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Accumulation of 5-Aminolevulinic Acid-Induced Protoporphyrin IX in Normal and Neoplastic Human Endometrial Epithelial Cells

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The aim of this study was to evaluate 5-Aminolevulinic acid (ALA)-induced fluorescence of normal and neoplastic endometrial epithelial cells for diagnosis and photodynamic treatment. Fluorescence of ALA-induced PpIX in vitro was measured by flow cytometry in two different human endometrial adenocarcinoma cell lines and in normal cells cultivated from fresh endometrial tissue of three premenopausal patients. The cells were analysed after incubation with different concentrations of ALA during 3, 6, or 24 hours. Both tumor cell lines showed a statistically significant higher fluorescence of PpIX than normal epithelial cells after incubation with 1 mg ALA per ml medium during 24 hours. The well-differentiated cancer cells produced significantly more PpIX than the poorly differentiated cancer cells. Relative PpIX intensity of the two cancer cell lines correlated with cell proliferation rate as measured by the doubling times of the cells.

Higher accumulation of Pp IX in neoplastic endometrium compared to normal endometrial epithelial cells may provide targeted biopsies and selective photodynamic destruction of neoplastic micro-lesions.

5-Aminolevulinic acid (ALA) is a precursor of Protoporphyrin IX (Pp IX) in the biosynthetic pathway of heme. Heme biosynthesis is essential to life and occurs in all aerobic cells.The administration of exogenous ALA induces accumulation of Pp IX (photosensitizer) which represents the fluorescenting substance (1). In general, malignant cells of epithelial origin accumulate more Pp IX than their normal counterparts (2,3,4,5,6). The capacity of neoplastic endometrial cells to accumulate ALA induced Pp IX has not been investigated until now. Since PpIX content may be increased in neoplastic endometrial cells, photodynamic diagnostic procedures becomes possible. Excitation of accumulated PpIX by light at a wavelength of 405 nm causes optimal fluorescence emission for hysteroscopic diagnosis. Even the smallest neoplasic lesions may be visualized for guided biopsies. Thereafter, therapeutic photodynamic procedures can be performed. When light of sufficient energy and appropriate wavelength interacts with the photosensitizer (PpIX), highly reactive oxygen intermediates are generated (7). These intermediates, primarily singlet molecular oxygen, irreversibly oxidize essential cellular components. The resulting photodestruction of crucial cell organelles and vasculature ultimately causes cell necrosis.

Because of the growing interest in photodynamic technique as a potential tool for diagnosis and treatment of endometrial neoplastic lesions, this study was designed to evaluate the potential of normal and neoplastic human endometrial cells to accumulate ALA-induced PpIX. A higher content of Pp IX in neoplastic endometrium may provide targeted biopsies and selective destruction of small lesions.

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MATERIALS AND METHODS

Primary cell cultures. The tissue dissociation and cell purification of both human epithelial and stromal endometrial cells were based on a combination of the methods of Satyaswaroop et al. (8) and Fleming et al. (9) with some modifications. Fresh endometrial tissue of 3 premenopausal patients not using oral contraceptives were obtained from pathologist immediately after curettage. A neoplastic disease was excluded in all specimens. The fresh normal tissues were stored on ice in transporting medium which is growth medium (DMEM with 4.5 mg/ml D-glucose) supplemented with 10% Fetal Bovine Serum (FBS), 0.2 U/ml human insulin (Novolin R by Novo Nordisk, Bagsvaerd, Denmark) and four times strength of antibiotics (10 μg/ml Amphotericin, 0.2 mg/ml Gentamycin, Sigma) as in plating medium. After washing three times with fresh transporting medium, the tissues were cut into 1 mm^3 pieces and subsequently transfered into 5 to 10 ml of growth medium containing 0.25% collagenase (GIBCO, 189 U/mg). The tissue pieces were treated by agitation in a 37°C water bath for 2 hours. A cell suspension consisting of stromal cells and epithelial fragments resulted by collagenase digestion. This cell suspension was gently dispersed using a 10 ml pipette and strained through a 250 μm-stainless steel sieve to remove the undigested material (retained by the sieve). The stromal cells and epithelial fragments were subsequently separated by filtering the cell suspension through a 45 μm-nylon mesh.

The stromal-enriched fraction consisted mostly single cells passed through this filter together with erythrocytes. The fraction was centrifuged at 1200 rpm for 10 minutes and washed with 50 ml plating medium (same as transporting medium with one fouth strength of antibiotics) and cultured in plating medium. After 1 to 2 h of plating, stromal cells had adhered to the culture dish, and erythrocytes were removed by medium change.

The majority of the epithelial fragments that remained as clumps was retained on the nylon mesh filter and was rinsed off into growth medium. After centrifugation (500 rpm, 10 minutes), the pellet was washed again and cultered in dishes with the plating medium. It required more time for epithelial fragments to adhere to culture dishes than stromal cells. After about 1 to 2 hours of plating, the stromal cells were adhered as described above, whereas epithelial fragments were still in suspension. This suspension was transferred into new dishes with plating medium. Between 6 to 24 hours after initial plating, epithelial cells were eventually spreaded out.

The cells were incubated in an atmosphere of 95% air and 5% CO_2 at 37°C. The cell culture medium of both stromal and of epithelial cells were renewed twice a week and subcultured on the fourth or fifth day with 0.125% Viokase (5% 4× pancreatin from Gibco and 1% EDTA). A single cell suspension was prepared for the flow cytometer experiments.

Cell lines. Well (ATCC HTB 112, HEC-1-A) and poorly (ATCC CRL 1622, KLE) differentiated human endometrial adenocarcinoma cells were obtained as established culture system from American Type Culture Collection (ATCC). HEC-1-A cells were grown in complete culture medium 199 with Earles salts (Irvine Scientific, Irvine CA) supplemented with 10 mM L-Glutamine (GIBCO), 100 μg/ml Streptomycin, 50 μg/ml Gentamycin (GIBCO) and 10% FBS. KLE cells were cultured in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F-12 (GIBCO) supplemented with 15 mM Hepes, 10 mM L-Glutamine, 100 μg/ml Streptomycin, 50 μg/ml Gentamycin and 10% FBS. Both HEC-1-A and KLE cells were subcultured with 0.25% trypsin/1 mM EDTA on a routine basis.

Cell doubling time. 1.5×10^5 HEC cells/dish and 0.6×10^5 KLE cells/dish were plated in several 35 mm culture dishes initially. Every 24 hours, the cells of one dish were subcultured and cell number was determined with a Haemocytometer (Neubauer chamber) until confluence is reached. The doubling time corresponds to the time taken for the culture to increase two-fold in the middle of the exponential or 'log' phase of growth (10). The following formula was used to determine the doubling time (DT) in our study:

$$DT = (t_2 - t_1)\log 2/(\log N_2 - \log N_1).$$

N_1 and N_2 are the cell numbers at the time points t_1 and t_2.

Incubation of 5-aminolevulinic acid. 5-Aminolevulinic Acid solution (Deprenyl USA Inc., Parsippany, NJ) was prepared according to concentrations desired in incubation medium and pH adjusted to 7.4. Concentrations of 0.01, 0.1, 1 and 10 mg of ALA per ml phenol red free growth medium were used for our experiments. Cells in exponential growth stage were incubated with ALA for 3, 6 or 24 hours. At the end of the incubation time, cells were detached from the culture dishes and fluorescence intensities were measured with the flow cytometer. The amount of Protoporphyrin IX containing in each cell is determined by the measured relative fluorescence intensity.

Flow cytometer system. All studies related to ALA uptake were performed on the Epics V flow cytometer from Coulter Electronics, Inc., equipped with Coherent Innova 90 Argon Laser. The samples were excited with 488 nm wavelength of light and fluorescence intensities were measured on Log Integrated Red Fluorescence (LIRF) channel for 10,000 cells per sample. Cells without ALA incubation were used as control. The LIRF channel numbers of each sample were converted to a linear scale according to the 3.00 decade channel conversion table provided by Coulter Electronics. Thereafter, control values (autofluorescence) were substracted from ALA-treated samples. Statistical analysis were performed subsequently and graphs plotted.

For studies with the normal epithelial gland cells, samples from three different premenopausal patients were used.

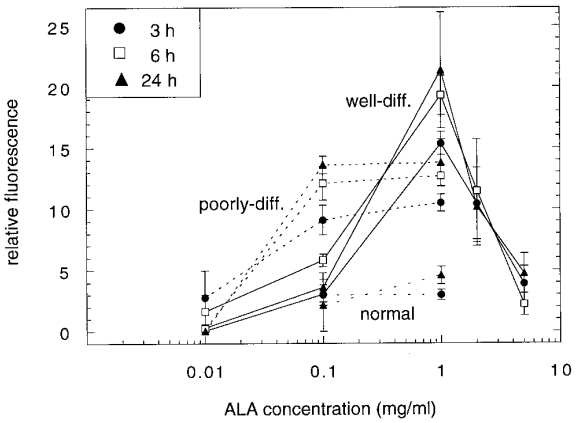

FIG. 1. ALA-induced relative fluorescence of PpIX in well (HEC)- and poorly (KLE) differentiated endometrial adenocarcinoma cells, and in normal endometrial epithelial cells determinated by flow cytometry. Cells were measured after incubation of different concentrations of ALA (0.01, 0.1, 1, 2, 5 mg/ml medium) during different time points (3, 6, 24 hours).

For experiments involving the cell lines HEC and KLE, each study was repeated three times. For all studies with flow cytometer, each measurement is carried out with two different culture dishes for each incubation time point and for each ALA concentration.

Statistic. Analysis of variance (ANOVA) was used to compare Pp IX-fluorescence of normal and neoplastic (well-differentiated, poorly-differentiated) endometrial epithelial cells (Fig.2). The difference of Pp IX-fluorescence of well- and poorly-differentiated tumor cells was evaluated with the unpaired Student t-test. Means and standard-errors were calculated for fluorescence data.

RESULTS

ALA-metabolism in neoplastic and normal endometrial cells. Fluorescence of ALA-induced PpIX in well (HEC)- and poorly (KLE)-differentiated endometrial cancer cells as well as in normal endometrial cells evaluated by flow cytometry after an incubation time of 3, 6 or 24 hours with different concentrations of ALA is stated in figure 1. Highest intracellular Pp IX accumulation was measured at ALA-concentrations of 1 mg/ml medium in HEC-cells and of 0.1 and 1 mg/ml medium in KLE-cells. At these ALA-concentrations, cells incubated for 24 hours revealed increased fluorescence intensity compared to 3 and 6 hours. A substantial decrease of fluorescence intensity was detected at ALA-concentrations of 2 and 5 mg/ml medium. In order to avoid possible cytotoxic effects of ALA at higher concentrations, KLE-cells were incubated only in media with 0.01, 0.1 and 1 mg ALA/ml.

The appropriate ALA concentration and incubation time for normal, non-pathological endometrial epithelial cells related studies, were determined based upon pharmacokinetic information obtained in the HEC- and KLE-cell studies. Therefore, normal endometrial cells were incubated in 0.1 and 1.0mg ALA/ml medium during 3 and 24 hours. Using these pharmacokinetic parameters, ALA-induced fluorescence capacity could not be increased nor changed in the endometrial cells.

Fluorescence measurements in normal and tumor epithelial cells incubated during 24 hours with 1mg ALA per ml medium are summarized in figure 2. Both tumor cell lines HEC and KLE showed a statistical significant higher fluorescence of PpIX than normal epithelial cells ($P=0.0004$). Comparing the relative fluorescence of the two tumor cell lines, the well-differentiated HEC-cells produced significantly more PpIX than the poorly-differentiated KLE-cells ($P=0.0299$).

Correlation of Pp IX synthesis with doubling time. Doubling time and relative PpIX-fluores-

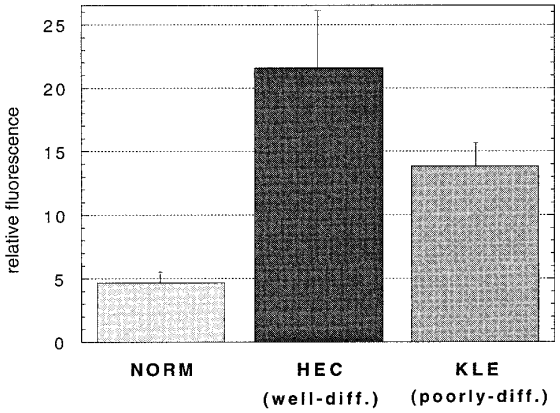

FIG. 2. ALA-induced relative fluorescence intensity of PpIX in normal and neoplastic (HEC, KLE) endometrial epithelial cells following 24 hours incubation with 1 mg ALA/ml medium. Statistical comparison of HEC-, KLE-, and normal cells: Analysis of variance: P=0.0004. Statistical comparison of HEC- and KLE-cells: unpaired Student t-test: P=0.0299.

cence of HEC- and KLE-cells following incubation with 1mg ALA per ml medium during 24 hours are shown in Tab.1. The doubling time in both cell-lines was inverted proportional to the ALA-induced relative fluorescence of PpIX. HEC-cells had a faster growth and a higher PpIX-fluorescence compared to KLE-cells.

DISCUSSION

The evaluation of ALA induced PpIX accumulation in neoplastic, epithelial endometrium was of substantial interest for photodynamic diagnosis. High intracellular Pp IX concentrations produces strong fluorescence which is important for photodynamic diagnostic procedures and for selective cell destruction. The influence of drug dose on intracellular Pp IX content was primary evaluated in well-differentiated adenocarcinoma cells (HEC) using several different ALA-concentrations. These dose-response studies indicate that there are significant concentration-dependent variations of Pp IX content. Fluorescence intensity was substantially elevated using an ALA-concentration of 1.0 mg/ml medium. At higher ALA concentrations (2 and 5 mg/ml), fluorescence intensities decreased in HEC cells due to the cell death caused by the cytotoxic effects as seen microscopically using the trypan blue test. Another explanation for the decrease in fluorescence at higher ALA concentrations may be nonfluorescent aggregates. Porphyrins are known for its tendency to aggregate in solution and in cells to produce aggregates of very poor fluorophores (11). Accordingly, we conducted dose-response studies in the poorly-differentiated adenocarcinoma cells (KLE) with lower ALA-concentrations (0.01/0.1/1.0 mg/

TABLE 1
Cell Doubling Time of HEC- and KLE-Cells and Their Relative PpIX Fluorescence after Incubation with 1 mg ALA/ml Medium during 24 Hours

Cell line	Doubling time (hours)	PpIX fluorescence (mean ± SE)
HEC (well-diff)	22.5	21.4 ± 4.8
KLE (poorly-diff)	36.9	13.8 ± 1.9

ml). Fluorescence was uniformly increased using 0.1 and 1.0 mg ALA/ml medium. However duration of cellular exposure to ALA affected the amounts of Pp IX content as well. Incubation time of 24 hours revealed highest fluorescence intensity at an ALA-concentration of 1 mg/ml in HEC-cells and of 0.1 mg/ml and 1 mg/ml in KLE-cells, respectively. ALA as a precursor of Pp IX in heme synthesis must be metabolized to the active photosensitizer Pp IX. This process may explain the delayed endometrial fluorescence elevation. ALA-concentrations and incubation times were restricted to 0.1 and 1.0 mg/ml, and, 3 and 24 hours respectively for non-pathological cells because of the limited amount of cells in curetted tissue. However fluorescence activity in non-pathologic human endometrial cells remained stable regardless of changing concentration and incubation time. The low amount to Pp IX may be explained by a low metabolism rate of normal endometrial epithelial cells. Conclusively, flowcytrometric data revealed a significantly higher ALA-induced accumulation of Pp IX in human endometrial adenocarcinoma cells compared to the normal, non-pathological endometrial epithelial cells. Bedwell et al. (6) showed that PpIX-fluorescence intensity of rat colonic tumor glands was about 6 times higher than normal glands after systemic administration of ALA. In general, metabolism in malignant cells seems to be highly activated producing increased accumulation of Pp IX following ALA-application.

Comparing the two neoplastic endometrial cell lines, photosensitizer concentration was highest in the well-differentiated cells (HEC) which exhibited the faster doubling time. Poorly-differentiated cells (KLE) showed fewer fluorescence corresponding to a longer doubling time. Rebeiz et al. (5) showed that stimulated splenocytes with rapid turnover produced more Pp IX compared to resting splenocytes. Iinuma et al. (12) evaluated different cell lines of varied origin (excluded endometrial cells) and compared the doubling time to PpIX cellular contents after ALA administration. Cells with high proliferative rates often, but not always synthesized more Pp IX.

Endometrial cancer foci visualized by high fluorescence become the target for guided biopsies using hysteroscopy for photodynamic diagnostics. Since accumulation of the photosensitizer Pp IX is increased in neoplastic endometrial cells, selective photodynamic destruction may be feasible. In the study of Kennedy et al. (1,13) and Divaris et al. (14) topical application of ALA to certain skin tumors and subsequent exposure to photoactivating light lead to selective destruction of such tumors without producing damage of normal skin due to increased PpIX content in tumors compared to normal skin. The intensity of the ALA-induced PpIX fluorescence in various skin structures correlated well with the degree of phototoxic damage after exposure of the skin to a standard dose of photoactivating light (14).

It is concluded that photodynamic techniques following intrauterine application of ALA may provide a new approach for guided biopsies and selective destruction of neoplastic microlesions of the endometrium.

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