Potential Activity of Ca^{2+}/Mg^{2+}-Dependent Endonuclease in Endometrial Hyperplasia and Cancer

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Potential activity of Ca^{2+}/Mg^{2+}-dependent endonuclease in hyperplastic endometrial tissues is lower than in normal endometrium and practically absent in endometrial cancer tissue. Thus, molecular mechanisms of apoptosis regulation are disturbed even at the stage of hyperplasia.

Key Words: *apoptosis; Ca^{2+}/Mg^{2+}-dependent endonuclease; endometrial hyperplasia; endometrial cancer*

At present, the role of apoptosis in the development of various pathological processes in humans is extensively studies in clinical, cytological, immunological, and molecular biology experiments. Apoptosis is a normal physiological process directed at elimination of waste or excessively proliferating cells on the organism and maintenance of cell homeostasis in various tissues, in particular endometrium [11]. Disturbances of programmed cell death lead to serious pathologies associated with either insufficient of excessive apoptosis. It is assumed that tumor growth and hyperplastic processes in the endometrium result not only from tumor transformation or enhanced cell proliferation, but also from impaired regulation of programmed cell death.

Morphological signs of apoptosis are described in detail [1,10]. The most characteristic feature of this process is chromatin degradation due to activation of cell endonucleases (dissociation of the chromatin into oligonucleosomal fragments) [16]. The final stage of DNA degradation, internucleosomal fragmentation, is catalyzed by Ca^{2+}/Mg^{2+}-dependent endonuclease (CME) [12] discovered in the nuclei of different tissues.

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Activity of endonucleases, in particular CME, changes in various physiological and pathological processes, therefore, the degree of DNA degradation to oligonucleosomal fragments is an important biological marker of the intensity of apoptosis.

In the present study we proposed a new approach to assessing CME activity and evaluated the possibility of apoptosis in endometrial cells in hyperplastic and neoplastic changes.

MATERIALS AND METHODS

Human endometrial samples from healthy donors (n=10) and patients with endometrial polyps (n=16), hyperplasia (n=17), and neoplasms (n=17) were examined.

The samples (10^7 cells) were homogenized in a Polytron homogenizer in 5 volumes of 2 M sucrose containing 50 mM Tris-HCl (pH 8.0), 10 mM $CaCl_2$, 5 mM mercaptoethanol, and 1 mM PMSF. The homogenate was centrifuged at 14,000g and 4°C for 20 min. The pellet was resuspended in sucrose solution (50% of the initial volume) and recentrifuged. The nuclei were suspended in 500 μl buffer containing 60 mM Tris-HCl (pH 8.5), 6 mM $MgCl_2$, 0.25 M sucrose, and 50% glycerol and stored at -20°C.

For activation of CME, the isolated cell nuclei were incubated in a buffer containing 50 mM Tris-HCl (pH 8.1-8.3), 10 mM $MgCl_2$, 1 mM $CaCl_2$, and 0.25 M

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sucrose at 37°C for 30 and 90 min. The reaction was stopped by adding EDTA to a final concentration of 80 mM. To the control samples EDTA was added before incubation. To release DNA from nucleoprotein complexes, pronase (10 mg/ml in a buffer containing 0.1 M Tris-HCl, (pH 7.8), 0.1 M EDTA, and 5% SDS was added to a final concentration of 1 mg/ml and incubated at 37°C for 30 min. The samples were deproteinated with chloroform:isoamyl alcohol mixture (24:1). The samples were vigorously shacked, proteins were removed by centrifugation at 14,000*g* for 10 min, and the upper aqueous DNA-containing fraction was collected. CME activity was assessed by DNA electrophoresis in 1% agarose. The gels stained with ethidium bromide were analyzed using a Gel Doc 1000 system and Molecular Analyst software (BioRad).

RESULTS

The applied methods were based on measuring CME activity after exogenous stimulation of purified nuclei with Ca^{2+} and Mg^{2+} followed by electrophoretic separation of DNA fragments in agarose gel [15].

Activity of CME was proportional to the number of DNA fragments after cleavage of the given DNA sample. Maximum CME activity resulted in predominance of the smallest products of internucleosomal dissociation of DNA from monomers on electrophoregrams (from 180-200-bp monomers to tetramers). Therefore, coefficients inverse proportional to the molecular weight of DNA fragments and reflecting its relative contribution to endonuclease activity (EA) were introduced into the formula for calculation of enzyme activity.

Areas on the electrophoregrams including DNA fragments consisting of 200-800, 800-3200, and 3200-12800 bp were determined and coefficients 16, 4, and 1 were assigned to these areas. The degree of DNA cleavage by endonuclease was assessed by distracting total fluorescence of the given area from the total fluorescence of the corresponding area on the control run (intact DNA, negative control). EA was calculated by the formula:

$$EA = \frac{[16(X3-Y3)+4(X2-Y2)+(X1-Y1)]}{X4-Y4} \text{ (arb. units)},$$

where X1, X2, and X3 are the total fluorescence of the 3 areas on electrophoregrams, Y1, Y2, and Y3 are the total fluorescence of the corresponding areas on the control run with intact DNA, X4 is the total fluorescence of all DNA samples, and Y4 is the total baseline fluorescence (Fig. 1).

Activation of endonuclease by adding cofactors Ca^{2+} and Mg^{2+} to isolated nuclei from normal and altered endometrium induced chromatin degradation of varying degree: from considerable (formation of oligonucleosomal DNA fragments typical of normal endometrium at the secretory phase of the menstrual cycle, runs *1-3*) to zero (endometrial adenocarcinoma, runs 7-9).

All samples of normal endometrium exhibited high CME activity (Fig. 3). The enzyme activity considerably decreased in endometrial hyperplasia, and was minimum or absent in endometrial cancer. The differences between all studied groups were significant in pairwise comparison (Student test) except for the polyps—normal endometrium pair. The significance of differences was close to 100% ($p<0.01$) and only

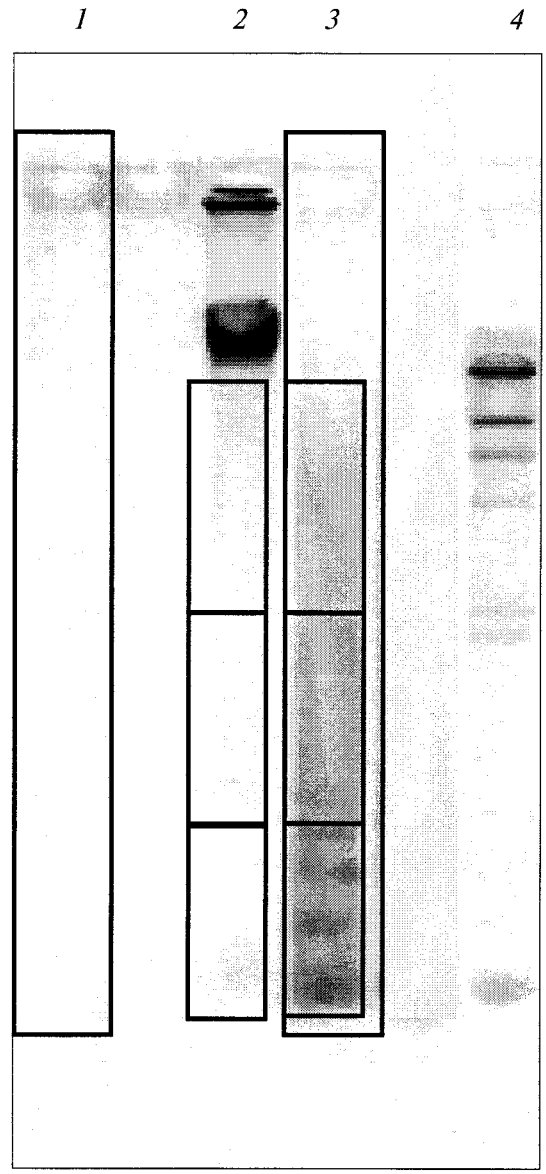

Fig. 1. Measurement of Ca^{2+}/Mg^{2+}-dependent endonuclease activity. DNA after (*1*) and without endonuclease activation (*2*) in isolated nuclei; λ Hind III (*3*); DNA-free run (*4*).

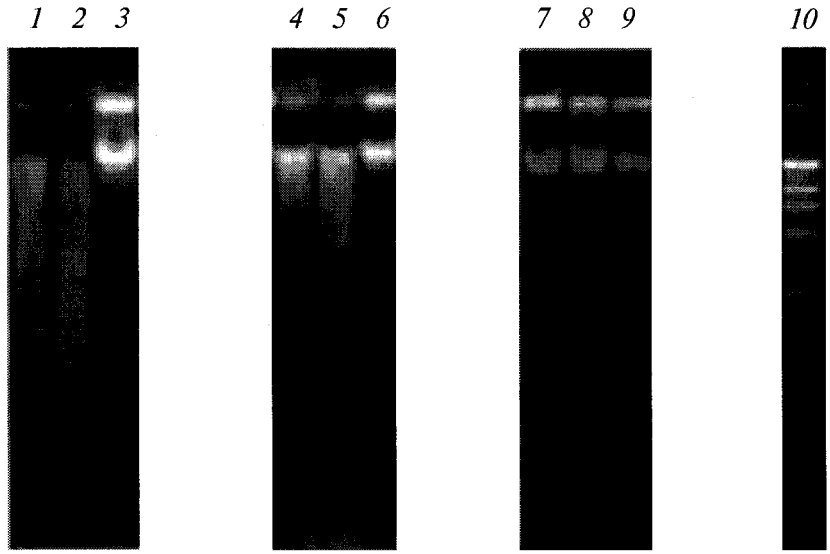

Fig. 2. Endonuclease activity in human endometrium cells (electrophoregram of DNA in 1% agarose) after activation of Ca^{2+}/Mg^{2+}-dependent endonuclease in isolated cell nuclei for 30 min (*1, 4, 7*), 90 min (*2, 5, 8*) and without activation (*3, 6, 9*). *1-3*) normal endometrium; *4-6*) endometrial hyperplasia; *7-9*) Endometrial adenocarcinoma; 10) DNA molecular weight standard (λ Hind III).

between hyperplasia and cancer samples it was below 99% ($p<0.05$).

In endometrial adenocarcinoma, a correlation was observed between CME activity and the degree of differentiation (Table 1). For these samples, arbitrary notations were introduced: low, middle, and high activity. Middle and low CME activity was observed in 2 and 4 samples of highly differentiated adenocarcinoma, respectively. In low differentiated adenocarcinoma, CME activities were absent in 4 samples and low in 2 samples. In moderately differentiated adenocarcinoma CME was low in 3 samples and absent in 2 samples.

The relation between hyperplastic processes and carcinogenesis in the endometrium and the transformation of hyperplasia into cancer are now intensively discussed. Since endometrium is a proliferative tissue, some markers are required for distinguishing between normal cell growth compensated by apoptosis and proliferation of transformed cells.

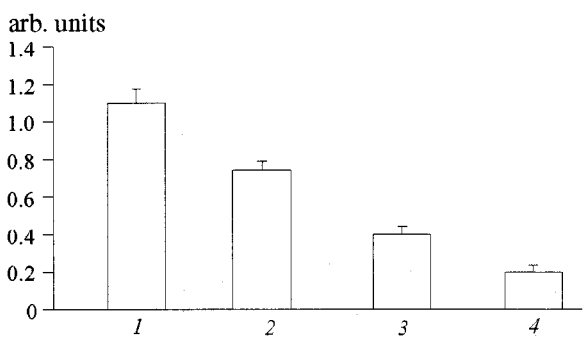

Fig. 3. Potential endonuclease activity in the endometrium. *1*) normal endometrium; *2*) polyps; *3*) endometrial hyperplasia; *5*) endometrial adenocarcinoma.

Overexpression of the most studied apoptosis inhibitor Bcl-2 produced by normal endometrium under hormonal control (regulators of the menstrual cycle [9]) cannot be used as a reliable marker of inhibition of programmed cells death, because of the existence of Bcl-2-independent regulation of apoptosis [5]. Moreover, previous studies demonstrated that intense expression of *bcl-2* in endometrial hyperplasia (primarily, non-atypical) was reduced in endometrial cancer samples, and the percentage of samples with high expression of this gene also decreased [4,14]. Both the intensity of gene expression and the number of *bcl-2*-positive samples decreased in the row: non-atypical (simple and complex) hyperplasia→atypical hyperplasia→highly differentiated adenocarcinoma→moderately differentiated adenocarcinoma→low differentiated adenocarcinoma.

Overexpression of wild-type or mutant p53 that normally inhibits proliferation and triggers apoptosis in cells with aberrant DNA also cannot serve as a prognostic factor of apoptosis, hyperplasia, and neoplastic transformation [3], because the role of p53 mutations in neoplastic processes is not established [13], while the correlation between p53 overexpression and endometrial tumor growth is still a matter of controversy [6].

The generally accepted criterion of apoptosis is the intensity of internucleosomal DNA splitting assessed by *in situ* TUNEL method [8] or by electrophoresis of DNA extract from the test sample [2]. However, evaluation of DNA degradation as the sole

TABLE 1. Correlation of EA and the Degree of Differentiation of Endometrial Adenocarcinoma

Degree of differentiation	EA, arb. units			
	no activity	low (0-0.3)	middle (0.3-0.6)	high (>0.6)
High	0	4	2	0
Middle	2	3	0	0
Low	4	2	0	0

assay of programmed cell death is sometimes insufficient, because characteristic DNA splitting is found in cells without morphological signs of apoptosis [7].

Measurement of potential CME activity is probably the most informative approach to quantitative evaluation of apoptosis in proliferating cells. These findings suggest the presence of apoptosis disturbances at the stage endometrial hyperplasia. The development of endometrial hyperplasia is associated with considerable reduction of apoptotic cell elimination, which can result in rapid uncontrolled proliferation of transformed cell. Minimum CME activity in adenocarcinoma samples indicates the resistance of tumor cells to programmed cell death, which agrees with our previous data on a considerable decrease in potential CME activity in Jurkat T lymphocytic leukemia cells. At the same time, similar EA in the endometrium in secretory and proliferative phases of the menstrual cycle confirms high potential CME activity of normal endometrium cells, which can be realized to a greater or lower extent depending on the intensity of physiological apoptosis. The observed decrease in CME activity in the endometrium involved in pathological processes suggests disturbances of apoptosis, rather than the decrease in its intensity, which normally varies in a wide range.

Thus, measurement of potential CME activity, a biological marker of apoptosis, is of practical value, because it allows to evaluate cell proliferation-elimination balance and the risk of uncontrolled cell proliferation in the endometrium. Prompt detection of this marker of disturbed programmed cell death will contribute to prevention of malignization.

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