

## Role of versican in the pathogenesis of peritoneal endometriosis

Hirohiko Tani<sup>1</sup>, Yukiyasu Sato<sup>2\*</sup>, Masashi Ueda<sup>1</sup>, Yumiko Miyazaki<sup>1</sup>, Koh Suginami<sup>1</sup>, Akihito Horie<sup>1</sup>, Ikuo Konishi<sup>1</sup>, Tamayuki Shinomura<sup>3</sup>

<sup>1</sup> Department of Gynecology and Obstetrics, Kyoto University Graduate School of Medicine; <sup>2</sup> Department of Obstetrics and Gynecology, Otsu Red Cross Hospital; <sup>3</sup> Tissue Regeneration, Department of Bio-Matrix, Tokyo Medical and Dental University Original article

**Context:** Sampson's theory cannot explain why only some cycling women develop peritoneal endometriosis. Few studies have focused on the pelvic peritoneum, which receives regurgitated endometrial tissues. We hypothesized that molecular alterations in the peritoneum are involved in the development of peritoneal endometriosis and conducted a microarray analysis to compare macroscopically normal peritoneums sampled from women with peritoneal endometriosis (endometriotic peritoneums) and those without (non-endometriotic peritoneums). Versican, a major proteoglycan component of the extracellular matrix, is one of the molecules up-regulated in endometriotic peritoneums.

**Objective:** To investigate the role of versican in peritoneal endometriosis.

**Design, Patients, and Main Outcome Measure:** Endometriotic peritoneums and non-endometriotic peritoneums were subjected to RT-PCR, immunostaining, and Western blotting. The versican V1 isoform was stably transfected into Chinese hamster ovary cells (CHO-V1) and the effects of CHO-V1-derived conditioned medium (V1-CM) on primary human endometrial stromal cells (ESCs) were investigated with attachment, invasion, and proliferation assays. The effects of peritoneal fluid collected from endometriotic women (endometriotic PF) or cytokines/growth factors, which were shown to be elevated in endometriotic PF, on versican expression in a human peritoneal cell line (HMrSV5) were also examined.

**Results:** Versican V1 expression levels were significantly higher in endometriotic peritoneums. *In vitro*, V1-CM promoted attachment to the HMrSV5 cell monolayer as well as the Matrigel invasion of ESCs. Although versican V1 expression was up-regulated by TGF- $\beta$ 1 in HMrSV5 cells, it remained unchanged in endometriotic PF.

**Conclusions:** Our results suggest the involvement of peritoneal versican in the development of peritoneal endometriosis.

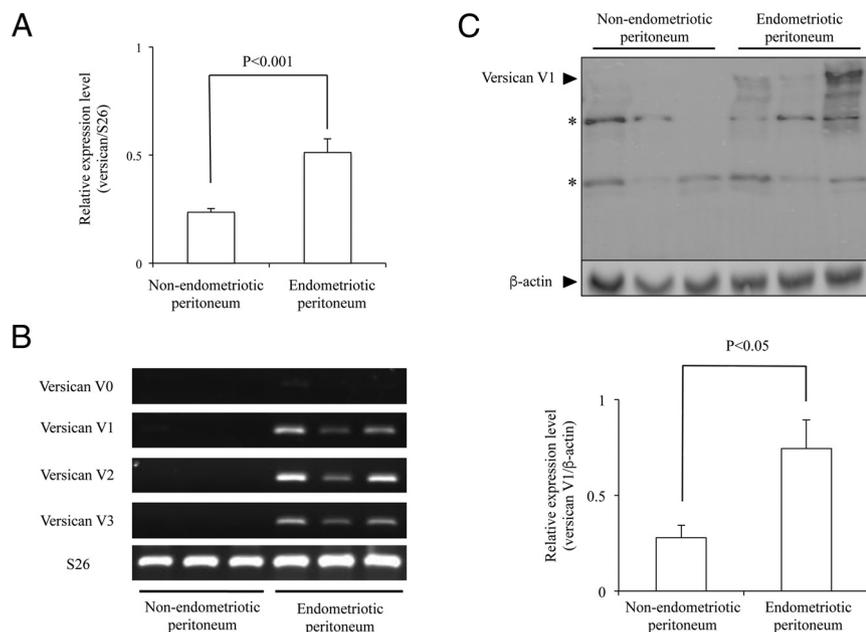
**E**ndometriosis is a chronic inflammatory disease defined as the presence of endometrial tissue outside the uterus, which is typically composed of endometrial-like stromal and epithelial cells. The incidence of endometriosis is estimated to be 6%–10% in the general population of females of reproductive age (1, 2). The pelvic peritoneum, particularly the recto-vaginal region (Douglas pouch), is one of the favored sites of endometriosis. Peri-

toneal endometriosis has been implicated in chronic pelvic pain, dysmenorrhea, and infertility (3).

The most widely accepted theory for the pathogenesis of peritoneal endometriosis is "Sampson's hypothesis", which states that endometrial tissue contained within menstrual blood escapes due to retrograde flow through the fallopian tubes into the pelvic cavity and attaches to the peritoneum, ultimately becoming the source of an endo-

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Abbreviations:



**Figure 1.** Expression of versican mRNA (A and B) and the versican protein (C) in macroscopically normal peritoneums from endometriotic women (endometriotic peritoneums) and those from nonendometriotic women (nonendometriotic peritoneums). (A) RT-PCR using primers that amplify all four of the versican isoforms to produce a single band. The expression level of versican relative to S26 (control) was significantly higher in endometriotic peritoneums ( $n = 6$ ) than in nonendometriotic peritoneums ( $n = 9$ ). Error bars indicate the standard error. (B) RT-PCR using isoform-specific primers. Representative images comparing nonendometriotic peritoneums and endometriotic peritoneums are shown. The versican V0 isoform (versican V0) was undetectable in all peritoneal samples. Note that the intensities of the bands for versican V1, V2, and V3 are all greater in endometriotic peritoneums than in nonendometriotic peritoneums. (C) Western blotting using the antibody specific to versican V0 and V1. Only versican V1 is detected (upper panel, asterisks indicate nonspecific bands). The intensity of the band for versican V1 relative to  $\beta$ -actin (control) was significantly greater in endometriotic peritoneums ( $n = 5$ ) than in nonendometriotic peritoneums ( $n = 5$ ) (lower panel). Error bars indicate the standard error.

metriotic lesion (4). However, this hypothesis does not account for why peritoneal endometriosis occurs in only 6%–10% of females of reproductive age, while retrograde menstruation has been documented in as many as 90% of these women (5). This finding suggests that the development of peritoneal endometriosis requires at least two steps. The first step is the direct contact of regurgitated endometrial tissues with the peritoneal surface, which occurs periodically at menstruation in most women of reproductive age. The second step involves the implantation, proliferation, and invasion of endometrial tissue attached to the peritoneum. This second step, which is required for the formation of endometriotic lesions, follows the first step in only a small number of cycling women. In most women, the attached endometrial tissues are cleared from the peritoneum over time. By elucidating the molecular mechanism underlying this second step, it may be possible to establish why peritoneal endometriosis only occurs in a small number of women with retrograde menstruation.

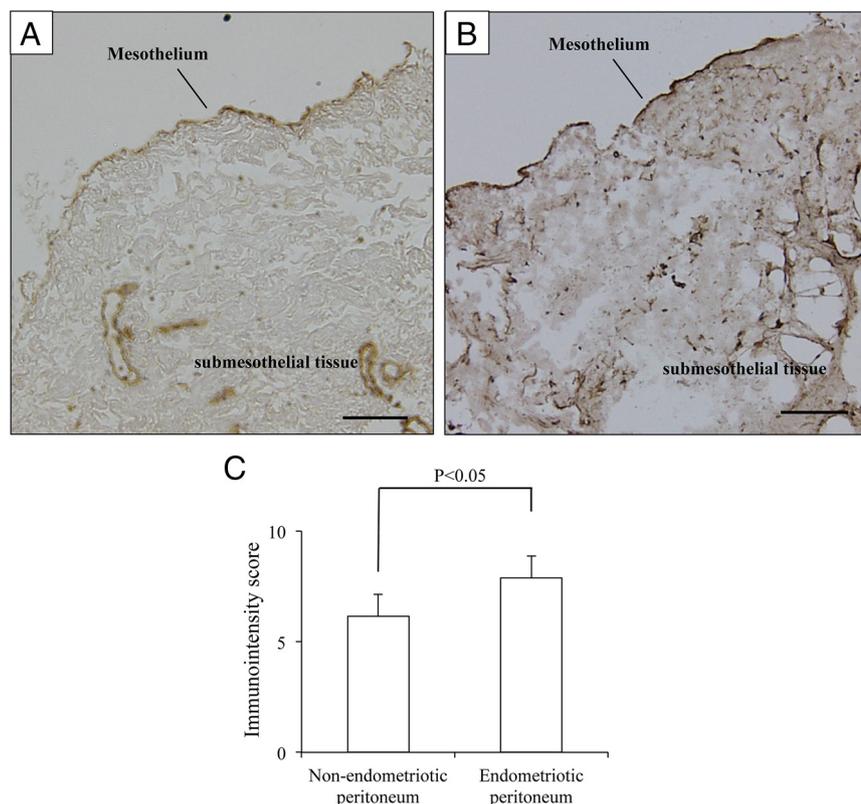
Numerous studies have focused on eutopic endometrial tissues derived from endometriotic women in an attempt to clarify the molecular mechanism responsible for the

second step in the development of peritoneal endometriosis. Most of these studies compared eutopic endometrial tissues between women with and without endometriosis (6, 7). The aberrant expression of cytokines/growth factors, cell adhesion molecules, aromatase activity, angiogenic activity, proteolytic activity, and proapoptotic activity as well as exaggerated immunological tolerance have been demonstrated in eutopic endometrial tissues derived from endometriotic women. In spite of these studies, it currently remains unclear whether these endometrial aberrations contribute to the progression of the second step in the development of peritoneal endometriosis.

In contrast, relatively fewer studies have focused on the pelvic peritoneum, which receives regurgitated endometrial tissues (8). A previous study demonstrated that menstrual effluent elicited epithelial-mesenchymal transition in peritoneal mesothelial cells (9). Another group found that the invasive capacity of endometrial stromal cells (ESCs) was markedly enhanced in the presence

of peritoneal mesothelial cells *in vitro* (10). Furthermore, macroscopically normal peritoneal samples derived from endometriotic women were found to express higher levels of IL-6 and lower levels of ferritin and IL-12 than those derived from nonendometriotic women (11). These findings strongly suggest that not only regurgitated endometrial tissues, but also the pelvic peritoneum play crucial roles in the development of peritoneal endometriosis.

In the present study, we hypothesized that some intrinsic alterations in the molecular characteristics of the peritoneum facilitate the second step of endometriotic development, ie, the implantation, proliferation, and invasion of endometrial tissues regurgitated through the fallopian tubes and attached to the peritoneum. In order to substantiate this hypothesis, we sampled peritoneums from cycling women during benign gynecological surgeries. The peritoneums that were adjacent to the upper end of the abdominal incision or the umbilical port sites and were devoid of any pathological findings including endometriosis were collected. We divided these samples into two groups based on the presence or absence of endometriotic



**Figure 2.** Immunostaining of endometriotic and nonendometriotic peritoneums using the antiversican antibody. (A) A representative photo of a nonendometriotic peritoneum immunostained with the antiversican antibody. Note the ‘weak’ staining and ‘very weak’ staining observed in the mesothelium and submesothelial connective tissue, respectively. An immunointensity score of 2 (weak) was given to the mesothelium and 1 (very weak) to the submesothelial connective tissue (see the Supplementary Materials for details). As a result, the immunointensity score for this nonendometriotic peritoneum was calculated as 3. Original magnification  $\times 100$ . The scale bar indicates  $100\ \mu\text{m}$ . (B) A representative photo of an endometriotic peritoneum immunostained with the antiversican antibody. Note the ‘strong’ staining observed in the mesothelium and submesothelial connective tissue. Immunointensity scores of 4 (strong) were given to both of these layers (see the Supplementary Materials for details). As a result, the immunointensity score for this endometriotic peritoneum was calculated as 8. Original magnification  $\times 100$ . The scale bar indicates  $100\ \mu\text{m}$ . (C) The immunointensity score was significantly higher in endometriotic peritoneums ( $n = 8$ ) than in nonendometriotic peritoneums ( $n = 7$ ). Error bars indicate the standard error.

lesions in the pelvic peritoneum: endometriotic peritoneums and nonendometriotic peritoneums. A microarray analysis identified “versican” as one of the top 50 genes with significantly higher expression levels in endometriotic peritoneums.

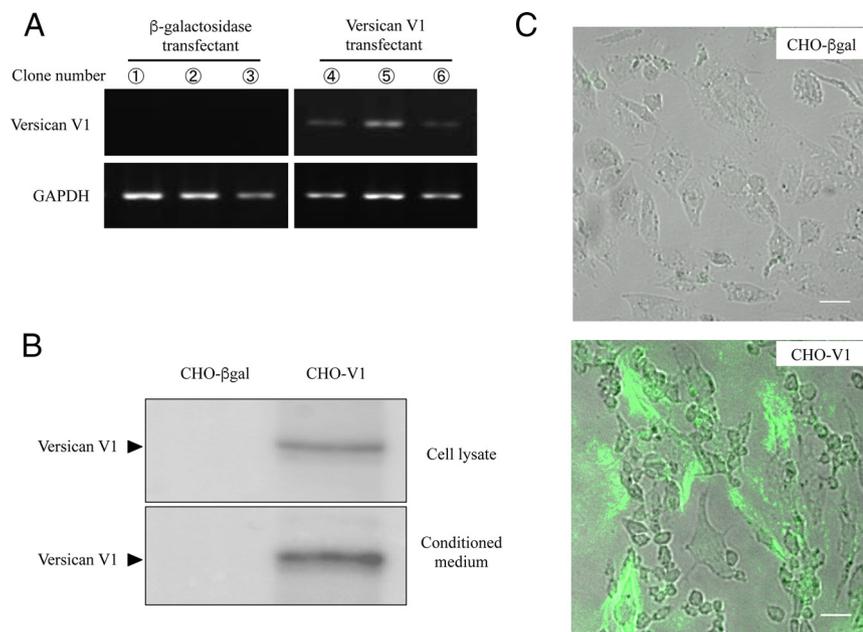
Versican is a large chondroitin sulfate proteoglycan that is found in the extracellular matrix of most soft tissues (12). It exists in at least 4 different isoforms due to alternative splicing of the major exons that encode 2 attachment domains for the chondroitin sulfate side chains designated as glycosaminoglycan (GAG)- $\alpha$  and GAG- $\beta$ . Three of these isoforms (V0, V1, and V2) contain chondroitin sulfate side chains, while the other isoform (V3) does not. In malignancies, elevated versican levels have been associated with a poor prognosis (13). Functional studies have shown that versican, which is mainly secreted

by peritumoral stromal cells, regulates cancer cell attachment, proliferation, and invasion, all of which are also key components of endometriotic development. Versican has also recently been shown to induce inflammation, which invariably coexists with peritoneal endometriosis, by interacting with myeloid/lymphoid cells to promote their adhesion and production of inflammatory cytokines (14). Notably, in the microarray analysis conducted by Aghajanova and Giudice, versican was found to be more strongly expressed in eutopic endometria derived from women with severe endometriosis than in those from women with mild endometriosis (15). These findings led us to speculate that the strong expression of versican in the endometriotic peritoneum may contribute to the development of peritoneal endometriosis. The specific aim of the present study is to investigate the possible roles of versican in peritoneal endometriosis.

## Materials and Methods

A detailed description of the materials and methods used in this study is provided in the Supplementary Materials. Briefly, macroscopically normal peritoneums were collected from cycling women during benign gynecological laparotomy or laparoscopy. Small pieces of the peritoneums ( $\sim 1\ \text{cm}^2$ ) that were adjacent to the upper end of the abdominal incision at laparotomy or the umbilical port site at laparoscopy and were devoid of any pathological findings including endometriosis were sampled. Based on the presence or absence of endometriotic lesions in the pelvic peritoneum, we divided these samples into endometriotic peritoneums and nonendometriotic peritoneums. Total RNA samples extracted from these peritoneums were subjected to a microarray analysis and RT-PCR. Frozen sections and protein lysates prepared from these peritoneums were subjected to immunohistochemistry and Western blotting, respectively.

The plasmid containing the full coding region of human versican V1 was transfected into Chinese hamster ovary cells (CHO-V1) and culture medium was collected as CHO-V1 conditioned medium (V1-CM). The effects of V1-CM on the behavior of primary human endometrial stromal cells (ESCs) were investigated with attachment, proliferation, and Matrigel invasion assays. The effects of peritoneal fluid collected from endometriotic



**Figure 3.** Selection and characterization of  $\beta$ -galactosidase-overexpressing Chinese hamster ovary cells (CHO- $\beta$ gal) and versican V1-overexpressing Chinese hamster ovary cells (CHO-V1). (A) RT-PCR showed that none of the three clones originating from pRc/RSV- $\beta$ gal-transfected CHO cells (clone 1–3) expressed a detectable amount of human versican V1 mRNA (left panel). Clone 2 was arbitrarily selected as ‘CHO- $\beta$ gal’. On the other hand, all three clones that originated from pRc/RSV-hM(V1)-transfected CHO cells (clone 4–6) expressed human versican V1 mRNA (right panel). Clone 5, which exhibited the strongest signal, was selected as ‘CHO-V1’. (B) Western blotting using the antiversion antibody. The expression of versican V1 was detected not only in the cell lysate, but also in conditioned medium derived from CHO-V1 cells. (C) Fluorescent immunocytochemistry using the antiversion antibody. Phase contrast images are overlaid. Although CHO- $\beta$ gal cells yielded no signal (upper panel), an intense fluorescent signal was detected in the extracellular spaces surrounding CHO-V1 cells (lower panel). Original magnification  $\times 100$ . Scale bars indicate  $100\ \mu\text{m}$ .

women (endometriotic PF) or various cytokines/growth factors (IL-1 $\beta$ , IL-6, TNF- $\alpha$ , CCL5, and TGF- $\beta$ 1) on the mRNA expression of versican V1 in HMrSV5 cells (human peritoneal cell line) were also examined. All quantitative experiments were performed more than 3 times. An unpaired *t* test or one-way ANOVA was used to assess the significance of differences. *P*-values of  $< 0.05$  were considered significant. All values are given as the mean with the bar showing the mean  $\pm$  standard error. All statistical analyses were performed using StatMate version 3.14 (ATOMS, Oita, Japan).

The study protocol was approved by the Ethical Committee of Kyoto University Hospital. Informed consent for taking and using endometrial and peritoneal samples was obtained from all donors.

## Results

### Stronger expression of versican in macroscopically normal peritoneums from endometriotic women than in those from nonendometriotic women

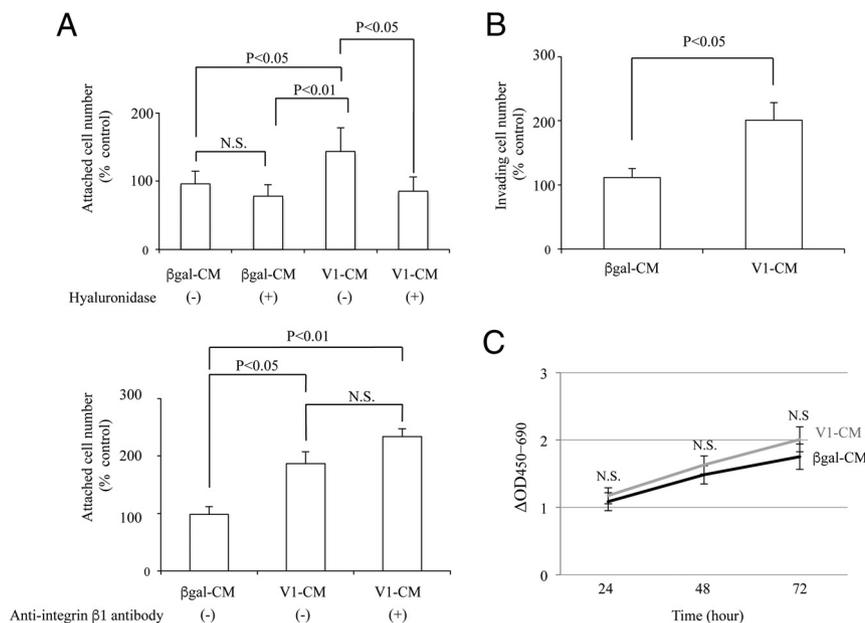
The microarray analysis comparing macroscopically normal peritoneums from women with peritoneal endometriosis (endometriotic peritoneum,  $n = 4$ ) and those from women without peritoneal endometriosis (nonendo-

metriotic peritoneum,  $n = 4$ ) revealed multiple genes that were up-regulated in the endometriotic peritoneum. Versican was identified as one of the top 50 up-regulated genes (Supplemental Table 1). The up-regulation of versican mRNA was validated by RT-PCR using other independent samples of the endometriotic peritoneum ( $n = 6$ ) and nonendometriotic peritoneum ( $n = 9$ ) (Figure 1A). In RT-PCR analyses using isoform-specific primer pairs, the mRNA expression of versican V1, V2, and V3, but not that of versican V0 was confirmed in endometriotic and nonendometriotic peritoneums. The expression of versican V1, V2, and V3 was stronger in endometriotic peritoneums than in nonendometriotic peritoneums (Figure 1B). Western blotting using anti-human versican pAb specific to versican V0 and V1 yielded specific bands at 440 kDa, which corresponded to the size of versican V1. Versican V1 showed a significantly higher signal intensity in endometriotic peritoneums ( $n = 5$ ) than in nonendometriotic peritoneums ( $n = 5$ )

(Figure 1C).

### Immunostaining of versican in macroscopically normal peritoneums

In macroscopically normal peritoneums, immunoreactive versican was detected in the mesothelium and submesothelial connective tissue. Although the expression of versican was moderately and markedly weak in the mesothelium and submesothelial connective tissue, respectively, of nonendometriotic peritoneums (Figure 2A), it was equally strong in both of these layers in endometriotic peritoneums (Figure 2B). As a result, the immunointensity scores from both the mesothelium and submesothelial connective tissue tended to be higher in endometriotic peritoneums than in nonendometriotic peritoneums (Supplemental Figure 1). When immunointensity scores from the mesothelium and submesothelial connective tissue were combined, endometriotic peritoneums ( $n = 8$ ) showed significantly higher immunointensity scores than those of nonendometriotic peritoneums ( $n = 7$ ) (Figure 2C).



**Figure 4.** Versican V1 promotes attachment to the monolayer of HMrSV5 cells (human peritoneal cell line) as well as Matrigel invasion by primary human endometrial stromal cells (ESCs). (A) Effects of conditioned medium derived from CHO-V1 cells (V1-CM) on the attachment of ESCs to the HMrSV5 monolayer. Conditioned medium derived from CHO-βgal cells (βgal-CM) was used as a control. The number of ESCs attached to the HMrSV5 monolayer was significantly larger in the presence of V1-CM than βgal-CM. An additional treatment with hyaluronidase reduced the number of attached ESCs increased by V1-CM (upper panel), while the increased number was not reduced by the treatment with the anti-integrin β1 neutralizing antibody (lower panel). Error bars indicate the standard error. (B) Effects of V1-CM on Matrigel invasion by ESCs. βgal-CM was used as a control. ESCs were allowed to invade a Matrigel-coated membrane filter for 24 hours in the presence of V1-CM or βgal-CM. The number of ESCs that reached the lower filter surface was significantly larger in the presence of V1-CM than βgal-CM. Error bars indicate the standard error. (C) Effects of V1-CM on the proliferation of ESCs. βgal-CM was used as a control. The treatment with V1-CM did not affect the proliferation of ESCs when incubated for up to 72 hours. Error bars indicate the standard error. N.S. stands for 'not significant'.

### Versican V1 expression in pRc/RSV-hM(V1)-transfected CHO cells

In the RT-PCR analysis, none of the three clones originating from pRc/RSV-βgal-transfected CHO cells (clones 1–3), the β-galactosidase activities of which were verified by X-gal staining (Supplemental Figure 2), expressed detectable amounts of human versican V1 mRNA (Figure 3A, left panel). Clone 2 was arbitrarily selected and designated as CHO-βgal. On the other hand, all three clones originating from pRc/RSV-hM(V1)-transfected CHO cells (clones 4–6) expressed human versican V1 mRNA (Figure 3A, right panel). Among them, clone 5 exhibited the strongest expression and was designated as CHO-V1.

Western blotting using antihuman versican pAb demonstrated that the cell lysate and conditioned medium derived from CHO-V1 cells (V1-CM) yielded a specific band at 440 kDa corresponding to the size of the versican V1 protein (Figure 3B). Immunocytochemistry using antihuman versican pAb produced an intense fluorescent signal in the extracellular space around CHO-V1 (Figure 3C).

### Versican V1 promotes attachment to the HMrSV5 cell monolayer and Matrigel invasion by ESCs

In order to evaluate the possible effects of versican V1 on the behavior of ESCs, we utilized conditioned medium derived from the CHO-V1 culture (V1-CM). Conditioned medium derived from the CHO-βgal culture (βgal-CM) was used as the control. The number of ESCs that had attached to the HMrSV5 cell monolayer was significantly higher in the presence of V1-CM than βgal-CM (Figure 4A). The number of attached ESCs that had been increased by V1-CM was reduced by the additional treatment with hyaluronidase (Figure 4A, upper panel), but not with the anti-integrin β1 neutralizing antibody (Figure 4A, lower panel). Similarly, the number of ESCs that had invaded Matrigel was significantly higher in the presence of V1-CM than βgal-CM (Figure 4B). On the other hand, the proliferation of ESCs was not affected by the V1-CM treatment (Figure 4C).

### The TGF-β1 treatment up-regulates versican V1 expression in HMrSV5 cells

The effects of IL-1β, IL-6, TNF-α, CCL5, and TGF-β1 on versican V1 expression in HMrSV5 cells were examined. Among these cytokines/growth factors, only TGF-β1 significantly increased the mRNA expression level of versican V1 in HMrSV5 cells (Figure 5A-E).

### Endometriotic PF does not affect versican V1 expression in HMrSV5 cells

The treatment with endometriotic PF did not alter the mRNA expression level of versican V1 in HMrSV5 cells from that in the PBS-treated control (Figure 6A). The bioactivity of endometriotic PF was verified by the treatment with the same endometriotic PF significantly up-regulating the mRNA expression of IL-6 and IL-8 (Figure 6B).

### Discussion

We herein demonstrated that versican is more strongly expressed in macroscopically normal peritoneums derived

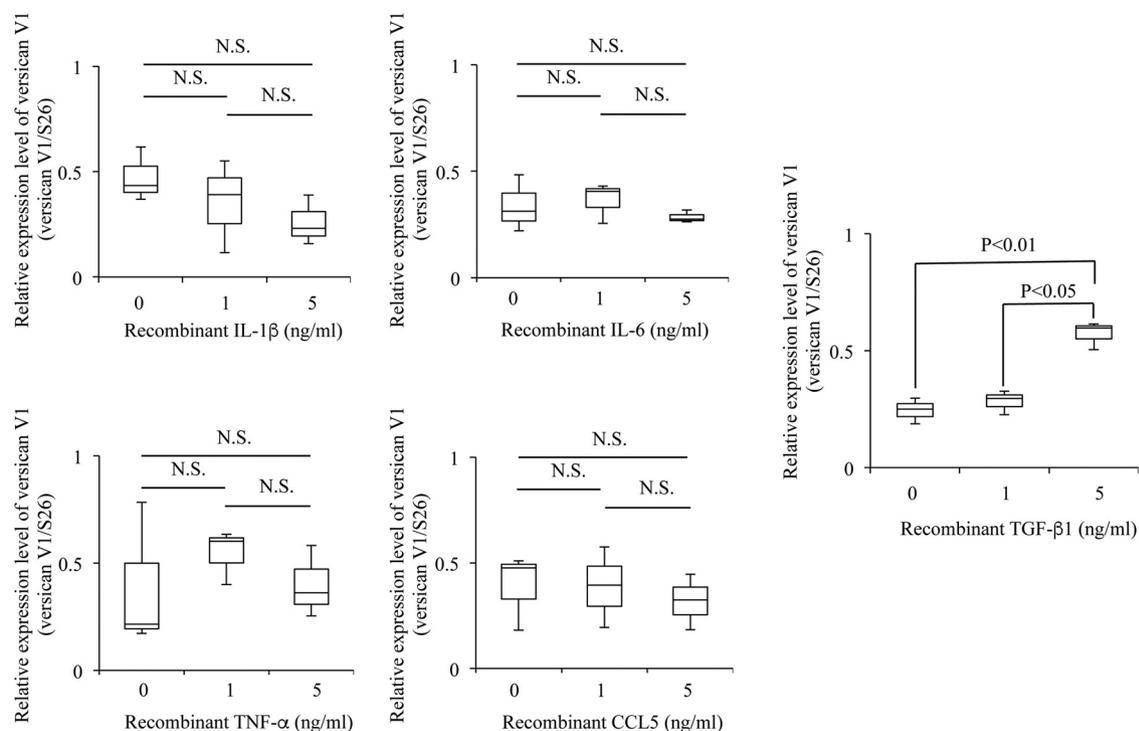
from endometriotic women (endometriotic peritoneums) than in those from nonendometriotic women (nonendometriotic peritoneums). Versican exists in at least 4 different isoforms due to the alternative splicing of exons that encode the glycosaminoglycan attachment domains (12). In RT-PCR using isoform-specific primers, the mRNA expression of versican V1, V2, and V3 was up-regulated in endometriotic peritoneums, whereas that of versican V0 was undetectable irrespective of the presence or absence of endometriosis. Western blotting using the antibody specific to versican V0 and V1 showed that the versican V1 protein was significantly up-regulated in endometriotic peritoneums.

Immunohistochemistry using the antibody specific to versican V0 and V1 revealed that the protein expression of versican, most likely versican V1, was detectable in the mesothelium and submesothelial connective tissue. The up-regulation of versican V1 protein expression in endometriotic peritoneums was observed in the mesothelium and submesothelial connective tissue. In most of the macroscopically normal peritoneal samples examined, versican V1 expression in the mesothelium was stronger in endometriotic peritoneums than in nonendometriotic peritoneums. Although intense versican V1 expression was observed diffusely in submesothelial connective tissue from endometriotic peritoneums, it was confined to blood vessels in submesothelial connective tissue from nonendometriotic peritoneums. These results suggest that me-

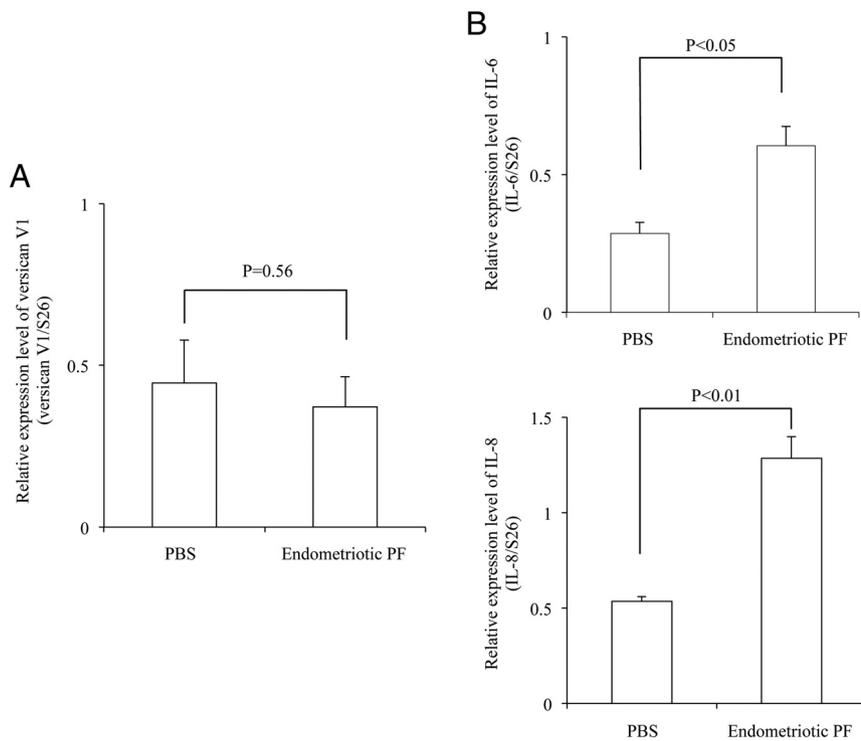
sothelial cells and the cells contained in submesothelial connective tissue such as fibroblasts and endothelial cells have the ability to produce versican V1. Consistent with this result, the secretion of versican was previously demonstrated in epithelial- and fibroblast-like mesothelioma cell lines (16). Collectively, our results suggest that peritoneal cells including the mesothelial cells and submesothelial fibroblasts of endometriotic women have a greater capacity to produce versican V1 than those of nonendometriotic women.

In order to investigate the possible roles of versican in peritoneal endometriosis, we used the normal human peritoneal cell line HMrSV5, which expresses endogenous versican at negligible levels (Supplemental Figure 3). We constructed versican V1-overexpressing CHO cells (CHO-V1) and utilized their conditioned medium (V1-CM). In the presence of V1-CM, the number of ESCs that had attached to the HMrSV5 cell monolayer was significantly increased. Moreover, V1-CM increased the invasion of ESCs through Matrigel. These results suggest that peritoneal versican acts to promote implantation to the mesothelium as well as subsequent invasion through the basement membrane beneath the mesothelium by regurgitated endometrial cells, both of which are crucial components of the second step in the development of peritoneal endometriosis.

The development of endometriosis shares many common features with the establishment of peritoneal dissem-



**Figure 5.** Effects of IL-1 $\beta$ , IL-6, TNF- $\alpha$ , CCL5, and TGF- $\beta$ 1 on versican V1 expression in HMrSV5 cells (human peritoneal cell line). Note that only the treatment with TGF- $\beta$ 1 significantly up-regulated versican V1 expression in HMrSV5 cells (rightmost panel). Error bars indicate the standard error. N.S. stands for 'not significant'.



**Figure 6.** Peritoneal fluid derived from endometriotic women (endometriotic PF) does not affect versican V1 expression in HMrSV5 cells (human peritoneal cell line). (A) Effects of endometriotic PF on the mRNA expression of versican V1 in HMrSV5 cells. Versican V1 expression in endometriotic PF-treated HMrSV5 cells was not significantly different from that in the PBS-treated control. Error bars indicate the standard error. (B) Effects of endometriotic PF on the mRNA expression of IL-6 (upper panel) and IL-8 (lower panel) in HMrSV5 cells. Endometriotic PF-treated HMrSV5 cells expressed higher levels of IL-6 and IL-8 than the PBS-treated control. Error bars indicate the standard error.

ination by cancer cells, ie, cell attachment and implantation to the peritoneal surface followed by proliferation and invasion through the basement membrane beneath the mesothelium. The importance of versican in the peritoneal dissemination of ovarian cancer cells was proposed by Ween et al (17). They found that versican induced the formation of a hyaluronan-rich pericellular matrix as well as the invasion of ovarian cancer cells. These effects of versican required the presence of hyaluronan and its receptor, CD44. Accordingly, they concluded that the formation of a hyaluronan/versican pericellular matrix may contribute to the strong adhesion of ovarian cancer cells to peritoneal cells, which is mediated by CD44, thereby providing the basis for subsequent ovarian cancer cell invasion. In the present study, we confirmed that HMrSV5 cells and ESCs both intensely expressed CD44 (Supplemental Figure 4). In addition, the treatment with hyaluronidase reduced the peritoneal attachment of ESCs that had been enhanced by versican. Collectively, our results suggest that versican-induced ESC attachment to the peritoneal cell monolayer is mediated by hyaluronan, and possibly by CD44.

The C-terminal domain of versican was previously reported to interact with integrin  $\beta 1$  (18). The interaction of

integrin  $\beta 1$  with versican activates focal adhesion kinase and promotes cell adhesion. Integrin  $\beta 1$  is known to be expressed on peritoneal mesothelial cells (19) and endometrial cells (20). We herein confirmed that HMrSV5 cells and ESCs intensely expressed integrin  $\beta 1$  (Supplemental Figure 4). However, the addition of the anti-integrin  $\beta 1$  neutralizing antibody did not reduce the peritoneal attachment of ESCs that had been enhanced by versican. Collectively, these results suggest that enhanced ESC attachment to peritoneal cells is mediated by the hyaluronan-CD44 complex, but not by integrin  $\beta 1$ .

Demir et al demonstrated that an incubation with the conditioned media of endometrial cells that had been isolated from menstrual effluent promoted the epithelial-to-mesenchymal transformation of peritoneal cells (9). More recently, Lessey et al reported the presence of endometrial cells in macroscopically normal peritoneums, and the attached endometrial cells had the ability to disrupt the integrity of the surrounding me-

sothelial lining, thereby exposing the underlying extracellular matrix (21). These findings strongly suggest that even macroscopically normal peritoneums in endometriotic women have already undergone certain phenotypic changes induced by regurgitated endometrial cells. We found that TGF- $\beta 1$ , which is produced by cultured endometrial cells (22), significantly increased versican expression in human peritoneal cells. Thus, the up-regulation of versican in endometriotic peritoneums may have merely resulted from the preceding effects of regurgitated endometrial cells on peritoneal cells. In the present study, we collected samples of macroscopically normal peritoneums from the upper region of the anterior abdominal wall, which was remote from the orifice of fallopian tubes or from endometriotic lesions in the pelvic peritoneum. Therefore, it appears unlikely that the endometriotic peritoneums used in this study had already been affected by regurgitated endometrial cells at the time of sampling.

Peritoneal fluid is often present in women with peritoneal endometriosis. A number of cytokines/growth factors are elevated in the peritoneal fluid derived from endometriotic women (endometriotic PF), including IL-1, IL-6, IL-8, IL-10, TNF- $\alpha$ , and VEGF (23). In women with peri-

toneal endometriosis, even macroscopically normal peritoneums may be exposed to endometriotic PF. Therefore, some constituents of endometriotic PF may have induced phenotypic changes in the macroscopically normal peritoneums sampled from endometriotic women. Treatments with cytokines contained in endometriotic PF such as IL-1 $\beta$  and TNF- $\alpha$  have been shown to up-regulate the expression of IL-6 and IL-8 in mesothelial cells (24, 25). In the present study, the treatment with endometriotic PF failed to alter versican V1 expression in HMrSV5 cells, whereas IL-6 and IL-8 were significantly up-regulated by the treatment with the same endometriotic PF. These results imply that the up-regulation of versican in the endometriotic peritoneum is not a secondary event to the presence of peritoneal fluid or to the influence of regurgitated endometrial cells.

In summary, we herein demonstrated that macroscopically normal peritoneums derived from women with peritoneal endometriosis expressed versican V1 more strongly than those derived from women without peritoneal endometriosis. An in vitro study using primary ESCs and a normal peritoneal cell line showed that versican V1 enhanced peritoneal attachment and Matrigel invasion by ESCs. These results suggest that peritoneal versican is one of the key factors involved in the development of peritoneal endometriosis.

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Address all correspondence and requests for reprints to: Yukiyasu Sato, M.D., Ph.D., Department of Obstetrics and Gynecology, Otsu Red Cross Hospital, 1-1-35 Nagara, Otsu 520-8511, Shiga, Japan, Tel.: 81-77-522-4131, Fax: 81-77-525-8018, E-mail: yukiyasu@kuhp.kyoto-u.ac.jp.

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