

Increased Numbers of Activated Mast Cells in Endometriosis Lesions Positive for Corticotropin-Releasing Hormone and Urocortin

Kempuraj D, Papadopoulou N, Stanford EJ, Christodoulou S, Madhappan B, Sant GR, Solage K, Adams T, Theoharides TC. Increased numbers of activated mast cells in endometriosis lesions positive for corticotropin-releasing hormone and urocortin. *AJRI* 2004; 52:267–275 © Blackwell Munksgaard, 2004

PROBLEM: Mast cells are critical in allergic and inflammatory diseases such as interstitial cystitis, which is often clinically associated with or mistaken as endometriosis. Mast cells had previously been reported to be increased at sites of endometriosis, and tryptase may contribute to the fibrosis and inflammation characterizing endometriosis.

METHOD OF STUDY: This is a pilot study of mast cell numbers and its activation in endometriosis biopsies ($n = 10$) by immunostaining for mast cell tryptase, corticotropin-releasing hormone (CRH) and urocortin (Ucn).

RESULTS: This is the first report that tryptase positive mast cells were not only increased (64–157 mast cells/mm²) in human endometriosis, but also highly activated (89%) in areas strongly stained positive for CRH/Ucn. Normal endometrium was weakly positive for both CRH/Ucn.

CONCLUSION: High numbers of activated mast cells are present in endometriosis sites that were strongly positive for CRH/Ucn. CRH and Ucn may activate mast cells and contribute to the fibrosis and inflammation in endometriosis.

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Key words: Corticotropin-releasing hormone, endometriosis, inflammation, mast cells, tryptase, urocortin

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Submitted March 8, 2004;
revised August 13, 2004;
accepted September 8, 2004.

INTRODUCTION

Endometriosis is a disorder characterized by the ectopic occurrence of endometrial tissue, primarily into the peritoneum; it is characterized by a wide variety of symptoms including pelvic pain.¹ Endometriosis is often associated with or mistaken for interstitial cystitis (IC) an inflammatory condition of the urinary bladder with chronic pelvic pain.² The ectopic endometrial tissue is surrounded by abundant fibrotic tissue and inflammatory infiltrate, but the triggering factors for these processes are not yet clearly understood. It was previously reported that mast cells were significantly

increased at sites of peritoneal endometriosis.³ Moreover, proteases secreted from mast cells play an important role in fibrogenesis.^{4,5} IC has increased and highly activated bladder mast cells,⁶ while endometrial mast cell activation was only reported in habitual abortions.⁷ Tryptase is a tetrameric serine protease that constitutes approximately 20% of the total protein within human mast cells. It is released in parallel with histamine from the cell upon activation and has been used as a marker for mast cells and their activation.⁸ Tryptase and chymase have been used as markers of distinct subpopulations of human mast cells, tryptase+ and chymase– (MC_T) and tryptase+ and chymase+ (MC_{TC}). The

release of tryptase from activated mast cells may stimulate the secretion of neighboring mast cells, thus providing an amplification signal for degranulation.⁹

Mast cells are essential for the elicitation of the allergic response, as well as the initiation of inflammatory reactions, by releasing several mediators including histamine, proteases, and several multifunctional cytokines such as interleukin-1 (IL-1), IL-6, IL-8, granulocyte macrophage-colony stimulating factor (GM-CSF), tumor necrosis factor- α (TNF- α), and transforming growth factor- β (TGF- β).¹⁰⁻¹² Inflammatory cytokines such as IL-1 α , IL-6, IL-8, IL-18, and TNF- α were reported to be increased in certain endometriosis.¹³⁻¹⁵ Tryptase stimulates selective synthesis of type 1 collagen in human lung fibroblasts⁴ and leads to the increased accumulation of type 1 collagen in endometriosis cases.³ Tryptase could also induce microvascular leakage⁹ or further inflammation through protease-activated receptors (PAR).¹⁶ Mast cell chymase cleaves type 1 procollagen to fibril-forming collagen molecules.⁵ Mast cells also synthesize and secrete corticotropin-releasing hormone (CRH), and its structurally related urocortin (Ucn).¹⁷ CRH and Ucn, may in turn, exert local effects in inflammatory disorders, especially those worsened by stress through activation of mast cells.^{11,18}

Here, we show for the first time that the increased mast cells in human peritoneal endometriosis tissue are highly activated and are found at sites strongly positive for CRH and Ucn. These results suggest a possible link between the local release of stress hormones, such as CRH, and mast cell activation.

MATERIALS AND METHODS

Sample Collection

Biopsies were obtained from 13 patients with ($n = 10$) or without ($n = 3$) endometriosis confirmed independently by the histologic presence of glands or stroma in the peritoneum. Multiple biopsies (2-4) were obtained from each of these patients; the biopsies analyzed from the endometriosis patients included endometriosis ($n = 10$) and unaffected peritoneum (UP, $n = 4$). In three patients with confirmed endometriosis (E), a peritoneal biopsy of presumed normal UP remote from the endometriosis implants was obtained, as well as a biopsy of UP in a patient without endometriosis. Additionally, we investigated uterine endometrial biopsies in two patients (all showing proliferative endometrium, PE). Some characteristics of these patients, presenting symptoms, medications as well as 'color' of the endometriosis implants (clear, red, brown, or black) are shown in Table I. Informed consent was obtained from each patient as approved by the relevant Human

Investigation Review Board. Patients in this study had failed medical therapy, including hormonal treatment for at least 3 months with no relief of symptoms. Patients had also not undergone any prior surgical procedures for at least 1 year. Tissues were fixed in freezing medium (Triangle Biomedical Sciences, Durham, NC, USA) immediately after collection. Cryostat sections (8 μ m) were prepared, dried and stored at -80°C until staining.

Immunohistochemical Staining for Tryptase

The sections were brought to room temperature (RT) and fixed with Carnoy's solution (60% ethanol, 30% chloroform, and 10% glacial acetic acid) for 3 min. The sections were then stained for mast cell tryptase by the alkaline phosphatase antialkaline phosphatase (APAAP) procedure using Dako APAAP Kit system (Dako Corporation, Carpinteria, CA, USA) as was reported previously.¹⁹ Briefly, the sections were incubated overnight at 4°C with mouse antihuman tryptase monoclonal antibody (Chemicon, Temecula, CA, USA) used at working dilutions of 1 $\mu\text{g}/\text{mL}$ in Tris-HCl-PBS (pH 7.6), plus 10% fetal bovine serum. The sections were then brought to RT and were first incubated with rabbit antiserum (Ig fraction) to mouse Ig for 30 min followed by incubation with the APAAP immune complex for another 30 min. Between each incubation, sections were rinsed in Tris-buffered saline (TBS pH 7.6) for 10 min. The reaction was finally developed with substrate solution (naphthol AS-MX phosphate, Fast Red and levamisole) for 20 min and then rinsed briefly in a water bath. Negative controls were performed either by the omission of the primary antibody or by using an isotype-matched mouse IgG1 antibody instead of the primary antibody.

Mast Cell Activation

Mast cells were counted by two different investigators, blind to the site, in high-power field ($\times 400$) from three non-consecutive sections of each sample; results are reported as mean \pm SD of mast cells/ mm^2 . Mast cell activation was assessed by the lack of uniform staining, and/or reduction in staining by $>30\%$ and the presence of extracellular tryptase as previously described.¹⁹ Mast cell activation was also confirmed by staining the samples metachromatically with acidified (pH 2) 0.1% Toluidine blue (TB) for 5 min at RT. TB binds to heparin in secretory granules and changes its color to red-purple (metachromasia).

Immunohistochemical Staining for CRH and Ucn

Sections were fixed with Carnoy's solution for 3 min at RT and were stained by the peroxidase method using Dako EnVision System, Peroxidase Kit (Dako Corp.), as previously reported.¹⁷ Briefly, sections were

TABLE I. Characteristics of the Biopsies

Patients	Age (years)	Gravida	Parity	Symptoms	Medications	Previous history	Biopsy sites	Biopsy color
JN	20	2	1	LLQ pain, dysfunctional uterine bleeding	Cyclic OC, antidepressant	–	Cul-de-sac	Black
JD	23	2	2	Bilateral pelvic pain, vulvar pain	Cyclic OC	IC	Parietal peritoneum over bladder, visceral peritoneum on large intestines	Black
KT	24	1	1	Bilateral pelvic pain	Daily OC, previously on amitriptyline	Failed DepoLupron	Right uterosacral ligament, right pelvic side wall	Black/tan
BD	21	2	1	Infertility, bilateral pelvic pain	–	–	Bilateral uterosacral ligaments	Red/black
DD	40	2	2	Pelvic pain	Paxil	–	Endometrium	Brown
RL	30	2	2	Pelvic pain	–	Vestibulitis	Endometrium Perimetrium Endometrium	Brown N/A N/A
AS	30	6	1	Pelvic pain	–	–	Endometrium Peritoneum	Brown N/A
SW	35	2	3	Pelvic pain	–	–	Endometrium Peritoneum	Brown N/A
LP	38	3	2	LLQ pain	Daily OC, elmiron	IC	Parietal peritoneum in the ovarian fossa	Red
JED	32	4	3	Vestibulitis	–	–	Endometrium Peritoneum	Clear N/A
JD	21	2	1	–	–	–	Endometrium Peritoneum	N/A N/A
MM	70	3	3	–	Doxepin	–	Peritoneum	N/A
SH	24	2	2	Vulvar pain Pelvic pain	–	IC	Endometrium Peritoneum	N/A N/A

LLQ, left lower quadrant; RLQ, right lower quadrant; IC, interstitial cystitis; OC, oral contraceptive.

incubated with peroxidase blocking reagent (0.03% hydrogen peroxide containing sodium azide) for 5 min and placed in TBS for another 5 min. Sections were then incubated either with rabbit anti-CRH (human) serum (Phoenix Pharmaceuticals, Inc. Belmont, CA, USA) or rabbit anti-Ucn (human) serum (Phoenix) at 1:500 dilution for 30 min. Sections were placed in TBS for 5 min and incubated with peroxidase-labeled polymer conjugated to goat anti-rabbit and anti-mouse Igs for 30 min. Sections were then rinsed with TBS and incubated with substrate 3,3'-diamino-benzidine chromogen solution for 5 min. All the incubations were carried out at RT. The presence of a brown-colored end product at the site of the target antigens indicated positive reactivity. Negative controls contained samples in which the primary antibody was omitted or replaced by normal rabbit serum.

Statistics

Statistical comparison of mast cell numbers/mm² between endometriosis and control (normal endometrium/UP) samples was made using unpaired Student's *t*-test. Statistical significance is denoted as $P < 0.05$. No statistics was performed between samples based on the biopsies color, as there was only one clear, one red, one red/black, one black/tan, four brown and two black samples ($N = 10$) as described in the tables.

RESULTS

In the present study, increased numbers of mast cells were identified in endometriosis tissue, by positive immunostaining for mast cell specific tryptase

TABLE II. Number of Mast Cells and State of Activation^a

Subjects (initials)	Bx site/diagnosis	Total mast cells (per mm ²)	Activated mast cells (per mm ²)	Percent mast cell activated (% total)	Biopsy color
JN	E	157 ± 5	143 ± 1	91.0	Black
JD	E	79.6 ± 35	74.3 ± 38	93.3	Black
KT	E	79.6 ± 25	73 ± 28	91.7	Black/tan
BD	E	119 ± 62	109 ± 56	91.5	Red/black
DD	E	112 ± 28	101 ± 18	91.0	Brown
RL	UP	20 ± 7	0	0	
	E	105 ± 14	92 ± 4	87.9	Brown
AS	E	121 ± 38	100 ± 41	81.5	Brown
	UP	24 ± 7.5	0	–	
SW	E	117 ± 21	103 ± 31	87.0	Brown
	UP	27 ± 24	6 ± 12	12.3	
LP	E	64 ± 3	56 ± 4	87.5	Red
JED	E	11 ± 1	0	–	Clear
JD	NE	0	–	–	–
MM	UP	17 ± 8	0	–	
SH	Vulvar	20 ± 7	0	–	
	PE	24 ± 17	2 ± 3	5.0	

^aHigh power field from three non-consecutive sections; mast cells expressed as mean ± SD; E, biopsy-proven endometriosis; NE, normal endometrium; PE, proliferative endometrium; UP, unaffected peritoneum; Bx=biopsy.

(Table II). Positive staining resulted in intense red color at sites of tryptase presence (Fig. 1A–C). A significant finding is that normal endometrium (Fig. 1C) or UP from the same patient with biopsy-proven endometriosis had fewer mast cells and negligible activation. Negative controls with omission of the primary antibody or incubated with an isotype matched mouse IgG1 antibody, instead of the primary antibody, did not show any positive staining for tryptase (Fig. 1D). The mean total number of mast cells in the nine endometriosis samples (excluding the clear lesion) studied varied from 157 ± 5 (black lesion) to 64 ± 3 (red lesion) mast cells/mm² as summarized in Table II. The mean total number of mast cells in lesions with black/brown lesions was 115 ± 25 cells/mm²; this was significantly higher as compared with 64 ± 3 mast cells/mm² in the single red lesion ($P < 0.05$). Most of the mast cells were highly activated (89.6%) and degranulated in endometriosis lesions (Fig. 1A,B) as compared with the control tissues studied. The mean total activated mast cells varied from 143 ± 1 (black lesion) to 56 ± 4 (red lesion) (Fig. 2 and Table II). The mean total mast cells in the endometriosis (106 ± 28 cells/mm²) excluding the clear lesion, was significantly ($P < 0.05$) higher when compared with control tissue (17.6 ± 9.4 cells/mm²), as shown in Fig. 2. Total activated mast cell in endometriosis lesions were 95 ± 25 cells/mm². Less than 10% of mast cells were activated at some of normal unaffected sites. Mast cell count and its

activation were also confirmed by staining of mast cells metachromatically with TB.

The endometriosis biopsies and normal endometrium samples were also immunostained for the presence of CRH and Ucn. Positive reactions resulted in brown-colored staining at the site of target antigens, CRH and Ucn. Sections from endometriosis lesions incubated with polyclonal rabbit antihuman CRH or polyclonal rabbit antihuman Ucn stained positively for CRH (Fig. 3A), as well as for Ucn (Fig. 3B). Normal endometrium was weakly positive for both CRH (Fig. 3C) and Ucn (data not shown), as compared with endometriosis lesions. Negative control sections incubated with normal rabbit serum did not show any positive reaction (Fig. 3D).

DISCUSSION

In this pilot study, we confirmed that endometriosis lesions contained more mast cells as compared with the UP or proliferative endometrium. Here we show for the first time that mast cells were highly activated in all the biopsy-proven endometriosis lesions except for the clear lesion. Black/brown lesions were strongly positive compared with the red lesion; mast cells in normal tissues were largely intact. Only one study in humans³ previously reported increased mast cells in 'black' as compared with 'red' endometriosis tissue, as compared with eutopic control endometrium. They also found

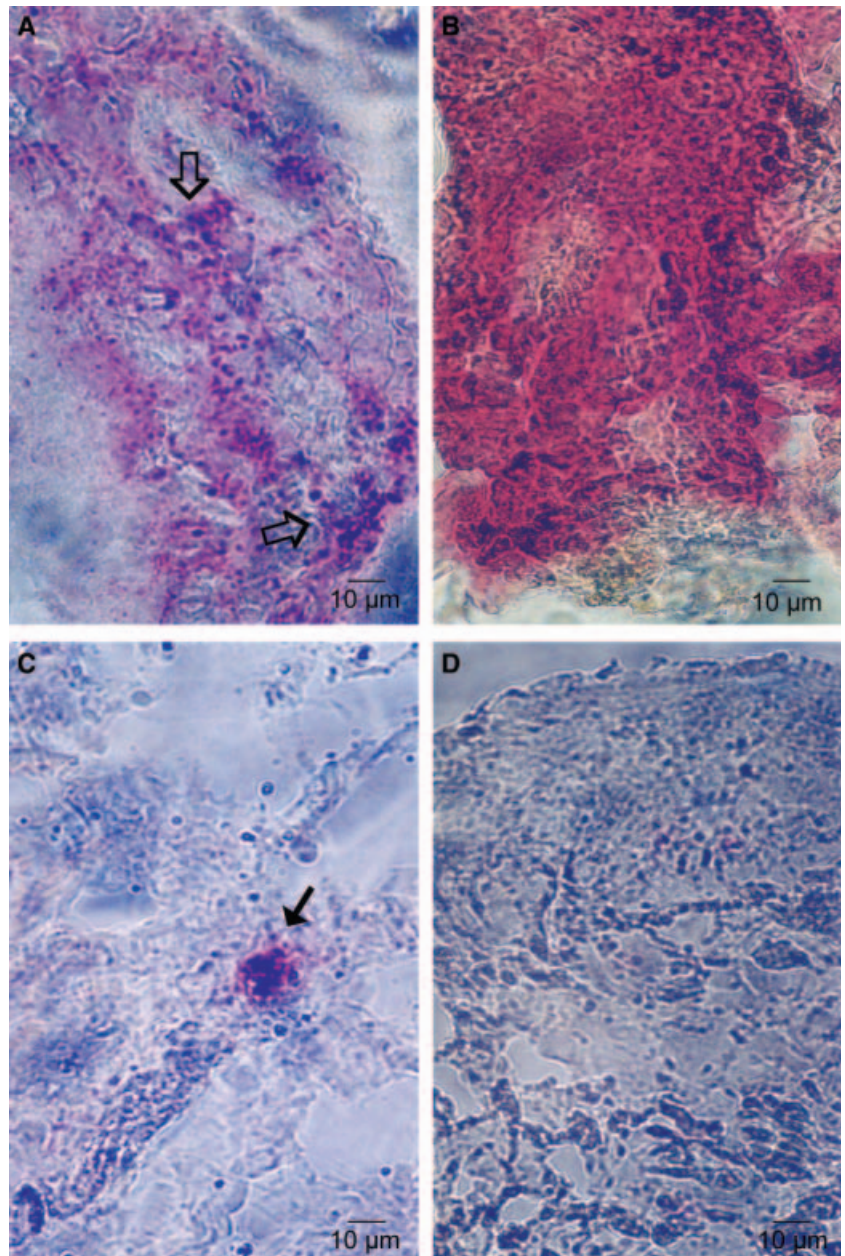


Fig. 1. Photomicrographs of endometriosis biopsies immunostained for mast cell tryptase. Cryostat sections were incubated with mouse antihuman tryptase monoclonal antibody. Positive staining resulted in bright red color at the site of the target antigen tryptase (A–C). Most of the mast cells were activated in endometriosis, as shown by the extracellular presence \Rightarrow of secretory granule material (A, B) due to mast cell degranulation. However, mast cells were intact \rightarrow in normal endometrial tissue (C). Negative controls incubated with an isotype-matched mouse IgG1 antibody, instead of the primary antibody, did not show any positive staining for tryptase (D).

that no cyclical change in mast cell density of the whole layer of eutopic endometrium from patients without endometriosis.³ In peritoneal endometriosis they reported markedly heterogeneous distribution of mast cells; however, they did not examine mast cell activation or the CRH/Ucn in these samples.³ Their report and our present results support the concept that black and red peritoneal lesions may be different stages of the spontaneous evolution of endometriotic implants, with red lesions as the first stage. The clear lesion, which was noted to be consistent with endometriosis with stroma on pathologic diagnosis, is presumably an early implant or a ‘drop implant’ and may represent a lesion that has not initiated a fibrotic

reaction as indicated by its low number of mast cells and the lack of activation.

The increased numbers, as well as the increased degree of activation of mast cells, in endometriosis lesions is higher than what has been reported in the painful bladder inflammatory condition IC, which is often present with or mistaken for endometriosis.⁶ The present study shows that mast cells in the UP and normal proliferative endometrium are not activated. Similarly, no activation of mast cells had been reported previously in normal endometrium.²⁰ Therefore, if the mechanism of action is that normal endometrium is deposited by retrograde menstruation into the peritoneal cavity, a local phenomenon

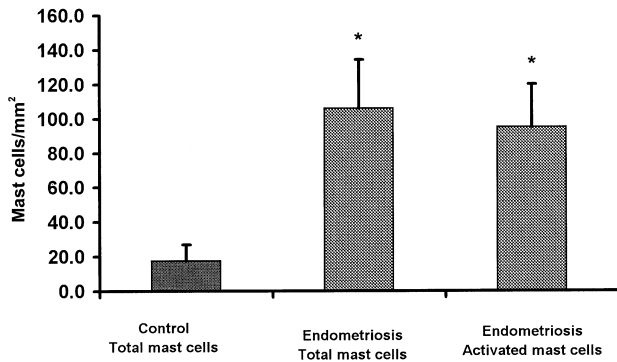


Fig. 2. Total mast cell numbers and its activation in endometriosis lesions. Cryostat sections of endometriosis biopsies were immunostained for mast cell tryptase. Both activated and intact mast cells were counted in three non-consecutive sections from each patient using high power field (mast cells per mm²). Endometriosis samples show significantly increased numbers, as well as highly activated mast cells as compared with control samples (* $p < 0.05$). Mast cells in control tissue did not show any significant activation.

occurs that increases mast cell number and causes activation presumably leading to the associated scarring and fibrosis seen with endometriosis. In another study, no significant difference was found in the number of mast cells in endometrial cysts at different stages of endometriosis;²¹ however, mast cells were numerous around blood vessels in the interstitium with fibrosis and appeared degranulated by electron microscope.²¹

Mast cells are recognized as key effector cells of immediate-type allergic reactions, but they might also be involved in IgE-independent inflammatory and tissue repair processes by releasing inflammatory mediators and cytokines; these include IL-1, IL-6, IL-8, GM-CSF, TNF- α , CRH, Ucn, TGF- β .^{10,11} Stem cell factor (SCF), a growth factor for human mast cells is increased in peritoneal fluid of patients with endometriosis,²² suggesting a role in mast cell proliferation in affected peritoneum. SCF has been shown to cause

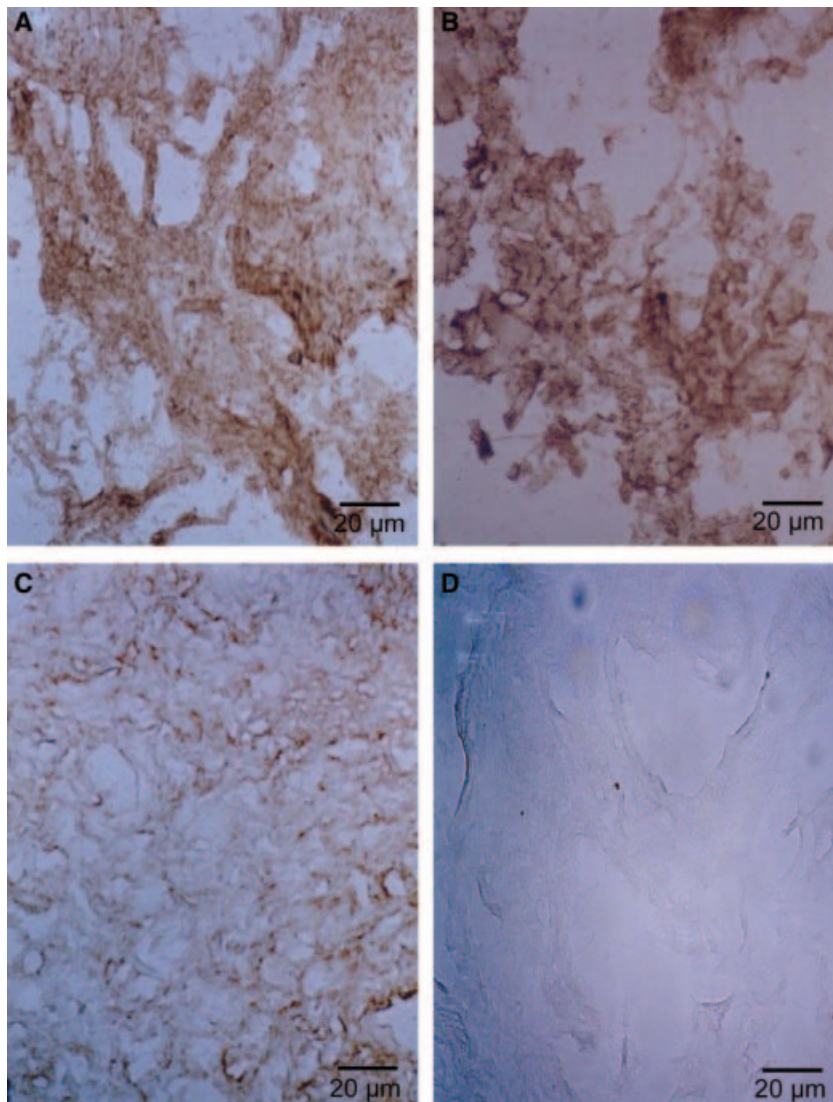


Fig. 3. Photomicrographs of endometriosis lesions immunostained for CRH and Ucn. Sections were stained with rabbit anti-CRH (human) serum or rabbit anti-Ucn (human) serum. Positive reactions resulted in brown-colored staining at the site of target antigens CRH/Ucn. Endometriosis biopsy showed strong positive staining reaction for CRH (A) and Ucn (B). However, normal endometrial tissue showed weak-positive staining for CRH (C) and Ucn (picture not shown). Negative control using normal rabbit serum did not show any positive reaction (D).

activation of mast cells *in vitro*, and the endometriotic tissue expresses SCF receptors.²² High follicular fluid histamine levels have also been reported in infertile women with pelvic adhesions, as compared with women without adhesions,²³ indicating activation of peritoneal mast cells. In addition, a rat endometriosis model developed by uterine autotransplantation to the peritoneum, showed proliferation and infiltration of mast cells in the peritoneal stromal tissue, as well as presence of other cells related to allergic reactions;²⁴ in this model, mast cells showed degranulation at 4 days after the implantation, and disappeared by day 14. In another study using experimental rat endometriosis, treatment with a leukotriene receptor antagonist suppressed infiltration and activation of mast cells, as well as stromal proliferation.²⁵

There is substantial evidence that immunologic factors play a role in the pathogenesis of endometriosis and endometriosis-associated infertility.^{15,26} Increased inflammatory cytokines such as IL-1 α , IL-6, IL-8, IL-18, TNF- α have been reported in endometriosis.¹³⁻¹⁵ Increased levels of IL-8, in particular, have been reported in the peritoneal fluid of women with endometriosis, as compared with healthy women, and IL-8 is an important cytokine in the recruitment of leukocytes to the endometrium.^{27,28} IL-8 may derive from endometrial epithelial cells,²⁸ but is also the most abundant cytokine of human mast cells.²⁹ During inflammation, tryptase can stimulate the release of IL-8 from epithelial cells.³⁰ TNF- α ^{31,32} and TGF- β ³² have also been reported to play a role in the establishment and maintenance of endometriosis. Mast cell chymase was shown to participate in the pathogenesis of pulmonary fibrosis that appeared to be mediated at least in part by TGF- β 1.³³ Most recently, it was shown that mouse mast cell protease-7, which is expressed in differentiated mast cells, is transcriptionally activated by activin A and TGF- β 1 in bone marrow derived cultured mast cells.³⁴

Mast cell tryptase stimulates selective synthesis of type 1 collagen in human lung fibroblasts⁴ and contributes to the accumulation of type 1 collagen in the stroma.³ Tryptase also induces microvascular leakage⁹ permitting exit of circulating inflammatory cells to the tissues. Moreover, tryptase cleaves PAR2 and induces widespread inflammation, partially through the release of the proinflammatory neuropeptide substance P (SP).¹⁶ A large proportion of primary spinal afferent neurons express PAR2 and contain SP.¹⁶ Mast cells from a variety of sites, such as endometrium, myometrium¹⁸ and the urinary bladder,³⁵ are closely associated with SP-positive neurons and respond to SP with histamine release,³⁶ as well as TNF- α gene expression and TNF- α production.³⁷ These findings support the concept that neuropeptide-

mediated mast cell cytokine release contributes to neurogenic inflammation.¹¹

This is the first report to our knowledge that sites of peritoneal endometriosis are strongly positive for both CRH and Ucn, while normal proliferative endometrium is weakly positive for both peptides. Expression of mRNA has been shown in normal endometrium throughout the menstrual cycle.^{38,39} CRH and CRH-binding protein was also measured in peritoneal fluid of patients with pelvic adhesions or with endometriosis. There were no significant differences compared with normal women; only patients from advanced stages of endometriosis had their peritoneal fluid CRH-binding protein levels higher than in healthy subjects or those patients with a lower grade of endometriosis.⁴⁰ This finding may suggest an attempt by the body to neutralize high CRH levels otherwise present in affected endometrium. Increased numbers of activated mast cells and high amount of locally produced CRH and Ucn were reported in products of conception from habitual spontaneous abortions.⁷ Moreover, CRH⁴¹ and Ucn⁴² were shown to induce skin mast cell degranulation and could activate mast cells in endometriosis. In fact, mast cells were recently reported to express a number of CRH receptor isoforms⁴³ and be a rich source of CRH/Ucn, themselves.¹⁷ These findings, together with the fact that endometrial cells express both types of CRH receptors 1 and 2,^{44,45} suggest a possible autocrine/paracrine role of CRH and Ucn in the regulation of endometrial pathophysiology.

CONCLUSIONS

Increased numbers of highly activated mast cells were identified in peritoneal endometriosis tissue, as compared with UP or proliferative endometrium from the same patients. Affected tissue also stained strongly for CRH or Ucn suggesting they may be associated with activated mast cells. These processes could contribute to the fibrosis, inflammation, low fertility or spontaneous abortions associated with endometriosis.

Acknowledgements

This work was supported in part by Theta Biomedical Consulting and Development Co., Inc. (Brookline, MA, USA) to TCT. We thank Ms Jessica Christian for her patience and word processing skills.

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