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ECTOPIC UTERINE TISSUE AS A CHRONIC PAIN GENERATOR

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Abstract—While chronic pain is a main symptom in endometriosis, the underlying mechanisms and effective therapy remain elusive. We developed an animal model enabling the exploration of ectopic endometrium as a source of endometriosis pain. Rats were surgically implanted with autologous uterus in the gastrocnemius muscle. Within two weeks, visual inspection revealed the presence of a reddish-brown fluid-filled cystic structure at the implant site. Histology demonstrated cystic glandular structures with stromal invasion of the muscle. Immunohistochemical studies of these lesions revealed the presence of markers for nociceptor nerve fibers and neuronal sprouting. Fourteen days after surgery rats exhibited persistent mechanical hyperalgesia at the site of the ectopic endometrial lesion. Intralesional, but not contralateral, injection of progesterone was dose-dependently antihyperalgesic. Systemic administration of leuprolide also produced antihyperalgesia. *In vivo* electrophysiological recordings from sensory neurons innervating the lesion revealed a significant increase in their response to sustained mechanical stimulation. These results are consistent with clinical and pathological findings observed in patients with endometriosis, compatible with the ectopic endometrium as a source of pain. This model of endometriosis allows mechanistic exploration at the lesion site facilitating our understanding of endometriosis pain.

Key words: endometriosis, chronic pain, progesterone, leuprolide, nociceptors, mechanical hyperalgesia.

INTRODUCTION

Endometriosis, a chronic pain syndrome associated with the presence of ectopic endometrial glands and stroma, affects approximately 10% of women in their reproductive years (Giudice, 2010). Most characteristically, pain associated with endometriosis is exacerbated by mechanical stimuli, associated with physical activity, generating symptoms of dyspareunia (painful sexual intercourse), dyschezia (painful defecation), dysuria (painful urination) and low back pain (Giudice, 2010; Roman et al., 2011). This pain can occur unpredictably, be related to the menstrual cycle or can be continuous (Giudice, 2010).

Pain is more common or severe if lesions have a cystic appearance, are located at certain anatomical sites and when tissue penetration is deep (Fauconnier et al., 2002; Dai et al., 2012). And, some clinical studies indicate a positive association between pain symptoms and lesions observed at surgical exploration (Porpora et al., 1999). Unfortunately, available medical and surgical treatments for endometriosis pain provide only limited relief, with pain recurring in up to 50% of women within 6–12 months after the completion of treatment (Giudice, 2010).

To explore the pathophysiology of endometriosis and to assay potential therapies, preclinical models have been developed. Most of these models are based on surgically implanted uterus ectopically, in the peritoneal cavity (Vernon and Wilson, 1985). Using this model in the rat, nociceptive responses compatible with pain symptoms observed in human endometriosis have been reported (Berkley et al., 2005), leading to the suggestion of the involvement of neuronal (Berkley et al., 2005) and immune (Umezawa et al., 2008) elements, as observed in human endometriosis (Giudice, 2010). However, while the relief of pain provided by surgical excision of symptomatic lesions (Jacobson et al., 2009; Giudice, 2010) and activity-induced pain symptoms (Giudice, 2010; Roman et al., 2011) strongly suggest a prominent role for peripheral mechanisms in endometriosis pain, there are no studies directly exploring the role of peripheral sensory processing in endometriosis pain. This results, in part, from the absence of preclinical models allowing direct stimulation of lesions and local interventions to modulate nociceptive processing or the ability to record neuronal activity arising from ectopic endometrial lesions. We report a new preclinical model of endometriosis pain in which one can explore

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Abbreviations: CGRP, calcitonin gene-related peptide; DAPI, 4′,6-diamidino-2-phenylindole; DiI, 1,1′-dioctadecyl-3,3,3′,3′-tertamethyllindocarbocyanine perchlorate; DRG, dorsal root ganglion; EGTA, ethylene glycol tetraacetic acid; FITC, fluorescein isothiocyanate; GAP43, growth associated protein 43; GnRH, gonadotropin-releasing hormone; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; IB4, isolectin B4; NGS, normal goat serum; PBS, phosphate-buffered saline; TX, Triton X.

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underlying mechanisms and the evaluation of mechanism-based therapies to determine if they act at the site of an endometriosis lesion.

EXPERIMENTAL PROCEDURES

Animals

Adult female Sprague–Dawley rats (200–220 g; Charles River, Hollister, CA, USA) were used in these experiments. They were housed in the Animal Care Facility at the University of California San Francisco, under environmentally controlled conditions (lights on 07:00–19:00 h; room temperature 21–23 °C) with food and water available *ad libitum*. Upon completion of experiments, rats were killed by pentobarbital overdose followed by cervical dislocation. Animal care and use conformed to NIH guidelines (NIH Guide for the Care and Use of Laboratory Animals). The University of California San Francisco Committee on Animal Research approved all experimental protocols. Concerted effort was made to minimize number and the suffering of experimental animals.

Chemicals

Unless otherwise stated, all chemicals used in these experiments were obtained from Sigma–Aldrich (St. Louis, MO, USA).

Determination of estrous cycle phases

The phase of the estrous cycle was assessed daily (8:00–9:00 AM). Briefly, rats were gently restrained and 20 μl of NaCl 0.9% was flushed 3–4 times into the vaginal cavity. The resulting fluid was then placed onto a slide and observed unstained at 100× magnification. The diagnostic criteria used to determine the cycle phase has been previously reported (Marcondes et al., 2002): in proestrus a predominance of round nucleated (epithelial) cells is observed; in estrus a predominance of polygonal/irregular anucleated (cornified) cells is observed; in diestrus a predominance of small round (neutrophil) cells is observed and in metestrus a similar proportion of small round cells, cornified cells and epithelial cells is observed.

Surgical induction of muscle endometriosis

In general terms, our model of surgically-induced muscle endometriosis was adapted from that used to induce peritoneal endometriosis in the rat (Vernon and Wilson, 1985). Only rats in proestrus were used for endometrial implantation surgery. Rats were anesthetized with a mixture of ketamine hydrochloride and xylazine (80 and 6 mg/kg, s.c., respectively) and anesthesia was maintained with isoflurane (1.5% in 98.5% oxygen). The fur on the abdominal and left calf regions was clipped and disinfected, and a midline abdominal anesthetic block performed by injecting 0.25% bupivacaine (0.2 ml, s.c.). Under aseptic conditions a midline incision approximately 4 cm in length was performed. After laparotomy, the abdominal cavity was examined and the right uterine horn identified, exposed and isolated using a sterilized cotton roll. With the aid of a surgical microscope, the right uterine artery and vein, and the uterine vessels from the ovarian artery were ligated at the level of the transition of the uterine horn to the oviduct, with a 5–0 nylon suture. This procedure was repeated 1 cm distally. The uterine horn bounded by these ligatures was sectioned perpendicularly to its axis, and a 1-cm segment removed and immediately placed in a Petri dish containing 0.9% NaCl. The distal stump of the uterine horn was then tied with 5–0 nylon suture. After confirming hemostasis, the musculature of the abdominal wall was closed with single cross stitches and the skin incision closed with horizontal mattress stitches, using 5–0 nylon. The excised uterine tissue was measured with a millimeter scale and opened longitudinally; a full thickness 3 × 3 mm square of uterine tissue was then removed and kept in physiologic saline. To perform the implant, the *biceps femoris* muscle was exposed by means of a 2-cm skin incision perpendicular to the long axis of the calf. Then, a 1-cm incision was performed in the *b. femoris* allowing exposure of the underlying gastrocnemius muscle. With the aid of a surgical microscope, the square of uterine tissue was sutured to the surface of the gastrocnemius muscle applying three to four single stitches using 5–0 nylon with the endometrial portion of the uterine tissue contacting the gastrocnemius muscle. After checking for hemostasis, the *b. femoris* muscle was closed with single stitches and the skin with single cross stitches, using 5–0 nylon. The sham surgical procedure was similar but the implant sutured to the surface of the gastrocnemius muscle consisted of a 3 × 3 mm square of peritoneal fat instead of uterine tissue. Postoperative recovery was assessed daily. Return of normal estrus cyclicity was found within one week of the procedure.

Measurement of muscle hyperalgesia

Mechanical nociceptive threshold in the gastrocnemius muscle was quantified using a digital force transducer (Chatillon DFI2; Amtek Inc., Largo, FL, USA) with a custom-made 7-mm diameter probe (Alvarez et al., 2010). Rats were lightly restrained in a cylindrical acrylic holder with lateral slats that allows for easy access to the hind limb and application of the force transducer probe to the site of implantation in the belly of the gastrocnemius muscle. The nociceptive threshold was defined as the force, in milliNewtons, required to produce a flexion reflex in the hind leg. Baseline withdrawal threshold was defined as the mean of three readings taken at 5-min intervals.

Intralesional and systemic injections

Rats were briefly anesthetized with 2.5% isoflurane to facilitate the injection of progesterone (Calbiochem®, La Jolla, CA, USA) into the endometrial implant located in the gastrocnemius muscle (20 μl). The injection site was previously shaved and scrubbed with alcohol. The precise location of the uterine implant was identified by palpation and the tip of the needle directed to the base of the implant. Immediately after injections the skin puncture site was marked with a fine-tip indelible ink pen, so that the mechanical nociceptive threshold of the underlying injection site in the muscle could be repeatedly tested. Solutions of progesterone (1 and 3 μg/μl dissolved in 10% ethanol in NaCl 0.9%) were freshly prepared immediately before injection. For systemic injections, rats were briefly anesthetized with 2.5% isoflurane and 0.25 ml of freshly prepared leuprolide acetate solution (concentration 4 mg/ml in sterile-filtered PBS containing 0.5% bovine serum albumin) were s.c. the base of the neck.

Single fiber *in vivo* electrophysiology

Rats were anesthetized with sodium pentobarbital (initially 50 mg/kg, intraperitoneally, with additional doses given to maintain areflexia throughout the experiment), their trachea cannulated to maintain patency of their upper airway and heart rate monitored. Anesthetized animals were positioned on their right side and an incision made on the dorsal skin of the left leg, between the mid-thigh and calf. Then the *b. femoris* muscle was partially removed to expose the sciatic nerve and gastrocnemius muscle. The edges of the incised skin were fixed to a metal loop to provide a pool that was filled with warm mineral oil that bathed the sciatic nerve and gastrocnemius muscle.