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Progesterone induction of chondroitin sulfate proteoglycan aggrecan expression in human endometrial epithelial cells

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ABSTRACT

Chondroitin sulfate (CS) is the most abundant glycosaminoglycan species in the human endometrium, but the expression profile of CS proteoglycans (PGs) in this mucosal tissue remains fully undetermined. In this study, we aimed to clarify the expression of CSPGs including aggrecan, neurocan, melanoma-associated CSPG, neuroglycan C, and brevican in the human cycling endometrium. By reverse transcription-polymerase chain reaction, the gene transcripts for aggrecan core protein were detected in all samples examined, while other CSPGs were not. Western blotting showed the immunoreactivity for aggrecan core protein at approximately 370 kDa size after enzymatic digestion of CS-A and CS-C side chains. The expression level of aggrecan core protein was significantly higher in the secretory phase than in the proliferative phase. The immunostaining for aggrecan was detected in the endometrial microvascular endothelium throughout the menstrual cycle. The immunostaining in the glandular epithelium was faint during the proliferative and early secretory phase, but distinct during the mid-to-late-secretory phase. Progesterone, but not 17β-estradiol, induced aggrecan core protein expression in cultured endometrial epithelial cells. The endometrial expression pattern of aggrecan was distinct from that of other known CSPGs, suggesting the unique role of this proteoglycan at the implantation site.

1. Introduction

Proteoglycans (PGs) are a group of heavily glycosylated proteins that play an essential role in the tissue maintenance and remodeling in various organs. PGs consist of one core protein and covalently attached glycosaminoglycan side chains, and are localized widely in the extracellular matrix or on the cell surface. According to the glycosaminoglycan composition, PGs are subclassified into heparan sulfate PG, keratan sulfate PG, dermatan sulfate PG, chondroitin sulfate (CS) PG, and heparin.

Studies have been demonstrating the involvement of PGs in the human endometrial integrity. For instance, the heparan sulfate PGs syndecan-1 and -4 and keratan sulfate PG mucin-1 in surface epithelial cells are able to bind a wide range of growth factors and adhesion molecules and regulate their bioactivity, which are important for endometrial shedding, proliferation, and embryo implantation [1–4]. In addition, the dermatan sulfate PG biglycan and CSPGs versican and serglycin are induced in the endometrial endothelial cells after ovulation and thought to play a central role in selective recruitment of circulating CD16(−) natural killer (NK) cells into endometrial stroma [5–8].

CS consists of the repeating disaccharide units of glucuronic acid and N-acetylgalactosamine that acquired structural diversity by sulfate modification. Despite that CS is the most abundant glycosaminoglycan species in the human endometrium across the menstrual cycle, the endometrial expression of the CSPG core proteins remains fully undetermined [5,9,10]. In this study, we aimed to clarify the expression of CSPGs in the human nonpathological cycling endometrium.

2. Materials and methods

2.1. Samples

Endometrium was obtained from 32 fertile women aged 36–44 years with regular menstrual cycles ranging from 28 to 35 days under informed consent. They had undergone hysterectomy for subserous leiomyoma ($n = 20$) or cervical intraepithelial neoplasia ($n = 12$), and not received any hormonal pretreatment. The study was approved by the Institutional Review Board. According to the dating criteria [11], they were classified into 15 proliferative phase, six early secretory phase, six mid-secretory phase, and five late-secretory phase. None of these samples showed any pathological findings such as polyps, hyperplasia, cancer or endometritis. After being washed thoroughly in phosphate-buffered saline (pH 7.4), one portion of all secretory and six proliferative phase samples

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was homogenized in a TRIzol reagent (Invitrogen, Carlsbad, CA). The total RNA and protein fraction were isolated according to the manufacturer's instructions. The remainder was fixed overnight in 4% paraformaldehyde (pH 7.3), embedded in paraffin, and cut into 4 μm sections. Nine proliferative phase samples were used for the isolation of the epithelial cells.

2.2. Reverse transcription-polymerase chain reaction

Total RNA (2 μg) was converted to 20 μl cDNA solution with 1 μg oligo deoxythymidine and reverse transcriptase (Invitrogen). In the presence of Taq DNA polymerase (Invitrogen), 1 μl of cDNA solution was amplified with 0.5 μM primer pairs for CSPG core proteins (aggrecan, 5′-aacagccacctccccaa and 3′-gaagtggcggtaacagtgg; neurocan, 5′-cacgtcacaacatggagacc and 3′-tcttcttccccactggacac; melanoma-associated CSPG, 5′-cctcctgctgcagctctact and 3′-ctgaggaggcgttcagaaac; neuroglycan C, 5′-gcaggactacatctggcaca and 3′-ccgtcttgagcaggtagagc; brevican, 5′-aaggtcaaaggggtcgtctt and 3′-gcatcacattgctcatagcc). Each cycle consisted of 60 s at 94 °C, 45 s at 60 °C, and 90 s at 72 °C. As an internal control, ribosomal protein L19 which is expressed constantly in the human endometrium throughout the menstrual cycle was simultaneously amplified [7]. The amplified products were electrophoresed on an agarose gel, stained with ethidium bromide, and verified on an ultraviolet transilluminator. The products were confirmed by a direct sequence analysis. Human melanoma cell line G361 lysate and brain total RNA (Clontech, Mountain View, CA) were used as a positive control. The products without reverse transcription were used for a negative control. The image of the gel was scanned into a computer, and the signal intensity of the products was measured densitometrically using National Institutes of Health Image 1.61 (Bethesda, MD, USA).

2.3. Enzymatic treatment of glycosaminoglycan side chains and Western blotting analysis

The protein fraction was solubilized in lysis buffer containing 2 μM aprotinin, 50 μM leupeptin, 125 μM bestatin, and 25 μM pepstatin A (Nacalai Tesque, Kyoto, Japan). The protein concentration was measured with a DC assay kit (Bio-Rad Laboratories, Hercules, CA, USA). In some experiments, the soluble protein was digested with 0.2 IU/ml chondroitinase ACII (degrading CS-A and -C, Seikagaku Corp., Tokyo, Japan) in 250 mM Tris–HCl, 300 mM Na-acetate (pH 8.0), 1% fetal calf serum, and 0.25% Triton X-100 at 37 °C for 2 h. Under non-reducing conditions, the soluble protein (50 μg) was separated on a polyacrylamide gel, and electrotransferred to a polyvinylidene difluoride membrane. After being soaked in 5% nonfat milk or fetal calf serum containing 0.1 M NaCl, 0.01 M Tris, and 0.1% Tween-20, the membrane was incubated with 0.4 μg/ml goat polyclonal IgG against human aggrecan core protein (AF1220, R&D Systems, Minneapolis, MN) or glyceraldehyde-3-phosphate dehydrogenase (A14, Santa Cruz Biotechnology, Santa Cruz, CA) for 30 min at room temperature, followed by washing and incubation with a horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology). Immunoreactivity was detected with an Enhanced Chemiluminescence Plus kit (Amersham-Pharmacia, Uppsala, Sweden). The signal intensity of the immunoreactivity was measured densitometrically as described above.

2.4. Immunohistochemistry

The sections were dewaxed in xylene and rehydrated in a graded series of ethanol. The sections were then immersed in 3% hydrogen peroxide for 5 min to quench endogenous peroxidase activity, and incubated with 10% fetal calf serum for 10 min to minimize nonspecific antibody binding. Sections were incubated with 10 μg/ml AF1220 in a moist chamber for 30 min at room temperature. After being washed, the sections were incubated with a horseradish peroxidase-conjugated secondary antibody (Dako, Kyoto, Japan). Sections were washed, developed with diaminobenzidine, and counterstained with hematoxylin. The specificity of the immunoreactivity was confirmed by an immunoabsorption test using recombinant human aggrecan (R&D Systems).

2.5. Stimulation of endometrial epithelial cells with ovarian steroids

Endometrial epithelial cells were isolated as previously described [12]. In brief, endometrium was minced into approximately 1-mm^3 pieces and incubated with 0.5% collagenase (Sigma–Aldrich, St. Louis, MO) for 1 h at 37 °C. The cell suspension was then passed through a nylon mesh (Becton Dickinson, San Jose, CA). The endometrial glands retained on the mesh were washed free of adhering stromal cells with Hanks' balanced salt solution, transferred by backwashing with the medium, and seeded on sterile plastic dishes. After allowing the remaining stromal cells to adhere to the dishes for 30 min, the unattached epithelial cells were collected. The purity of the cell preparations was confirmed by staining with mouse anti-human cytokeratin-7 IgG_1 antibody (OV-TL12/30, Dako). In a pilot study, the gene transcript expression for ovarian steroid receptors was confirmed by reverse transcription-polymerase chain reaction. The cells were incubated in phenol-red free Dulbecco's modified Eagle medium (Invitrogen) supplemented with 10% charcoal-stripped fetal calf serum at 37 °C in 5% CO_2 with or without 10^{-6}–10^{-9} M 17β-estradiol or 10^{-6}–10^{-9} M progesterone (Sigma–Aldrich) dissolved in dimethylsulfoxide. After 48 h, the cells were subjected to Western blotting as described above.

2.6. Statistics

The datasets were compared with Bonferroni/Dunn test. A p value less than 0.05 was regarded as significantly different.

3. Results

3.1. Expression of gene transcripts for CSPGs in human cycling endometrium

Two bands corresponding to the gene transcript variants for aggrecan core protein (250 base pairs and 136 base pairs) and the single band for the internal control ribosomal protein L19 were detected in all endometrial samples examined as well as in the positive control human brain (Fig. 1A). By sequence analysis, the 136 base pair splicing variant lacked 114 base pairs of epidermal growth factor-like sequence, which was preserved in the 250 base pair variant. In all samples examined ($n = 13$), the densitometrical signal intensity for 136 base pair variant was significantly higher ($p < 0.00002$, mean 2.51-fold) than that for 250 base pair variance. There was no significant ($p > 0.37$) menstrual cycle-dependent fluctuation in the signal intensity ratio for 136 base pair variant versus 250 base pair variant (Fig. 1B). The gene transcripts for neurocan, neuroglycan C, brevican, and melanoma-associated CSPG were detected in the positive controls, but not in any of the endometrial samples.

3.2. Expression of aggrecan in human cycling endometrium

The immunoreactivity for housekeeping glyceraldehyde-3-phosphate dehydrogenase was detected as a 37 kDa band in all endometrial samples examined, while that for aggrecan core protein was not detectable in any of the undigested samples (Fig. 2A). After enzymatic digestion of CS-A and -C side chains with

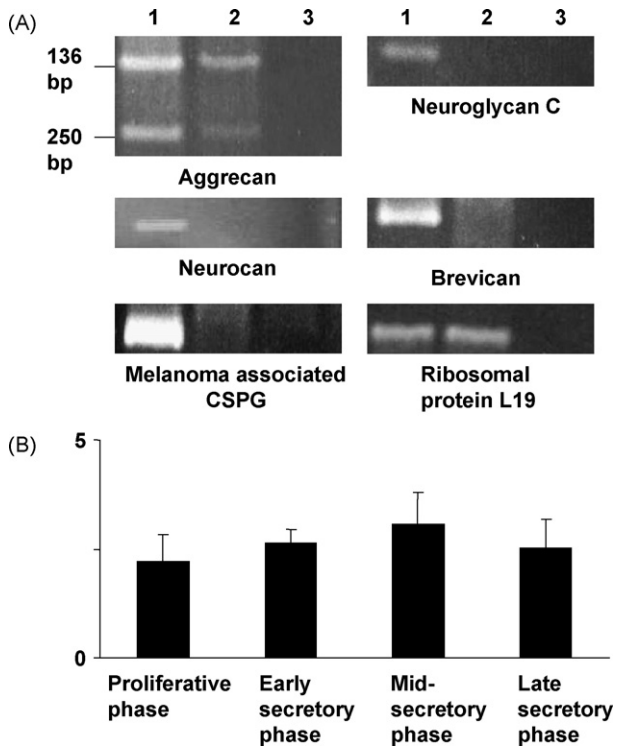

Fig. 1. Expression of CSPGs gene transcripts in human cycling endometrium. (A) Representative microphotographs of gene transcript expression. Lane 1 represents the electrophoresis image of the positive control G361 cell lysate (for melanoma-associated CSPG) and brain total RNA (for aggrecan, neurocan, neuroglycan C, and brevican). Lanes 2 and 3 represent the image of the endometrial sample (in the mid-secretory phase) and the products without reverse transcription, respectively. (B) Densitometrical signal intensity ratio for 136 base pair variant versus 250 base pair variant of aggrecan core protein in the proliferative phase ($n=4$), early secretory phase ($n=3$), mid-secretory phase ($n=3$), and late-secretory phase ($n=3$).

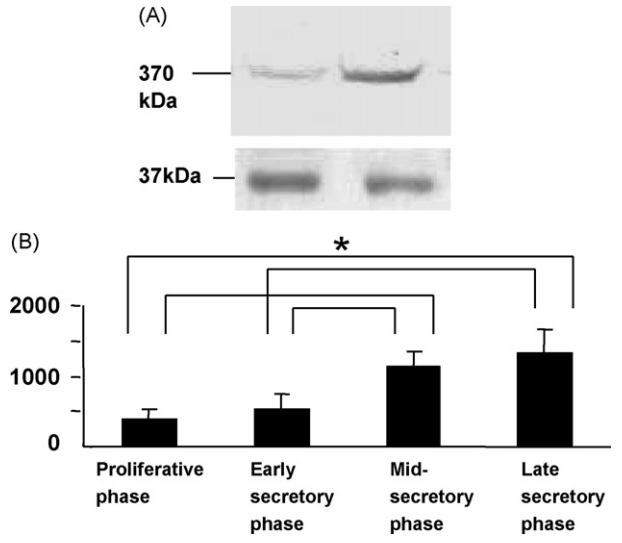

Fig. 2. Expression of aggrecan core protein in human cycling endometrium. (A) A representative microphotograph of Western blotting analysis for aggrecan core protein expression after chondroitinase ACII treatment (upper panel). Lower panel represents the housekeeping glyceraldehydes-3-phosphate dehydrogenase. The left lane and right lane of each panel represent the electrophoresis of one proliferative and mid-secretory phase sample, respectively. (B) Densitometrical signal intensity (arbitrary units) for aggrecan core protein expression in 50 μg endometrial soluble protein fraction in the proliferative phase ($n=6$), early secretory phase ($n=6$), mid-secretory phase ($n=5$), and late-secretory phase ($n=5$). $^{*}p<0.01$.

chondroitinase ACII, the immunoreactivity appeared as an approximately 370 kDa band (Fig. 2A). When the same amount of the endometrial soluble protein fraction (50 μg) was electrophoresed in each lane and transferred to the membrane, the densitometrical band intensity for immunoreactive aggrecan core protein was significantly ($p<0.0073$) higher in the mid- and late-secretory phase than in the proliferative and early secretory phase (Fig. 2B).

In the proliferative phase, the immunostaining for aggrecan core protein was detected distinctly in the microvascular endothelium, particularly in the endometrial functional layer. In contrast, the immunostaining in the surface and glandular epithelium was faint in this phase (Fig. 3A). The immunostaining pattern in the early secretory phase was similar to that in the proliferative phase, except for some additional diffuse staining in the stromal compartment (Fig. 3B). In the mid- to late-secretory phase, the membranous immunostaining in the epithelium became distinct, while the immunostaining in the microvascular endothelium remained constant (Fig. 3C and D). In addition, some stromal cells showed focal patchy staining in the late-secretory phase (Fig. 3D). The specificity of the immunoreactivity was confirmed by an immunoabsorption test (Fig. 3E).

3.3. *Effect of ovarian steroids on aggrecan core protein expression in endometrial epithelial cells*

In an in vitro system, 10^{-6}–10^{-9} M 17β-estradiol ($n=6$) failed to induce aggrecan core protein expression in isolated endometrial epithelial cells as well as vehicle control dimethylsulfoxide (Fig. 4A). On the contrary, 10^{-6} M progesterone significantly ($p<0.0001$, $n=6$) induced its expression in endometrial epithelial cells compared with vehicle control or 10^{-6} M 17β-estradiol (Fig. 4A and B). The effect of progesterone was detected at the concentration of more than 10^{-8} M (Fig. 4C) and was dose-dependent between 10^{-7} and 10^{-9} M ($n=3$) (Fig. 4D).

4. Discussion

Studies have been focusing on aggrecan in the chondrocytes, but several investigators demonstrated its expression in the organs outside the cartilage. For example, aggrecan is a component of human cerebral cortex and sclera, and contributor to the maintenance and rearrangement of the structural integrity in these tissues [13,14]. The dermal part of the hair follicle is also capable of producing extracellular aggrecan, suggesting its role in nutrient diffusion, cell proliferation and differentiation, and hair protection [15]. In this study, we demonstrate the expression of aggrecan in the human cycling endometrium, whereas other CSPGs including neurocan, melanoma-associated CSPG, neuroglycan C, and brevican were not detectable throughout the menstrual cycle.

Early studies demonstrated that aggrecan core protein gene generates two transcriptional splicing variants [16]. One is the transcript containing epidermal growth factor-like sequence and the other is the one lacking the sequence. Although the exact roles of these variants remain undetermined, their relative abundance is known to vary among the tissues, for instance, between the human cartilage and brain [14,16]. In this study, we detected both variants in the human cycling endometrium. Regardless of the menstrual cycle, the expression levels of the variant lacking the sequence were predominant over those of the variant containing the sequence, but the finding may reflect not only the relative abundance of two variants but the amplification efficiency. Aggrecan may regulate the growth and differentiation of the endometrial component cells directly via its epidermal growth factor-like domain or indirectly by presenting other extracellular matrix proteins such as tenascin and fibulin-1 [17–19].

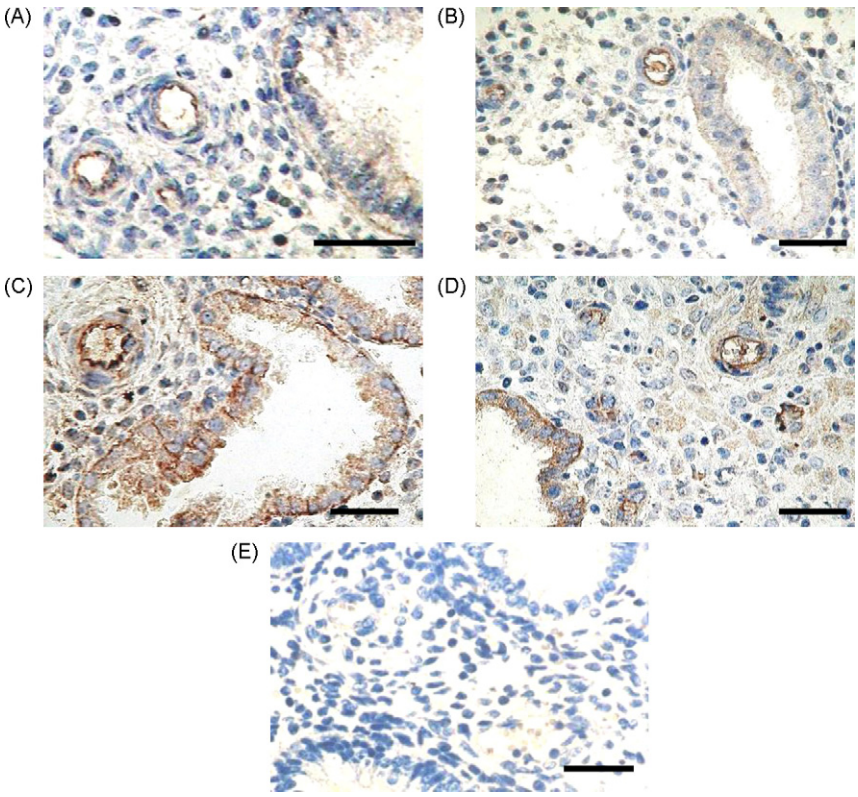

Fig. 3. Localization of aggrecan in the human cycling endometrium. Representative microphotographs (400× magnification) of immunohistochemistry in the proliferative phase (A), early secretory phase (B), mid-secretory phase (C), late-secretory phase (D), and immunoabsorption test using recombinant aggrecan protein (E). Scale bars indicate 50-μm.

The immunoreactivity for aggrecan core protein was detectable in the endometrium after enzymatic digestion of CS-A and -C. The glycosaminoglycan side chains of aggrecan contain not only CS but also some keratan sulfate in the human cartilage and sclera. While keratan sulfate digestion with a combination of enzymes, such as keratanase and endo-β-galactosidase [13,14], was required to detect aggrecan core protein in these organs, pretreatment with these enzymes was unnecessary for endometrial samples. The findings indicate that the side chains of the endometrial aggrecan are comprised mainly of CS-A and -C, and less keratan sulfate. This may resemble a novel isoform recently identified in the perineuronal nets of the human adult cerebral cortex [14].

Although CS is the most abundant glycosaminoglycan species both in the human endometrial epithelium and stroma across the menstrual cycle, some studies found the difference in localization of some CSPGs in this mucosal tissue. The expression of CD44, a CSPG that binds hyarulonan, is confined in the human endometrial surface epithelium only during the secretory phase [9], while versican and serglycin are distributed throughout the tissues with some menstrual cycle-dependent variation [5,6].

The expression levels of aggrecan in the endometrial epithelium increased in the mid-to-late-secretory phase, suggesting the ruling effect of the ovarian steroids. Using isolated endometrial epithelial cells, we found that progesterone, but not estradiol, is a positive regulator of aggrecan expression in vitro. In contrast to immunohistochemistry, aggrecan expression in isolated epithelial cells was very weak or undetectable in some samples, which may be due to disintegration in the process of the cell isolation. CD62L is a CS ligand that is expressed on human blastocysts and postulated to play a critical role in embryo implantation [20]. Upregulation of aggrecan in endometrial epithelial cells in the late stage of the cycle and by progesterone stimulation suggests the potential role of this PG in embryo implantation.

In addition, aggrecan was expressed distinctly in the endometrial microvascular endothelium throughout the menstrual cycle. Unique CD16(−) NK cells increase in number in the endometrial stroma as the menstrual phase progresses [21]. CS-A and -C are the ligands for CD44, an adhesion molecule expressed highly on these lymphocytes. The CS side chains of aggrecan may be associated with extravasation of circulating CD16(−) NK cells into endometrium [5,8]. The constant expression of aggrecan in the microvascular endothelium, however, may imply the involvement of aggrecan in other phenomena.

Moreover, some endometrial stromal cells showed patchy immunoreactivity for aggrecan in the late-secretory phase, which resembled the staining pattern of serglycin in our previous study [6]. Aggrecan core protein has a capacity to bind more than 100 CS chains, which plays a critical role in water retention and elasticity preservation of the cartilage [22]. The appearance of aggrecan in the endometrial stroma in the late-secretory phase may be associated with the stromal edematous change seen during this period.

In summary, we demonstrated aggrecan core protein expression in the human cycling endometrium along with CS-A and CS-C side chains. Its expression in the endometrial epithelium increased in the mid-secretory phase and by in vitro progesterone stimulation. The CSPGs neurocan, melanoma-associated CSPG, neuroglycan C, and brevican were not detectable in this mucosal tissue. The aberrant CS expression has been reported in some endometrial pathology. For example, the dense expression of CS was found in the deep-infiltrating endometriosis foci [23]. Moreover, the correlation between the high versican expression and poor prognosis (extrauterine metastasis and space involvement) was shown in endometrial cancer [24]. The excessive CS expression may relate to uncontrolled ectopic proliferation of endometrial cells.

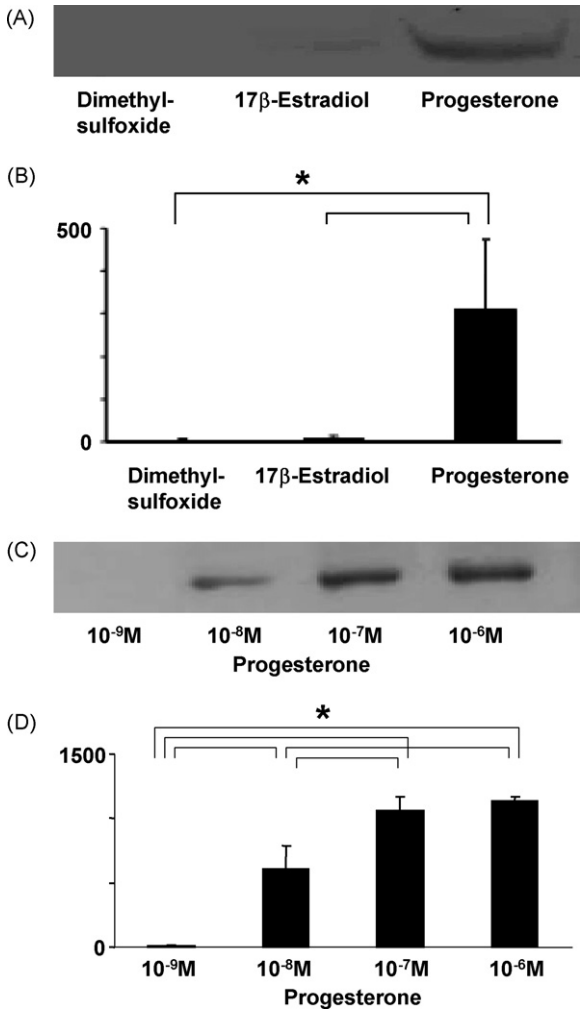

Fig. 4. Aggrecan core protein expression in 50 μg soluble protein fraction extracted from isolated endometrial epithelial cells incubated with ovarian steroids for 48 h. Representative microphotograph of Western blotting analysis (A) and densitometrical signal intensity (arbitrary units, $n = 6$) (B) for aggrecan core protein expression in the presence of 10^{-6} M 17β-estradiol or 10^{-6} M progesterone dissolved in dimethylsulfoxide, which was also used as the vehicle control. $^{*}p < 0.0001$. Representative microphotograph of Western blotting analysis (C) and densitometrical signal intensity (arbitrary units, $n = 3$) (D) for aggrecan core protein expression in the presence of 10^{-6}–10^{-9} M progesterone. $^{*}p < 0.0001$.

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