliferation. **P < 0.01. Error bars, SEM (n = 4); (B) the cells were also seeded on tissue cultures plates in serum-free medium. The number of living cells was counted with trypan blue staining using a haematocytometer for the duration of six days. Expression of V2 enhanced cell survival. **P < 0.01. Error bars indicate SEM (n = 4); (C) the cells were also subjected to cell cycle analysis. An increased number of V2 cells were detected in the G1 phase as compared with the control cells; (D) the culture medium was subject to western blot analysis for versican expression to confirm secretion of the versican product.
transfected with the V2 or control vector. We found that cells transfected with V2 grew much slower relative to the control cells (Fig. 2A). In soft agar, the V2-transfected cells formed smaller colonies than the control cells (Supplementary Fig. S1). We thus conducted an analysis of cell cycle progression and observed that more V2-transfected cells were detected in the G1 phase than in the G2 or S phases (Fig. 2B). This result indicated that the V2 cells turned over slower than the control cells, confirming the anti-proliferative function of versican V2.

We also tested whether or not expression of V2 affected cell survival. The cells were cultured in serum-containing DMEM overnight. The next day, the culture media was removed and the cells were cultured in serum-free medium. The overnight culture in serum-containing medium allowed the cells to intake serum and growth factors, resulting in continued growth. Continued culturing without serum however resulted in cell detachment and cell death. Interestingly, while the V2-transfected cells grew slower than the control cells, they displayed a much greater capacity to survive in serum free conditions and resist serum-free-induced cell detachment and cell death (Fig. 2C). Secretion of the V2 product was confirmed on Western blot by using the culture medium harvested from the V2-transfected cells (Fig. 2D).
Fig. 4. V2 upregulates fibronectin expression. (A) cell lysates prepared from V2- and vector-transfected cells or from the parental cells U87 were subject to western blot analysis probed with anti-fibronectin antibody. The same membranes were also probed with anti-β-actin antibody serving as a loading control. Expression of V2 construct increased fibronectin levels compared with the vector-transfected cells or the U87 cells; (B) culture medium was analyzed by Western blotting for fibronectin levels. The cells transfected with the V2 construct secreted larger amounts of fibronectin than the control cells; (C) tumor lysates were analyzed on Western blot for fibronectin expression. Transfection with the V2 construct increased fibronectin level; (D) tumor sections were subject to immunohistochemistry to confirm up-regulation of fibronectin expression in the tumors formed by the V2-transfected cells; (E) cell lysates prepared from siRNA constructs targeting fibronectin were analyzed on Western blot and probed with an anti-fibronectin antibody, confirming silencing of fibronectin; (F) cells transfected with siFN-32 or a control vector were mixed with YPEN cells, followed by inoculation in Matrigel for tube formation assay. Transfection with siFN-32 decreased the complexity of the tube-like structures; (G) cells transfected with siFN-32 or a control vector were also mixed with EOMA cells for tube formation assay. Silencing fibronectin decreased the formation of tube-like structures; (H) typical photographs of tube-like structures formed by EOMA cells mixed with cells transfected with siFN-32 or a control vector are shown.
3.3. V2 expression facilitates endothelial-tumor cell interaction

Subcutaneous injection into nude mice revealed that the tumors formed by V2-expressing cells harbored significantly more blood vessels than those formed by control cells. We examined whether the V2-expressing cells and the control cells might display differential capacities in interacting with endothelial cells, the major component of blood vessels. U87 cells transfected with or without the V2 construct were mixed with EOMA mouse endothelial cells and inoculated into tissue culture plates. The cells were co-cultured for five days. Cell–cell interactions were examined carefully by light microscopy. We found that EOMA cells, when mixed with the vector-transfected cells or the parental cells, tended to grow as colonies, with a clear delineation between the two cell types (Fig. 3A). In contrast, the V2-expressing U87 cells showed high levels of interaction with EOMA cells. These results indicated that the V2-expressing cells had a high affinity to endothelial cells, potentially linking the V2-expressing U87 cells with endothelial cell recruitment for blood vessel formation. We confirmed these results by co-culturing the V2- or vector-transfected U87 cells with YPEN rat endothelial cells and obtained a similar result (Fig. 3B, Supplementary Fig. S2).

As an angiogenic assay, we then tested whether V2-expressing U87 cells could facilitate the formation of tube-like structures in Matrigel. The V2- and vector-transfected cells were mixed with YPEN cells and cultured in Matrigel, followed by examination of tube-like structure formation. We found that YPEN cells formed complex tube-like structures when mixed with the V2 cells, which did not occur in culture with the control cells (Fig. 3C). Analysis of tube length and tube complexity was performed by ImageJ software. Both measures of tube formation were significantly increased when endothelial cells were cultured with V2-expressing tumor cells. Typical photographs of tube-like structures are shown (Fig. 3D). Similar results were obtained with EOMA cells (Fig. 3E). These results indicated that the V2-expressing cells were better able to facilitate the production of tube-like structures through the recruitment of endothelial cells. Through the release of adhesion factors and proangiogenic signals, versican V2 may be a critical mediator of tumor angiogenesis.

3.4. V2 upregulates fibronectin expression

We next analyzed levels of fibronectin, an adhesion molecule, by Western blot. Our results showed that the V2-expressing cells expressed much higher levels of fibronectin than the control cells (Fig. 4A). Increased levels of fibronectin were also detected in the medium of the V2-culture (Fig. 4B). Tumor lysates were subject to Western blot analysis for fibronectin levels, and we confirmed that the tumor lysates derived from the V2-transfected cells showed higher levels of fibronectin than from the control cells (Fig. 4C). Immunohistochemistry confirmed this result (Fig. 4D). Our results suggested that the versican V2 isoform might up-regulate fibronectin expression and enhance interaction of tumor cells and endothelial cells, leading to increased angiogenesis.

To test whether fibronectin played a role in mediating V2 functions, we used a siRNA approach to silence fibronectin expression. Cells transfected with four siRNA constructs targeting fibronectin were found to produce lower levels of fibronectin (Fig. 4E). The cells transfected with one of the siRNA constructs (siFN-32) or a control vector were subjected to tube-like structure formation assay. The experiment indicated that cells transfected with siRNA targeting fibronectin displayed decreased complexity of tube-like structure as compared with cells transfected with the V2 construct (Fig. 4F). A similar result was obtained with EOMA cells. Silencing fibronectin expression led to a decreased formation of tube-like structures (Fig. 4G), which could be clearly observed by light microscopy (Fig. 4H). These results indicated that fibronectin played an important role in mediating versican V2 function associated with angiogenesis.

Both versican and fibronectin are extracellular molecules and play important roles in enhancing cell adhesion. Although all versican isoforms contain the N-terminal G1 domain and a C-terminal G3 domain, the expression and functions of each isoform appear to be differentially regulated. Previous studies have demonstrated that the V1 isoform can enhance cell proliferation while the V2 isoform can inhibit cell growth [7]. The V1 isoform can also stimulate tumor growth [34]. However, the effect of the V2 isoform in tumor development has not yet been elucidated. Since the V2 isoform has been shown to be the primary isoform expressed in the mature brain, reports on versican expression and function in the brain are likely referring to this isoform. In the brain, versican is mainly expressed in the submeningeal layers of the cortex, around the blood vessels, and in a layer of cerebellum where it is observed to co-localize with tenascin and hyaluronan, two adhesion molecules [2,35,36]. In brain tumors, versican is detected in the interstitial tissues at the invasive margins, and in the perivascular elastic tissues associated with tumor invasion [26]. Increased versican immunostaining is detected in tumor blood vessels [29]. Elevated versican expression is also observed in the mesenchymal tissues between the invasive clumps of carcinoma cells at the margins of infiltrating ductal carcinoma. Collectively, it is highly likely that versican may play an important role in tumor development and blood vessel formation. Because the versican V2 isoform is the predominant isoform in the mature brain, we investigated the role of V2 isoform in tumor angiogenesis. Our study directly demonstrated that versican V2 isoform promoted tumor angiogenesis. This occurred through increased levels of interactions between tumor cells and endothelial cells, facilitating the formation of tube-like structures.

The ability of V2-expressing cells to survive under stressed conditions (i.e., deprivation of serum from the medium) may be key to its angiogenic role. Despite a reduced proliferative ability, the V2-expressing cells were able to survive much better than the control cells. This may be suggestive of a role for tumor side-population cells and vasculogenic mimicry, although this awaits further investigation.

Up-regulation of fibronectin expression appears key in angiogenesis. Fibronectin is an adhesion molecule playing a role in angiogenesis [33]. Since the versican G3 domain has been shown to bind to fibronectin, the V2 isoform may play the role in angiogenesis by up-regulating and binding to fibronectin. To confirm this, we have demonstrated that silencing fibronectin expression by siRNA abolished V2’s effect in enhancing tube-like structure formation. Taken together, our results demonstrated that the versican V2 isoform played a role in tumor angiogenesis by enhancing tumor cell survival, facilitating interactions between tumor and endothelial cells leading to the formation of tube-like structures, and up-regulating fibronectin expression. Regulating the versican V2 isoform and fibronectin may be potential targets for the intervention of tumorigenesis and angiogenesis in brain cancer.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2012.11.023.

References


