EXPERIMENTAL METHODS FOR CLINICAL PRACTICE

Synthesis of Highly Tritium Labeled Steroids for Evaluation of Aromatase and Steroidsulfatase Activities in Endometrial Tumors

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Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 142, No. 10, pp. 462-464, October, 2006
Original article submitted September 7, 2005

Data on activities of estrogen-synthesis enzymes (aromatase and steroidsulfatase) in endometrial tumors were obtained by sensitive and highly precise radioisotope methods. The results clear out some pathogenetic aspects in the development and progress of malignant tumors in the endometrium.

Key Words: *tritium-labeled steroids; aromatase; steroidsulfatase; endometrial cancer*

In most cases estrogens are essential for initiation and maintenance of malignant tumor growth in the endometrium. They can be synthesized in the endometrium from androgens under the effect of aromatase and from sulfated estrogens (mainly from estrone sulfate) with participation of steroid sulfatase (SSF). Estrogens delivered into the uterus from the peripheral blood and newly synthesized estrogens induce excessive proliferation of the endometrium through stimulation of estrogen receptors, and under appropriate conditions lead to the development of endometrial cancer (promotor variant of hormonal carcinogenesis). In addition, estrogens are involved in the realization of the genotoxic variant of hormonal carcinogenesis [1,10]. Associations of aromatase and SSF activities in the tumor and clinical and morphological parameters were detected in patients with endometrial cancer, which can be significant for prediction of the disease course and outcome [1,4,7-9]. However, these data require more detailed verification on more extensive clinical material.

We found no published data about complex evaluation of aromatase and SSF activities in endometrial tumors. Evaluation of estrogen synthesis enzymes (aromatase and SSF) in oncohematological practice by available laboratory methods seems to be an important problem.

The aim of our study was development of methods for evaluation of aromatase and SSF activities in endometrial tumors using tritium high-labeled steroids.

MATERIALS AND METHODS

Tritium-labeled reagents: estrone (molar radioactivity 5.85 PBq/mol), estradiol (3.52 PBq/mol), [1,2-^{3}H]androst-4-ene-3,17-dion and [1,2-^{3}H]progesterone (1.4-1.5 PBq/mol) were prepared at Institute of Molecular Genetics. Chromatographic data were

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processed using a MultiChrom programmed complex. Radioactivity was measured in a dioxane scintillator with 30% tritium registration efficiency.

In order to remove the label in position 2 of [1,2-^{3}H]androst-4-ene-3,17-dion, the steroid (0.5 GBq) was dissolved in 0.5 ml methanol:water mixture (5:1) with 1 mg caustic soda and exposed for 5-10 h at room temperature with stirring. The solution was then neutralized with 0.1 ml acetic acid, evaporated until dry, and the labeled preparation was isolated by HPLC on a Kromasil 7 C_{18} column (4×150 mm) in a methanol:water (75:25) system at the eluent flow rate of 1 ml/min, retention time 3.9 min. The labeled preparation output was 50%, molar radioactivity 0.9 PBq/mol.

The synthesis of tritium-labeled estrone sulfate was carried out as follows: 6 mg SO_3-pyridine complex was added to solution of a mixture of 0.125 mg labeled estrone (5.85 PBq/mol) and 0.125 mg unlabeled estrone in 0.5 absolute pyridine and left overnight at room temperature. The reaction solution was evaporated, 0.1 ml chloroform:methanol (2:1) mixture was added to the residue, applied onto a Sorbfil silica gel plate, and developed in the chloroform:methanol:10 n ammonium (15:5:1) mixture by thin layer chromatography (TLC). The zone containing tritium-labeled estrone sulfate (R_f 0.65) was cut and the labeled preparation was extracted in methanol (4×2 ml). Methanol extracts were pooled and evaporated. Final purification was carried out by HPLC on a Kromasil 100 C_{18} column (7 μ, 4×150 mm) in methanol:5 mM sulfuric acid (70:30) system at eluent flow rate of 1 ml/min, estrone sulfate retention time 4.23 min. Radiochemical purity was evaluated on the same column in methanol:50 mM ammonium phosphate buffer pH 2.8 (60:40) with 1 mM tetrabutylammonium fluoride, at eluent flow rate 1 ml/min, estrone sulfate retention time 4.73 min. Labeled estrone sulfate output was 65-70%, molar radioactivity 2.75-2.95 PBq/mol, radiochemical purity 95-97%.

Biomedical studies were carried out in 64 patients with endometrial cancer (mean age 57±2 years). Clinical morphological study included evaluation of age, menstrual and reproductive function, disease stage, histological type, differentiation degree, and depth of tumor invasion in the myometrium. The stage of tumor process was evaluated in accordance with FIGO and TNM international classifications [6]. Morphological analysis detected endometrioid carcinomas of different differentiation degree in 55 patients and non-endometrioid tumors in 9.

Material for enzyme assay was obtained during interventions. Resected tumor specimens (50-80 mg) were crushed in liquid nitrogen, homogenized in 50 mM phosphate buffer (pH 7.0). Aromatase activity was evaluated by tritium water formation from [1-^{3}H]androstenedione (AS) [4]. Tritium water and unlabeled estrogens are forming during enzymatic reaction, while nonreactive labeled AS is easily separated. The use of other AS isotopomers, for example, commercial [1,2,6,7-^{3}H]AS (Amersham Bioscience) does not provide so unambiguous quantitative estimations of aromatase activity. In addition, measurement of aromatase activity is associated with nonenzymatic wash out of tritium label from [1,2-^{3}H]AS position 2, which makes the results unreliable.

[1-^{3}H]AS was obtained by 10-h exposure of water-methanol solution of [1,2-^{3}H]AS in alkaline medium. Tritium removal from AS position 2 (α-position for ketone-3) is related to the possibility of ketone enolization [2]. Exposure of [1-^{3}H]AS under alkaline conditions for 48 h does not reduce its molar radioactivity, this indicating tritium stability in AS position 1. These properties of [1-^{3}H]AS rule out the nonenzymatic loss of tritium during incubation of the labeled preparation.

Labeled estrone was sulfated using SO_3-pyridine [5]. Purification of the resultant labeled preparation should be carried out by not only HPLC, but by TLC as well, as many impurities, for example, estradiol sulfate, can be separated with guarantee only by this method. Activity of SSF was measured by the radiometric method [3] on a MICROBETA liquid scintillation counter (Perkin Elmer). Protein was measured by Lowry's method.

The results were statistically processed using Mann—Whitney nonparametric test. Spearman's ranked coefficient of correlations was calculated. The differences were significant at $p<0.05$.

RESULTS

Tumors in perimenopausal women were characterized by high aromatase and low SSF activities; the ratio of enzyme activities was opposite in postmenopausal patients (Table 1). Activities of estrogen synthesis enzyme varied depending on the tumor stage. The difference in SSF activity in preinvasive and invasive endometrial cancer is sufficiently demonstrative. Aromatase activity increased significantly if the tumor process involved the cervix uteri (stage II). No correlation between aromatase and SSF activities in endometrial tumors was detected.

Aromatase and SSF play a role in tumor growth and invasion, but at different stages of the tumor process. Aromatase activity does not depend on the

TABLE 1. Aromatase and SSF Activities in Endometrial Tumors and Clinical Morphological Parameters

Parameter	*n*	Aromatase, fmol AS/mg protein/h	SSF, fmol estrone sulfate/mg protein/h
Menopausal status:			
reproductive age	9	15.1 (6.0-33.1)	32.0 (10.0-55.1)
perimenopausal	13	26.0 (14.2-34.1)	15.4 (3.0-40.0)
postmenopausal	42	12.5 (7.1-22.8)**	109 (38.0-140.0)*
Disease stage:			
Ca *in situ*	10	26.0 (9.4-34.0)	15.4 (10.2-21.3)$^{+}$
I	40	11.4 (5.5-21.3)	60.0 (14.2-100.0)
II	14	26.1 (8.6-43.1)$^{+}$	28.0 (20.3-40.8)
Differentiation degree:			
G1	15	18.2 (9.0-32.7)	70.0 (33.2-110)
G2	27	13.4 (5.5-21.8)	37.0 (4.3-60.0)
G3	13	14.7 (8.4-34.0)	19.2 (4.0-36.9)^{o}

Note. *$p<0.01$, **$p<0.05$ compared to perimenopausal patients; $^{+}p<0.05$ compared to patients with stage I disease; $^{o}p<0.01$ compared to G1.

differentiation degree [1,9]. Immunohistochemical methods also revealed no relationship between SSF expression in endometrial tumors and differentiation degree [8]. There is a correlation between SSF activity and tumor differentiation degree. Our data on activities of these enzymes are in line with previous reports [8].

Hence, improvement of technologies for the synthesis of tritium-labeled steroids making use of sensitive and highly precise radioisotope methods provided data on activities of estrogen synthesis enzymes in endometrial tumors. These results clear out some pathogenetic aspects in the development and progress of malignant tumors of the endometrium.

The study was supported by the Program of Russian Academy of Sciences "Molecular and Cellular Biology" and grant "Scientific Schools" (No. NSh-2150.2003.4).

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