Photodynamic diagnosis (PDD) using 5-aminolevulinic acid-supplemented cultures of human endometrial epithelial cells

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Abstract

Purpose: The studies were aimed at monitoring 5-aminolevulinic acid (5-ALA)-dependent accumulation of endogenous protoporphyrin IX (PpIX) in epithelial cells originating from normal endometrium or endometriotic foci, as related to steroid treatment.

Material and methods: Epithelial cells were cultured in presence of estradiol-17 beta (E2) and progesterone (P) in concentrations typical for the follicular stage (E2 alone, 220 pg/ml) or the luteal stage (E2 100 pg/ml and P 2 ng/ml) or in presence of progesterone alone (2 ng/ml) for a period of 24, 48 or 72 h. Effect of 5-ALA concentration on the accumulation of PpIX was defined in the cells incubated with 2.0 mmol/l 5-ALA for 2 h. PpIX fluorescence was detected using a confocal microscope.

Results: After hormonal stimulation, intensity of PpIX-specific fluorescence was only slightly increased in epithelial cells originating from normal endometrium. Cultures of epithelial cells from endometriosis foci showed higher concentration of PpIX than did the cells originating from normal endometrium. The highest peak of PpIX fluorescence was noted in epithelial endometriotic cells after 48h incubation with progesterone.

Conclusions: The data on PpIX accumulation in epithelial cells in the presence of estradiol-17 beta or progesterone may provide indications as to the menstrual cycle phase(s) in which photodynamic therapy for endometriosis should be performed. It is concluded that hormonal condition of female body must be taken into account for diagnosis and treatment of endometriosis.

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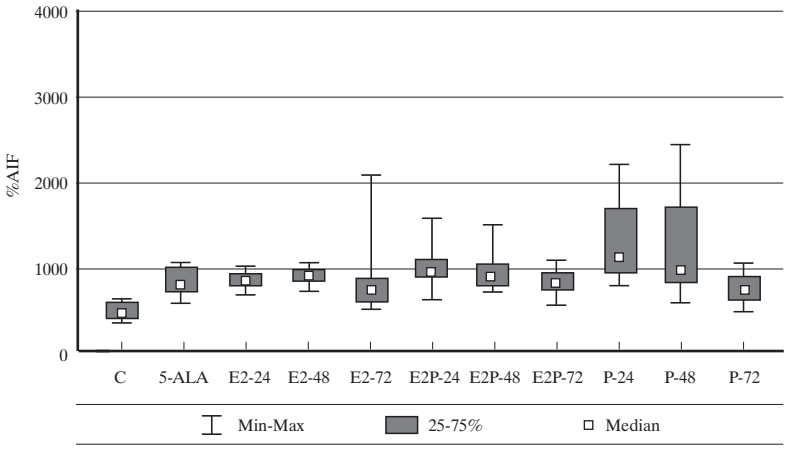
Introduction

Endometriosis is a disease of endometrial glands and stromal cells, developing out of the uterine cavity. It is appraised to affect 3-10% women in the generative age but among infertile women and in women with pain in the pelvis the incidence is supposed to reach 20-90% [1]. In clinical practice the only reliable way to diagnose endometriosis is to visualize its typical lesions in the course of laparoscopy or laparotomy and to confirm the diagnosis by histopathology. In recent years a photodynamic technique has been introduced to clinical practice, both for diagnostic and therapeutic purposes. It uses fluorescent drugs that concentrate preferentially in tumours and other hyperproliferative tissues. At present, among substances applied in the photodynamic approach particular attention is focused on 5-aminolevulinic acid (5-ALA), the presence of which represents a physiological requirement for heme production in cells protoporphyrin IX (PpIX) represents one of the compounds which arise during heme biosynthesis and is used in photodynamic therapy (PDT) as a photosensitizer [2].

Several studies have demonstrated the capacity of endometrium to accumulate more ALA-dependent PpIX as compared to other tissues [3-10]. The obtained data allow us to suggest that this compound can be used for diagnosis and treatment of endometriosis, when hormonal condition of female body is taken into account. Recognition of PpIX accumulation in isolated cells of endometrial epithelium (from uterine cavity) and of endometriotic foci, as affected by estrogen and progesterone, may be practical significance for definition of requirements of photodynamic diagnosis of endometriosis and its therapy.

Figure 1. **Alterations in PpIX content in epithelial cells isolated from normal endometrium during incubation with steroids and 5-ALA. C: control cells, 5-ALA: 2 h incubation with 2 mmol/l 5-aminolevulinic acid; E2-24/48/72: time of preincubation with estradiol subsequently 24, 48 or 72 h and 5-ALA-2 h; E2P-24/48/72: time of preincubation with estradiol and progesterone for 24, 48 or 72 h and 5-ALA-2 h; P-24/48/72: time of preincubation with progesterone for 24, 48 or 72 h and 5-ALA-2 h**



Material and methods

The studies were performed on human primary epithelial cells, originating from normal uterine cavity or from genital or and ovarian endometriotic foci in five patients. The cells were isolated and cultured as described by Ryan et al., with some modifications [11]. Immediately after biopsy, tissue material was stored in F12 fluid (Sigma) supplemented with antibiotics: 10 μg amphotericin/ml (Sigma) and 0.2 mg gentamycin/ml (Sigma). The material was dissected into fragments of 1 mm^2 in area and subsequently transferred into F12 medium containing collagenase (0.25%, GIBCO, 189 U/mg). The tissue pieces were subjected to shaking in 37°C water bath for 2 h. Cells of the glands were separated from stromal and blood cells by filtration through sieves of 38 to 105 μm pore diameters. Glands were recovered from the sieves and placed into F12 medium with trypsin (0.25%, 15 minutes). The epithelial cells were incubated in F12 growth medium, containing 100 μg/ml streptomycin, 100 U/ml penicillin, 2 mmol/l L-glutamine and 10% foetal calf serum (FCS). All the subsequent experiments on the cells were conducted following 4 days of culture.

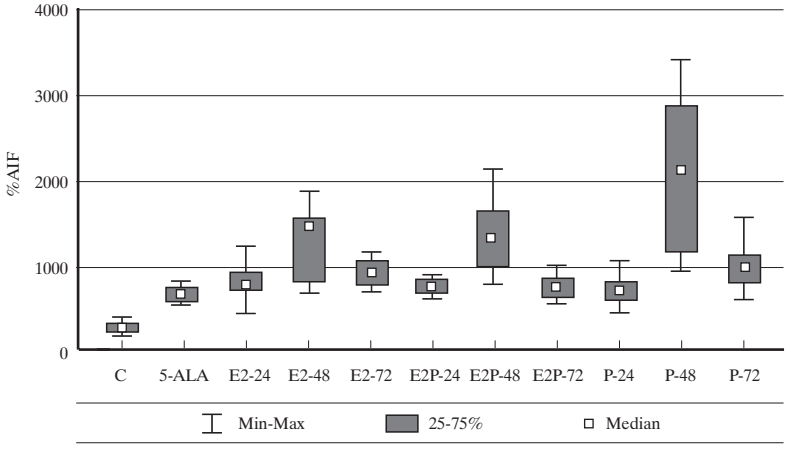
Cultures of epithelial cells, isolated from normal endometrium and endometriosis, were conducted in presence of estradiol-17 beta (E_2) and progesterone (P) in concentrations typical of the follicular stage (E_2 alone, 220 pg/ml) or the luteal stage (E_2 100 pg/ml and P 2 ng/ml) for a period of 24, 48 or 72 h (the hormone doses were selected to correspond to their blood levels during normal menstrual cycles in women) [12]. Effect of 5-aminolevulinic acid (5-ALA) concentration on accumulation of protoporphyrin IX (PpIX) in cells was defined in cells following their incubation with 2.0 mmol 5-ALA per l ml culture medium for a period of 2 h (5-ALA concentration and duration of incubation were selected on the basis of published data and our preliminary results) [2,4,13]. Following that time, PpIX content in cells was evaluated using a confocal microscope (LSM 510, Zeiss). The estimations took advantage of PpIX-exciting laser beam of 458 nm wavelength (argon laser, HFT 458), while the emitted light was analysed using 585 nm filter (LP 585). PpIX content was evaluated using the CytFlu 1.2 software and expressed as a product of an average intensity of fluorescence and percentage surface bearing area over the level of background (%AIF). In each experiment, cells of control group were evaluated, incubated in the same way but in the culture medium devoid of 5-ALA. Statistical evaluation of the obtained results involved the nonparametric U-Mann Whitney's test, performed using Statistica ver. 5 software. P value <0.05 was considered to represent threshold of significance.

Results

Results of PpIX-specific fluorescence estimation in a confocal microscope following preincubation of normal epithelial cells with steroids and incubation with 5-ALA are presented in *Fig. 1*. Protoporphyrin IX content, following 2h incubation with 2 mmol/l ALA without steroid treatment, was 675 %AIF. After hormonal stimulation with estradiol-17 beta and estradiol-17 beta plus progesterone, intensity of PpIX-specific fluorescence was only slightly increased in epithelial cells originating from normal endometrium. The maximum degree of PpIX accumulation (1023% AIF) was noted after 24 h preincubation with progesterone (*Fig. 1*).

A separate cycle of experiments was devoted to alterations in PpIX content in epithelial cells isolated from endometriotic foci, which were preincubated with steroids and, then, transferred to the 5-ALA-containing medium. The obtained results are illustrated in *Fig. 2*. The cultured epithelial cells from endometriotic foci showed higher concentration of PpIX than the cells originating from normal endometrium, especially in the case of 48 h preincubation with hormones. Following the 48 h/estradiol-17 beta treatment of the epithelial cells, intensity of the PpIX-specific fluorescence significantly increased

Figure 2. **Alterations in PpIX content in epithelial cells isolated from endometriotic foci during incubation with steroids and 5-ALA. C: control cells, 5-ALA: 2 h incubation with 2 mmol/l 5-aminolevulinic acid; E2-24/48/72: time of preincubation with estradiol subsequently 24, 48 or 72 h and 5-ALA-2 h; E2P-24/48/72: time of preincubation with estradiol and progesterone for 24, 48 or 72 h and 5-ALA-2 h; P-24/48/72: time of preincubation with progesterone for 24, 48 or 72 h and 5-ALA-2 h**



($p<0.05$) but was followed by a significant decrease ($p<0.05$) after 72h (*Fig. 2*). Likewise, in the case of estrogen plus progesterone treatment a significant increase ($p<0.05$) in cellular PpIX content was noted after 48h. However, the highest peak of protoporphyrin IX fluorescence (2115% AIF) was observed after progesterone treatment in 48th h of the experiment (*Fig. 2*). PpIX-specific fluorescence in cells of the control group did not significantly change in the course of the entire experiment and never exceeded the value of 240%AIF.

Discussion

For a properly conducted photodynamic therapy, an appropriate photodynamic diagnosis is pre-required. The finding that 5-aminolevulinic acid (5-ALA), which basically is not a photosensitizer itself, induces accumulation in cells (particularly in tumour cells) of endogenous protoporphyrin IX has proved especially significant. 5-ALA is a naturally occurring metabolite in the heme biosynthesis pathway. It is synthesized in the mitochondrial matrix from glycine and succinyl-CoA under the effect of 5-ALA-synthase. Subsequently, ALA finds its way to the cytoplasm, in which in the presence of 5-ALA-dehydratase (ALA-D) it becomes condensed to porphobilinogen. As the result of subsequent reactions (deamination, decarboxylation and oxidation), catalysed by appropriate enzymes (porphobilinogen-deaminase, uroporphyrinogen-decarboxylase, coproporphyrinogen-oxidase and protoporphyrinogen-oxidase) PpIX is formed, from which, following addition of iron (mitochondrial ferrochelatase), heme is produced [14]. In cases of augmented heme production, activity of 5-ALA-synthase is inhibited due to a negative feedback [15]. Excess exogenous 5-ALA circumvents feedback inhibition, leading to accumulation of highly fluorescent and photosensitizing porphyrins, mainly protoporhyrin IX. Accumulation of PpIX in cells under effect of ALA used to be evaluated by analysis of fluorescence intensity. In our studies, the measurement of fluorescence intensity was performed using confocal microscope. The results have demonstrated that incubation in media containing steroids (estradiol-17 beta, progesterone) and 5-aminolevulinic acid induce accumulation of PpIX in the isolated epithelial normal cells and epithelial cells isolated from endometriotic foci. The evident decrease in the fluorescence intensity was noted always following 72 h incubation with steroids. The decrease in PpIX content in the studied cells might have reflected efflux of the compound or increased activity of ferrochelatase, which catalyses binding of PpIX with iron. Moreover, application of progesterone for 48 h and ALA for 2h resulted in a twofold increase in PpIX-related fluorescence in epithelial cells isolated from endometriotic foci only. At the moment, the mechanism of this phenomenon remains difficult to interpret but one should keep in mind the potential for induction of enzymes responsible for ALA synthesis and possibly lower efflux of the compound from the cells. It is certainly important for future investigations; it seems that in certain conditions the cell may preferentially switch to an endogenous mechanism under progesterone stimulation, inducing a higher intracellular accumulation of the sensitizer.

The data on PpIX accumulation in endometrial cells as related to presence of estradiol-17 beta or progesterone in incubation medium may provide indications as to the menstrual cycle phase(s) in which PDD and/or PDT for endometriosis treatment should be performed. It is concluded that for diagnosis and treatment of endometriosis hormonal condition of female body must be taken into account.

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