CYCLIC CHANGES OF ALKALINE PHOSPHATASE IN THE HUMAN ENDOMETRIUM: HISTOCHEMICAL AND BIOCHEMICAL ANALYSES

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The changes of the endometrial alkaline phosphatase (ALP) along with menstrual cycles were investigated by both enzyme histochemistry and biochemistry. In order to avoid biassed views which may come mostly from insufficient observation on the limited number of cases, the endometrial specimens were obtained from over 180 patients without known abnormalities. Very fine cyclic changes of the ALP, including changes of the intracellular localization pattern, were clearly evidenced by histochemical observations, which were intimately substantiated by biochemical assay. In the proliferative phase, the ALP activity gradually increased to reach its peak at the early secretory phase, right after ovulation. In the mid to late secretory phase, a rather abrupt decrease of the enzyme was evidenced by an inverse increase of the mucin secretion which could be observed by alcian blue staining. On the basis of this clear cyclic change of the endometrial ALP, the mode of hormonal regulation, either by estrogen or by progesterone, on the enzyme activity was discussed.

It is a well documented fact that human endometrial alkaline phosphatase (ALP) activity shows characteristic changes which are dependent on the menstrual cycle. However, definite or unequivocal explanations have not yet been given to such changes, as the results reported by several investigators (1, 2, 3, 6, 7, 10) have not agreed in details. For instance, the highest activity was recorded in the proliferative phase in some reports (1, 6, 10), while the highest activity was exhibited in the early secretory phase in another report (3). Such complexity may be attributed to the fact that most of those reports dealt with a rather limited number of cases and the enzyme activity is quite variant from one patient to the other. In the present study, the endometrial specimens for both histochemical and biochemical analyses on the ALP were sampled from a large number of carefully selected patients in order to avoid obtaining equivocal results.

MATERIALS AND METHODS

Specimens of endometrium were secured by curettage or hysterectomy from 181 patients aged 22–44 years. Among these, only the endometrium obtained from
patients who showed normal regular menstrual cycles (25–35 days) were employed in the present study, while those of patients who had been placed on hormone preparations or who had endometrial hyperplasia or similar disorders were excluded. Ninety-nine were subjected to biochemical determination and 74 to routine histological and enzyme histochemical observations. The intervals of the menstrual cycles were determined by either histological dating or basal body temperature, which were converted into the intervals of the 28 days' cycle.

For histological observation, formalin (10% formol commercially obtained) fixed and deparaffinized sections were stained by "hematoxylin and eosin (H & E)"; "periodic acid Schiff's (PAS) reaction" and "alcian blue".

For enzyme histochemical observation of ALP, the tissues were fixed with cold 4% Baker's formol-calcium overnight, and then washed by 0.88 M Holt's gum-sucrose solution for 24–48 hr. The tissues were cut at 6 μm thickness in a cryostat. The histochemical staining was effected by a modified Burstone's azo dye method with naphthol AS-BI phosphate as the substrate (4). The incubation was carried out at room temperature for 10 min. The nuclear counterstaining was done with either Azur A-Schiff or methylgreen.

For biochemical assay of ALP, the tissues were immediately cleaned of all blood

![Fig. 1-5. Enzyme histochemical localization of the alkaline phosphatase (ALP) in the normal human endometrium. The ALP localization is shown as fine dark deposits along the cell membrane. Fig. 1. Early proliferative glands from the endometrium of a 40-year-old woman on the 8th day of the cycle. ALP staining is recognized on the apical and lateral surfaces (arrows) of the endometrial glandular epithelia. The endothelia of the stromal capillaries (paired arrows) are rather intensely stained. ×150](image-url)
traces by washing with 0.1 M Tris-HCl buffer, pH 7.4, and then were homogenized in the same buffer containing 1 mM MgCl₂ at 0°C. The homogenates were incubated in the following reaction medium for 30 min, at 37°C; 2 mM p-nitrophenyl phosphate and 1 mM MgCl₂ in 25 mM NaHCO₃, pH 10.0. The reaction was stopped by adding 250 μl of 1 N-NaOH, and the amount of p-nitrophenol released was determined by its absorbency at 405 μm. The specific activity was expressed as micromoles of p-nitrophenol liberated per mg of protein which was determined according to the Lowry procedure (9).

RESULTS

Histochemical observation of endometrial ALP

In the normal endometrial tissue ALP activity can be seen as fine granules on the plasma membrane of the endometrial glandular cells. No ALP activity was observed in any cells of the underlying interstitium except in the endothelial cells of the capillaries. Endometrial ALP activity exhibited a remarkable cyclic change along with the menstrual cycle.

In the early proliferative phase, approximately 50 to 70% of the total glandular elements showed relatively weak ALP staining. In those ALP positive glandular epithelial cells, the apical portions were most conspicuously stained and then the lateral membranes. The basal membranes were only occasionally stained (Fig. 1).

In the mid and late proliferative phases of the cycle, the enzyme staining

Fig. 2. Late proliferative glands from the endometrium of a 42-year-old woman on the 12th day of the cycle. The ALP activity is markedly increased, showing the positive staining on the entire (apical, lateral and basal) (arrows) surfaces of the glandular epithelia. ×300
intensity markedly increased. As Fig. 2 shows, a positive ALP reaction was exhibited on almost the entire cell surfaces though the apical membranes were stained most intense. In this mid to late proliferative phase, the upper two-thirds of the endometrium ("functional layer") consisted of strongly ALP positive glands as illustrated in Fig. 2, while its lower one third ("basal layer") was mostly composed
of weakly ALP positive glands as illustrated in Fig. 3.

In the early secretory phase, almost the entire layer of the thickened endometrium was occupied by the strongly ALP positive glands as shown in Fig. 4. In those endometrial glands, which show "subnuclear vacuoles" (arrows in Fig. 4), positive ALP staining was strictly confined in their apical portions (the lateral and basal membranes of the epithelial cells are free of enzyme activity.).

In the late secretory and the premenstrual phase of the cycle the enzyme activity diminished dramatically leaving barely detectable staining on the apical membranes of some glandular cells. In contrast to the change of the glandular cells, the ALP staining of the capillary endothelia was unaltered (Fig. 5).

**Alcian blue staining for the endometrial mucins**

Alcian blue staining is known to stain the compound of acid mucopolysaccharides including sialomucin.

In the proliferative glands, only apical membranes of the epithelial cells were

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**Fig. 4.** Early secretory glands from the endometrium of a 31-year-old woman on the 17th day of the cycle. Extremely intense ALP staining is seen mainly on the lumenal surfaces of the glands. "Subnuclear vacuoles" are clearly evident (arrows). × 150

**Fig. 5.** Late secretory glands from the endometrium of a 35-year-old woman on the 28th day of the cycle. Only faint staining is noted in the degenerative glands (arrows). The glands seen in the upper part of the figure (G) are almost completely devoid of ALP staining. × 150

**Fig. 6.** Alcian blue staining applied on the section of the same endometrial glands of Fig. 5. Very intense alcian blue positive staining is seen on the lumenal surfaces of the endometrial glands (arrows). Alcian blue positive staining is usually not so conspicuous in monochrome pictures, especially on the sections counterstained for the nuclei. × 150
weakly stained in linear form.

In the early secretory glands, apical membranes of the glandular epithelia stained similarly to those of proliferative glands. In addition “subnuclear vacuoles” were also stained with alcian blue.

In contrast to these, the endometrial glands of the late secretory phase exhibited very intense staining along their apical surfaces (Fig. 6). That is, the apical portion of the glandular epithelia was covered by much thicker alcian blue positive substance and the lumina was often filled with alcian blue positive secrates (Fig. 6).

**Biochemical determination of endometrial ALP**

The intervals of the menstrual cycles were determined by either histological dating or basal body temperature, which were converted into the intervals of the 28 days’ cycle. The values of the endometrial ALP activity of each individual were plotted at such converted intervals indicated on Fig. 7.

In the proliferative phase, the ALP activity increases steadily to reach its peak at about the 16th day of the menstrual cycle, that is, a day or two later than ovulation (very early secretory phase). After that, a rather abrupt decrease of the enzyme activity was noted and the lowest activity was measured at the late secretory or premenstrual phase (Fig. 7).
DISCUSSION

Endometrial ALP and menstrual cycle

On enzyme histochemical observation, the ALP staining in the endometrial glandular epithelia gradually increased in its intensity and its positive range in the proliferative phase. The epithelial cells in the latest proliferative phase, which were entirely encircled with ALP positive cell membranes (Fig. 2), showed the most intense staining histochemically. In contrast to this, the glandular epithelia in the early secretory phase exhibited very intense ALP staining only on their lumenal surfaces but not on the lateral and basal surfaces of their epithelial cells. According to our knowledge at present, the reason is obscure as to why such change in the cell surface distribution of ALP occurred along with the menstrual cycle. In this early secretory phase, however, nearly all glandular epithelia of the entire endometrial layer showed strong ALP staining, while only the upper two-thirds of the endometrial layer were strongly positive for ALP in the latest proliferative phase. This evidence might be reflected in the biochemical analysis of the endometrial ALP which showed the highest activity in the former stage (the early secretory phase).

Hormonal regulation on the endometrial ALP

Biochemical analysis on the endometrial ALP, as Fig. 7 shows, proved that the ALP activity underwent a rather clear cyclic change along with menstrual cycles reaching its peak on the 16th day of the cycle, just after ovulation. This finding roughly corresponds to that of Gautray et al. (5) and suggests that the enzyme activity would be induced by estrogen as was also pointed out by these authors. Our previous histochemical observations (14) on the rat endometrial ALP also proved that the ALP activity was clearly increased by estrogen. That is, almost completely abolished ALP activity in rat endometrial glands by castration was sharply recovered by a single injection of estradiol-17β.

A rapid fall of the endometrial epithelial ALP activity, which once reached its peak in the early secretory phase (16th day of the menstrual cycle), was evidenced in the later secretory phase by both histochemical and biochemical observations. In contrast to this rapid fall of the endometrial ALP, the ALP of the stromal capillaries was almost unaltered (histochemical observations). It is quite conceivable that the endometrial ALP would also be quite sensitive to progesterone of which secretion is markedly increased after ovulation. In this case, progesterone may be working as an inhibitor against the endometrial ALP, while estrogen could be activating the enzyme. The direct dependency of the ALP activity on progesterone was strongly suggested by our previous in vitro experiment using endometrial carcinoma cell line (12, 13). The ALP activity of the cell line was markedly suppressed by the administration of progesterone into the culture media. And the ALP of these cell lines was proved to be identical to that of the normal endometrium by checking biochemical and immunoelectrophoretical properties of the enzyme (11).

It is also well known fact that progesterone stimulates mucin secretion in the endometrium. In the early secretory phase, however, mucin is mostly confined in the cytoplasms of the epithelial cells as “subnuclear vacuoles”. In this phase, the ALP was concentrated on the lumenal surfaces of the epithelial cells showing its most intense activity. After the mid secretory phase, the mucin accumulated in the cytoplasms is excreted into the lumina of the endometrial glands covering the
lumenal surfaces of the epithelial cells (Fig. 6) where the ALP was once localized in the previous periods. It is of great interest that the ALP on the lumenal surfaces was remarkably inactivated with concomitant increase of the mucin secretion. According to Komoda et al. (8), the sialic acid inactivated quite effectively the liver ALP which was proved to be the same isoenzyme of the endometrial ALP (11, 15). It is quite conceivable that the mucin (mostly sialic acid) excreted onto the lumenal surfaces inactivated the ALP which was concentrated on the same areas (the indirect suppression of the ALP by progesterone). In order to verify this latter concept, we are now purifying human endometrial ALP to investigate the immunohistochemical localization of this enzyme which must be compared with the enzyme-histochemical one. That is, in our presumption, the enzyme protein itself of the ALP, which can be detected by the immunohistochemistry, would still remain on the lumenal surfaces even after the mid secretory phase when its activity, which can be demonstrated by the enzyme-histochemistry, would be inactivated by the excreted mucin.

At any rate, it is noteworthy that the activity of the ALP in the endometrium, which is one of the target organs against sex steroid hormones, is definitely dependent on these hormones. The usefulness of the ALP as a sensitive marker for certain sex steroid hormone actions on the endometrium was thus pointed out.

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