Induction of the Neurokinin 1 Receptor by TNFα in Endometriotic Tissue Provides the Potential for Neurogenic Control Over Endometriotic Lesion Growth

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Context: Endometriosis is characterized by the growth of ectopic endometrial tissue. Nerve fibers are frequently associated with ectopic lesions, and neurogenic inflammation may play a role in endometriosis.

Objective: The purpose of this study was to determine the presence of tachykinin receptors in endometriotic lesions and the role of TNFα on their expression.

Design: This study was an assessment of matching eutopic and ectopic endometrial tissue and peritoneal fluid from patients with endometriosis and an in vitro analysis of primary endometrial cells.

Setting: The setting was a university hospital.

Patients: Participants were premenopausal women undergoing laparoscopy.

Interventions: Endometriotic lesions were removed surgically.

Main Outcome Measures: Tachykinin mRNA (TACR1/2) and protein (neurokinin 1 receptor [NK1R]) expression in both eutopic and ectopic endometrial tissue from patients with endometriosis and the correlation to peritoneal fluid TNFα were measured. Primary endometrial epithelial and stromal cells were assessed in vitro to determine the induction of TACR1/2 and NK1R expression after TNFα treatment. Cell viability of endometrial stromal cells after substance P exposure was also assessed.

Results: Expression of both TACR1 and TACR2 mRNA was significantly higher in the ectopic than in the eutopic tissue. Both TACR1 mRNA and NK1R protein expression was significantly correlated with peritoneal fluid TNFα, and in vitro studies confirmed that TNFα treatment induced both TACR1 mRNA and NK1R protein expression in endometrial stromal cells. In endometrial stromal cells, substance P treatment enhanced cell viability, which was inhibited by a specific NK1R antagonist.

Conclusions: NK1R expression is induced in ectopic endometrial tissue by peritoneal TNFα. Induction of NK1R expression may permit endometriotic lesion maintenance via exposure to substance P. **(*J Clin Endocrinol Metab* 98: 2469–2477, 2013)**

Endometriosis is characterized by the growth of endometrial epithelial and stromal cells outside the uterine cavity. The growth of ectopic endometrial lesions can result in severe dysmenorrhea, chronic pelvic pain, and reduced fecundity. It is a prevalent disease affecting approximately 10% of women during their reproductive years (1) and represents a significant reduction in the quality of life and a burden on health care systems (2). The mechanisms

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Abbreviations: BSA, bovine serum albumin; DAPI, 4,6-diamidino-2-phenylindole; EEC, endometrial epithelial cell; ESC, endometrial stromal cell; NK1R, neurokinin 1 receptor; NK2R, neurokinin 2 receptor; PBS, phosphate-buffered saline; PBST, phosphate-buffered saline with 0.1% Tween 20; RVS, rectovaginal septum; SP, substance P.

that underlie both the pathophysiology of growth of lesions and the pain they cause are unknown, although an aberrant immune response after retrograde menstruation plays an important role (3).

Sensory nerve fibers can innervate endometriotic lesions, and thus neurogenic inflammation may have a role in the pathogenesis of endometriosis. Although early studies found no difference in the number of nerve fibers between women with and without endometriotic lesions in either the peritoneal wall (4, 5) or the rectovaginal septum (RVS) region (6), there was a significant difference reported in the degree of intraneurial and perineurial invasion of RVS lesions (6) and an increased density of nerve fibers proximal to endometriotic lesions on the peritoneal wall (7), a high proportion of which were confirmed as sensory nerve fibers (5, 7). Sensory nerve fibers transmit afferent pain signals and can also elicit a local response via neurogenic inflammation. Substance P (SP), a member of the tachykinin family, is one of the strongest mediators of neurogenic inflammation (8, 9) and is secreted by afferent sensory neurons (10). It has previously been detected in sensory nerve fibers close to endometriotic lesions (5, 7) and is present in peritoneal fluid (11).

SP can activate neurogenic inflammation by binding to the neurokinin 1 receptor (NK1R). NK1R is up-regulated by inflammation (12) and is involved in a number of inflammatory conditions (13, 14). The presence of NK1R or the gene that encodes it, *TACR1*, has not been reported previously in endometriotic lesions despite the importance of inflammation to the progression of the disease. We proposed that endometriotic lesions express NK1R, that this expression is related to the inflammatory microenvironment, and that activation of NK1R will affect endometriotic lesion viability. We therefore examined the expression of NK1R in both eutopic and ectopic endometrial tissue and determined whether this expression was related to inflammatory cytokines present in the peritoneal fluid. In addition we also assessed the viability of endometrial cells in vitro after exposure to SP.

Materials and Methods

Sample collection and patient data

Institutional review board approval was obtained from the ethics committee before commencement of the study. During laparoscopic surgery to investigate pelvic pain or infertility, any endometriotic lesions identified were removed, their location was noted (peritoneum, ovary, or RVS), and they were stored in either dimethyl sulfoxide (Sigma, Buchs, Switzerland) or RNA*later* (Invitrogen Life Technologies, Zug, Switzerland) or frozen in liquid nitrogen. Peritoneal fluid was collected from the pouch of Douglas (cul-de-sac), clarified by centrifugation, and stored at −80°C. Eutopic endometrial biopsy samples were obtained using a soft curette (Pipelle de Cornier, Laboratoire C.C.D., Paris, France) and treated the same way as the lesions. Exclusion criteria for the study included prior or current infections, liver dysfunction, or the use of GnRH analogs within the past 3 months. All laparoscopies were performed during the proliferative phase of the menstrual cycle.

During the study period, 35 distinct endometriotic lesions were collected with a matching eutopic endometrial biopsy sample during the same operation. The major indications for surgery included dysmenorrhea (19 samples), pelvic pain (9 samples), dyspareunia (2 samples), and infertility (5 samples). Of these 35 samples, 21 were stored in RNA*later* and 14 were stored fresh-frozen. The ectopic samples stored in RNA*later* included 8 lesions from the peritoneum, 9 lesions from the ovaries, and 4 lesions from the RVS. The fresh-frozen ectopic samples included 3 lesions from the peritoneum, 7 lesions from the ovaries, and 4 lesions from the RVS.

Isolation and culture of endometrial epithelial and stromal cells

Primary endometrial epithelial cells (EECs) and endometrial stromal cells (ESCs) were isolated from endometrial biopsy samples collected during the proliferative phase and stored in dimethyl sulfoxide. Separation was performed via collagenase digestion and size exclusion membranes as described previously (15). A total of 6 primary cell isolations were performed, and sufficient EECs and ESCs were isolated in 4 and 6 preparations, respectively. All preparations were from women with confirmed endometriosis, and no hormonal therapy for at least 3 months before surgery.

Isolated EECs and ESCs were maintained in Dulbecco's modified Eagle's medium (Invitrogen Life Technologies) supplemented with 10% fetal calf serum (Invitrogen Life Technologies) and 1% antibiotic/antimyotic (Invitrogen Life Technologies). Cells were seeded into 6-well (ESCs) or 24 well plates (EECs) for subsequent RNA collection or onto sterile coverslips for immunofluorescence. Before treatments, cells were serum-starved overnight (Dulbecco's modified Eagle's medium with 0.5% fetal calf serum) and treatment was with either 0, 10, or 100 ng/mL TNFα for 6 hours.

Determination of gene expression in eutopic and ectopic tissue

Approximately 30 mg of tissue from both the eutopic endometrial biopsy samples and ectopic endometriotic lesions was excised and homogenized in FastPrep 120 tissue homogenizer (30 seconds at 4.0 m/s) in cell lysis buffer (Qiagen, Düsseldorf, Germany). The remaining RNA isolation was performed with an RNAeasy mini kit (Qiagen) and TURBO DNase (Ambion Life Technologies, Zug, Switzerland) for genomic DNase digestion. One microgram of total RNA was reverse transcribed in a 25-μL reaction with Moloney murine leukemia virus reverse transcriptase (Promega, Dübendorf, Switzerland) and random primers. The resulting cDNA was diluted 1:20, and the absence of genomic DNA was confirmed with a reverse transcriptase control.

Quantitative real-time PCR was performed with TaqMan Fast Advanced Master Mix (Invitrogen Life Technologies) and a Rotor-Gene RG 2000 (Corbett Research, Sydney, NSW, Australia), under the following conditions: 50°C for 2 minutes and then 95°C for 20 seconds, followed by 40 cycles of 95°C for 1

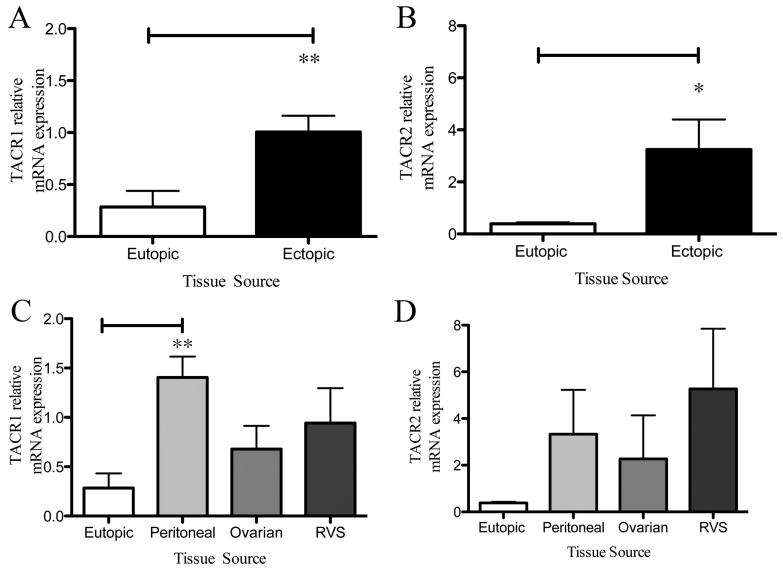

Figure 1. TACR1 and TACR2 expression in eutopic and ectopic tissue from patients with endometriosis. In matching ectopic and eutopic endometrial tissue from 21 patients, the expression of TACR1 (eutopic = 0.2848 ± 0.1543 vs ectopic 1.006 ± 0.1563, n = 21, P = .0021) (A) and TACR2 (eutopic = 0.3919 ± 0.04818 vs ectopic = 3.244 ± 1.153, n = 21, P = .0178) (B) was significantly stronger in the ectopic lesions than in the eutopic tissue. C, When ectopic lesions were separated based on lesion location, there was a significant variation between TACR1 expression, as determined by a 1-way ANOVA ($F_{3,39}$ = 5.659, P = .0026). A Bonferroni post hoc test confirmed that TACR1 mRNA expression in peritoneal lesions (1.405 ± 0.2110, n = 8, $P < .01$) was significantly higher than expression in the eutopic endometrium. No significant difference was observed, however, between the ovarian lesions (0.6789 ± 0.2361, n = 9, $P > .05$) or the RVS lesions (0.9425 ± 0.3541, n = 4, $P > .05$). D, In contrast, TACR2 mRNA expression did not show significant variation ($F_{3,39}$ = 2.744, P = .0560) between eutopic endometrium and ectopic lesions on either the peritoneal wall (3.329 ± 1.904, n = 8, $P > .05$), the ovaries (2.268 ± 1.867, n = 9, $P > .05$), or the RVS (5.273 ± 2.578, n = 4, $P > .05$). All values are presented as means ± SEM. $*P < .05$, $**P < .01$.

second and 60°C for 20 seconds. Product size was confirmed on a 4% agarose gel.

A TaqMan gene expression assay was used for the genes of interest: *TACR1* (NM_001058; Hs00185530_m1), *TACR2* (NM_001057; Hs00169052_m1), and *TACR3* (NM_001050.1; Hs00357277_m1) and the reference genes *GAPDH* (NM_002046; Hs00266705_g1), *ACTB* (NM_001101; Hs01060665_g1), *YWHAZ* (NM_003406; Hs03044281_g1), and *RPL13A* (NM_012423; Hs04194366_g1) (Invitrogen Life Technologies). The number of and most stable reference genes were selected via the geNORM software program, which is part of the qBASE software suite (Biogazelle, Zwijinaarde, Gent, Belgium), and a geometric mean of all 4 reference genes was used to normalize *TACR1/2* expression for each tissue type (16). The reaction efficiency of each assay was determined via linear regression (17), and the fold change was calculated with qBASEplus software.

Determination of NK1R protein expression in ectopic endometrial tissue

Whole cell extracts were prepared from fresh-frozen ectopic endometrial tissue by homogenization with the FastPrep 120 tissue homogenizer (30 seconds at 4.0 m/s) in radioimmunoprecipitation assay buffer (50 mM Tris · Cl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate, and 1% protease and phosphatase inhibitor cocktail [Cell Signaling Technology, Danvers, Massachusetts]). Protein concentrations were determined by the bicinchoninic acid assay (QuantiPro BCA; Sigma), and approximately 20 μg of protein was diluted in LDS Sample Buffer (Invitrogen Life Technologies), heated to 70°C for 10 minutes, and separated on a 4% to 15% Novex NuPAGE Bis/Tris gel (Invitrogen Life Technologies).

Proteins were transferred to a 0.45-μm nitrocellulose membrane in 4-morpholinepropanesulfonic acid buffer (MOPS; Invitrogen Life Technologies), and nonspecific staining was blocked by incubation overnight with 5% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) with 0.1% Tween 20 (PBST). Membranes were probed with both rabbit antihuman NK1R antibody (Thermo Scientific, Lausanne, Switzerland) and mouse antihuman actin-β antibody (Abcam, Cambridge, UK) diluted 1:5000 and 1:1000, respectively, in 5% BSA in PBST overnight. Secondary antirabbit (GE Healthcare, Opfikon, Switzerland) and antimouse (Sigma) antibodies conjugated to horseradish peroxidase were diluted 1:50 000 and 1:120 000, respectively, in 5% BSA in PBST, and immunocomplex detection was performed with the SuperSignal West Femto Kit (Pierce; Thermo Scientific) and a Bio-Rad ChemiDoc XRS+ system (Bio-Rad Laboratories AG, Cressier, Switzerland). Band densitometry was analyzed with Quantity One analysis software (Bio-Rad Laboratories AG). NK1R expression was normalized to actin-β expression, and a positive control reference sample was included on each gel for comparison across membranes.

Determination of peritoneal fluid cytokine concentrations

To confirm that material was taken during the proliferative phase, the progesterone concentrations in the peritoneal fluid were determined via a RIA (Coat-A-Count, DPC; Buhlmann Laboratories, Allschwil, Switzerland) with a cutoff value of 27 nmol/L. The protein concentration in the peritoneal fluid was determined via the BCA assay and the concentration of TNFα was calculated with an ELISA as described previously (3, 4).

Immunofluorescence

EECs and ESCs were seeded onto coverslips, treated with TNFα (0, 10, and 100 ng/mL) for 6 hours, and fixed in 4% paraformaldehyde in PBS for 10 minutes. Cell membranes were permeabilized with 2% Triton X-100 in PBS for 15 minutes and then were incubated with either rabbit antihuman NK1R antibody (Thermo Scientific) alone (ESCs) or in combination with a mouse anti-pan cytokeratin antibody (Abcam) (EECs) diluted 1:200 and 1:250, respectively, in 0.1% BSA in PBS for 1 hour. After brief washing, cells were incubated with goat antirabbit IgG DyLight 488 and antimouse IgG DyLight 594 secondary antibodies (Thermo Scientific) diluted 1:200 in 0.1% BSA in

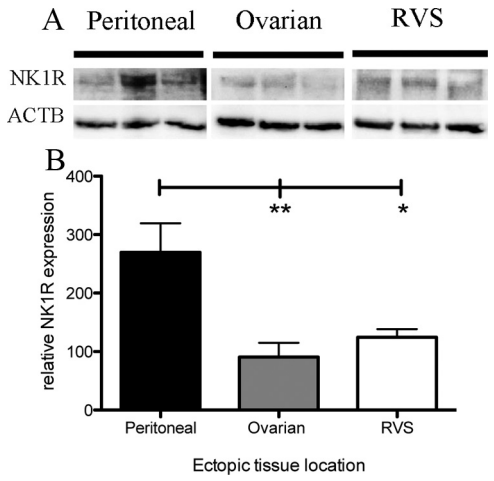

Figure 2. NK1R protein expression in endometriotic lesions. A, Western blot analysis with a specific rabbit antihuman NK1R antibody confirmed that NK1R protein was present in the ectopic endometriotic lesions. NK1R expression was calculated by normalization within tissue to actin-β and across tissue with a positive control reference sample. B, Semiquantitative analysis of band densitometry using a 1-way ANOVA showed a significant difference ($F_{2,11} = 9.046$, $P = .0048$) among the 3 groups based on lesion location. A Bonferroni post hoc test indicated that expression of NK1R was significantly stronger in the peritoneal lesions (269.90 ± 49.46, n = 3) than in either the RVS (124.40 ± 14.01, n = 4, $P < .05$) or ovarian lesions (90.72 ± 24.23, n = 7, $P < .01$). All values are expressed as means ± SEM. *$P < .05$, **$P < .01$.

PBS. Cells were incubated in 4,6-diamidino-2-phenylindole (DAPI), 1:5000 for 5 minutes, washed, and mounted onto slides with ProLong Gold antifade (Invitrogen Life Technologies).

Images were captured with a Zeiss Axiovert 40 inverted transmitted-light microscope with a ×20 objective and an AxioCam MRm camera and AxioVision 4.6.3 software (Carl Zeiss, Goettingen, Germany).

Measurement of cell viability

ESCs were plated into 96-well plates at a density of 6000 cells/well and grown for a period of 4 to 6 days until approximately 80% confluent. Cells were serum starved overnight before any treatment. Cells were pretreated with 100 ng/mL TNFα for 6 hours and treated with 10 nM SP, either in the presence or absence of 100 nM of L733 606, a specific antagonist for NK1R. After 24 hours of treatment, cell viability was measured by the CellTiter 96 AQ_{ueous} One Solution Cell Proliferation Assay (Promega) according to the manufacturer's instructions. All treatments were performed in triplicate for each ESC preparation isolated from 6 different women. Absorbance values were normalized to a reference sample with no treatment.

Statistical analysis

All statistical analyses were performed using GraphPad Prism (version 5.0 for Mac OS; GraphPad Software Inc., San Diego, California). The comparison of 2 groups was performed with a Student t test. For analysis of more than 2 groups, a 1-way ANOVA was performed with the Bonferroni post hoc test to compare each pair. To determine whether a significant correlation existed between 2 variables, the Pearson r was used. For all statistical tests, significance was set at $P < .05$.

Results

TACR1 and TACR2 mRNA and NK1R protein are present and up-regulated in endometriotic lesions

Expression of TACR1 (Figure 1A) was significantly higher in ectopic tissue than in eutopic tissue. Similarly, TACR2 expression (Figure 1B) was also significantly higher in the ectopic tissue than in the matching eutopic tissue. When ectopic tissue was separated based on lesion location, a 1-way ANOVA confirmed that a significant difference existed between TACR1 expression in the eutopic tissue and the ectopic tissue that was separated based on location. A Bonferroni post hoc test indicated that expression of TACR1 was significantly increased in eutopic tissue from peritoneal lesions but not that from ovarian or RVS lesions (Figure 1C). No significant difference was observed in TACR2 expression between eutopic endome-

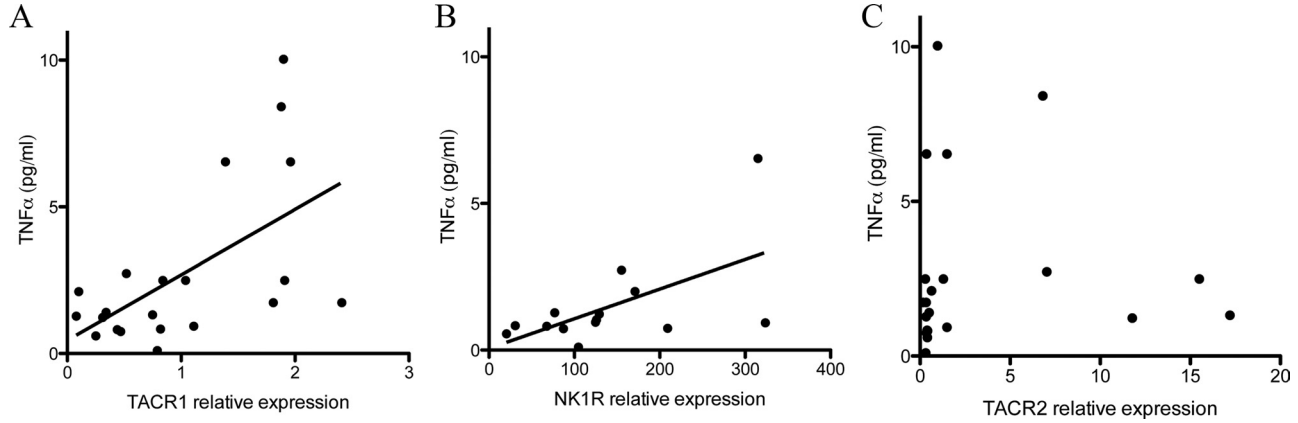

Figure 3. Correlation of NK1R expression with peritoneal fluid TNFα. Using the ectopic endometriotic lesions and the matching peritoneal fluid, a significant positive correlation was observed between the TNFα concentration in the peritoneal fluid and both TACR1 mRNA expression ($r = 0.5817$, n = 21, $P = .0057$) (A) and NK1R protein concentrations ($r = 0.5845$, n = 14, $P = .0282$) (B) in the endometriotic lesions. C, No significant correlation was observed between TACR2 mRNA and peritoneal fluid TNFα concentrations ($r = 0.2940$, n = 21, $P = .1957$). All values represent means ± SEM.

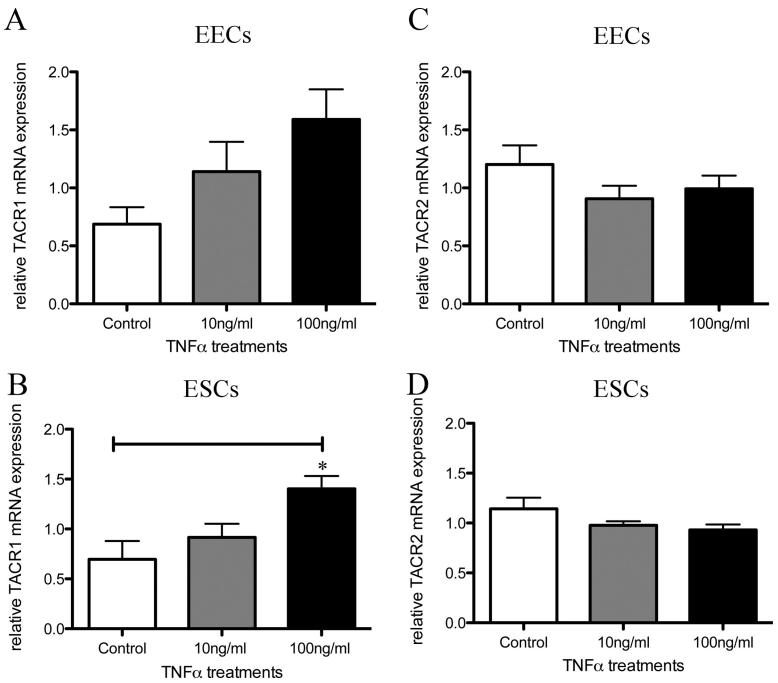

Figure 4. TACR1 and TACR2 mRNA expression in epithelial and stromal cells after TNFα treatment. A, 1-way ANOVA comparing the control (0.6875 ± 0.1465, n = 4), 10 ng/mL TNFα (1.1400 ± 0.2567, n = 4), and 100 ng/mL TNFα (1.590 ± 0.2598, n = 4) treatments showed no significant variation in the TACR1 mRNA expression in EECs ($F_{2,9} = 3.944$, $P = .0589$). B, Similar analysis showed a significant difference in the TACR1 mRNA expression in ESCs ($F_{2,14} = 5.850$, $P = .0142$), between the control (0.6967 ± 0.1831, n = 6), 10 ng/mL TNFα (0.9160 ± 0.1372, n = 5), and 100 ng/mL TNFα (1.403 ± 0.1273) conditions. A Bonferroni post hoc test confirmed that a significant increase in TACR1 expression occurred after 100 ng/mL TNFα compared with that in the control ($P < .05$). No significant difference was observed in TACR2 mRNA expression in either EECs ($F_{2,9} = 1.327$, $P = .3126$; control = 1.2030 ± 0.1644, n = 4; 10 ng/mL TNFα = 0.9075 ± 0.1111, n = 4; 100 ng/mL TNFα = 0.9925 ± 0.1129, n = 4) (C) or ESCs ($F_{2,15} = 2.200$, $P = .1452$, control = 1.1430 ± 0.1112, n = 6; 10 ng/mL TNFα = 0.9767 ± 0.0407, n = 6; 100 ng/mL TNFα = 0.9300 ± 0.0560, n = 6) (D) at both concentrations tested. All values represent means ± SEM. *$P < .05$, **$P < .01$.

trium and ectopic endometriotic lesions stratified by lesion location (Figure 1D).

The presence of NK1R protein in endometriotic lesions from all locations was confirmed by the presence of a specific band of 58 kDa (18) in Western blot analysis (Figure 2A). Semiquantitative analysis of band densitometry using a 1-way ANOVA showed that a significant difference existed for NK1R expression based on lesion location. A Bonferroni post hoc test indicated that expression of NK1R was significantly stronger in the peritoneal lesions than in either the ovarian or RVS lesions (Figure 2B). Neurokinin 1 receptor (NK2R) protein expression was not analyzed.

Peritoneal fluid TNFα correlates with TACR1 mRNA and NK1R protein expression in endometriotic lesions

There was a significant, positive correlation between peritoneal fluid TNFα and TACR1 mRNA (Figure 3A) and NK1R protein (Figure 3B). No significant association was observed between peritoneal fluid TNFα and TACR2 (Figure 3C). The association between NK2R and TNFα peritoneal fluid was not analyzed.

TNFα treatment of endometrial stromal cells induces TACR1 but not TACR2 mRNA expression

A 1-way ANOVA showed no significant variation in the TACR1 mRNA expression in EECs (Figure 4A). A similar analysis with ESCs, however, showed a significant difference in the TACR1 mRNA expression after TNFα treatments. A Bonferroni post hoc test confirmed that a significant increase in TACR1 expression occurred after 100 ng/mL TNFα compared with that of the control ($P < .05$) (Figure 4B). However, no significant difference was observed in the TACR2 mRNA concentration after treatment with either 10 or 100 ng/mL in the EECs (Figure 4C) or ESCs (Figure 4D).

TNFα treatment of endometrial epithelial and stromal cells induces NK1R expression

Small amounts of NK1R could be detected by immunofluorescence in EECs (Figure 5A, green staining and arrows) without TNFα stimulation. Treatment with 100 ng/mL TNFα increased the number of cells positive for NK1R protein (Figure 5B). Pan cytokeratin costaining was used to confirm that cells were epithelial (Figure 5, C and D, red staining and arrows). A merged image of the antirabbit NK1R antibody staining and the antimouse pan cytokeratin antibody staining with the nuclear DAPI stain confirmed expression of NK1R in EECs (Figure 5, E and F, blue).

In ESCs, there was some positive signal for NK1R staining under control conditions (Figure 5G, gray arrows). After treatment with 100 ng/mL TNFα, an increase in NK1R positive immunoreactivity was observed (Figure 5H, gray arrows). This staining was predominantly cytoplasmic.

SP enhances endometrial stromal cell viability, whereas L733 606 reduces cell viability

A 24 hours, 10 nM SP treatment of ESCs resulted in a significant increase in the number of viable ESCs compared with that for no treatment (Figure 6A). To induce

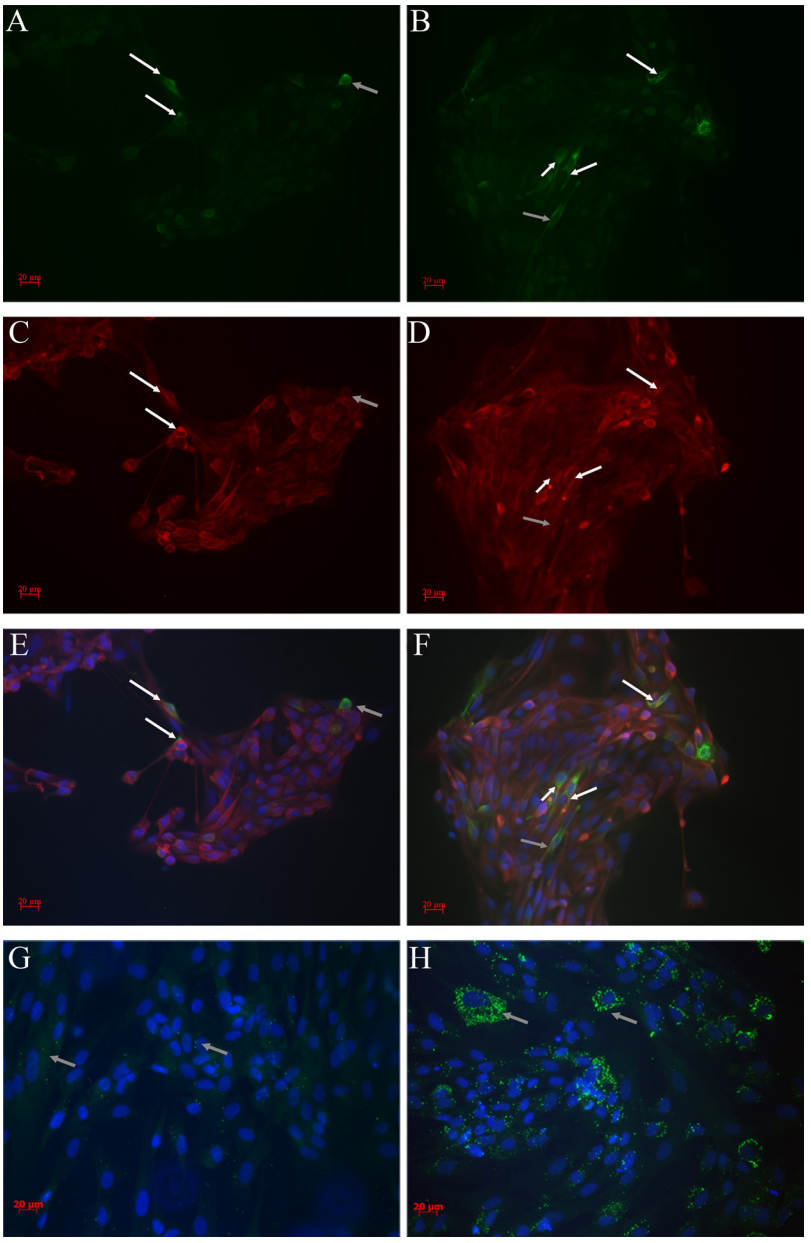

Figure 5. NK1R expression in endometrial epithelial and stromal cells after TNFα treatment. EECs (A–F) and ESCs (G and H) were treated for 6 hours as either control (A, C, E, and G) or with 100 ng/mL TNFα (B, D, F, and H). A–D, Double staining of endometrial epithelial cells with a rabbit anti-NK1R antibody followed by an antirabbit DyLight 488 antibody (green; A and B) and a mouse anticytokeratin antibody followed by an antimouse DyLight 594 antibody (red; C and D). E and F, Merged image of NK1R and cytokeratin staining with a DAPI nuclear (blue) stain. G and H, Single staining of ESCs with rabbit anti-NK1R antibody followed by an antirabbit DyLight 488 antibody (green) and a nuclear DAPI stain. White arrows indicate cells costained for both NK1R and cytokeratin (A–F), and gray arrows indicate regions of positive NK1R cytoplasmic staining (G and H).

NK1R expression, ESCs were pretreated with 100 ng/mL TNFα before treatment with either 10 nM SP alone, with 100 nM L733 606 alone, or with a combination of SP and L733 606 together. A 1-way ANOVA showed that there was a significant difference between the means of the 5 treatment groups. A Bonferroni post hoc comparison between all 5 groups indicated that treatment with 100 nM L733 606 significantly reduced the number of viable cells compared with either TNFα treatment alone or TNFα treatment in combination with 10 nM SP. No significant difference was observed between the remaining pairs.

Discussion

The presence of the tachykinin receptors in endometrial tissue has not been reported previously. The results of this study show that both *TACR1* and *TACR2*, genes that encode tachykinin receptors, are expressed in eutopic endometrial tissue and are significantly up-regulated in endometriotic lesions derived from the same women. The expression of both the *TACR1* gene and the protein it encodes, NK1R, was related to peritoneal fluid TNFα concentrations, and in vitro experiments confirmed that TNFα induced both TACR1 and NK1R in eutopic ESCs. Exposure to SP also increased the viability of ESCs, whereas specifically blocking NK1R with an antagonist reduced the viability of ESCs that had previously been exposed to TNFα. This induction of NK1R in endometriotic tissue by the inflammatory microenvironment and the response to SP and NK1R antagonists may represent the potential for neurogenic control over the maintenance of ectopic lesions.

Based on the theory of retrograde menstruation (19), ectopic lesions are derived from refluxed eutopic tissue and pathogenic changes in this tissue give rise to the ensuing lesions. In this study, we used matched eutopic and ectopic endometrial tissue from the same women and observed an increase in NK1R expression in the endometriotic lesion. By analyzing matched tissue, these results suggest that a direct induction of NK1R occurs only once the tissue became abnormal, and by using peritoneal fluid samples, also from the same women, we established that exposure to the inflammatory microenvironment and, in particular, to TNFα, was important for this induction. Furthermore, for the in vitro studies we used cells isolated from eutopic

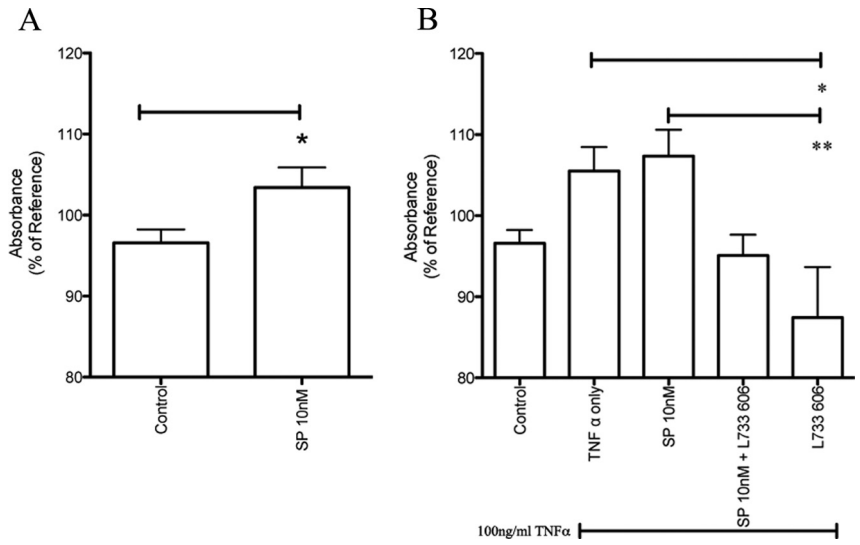

Figure 6. Proliferation of endometrial stromal cells after treatment with SP. A, Treatment of ESCs with 10 nM SP resulted in a significant increase in the number of viable cells compared with no treatment after a 24-hour period (control = 96.59 ± 1.66%, n = 6 vs 10 nM SP = 103.40 ± 2.45%, n = 6; $P = .0438$). B, When cells were pretreated with 100 ng/mL TNFα a 1-way ANOVA test found a significant difference ($F_{4,25} = 4.917$, $P = .0046$) between the 5 groups, which included control (96.59 ± 1.66%, n = 6), 100 ng/mL TNFα only (105.50 ± 2.94%, n = 6), 100 ng/mL TNFα and 10 nM SP (107.40 ± 3.25%, n = 6), 10 nM SP and 100 nM L733 606 (95.07 ± 2.59%, n = 6), or 100 nM L733 606 alone (87.42 ± 6.25%, n = 6). A Bonferroni post hoc comparison indicated a significant reduction in the number of viable cells that occurred when ESCs were treated with 100 nM L733 606 alone compared with either the 100 ng/mL TNFα group ($P < .05$) or the 100 ng/mL TNFα and 10 nM SP group ($P < .01$). All values are reported as means ± SEM. *$P < .05$, **$P < .01$.

endometrial tissue and confirmed that TNFα can significantly increase NK1R expression in these cells. Therefore, the inflammatory microenvironment may induce an up-regulation of NK1R expression in refluxed eutopic cells, and this may contribute to a pathogenic transformation.

Because lesions in the peritoneum, ovary, and RVS could be considered distinct but related conditions (20), we also compared TACR1 and NK1R expression among lesions from different locations. At the RNA level, we found that only lesions in the peritoneal wall had significantly increased expression above that of the eutopic tissue, whereas at the protein level NK1R expression in the peritoneum was significantly higher than that observed in either the ovarian or RVS lesions. Peritoneal lesions are the most likely to have significant exposure to peritoneal fluid and the inflammatory mediators contained within. Increased exposure to inflammation might explain the elevated expression of NK1R in these lesions and suggests that these lesions in particular could be more susceptible to neurogenic control.

An increase in NK1R expression has been noted previously in other inflammatory diseases, and TNFα, a cytokine related to endometriosis progression (21), was an important mediator of this increase. There was a significant increase in NK1R expression in colonic mucosal biopsy samples from patients with irritable bowel syndrome (22), which was induced in vitro by a cocktail of TH-1 cytokines including TNFα (23). A significantly increased NK1R expression was observed in *Clostridium difficile*–induced enterocolitis (24) and interstitial cystitis (25). NK1R expression was also induced in rheumatoid arthritis synoviocytes (26) by TNFα (13). Both IL-1β and TNFα can induce NK1R in monocytes (14). TNFα concentrations are significantly increased in the peritoneal fluid of women with endometriosis (27–29) and given that the average delay in diagnosis for endometriosis is between 3 and 11 years after the onset of symptoms (30), the ectopic tissue could be exposed to high TNFα levels for a considerable period of time. A long-term exposure to even small concentrations of TNFα could be sufficient to induce NK1R expression in ectopic lesions.

A significant induction of the *TACR2* gene in ectopic tissue was also observed, although as opposed to *TACR1*, this was not specific to lesions from any particular region. The role of NK2R in endometriotic tissue is not clear but deserves further attention. Previous evidence indicates that NK2R can also be up-regulated by TNFα in synoviocytes (13), although in endometrial tissue another substance is likely to be responsible because it was not correlated with peritoneal fluid TNFα nor was the expression up-regulated in primary cells after TNFα treatment in this study. *TACR3* showed very little or no expression in both the eutopic and ectopic endometrial tissue and was not investigated further.

Both the RNA results and the immunofluorescent images, although not quantitative, suggest that NK1R induction by TNFα occurred predominantly in the stromal cells. Notably, however, treatment of ESCs with SP even without any prior in vitro exposure to TNFα still significantly increased the viability of these cells. The RNA and protein analysis showed that some basal expression, although low, exists in both the EECs and ESCs, which suggests that even low basal expression of NK1R in eutopic tissue may be sufficient for the maintenance of these cells when exposed to SP. Many previous studies have shown that women with endometriosis have variable gene expression in their eutopic endometrium that may predispose them to development of endometriosis or infertility

through implantation failure (31). Because the ESCs used in this study were isolated only from women with confirmed cases of endometriosis, it is possible that a susceptibility to SP may be specific to the eutopic endometrium of women with endometriosis. Recent studies have suggested that nerve fibers are found in the eutopic endometrium of women with endometriosis (32, 33). Because of these nerve fibers, women with endometriosis may have SP and SP-mediated mechanisms already functioning within the eutopic endometrium with the potential for neurogenic control within this tissue. A comparison of TACR1 and NK1R expression and the ability of SP to support cell viability in the eutopic endometrium between women with and without endometriosis is an interesting avenue for future studies.

In addition, the inclusion of a specific antagonist for NK1R, L733 606, provided strong evidence that blocking of the NK1R-SP pathway in cells that have been exposed to TNFα can reduce the viability of ESCs. NK1R-SP binding has previously been shown to contribute to cell viability by inducing proliferation or preventing apoptosis in both normal and pathological cells such as epithelial cells of the intestines (34), lymphocytes (35), and glioblastoma cells (36). Therefore, it may also represent a possible avenue to reduce cell viability and the size of endometriotic lesions by using specific NK1R antagonists such as aprepitant, although further studies will be needed to confirm this.

The combination of induced expression of NK1R by exposure to an inflammatory environment, the ability of SP to support ESC viability, and the ability of specific NK1R antagonists to reduce it suggests that NK1R may contribute to endometriotic lesion maintenance. In addition, it is an intriguing possibility that the pain response itself may have a role in lesion maintenance. SP is released from sensory nerve fibers, and this secretion can be upregulated in response to nerve damage (37) and inflammation (38). Nerve fibers proximal to endometriotic lesions can be activated both physically, by infiltration of the lesion (6, 39), or chemically, by pain-associated prostaglandins (40, 41). The activation of these nerve fibers could not only send an afferent nerve signal but also release SP locally. Locally secreted SP could bind to NK1R and assist in maintaining cell viability of the ectopic lesions. A similar mechanism has been postulated for gastric cancer (42). In addition, because evidence is beginning to show that pain itself can become independent of the disease through activation of the central and peripheral nervous system, researchers have suggested that the focus of endometriosis research should be more on pain rather than on morphological changes (43), potentially leading to clinical treatments that focus on chronic pain management. The expression of NK1R and the ability of tachykinins to activate it may serve as a link between the morphology of the disease and the production of sustained painful symptoms.

The neuroimmune axis is a bidirectional pathway of intersystem communication. Endometriotic lesions are characterized by the growth of ectopic endometrial tissue. They also have neurotrophic properties (44) and are frequently found to be innervated. The results of this study suggest that ectopic endometrial tissue expresses NK1R and that this receptor can be induced by an inflammatory microenvironment. The presence of this receptor in an innervated environment may allow a positive feedback loop to exist between the pain-generating aspects of the disease and the maintenance of the ectopic tissue.

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