Histol Histopathol (2012) 27: 1495-1502
DOI: 10.14670/HH-27.1495

http://www.hh.um.es

Histology and Histopathology
Cellular and Molecular Biology

Expression of matricellular proteins in human uterine leiomyomas and normal myometrium

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Summary. Growth of human leiomyomas can probably be initiated as a response to injury, in a way similar to the development of keloids. Among many bioactive molecules, which are implicated in tissue repair, a pivotal role is attributed to matricellular proteins. The aim of the current study was to evaluate the immunohistochemical expression of tenascin-C (TNC), thrombospondin-1 (TSP-1), SPARC/osteonectin and tenascin-X (TNX) in human uterine leiomyomas and normal myometrium. Immunostaining was performed on 33 pairs of paraffin-fixed sections and 9 cell-lines derived from uterine leiomyomas and normal myometrium. Fifteen (45.5%) leiomyomas investigated were positive for TNC, whereas all normal myometrial samples were immunonegative (χ^2=19.41; p<0.001). Immunostaining for TSP-1 was observed in 20 (60.6%) uterine fibroids and in 12 (36.4%) control samples (χ^2=3.88; p<0.05). The expression of SPARC/osteonectin protein was more frequently found in leiomyomas than in normal myometrium, but this difference was not significant. Apart from one fibroid culture and one myometrial culture, all the others revealed strong TNC immunostaining. Expression of TSP-1 and SPARC/osteonectin was weak to moderate in all established cell-lines. None of the tissues or cell lines investigated showed positive staining for TNX. In conclusion, TSP-1 and TNC are likely to play important roles in the pathogenesis of uterine leiomyomas, presumably affecting cell proliferation and/or extracellular matrix deposition.

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Key words: Uterine leiomyoma, Matricellular proteins, Thrombospondin-1, Tenascin-C

Introduction

Uterine leiomyomas are the most common tumors in women, being the most prevalent reasons for surgical intervention within the female genital tract (Ioffe et al., 2005). Despite their high incidence and the fact that they pose a significant health problem, the etiology and biology of uterine leiomyomas remain poorly understood (Zaloudek and Hendrickson, 2002; Ioffe et al., 2005). Several theories have been advanced to clarify the pathogenesis of uterine leiomyomas, most of them underlining the crucial role of sex steroids (Zaloudek and Hendrickson, 2002; Flake et al., 2003; Blake, 2007; Fleischer et al., 2008; Okolo, 2008). However, one interesting hypothesis proposes that growth of these neoplasms may be initiated as a response to injury, in a way similar to the development of human keloids (Leppert et al., 2006). According to this hypothesis, ischemia related to the release of vaso-constrictive substances during menses may potentially harm the myometrial cells. Abnormal response to the injury may, in turn, result in disordered healing and formation of altered extracellular matrix, leading to growth of leiomyomas (Flake et al., 2003).

Among many bioactive molecules, which are implicated in tissue repair, a pivotal role is attributed to matricellular proteins (Bornstein and Sage, 2002). These are composed of a group of structurally unrelated proteins, which are associated with cell-surface receptors, extracellular matrix (ECM) and growth factors, modulating cell-cell and cell-matrix interactions

(Midwood et al., 2004; Chiodoni et al., 2010). Matricellular proteins are abundantly expressed during embryogenesis, whereas in post-natal life they are up-regulated almost exclusively under pathological conditions in processes associated with enhanced ECM remodeling, such as inflammation, tissue renewal, wound healing and/or human tumorigenesis (Bornstein and Sage, 2002; Midwood et al., 2004; Chiodoni et al., 2010; Llera et al., 2010; Matsui et al., 2010). Interestingly, Howard and co-investigators (2010) suggested that matricellular protein SPARC/osteonectin level declines with patients' age, being probably implicated in the pathogenesis of age-related macular degeneration.

The aim of the current research was to assess the expression of matricellular proteins - thrombospondin-1 (TSP-1), tenascin-C (TNC), tenascin-X (TNX) and SPARC/osteonectin in tissues and cell-lines derived from human uterine leiomyomas and normal myometrium. Additionally, we compare the expression pattern of matricellular proteins in uterine fibroids with immunoreactivity of these proteins found in normal myometrial slides.

Materials and methods

Specimens of uterine leiomyomas and normal myometrium were obtained from 33 peri-menopausal patients (mean age 47 years, ranged between 43–53 years) who had undergone hysterectomy at the IInd Department of Gynecology Medical University of, Lublin, Lublin, Poland. All women were Caucasians and had not received hormonal treatment for at least 3 months prior to surgery. Indication for the surgery were heavy and prolonged menstrual bleedings, caused by multiple fibroids. Additionally, 5 patients reported dysmenorhoea and pelvic pain. Patients were subjected to total or subtotal hysterectomies within 12 day from the first day of the menstrual cycle. In order to obtain comparable samples for the study we decided to sample fibroids of similar size and localization (intramural ones sized between 1 and 1.5 cm). Since uterine specimens contained multiple leiomyomas of various localization, shortly after removal they were cut and suitable fibroids identified and sampled. None of the tumors showed signs of degenerative changes. Surrounding normal myometrial tissue served as a control. Nine pairs of uterine fibroids and control myometrial tissues were used to establish cell cultures. The study was approved by the Ethical Committee of Medical University of Lublin, Lublin, Poland.

Tissue specimens were immediately fixed in 10% buffered formalin for 48 hours and embedded in paraffin. Paraffin-embedded sections were cut at 4 μm and placed on positively-charged microscope slides (Superfrost© Plus, Fisher Scientific, Ottawa, Canada). Immunohistochemical staining was performed by the avidin-biotin-peroxidase complex technique using VECTASTAIN® Elite ABC Kit (Vector Laboratories, Burlingame, CO, USA). Antigen retrieval was performed by boiling the slides in 10 mM citrate buffer (pH 6.0) three times at 750 W for 5 minutes in a microwave oven (Hayat, 2002; Semczuk et al., 2005). The slides were exposed to 10% non-immunized serum in PBS for 10 minutes, and then sections were incubated overnight at 4°C with primary antibodies.

The primary antibodies (diluted 1:100) were as follows: thrombospondin-1 (clone A6.1), tenascin-C (clone B324.1), tenascin-X (clone H-90) and SPARC/osteonectin (clone PP16) (all were purchased from Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA).

Known positive controls were included in each experiment. Control tissue were as follows: human placenta for TSP-1, human tonsil for TNC, human skin for TNX and SPARC/osteonectin. As a negative control, the same procedure was applied, but omitting the primary antibody. Samples of positive controls and negative specimens of leiomyoma and myometrium are shown in Figure 1. The immunoreactivity was judged independently by two investigators (M.B. and A.S.) who reached full agreement in 85% of the slides analyzed. Tissue slides were classified as positive or negative, regardless of the intensity of staining. To ensure the reproducibility of the data all slides showing negative results were processed twice.

For cell cultures, tissues were collected in PBS, cut into small pieces and then transferred to cell culture flasks. Cultures were maintained in DMEM without phenol red (Sigma, St. Louis, MO) supplemented with 10% FBS (Sigma, St. Louis, MO, USA), penicillin (100 U/mL) (Sigma, St. Louis, MO, USA) and streptomycin (100 μg/mL) (Sigma, St. Louis, MO, USA) at 37°C in a humidified atmosphere of 95% air and 5% CO_2. Cell monolayer cultures were trypsinized and frozen in liquid nitrogen. Cells were then plated on Lab-tek chamber slides (Nunc, Rochester, NY, USA) at a density of $1x10^5$ cells/mL and grown for 48 hours under standard conditions. They were then fixed with cold 4% para-formaldehyde for 15 min and placed in 75% ice-cold ethanol for 24 hours at 4°C. The Lab-tek slides were then washed with PBS and staining procedure was performed using the same primary antibodies and detection system as previously reported. The intensity of staining was classified as strong, moderate or weak.

Statistical analysis was performed applying the Statistica Statsoft vs 8 software (Statsoft Inc,. Tulsa, Okla, USA) using χ^2 test. p value <0.05 was considered statistically significant.

Results

Immunohistochemical staining for TNC, TSP-1 and SPARC/osteonectin was seen in the cytoplasm, cellular membranes and extracellular matrix (Figs. 1-4). The staining was focal and confined to three areas in the section. Immunostaining for TSP-1 was observed in 20 (60.6%) out of 33 uterine fibroids and in 12 (36.4%) out

of 33 control samples (χ^2=3.88; p<0.05) (Fig. 2). Fifteen (45.5%) out of 33 uterine leiomyomas investigated were positive for TNC (Fig. 3), whereas all normal myometrial samples were immunonegative (χ^2=19.41; p<0.001). The expression of SPARC/osteonectin was found more frequently in uterine leiomyomas than in the normal myometrium (18 and 11 samples, respectively; Fig. 4), but this difference was not significant. None of the samples investigated revealed positive TNX immunoreactivity.

In cultured cell-lines, positive signals for TSP-1, TNC and SPARC/osteonectin were detected in the cytoplasm and/or cellular membranes (Fig. 5). Apart from one fibroid culture and one myometrial culture, all the others revealed strong TNC immunoreactivity. Expression of TSP-1 and SPARC/osteonectin was weak to moderate in all established cell-lines. All cell-lines investigated were negative for TNX.

Discussion

Uterine leiomyomas are benign neoplasms composed of interlacing bundles of spindle-shaped or stellate cells which, as normal myometrial cells, display features consistent with smooth muscle cells (Kobayashi et al., 1996; Fleischer et al., 2008). Cytogenetic studies provide clear evidence that uterine fibroids develop monoclonally from one single smooth muscle cell (Mashal et al., 1994; Quade, 1995; Ligon and Morton, 2000). Leiomyomas may increase in size during hormonal (estrogen) therapy, whereas they may decrease in size during GnRh agonist treatment (Cook and Walker 2004; Lethaby and Vollenhoven 2008). It is worth mentioning that the growth of these tumors is associated with low mitotic activity (less than 5/10 MF) and accumulation of ECM comprising up to half of tumor masses (Zaloudek and Hendrickson, 2002; Flake et al.,

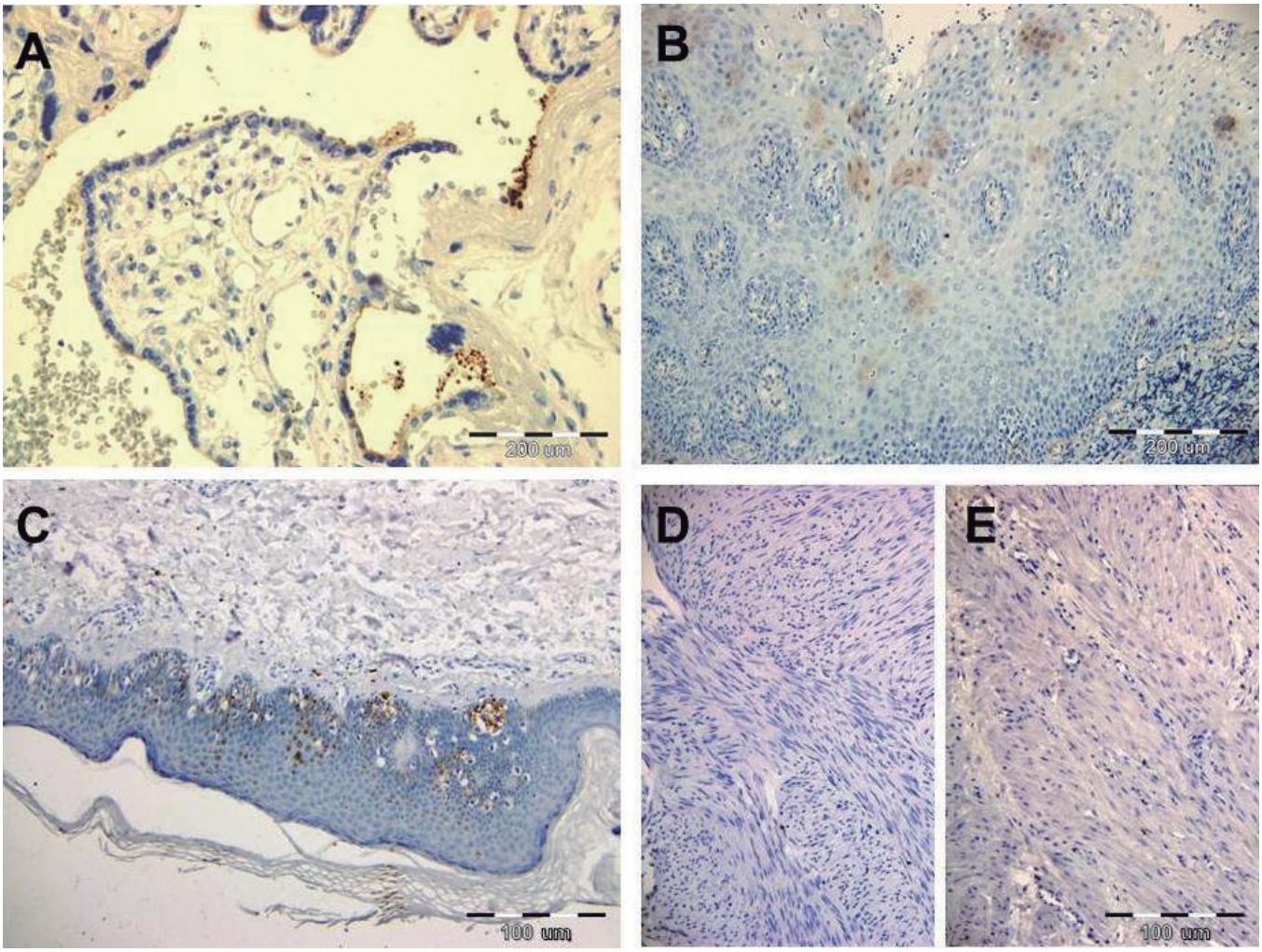

Fig. 1. Positive immunostaining for thrombospondin-1 in human placenta **(A)**, for tenacin-C in human tonsil **(B)**, for SPARC/osteonectin in human skin **(C)**. Samples of immunonegative leiomyoma **(D)** and myometrium **(E)**.

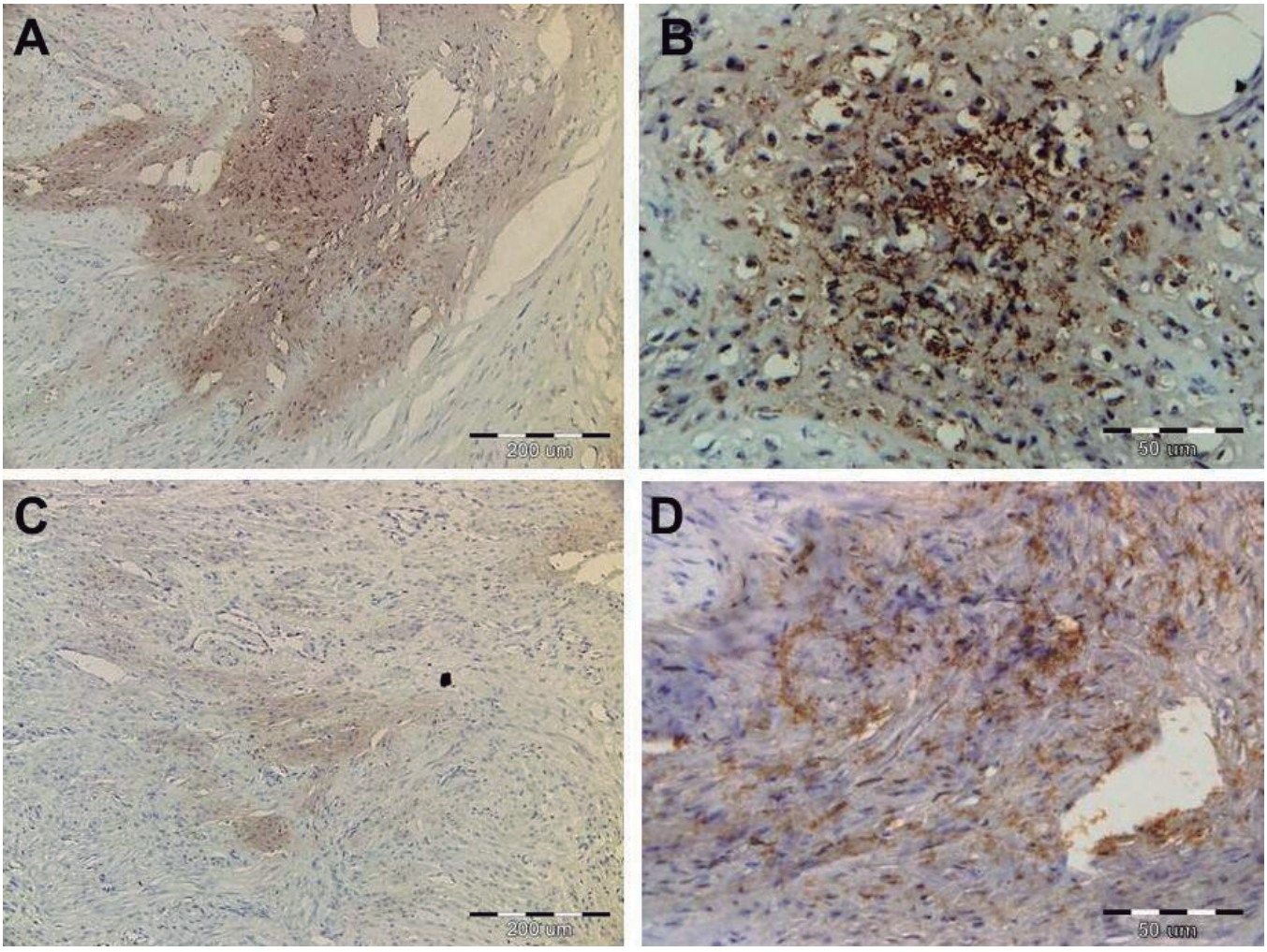

Fig. 2. Expression of thrombospondin-1 in the leiomyoma **(A, B)** and normal myometrium **(C, D)**.

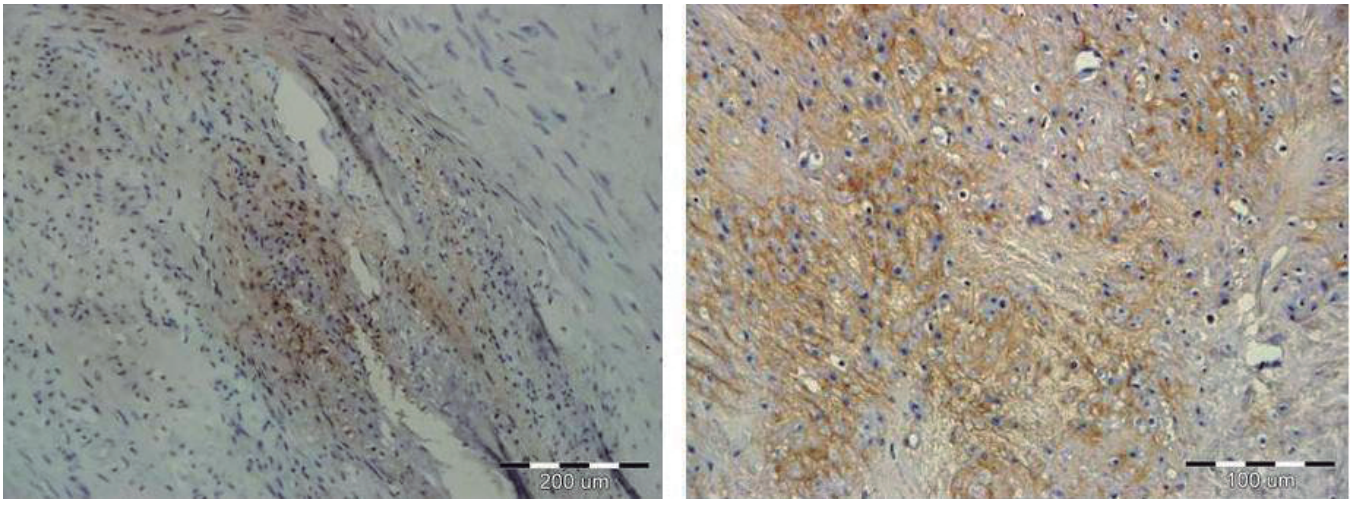

Fig. 3. Expression of tenascin-C in the uterine fibroids. All investigated myometrial samples were negative for tenscin-C.

2003; Ioffe et al., 2005). A hypothesis proposed by Leppert and co-workers (2006) suggests that fibroid cells may originate from normal myometrial cells that undergo altered growth in response to disordered extracellular signals. This is accompanied by changes in phenotype, leading to a transition of smooth muscle myometrial cells into myofibroblasts. Myofibroblasts secrete components of ECM and their abnormal function may cause fibrosis. According to this hypothesis, the growth of uterine leiomyomas seems to be similar to abnormal wound healing resulting in the formation of keloids.

In the current study, we investigated the expression of matricellular proteins in uterine fibroids and normal myometrial samples collected from patients operated on for benign uterine disorders. Our results revealed that two of the proteins investigated (thrombospondin-1 and tenascin C) are significantly overexpressed in uterine fibroids compared to the normal myometrial slides. Surprisingly, the pattern of TSP-1 expression differs from data published by Behera and co-investigators (2007), who showed that this protein is under-expressed in uterine fibroids. Differences of these results may be due to uterine fibroid sizes selected for investigation.

For this study we selected only intramural leiomyomas sized from 1 to 1.5 cm, considering that such small fibroids are less prone to hypoxia or degeneration than ones of larger size or from other locations. It is also believed that small myomas represent tumors in the early growth stage, although due to inaccuracy of measurement of small fibroids by imaging techniques this assumption cannot be proven (Peddada et al., 2008). The growth of fibroids larger than 1.5 cm seems unpredictable and independent of size and location (Peddada et al., 2008). Moreover, there are differences among tumors from the same woman (Peddada et al., 2008; Baird et al., 2011), although myomas larger than 5 cm increase their size faster than

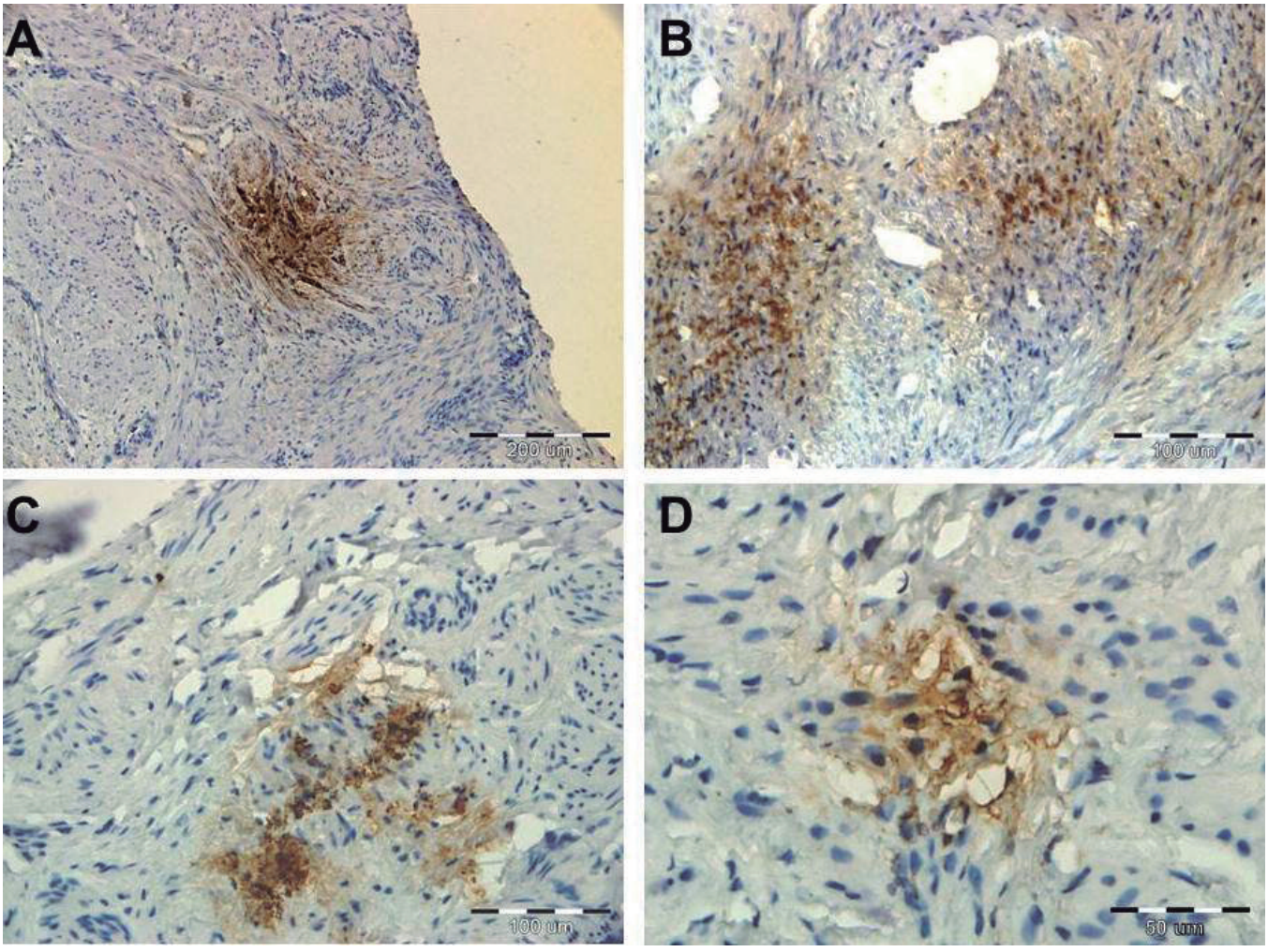

Fig. 4. Expression of SPARC/osteonectin in the leiomyoma and normal myometrium.

smaller ones (Baird et al., 2011). Overall, the findings of our study suggest that matricellular proteins are expressed in the initial stages of fibroid development and need further investigation.

Typically, expression of TSP-1 is higher in proliferating cells than in quiescent ones, and tumor cell-lines exhibit lower expression compared to normal or benign cell-lines (Kapiteijn et al., 2001; Sargiannidou et al., 2001). Its expression is also lower in metastatic cell-lines compared to cell-lines shearing a low-metastatic potential (Kapiteijn et al., 2001; Campbell et al., 2010). These observations suggest TSP-1 expression is inversely related to tumor aggressiveness, probably due to suppression of angiogenesis (Campbell et al., 2010). In line with widespread pattern, TSP-1 was stronger in uterine leiomyomas than in smooth muscle tumors of uncertain malignant potential and leiomyosarcomas (Bodner-Adler et al., 2006). Interestingly, immunostaining for TSP-1 was higher in uterine fibroids than in normal myometrial samples reported herein.

The role of TSP-1 in fibroid biology may be associated with inactivation of TGF-ß, inhibition of angiogenesis and/or de-adhesions. TSP-1 is a major activator of TGF-ß *in vivo* (Crawford et al., 1998). In turn, TGF-ß has been shown to play an important role in the growth of uterine leiomyomas (Ciarmela et al., 2011). Both TGF-ß and the components of its receptor signaling pathway are over-expressed in uterine fibroids as compared with normal myometrial samples (Fleischer et al., 2008; Okolo, 2008). Increased TGF-ß signaling promotes growth and migration of cells, production and deposition of ECM, and modifies the expression of proteases and their activity (Branton and Kopp, 1999). All of these processes leading to excessive fibrosis are

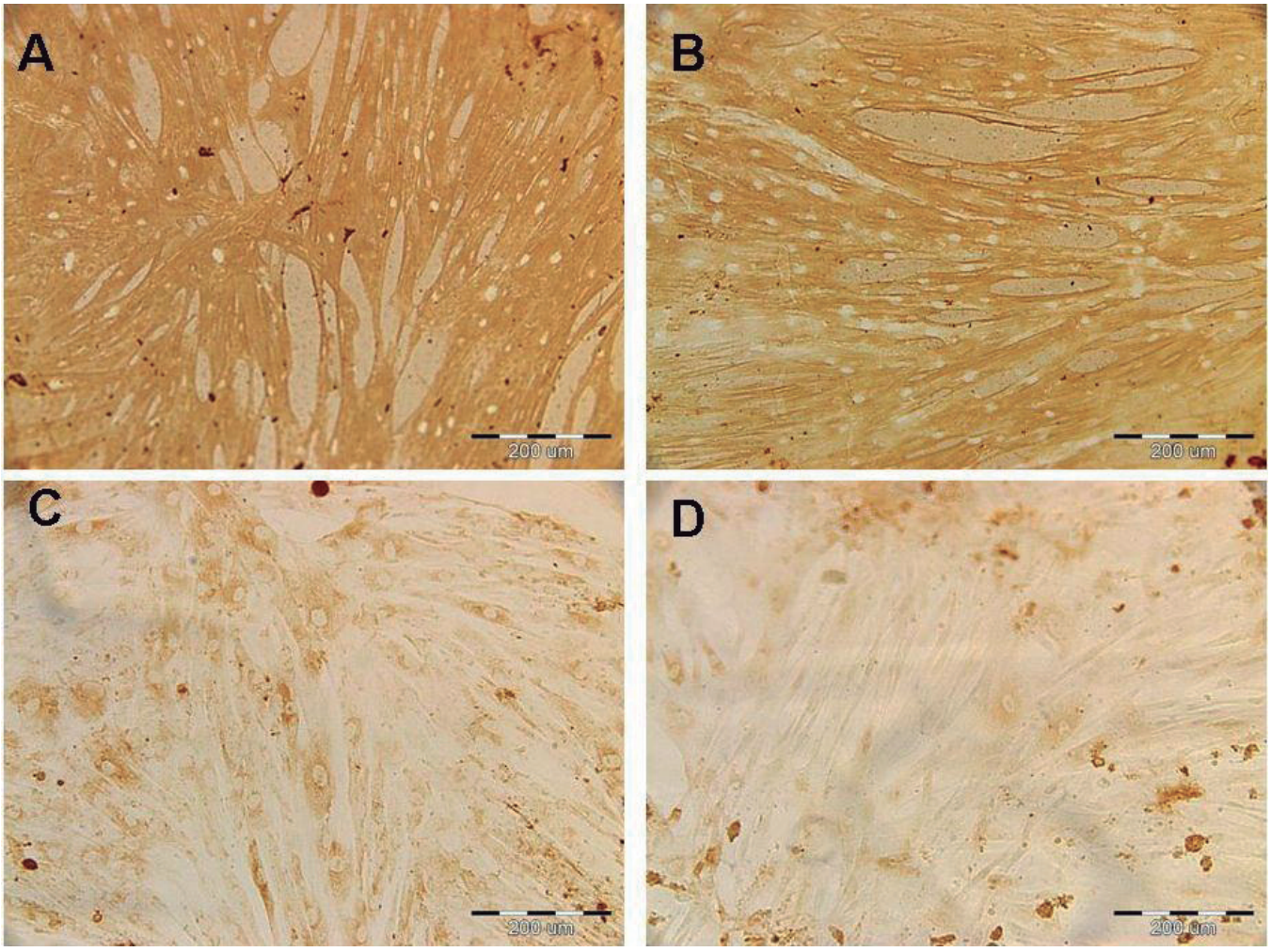

Fig. 5. Extracellular protein immunoreactivity in the cultured cells derived from uterine fibroids and normal myometrium. Expression of tenascin-C in the cultured leiomyoma cells **(A)** and normal myometrial **(B)** cells. Expression of thrombospondin-1 **(C)** and SPARC/osteonectin **(D)** in the cultured leiomyoma cells.

believed to stimulate growth of uterine leiomyomas (Branton and Kopp, 1999; Fleischer et al., 2008; Okolo, 2008).

Enhanced angiogenesis is a prerequisite for growth of neoplastic tissue (Carmeliet and Jain, 2000; Mazurek and Kuc, 2005). However, in contrast to most malignant tumors, small uterine leiomyomas are less vascularized compared to the surrounding normal myometrium. Vascular density, as well as vascular diameter, is smaller in small uterine fibroids than in the myometrium (Casey et al., 2000). Moreover, a lack of well-developed muscularized vessels and no evidence of spiraling have been reported in small fibroids, suggesting faulty vascular maturation (Aitken et al., 2006). It is well established that TSP-1 acts as a potent inhibitor of angiogenesis (Campbell et al., 2010). Therefore its stronger expression in uterine fibroids in comparison with normal myometrium may reflect the inhibition of tumor angiogenesis.

De-adhesion is a term that describes the reversal of the adhesive processes in which a cell moves from a state of a stronger adherence to a state of weaker adherence. This is observed during several processes in humans, including morphogenesis, wound healing, cellular metaplasia, cell proliferation and the development of metastases (Murphy-Ullrich, 2001). During de-adhesion a cell undergoes a transition from strong adherence to intermediate adherence, which is associated with the reorganization of actin stress fibers and disassembly of focal adhesion with minimal effects on cell shape (Murphy-Ullrich, 2001; Makrilia et al., 2009). TSP-1, TNC and SPARC/osteonectin have been shown to induce an intermediate adhesive state (Makrilia et al., 2009). Interestingly, cultured fibroma and myometrial cells show a distinctive assembly of actin fibers. In myometrial cells, positive staining for α-smooth muscle actin was found in filaments running parallel to the major axis of the cell, whereas in cells derived from leiomyomas filaments often overlapped and crossed intricately. Therefore, one might speculate that de-adhesion of myometrial smooth muscle cells, mediated by TSP-1 and TNC, may play an important role in initiation of uterine leiomyoma growth.

TNC participates in many processes associated with enhanced matrix remodeling (Imanaka-Yoshida et al., 2004). It is overexpressed in the majority of malignant solid tumors, participating in tumor growth, angiogenesis and immunosupression (Orend, 2005; Orend and Chiquet-Ehrismann, 2006; Midwood and Orend, 2009). Our observations of abundant expression of TNC protein in cell-lines derived both from uterine fibroids and normal myometrial cells suggest that this protein is likely to be associated with proliferation of fibroid and myometrial cells. Moreover, as TNC mediates fibrotic processes, it may also play a role in ECM deposition within uterine leiomyomas. TNC regulates deposition of fibronectin (Midwood et al., 2004) and its expression by cultured cells is up-regulated by TGF-ß1 (Tucker and Chiquet-Ehrismann, 2009). Furthermore, the essential role of TNC in fibrotic processes is supported by the observation that mice lacking TNC are protected from pulmonary and liver fibrosis (El-Karef et al., 2007; Carey et al., 2010).

In conclusion, our study showed that thrombospondin-1 and tenascin C were expressed in uterine leiomymas as compared with normal myometrium, thus being likely to mediate the growth of these tumors. Expression of SPARC/osteonectin does not differ significantly between uterine fibroids and normal myometrium, while tenascin X is expressed neither by leiomyomas nor by the myometrium.

Acknowledgements. The study was supported by grant MNiSW Nr NN407289533 (M.B).

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Accepted June 18, 2012