Acta Histochemica xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Acta Histochemica

journal homepage: www.elsevier.com/locate/acthis

Endocannabinoids modulate apoptosis in endometriosis and adenomyosis☆

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ARTICLE INFO

Keywords:
Endocannabinoids
Endometriosis
Adenomyosis
Apoptosis

ABSTRACT

Adenomyosis that is a form of endometriosis is the growth of ectopic endometrial tissue within the muscular wall of the uterus (myometrium), which may cause dysmenorrhea and infertility. Endocannabinoid mediated apoptotic mechanisms of endometriosis and adenomyosis are not known. We hypothesized that the down regulation of endocannabinoid receptors and/or alteration in their regulatory enzymes may have a direct role in the pathogenesis of endometriosis and adenomyosis through apoptosis. Endocannabinoid receptors CB1 and CB2, their synthesizing and catabolizing enzymes (FAAH, NAPE-PLD, DAGL, MAGL) and the apoptotic indexes were immunohistochemically assessed in endometriotic and adenomyotic tissues. Findings were compared to normal endometrium and myometrium. Endometrial adenocarcinoma (Ishikawa) and ovarian endometriosis cyst wall stromal (CRL-7566) cell lines were furthermore cultured with or without cannabinoid receptor agonists. The IC50 value for CB1 and CB2 receptor agonists was quantified. Cannabinoid agonists on cell death were investigated by Annexin-V/Propidium iodide labeling with flow cytometry. CB1 and CB2 receptor levels decreased in endometriotic and adenomyotic tissues compared to the control group ($p = 0,001$ and $p = 0,001$). FAAH, NAPE-PLD, MAGL and DAGL enzyme levels decreased in endometriotic and adenomyotic tissues compared to control ($p = 0,001$, $p = 0,001$, $p = 0,001$ and $p = 0,002$ respectively). Apoptotic cell indexes both in endometriotic and adenomyotic tissues also decreased significantly, compared to the control group ($p = 0,001$ and $p = 0,001$). CB1 and CB2 receptor agonist mediated dose dependent fast anti-proliferative and pro-apoptotic effects were detected in Ishikawa and ovarian endometriosis cyst wall stromal cell lines (CRL-7566). Endocannabinoids are suggested to increase apoptosis mechanisms in endometriosis and adenomyosis. CB1 and CB2 antagonists can be considered as potential medical therapeutic agents for endometriosis and adenomyosis.

1. Introduction

Endometriosis and adenomyosis are defined as the unusual location of the endometrial tissue at ectopic sites and in the myometrium. They can cause pelvic pain, dyspareunia, amenorrhea, dysmenorrhea and infertility (Irving and Clement 2011; Kruse et al., 2012; Lin et al., 2014; Lo Monte et al., 2013; Sznurkowski and Emerich, 2008; Gao et al., 2006; Vannuccini et al., 2016; Yang et al., 2013; Yamanaka et al., 2014). Endometriosis affects a large population and decreases quality of life. The pathogenesis of the disease remains unclear, although it is believed to relate with the quite aggressive behavior of endometriotic cells at migrating ectopic locations and the resistance of these cells to apoptosis (Agic et al., 2009; Nasu et al., 2011; Sbracia et al., 2016). Pathogenesis of adenomyosis is similar to endometriosis; adenomyotic cells have resistance to apoptosis as well (Yamanaka et al., 2014). The relationship between the endocannabinoids and adenomyosis is not yet studied.

Endocannabinoids, which are mostly located in the central nervous system and also in other organ systems, are Cannabis ligands that specifically act through their CB1 and CB2 receptors (Alger and Kim,

☆ Various data of this study was presented at the XII. National Congress of Histology and Embryology, Ankara, Turkey, May 27–30, 2014, and at the XIII. National Congress of Histology and Embryology, İzmir, Turkey, April 30–May 3, 2016.

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http://dx.doi.org/10.1016/j.acthis.2017.05.005
Received 24 October 2016; Received in revised form 9 May 2017; Accepted 9 May 2017

Table 1
Age-matched control and experimental groups are presented with mean ± standard deviation/median (min–max) of their immune reactivities and apoptotic indexes.

Control	n	AGE mean ± SD	CB1 mean ± SD	CB2 mean ± SD	FAAH mean ± SD	NAPE-PLD mean ± SD	MAGL mean ± SD	DAGL median (min-max)	TUNEL median (min-max)
Proliferative	12	37,5 ± 1,27	0,80 ± 0,11	0,79 ± 0,10	0,79 ± 0,07	0,73 ± 0,15	0,69 ± 0,09	0,64 (0,49–0,79)	0,63 (0,0–0,85)
Secretory	7	41,4 ± 2,5	0,80 ± 0,11	0,76 ± 0,10	0,83 ± 0,11	0,71 ± 0,11	0,76 ± 0,10	0,72 (050–0,82)	0,65 (0,4–0,91)
Endometriosis									
Non-cystic	11	40,4 ± 2,07	0,35 ± 0,16	0,35 ± 0,21	0,37 ± 0,26	0,27 ± 0,23	0,22 ± 0,29	0,60 (0,0–0,66)	0,0 (0,0–0,70)
Cystic	9	35,6 ± 2,5	0,39 ± 0,20	0,40 ± 0,25	0,34 ± 0,34	0,32 ± 0,29	0,38 ± 0,24	0,52 (0,48–0,74)	0,11 (0,0–0,66)
Adenomyosis	17	42,5 ± 0,9	0,37 ± 0,19	0,34 ± 0,32	0,41 ± 0,21	0,43 ± 0,25	0,29 ± 0,26	0,48 (0,0–0,81)	0,05 (0,0–0,69)

2011; Coskun and Bolkent, 2014; Mercati et al., 2012; Muccioli, 2010; Scotchie et al., 2015; Yazulla, 2008). The most well-known endocannabinoids are anandamide (AEA) and di-arachidonoylglycerol (2-AG) (Alger and Kim, 2011; Muccioli, 2010; Scotchie et al., 2015; Yazulla, 2008). AEA is known to act more over the CB1 receptor in the female genital system, while 2-AG displays its effects usually through the CB2 receptor (Maccarrone, 2009; Taylor et al., 2010). In the female genital system, endocannabinoids and their receptors are generally located in the endometrium, the myometrium, the ovarian cortex and the medulla and the uterine tubes; they have critical roles in menstrual cycle, ovarian maturation, embryo transplantation and implantation (Brighton et al., 2011; El-Talatini et al., 2009, 2010; Karasu et al., 2011; Maccarrone, 2009; Scotchie et al., 2015; Sun et al., 2009; Taylor et al., 2010).

Recent researches suggested that endocannabinoids are involved in the pathophysiology of endometriosis in a variety of ways. Endocannabinoid agonists have anti-proliferative and analgesic effects on endometriotic cells or patients. Endometriosis-associated pain is shown to decrease by WIN 55212-2, CB1 and CB2 receptor agonist in experimental studies or by palmitoylethanolamide in patients with endometriosis (Cobellis et al., 2011; Dmitrieva et al., 2010; Giugliano et al., 2013; Indraccolo and Barbieri, 2010; Lo Monte et al., 2013). Cell proliferation in deep infiltrating endometriosis decreased with WIN-55212-2, both in vitro and in vivo (Leconte et al., 2010). In vitro stimulatory effect of endocannabinoid agonists on cell migration moreover was presented (Gentilini et al., 2010; McHugh et al., 2012). According to this, enhanced endometrial stromal cell migration via CB1 and GPR18 receptor with use of methanandamide, which is another endocannabinoid agonist, or *N*-arachidonyl glycine, which is an endogenous metabolite of anandamide, were shown through the activation of PI3K/Akt, ERK1/2 or MAPK pathways (Gentilini et al., 2010; McHugh et al., 2012). Although some activities of endocannabinoids are defined, we still do not know the effects of endocannabinoids on apoptosis in endometriosis. Anandamide leaded to apoptosis through CB1 receptor and p38 pathway on decidual cells (Almada et al., 2015; Fonseca et al., 2009).

Given the apoptotic and anti-proliferative effects of endocannabinoids, we hypothesized that the down regulation of endocannabinoid receptors and/or alteration in their regulatory enzymes may have a direct role in the pathogenesis of endometriosis and adenomyosis through apoptosis. We aimed to define the potential apoptosis related classical receptor mediated effects of endocannabinoids on endometriosis and adenomyosis. We investigated the differences of immune labelings of CB1 and CB2 receptors, AEA and 2-AG catabolizing and synthesizing enzymes, as well as apoptotic index between the endometriotic and adenomyotic patients and age-matched controls. Depending on the supposedly pro-apoptotic effects of endocannabinoids (Almada et al., 2015; Siegmund et al., 2016), endometrial adenocarcinoma cell line (Ishikawa) and ovarian endometriosis cyst wall stromal cell line (CRL 7566) were cultured with or without cannabinoid classical receptor agonists. The xCELLIgence cell impedance based system was used to calculate the IC50 value for CB1 and CB2 receptor agonists. Cannabinoid agonists' effect on cell death was investigated with flow cytometry by Annexin-V/propidium iodide labeling. Outcomes of these experiments may explain endocannabinoid effects of cell survival and death mechanisms in endometriosis.

2. Materials and methods

2.1. Design

A double blind randomized experimental study was designed. We received endometrial archive samples belonging to patients having been diagnosed as endometriotic and adenomyotic from January 2010 to July 2012. Age-matched paraffin endometrial tissue blocks of 20 endometriosis, 17 adenomyosis patients and 19 normal controls between 24 and 52 years were obtained from Hacettepe University Pathology Department (Table 1). Control tissues were obtained from patients undergoing dilatation and curettage surgery for benign gynecological conditions other than endometrial disease. Control endometrial tissues were sub-grouped according to the phase of menstrual cycle as proliferative (n = 12) and secretory phases (n = 7). The endometriotic tissues were also sub-grouped as cystic (n = 9) and non-cystic (n = 11). The use of endometriotic cells and the paraffin blocks of endometrial tissue was approved by the Hacettepe University Non-invasive Clinical Researches Ethical Committee (TBK 12/05-08), Ankara, Turkey.

2.1.1. CB1 and CB2 receptors and FAAH, NAPE-PLD, MAGL, DAGL enzymes immune labeling

5–6 μm Thick paraffin sections were deparaffinized. Endogenous peroxidase activity was blocked by 3% hydrogen peroxide (cat# 216763, Sigma-Aldrich, St. Louis, USA) after antigen retrieval. Nonspecific binding was blocked with 5% normal mouse serum (cat#M5905, Sigma-Aldrich, St. Louis, USA) for 30 min. Slides were incubated with following primary antibodies overnight at 4 °C: CB1(cat#C2866, rabbit polyclonal;1/100 dilution; Sigma-Aldrich, St. Louis, USA), CB2 (cat#HPA028718, rabbit polyclonal; 1/100 dilution; Sigma-Aldrich, St. Louis, USA), FAAH (cat#HPA007425, rabbit polyclonal, 1/50 dilution; Sigma-Aldrich, St. Louis, USA), MAGL (cat#100035, rabbit polyclonal, 1/100 dilution, Cayman, Michigan, USA), DAGL (cat#ab106979, rabbit polyclonal, 1/100 dilution, Abcam, Cambridge, USA). Incubation with NAPE-PLD (cat#HPA019832, rabbit polyclonal, 1/100 dilution, Sigma-Aldrich, St. Louis, USA) was performed overnight at RT. The secondary antibody incubation (cat#EXTRA3, mouse monoclonal, Sigma-Aldrich, St. Louis, USA) was performed for 30 min at RT at 1/20 dilution. After washing slides and incubating with DAB (cat#D3939, Sigma-Aldrich, St. Louis, USA) we used haematoxylin for counterstaining. Digital images were analyzed and captured using the Leica DM6000B microscope equipped with a DFC480 digital camera.

2.1.2. Image analysis

Two pathologists according to pathological criteria for the diseases selected endometriotic and adenomyotic foci under the microscope (Fu et al., 2013; Yu et al., 2015).