

Total tissue lactate dehydrogenase activity in endometrial carcinoma

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Abstract. Šimaga Š, Abramić M, Osmak M, Babić D, Ilić-Forko J. Total tissue lactate dehydrogenase activity in endometrial carcinoma. *Int J Gynecol Cancer* 2008.

Lactate dehydrogenase (LDH) is essential for continuous glycolysis necessary for accelerated tumor growth. The aim of this study was to reconsider if assay of total tissue activity of this enzyme could be useful as marker for endometrial carcinoma (EC). Activity of LDH was measured spectrophotometrically in homogenate supernatants of uterine tissue samples of 40 patients (10 normal endometria, 27 normal myometria, and 33 EC), including 30 matched pairs. Data obtained were analyzed in relation to clinical and histopathologic findings and compared with our previously published results on the tissue levels of the same enzyme in ovarian cancer and on the proteolytic activity of dipeptidyl peptidase III (DPP III) in EC (suggested biochemical indicator of this malignancy). Significantly increased (1.8–3.0 times; $P < 1 \times 10^{-4}$) LDH activity was observed in EC samples if compared with normal uterine tissues. This rise was not related to the clinicopathologic findings, however. In contrast to previous results on LDH in ovarian carcinomas, a significant rise in LDH activity was found already in grade 1 EC. Using the cutoff value of 1.06 U/mg, diagnostic sensitivity of 82%, specificity of 100%, and accuracy of 91% for total tissue LDH assay have been calculated. A correlation of tissue's LDH and DPP III activities was found, and their combined assay for EC showed increased diagnostic sensitivity (94%) and accuracy (96%).

KEYWORDS: dipeptidyl peptidase III (DPP III), endometrial carcinoma, lactate dehydrogenase (LDH).

Cancer of the corpus uteri is the eighth most common malignant neoplasm in women worldwide and endometrial cancer constitutes about 95% of all malignant lesions of uterine cavity⁽¹⁾. The prevailing form of endometrial cancer is endometrial carcinoma (EC), tumor originating from the glandular epithelium of uterine endometrium. EC arises through a series of precursor lesions, which are thought to develop and be promoted in response to unopposed and prolonged stimulation by estrogen. On the other hand, some types of EC are estrogen independent⁽²⁾. EC is usually postmenopausal disease with peak incidence between age 50 and 60 years. Prognosis of EC is fairly good since overall 5-year survival rate is 83% and for the early stage of the disease, about 90%⁽¹⁾. This is mainly

due to early diagnosis indicated by abnormal bleeding and based on mandatory endometrial biopsy, which is strengthened by transvaginal ultrasonography, hysteroscopy, vaginal and endometrial cytology, and biochemical clinical tests. One of these tests, assay of lactate dehydrogenase (LDH), is still under clinical evaluation in gynecological oncology.

LDH [(S)-lactate:NAD⁺ oxidoreductase Enzyme Commission 1.1.1.27], is one of the major glycolytic enzymes that catalyzes the last step of glycolysis, conversion of pyruvate to lactate. It is a tetrameric protein composed of two immunologically distinct subunits, "M" (muscle) and "H" (heart) type, which combine to form five isoenzymes⁽³⁾.

LDH is ubiquitous cytosolic enzyme present in all tissues, exhibiting origin- and tissue-specific isoenzymatic pattern⁽⁴⁾. Routine serum measurement of this enzyme is of clinical use in the diagnosis and monitoring of certain diseases including cancer, but is of low diagnostic value for gynecological malignancy⁽⁵⁾. In response to the need for more specific

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diagnostic and prognostic tools, attempts have been made also to measure either activity or protein level (by immunohistochemistry) of LDH in other body fluids^(6,7), cavity washings⁽⁸⁾, and uterine tissues^(9–16).

Recently, we studied separately activities of total LDH and of another “housekeeping” enzyme, metallo-peptidase dipeptidyl peptidase III (DPP III) in human ovarian tissues^(17,18). This last enzyme, responsible for intracellular peptide catabolism⁽¹⁹⁾, was previously shown by us to be a biochemical indicator for ovarian and endometrial cancer^(18,20), and subsequent complementary DNA microarray-based expression profiling of ECs⁽²¹⁾ showing alteration in DPP III gene expression (overexpression) additionally strengthened this suggestion. Interestingly, we found significant enhancement of ovarian LDH and DPP III activity only in grade 2 and grade 3 tumors, but not in well-differentiated (grade 1) tumors. Since this indicated similarity in regulation of LDH and DPP III in tumors of epithelial origin, we extended our research on LDH to EC and compared the data to our published data on human DPP III in the same type of malignant tumors.

Materials and methods

Patients and samples

This study covered 40 patients undergoing surgical treatment at the Department of Obstetrics and Gynecology, School of Medicine, University of Zagreb, Croatia. The mean age of patients was 60.6 ± 1.7 (mean $\pm$ SEM) years. The consecutive specimens of uterine tissues obtained at surgery or biopsy comprised ten samples of normal uterine endometrium (NE), 33 samples of EC, and 27 samples of normal uterine myometrium (NM). Among them were 30 matched pairs (EC /normal uterine tissue) originating from the same patient and having NE ($n = 5$, “true” pairs) or NM ($n = 25$, “virtual” pairs) as EC’s counterpart. Patients were untreated for EC before the sampling.

Histopathologic classification of EC samples was based on the FIGO-staging system. Additional histopathologic characteristics included determination of the degree of leukocyte infiltration. Clinical informations were obtained after completion of biochemical assays. The protocol was approved by the Ethics of the Research Committee at the School of Medicine, University of Zagreb.

Tissue sampling and processing

Samples of uterine tissue were frozen within 10 min in liquid nitrogen and kept at -196°C until use. For bio-

chemical assays, tissues were minced, suspended in a buffer (50 mM Tris-HCl, 250 mM sucrose, 134 mM KCl, pH 7.6), and homogenized on ice (Ultra-Turrax T 25 homogenizer, Janke&Kunkel, Ika-Labortechnik, Germany) for three 5-sec bursts. Supernatants obtained after centrifugation (4°C , 15 min, $15,000 \times g$) were used for analysis.

Biochemical assays

Total tissue LDH activity was determined by following initial rate of pyruvate reduction to lactate, using slightly modified procedure⁽²²⁾. Assay mixture of 1 mL was buffered by 100 mM potassium phosphate pH 7.0 and contained finally 0.096 mM pyruvate and 0.060 mM β -nicotinamide adenine dinucleotide, reduced (NADH). Reaction was started by the addition of enzyme sample (up to 20 μL supernatant of tissue homogenate), and followed spectrophotometrically at room temperature (25°C) for 3 min by measuring decrease in absorbance of NADH at 340 nm. Initial velocity was calculated using the linear regression method. The specific activity of LDH was expressed in units per milligram of the sample protein. One unit of enzyme activity was defined as the amount of enzyme which transforms (reduces pyruvate or oxidizes NADH) 1 μmol of substrate in 1 min at 25°C and pH 7.0.

Specific activity of the DPP III was determined as described elsewhere⁽²⁰⁾. Protein concentrations were measured by the protein dye-binding assay⁽²³⁾.

Statistical analysis

The results were analyzed statistically using the STATISTICA (StatSoft Inc., 1984–1995, Version 5.0) software, by evaluating groups consisting of at least five pieces of data. The significance of differences between the mean values was evaluated by t tests when the groups consisted of at least ten data per group; for independent samples, (independent), two-samples t test was used, and for matched pairs, paired t test was applied. The comparison of median values for groups with number of data less than ten was performed by appropriate nonparametric tests, Mann-Whitney U test was used for independent samples, and Wilcoxon’s signed rank test for two paired (dependent) samples was applied. The correlation coefficients were determined by simple linear regression analysis. Two-tailed probability values of less than 0.05 were considered to be significant. The cutoff values of enzyme activities, as mean + 2 SD of the control group (normal uterine tissue consisting of endometrium plus myometrium) sample and

diagnostic parameters for LDH and DPP III assay were calculated according to Schneider *et al.*⁽⁸⁾

Results

Tables 1 and 2 and Figure 1 present the results of total LDH activity determination in samples of normal uterine tissues and EC. These data show that NM had the lowest LDH enzymatic level (mean = 0.544 U/mg, $n = 27$), which was about 30% lower than that found in NE (mean = 0.808 U/mg, $n = 10$).

When all EC samples have been compared with all normal uterine tissues, considerably higher levels (1.8- to 3-fold) of LDH activity (mean = 1.525 U/mg, $n = 33$) have been observed in malignant than in normal uterine tissue (Table 1; Fig. 1). Similar values were also obtained when matched pairs of EC and their normal counterpart tissue were compared (Table 2). There was no overlapping in total tissue LDH activity based on 95% confidence intervals (Table 1); thus, the enzyme level was clearly distinguishable in each type of uterine tissue and significantly higher in the malignant one. Total LDH activity of EC tissues apparently

Table 1. Total tissue LDH activity in normal uterine tissue and in primary ECs: distribution according to the clinical and histopathologic findings

Findings	<i>n</i>	Total tissue LDH (U/mg protein)			
		Mean	SD	Median	95% Confidence interval
Normal uterine tissue	37	0.616	0.222	0.580	0.542–0.690
Endometrium	10	0.808	0.145	0.800	0.704–0.912
Myometrium	27	0.544	0.203	0.480	0.464–0.625
EC	33	1.525	0.633	1.460	1.300–1.749
Clinical stage					
IA	4			N/A ^a	
IB	13	1.710	0.694	1.500	1.290–2.130
IC	16	1.467	0.577	1.480	1.159–1.775
Tumor grade					
1	16	1.584	0.727	1.425	1.196–1.971
2	15	1.478	0.544	1.500	1.177–1.779
3	2			N/A ^a	
Carcinoma type					
Endometrioid	23	1.441	0.508	1.600	1.221–1.661
Mixed	8	1.694	0.967	1.810	0.885–2.502
Others ^b	2			N/A ^a	
Inflammation					
Present	16	1.680	0.584	1.680	1.369–1.991
Absent	17	1.379	0.660	1.240	1.040–1.718
Age of the patients (years)					
<50	5	1.356	0.411	1.250	0.845–1.867
>50	28	1.555	0.666	1.480	1.297–1.813

^aN/A, numbers too low for statistical analysis.

^bClear cell carcinoma 1 and undifferentiated carcinoma 1.

Table 2. The comparison of total tissue LDH activity in malignant versus normal uterine tissues

Sample	(n1/n2)	Ratio ^a	<i>P</i>
I. EC versus ^b			
Normal endometrium	33/10	1.825	0.000148 [†]
Normal myometrium	33/27	3.042	<10 ^{-6*}
Normal endometrium plus normal myometrium	33/37	2.517	<10 ^{-6*}
II. Paired samples ^c			
"True" pairs	5	2.128	0.043 ^{††}
"Virtual" pairs	25	2.775	<10 ^{-6**}
"All pairs"	30	2.626	<10 ^{-6**}

^aRatio of mean or median values of LDH activity.

^b*P* calculated for independent samples by "t test" (*) or by "U test" (†).

^cEC versus normal tissue sampled from the same patient, where counterpart is normal endometrium ("True" pairs), normal myometrium ("Virtual" pairs), or normal endometrium plus normal myometrium ("All" pairs). *P* calculated for dependent samples by "t test" (**) or Wilcoxon's matched pairs test (††).

did not depend on the age of patients and seemed not to be related to the clinical stage, tumor grade, histologic type of tumor, or to the extent of tumor inflammation (Table 1).

We further correlated the activity of this enzyme in the normal uterine tissues and EC with levels of proteolytic enzyme DPP III determined earlier by us⁽²⁰⁾ (Table 3). Moderate (Pearson's "*r*" ~ 0.5) but significant association of LDH activity with DPP III ($P = 1 \times 10^{-3}$) activity was found. For both of these assays, cutoff values have been determined and diagnostic parameters for separate and combined tests have been calculated (Table 4). Both assays showed similar specificity, positive predictive value, and diagnostic accuracy, but DPP III assay seems to be superior to the LDH test concerning sensitivity and negative predictive value. Combined results of the assay of these two enzymes improved diagnostic parameters of LDH measurement alone, resulting in at least 96% reliability to discriminate EC from normal uterine tissue.

Discussion

LDH is a ubiquitous cytoplasmic enzyme, and its appearance in body fluids is recognized as a pathologic manifestation that can be used as a measure of cell or tissue injury. The determination of serum LDH, routinely used for diagnostic purposes for at least 30 years, was established as relevant in the diagnosis of myocardial infarction (late detection), hemolytic anemia, ovarian dysgerminoma, and testicular germ cell tumor⁽²⁴⁾.

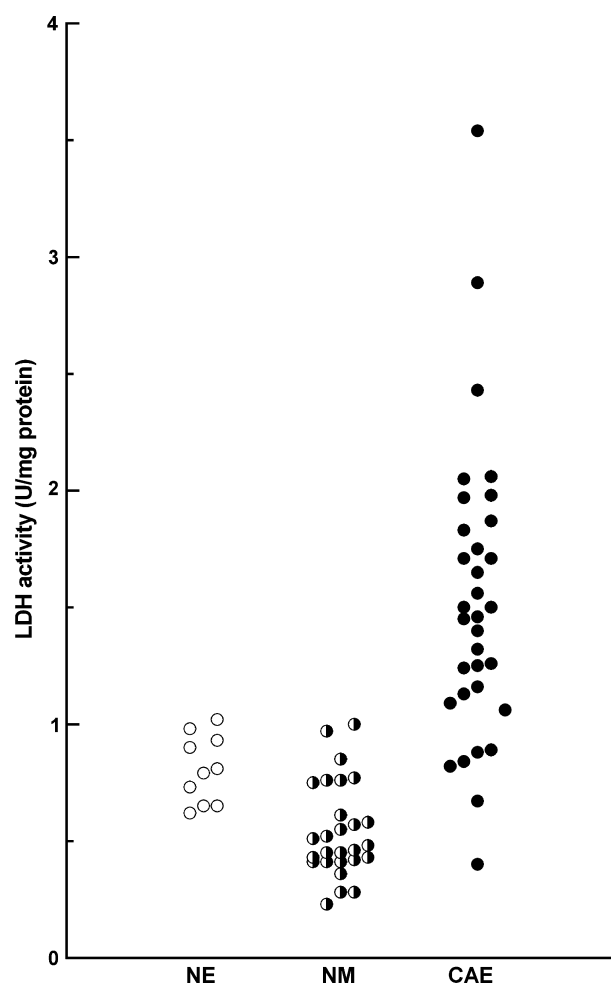


Figure 1. Total LDH levels in normal and malignant uterine tissue. NE ($n = 10$), NM ($n = 27$), and EC ($n = 33$). Enzyme activity was measured as described under "Materials and methods."

Being necessary to enable continuous glycolysis for accelerated growth rate of malignant tissue, LDH has been subject of many investigations in tumor metabolism, and its clinical use as a possible tumor marker has been suggested. Increased total serum LDH activity and isoenzymatic "shift" toward "M" isoforms are reported for most malignancies^(25,26). Assay of LDH activity has been evaluated and in the diagnosis and monitoring of gynecological malignancies, contributed

mostly by serum data and much less by other body fluids or tissue extracts. Increased total LDH activities have been reported for sera of the patients with ovarian⁽²⁷⁾ or cervical⁽²⁸⁾ cancer and some tumors of uterine cavity⁽²⁹⁾. Similar increase has been observed also for other body fluids and cavity washings of patients with gynecological malignancies—vaginal⁽⁷⁾, uterine⁽⁶⁾, and peritoneal fluid⁽⁸⁾. With exception for ovarian dysgerminoma where increased levels of total serum LDH activity and its "H" isoforms have been well documented and accepted as useful in the managing of this disease⁽²⁴⁾, relevant diagnostic utility of LDH assay is not firmly established, however, since observed changes are not sensitive and specific enough. All that is mainly due to different rates of clearing of LDH isoenzymes from the circulation⁽²⁵⁾, which consequently does not reflect true tissue enzyme activity.

Normal uterine tissue, cyclically influenced by sex hormones, differs in LDH level during menstrual cycle. Total LDH activity of NM remains stable and is altered only by prolonged hormonal stimulation in pregnancy or postmenopause and in the malignancy⁽³⁰⁾. On the contrary, total LDH activity of NE gradually increases in almost linear fashion over the entire period of menstrual cycle⁽³¹⁾, being lowest in early proliferative phase and highest in the late secretory phase⁽³²⁾.

Our results of measurement of total LDH activity in matched normal and malignant uterine tissues corroborate that neoplastic transformation of human endometrial tissue significantly increases activity of this important glycolytic enzyme. Endometrial hyperplasia, which is considered as premalignant neoplasm⁽¹⁾, is characterized by two to fourfold higher in total tissue LDH level than that found in normal secretory endometrium⁽¹¹⁾. Even higher activities of this enzyme occasionally have been reported for limited number of EC samples studied up to now—the four- to eightfold rise has been observed in (totally) 17 ECs studied previously^(9,12,14), and these findings were confirmed later^(10,11,13) on additional several tenths of similar samples. This rise was proposed to represent an adaptation mechanism of energy supply and glycolysis to an increased demand for energy at a time when the normal capacity of oxygen-consuming pathways becomes inadequate to satisfy the needs of proliferating malignant cells⁽¹⁰⁾.

To improve the knowledge on this tumor biology, other molecular markers are under study. Recent findings suggest possible use of some enzymes as tumor markers, among which are glutathione S-transferase⁽³³⁾ that is involved in detoxification system and proteases

Table 3. Correlation^a of total tissue LDH activity with activity of tissue DPP III^b

Uterine tissue	<i>n</i>	<i>r</i>	<i>P</i>
Normal endometrium and myometrium	37	0.543	0.001
EC	33	0.546	0.001

^aPearson's product-moment correlation, at significance level of 0.05.

^bCalculated from the published data⁽²⁰⁾.

Table 4. Diagnostic value of the total tissue LDH and tissue DPP III assays for detecting EC

Assay	Cutoff value (U/mg) ^a	Sensitivity (%)	Specificity (%)	Positive predictive value (%)	Negative predictive value (%)	Diagnostic accuracy (%)
LDH	1.06	81.8	100	100	86.0	91.4
DPP III	20.60 ^b	90.9	97.3	96.8	92.3	94.3
LDH + DPP III	—	93.9	97.3	96.9	94.7	95.7

^aCalculated on the basis of "all normals" (normal endometrium plus normal myometrium) sample, as described under "Materials and methods."

^bCalculated from the published data⁽²⁰⁾.

that participate in the degradation of the basement membrane and digestion of extracellular matrix in the course of invasion and metastasis. These last comprise collagenase⁽¹⁾ and cathepsin D⁽³⁴⁾ which were found to be elevated not only in malignant endometrial tissue but also even in the hyperplastic⁽¹⁾ one, when these have been compared with normal or benign tissues^(1,34). The increased level of another proteolytic enzyme, DPP III has been reported in endometrial carcinomatous tissue by us, but its role in malignant growth is not elucidated⁽²⁰⁾. One possibility is that DPP III enzymatic activity may initiate or terminate some biological events (eg, activation or inactivation of peptide hormone with regulatory effect on the behavior of malignancy).

Increased glycolysis of the malignant tissue with the pronounced role of LDH together with the convenience of its measurement makes this enzyme still current in the evaluation as a tumor marker in gynecological malignancy. In spite of that, diagnostic utility of LDH assay has not been (except for ovarian dysgerminoma) yet firmly established. We intended to investigate the endometrial cancer biology by measuring total LDH enzymatic activity in homogenates of normal uterine tissues and EC and by correlating obtained data with routine clinical and histopathologic findings and with tissue levels of DPP III, a suggested marker of EC. Due to marked variability within human normal and neoplastic tissues, which requires evaluation of paired specimens⁽³⁵⁾, we also analyzed separately 30 "matched pairs" in which malignant tissue and normal counterpart originated from the same patient. Determination of LDH by continuously monitoring consumption of NADH, while pyruvate is converted to lactate is generally accepted by most of the European Societies for Clinical Chemistry⁽²⁵⁾ and was used in this study. Our results on assay of total LDH enzymatic activity in uterine tissues are consistent with earlier findings, yet with a few distinctions.

First, similar to the findings of others, we observed significant increase in total tissue LDH activity when

normal endometrium underwent malignant transformation to EC. This elevation was, however, far from some extreme values reported earlier^(9–11,14), and the difference could be probably result of the analytical and/or sampling methods applied. Second, thorough analysis of the obtained data showed no correlations with clinicopathologic findings, irrespectively of which EC samples (paired or unpaired) were examined. This indicated that total tissue LDH assay may not be of prognostic value for EC. High values of its diagnostic parameters (Table 4) suggest, however, that this assay could be useful in the diagnosis of EC.

The importance of LDH in tumor biology, including that of EC, has been extensively studied and confirmed (reestablished) lately by the "Tumour and Angiogenesis Research Group" of Democritus University of Thrace (Alexandroupolis, Greece). Most recently, Giatromanolaki *et al.*⁽¹⁵⁾ investigated immunohistochemically normally cycling endometrium and endometrial adenocarcinoma tissue of the endometrioid cell type for the expression of LDH-5 isoenzyme, for which they found earlier⁽¹⁶⁾ that it is expressed preferentially in cells of epithelial malignant tumors, compared to the normal tissues. They found that this isoenzyme is consistently expressed in cytoplasm and nuclei of normal glandular endometrial cells, unlike in other normal epithelia. LDH-5 was found expressed in all carcinoma tissues, and 31 of 68 examined ECs (45.5%) displayed high LDH-5 expression, taking into account both the cytoplasmic and the nuclear staining pattern.

Our results, based on the quantitation of total LDH activity, compare well with Giatromanolaki *et al.*⁽¹⁵⁾ finding that LDH-5 is not related to any histopathologic parameters (grade and depth of myometrial invasion). Furthermore, these authors have shown, using the same immunohistochemical approach, significant association of LDH-5 with the expression of phosphorylated vascular endothelial growth factor-2 (VEGFR2) receptors in both cancer cells and tumor-associated vasculature and revealed by the multivariate analysis that the expression of LDH-5 was independent

prognostic marker in endometrial cancer, linked with impaired host immune response and activation of VEGFR2 receptors in both cancer cells and tumor-associated vasculature. This finding encourages examination of the prognostic value of total tissue LDH activity for EC, when sufficient follow-up data will be available.

In precedent study⁽¹⁷⁾, we found that grade 1 ovarian carcinoma, contrary to the grade 2 and grade 3 ovarian tumors, did not differ in total tissue LDH activity from normal ovarian tissue. In the present study, significantly enhanced total tissue LDH activity was measured in EC samples of grade 1 and grade 2, and no difference was observed between these two subgroups. The same we found previously for DPP III—while its activity was dependent on the grade of ovarian tumors, this was not the case when ECs were studied^(18,20). Our present results on well-differentiated ECs point to the complexity and the difference in regulation of LDH in malignant gynecological tissues.

No single marker has proved sufficient to meet the full requirements of clinical application, and many workers have reported that a combination of more than one marker ("biochemical index") would prove more effective than any single assay⁽²⁸⁾. Therefore, we compared the levels of LDH and DPP III, (suggested biomarker for ovarian⁽¹⁸⁾ and endometrial⁽²⁰⁾ cancer) and found a significant correlation of LDH with DPP III in normal as well as in transformed uterine tissues (Table 3). Biochemical index consisting of the results of LDH and DPP III assays showed diagnostic reliability of at least 96% to detect EC (Table 4).

The data presented in this study establish the cutoff value between LDH activity in normal and malignant endometrial tissue and show high diagnostic value of this assay in EC, very close to those of a (suggested) new tumor marker DPP III. In contrast to our previous results on LDH in ovarian tumors of epithelial origin, a significant rise in LDH activity was found already in grade 1 EC.

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