A Histological and Immunohistochemical Study of the Cyclic Human Endometrial Angiogenesis

Hanan A. Amin[1,2] and Siham K. Abunasef[1,3]

[1]Anatomy Department – Faculty of Medicine - King Abdulaziz University -KSA
Histology Department – Faculty of Medicine - [2] Cairo University- [3]Ain Shams University- - Egypt
abunasef2@hotmail.com

Abstract: Background: All platelet-derived endothelial cell growth factors (PDGFs) had important roles in embryogenesis and adult maintenance, in addition to sharing in the phenotypes of different diseases and malignancies. They were considered as activating factors in angiogenesis in the endometrium. **Aim of the work:** The current study aimed at describing and evaluating the immunohistochemical and morphometrical expression of thymidine phosphorylase (TP) or (PDGF.44C) in the normally cycling human endometrium. **Materials and Methods:** Thirty two normal endometria were studied. The endometria were "dated" on haematoxylin and eosin stained sections. TP expression was assessed with the platelet-derived endothelial cell growth factor (PDGF.44C) monoclonal antibody, using the Avidin Biotin Complex (ABC) method. The mean area percent and the optical density of the PDGF.44C positive reaction were morphometricaly determined using Leica Qwin image analysis system. The collected data was statistically analysed. **Results:** In normal proliferative endometrium PDGF.44C was found invariably patchy. Expression was cytoplasmic in glandular epithelium, and nuclear in stromal cells. This immunohistochemical picture remained almost unaltered during the early secretory phase of the normal menstrual cycle but, most impressively, PDGF.44C was expressed uniformly in the epithelium of all endometrial glands towards the end of the cycle. At this stage, expression was mixed nuclear/cytoplasmic and there was very little stromal nuclear staining. **Conclusion:** PDGF.44C was expressed consistently in normal endometrium, suggesting a role in physiological angiogenesis. It had a definite pattern of distribution, which was dependent on the phase of menstrual cycle shifting from the endometrial stroma to the endometrial glands with progress of the cycle.
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1.Introduction

Human endometrium undergoes cyclical changes, approximately each month, during the reproductive years [(1)]. These endometrial changes, which are under the control of the ovarian hormones, constitute the menstrual cycle and include the regeneration and growth of the shed endometrium, its maturation and secretory transformation [(2)].

The high metabolic activity of the endometrium is ensured by a rich arterial supply. The integral part of the menstrual cycle is the phenomenon of angiogenesis-the formation of new blood vessels from a pre-existing vascular network in the basalis [(3)]. Angiogenesis is a complex multi-step process involving extracellular matrix remodeling, endothelial cell proliferation and migration, capillary differentiation and anastomosis Angiogenesis plays a major role in tumor progression, and thus inhibiting angiogenesis is a promising strategy for treatment of cancer [(4)].

Factors promoting angiogenesis in the endometrium include basic fibroblast growth factor (bFGF), transforming growth factor (TGF), vascular endothelial growth factor (VEGF), and thymidine phosphorylase (TP), also known as platelet derived endothelial cell growth factor (PD-ECGF) [(5)]. This enzyme is a potent angiogenic molecule that induces endothelial cell migration and proliferation. It is a strong reducing sugar, that generates oxygen radicals involved in early stages of protein glycation [(6)].

TP is expressed in normal tissues including the human endometrium [(6, 7)]. However, although the morphological and functional changes in the normal endometrium, together with the related fluctuating values of the ovarian hormones and their receptors, have been studied extensively [(8)], little is known about the immuno-histochemical expression of angiogenic factors in this tissue during the various phases of a normal menstrual cycle[(9)].

The present study set out to investigate the cellular and tissue distribution of PDGF.44C in the normally cycling endometrium using immunohistochemical and morphometrical methods.

2. Materials and Methods

The present study was based on endometrial samples obtained from thirty two adult females with a history of regular menstrual cycles and their ages ranged from 25 to 42 years.

These cases were collected from the Obstetrics and Gynaecology Department of the National Cancer Institute. They had undergone hysterectomy for non-endometrial disease, usually for carcinoma *in situ* of the cervix and as a routine step in anterior pelvic excentration for carcinoma of the urinary bladder. In all cases, the date of the last menstrual period was available. None of the patients had received hormone treatment.

The specimens comprised: 17 normal endometria of early (ten), late (seven) proliferative phase; 15 normal endometria of early (eight), late (seven) secretory phase. They were fixed in 10% formalin and were processed to paraffin blocks. Sections were cut at 5µm thickness and stained with haematoxylin and eosin (H&E) and alcian blue staining.

The endometria were "dated" on haematoxylin and eosin stained sections using the histological criteria of MacLusky [10].

Immunohistochemical Technique:

Immunohistochemical staining was done using the three layer technique. Mouse monoclonal antibody against thymidine phosphorylase; Ab-1, clone PDGF.44C (NeoMarkers, Westinghouse, USA) was used as the first layer. This was followed by incubation with biotinylated secondary antibody as the second layer. The slides were incubated with labeled Avidin-Biotin-Peroxidase complex (ABC) that binds to the biotin on the secondary antibody forming the third layer. The peroxidase in the ABC complex converts DAB (diamino-benzedine-tetrahydro-chloride) from Dako[@] into brown colour. The sections were counterstained with haematoxylin.

Control slides:

Positive control slides (breast carcinoma supplied by Neomarkers, Westinghouse, USA) were included in each staining session. As a negative control, sections were processed in the above sequence but the primary antibodies were not added and instead phosphate buffer was used in this step.

Morphometrical Study:

Leica Qwin 500 Ltd image analysis system was used to determine the area percent and the optical density of PDGF.44C positive reaction using the binary mode (Figs.1a, 1b, 1c). For this study 10 immunostained sections for each case were examined. Within each slide, 10 randomly chosen fields (LPF X200) were studied. The collected data were tabulated and statistically analyzed.Statistical analysis of the data using Duncan's test [11] was done.

3.Results

A) Histological and Immunohistochemical Results

Early Proliferative Phase Endometrium:

It was revealed in ten cases and assigned by the presence of non-branching glands having a regular contour. The glands were also evenly distributed in the stroma (Fig.2-a). The glandular cells were quite regular, tall columnar with basal oval nuclei. Stromal cells were compact, spindle shaped with scanty cytoplasm resembling fibroblasts. Few mitotic figures were detected in both glandular and stromal cells (Fig.2-b). No glandular luminal secretions could be observed as proved by negative alcian blue reaction (Fig.3).

The immunostained sections showed that PDGF.44C staining was characteristically patchy (Fig.4-a). The glandular epithelial cells showed weak cytoplasmic immunostaining whereas, the periglandular stromal cells revealed strong nuclear immunostaining (Fig.4-b).

Late Proliferative Phase Endometrium:

Seven cases were found to be in the late proliferative phase. This was recognized by the presence of tall columnar non-vacuolated ciliated epithelial cells with ovoid basal condensed nuclei lining the glands (Fig.5a). Areas of pseudostratification were also seen. On the other hand, the stromal cells were slightly separated and mitotic figures were evident in both glandular and stromal cells (Fig.5-b). Although an eosinophilic material was detected in the lumen of some glands in H&E sections, yet this material showed a negative reaction with alcian blue (Fig.6).

In TP immunostained sections, the reaction remained cytoplasmic in glandular cells and nuclear in the periglandular stroma (Fig. 7).

Early Secretory Phase Endometrium:

It could be demonstrated in eight cases. This phase was characterized by irregular glandular outline. The glands were lined by columnar cells with vesicular nuclei. The subnuclear (basal) vacuolations of the glandular cells were the characteristic feature of this phase. Stromal cells had condensed nuclei (Figs.8-a, 8-b). Glandular secretion could be detected in some glands and were positively stained by alcian blue (Fig. 9).

The immunohistochemical stained sections remained unchanged during this stage (Fig. 10).

Late Secretory Phase Endometrium:

The remaining seven cases were in this phase as evident by the presence of the saw-toothed appearance of the glands (wide glands with serrated luminal margins) which contain copious secretions (Figs. 11-a, 11-b). Most of the stromal cells were large having a considerable amount of pinkish cytoplasm whereas, others had vacuolated cytoplasm. Stromal oedema with extravasated blood and leucocyte infiltration were also evident (Fig. 11-c). The spiral arterioles were prominent, congested and the stromal cells were concentrically arranged around them (Fig. 12).The lumina of most of the glands were full of secretion that gave a strongly positive alcian blue reaction (Fig. 13).

TP was expressed uniformly by all epithelial cells in all endometrial glands throughout the endometrium (Fig.14). At this stage the pattern of enzyme Expression was mixed nuclear/cytoplasmic, and the reaction was strong, in contrast to the remaining phases of the menstrual cycle. Stromal reactivity was not a feature.

In many cases, there was some variation of immunostaining between individual epithelial cells within glands and also from one gland to another in any specimen.

Endothelial and smooth muscle cells in the media of occasional blood vessels were positive for PDGF.44C (Figs. 15-a, 15-b). No association between staining of blood vessels or myocytes and the phase of the menstrual cycle was observed.

B) Morphometric Results:

The image analysis computer system was used to measure and compare the mean area percent and optical density in the different endometrial phases.

The results are shown in (Table 1) and (Figs.16, 17). The mean area % and optical density were increasing during the cycle. The only significant difference was between the mean area% and optical density of the late secretory phase with that of the other phases.

Table (1): The Mean Area% and Optical Density in Different Endometrial Phases.

Endometrial Phase	Number of cases	Mean Area % mean ± SD	Optical Density mean ± SD
Early Proliferative (EP)	10	14.79 ± 2.89	0.45 ± 0.015
Late Proliferative (LP)	7	24.11 ± 3.90	0.50 ± 0.014
Early Secretory (ES)	8	27.99 ± 3.30	0.52 ± 0.009
Late Secretory (LS)	7	49.55 ± 2.20	0.61 ± 0.013

- Significance was considered $P \leq 0.05$

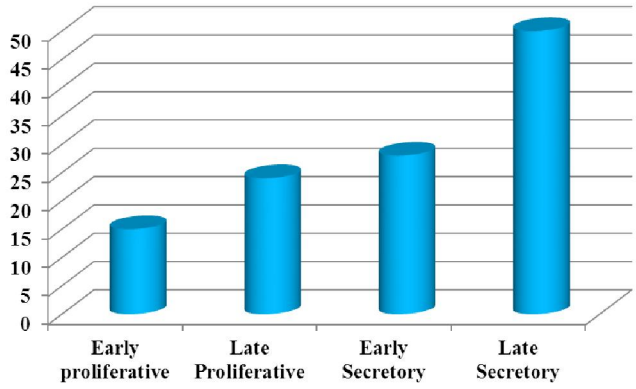

Fig. (16): Showing the mean values of the area percent of the PDGF.44C immune expression in the different endometrial phases.

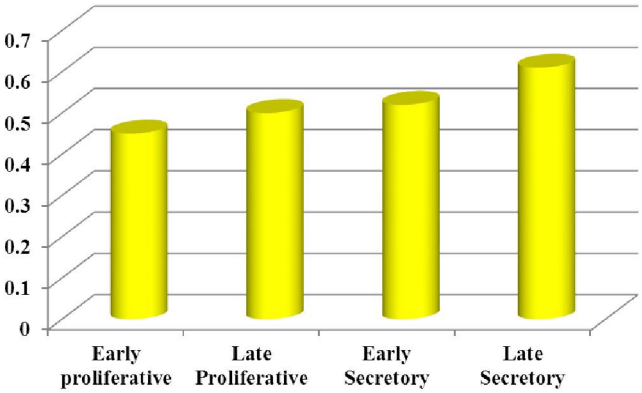

Fig. (17): Showing the mean values of the optical density (mean grey) of the PDGF.44C immune expression in the different endometrial phases.

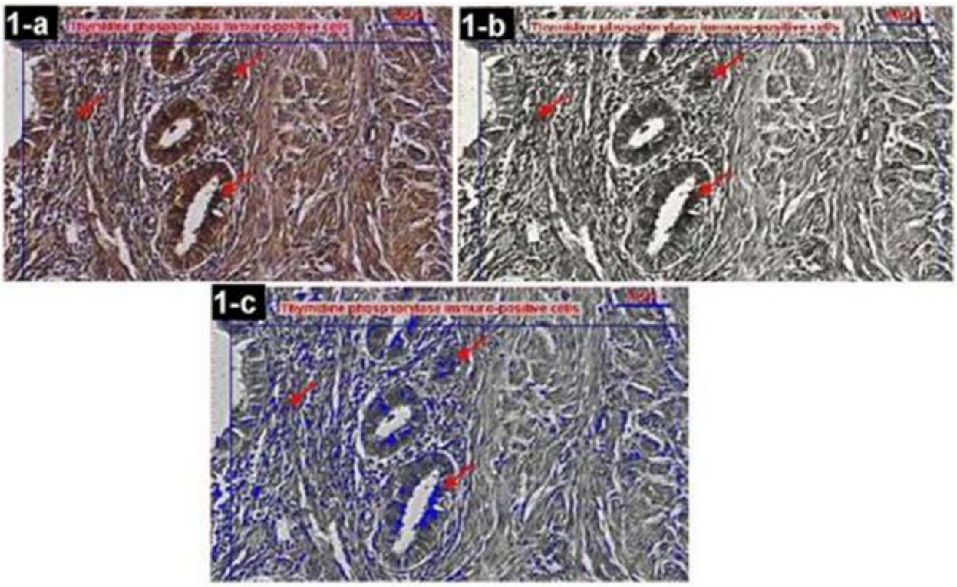

Fig. (1): Photomicrographs of display seen on the monitor's screen of the image analyzer showing:
(a): thymidine phosphorylase immunopositive cells (↑) (PDGF.44C immunostaining; X200)
(b): grey image of thymidine phosphorylase immunopositive cells (↑) (PDGF.44C immunostaining; X200)
(c): thymidine phosphorylase immunopositive cells masked by blue binary colour (↑) (PDGF.44C immunostaining; X200)

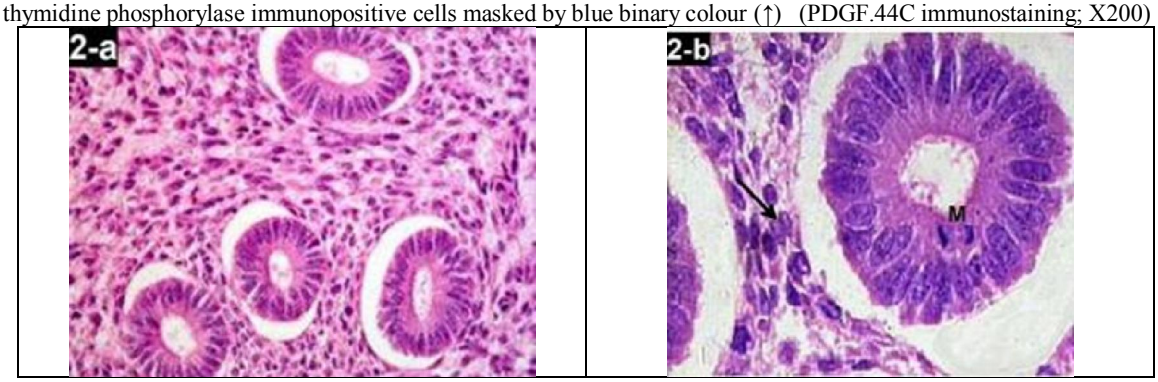

Fig. (2): Photomicrographs of sections in the endometrium in the early proliferative phase (age 32, day of cycle 7) showing:
(a): non-branching endometrial glands with regular contour. *(H & E; X 200)*
(b): the glands are lined by non-vacuolated tall columnar cells with basal oval nuclei. Stromal cells are fibroblast-like. Few mitotic figures are also evident in the glandular (M) and stromal (↑) cells. *(H & E; X 1000)*

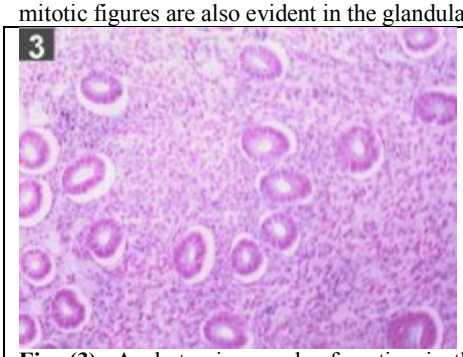

Fig. (3): A photomicrograph of section in the endometrium in the early proliferative phase (age 33, day of cycle 8) showing nearly negative alcian blue reaction in the luminal contents of the glands. *(Alcian blue; X 100)*

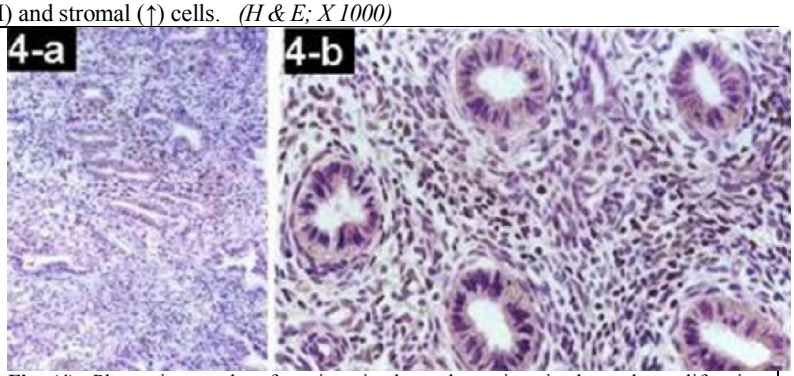

Fig. (4): Photomicrographs of sections in the endometrium in the early proliferative phase (age 32, day of cycle 7) showing:
(a): PDGF.44C immunostaining is patchy (*PDGF.44C immunostaining; X100)*
(b): weak cytoplasmic immunostaining in the glandular epithelium and strong nuclear staining in the stromal cells *(PDGF.44C immunostaining; X200)*

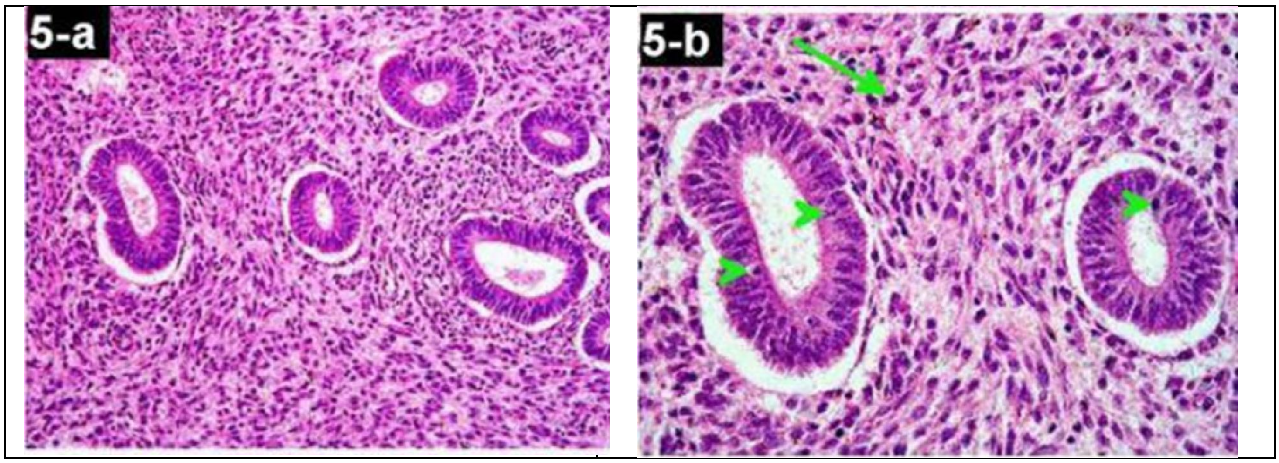

Fig. (5): Photomicrographs of sections in the endometrium in the late proliferative phase (age 35, day of cycle 11) showing:
(a): pseudostratification of glandular epithelial lining *(H & E; X 200)*
(b): higher magnification of the previous section revealing the non-vacuolated and ciliated glandular cells. Multiple mitotic figures in both glandular (>) and stromal (↑) compartments *(H & E; X 400)*

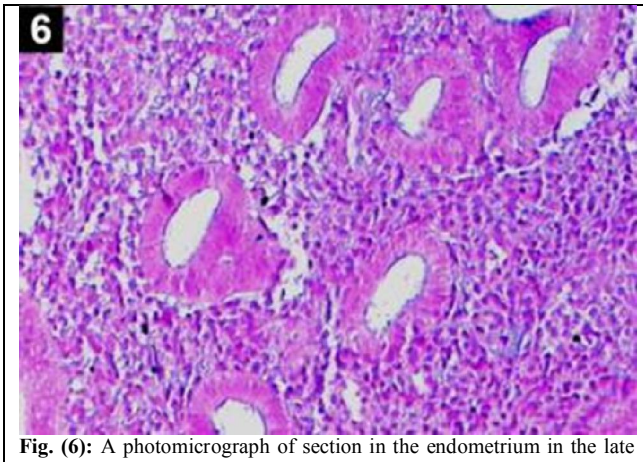

Fig. (6): A photomicrograph of section in the endometrium in the late proliferative phase (age 35, day of cycle 11) showing negative alcian blue reaction in the glandular lumina *(Alcian blue; X 200)*

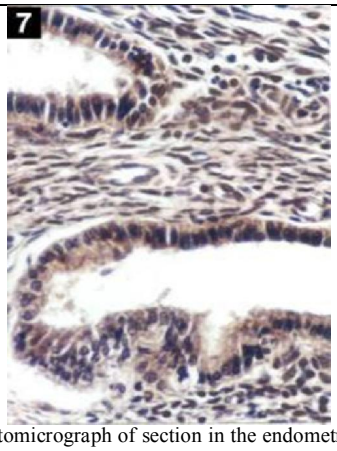

Fig. (7): A photomicrograph of section in the endometrium in the late proliferative phase (age 35, day of cycle 11) revealing cytoplasmic PDGF.44C immunostaining in the glandular epithelium and nuclear staining in the stromal cells *(PDGF.44C immunostaining; X400)*

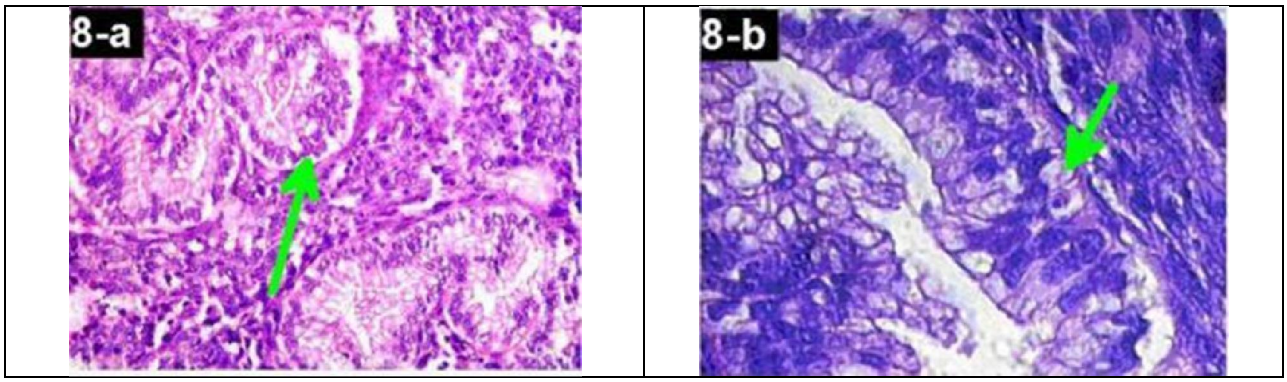

Fig. (8): Photomicrographs of sections in the endometrium in the early secretory phase (age 38, day of cycle 17) showing:
(a): irregular outline of the glands and vacuolations in the glandular cells (↑) *(H & E; X 200)*
(b): infranuclear vacuolations which are the characteristic feature of this phase (↑). Supranuclear vacuolations are also noted. Stromal cells are slightly separated from each other and their nuclei are condensed. *(H & E; X 1000)*

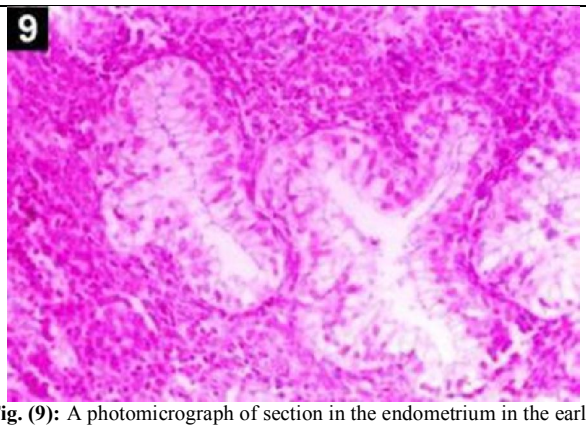

Fig. (9): A photomicrograph of section in the endometrium in the early secretory phase (age 38, day of cycle 17) showing weak alcian blue reaction in the lumen of some glands.

(Alcian blue; X 200)

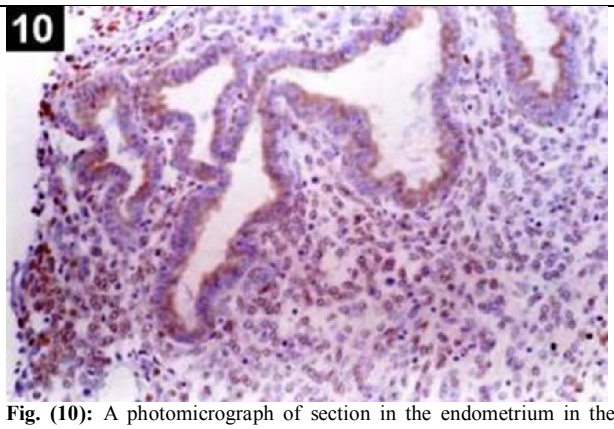

Fig. (10): A photomicrograph of section in the endometrium in the early secretory phase (age 38, day of cycle 17) revealing cytoplasmic PDGF.44C immunostaining in the glandular cells whereas nuclear staining in the stromal cells.

(PDGF.44C immunostaining; X400)

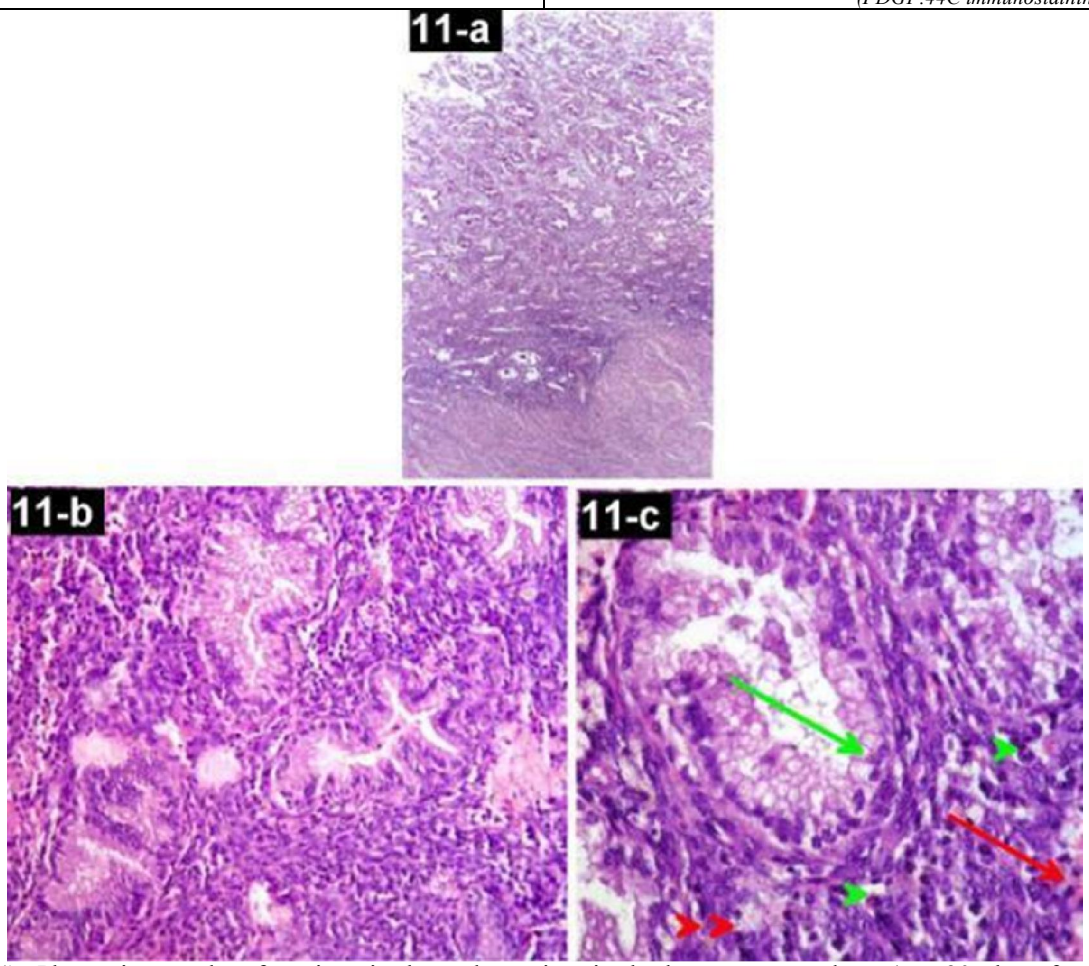

Fig. (11): Photomicrographs of sections in the endometrium in the late secretory phase (age 30, day of cycle 25) showing:

(a): the saw-tooth appearance of the glands (wide glands with serrated luminal margins) which contain copious secretions *(H & E; X 100)*

(b): wide glands with serrated luminal margins which contain copious secretions. *(H & E; X 200)*

(c): the glandular cells exhibit supranuclear vacuolations (green↑). Some of the stromal cells have vacuolated cytoplasm (>>). stromal extravasation of blood (red↑) and leucocytic infiltration (>) are seen. *(H & E; X 400)*

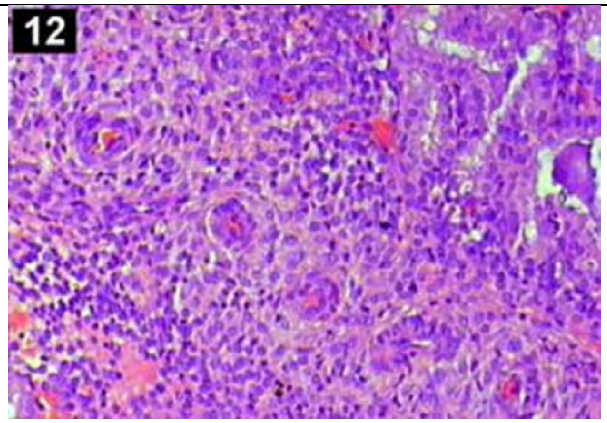

Fig. (12): A photomicrograph of section in the endometrium in the late secretory phase (age 30, day of cycle 25) showing prominent congested spiral arterioles with stromal cells concentrically arranged around them *(H & E; X 400)*

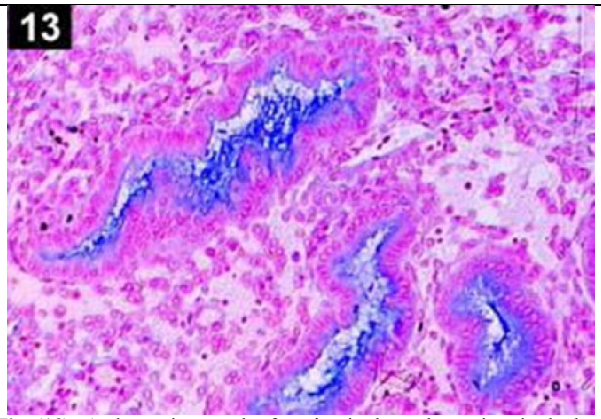

Fig. (13): A photomicrograph of section in the endometrium in the late secretory phase (age 30, day of cycle 25) showing glandular secretions with positive alcian blue staining

(Alcian blue; X 200)

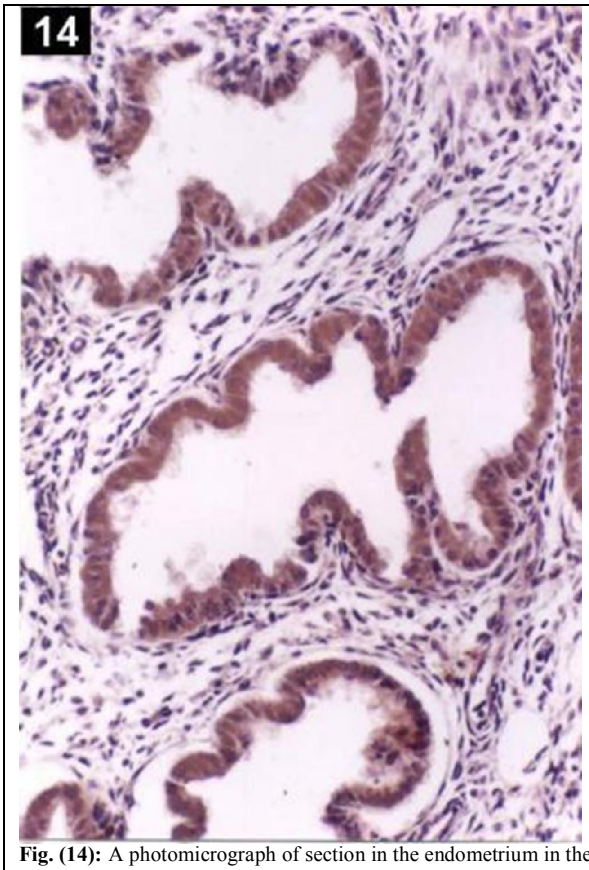

Fig. (14): A photomicrograph of section in the endometrium in the late secretory phase (age 30, day of cycle 25) revealing the PDGF.44C immunostaining is mixed nuclear/cytoplasmic in glandular cells whereas stromal immunostaining is not a feature

(PDGF.44C immunostaining; X400)

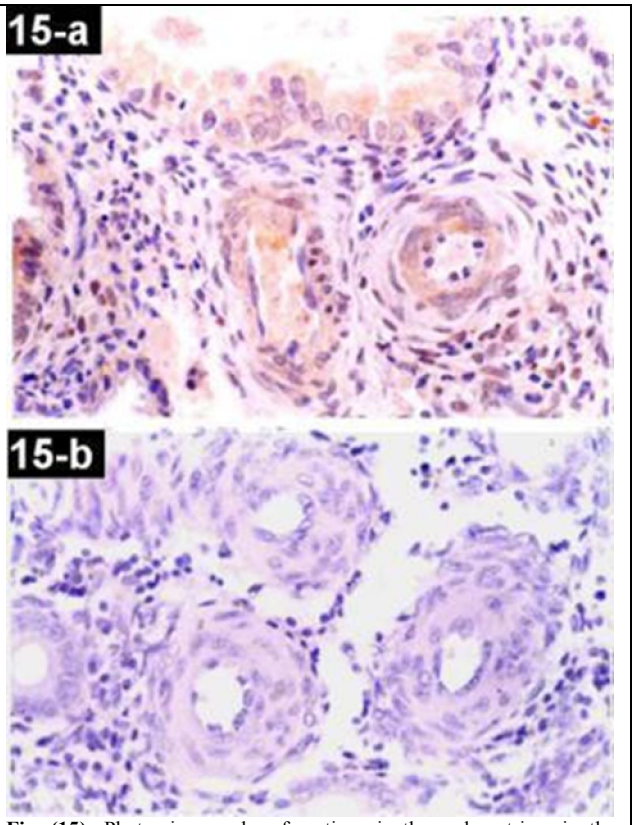

Fig. (15): Photomicrographs of sections in the endometrium in the early secretory phase (age 40, day of cycle16) showing:
(a) TP immunopositive endothelial and smooth muscle cells in the media of blood vessels *(PDGF.44C immunostaining; X400)*
(b) PDGF.44C immunonegative endothelial and smooth muscle cells in the media of blood vessels *(PDGF.44C immunostaining; X400)*

4. Discussion

Several polypeptide growth factors regulate epithelial and stromal development in endometrium under the influence of oestrogen and progesterone during menstrual cycle. However, little is known about the angiogenic growth factors that may affect endometrial vasulature. PDGF.44C was suggested to be a potent angiogenic growth factor in the female reproductive tract**.**

In the present work, the expression of PDGF.44C in the endometrium was detected by an immunohistochemical technique, using a specific monoclonal antibody to TP. Thymidine phosphorylase was invariably expressed in the normal endometrium. It had a cyclical pattern, which mirrored the endometrial phases of the menstrual cycle. Thus, at the beginning of the cycle, PDGF.44C was found in a patchy manner of positively stained endometrial glands and stroma. The reaction was weak cytoplasmic in glandular epithelial cells and strong nuclear in stromal cells. Towards the end of the menstrual cycle, epithelial expression was extended throughout the entire endometrium, involving all glands. The reaction was mixed nuclear and cytoplasmic and there was very little stromal nuclear immunostaining.

Moreover, quantification of immunohistochemical results in the present study revealed that the mean area percent and optical density of PDGF.44C expression were significantly higher in late secretory phase than the other phases.

Similar menstrual cycle related changes were reported by Zhang *et al.* [7] and Siviridis *et al.* [12]. Both described a shift in TP expression from the endometrial stroma to the endometrial glands as the cycle advances. Siviridis *et al.* [12] added that the intensity of the epithelial staining was greatest in the basalis adjacent to myometrium and diminished towards the endometrial surface. Another report from Fox *et al.* [6] who studied TP expression in a series of normal tissues, including human endometrium, showed that the enzyme was found in both endometrial glands and periglandular stroma, and observed both cytoplasmic and nuclear staining. Zhang *et al.* [7] suggested that these cyclical changes in TP expression might be under the control of ovarian hormones. Later on, Fujiwaki *et al.* [9] reported that TP expression in the endometrium seemed to be inversely correlated with estradiol concentrations during the menstrual cycle.

On the other hand, Osuga *et al.* [13] reported that TP expression in the human endometrium was predominately in the stroma and not the glands during the late secretory phase of the cycle. The reason for this discrepancy between this research and the present work is not known but may arise as a result of using different antibodies and staining techniques.

TP is not the only angiogenic factor whose concentrations vary during the course of the menstrual cycle; cyclical pattern of endometrial activity for VEGF was also reported [14]. VEGF was expressed in normal glandular epithelium and, to a lesser extent, in stromal cells throughout the cycle; its activity was highest at the beginning (early proliferative phase) and towards the end of the menstrual cycle (late secretory phase).

TP as an angiogenic factor promotes the formation of new blood vessels from a pre-existing network of capillaries [15, 16]. The new blood vessels, which are most important for the regeneration and growth of the shed endometrium, arise from the straight arteries in the region between myometrium and basal layer [12]. At this part of the endometrium, coordinated nuclear and cytoplasmic TP enzymatic activity, regulated by stromal and epithelial cells, respectively, seems to be needed for the formation of new spiral arteries [12]. This is consistent with the finding that PDGF.44C expression of the endometrial stroma, is maximal in areas of poor perfusion and necrosis, where hypoxia prevails; that is, in the endometrium after menstruation [17].

Later in the course of the menstrual cycle and, specifically during the late secretory phase, maturation of the muscular coat occurs [8], which may be also dependent on combined nuclear/cytoplasmic secretion of thymidine phosphorylase, this being produced mainly by the glandular epithelium. Fujiwaki *et al.* [9] suggested that TP in the nucleus might regulate thymidine concentrations for DNA synthesis, whereas in the cytoplasm it might control other effects via different enzyme systems, such as thymidylate synthase and ribonucleotide reductase. Whether cytoplasmic or nuclear, the enzyme is located intracellularly, whereas its effects are extracellular—on the nearby capillary endothelium; therefore, the angiogenic stimulus may be mediated by a paracrine pathway [18].

The present work did not investigate microvessel density; however, in the light of the report of Morgan *et al.* [19] it appears that normal secretory phase endometrium is more vascular than normal proliferative endometrium. This finding is in accordance with the current observation that epithelial/cytoplasmic PDGF.44C expression increases from proliferative to secretory phase endometrium.

The fact that stromal/nuclear PDGF.44C expression prevails of the early proliferative endometrium suggests that this type of enzymatic activity is important in the induction of neo-angiogenesis, in normal endometrium. In this context, it appears that stromal PDGF.44C positivity is oestrogen, rather than cycle (time) related. This observation was promoted by Siviridis et al. [12], who reported stromal nuclear TP expression in endometrial hyperplasia.

Furthermore, enzyme production might not only be regulated by ovarian hormones, but might also be regulated by cytokines released at high concentrations by endometrial leucocytes, which are a common feature during the secretory phase [6, 7].

Fujiwaki *et al.* [9] showed that high ratio of TP was found in endometrial cancer than in normal endometrium in any endometrial phase. Moreover, Giatromanolaki *et al.* [20] confirmed the hypothesis that

TP over-expression correlated with poor prognosis in endometrial carcinoma. It was suggested that TP facilitated tumour growth and metastasis via associated angiogenesis [4,18]. On the other hand, Ikeda *et al.* [21] found that TP inhibited apoptosis induced by anticancer agent. Hence, specific inhibitors of TP and other angiogenic factors may be a useful therapeutic intervention in endometrial carcinoma. It has become a target of medicinal chemistry.

Another study stated that the cytoplasmic TP activity in endometrial cancer is significantly higher than in normal endometrium There was no relation to the stage and grade of tumors, but correlates with the PDECGF/TP protein expression may therefore be associated with favorable prognosis in patients treated with chemo- or radiotherapy after surgery [22].

The synthesis and evaluation of a 6-methylene bridged uracil derivative as inhibitor of human TP to enhance the in vivo anti tumour activity of cytotoxic drugs was investigated. They stated that the process of angiogenesis is closely related to metastatic ability. One of these compounds (TPI) may have an significant impact on the treatment of patients with advanced cancers [23]. Also, another study stated that, TP was inhibited by 5'-O-trityl-inosine (KIN59) and related compounds, 2-deoxy-L-ribose and glycosides isolated from the bark of Symplocos racemosa [24.25]. This could pave the way for establishing new therapeutic modalities for endometrial carcinoma.

In conclusion, the expression of TP (PDGF.44C) in the normal endometrium has well defined cellular and tissue patterns that are dependent on the phase of the menstrual cycle. The consistent expression of PDGF.44C in the endometrium is suggestive of its role in physiological angiogenesis. Other studies are still required to provide further understanding of the regulation of endometrial angiogenesis.

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