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Reinnervation of rat endometrium in the anterior eye chamber model of experimental endometriosis: Old methods for new questions

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ABSTRACT

Endometriosis is a benign estrogen-dependent chronic gynecological disease characterized by the presence of endometrial-like tissue outside the uterine cavity. In both women and experimental endometriotic rats, endometriosis lesions endow autonomic and sensory nerves, which are thought to contribute to the disease-associated pain. Some evidence indicates that the reinnervation of lesions is regulated by factors produced by the endometrial tissue as well as by environmental factors from the peritoneum. In this study, we examined the reinnervation of the rat endometrial tissue in an ectopic environment different from the peritoneum employing the anterior eye chamber model of experimental endometriosis. At 3 and 6 weeks following transplantation, endometrial grafts retained many histological features of the eutopic tissue. Both sympathetic and sensory nerves reinnervated endometrial grafts and distributed in the stroma-like tissue, around blood vessels and in close proximity to the glands and lining epithelium. Sympathetic innervation was more robust than sensory innervation. No significant topographical relationship between sympathetic nerves and macrophages was observed. These results suggest that the rat endometrium possesses intrinsic neuritogenic capacities and can be reinnervated by sympathetic and sensory nerves in ectopic sites different from the peritoneum.

1. Introduction

Endometriosis is an estrogen-dependent chronic inflammatory disease affecting about 10% of reproductive-aged women, causing chronic pelvic pain and infertility. It is defined by the presence of viable growths of endometrial-like tissue outside the uterine cavity, usually implanted over pelvic visceral and peritoneal surfaces (Giudice and Kao, 2004). Studies developed in the last decade revealed that endometriotic lesions are endowed with autonomic and sensory nerves (Tokushige et al., 2006; Mechsner et al., 2007) which are likely to contribute to endometriosis-associated pain (Medina and Lebovic, 2009; Fraser, 2010; Morotti et al., 2014; Barcena de Arellano and Mechsner, 2014). Like in human endometriosis, experimental endometriotic-like cysts developed in rats by autotransplantation of pieces of uterine wall on abdominal arteries are innervated (Berkley et al., 2004, 2005). Consistently, endometriotic rats exhibit vaginal and abdominal muscle hyperalgesia (Berkley et al., 2007; McAllister et al., 2009, 2012). Interestingly in human adenomyosis, foci of endometrial-like tissue located at least 2.5 mm below the endometrial-myometrial barrier are not innervated (Quinn, 2007; Barcena de Arellano et al., 2013). This suggests that not only intrinsic properties of the endometrial tissues but also environmental factors contribute to regulate the innervation of the endometrium in ectopic sites (Anaf et al., 2002; Barcena de Arellano et al., 2011). However, the nature and relative contribution of these factors are not fully understood.

Previous studies carried out in Rhesus monkeys (Markee, 1940), rabbits (Rock et al., 1993) and sheep (Garry, 2010) showed that the anterior eye chamber transplantation method is a suitable model of experimental endometriosis. This approach might therefore provide the opportunity to analyze the reinnervation of the endometrial tissue in an ectopic site different from the peritoneum. Since the anterior eye chamber is an immunologically-privileged site, it might mimic to some extent the immunological tolerance observed in the peritoneum of patients with endometriosis (Siristatidis et al., 2006).

The in oculo transplantation model has been widely used to study trophic interactions between neurons and their targets. Using this approach, different tissues have been shown to exert a profound influence over the organotypic pattern and density of their innervation (Olson and Malmfors, 1970; Burnstock, 1974; Gavazzi et al., 1992; Todd, 1986). Indeed, this method allowed demonstrating the role of target-derived signals in the regulation of plasticity in myometrial sympathetic nerves in response to estrogen and pregnancy (reviewed in Brauer and Smith, 2015; Brauer, 2016).

On these grounds, we carried out allogenic transplants of isolated rat endometrium into the anterior eye chamber of adult cyclic female rats. The reinnervation of intraocular grafts by sympathetic and sensory nerves was examined using immunohistochemistry. Considering that

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in human endometriotic lesions, the density of innervation correlates with the density of macrophages (Tran et al., 2009; Greaves et al., 2015), in the current study, we assessed quantitatively the relationship between macrophages and nerve fibers in endometrial intraocular grafts using double-labeling immunohistochemistry.

2. Materials and methods

2.1. Animals

Studies were conducted on female Wistar-derived albino rats from the breeding colony held at the Instituto de Investigaciones Biológicas Clemente Estable (IIBCE, Montevideo, Uruguay). Animals were maintained under controlled conditions of temperature (21–22 °C) and illumination (12 h light/dark cycles) with water and food provided ad libitum. All animal procedures were conducted in accordance with standards of the Ethics Committee on the Use of Laboratory Animals (CEUA-IIBCE) regulated by the National Law No. 18611.

2.2. In oculo transplantation

2.2.1. Donors

Four cycling rats at diestrus were euthanized by a lethal dose of sodium pentobarbital (60 mg/kg; Sigma, USA). When the heart stop beating; the uterine horns were removed under aseptic conditions and placed in sterile ice-cold DMEM/F12 for dissection (Invitrogen, USA). The uterine horns were opened longitudinally and pinned stretched on Sylgard (Dow Corning, UK) using micropins. The endometrium was carefully separated from the myometrium, and after removing debris of the circular myometrial layer, endometrial tissues were cut into 1 mm width and 2 mm long strips.

2.2.2. Hosts

Twenty adult cycling rats were anesthetized with 90 mg/kg ketamine (Unimedical, Uruguay) plus 10 mg/kg xylazine (Unimedical) administered intraperitoneally, followed by local administration of 0.5% proparacaine hydrochloride solution (Anestalcon, Alcon-Argentina). Mydryasis was achieved by application of a drop of 10 mg/ml atropine sulphate to the cornea. After making a small slit in the pupillary region of the cornea using a microsurgical blade (Becton Dickinson, USA), endometrial transplants were inserted into the eye with the endometrial epithelium facing the iris and manipulated by gentle pressure on the cornea into the posterior iridocorneal angle of the eye (Brauer et al., 2000). Transplants were left in oculo for 3 or 6 weeks after which host animals were terminally anesthetized and transplants removed attached to a small portion of the iris. Considering that previous studies showed that in experimental endometriotic rats, pain symptoms were exacerbated by estrogen (Berkley et al., 2007); in the current study, hosts rats were euthanized at proestrus, when circulating levels of estrogen are at their highest cyclical levels.

2.3. Immunohistochemistry and histology

Transplants were fixed by immersion in 4% paraformaldehyde (Sigma) for 1 h at 4 °C. After fixation, tissues were washed in phosphate-buffered saline (PBS) and stored in 12% sucrose in PBS at 4 °C. Endometrial grafts were pooled and embedded together in tissue freezing medium (Shandon, USA). Serial cryostat tissue sections (12 μm) were mounted onto gelatin-subbed slides and processed for immunohistochemistry. Depending on their size, individual transplants yielded between 20 and 40 tissue sections. The identity of each transplant could be easily followed in different sections by their morphological profile. Sympathetic and sensory nerves were demonstrated respectively with rabbit anti-tyrosine hydroxylase (TH, final dilution 1:400; Pierce Biotechnology, USA) or rabbit anti-calcitonin gene-related peptide (CGRP, final dilution 1:600, Biomol International, USA), (incubation: overnight at RT). Immunohistochemical demonstration of sensory nerves by anti-CGRP required antigen-retrieval, consisting in the incubation of slides in pre-heated sodium citrate 10 mM (pH 9) at 80 °C for 30 min. Slides were incubated for 1.5 h at room temperature with goat anti-rabbit IgG conjugated with FITC (final dilution 1:400; Chemicon, USA), washed in PBS and mounted in antifade mountant (Citifluor, UK). For the demonstration of macrophages, sections were incubated with the pan-macrophage marker CD68 (anti-mouse CD68; ED1; 1:150, Chemicon), followed by anti-mouse IgG conjugated with Alexa-Fluor 568 (final dilution 1:400, Molecular Probes, USA). Sections of rat spleen were used as a positive control. In all cases, the specificity of the immunostaining was checked by omission of the primary antibody. Sections were examined with a Nikon E800 microscope equipped with epifluorescence. Images were captured with a CoolSNAP-Pro Digital camera using the Image Pro Plus software (Media Cybernetics, USA). After imaging, some slides were un-mounted, washed in PBS, fixed for 10 min in 4% PFA and stained with hematoxylin and eosin.

2.4. Macrophages count and sympathetic nerve density

The relationship between macrophages and sympathetic nerves was assessed on transplants left into the anterior eye chamber for 3 weeks. Studies on the relationship between macrophages and sensory nerves were not performed because the antigen-retrieval required for CGRP immunostaining interfered with CD68 staining. On images captured at 20×, a stereological grid with an area of 0.25 mm^2 and line intersects at 20 μm intervals was superimposed on different regions of each transplant section ($n = 45$ serial sections separated by 24 mμ). The number of macrophages and sympathetic nerve profiles in the squares was counted and averaged. The resulting number was multiplied by the total area of the transplant in each section, to obtain the density of macrophages and nerve fibers per square millimeter (Chávez-Genaro et al., 2002). Statistical comparisons were carried out on images taken from 6 transplants.

Following the method reported by Tran et al., 2009, values of the number of macrophages per mm^2 were divided into two equal groups around the Median (group A, <91 macrophages/mm^2; group B, ≥91 macrophages/mm^2). Following the demonstration of the non-Gaussian distribution of data by the Kolmogoron-Smirnov test, the density of nerve fibers in groups A and B was compared using the Mann Whitney non-parametric test. Data are expressed as the Median (quartiles 25th -75th). Values of $p < 0.05$ were considered statistically significant.

3. Results and discussion

3.1. Morphology of intraocular endometrial transplants

After 3 weeks in oculo, endometrial transplants were attached to the host irises by a variable number of connecting tissue bridges and well revascularized (Fig. 1A,B). As long as the eye was not damaged during surgery, all intraocular endometrial grafts remained viable. Most transplants contained both stroma-like tissue and glands (Fig. 1C) while others were mostly composed by stroma-like tissue (Fig. 1D). Some transplants presented a large central cavity lined by columnar epithelium and surrounded by stroma-like tissue and glands (cysts-like transplants), (Fig. 1C, E). These structures resemble peritoneal cystic lesions observed in experimental endometriotic rats (Berkley et al., 2004). At 6 weeks, some transplants presented areas largely composed of extracellular matrix (Fig. 1F). No signs of immune rejection were observed. The morphological variability observed in our intraocular grafts might be explained by differences in the proportions of stroma and glands in individual tissue fragments used for transplantation. Also, small differences in the size of transplants might contribute to this variability. Indeed, the size of uterine tissue grafts transplanted on the peritoneum