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Expression of Nerve Injury-Induced Protein1 (Ninj1) in Endometriosis

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Abstract

Objective: The aim of this study was to clarify the expression of Ninj1 in endometriosis and adenomyosis lesions, and its inductive factor in human endometriotic stromal cells (ESCs). **Background:** Nerve injury-induced protein 1 (Ninj1) is a molecule originally identified in dorsal root ganglion neurons and Schwann cells after nerve injury and promotes neurite outgrowth. The aim of this study was to clarify the expression of Ninj1 in endometriosis and adenomyosis lesions, and its inductive factor in human endometriotic stromal cells (ESCs). **Materials and Methods:** Tissues were obtained with consent from patients diagnosed with ovarian endometrioma (n = 15 in total), peritoneal endometriosis (n = 5), adenomyosis (n = 5), and other gynecological disorders (n = 5, control) during surgery. Immunohistochemistry was conducted in order to detect Ninj1 protein expression in the lesion of endometriosis, adenomyosis, and eutopic endometrium. Nerve fibers in the ovarian endometrioma were detected by positive staining of PGP-9.5. To evaluate the effects of IL-1β on Ninj1 gene expression in endometriosis, ESCs isolated from ovarian endometrioma (n = 5) were treated with IL-1β (5 ng/mL) for 3 or 6 hours. Messenger RNA (mRNA) expression for Ninj1 was examined using quantitative RT-PCR. **Results:** The Ninj1 protein was expressed by ovarian endometrioma, peritoneal endometriotic, and adenomyotic tissue. Nerve fibers were found in the areas of positive staining for Ninj1 in ovarian endometrioma. IL-1β, an indicator of inflammation in endometriosis, significantly increased Ninj1 mRNA expression by ESC. **Conclusion:** Our study demonstrates that Ninj1 is expressed in endometriosis and adenomyosis and is induced by the inflammatory stimuli. Given the neurogenetic property of Ninj1, our results imply that Ninj1, induced by inflammation in endometriosis lesion, may contribute to the pathogenesis of pain symptoms characteristic of endometriosis.

Keywords
Ninjurin-1, Ninj1, nerve fiber, endometriosis, adenomyosis, inflammation

Introduction

Endometriosis is a disease characterized by endometrial growth beyond the uterus, commonly into the ovary and/or peritoneal cavity.[1] Adenomyosis is a benign uterine disorder defined by the presence of endometrial tissue within the myometrium.[2] Both conditions may cause dysmenorrhea, dyspareunia, and chronic pelvic pain, and thereby remarkably deteriorate women's health.[3] Chronic inflammation is involved in its pathogenesis, and inflammatory cytokines such as IL-1β are highly expressed in endometriosis and adenomyosis lesions.[4,5] Regarding the cause of pain, it has been demonstrated that the inflammatory milieu and subsequent hyperinnervation is involved in the pathophysiology of pain generation.[6] Indeed, nerve fibers are abundant in endometriotic lesions such as ovarian endometrioma, deep infiltrating endometriosis, peritoneal lesion, and adenomyotic lesion.[7-10] High levels of expression of various neurotransmitters such as substance P (SP), calcitonin gene-related peptide (CGRP), acetylcholine (Ach), tyrosine hydroxylase (TH), and nerve growth factor (NGF)[7,9] are also present in these tissues.

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Nerve injury-induced protein 1 (Ninjurin, Ninj1), an adhesion molecule originally identified in dorsal root ganglion neurons and Schwann cells after nerve injury,[11] has been shown to mediate hemophilic adhesion and promote neurite outgrowth.[11,12] The Ninj1 is also expressed by other cell types (eg, myeloid cells,[13] epithelial cells,[11] endothelial cells,[14] keratinocytes and dermal fibroblasts,[15] hepatocellular carcinoma cells,[16] and leukemia cells[17]) and is induced under inflammatory conditions and controls neural generation.[12] Until now, the expression of Ninj1 in female reproductive organs has not been investigated. Further, there has been no study analyzing the expression of Ninj1 in endometriosis and adenomyosis, in contrast to the expressions of abovementioned, well-documented neurotransmitters such as SP, CGRP, Ach, TH, and NGF.

Given the inflammatory milieu and subsequent hyperinnervation status in endometriosis and adenomyosis lesions, together with the abovementioned properties of Ninj1, we asked whether Ninj1 is expressed in endometriosis and adenomyosis lesions, and if so, whether Ninj1 is induced by inflammation. The aim of this study was (1) to confirm whether or not Ninj1 protein is expressed in endometriosis and adenomyosis lesions, (2) to evaluate the association between the Ninj1 positive area and the presence of nerve fiber in ovarian endometrioma, and (3) to clarify whether or not the inflammatory stimuli enhances the Ninj1 expression in human endometriotic stromal cells (ESCs).

Materials and Methods

The experimental procedures were approved by the institutional review board of the University of Tokyo (registration number: 0324-4), and written informed consent was obtained from each patient.

Collection of tissues of ovarian endometrioma, peritoneal endometriosis, adenomyosis and eutopic endometrium, and preparation of tissue sections for immunohistochemistry

Ovarian endometrioma tissues were obtained from patients during laparoscopy or abdominal salpingo-oophorectomy (n = 10, aged 42.6 ± 3.62 years, mean ± SD, revised American Society for Reproductive Medicine (rASRM) score 44.8 ± 19.9). Peritoneal endometriosis tissues were obtained from patients during laparoscopy or abdominal surgery for removal of endometriosis (n = 5, aged 34.4 ± 5.77 years, rASRM score 25.0 ± 15.05). Adenomyosis tissues were obtained from patients during laparoscopy or abdominal hysterectomy (n = 5, aged 45.3 ± 2.87 years). As control samples, normal eutopic endometrial tissues were obtained from patients with other gynecological disorder such as cervical cancer (n = 5, aged 36.7 ± 5.77 years) during hysterectomy. Eutopic endometrial tissues were also obtained from patients of adenomyosis (abovementioned) during hysterectomy.

Prior to surgery, all patients were experiencing regular menstrual cycles and they had not received any hormonal treatment for at least 3 months. All tissues were fixed in neutral-buffered formalin and embedded in paraffin blocks, and 5-μm serial sections were prepared. Sections were deparaffinized and then rehydrated in decreasing concentrations of ethanol.

Immunohistochemistry

Immunohistochemistry was conducted for detecting protein expression of Ninj1 using anti-Ninj1 antibody, and detecting nerve fibers using anti-PGP-9.5 antibody using a set of serial sections. The following antibodies were used; monoclonal mouse antihuman Ninj1 antibody (MAB51051; R&D Systems, Minneapolis, Minnesota) at a 1:250 dilution and polyclonal rabbit anti-PGP-9.5 antibody, (Z511601), (Dako, Carpinteria, California) at a 1:100 dilution. Rehydrated sections were treated with 0.3% hydrogen peroxide (H_2O_2) for 5 minutes, to neutralize endogenous peroxidases, and rinsed for 5 minutes with distilled water. Antigen retrieval was performed using Target Retrieval (Dako). After washing with phosphate-buffered saline, slides were incubated in a moist chamber with primary antibody at 4°C overnight. To detect Ninj1 protein, slides were incubated with antimouse immunoglobulin G (IgG)-labeled polymer (Dako). To detect PGP-9.5 protein, slides were incubated with antirabbit IgG-labeled polymer (Dako). All sections were prepared using Liquid DAB+ (Dako) for substrate, followed by hematoxylin counterstaining and analyzed with a light microscope.

Isolation and Culture of Human ESC

Type I collagenase and deoxyribonuclease I (DNase I) were purchased from Wako (Tokyo, Japan). Antibiotics (a mixture of penicillin, streptomycin, and amphotericin B) were obtained from Sigma (St. Louis, Missouri). Dulbecco's modified Eagle's medium (DMEM)/F-12 medium, 2.5% Trypsin, HEPES and 0.25% Trypsin-EDTA were from Gibco (Grand Island, New York).

Endometriosis tissues were obtained from patients with ovarian endometrioma (n = 5, aged 42.6 ± 5.55 years, mean ± SD, rASRM score 31.1 ± 10.05) during excision of lesions. These patients had not received hormones or GnRH agonist for at least 3 months prior to surgery. Endometriosis tissue samples were obtained from the cyst wall of ovarian endometrioma under sterile conditions and transported on ice in DMEM/F-12 to the laboratory.

Isolation and culture of human ESCs were conducted as described previously.[18] Endometriosis tissue was minced into small pieces, incubated in DMEM/F-12 with 0.25% type I collagenase, 15 U/mL DNase I, 0.006% trypsin, and 0.02 mol/L HEPES for 1 to 2 hours at 37°C, and filtered through 100 μm (aperture size), then 70 μm nylon cell strainers. The ESCs were cultured in DMEM/F-12 containing 5% FBS and antibiotics. The purity of the stromal cells was greater than 98%; assessed by positive cellular staining for vimentin and negative cellular staining for cytokeratin and CD45. Further, by immunostaining the cells with anti-CD10 antibody, greater

than 95% of the cells were identified as ESCs.[19] At the first passage, the cells were plated at a density of 2×10^5 cells/well into 12-well culture plates, and incubated at 37°C in a humidified 5% CO_2/95% air environment.

Treatment of ESC With IL-1β

To evaluate the effects of IL-1β on Ninj1 gene expression, the cells were treated with IL-1β (5 ng/mL; Sigma) and incubated. Three or 6 hours after the IL-1β treatment, cells were harvested, and RNA was extracted.

RNA Extraction, Reverse Transcription, and Real-Time PCR

Total RNA was extracted using an RNeasy Mini Kit (Qiagen, Hilden, Germany). One microgram of total RNA was reverse transcribed in 20 μL using an RT-PCR kit (Toyobo, Japan). Real-time quantitative PCR was conducted using a LightCycler (Roche Diagnostic, Mannheim, Germany) according to the manufacturer's instructions. All PCR reactions of each sample were run in duplicate. Expression of each messenger RNA (mRNA) was normalized for RNA loading for each sample using human glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA as the internal standard. The Ninj1 primers (sense, 5′-TACGACCTTAACAACCCGGC-3′; antisense, 5′-CCTCACAGGTATGGCGACTC-3′) were chosen to amplify a 224-bp fragment. The GAPDH primers (sense, 5′-ACCACAGTCCATGCCATCAC-3′; antisense, 5′-TCCACCACCCTGTTGCTGTA-3′) were chosen to amplify a 450-bp fragment. The PCR conditions were as follows for Ninj1: 30 cycles of 95°C for 10 seconds, 66°C for 10 seconds, and 72°C for 10 seconds and for GAPDH: 30 cycles of 95°C for 10 seconds, 65°C for 10 seconds, and 72°C for 18 seconds. All PCR procedures were followed up with melting curve analysis.

Statistical Analysis

Data were evaluated using JMP software (version 10.0; SAS Institute Inc, Cary, North Carolina). The statistical differences between 2 samples were calculated using Student *t* test. Data are expressed as the mean ± SEM. A *P* value of <.05 is considered as significant.

Results

Nerve Injury-Induced Protein 1 Expression in Ovarian Endometrioma Tissue

Figure 1A and B are representative pictures of the 5 specimens. Positive staining for Ninj1 was observed in the nuclei of both epithelial and stromal cells. Positive staining for Ninj1 was observed in the nuclei and cytoplasm of both epithelial and stromal cells; however, more epithelial cells were positively stained than stromal cells.

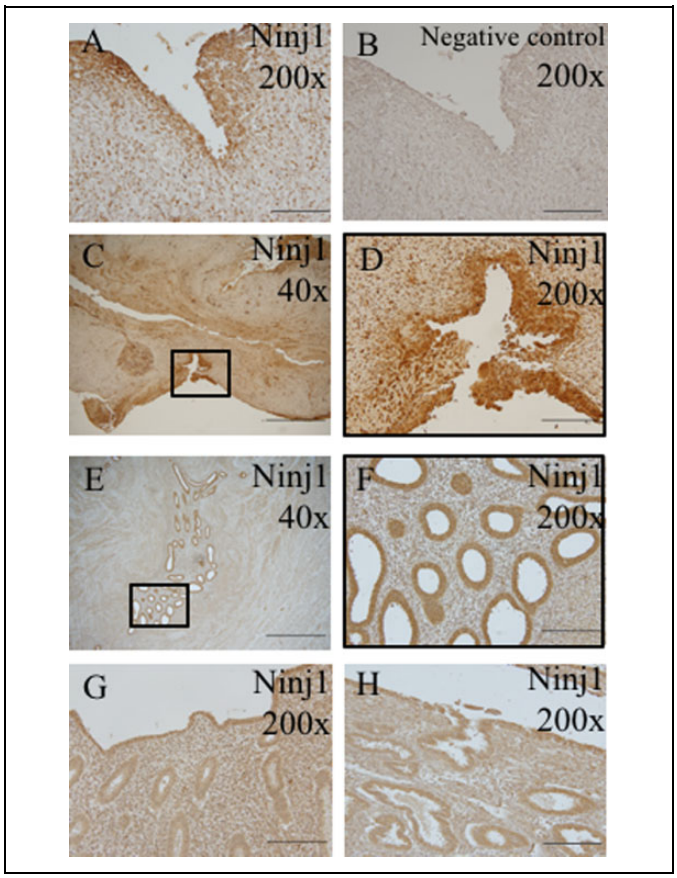

Figure 1. Representative results for immunohistochemical staining for Ninjurin-1 (Ninj1) in human ovarian endometrioma (A), peritoneal endometriosis (C, D) adenomyosis (E, F), and eutopic endometrium from control (G) and adenomyosis case (H). D and F are higher magnification images of the area indicated with a square in C and E, respectively. The negative control image using the isotype antibody is shown in (B). Cell nuclei were counter-stained using hematoxylin. Positive staining for Ninj1 was observed in the nuclei and cytoplasm of both epithelial and stromal cells; however, positive staining was more frequently observed in epithelial cells than in stromal cells. Black bars indicate 200 μm (C and E) or 40 μm (A, B, D, F, G, and H). Original magnification: ×40 (C and E), ×200 (A, B, D, F, G, and H).

Nerve Injury-Induced Protein 1 Expression in Peritoneal Endometriosis Tissue

Positive staining for Ninj1 was observed in the nuclei of both epithelial and stromal cells. Positive staining for Ninj1 was observed in the nuclei and cytoplasm of both epithelial and stromal cells; however, more epithelial cells were positively stained than stromal cells (Figure 1C and D).

Ninj1 Protein Expression in Adenomyosis Tissue

Figure 1E and F are the representative pictures of the 5 specimens. Positive staining for Ninj1 was observed in the nuclei of cells from an adenomyotic lesion. Positive staining for Ninj1 was observed in the nuclei and cytoplasm of both glandular epithelial and stromal cells; however, more epithelial cells were positively stained than stromal cells.

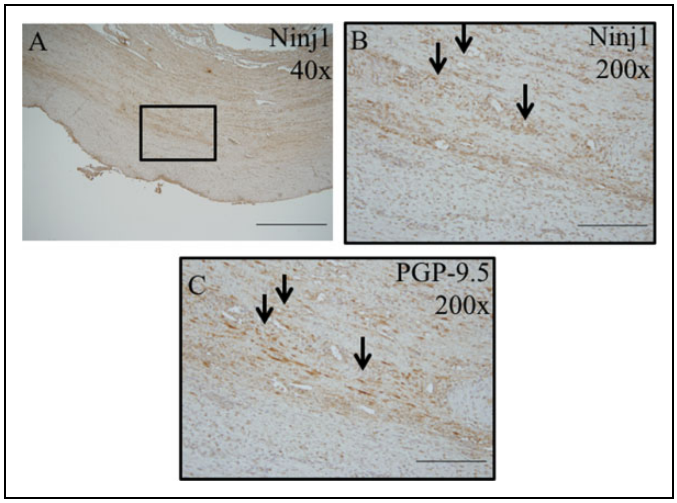

Figure 2. A representative result of immunohistochemical staining for Ninjurin-1 (Ninj1, A) in human ovarian endometrioma. B is a higher magnification image of the area indicated with a square in A, and the serial section was stained for PGP-9.5 (C). Cell nuclei were counter-stained using hematoxylin. PGP-9.5 positive cells were found in the area of positive staining for Ninj1. Black bars indicate 200 μm (A) and 40 μm (B, C). Magnification: ×40 (A), ×200 (B and C).

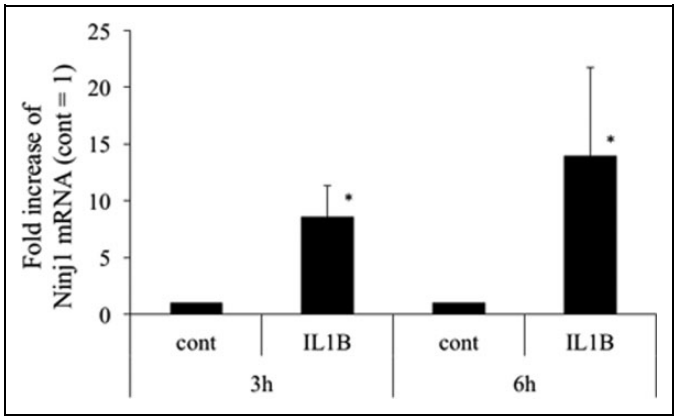

Figure 3. Ninjurin-1 (Ninj1) messenger RNA (mRNA) expression by endometriotic stromal cells (ESCs) stimulated with IL-1β for 3 or 6 hours. Stimulation of ESC with IL-1β (5 ng/mL) increased Ninj1 mRNA expression. *$P < .05$ versus nontreated control (n = 5).

Nerve Injury-Induced Protein 1 Protein Expression in the Eutopic Endometrial Tissue

Positive staining for Ninj1 was also confirmed in the eutopic endometrium from the control (Figure 1G) or patients with adenomyosis (Figure 1H). The intensity of staining, however, seemed to be slightly weaker in the eutopic endometrium than in endometriosis or adenomyosis lesions.

Presence of Nerve Fibers in Ovarian Endometrioma Tissue

Nerve fibers, identified by positive staining for PGP-9.5, were present in the areas of positive staining for Ninj1 in ovarian endometrioma sections (Figure 2).

The Effect of IL-1β on Ninj1 mRNA Expression by ESC

IL-1β significantly increased Ninj1 mRNA expression at 3 and 6 hours post treatment (8.62 ± 2.73, 13.99 ± 7.76, respectively, fold increase, n = 5; $P < .05$; Figure 3).

Discussion

In this study, we have made the novel discovery that Ninj1 protein is expressed in ovarian endometrioma, peritoneal endometriotic, and adenomyotic tissue. Furthermore, we showed that nerve fibers were present in the positive staining area for Ninj1 in ovarian endometrioma. We also demonstrated that IL-1β, a key inflammatory molecule in endometriosis, significantly increased Ninj1 mRNA expression in ESCs.

The Ninj1 protein is expressed in ovarian endometrioma, peritoneal endometriotic, and adenomyotic lesions. Many studies have demonstrated that neurotransmitters such as NGF, tyrosine kinase-A, SP, CGRP, Ach, and tyrosine TH are expressed in endometriosis[8,9,20] and adenomyosis,[21-23] and that these factors are involved in the generation of pain in these conditions. The current study's findings add the novel neurotransmitter, Ninj1, to the list of factors that may contribute to these pathologies, although more quantitative studies are warranted in order to compare its expression with other diseases or normal tissues.

The current study also demonstrated that nerve fibers were present in areas of positive staining for Ninj1 in endometriotic lesions. A significant feature of endometriotic and adenomyotic lesions is hyperinnervation, which is associated with the pain symptoms characteristic of these conditions.[7,9,24,25] The abovementioned neurotransmitters are colocalized with nerve fibers[7,9,20,26] and are believed to contribute to innervation. The current findings, together with evidence that Ninj1 promotes an increase in neurite outgrowth,[12] suggest that Ninj1 plays a role in their innervation and is thereby involved in the pathogenesis of pain symptoms inherent to endometriosis and adenomyosis.

Regarding the mechanism for the induction of Ninj1 by endometriotic tissue, our study indicates that IL-1β, a major pro-inflammatory cytokine enhanced in endometriosis,[4] significantly increases Ninj1 expression by ESCs. Endometriosis creates an inflammatory environment, but we are only beginning to elucidate the role of inflammation in stimulating peripheral nerve growth.[27] For instance, NGF is induced by tumor necrosis factor-α and IL-1β in immortalized human endometrial epithelial cells.[28] It has been demonstrated that Ninj1 is expressed in various inflammatory diseases such as allergic encephalomyelitis and multiple sclerosis[13] and modulates the nerve system in the inflammatory milieu.[13] The present findings imply that Ninj1 is also induced by the inflammatory environment and given its role in neurite outgrowth could stimulate nerve growth and thereby pain, a common and deleterious symptom of endometriosis.

Neurogenesis aside, Ninj1 has been shown to promote angiogenesis,[29] modulate p53-dependent cell survival and senescence,[30] mediate leukocyte migration and in turn mediate inflammation.[31] These properties are all pivotal in the pathogenesis of endometriosis, and therefore, Ninj1 may affect not only pain symptoms but also the progress of endometriosis per se. Further studies to explore the full effects of Ninj1 are warranted to fully appreciate its actions in endometriosis.

In conclusion, our study demonstrates for the first time that Ninj1 is expressed in endometriosis and adenomyosis and induced by the inflammatory milieu characteristic of endometriosis. Given its neurogenetic property, Ninj1 could induce nerve growth and thereby may contribute to the pathogenesis of pain symptoms commonly experienced by women with this disease. It is possible that Ninj1 is involved in the pathogenesis of endometriosis per se, although further studies are warranted.

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Declaration of Conflicting Interests

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