Title
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Regeneration processes in rabbit endometrium: a photodynamic therapy model

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The origin and process of regeneration in rabbit endometrium was evaluated following photodynamic epithelial destruction using topically applied aminolevulinic acid (ALA). Selective destruction of endometrial epithelium was performed using photodynamic therapy (PDT). ALA was diluted to 200 mg/ml dextran 70 shortly prior to administration. A volume of 1.2 ml was injected into the left uterus. Intrauterine illumination (wavelength 630 nm, light dose 40–80 J/cm^2) was performed 3 h after drug administration. Tissue morphology was evaluated by light and scanning electron microscopy 1, 3, 7 and 28 days post-treatment (three animals at each time-point). Regeneration of the endometrium following epithelial ablation by PDT was fully activated after 24 h and was completed after 72 h. Endometrial surface generation occurred by proliferation, originating primarily in deeper regions of the glands. Findings from our morphological follow-up study support the origin of endometrial regeneration being mainly from undifferentiated stem cells and residual glandular epithelium.

Key words: endometrium/photodynamic therapy/regeneration

Introduction

The regenerative capacity of the endometrium is regarded as unique and as one of the most dynamic phenomena in humans. It is characterized by cyclic proliferation, differentiation and cell death every menstrual cycle. The duration of menstrual surface re-epithelialization is ~48 h and begins on cycle days 2 or 3 (Ferenezy, 1977). Tissue regeneration at comparable rates is known only in the haematopoietic (Lajtha, 1973), intestinal (Hagemann and Lesher, 1973) and epidermal systems (Lavker and Sun, 1983). Several morphological studies on endometrial regeneration in humans (McLennan and Rydell, 1965; Baggish *et al.*, 1967; Ferenezy, 1976a,b) and rabbits (Schenker *et al.*, 1971; David *et al.*, 1973; Beier and Mootz, 1978) have already demonstrated its regenerative potential.

To understand the biological principles on which quickly renewing tissues are based, several hypotheses have been offered: (i) a small pool of multipotent 'undifferentiated stem cells' located near or within the endometrial–myometrial junction give rise to epithelial, stromal or vascular cells (Cairnie *et al.*, 1976; Prianishnikov, 1978); (ii) the regenerating surface epithelium originates from the residual epithelium of gland stumps or undamaged bordering epithelium (Bartelmez, 1933; McLennan and Rydell, 1965); (iii) stromal cells transform to endometrial epithelium, thereby relining the surface (Papanicolaou, 1933; Craig, 1963); and (iv) rupture of capillaries gives rise to migration and transformation of the endothelial cells.

Photodynamic therapy (PDT) offers a new approach to the study of endometrial epithelial regeneration because it offers the selective targeting and destruction of cells within the endometrium. The technique typically involves the i.v. or topical administration of a photosensitizing drug. When light of sufficient energy and appropriate wavelength interacts with the sensitizer, highly reactive oxygen intermediates are generated (Kimel *et al.*, 1989). These intermediates, primarily singlet molecular oxygen, irreversibly oxidize essential cellular components. The resulting photodestruction of crucial cell organelles and vasculature ultimately causes cell necrosis. In this study, aminolevulinic acid (ALA) was used to target the endometrium. ALA is a precursor of protoporphyrin IX (PpIX) in the biosynthetic pathway of haemoglobin. Haemoglobin biosynthesis is essential to life and occurs in all aerobic cells.

The slowest step in this process is the conversion of PpIX to haemoglobin. Therefore, the administration of exogenous ALA induces the accumulation of PpIX, a strong photosensitizer. Because only certain types of cell have a large capacity to localize PpIX, the use of ALA for PDT provides potential selectivity. After the intrauterine application of ALA, PpIX has been found to be metabolized more readily in endometrial glands than in the surrounding stroma (Wyss *et al.*, 1994b). Therefore, selective targeting and damage of the endometrial epithelium is possible, especially by using light doses under the threshold of complete and permanent endometrial destruction. The aim of this study was to test several hypotheses for the origin of endometrial regeneration by using PDT to observe the regenerative process of rabbit endometrium following epithelial ablation. If successful, this concept might offer a new approach to the study of endometrial physiology.

Materials and methods

Animals

A total of 15 mature female New Zealand White rabbits (n = 15) weighing 3600–4300 g were placed in a controlled environment with free access to food and water. Three rabbits were killed to evaluate histological changes at 1, 3, 7 and 28 days following PDT after

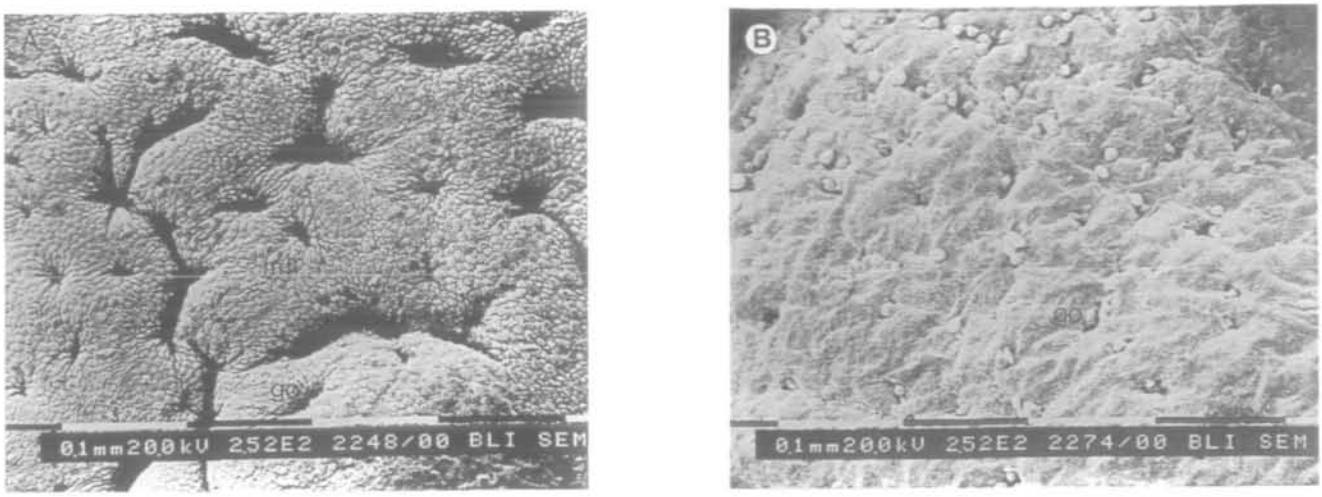

Figure 1. Scanning electron micrographs (original magnification ×252) (**A**) An untreated endometrial surface with mucosal folds (mf) and gland openings (go) at oestrous stage. (**B**) The same aspect 24 h after photodynamic therapy The columnar surface epithelium is missing, while the gland openings are still visible.

intrauterine ALA instillation and 28 days after intrauterine benzoporphyrin derivative administration. Rabbits are induced ovulators, therefore no oestrous monitoring was required.

Photosensitizer

Crystallized 5-ALA (DUSA USA Inc., Parsippany, NJ, USA) and vials of liposomally formulated benzoporphyrin derivative MA (BPD) (Quadra Logic Technologies Inc., Vancouver, British Columbia, Canada) were stored in the dark at a temperature of 4°C. ALA was diluted to 200 mg/ml and BPD to 2 mg/ml in Hyskon® (dextran 70; Kabi Pharmaceuticals Inc , Clayton, NJ, USA) shortly prior to administration. To minimize acidity, ALA/Hyskon solutions were titrated with 10 and 1 N NaOH to pH 5.5. The injected volume was 1 2 ml in rabbit uteri (left one only)

Procedures

Animals were anaesthetized with 0 75 ml/kg ketamine/xylazine (2 1) i.m., and isofluorane was added during the surgical intervention. Intrauterine drug application was performed through a midline incision. The photosensitizer was injected with a 20 gauge needle into the uterus 3–5 mm distal to the uterine bifurcation. Abdominal walls were closed in three layers [Dexon 4-0 (Davis & Geck, Wayne, NJ, USA) and staples].

At 3 h after ALA application and 1 5 h following BPD administration, re-laparotomy was performed. Light from an argon-pumped dye laser operating at 630 nm for ALA and 690 nm for BPD (Spectra Physics, Mountain View, CA, USA) was delivered into the uterine cavity via a 400 mm diameter quartz optical fibre terminated with a 3.0 cm long cylindrical diffusing tip (model 4420-A02, PDT Systems, Buellton, CA, USA). A clinical Hartridge reversion spectroscope (Ealing Electro-Optics, South Natick, MA, USA) was used to verify the wavelength. Because the length of the rabbit uterus was 12–15 cm, multiple (four or five) incremental irradiations were required. A total of 185 mW was launched into the fibre (65 mW/cm fibre tip) during 800 s, resulting in a variable tissue dose which, depending on geometry, ranged from 40 to 80 J/cm^2.

Specimen retrieval

The rabbits were first anaesthetized with isofluorane and then underwent euthanasia by intracardiac injection of 1 5 ml Euth-6, a barbituric acid derivative/central nervous system depressant used only for euthanasia of animals (Western Medical Supply, Arcadia, CA, USA) Uteri were retrieved immediately following euthanasia. Specimens were sectioned in four blocks of 3–4 mm each and fixed in 10% formaldehyde.

Plastic-embedded section for light microscopy

Samples were fixed in Karnovsky's fixative (2% paraformaldehyde, 3% glutaraldehyde in 0 1 M cacodylated buffer) for 24 h at room temperature and then rinsed in a 0 1 M cacodylated buffer. The samples were then post-fixed in 1% osmium tetroxide in 0.1 M cacodylate buffer for 1 h, rinsed with double-distilled water and stained *en bloc* in Kellenbeger's uranyl acetate for 2 h. Dehydration was performed with progressive concentrations of ethanol–water in 10 min steps (30, 50, 70, 80 and 100%) and also in progressive ethanol–propylenoxide in 10 min steps. Infiltration was performed with progressive propylenoxide (Poly/Bed 872; Polysciences, Warrington, PA, USA) in 30 min steps Each sample was embedded carefully in flat mold. Sections, 500 nm thick, were cut using a histodiamond knife (Diatome US, Fort Washington, PA, USA) on an ultramicrotome and stained with Richardson's stain

Scanning electron microscopy (SEM)

Samples were fixed in 10% formalin in phosphate buffer at room temperature for 24 h, post-fixed in 10% osmium tetroxide, dehydrated in graded acetone critical point-dried (Ladd critical point dryer, Ladd Research Industries Inc., Burlington, VT, USA) and sputter coated with gold (Pelco PAC-1 evaporating system; Ted Pello Inc , Redding, CA, USA) Micrographs were taken on a SEM (SEM 515, Philips Electronic Instrument Company, Mahwake, NJ, USA)

Results

The morphological changes following selective epithelial damage (glands and luminal epithelium) in rabbit endometrium by photochemical effects are demonstrated in a sequence of images. The luminal surface of an untreated uterus showed mucosal folds covered by polygonal bordered (columnar) epithelial cells and several gland openings (Figure 1A). The light microscopy image of a transverse aspect of the endometrial structures (Figure 2A) exhibited a columnar epithelium surfacing the lumen and a gland. The underlying stroma consisted of loosely structured fibroblasts with extracellular

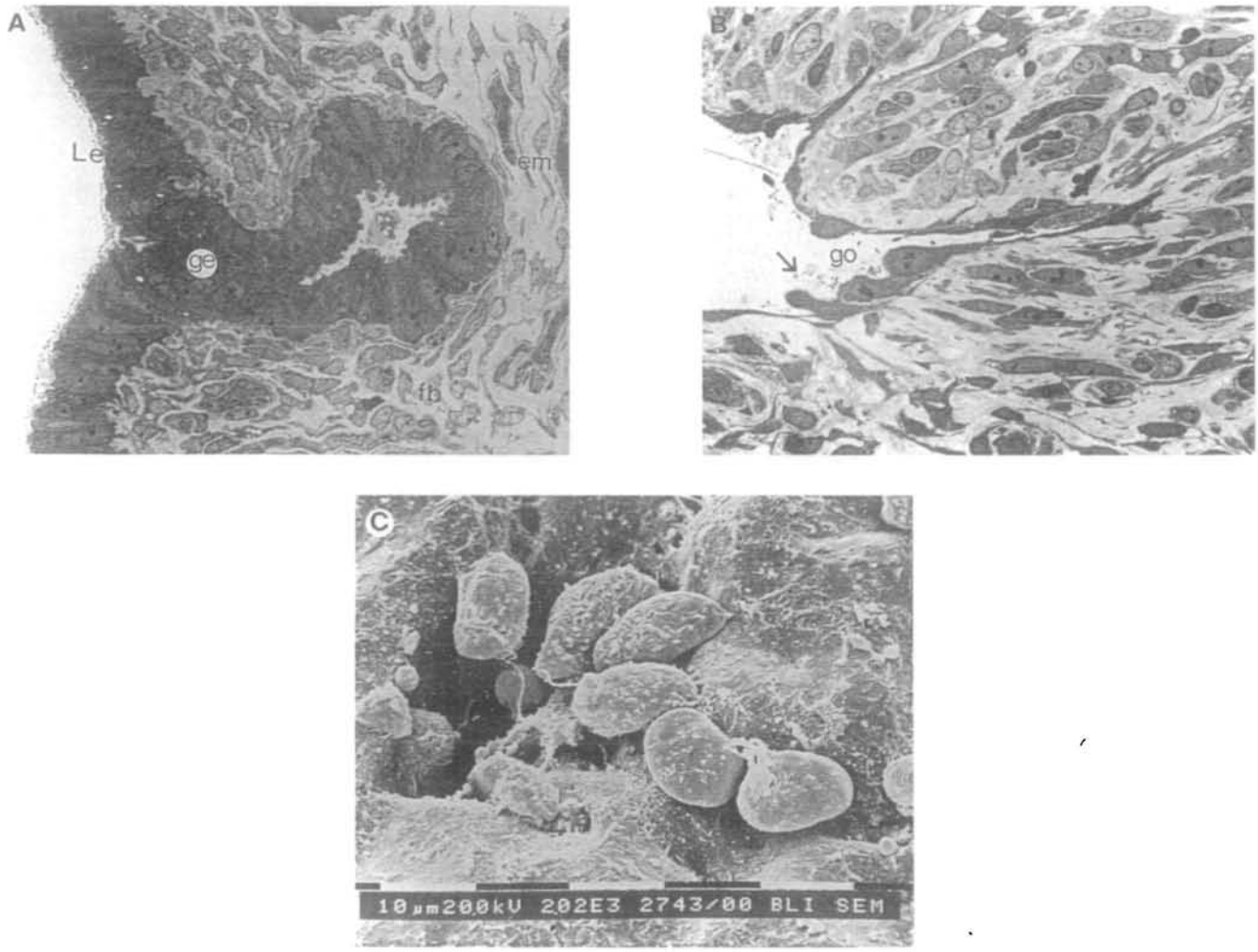

Figure 2. Light microscopic images (**A** and **B**, original magnification ×790); scanning electron micrograph (**C**, original magnification ×2020). (**A**) Transverse aspect of an untreated uterus (endometrium) exhibiting luminal (le) and glandular columnar epithelium (ge), stroma with fibroblasts (fb) and extracellular matrix (em). (**B**) Same endometrial aspect 24 h after photodynamic therapy (PDT). Columnar surface epithelium is absent. Round, flattened cells [see arrows, are protruding out of the gland openings (go)]. Cellular compactness in the stroma is decreased, whereas extracellular matrix is more abundant. (**C**) Chain of recovering flattened cells (ch) at a gland opening (go) (24 h after PDT)

matrix (collagen fibres, proteoglycan–glycoprotein ground substances) and capillaries. Stromal cellular density was higher near the uterine lumen than in the deeper endometrial regions. Myometrium (not shown) consisted of an internal circular and an external longitudinal muscle layer, and displayed no morphological change after PDT.

Endometrial morphology changed completely 24 h after PDT. In these specimens, mucosal folds were flattened, the epithelial layer was absent but the gland openings were still visible (Figure 1B). A few spherical cells were located primarily in and around the gland openings. The transsection of a gland (Figure 2B) 24 h after treatment showed flattened 'spindle' cells covering the luminal surface. Columnar epithelium was absent in these cells and their contour was more rounded at the gland opening. Cellular density in the stroma was reduced, while extracellular matrix was more abundant. Fibroblasts were directed towards the luminal surface. The superficial aspect of a similar gland opening (Figure 2C) displayed a chain of these flattened epithelial cells protruding out of the gland. These cells appeared to be more spherical when distant from the gland opening. The subjacent ground may represent the basal membrane. Figure 3A and B demonstrates the morphological difference between untreated luminal epithelium and the migrating regenerative epithelial cells in high magnification (SEM magnification ×5000). Normal oestrous endometrial epithelium consisted of ciliated cells surrounded by non-ciliated microvillous (secretory) cells. Regenerating epithelial cells exhibited a smooth surface with scant cilia or microvilli and appeared to lack functional specialization.

In a few of the images taken at 24 h after PDT, slightly pale cells containing large pale nuclei with irregular chromatin could be observed at the luminal surface (Figure 4). In these cells the nucleoli were not evident and the cell borders were poorly defined. The morphology of these cells was more similar to fibroblasts than to epithelial cells. Underlying stroma had abundant extracellular matrix, and stromal cells were directed towards the fibroblast-like cells which were likely to be participating in the surface regeneration process.

Capillaries were sometimes very close to the epithelial structures of the endometrium. In a small number of images

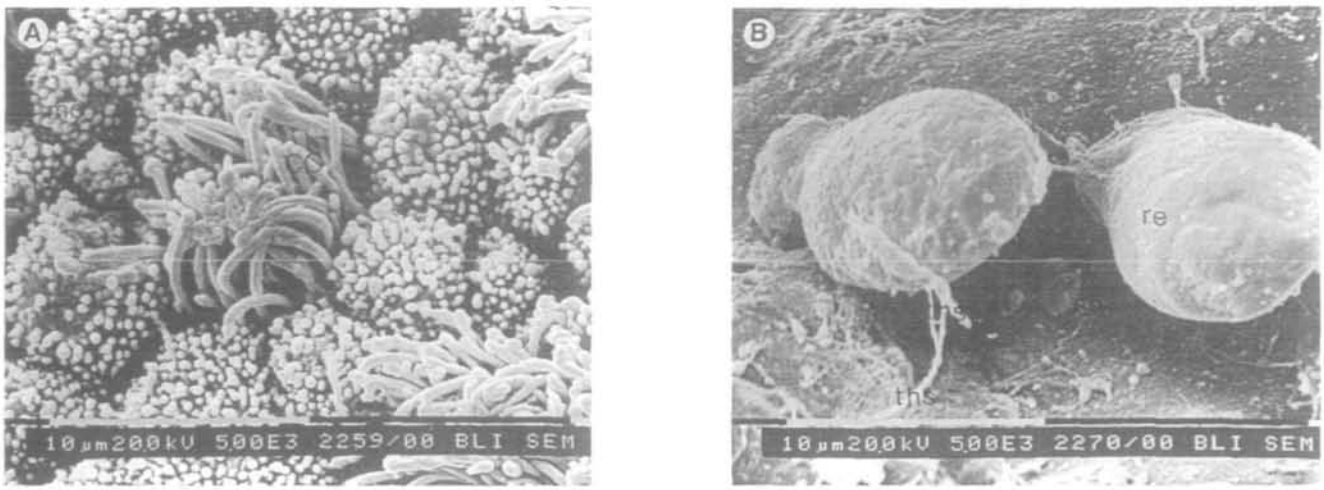

Figure 3. Scanning electron micrographs (original magnification ×5000). (**A**) Untreated endometrial surface (oestrous stage) with a ciliated cell (cc) surrounded by microvillous (secretory) cells (mc). (**B**) Flattened re-epithelializing cells (re) containing thread-like structures (ths) communicating with each other and with the base (24 h after photodynamic therapy).

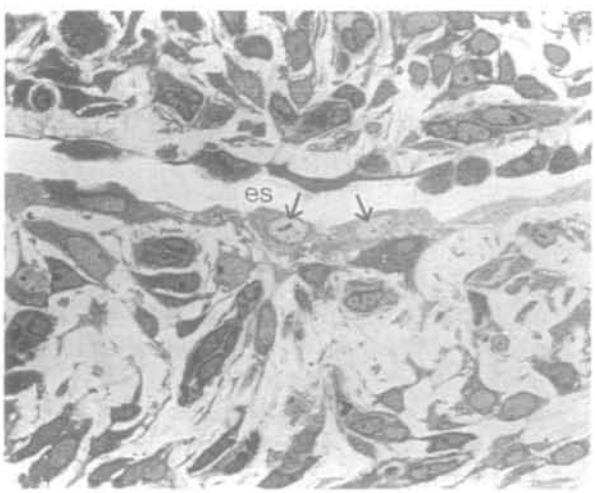

Figure 4. Light microscopic image (original magnification ×790). Slightly pale fibroblast-like cells (see arrows) with large pale nuclei were presumably participating in regeneration of the endometrial surface (es) (24 h after photodynamic therapy).

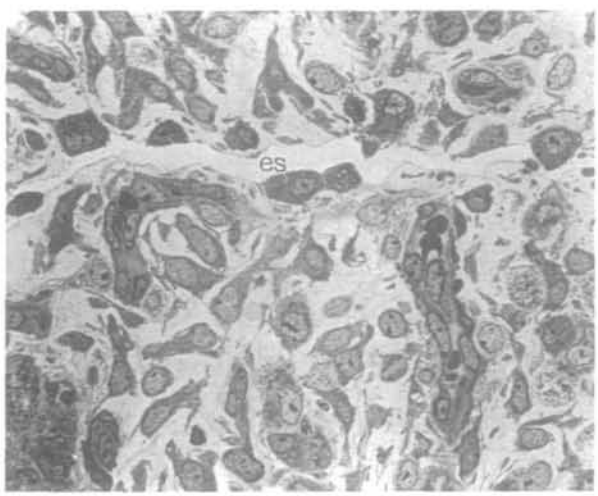

Figure 5. Light microscopic image (original magnification ×790). Some images exhibited endothelial cells of capillaries (see arrows) separated only by a thin layer of extracellular matrix from the basal membrane (bs) and the endometrial surface (es) (24 h after photodynamic therapy). Their morphological participation in the regenerative process could not be documented microscopically.

taken 24 h post-treatment, endothelial cells were separated by only a thin layer of extracellular matrix and the basal membrane from the de-epithelialized endometrial surface (Figure 5). A direct participation of endothelial cells in the re-epithelializing process could not be detected microscopically.

By 3 days after photodynamic damage of the epithelial structures using relatively low light levels, complete recovery of the endometrial surface was observed (data not shown). Ciliated cells were also distinguishable from microvillous secretory cells, but there were less mucosal folds visible. At 1 and 4 weeks after PDT, endometrial folds were reconstructed and the regenerated endometrium was identical to the untreated one.

In contrast, complete loss of the epithelial structures was observed in uteri 4 weeks following PDT using BPD as a photosensitizer. The surface was replaced by a collagen network resembling scar tissue (Figure 6).

Discussion

The endometrium of humans and mammals displays an astonishing regenerative capacity (Hartman, 1944; McLennan and Rydell, 1965; Schenker *et al.*, 1971; Ferenezy, 1977; Prianishnikov, 1978; Kaiserman-Abramof and Padykula, 1989; Ludwig *et al.*, 1990). This capacity is based on essential intrinsic endometrial tissue mechanisms that are independent of the hormonal influences of the reproductive system (Ferenezy, 1976a,b). Several hypotheses are proposed to explain the cellular origin of endometrial regeneration. This study was designed to use the principles of PDT to elaborate on the regenerative mechanism of the endometrium with suboptimal light doses for reversible selective destruction of the epithelial structures.

At 24 h after PDT, our microscopic examination demonstrated significant damage with the initial phase of regeneration

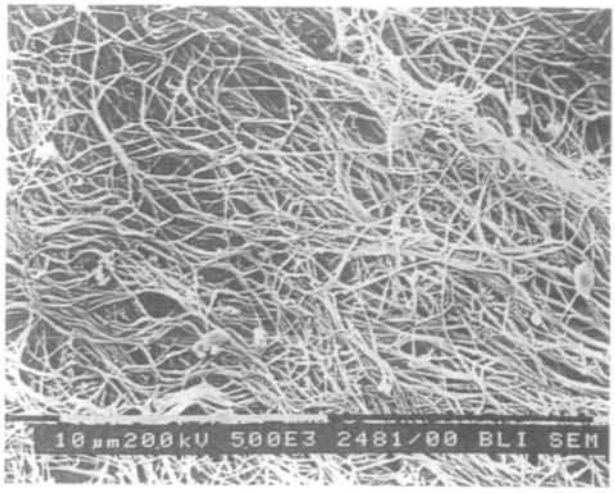

Figure 6. Scanning electron micrograph (original magnification ×5000) Complete loss of luminal columnar epithelium and glandular openings 4 weeks following photodynamic therapy using benzoporphyrin derivative and sufficient light dose. The surface appeared to be replaced by a collagen network resembling scar tissue or perhaps basal membrane [Reprinted with permission (Wyss *et al.*, 1994a).]

(Figure 2B and C). At this stage, most of the recovering cells were located at the gland opening. These observations indicate that endometrial surface generation after epithelial destruction occurs by proliferation originating primarily in the glands. Several studies of endometrial regeneration following menstruation, abortion, and mechanical and pharmacological destruction have suggested that the regenerating surface epithelium originates from the residual glands and epithelial structures (Bartelmez, 1933; McLennan and Rydell, 1965; Ferenezy, 1977; Beier and Mootz, 1978). However, endometrial stem cells for epithelial, stromal and vascular components are supposed to be located near to or within the endometrial–myometrial junction where the basal regions of the endometrial glands interdigitate with the myometrium (Padykula, 1989). A small pool of these undifferentiated multipotential cells is assumed to be the main source of the endometrial regenerative potency (Padykula, 1989).

Interestingly, the post-ovulatory epithelial mitotic activity in the deeper endometrial regions (called basalis IV) escapes inhibition by progesterone and may already be prepared for regenerative activity during menstruation (Kaiserman-Abramof and Padykula, 1989). Studies of the gastrointestinal mucosa (Hagemann and Lesher, 1973) and the epidermis (Lavker and Sun, 1983) have suggested that stem cells are more likely to be localized in the crypts. The location of these tissues in regions deeper than the lumen provides some protection and may also maintain the ability to regenerate after injury. The identification of stem cells in the endometrium is difficult because they have not been characterized clearly.

Other investigators have advocated a stromal origin of endometrial re-epithelialization (Papanicolaou, 1933; Baggish *et al.*, 1967). The main argument supporting the hypothesis of transformation of the stromal cells to epithelial cells is the common embryological origin of both cell types. Both cell types are presumed to arise from the intra-embryonic mesoderm that is built up by mesenchymal cells (Hinrichsen, 1990). The Müllerian (paramesonephric) system is developed within and by the mesoderm. This process includes the appearance of the intra-embryonic coelom, a cavity in the mesoderm covered by the mesothelial cells, and the invagination of coelomic surface building up the Müllerian duct. The duct consists of coelomic mesothelium (epithelial) surrounded by mesodermal mesenchyme. The mesothelium generates the endometrial epithelium and the mesenchyme generates the endometrial stroma and myometrium. Interestingly, the differentiation into endometrial epithelium is specified by mesenchyme (Cunha and Fujii, 1981). Because stromal fibroblasts are able to degrade collagen and elastic fibres, cellular migration through extracellular matrix and basal membranes should be possible. Our light microscopy images show cells relining the endometrial surface, which correspond morphologically to fibroblasts (Figure 4). Re-epithelializing cells are often described as 'fibroblastoid' or 'spindle' cells (Baggish *et al.*, 1967). However, electron microscopic studies could not support the hypothesis that surface epithelial cells may be derived from stromal fibroblasts (Ferenezy, 1976a,b). In addition, DNA synthesis in the stromal fibroblasts during the period of repair did not deviate from the normal values (Ferenezy, 1977), except by a short increase during maximum epithelial regenerative activity 48 h after injury. This suggests that the transformation of fibroblasts for epithelial regeneration is unlikely.

The first embryonic blood vessels arise in the extra-embryonic mesenchyme (Hamilton and Mossman, 1972) Intra-embryonic mesenchyme (mesodermal cells) is supposed to generate embryonic vessels as well (Reagan, 1917). Mesenchymal cells form angioblasts, which are generating endothelial cells. Therefore, the endothelium, the stroma and the epithelium of the endometrium may have the same mesodermal origin. Endometrial destruction with vascular damage (haemorrhagic) activates endothelial regeneration. Based on embryo development, precursors of endothelial cells may be transformed into stromal cells and be involved in the relining process of the endometrial epithelium. Capillaries were located just below the basal membrane (Figure 5) A direct participation of endothelial cells in epithelial regeneration could not be observed in our microscopic study.

Regeneration of the endometrium following selective epithelial destruction by PDT was fully activated at 24 h and completed after 72 h, resembling that of the non-treated control. In another study, following curettage of the rabbit endometrium, regeneration of the luminal epithelium was rapidly initiated at 3 h and completed at 72 h (Schenker *et al.*, 1971). Regeneration of human endometrium starts immediately after the onset of menstrual bleeding and a continuous layer of fusiform cuboidal epithelial cells is produced up to day 6 (Ludwig and Metzger, 1976).

In conclusion, the regenerative capacity of endometrial epithelium may originate in different cell types. Multipotent undifferentiated stem cells, residual endometrial epithelium, stromal fibroblasts and endothelial cells of ruptured capillaries are all possible participants in this regeneration.

Interestingly, regeneration of the epithelial structures did not occur using the BPD as a photosensitizer (Wyss *et al.*,

1994a) at the same light dose as for ALA. The absorption peak of BPD at 690 nm may offer a longer wavelength, with the deeper penetration depth of light in human endometrium increasing the light dose at the endometrial–myometrial conjunction where stem cells are supposed to be located. An augmented phototoxicity of BPD to regenerating cells may optimize photodynamic efficacy as well.

The genuine mechanism of endometrial regeneration is difficult to determine by microscopic investigations of cell morphology. Our investigation was limited to the histological localization of the definitive origin of regeneration in the deepest portions of the endometrium. Even functional studies may not define the final mechanism (Ferenezy, 1977; Padykula, 1989). Based on the embryological background, it is more likely that several processes may participate in regeneration of the endometrium, and none of the aforementioned hypotheses may be definitely excluded. Our morphological observations support the assertion that endometrial regeneration originates in undifferentiated stem cells and residual glandular epithelium.

Recent studies in our laboratories have demonstrated that different photosensitizers can target specific areas such as the endometrial glands or the stroma (Wyss *et al.*, 1994a,b). Various combinations of drugs and light may offer a new approach to the study of endometrial regeneration. Similar concepts may be applicable for studying mechanisms of embryo implantation, as demonstrated in our previous study in which no visible histological damage at implantation failure following PDT was demonstrated in rats (Steiner *et al.*, 1995).

Acknowledgements

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