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Composition of sulfated glycosaminoglycans and immunodistribution of chondroitin sulfate in deeply infiltrating endometriosis affecting the rectosigmoid

Plínio T. Berardo [a], Maurício S. Abrao [b], Maísa L.S. Souza [c,d], Daniel E. Machado [a], Luiz-Cláudio F. Silva [c], Luiz E. Nasciutti [a,*]

[a] Departamento de Histologia e Embriologia, Instituto de Ciências Biomédicas, Universidade Federal do Rio de Janeiro, Cidade Universitária-Ilha do Fundão, 21941-590 Rio de Janeiro, RJ, Brazil
[b] Departamento de Obstetrícia e Ginecologia, Faculdade de Medicina, Universidade de São Paulo, São Paulo, SP, Brazil
[c] Laboratório de Tecido Conjuntivo, Hospital Universitário Clementino Fraga Filho e Instituto de Bioquímica Médica, Universidade Federal do Rio de Janeiro, Rio de Janeiro, RJ, Brazil
[d] Centro Federal de Educação Tecnológica de Química de Nilópolis - CEFETQ, Brazil

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ABSTRACT

The composition of sulfated glycosaminoglycans (GAGs) and the tissue distribution of chondroitin sulfate (CS) were analyzed in deeply infiltrating endometriosis (DIE) of rectosigmoid, using metachromatic staining, and biochemical analysis employing electrophoresis before and after specific enzymatic or chemical degradations, and immunostaining with an antibody against CS. The sulfated GAGs were characterized as dermatan sulfate (DS), heparan sulfate (HS) and CS; and DS strongly predominated compared to HS and CS. Immunostaining procedures showed that CS was concentrated in the endometriosis foci, distributed throughout the stroma around the glands. This is the first report describing the composition of sulfated GAGs and the tissue location of CS in DIE by means of histochemical, biochemical and immunohistochemical analyses. These results confirmed that in DIE of rectosigmoid, as in eutopic endometrium [Nasciutti, L.E., Ferrari, R., Berardo, P.T., Souza, M.L.S., Takiya, C.M., Borojevic, R., Abrao, M.S., Silva, L.C.F., 2006. Distribution of chondroitin sulfate in human endometrium. Micron 37, 544–550], CS was the dominant sulfated GAG in stroma of the lesion foci.

1. Introduction

In humans, endometrial tissue undergoes cyclic morphological and functional modifications, stimulated by estrogen and progesterone, and involving rearrangement of components of the extracellular matrix (ECM), including the glycosaminoglycans (GAGs). GAGs consist of hexosamine [D-glucosamine or D-galactosamine] and either hexuronic acid [D-glucuronic or L-iduronic acid] or galactose units that are arranged in an alternating unbranched sequence and carry sulfate substitutions in various positions. The common GAGs include chondroitin sulfate (CS), dermatan sulfate (DS), heparan sulfate (HS), heparin, keratan sulfate (KS) and hyaluronic acid (HA). DS, HS and heparin contain both glucuronic acid and iduronic acid units, whereas CS and HA have glucuronic acid as the only hexuronic acid. In the tissue, GAGs are covalently bound to a protein core, forming a structure knowing as proteoglycan (PG). The only exception is the HA, a non-sulfated GAG, which exists as a protein-free polysaccharide on cell surfaces and in the ECM (Salamonsen et al., 2001). The strongly negatively charged GAG side chains attract osmotically active cations and an accompaniment of water-endowing PGs with the ability to contribute to tissue hydration and expansion. Owing to the variability in sulfate substitution, all GAGs display considerable sequence heterogeneity, and it is believed that structural differences are responsible for highly specific interactions of GAGs with other macromolecules (Powell et al., 2004; Coombe and Kett, 2005). HA distribution was analyzed in biopsy specimens from the female reproductive tract, using a biotinylated hyaluronan-binding protein (HABP) as a histochemical probe (Edelstam et al., 1991). In normal endometrium, the staining was confined to vessel walls and showed a weak and patchy distribution in connective tissue, and no cyclic changes in HA content were observed. In addition to HA, another GAG molecule, KS, was also detected in human endometrium. Immunohistochemistry analysis indicated that KS is associated with glandular epithelium throughout the normal human endometrium cycle, increasing in

* Corresponding author. Fax: +55 21 2562 6483.
E-mail address: luiz.nasciutti@histo.ufrj.br (L.E. Nasciutti).

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the secretory phase of the cycle, inside the epithelial cells and secretions, and is probably correlated with the regulation of embryo attachment (Graham et al., 1994; Aplin et al., 1988).

Recently, we showed that CS is the major sulfated GAG species, followed by HS and DS, present in the proliferative and secretory phases of the human endometrial cycle (Nasciutti et al., 2006). CS is present throughout the stroma and is concentrated around blood vessels and glands. In the secretory phase, which is concomitant with the implantation of the conceptus in a fertile cycle, CS is present at the epithelial surface and inside the lumen of glands. Interestingly, Yamaguchi et al. (2006) reported a homogeneous immunoreactivity for chondroitin sulfate proteoglycan 2 (CSPG2) distributed throughout the epithelium, stroma and endothelium of proliferative and secretory human endometrium. The immunoreactivity for CSPG2 is membranous, cytoplasmatic, and homogeneous in the epithelium. The immunostaining intensity in the stroma is equivalent to that in the epithelium, which elevated from the proliferative phase to the secretory phase (Yamaguchi et al., 2006).

Endometriosis is a prevalent condition in which endometrium-like glandular and stromal tissue exists at ectopic sites outside the uterus (Vinatier et al., 2001). Ectopic lesions exhibit recurrent menstruation-like bleeding that result in blood-filled cysts, local inflammatory reactions and fibrosis (Itoga et al., 2003). Endometriosis affects approximately 10% of the female population in their reproductive years (Wheeler, 1989), and women with endometriosis present characteristic signs and symptoms: dysmenorrhea, dyspareunia, chronic pelvic pain or sub-fertility.

Deeply infiltrating endometriosis is defined as pelvic endometriosis infiltrating deeper than 5 mm below the peritoneum, and the major component of the lesions is fibromuscular tissue (Cornillie et al., 1990). Bowel endometriosis is one of the greatest concerns of specialists treating deep infiltrative endometriosis because of the severity of its symptoms, concomitant infiltration of pelvic organs, the possibility of bowel obstruction resulting from the progression of the disease, and the technical difficulties in surgical removal (Abrao et al., 2003).

In the present study, we investigated the composition of sulfated GAGs and the tissue distribution of CS in DIE, using histochemical, biochemical and immunohistochemical analyses.

2. Materials and methods

2.1. Human endometrial and endometriotic tissues

This study was conducted following approval by the Internal Review Board of the Clementino Fraga Filho Teaching Hospital of the Federal University of Rio de Janeiro, Brazil (No. 051/01, 2001). All patients read and signed an informed consent form prior to enrollment in the study.

The patient groups were similar and between menarche and menopause, and the mean age was 34 years (range 25–43 years). Tissues from women without endometriosis consisted of 20 normal endometrium, of which 10 were in the proliferative phase of the menstrual cycle (day 10–12 of the cycle) and 10 in the secretory phase (day 21–25). 10 rectosigmoid endometriotic and 5 normal rectosigmoid tissue samples were used. All patients with endometriosis had stages III or IV of the disease, as classified by the American Society for Reproductive Medicine (ASRM). Samples of normal endometrium tissue were obtained from patients submitted to a total hysterectomy for myoma in patients with proven fertility and without endometriosis, and endometrial states were established according to the Noyes criteria (Noyes et al., 1950). The biopsy specimens of endometriosis were collected from patients undergoing videolaparoscopy or laparotomy for the diagnosis and treatment of endometriosis, and the lesions were confirmed according to the presence of endometrial glands and/or stroma. Normal rectosigmoid tissue samples were collected from disease free margin of intestinal resection of patients with intestinal carcinoma and endometriosis. Histopathology study was done to confirm the absence of microscopic invasion as well as endometriosis. All patients had regular (26–30 days) menstrual cycles, and none had received hormone therapy during the 3 months prior to the surgery, or gonadotrophin release hormone (GnRH) analogues in the preceding 6 months.

2.2. Material

CS, DS, HS, twice-crystallized papain (15 U/mg protein), and deoxyribonuclease I from bovine pancreas were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Chondroitin AC lyase (EC 4.2.2.5) from *Arthrobacter aurescens*, and chondroitin ABC lyase (EC 4.2.2.4) from *Proteus vulgaris* were purchased from Seikagaku American Inc. (Rockville, MD, USA). For immunohistochemical staining we used a mouse monoclonal anti-CS clone CS-56 (Sigma Chemical Co., St. Louis, MO, USA).

2.3. Metachromatic staining of sulfated GAGs

Tissues from endometrium and rectosigmoid (endometriosis and normal area) were fixed overnight in 4% paraformaldehyde (PFA) in Sorensen phosphate buffer (0.1 M, pH 7.4), at 4 °C. After fixation and washing, the tissues were dehydrated in ethanol and embedded in paraffin. Tissue sections (7 μm) were collected on polylysine-coated slides and stained with the cationic dye 1.9-dimethylmethylene blue (DMB) in 0.1N HCl, containing 0.04 mM glycine and 0.04 mM NaCl (Nasciutti et al., 2006). The sections were then examined and photographed using a light microscope (Zeiss, Axioskop 2). The positive reaction reveals GAGs as metachromatic structures (stained purple).

2.4. Isolation of GAGs

For these experiments, samples of rectosigmoid tissue that contained foci of endometriotic lesions were collected. Both normal and endometriotic rectosigmoid tissues were incubated with acetone for 24 h at room temperature and then dried. The tissues were suspended in sodium acetate buffer, pH 5.5, containing 40 mg papain in the presence of 5 mM of ethylenediamine tetraacetic acid (EDTA) and 5 mM cysteine, and incubated at 60 °C for 24 h. The incubation mixture was centrifuged at 2000 × *g* for 10 min at room temperature, and the supernatant, which contained the GAGs, was retained. A 10% cetylpyridium chloride solution was added to the supernatant to a final concentration of 0.5%, and the mixture was left to stand at room temperature for 24 h. The solution was centrifuged at 2000 × *g* for 10 min at room temperature, and the pellet was washed with 10 mL of 0.05% cetylpyridium solution. This pellet, a GAG–cetylpyridium complex, was dissolved in 3.7 mL of a solution of 2 M NaCl/absolute ethanol (100:15, v/v), and the GAGs were precipitated with the addition of 6 mL of absolute ethanol. After 24 h at 4 °C, the precipitate was collected by centrifugation and washed twice with 10 mL of 80% ethanol, followed by the same volume of absolute ethanol. The final pellet, which constituted the total tissue GAG preparation, was dissolved in 2 mL phosphate-buffered saline containing approximately 0.5 mg deoxyribonuclease I and incubated for 12 h at 37 °C. Finally, the incubation mixture was lyophilized and dissolved in 0.2 mL distilled water.

2.5. Identification of sulfated GAGs

Agarose gel electrophoresis was carried out as previously described (Rocha et al., 2000). Approximately 10 μg of tissue

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sulfated GAGs, before and after chondroitin lyase digestion or deaminative cleavage with nitrous acid (see below), as well as a mixture of standard CS, DS, HS (10 μg of each) were applied to 0.5% agarose gels in 0.05 M 1,3-diaminopropane:acetate (pH 9.0). After electrophoresis, sulfated GAGs were fixed in the gel with 0.1% N-acetyl-N,N,N-trimethylammonium bromide in water, and stained with 0.1% toluidine blue in acetic acid:ethanol:water (0.1:5:5, v/v).

Digestion with chondroitin AC or ABC lyases was carried out according to Saito et al. (1968). Approximately 100 μg of tissue sulfated GAGs was incubated with 0.3 units of chondroitin AC lyase or chondroitin ABC lyase for 8 h at 37 °C in 100 μL of 50 mM Tris:HCl (pH 8.0) containing 5 mM EDTA and 15 mM sodium acetate.

Deamination by nitrous acid at pH 1.5 was performed as described by Shively and Conrad (1976). Briefly, approximately 100 μg of tissue sulfated GAGs was incubated with 200 μL freshly generated HNO_2 at room temperature for 10 min. The reaction mixture was then neutralized with 1.0 M Na_2CO_3.

2.6. Immunodetection of CS

Tissues from endometrium and normal and endometriosis of rectosigmoid were prepared as described above for histochemistry. The endogenous peroxidase activity was blocked with 3% hydrogen peroxide. The sections were then incubated with goat normal serum and 1% bovine serum albumin (BSA) to reduce nonspecific antibody binding. After washing in 0.01 M phosphate-buffered saline (PBS) pH 7.5, the sections were incubated overnight at 4 °C with monoclonal anti-CS antibody (mouse IgM isotype) (Sigma) at 1:200 dilution. The reaction was revealed with StreptAB Complex/HRP Duet Kit (Dako Corporation, Carpinteria, CA, USA) and 3,3′ diaminobenzidine (DAB) tablets. Counterstaining was

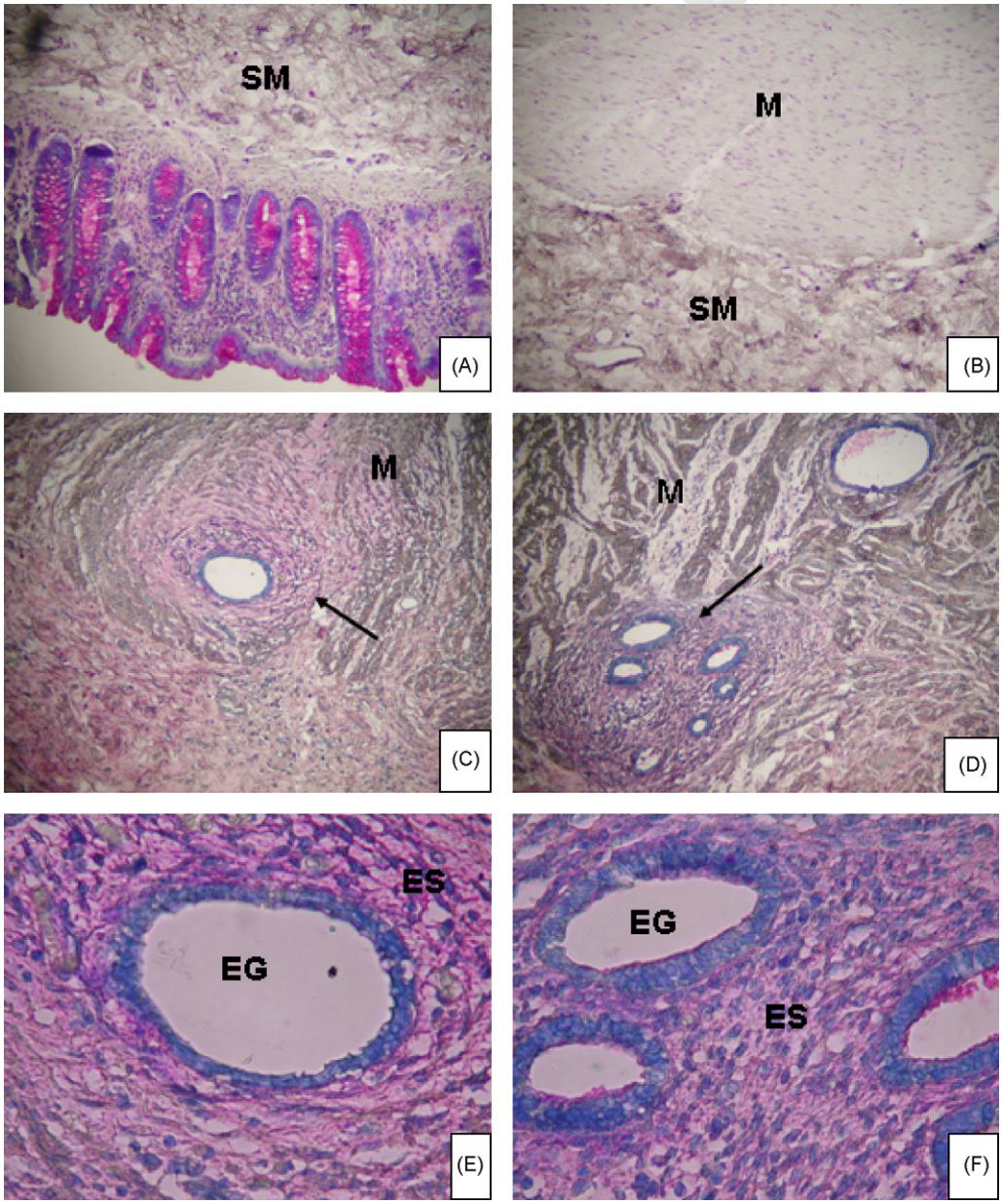

Fig. 1. Photomicrographs of normal rectosigmoid (A and B) and rectosigmoid endometriosis (C–F) tissues stained with DMB. In normal rectosigmoid tissue, metachromatic material was present mainly in the epithelial intestinal glands. In ectosigmoid endometriosis, abundant purple metachromatic material was distributed throughout the endometrial stroma (ES), and around endometriosis foci (line). Note that the metachromasia is more intense immediately around the endometrial glands (EG). Muscle layer (M), sub-mucosa layer (SM). A–D: 100×; E and F: 400×.

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carried out with Harris' haematoxylin. Negative controls were obtained omitting the primary antibody (incubating the sections in 0.01 M phosphate saline buffer pH 7.5), as previously reported (Nasciutti et al., 2006). The positive reaction reveals CS in brown.

2.7. Histomorphometry of CS immunodistribution

All tissue sections were examined by two observers blinded to the study using a 40× objective lens of a Nikon light microscope (Nikon, Tokyo, Japan) connected to a digital camera (Coolpix 990, Nikon, Japan). Ten fields of a CS-immunostained section were chosen at random and captured from each specimen. Quantification was assessed from captured high-quality images (2048 × 1536 pixels buffer) using the Image Pro Plus 4.5.1 (Media Cybernetics, Silver Spring, MD, USA). Data were stored in Adobe Photoshop, version 3.0, to enable uneven illumination and background color to be corrected.

2.8. Statistical analyses

All statistical calculations were carried out using the StatXact-5 program (CYTEL Software Corporation, Cambridge, MA, USA). The differences between groups were calculated using nonparametric analyses (Mann–Whitney *U*-test). A P value <0.05 was established as statistically significant.

3. Results

3.1. Metachromatic staining of sulfated GAGs

The endometrium stained with DMB cationic dye confirmed the presence of abundant metachromatic (purple) material distributed throughout the stroma, as described by Nasciutti et al. (2006) (data not shown). In the proliferative and secretory phases, metachromatic staining was similar, intense, and homogeneous among the connective cells in the stroma.

In rectosigmoid endometriosis, metachromatic staining was also intense and homogeneous in the stroma around the glands of endometriotic foci (Fig. 1C–F). In the fibrotic area far from the lesion, the reaction was less intense and spread between distorted muscle fibers. In normal rectosigmoid tissue, metachromatic staining was strongly concentrated in the epithelium of intestinal glands (Fig. 1A), with almost no purple coloration in the muscle layer (Fig. 1B).

3.2. Characterization of rectosigmoid endometriosis sulfated GAGs

The GAGs were characterized through their migration on agarose gel and digestion with specific lyases or deamination with nitrous acid. In normal (Fig. 2B) and endometriotic rectosigmoid tissue (Fig. 2C), the preponderant GAG migrated on agarose gel similarly to the DS standard, and it resisted the action of chondroitin AC lyase, but disappeared totally from the gel after chondroitin ABC lyase digestion. Two other GAGs, found in smaller amounts, migrated similarly to the HS and CS standards, which disappeared totally from the gel after deaminative cleavage with nitrous acid and digestion by chondroitin AC lyase, respectively. These experiments characterized these sulfated GAGs in the normal and endometriotic rectosigmoid tissues as DS, HS and CS, and indicated a strong dominance of DS compared to HS and CS.

3.3. CS immunodistribution in the endometriotic lesion foci

Because we have shown previously that CS is the major sulfated GAG present in eutopic endometrium, we decided to investigate if the CS immunodistribution in the endometrioctic lesion foci is similar. First, we located this molecule in the proliferative and secretory phases of endometrium. As expected, CS was observed in both endometrial phases, distributed throughout the stroma. In the proliferative phase (Fig. 3A and B), the peroxidase deposit was intense and homogeneous among the stromal cells, and more concentrated in the basal layer close to the myometrium. In the secretory endometrium, the CS immunodistribution was similar. Because in this phase the stroma is edematous, the immunoreaction was more diffuse and more concentrated in the basal membrane region of the epithelial cells (Fig. 3C and D). The histological scores of the CS immunodistribution area were significantly higher in proliferative than in secretory endometrium (Table 1).

In rectosigmoid endometriosis tissue, the CS immunoperoxidase reaction was very intense and homogeneous in the endometrial stroma around the glands of endometriotic foci (Fig. 4B–D). In the fibrotic area far from the lesion, as in DMB metachromatic staining, the immunoreaction was less intense and spreading between distorted muscle fibers (Fig. 4E and F). In normal rectosigmoid tissue, the reaction was very weak in the muscle layer (Fig. 4A). Comparing the different areas of rectosigmoid endometriosis and the proliferative and secretory endometrium, the histological scores of CS immunodistribution

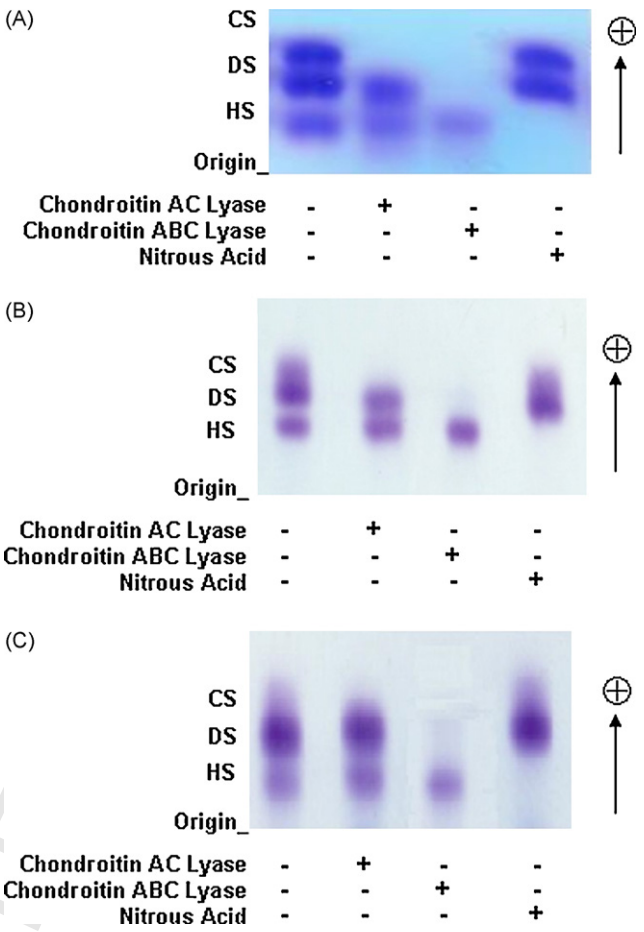

Fig. 2. Representative electrophoretograms of sulfated GAGs from normal (B) and endometriotic (C) rectosigmoid tissue, before (−) and after enzymatic degradation with chondroitin AC and ABC lyases (+) or deaminative cleavage by nitrous acid (+). The agarose gel electrophoresis was performed as described in Section 2. Sulfated GAGs were detected on gels by staining with toluidine blue. In (A) a mixture of standard GAGs containing heparan sulfate (HS), DS and chondroitin sulfate (CS) was analyzed by agarose gel electrophoresis as described above. Note that chondroitin AC lyase degrades CS standard, whereas chondroitin ABC lyase degrades both the CS and DS standards. Treatment with nitrous acid specifically degrades the HS standard.

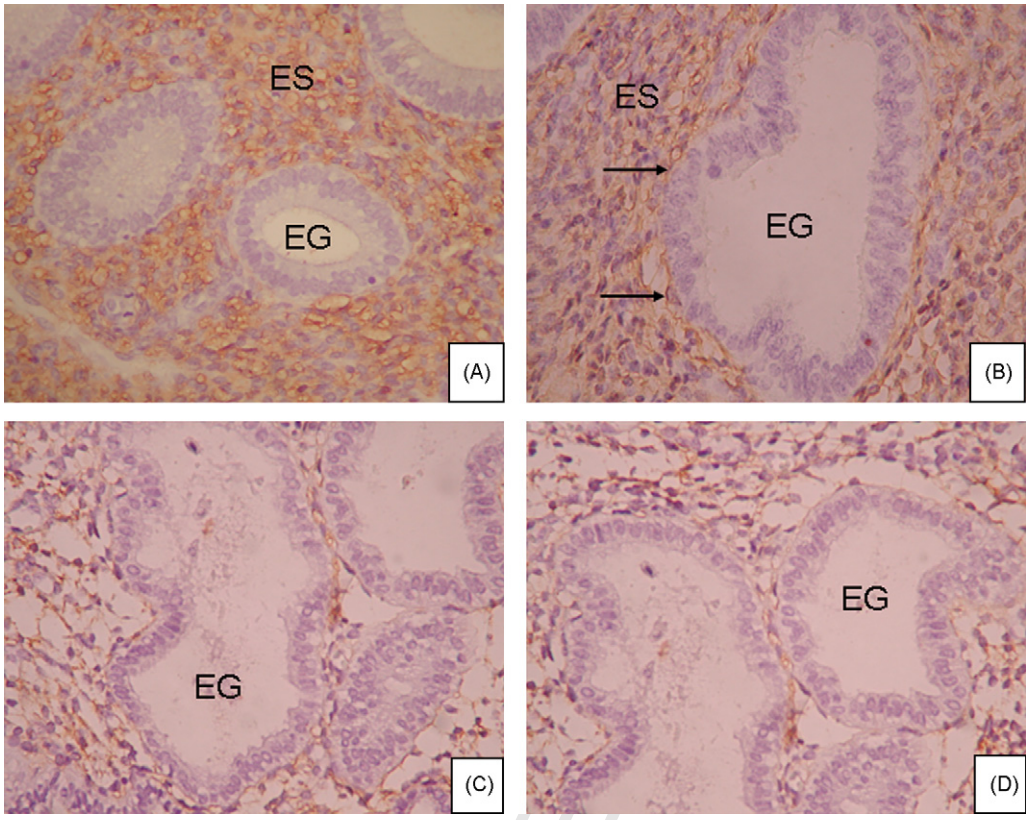

Fig. 3. Photomicrographs of human endometrium in proliferative (A and B) and secretory (C and D) phases of the menstrual cycle, immunostained with an antibody against CS. The peroxidase product is distributed throughout the stroma (ES) and is concentrated around the endometrial glands (EG). In B, the CS immunodistribution is also concentrated in some regions of the basal membrane (arrows). A: 200×; B–D: 400×.

Table 1
Histogram of histological scores of anti-CS immunostaining in proliferative and secretory endometrium, in normal rectosimoid and rectosigmoid with endometriosis. Values are mean ± standard error.

Tissue	CS (% of positive staining area)
Proliferative endometrium	12.43 ± 1.12[a]
Secretory endometrium	5.74 ± 0.72
Normal rectum	0.28 ± 0.11
Rectal endometriosis foci	17.57 ± 0.54[b]
External area of rectal endometriosis	4.93 ± 0.34

[a] $P < 0.05$ (the histological scores of CS immunodistribution area were statistically higher in proliferative than in secretory endometrium).
[b] $P < 0.05$ (the scores in the semi-quantitative evaluation of immunohistochemical staining for CS are significantly higher in rectal endometriosis than in normal rectum and in external area of rectal endometriosis, and also than the proliferative and secretory endometrium ($^*P < 0.05$)).

were significantly higher in rectosigmoid endometriosis foci than in the other sites (Table 1).

4. Discussion

Deeply infiltrating endometriosis is a particular form of the disease that is strongly associated with pelvic pain-related symptoms such as dysmenorrhea, deep dyspareunia, chronic pelvic pain and painful defecation (Chapron et al., 2005). The intensity of these symptoms seems to be correlated with the depth of infiltration, the location and the presence of increased activated and degranulating mast cells close to neuronal structures (Anaf et al., 2006).

Cell behavior is dependent on its environment. ECM composition can dictate cell proliferation, movements and morphology. GAGs such as DS, CS and HS are important ECM components that can influence these cell behaviors. The present study showed that in DIE of rectosigmoid, as well as in eutopic endometrium, CS concentrates significantly in the lesion foci.

The rectosigmoid tissues stained with DMB cationic dye contained abundant metachromatic material distributed throughout the stroma around the glands of endometriosis foci. In contrast, in the fibrotic area far from the lesion and in normal rectosigmoid tissue, the reaction was less intense or almost absent. These results indicate that in rectosigmoid endometriosis, the foci lesions contain abundant sulfated GAGs, similar to previous observations of normal endometrium tissue (Nasciutti et al., 2006).

Through biochemical analysis it was shown that the sulfated GAGs present in rectosigmoid tissues were DS, HS and CS, with a strong dominance of DS in comparison with HS and CS. Although this was not the same pattern of GAGs distribution found in normal endometrium tissue, where CS is the preponderant GAG (Nasciutti et al., 2006), the presence of CS was also confirmed in rectosigmoid tissues. These differences in concentrations of GAGs in rectosigmoid endometriosis and in eutopic endometrium could be explained by the difficulty, in rectosigmoid, of obtaining only the material from the endometriotic lesion area. For these experiments, we collected large amounts of rectosigmoid tissue that contained foci of endometriotic lesions and also normal areas.

Taking these results into account, we decided to assess the immunodistribution of CS in endometriosis and in normal rectosigmoid tissues. In this case, CS was observed in the same regions as the DMB metachromatic staining: the immunoreaction was concentrated in the endometriosis foci, distributed throughout the stroma around the glands. In the regions far from the lesions and in normal tissue, the immunoreaction was also very weak or absent. These results confirmed that in DIE of rectosig-

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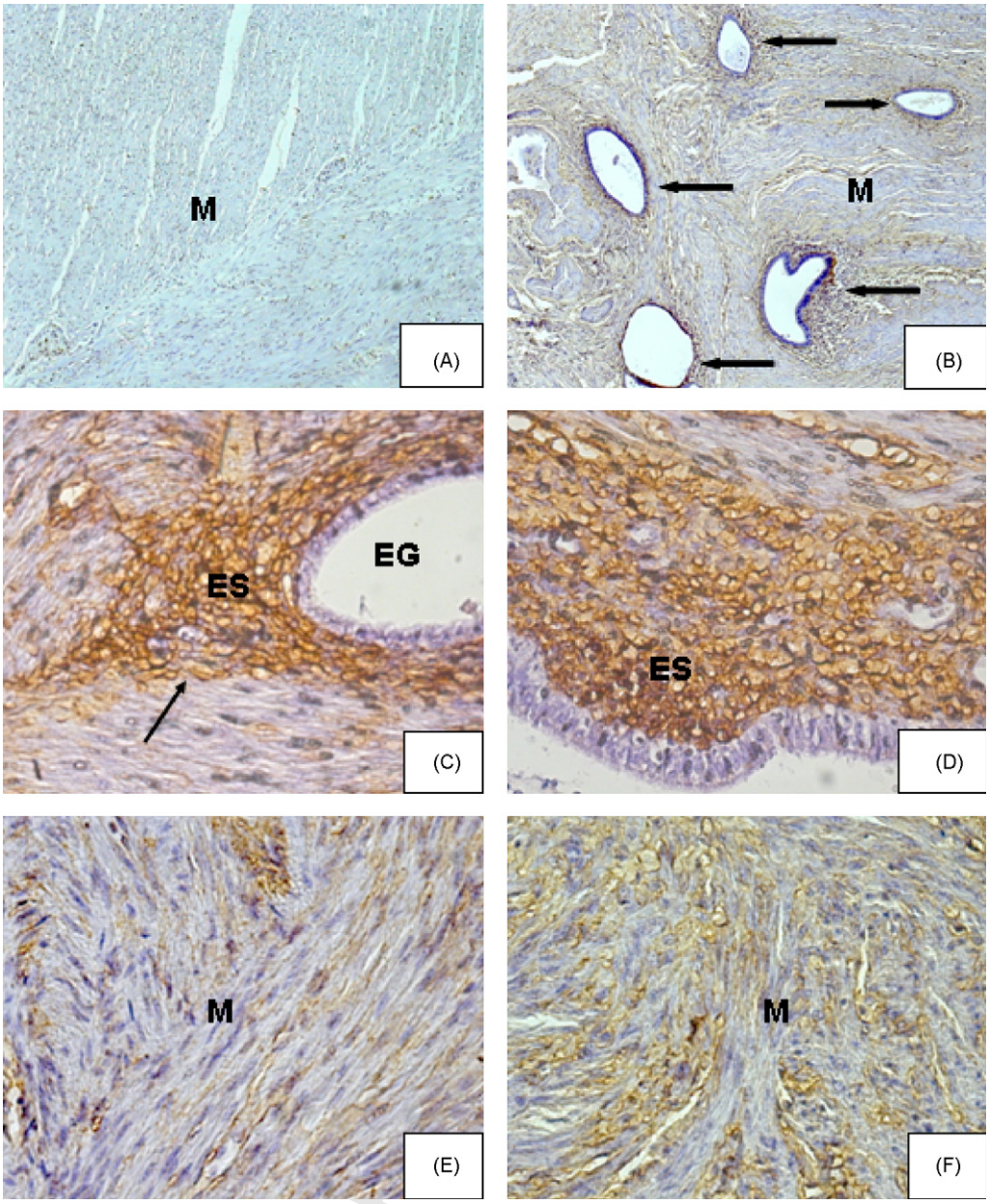

Fig. 4. Photomicrographs of normal (A) and endometriotic (B–F), rectosigmoid tissues immunostained with an antibody against CS. In normal rectosigmoid tissue (A), a very weak immunoreaction in the stroma is observed. In B, note 5 endometriosis foci (arrows), with the endometrium gland and stroma. In the endometriosis foci (C and D), the peroxidase product is concentrated and distributed throughout the endometrial stroma (ES), around the gland (EG). In the external area (EA) of endometriosis foci (C, E, F), the stroma immunoreaction is less intense. A and B: 100×; C–F: 400×.

moid, as in eutopic endometrium, CS was the predominant sulfated GAG present in the lesion foci.

The GAGs have documented associations with the growth and progression of malignant tumors. In various gastrointestinal carcinomas, the composition of GAGs in disaccharides was compared with those of normal tissues, and revealed an increase of non-sulfated and 6-sulfated disaccharides in the malignant tissues, suggesting a close relationship between the content of these GAGs in the malignant phenotype, the metastatic ability, and the survival time (Theocharis and Theocharis, 2002). Although endometriosis is not considered a malignant disease, the high concentration of CS in the lesion foci of deeply infiltrating rectosigmoid endometriosis could also be related to the aggressive and malignant-like condition of this pathology.

Endometriosis is considered a noncancerous gynecological condition, but it may be associated with extensive fibrosis. In rectovaginal endometriosis, the presence of fibrosis and aggregations of smooth muscles unassociated with blood vessels is correlated with the proliferation of endometriotic tissue and increased severity of the disease (Itoga et al., 2003). In the case of endometriosis, fibromuscular lesions have been considered to be the major cause of pelvic pain and sexual disfunction (Angioni et al., 2006). Ovarian tissue from patients with endometriosis showed a significant concentration of C-6S, produced by the myofibroblasts associated with tumor stroma (Nash et al., 2002). Similarly, we can suggest that in endometriosis, and especially in DIE of rectosigmoid, CS and DS concentrations in the stroma could play a role in the proliferation and differentiation of the cells in the lesion, and also could contribute to the modulation of fibrosis.

Recent studies have shown that the level of expression of CS PG 2 versican in endometrial endothelium is higher throughout the secretory phase of the cycle than in the proliferative phase, and it

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may be implicated in the interaction between selectin L and selectin L ligands functions in the postovulatory selective recruitment of peripheral blood CD16(−) natural killer (NK) cells into the human endometrium (Yamaguchi et al., 2006). In addition, Yasuo et al. (2008) showed that CS proteoglycan serglycin immunostaining was detected in stromal and endothelial cells, especially in the secretory phase, and suggested that serglycin may be a potential CS PG involved in extravasation of these NK cells into human endometrium, by the interaction between haematopoietic CD44 (CD44H) in peripheral blood CD16(−) NK cells and CS in endometrial endothelial cells. These findings provide new information regarding the role of CS in the human endometrium, and raise the question of whether CS in endometriotic lesions may also be related to the extravasation of NK cells. This delineable approach could be explored.

In addition, there is growing consensus that proteoglycans play an important role in the maintenance of vascular integrity. A recent study showed that proteoglycans are involved in angiogenesis by presenting and modulating a wide range of growth factors such as fibroblast growth factor-2 and -10 and vascular endothelial growth factor on their glycosaminoglycan side chains (Kirn-Safran et al., 2008). In our angiogenesis studies, we have demonstrated that vascularization and VEGF and its receptor Flk-1 expression are significantly higher in cases of endometriosis, particularly in DIE affecting the rectum, compared with the control samples and with the eutopic endometrium (Machado et al., 2008). Taken together, these studies suggest that the presence of CS in DIE could be related to the angiogenesis process, fundamental to the endometriosis establishment and growing.

In conclusion, this is the first report describing the pattern of histochemical and biochemical characterization of sulfated GAGs and the immunohistochemical location of CS in DIE of rectosigmoid demonstrating a significant concentration of CS in the stroma of endometriotic foci.

Acknowledgements

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