Monitoring of Intracellular Enzyme Kinetic Characteristics of Peripheral Mononuclear Cells in Breast Cancer Patients

Elena Afrimzon,[1] Naomi Zurgil,[1] Yana Shafran,[1] Judith Sandbank,[2] Ruben Orda,[3] Shlomo Lalchuk,[4] and Mordechai Deutsch[1]

[1]The Biophysical Interdisciplinary Jerome Schottenstein Center for the Research and the Technology of the Cellome, Department of Physics, Bar-Ilan University, Ramat Gan; [2]Departments of Pathology and Cytology, and [3]Surgery "A," Assaf Harofeh Medical Center, Zerifin; and [4]Department of Surgery, Rabin Medical Center, Petach Tikva, Israel

Abstract

A new methodology for the detection of functional response of peripheral blood mononuclear cells against breast cancer (BC) antigens was developed. The method is based on cellular enzymatic activity measurements, using a fluorogenic substrate. We used this method to estimate the kinetic activity of lymphocytes derived from cancer patients and healthy donors. The aim of the study was to determine a possible correlation between the basic characteristics (K_m and V_{max}) of biochemical enzymatic reactions in live peripheral white mononuclear cells and common clinical-pathological characteristics in BC patients. Our method shows that the enzymatic activity, upon interaction with mitogen or tumor antigens, of the peripheral blood cells in BC patients is different from the enzymatic reactions in healthy individuals. This holds true in the early stages, and the difference persists throughout all of the stages of the disease. This difference is manifested, primarily, by an increase in the K_m values after cell incubation with tumor tissue. It was also demonstrated that higher K_m values of tumor tissue-activated peripheral blood mononuclear cells are associated with a better prognostic status of the BC patients (lymph node-negative tumors, hormone receptor preservation, and the absence of Her-2/*neu* protein overexpression). Thus, the present methodology may serve as an additional criterion for prognosis and monitoring, both in BC patients, and in individuals associated with high cancer risk.

Introduction

Because early detection of cancer is now recognized to greatly contribute to more successful treatment and, thus, survival of cancer patients (1), numerous attempts have been made to develop sensitive and specific assays for diagnosis, monitoring, and surveillance of breast cancer (BC; Refs. 2–4). However, the methods of clinical screening and diagnosis (in particular, the screening mammography) are limited in their prognostic capacity due to significant rates of false results. A great number of markers (tumor size, lymph node involvement, tumor cell differentiation patterns, nuclear grade, DNA S phase profiles, the estrogen and progesterone receptor status, and Her-2/*neu* protein overexpression) used currently for detection and prognosis of BC are only suitable for predicting the life quality of patients who already undergo advanced stages of the disease. These markers are characteristic of advanced tumors and advanced anticancer responses, and may be obtained only after surgery (5, 6).

The study of the multistep process of BC tumorigenesis demonstrated a sustained, successive accumulation of genetic, biochemical, and immune abnormalities during the disease progression before the appearance of clinically determinable tumors (7–13). Because these qualitative and quantitative changes precede the cancer clinical manifestations there has been an ongoing effort to use them for early cancer detection.

The conditions of the lymphocyte membrane and cytoplasm differ structurally and functionally between individuals with and without malignancies. Antigenic stimulation by the classic mitogens phytohemagglutinin (PHA) and concanavalin A, as well as by specific tumor-associated antigens induces changes in the structure of the cytoplasmic matrix, detectable via fluorescein diacetate (FDA) staining (14–18). Specifically, mitogenic stimulation of peripheral lymphocytes results in changes in their intracellular microviscosity, which are manifested in a characteristic kinetic behavior of FDA hydrolysis leading to an increase in the fluorescence intensity of intracellular fluorescein (19). Moreover, these changes in microviscosity precede other alterations due to activation, such as gene, receptor, and protein expression.

We have developed a novel methodology for detection of early lymphocyte activation signs based on steady state measurements of the fluorescence intensity in fluorescein-labeled peripheral blood mononuclear cells (PBMCs; Refs. 15, 19). The estimation of the kinetics of this process was conducted via the Michaelis-Menten constants. Our preliminary results have shown that early activation events, which occur upon cell incubation with and without either phytohemagglutinin or autologous tumor tissue, may be demonstrated by the general biochemical kinetic parameters (the Michaelis-Menten constant, K_m, and the maximal velocity, V_{max}) that display the differences in intracellular processes in the lymphocytes, related to the enzymatic activity as well as to the cell size, the cytoplasm membrane permeability, and so forth (15, 16, 19).

The aim of our study was to determine possible correlations between basic characteristics (K_m and V_{max}) of biochemical enzymatic reactions in live PBMCs and common clinical-pathological signs in BC patients.

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Materials and Methods

Case Material. Twenty eight patients with BC, 10 patients with colon cancer, and 23 healthy volunteers were examined, using our methodology of measurement and analysis of apparent K_m (Michaelis-Menten constant) and V_{max} values for individual cells. The blood and tissue samples were obtained from surgical departments of Assaf Harofeh Medical Center and Rabin Medical Center. The research was conducted in accordance with the principles of the Declaration of Helsinki. The pathological tumor tissue examinations were carried out by the pathology departments of these hospitals. The tumor tissue samples were kept in liquid nitrogen until their use. The clinical and histological characteristics of BC patients are shown in Table 1. We used the colon cancer patients group for the comparison of the basic enzymatic characteristics of PBMCs in patients with fundamentally different cancer types, hormone-related (BC) and hormone-unrelated (colon) tumors.

Cell Separation and Experimental Procedure. Peripheral blood was collected in heparinized tubes at the time of surgery. After removing the iron-absorbing cells, the remaining mononuclear cells were separated by Ficoll-Hypaque (Sigma, St. Louis, MO) density gradient centrifugation. The cells were subsequently harvested, washed twice in PBS (pH 7.2), and resuspended in complete RPMI 1640. The rate of FDA hydrolysis by esterases was measured in PBMCs following three different treatments, after 30 min of incubation, without any stimulant, or with the standard nonspecific mitogen PHA (90 μg/ml) in PBS, or with a small piece (1 mm^3) of intact unfractionized auto- or homologous tumor tissue. The autologous tumor tissue was used in the majority of patients (58%), whereas the homologous tumor tissue was used in healthy donors and in the rest of the patients.

Table 1 Clinicopathological characteristics of the examined breast cancer patients

Clinical characteristics	Grades	% of patients
Age (years)	<40	3.5
	40–49	7.0
	50–59	21.5
	60–69	18
	70–79	32
	80+	10
Histopathological grades	1 grade	25
	2 grade	50
	3 grade	25
Cellular characteristics	Ductal carcinoma	72
	Lobular carcinoma	12
	Tubular carcinoma	4
	Ductal carcinoma *in situ*	36
Tumor size	<1.0	29
	1.1–2.0	22
	2.1–3.0	35
	>3.1	17
Axillary lymph nodes status	Metastasis negative	72
	Metastasis positive	28
Estrogen receptor status	Positive	64
	Negative	16
	No data	20
Progesterone receptor status	Positive	56
	Negative	24
	No data	20
Her-2/*neu* status	Positive	16
	Negative	56
	No data	28
Ki67 status	Positive	64
	No data	35

Measurements. The enzymatic hydrolysis of FDA and the intracellular accumulation of fluorescein by individual PBMCs were measured by the Individual Cell Scanner on a cell tray as described previously (18). The control of the cell uniformity was performed by microscopic examination on the cell tray developed and patented by our labs. In this tray (total capacity of 10,000 cells/tray), individual cells were separated by 20 μm in a matrix array. The remainders of the tumor tissue were removed by multiple PBS washing. Using the fluorogenic FDA substrate, we measured the kinetic parameters of esterase activity of PBMCs derived from cancer patients and healthy donors. The main kinetic characteristics K_m (the Michaelis constant) and V_{max} (maximal velocity) of biochemical reaction for individual cells (maximum 300 cells/patient) were calculated after repeated periodical measurements (24 times) of the same cells, which were sequentially exposed to various increasing fluorogenic substrate concentrations (0.6 μM, 1.2 μM, 2.4 μM, and 3.6 μM) of FDA in PBS staining solutions. Cells were measured six times per each substrate concentration at time intervals of ~40 s. The time intervals for the replacement of FDA solutions were 50 s. Under the sequential FDA exposure, the fluorescent intensity slope between successive FDA concentrations was almost zero, which means that V_0 dropped back to zero (18). The duration of all 24 of the measurements did not exceed 1,000 s. Damaged and dead cells, which did not show an increase in fluorescent intensity on exposure to higher concentrations of FDA, were excluded from the mathematical analysis. For each subject, six trays were used, two trays per each type of cell treatment.

Mathematical Calculations. The main biochemical constants (K_m and V_{max}) were derived by applying the so-called Lineweaver-Burk plot, also known as the double reciprocal plot

$$\frac{1}{\nu_0} = \frac{K_m}{V_{max}}\frac{1}{[S]} + \frac{1}{V_{max}}$$

that is relatively simple in comparison to other known methods and more suitable for the present experimental conditions (20).

All of the values are presented as average ± SE. The comparisons between groups were performed by Student's *t* test.

Results

Cells localized on the cell tray were sequentially exposed to four different FDA concentrations and scanned six times per concentration, at time intervals of ~40 s. A representative chart of a complete measurement procedure performed on 20 individual PBMCs derived from a BC patient is shown in Fig. 1. K_m and V_{max} coefficients were calculated for each individual cell within the population of PBMCs after incubation with or without tumor tissue. Average K_m values were found to be 1.51 ± 0.3 μM in untreated cells and 4.0 ± 1.9 μM in tumor tissue-treated cells. The corresponding V_{max} values were found to be 548.7 ± 113 au in untreated cells and 587 ± 213 au in tumor tissue-treated cells. This indicates an increase of 165% in K_m and 7% in V_{max} on exposure of the cells to cancer tissue.

The same procedure was performed to extract mean K_m and V_{max} values for all of the cancer patients and healthy donors. The average K_m values of untreated cells were found to be 1.73 ± 0.182 μM, 1.93 ± 0.24 μM, and 2.02 ± 0.208 μM for healthy donors, BC patients, and colon cancer patients, respectively (the K_m values and corresponding V_{max} values are pre-

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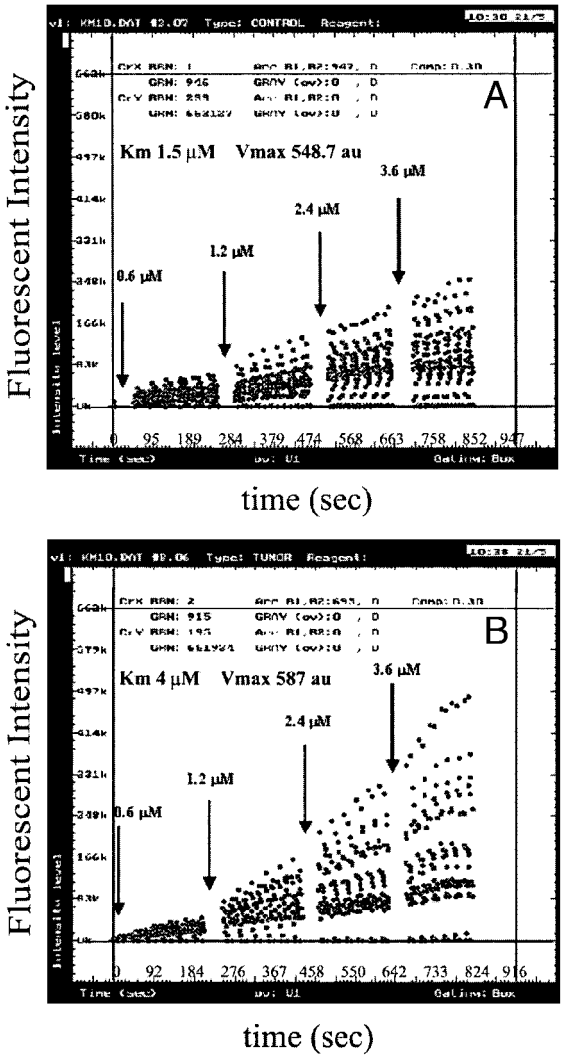

Fig. 1. The intracellular enzymatic reaction as monitored by fluorescent intensity of accumulated fluorescein (the ordinate) in individual peripheral blood mononuclear cells in one of the breast cancer patients (infiltrating ductal carcinoma, grade 1, without metastases). *A*, untreated cells; *B*, cells incubated with autologous tumor tissue. Each cluster is defined by six fluorescent intensity measurements taken at six time points for the same individual cells (20 cells total) when exposed to the appropriate substrate concentrations that are shown by *arrows* above (thus, each of the 20 cells was scanned 24 times). The spaces between clusters represent the duration of the staining solutions replacement.

sented in Table 2). An increase in average K_m values in cancer patients in comparison with healthy donors was not distinguishable statistically ($P > 0.05$). However, the cell stimulation with the standard mitogen or tumor tissue revealed a difference in the cells status. The nonspecific mitogenic PHA stimulation of PBMCs increased the K_m and V_{max} values both in healthy donors and in cancer patients as compared with measurements of the untreated cells (Table 2). Still, the increase of V_{max} values after PBMC activation by PHA in the BC patients (1094.5 ± 131.77 au) was less significant in comparison with healthy individuals ($P < 0.03$), whose average V_{max} value amounted to 1995.58 ± 360.35 au ($P < 0.00006$).

Incubation of PBMCs with tumor tissue effected a change in the K_m values different in cancer patients and healthy control subjects (Table 2); the K_m values sharply increased ($P < 0.004$), as compared with untreated cells, in cancer patients (from 1.93 ± 0.24 μM to 2.95 ± 0.47 μM in BC patients, and from 2.02 ± 0.208 μM to 3.45 ± 0.72 μM in colon cancer patients), and almost did not change in healthy individuals (1.73 ± 0.182 μM in untreated cells and 1.56 ± 0.127 μM after cell incubation with tumor tissue). Alterations in the V_{max} values were small in comparison with untreated cells.

Because the purpose of the present study relates primarily to the BC problematics, we studied the parameters of enzymatic reactions in different clinical-pathological groups of cancer patients, taking into account general solid tumor characteristics, histological grade, lymph node status, steroid hormone receptors (to estrogen and progesterone hormones), and HER-2/*neu* protein expression that shows the proliferation rate of tumor cells. The clinical and histological characteristics of BC patients are shown in Table 1.

Tumors are generally classified according to their histopathological grade. The more differentiated the tumor cells are, the better the prognosis. The present study indicated that the increase in the average K_m values of PBMCs, after exposure to tumor tissue, is higher in the groups with different tumor grades as compared with that in healthy individuals (Fig. 2). However, the increase of the average K_m after incubation with tumor tissue was significantly higher ($P < 0.03$) as compared with healthy donors, only in patients with moderately (grade 2) differentiated tumors (3.58 ± 0.75 μM and 1.56 ± 0.127 μM, respectively). We also found a statistically significant ($P < 0.009$) difference in K_m values in untreated cells between well- (grade 1) and moderately differentiated tumors (Fig. 2).

The average V_{max} value after incubation with tumor tissue did not change significantly in either healthy donors or in the general group of patients (Table 2). The analysis of the V_{max} value in PBMCs after tumor tissue incubation in the different histopathologic subgroups of BC patients did not reveal any significant changes (Table 3). At the same time, incubation with the nonspecific standard mitogen PHA (Table 3) led to an increase in the average V_{max} values in patients with low-differentiated tumors (from 321.5 ± 106.0 au in untreated cells to 1109.2 ± 254.9 au; $P < 0.02$) and well-differentiated tumors (from 224.9 ± 64.8 au to 1071.8 ± 316.9 au; $P < 0.02$). This increase was comparable in magnitude to that in healthy individuals (in 3.5, 4.8, and 3.1 times in low-differentiated, well-differentiated tumor groups, and healthy donors, respectively), which may indicate the preservation of the nonspecific functional competence of these cells. An increase of average V_{max} values in patients with moderately differentiated tumors (from 648.6 ± 186.3 au in untreated cells to 1124.8 ± 193.9 au in

Table 2 The general enzymatic characteristics of PBMCs[a] in cancer patients and healthy donors

Parameters	Healthy donors (n = 23)	Breast cancer (n = 28)	Colon cancer (n = 10)
K_m (μM)			
Untreated	1.73 ± 0.18	1.93 ± 0.24	2.02 ± 0.2
PHA	2.41 ± 0.21	2.52 ± 0.25[b]	3.22 ± 0.4[b]
Tumor tissue	1.56 ± 0.12	2.95 ± 0.47[b]	3.45 ± 0.72[b]
V_{max} (au)			
Untreated	633.8 ± 87.9	476.7 ± 99.2	338.9 ± 80.1
PHA	1995.5 ± 360.3[b]	1094.5 ± 131.8[b]	513.6 ± 94.8
Tumor tissue	612.5 ± 112.6	722.9 ± 269.1	422.1 ± 96.3

[a] PBMC, peripheral blood mononuclear cell; PHA, phytohemagglutinin.
[b] $P < 0.05$ represents the significance of difference between untreated cells and cells incubated with PHA or tumor tissue.

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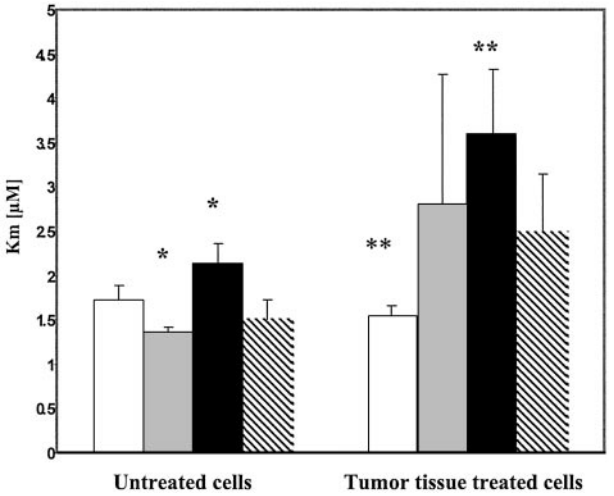

Fig. 2. Average K_m values in groups with different tumor histopathological grades. A significant difference in K_m values of untreated cells (the *Untreated cells* group) was found between breast cancer patients with high (grade 1, ▨) and moderately (grade 2, ■) differentiated tumors (*, $P < 0.0009$). The increase of K_m values after incubation with tumor tissue (the *Tumor tissue treated cells* group) is statistically significant (**, $P < 0.03$) in breast cancer patients with moderately differentiated (grade 2 ■) tumors, as compared with the healthy donor cells (□). In patients with low differentiated tumor (▧) the increase of average K_m value after tumor tissue exposure was not statistically significant. Results (mean) are expressed as substrate concentration (K_m) in μM; *bars*, ±SE. Student's *t* test was performed to discriminate between the results obtained in the different groups.

PHA-incubated cells) was observed, but it was not significant in comparison with untreated cells.

The most commonly used prognostic markers for cancer patients, and particularly BC patients, are tumor size and lymph node involvement. We tested the K_m and V_{max} alterations in different subgroups of BC patients according to the tumor size, bearing in mind that ductal carcinoma *in situ* is the microinvasive breast carcinoma with a better prognosis. It was shown that there is no significant difference in average K_m values between patients having ductal carcinoma *in situ* (1.96 ± 0.21 μM) and patients with other histological tumors (1.61 ± 0.2 μM). However, the increase in the average K_m values after tumor tissue incubation was more considerable in ductal carcinoma *in situ* patients (4.3 ± 1.07 μM; $P \leq 0.05$) than in patients with other histological BC types (2.84 ± 0.5; $P < 0.04$). On the other hand, there is a tendency of increase in the average V_{max} values after tumor tissue incubation in ductal carcinoma *in situ* patients only (1282.4 ± 573.2 au in comparison with 567.1 ± 185.9 au in untreated cells).

The involvement of axillary nodes in tumor development has long been recognized as a key feature in determining prognosis for BC patients. In patients without metastases in lymph nodes, average K_m values, after incubation with tumor tissue, were significantly higher as compared with untreated cells of these patients. In the subgroup of patients with lymph node involvement, no significant rise in K_m after incubation with tumor tissue was observed (Table 4).

Valuable parameters for determining the prognosis for BC patients are hormone receptor levels and proto-oncogene overexpression. We investigated the alterations of the main kinetic characteristics (K_m and V_{max}) of the enzymatic reaction according to estrogen and progesterone receptor status (Table 4). The results indicate that in subgroups of patients with better-differentiated tumor cells (progesterone and estrogen receptor-positive tumors) average K_m values were significantly higher ($P < 0.05$) after tumor tissue exposure compared with non-activated cells. Apart from this, in the subgroup of patients with preserved progesterone receptors, the average K_m values in untreated cells (without nonspecific or specific stimulation) were significantly higher than in patients with loss of progesterone receptors, whereas the average V_{max} values in these different subgroups were not significantly different in all of the experimental conditions (data not shown).

The level of the protein Her-2/*neu* is known to be a marker for poor outcome and is associated with typically poor response to chemotherapy by tamoxifen. We studied the K_m and V_{max} in the patient subgroups dependent on the Her-2/*neu* protein expression. It was shown that the average K_m values after incubation with tumor tissue increased significantly in comparison with untreated cells only in patients with Her-2/*neu* protein-negative tumors (Table 4). V_{max} values were similar in these two BC patient subgroups (data not shown).

Of special interest is the use of autologous or homologous tumor tissue as the specific or nonspecific stimuli for detection of functional response against BC based on periodical measurements of antigenic lymphocyte activation of peripheral blood cells. This interest derives from the observation that autologous tumor antigen is not present in all of the patients (21). As mentioned, we used autologous tumor tissue for the major part of BC patients (58%), whereas homologous tumor tissue was used in healthy donors and in other patients. It was shown that the average K_m values tended to rise after incubation with tumor tissue both in cells exposed to autologous and to homologous tumor tissue (Fig. 3). K_m pattern values in the patients were higher than in healthy donors (3.51 ± 0.9 μM in patient cells after autologous tumor tissue incubation, 2.41 ± 0.49 μM in patient cells after homologous tumor tissue incubation, and 1.56 ± 0.12 μM in healthy donors after homologous tumor tissue incubation), but the difference between average K_m values was statistically significant only in patients whose PBMCs were exposed to autologous tissue ($P < 0.05$). In patients whose lymphocytes were activated by homologous tumor tissue, the average K_m values had only a tendency of increase. Although there is no statistically significant difference in K_m after homologous tumor tissue exposure as compared with healthy donors, the presence of the similar tendencies of K_m increase may point out to the possible similarity of the antigens causing this response or the common mechanisms involved in the activation by both autologous and homologous tumor tissue. Our present results indirectly evidence that an increase of K_m values in cells incubated with autologous tumor tissue, in comparison with untreated PBMCs, may serve as a cautionary feature in the monitoring of BC patients in the postoperative period or during the periods between chemotherapy courses, whereas homologous tumor tissue can be used for screening individuals with high cancer risk.

Table 3 The changes of V_{max} (au) in the various groups of breast cancer patients depending on the tumor histopathological grade

	Untreated cells	Tumor tissue treated cells	Phytohemagglutinin activated cells
Well-differentiated tumors	224.9 ± 64.8	254.9 ± 93.7	1071.8 ± 316.9[a]
Moderately differentiated tumor	648.6 ± 186.3	1111.7 ± 479.8	1124.8 ± 193.9
Low-differentiated tumor	321.5 ± 106.0	303.5 ± 123.7	1109.2 ± 254.9[a]

[a] $P < 0.02$ represents the significance of difference between untreated cells and cells incubated with phytohemagglutinin.

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Table 4 The general biochemical characteristics in the various groups of breast cancer patients depending on risk factors

Risk factors	Mean K_m (μM) values					
	Positive			Negative		
	Untreated cells	Tumor tissue	P^a	Untreated cells	Tumor tissue	P^a
Metastasis	1.74 ± 0.16	1.81 ± 0.37	<0.85	1.68 ± 0.16	3.7 ± 0.67	<0.009
Her-2/*neu* protein overexpression	1.41 ± 0.27	2.43 ± 0.7	<0.20	1.9 ± 0.18	3.37 ± 0.67	<0.04
Estrogen Rc	1.85 ± 0.16	2.94 ± 0.47	<0.04	1.31 ± 0.24	5.28 ± 2.01	<0.09
Progesterone Rc	2.0 ± 0.17	3.2 ± 0.57	<0.04	1.2 ± 0.21	4.21 ± 1.27	<0.09

[a] *P* represents the significance of difference between untreated cells and cells incubated with tumor tissue.

Discussion

FDA staining has been long used in studying the cellular kinetic parameters, measurement of total population, and subset characteristics, and for the estimation of different cellular physiological states, such as activation and apoptosis (19, 22–27). It has been shown that cell populations exhibit wide heterogeneity with respect to their ability to use FDA, dependent on their type, cell cycle stage, and the cell surface status (28–31). The simplicity of FDA staining and the reliability of observation make this method handy and promising. Moreover, this novel methodology, which involves the estimation of classic kinetic parameters (K_m and V_{max}) of enzymatic reactions in individual cells, has several additional advantages over other techniques. First of all, it provides the ability to use different increasing FDA concentrations (18) and measure repeatedly the fluorescence of the same individual living cells within a population. The staining rate, which practically reflects the conversion rate of the nonfluorescent substrate to the fluorescent product, is measured for each cell at each dye concentration, yielding a series of staining rates for the same cell. In the preliminary experiments (18), the individual cell coefficient of variance obtained in >10 successive measurement scans of a 10 × 10 cell field never exceeded 2% for fluorescent intensity. Using these data, V_{max} and apparent K_m values can be calculated for each cell, giving the distribution of K_m and V_{max} of the entire population. These technique and analytic tools are applied to trace differences in distributions of K_m and V_{max} over a population of individual cells. Secondly, unfractionized tumor is used for the induction of anticancer functional response. It has been known that autologous tumor antigens, even if they are expressed on the tumor cell surface, are not accessible to immune surveillance in most solid tumors (21). Frequently arising problems of cancer cell recognition and development of the antitumor immune response are due to the low immunogenicity of tumor antigens, their low expression, the lack of HLA and other differentiated antigen expression on the tumor cells surface, and so forth (2, 9, 32). In view of this, the majority of studies dealing with the potential ability of breast tumor antigens to serve as prognostic markers for early detection and monitoring of tumor progression, use cell lines, purified cells, or their lysates (21, 33–35). However, advanced structured solid tumor includes not only cancer cells, but also stromal, immune, and inflammatory cells and elements, and their vital products. Therefore, we used the PBMC activation by unfractionized tumor tissue for creating an experimental *in vitro* model similar to *in vivo* tumor-host interaction, whereas the host responds to the entirety of cellular and noncellular tumor components. On the other hand, the methodology using frozen tumor tissue may introduce a potential degree of variability, related to a heterogeneous not precisely defined composition of the material used. We performed duplicate measurements to overcome part of these variations. Thirdly, the use of peripheral blood allows for periodical evaluations during an extensive pre- and postoperative period, thus allowing the monitoring of long-term effects in BC patients (by use of autologous tumor tissue) and the control of persons with high cancer risk (by use of homologous tumor tissue).

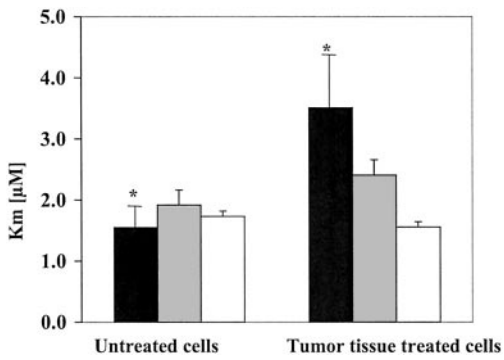

Fig. 3. Average K_m values in untreated peripheral blood mononuclear cells and in cells incubated with tumor tissue: ■, breast cancer (BC) patient cells incubated with autologous tumor tissues; ▨, BC patient cells incubated with homologous tumor tissues; and □, healthy donor cells incubated with BC tissues. Results (mean) are expressed in μM; *bars*, ±SE. Student's *t* test was performed to discriminate between the untreated and tumor tissue treated cells (*, $P < 0.05$).

Our observations indicated that there is an essential difference between peripheral mononuclear cells of BC patients and healthy subjects with respect to the overall esterase enzymatic activity. First of all, both mitogenic activation and tumor tissue exposure resulted in an increase of K_m, which indicated that this biochemical parameter is probably involved in lymphocyte stimulation. Moreover, a significant difference between cells of BC patients and healthy subjects was clearly demonstrated after the exposure of peripheral cells to tumor tissue; the apparent K_m values in BC patients significantly increased, whereas in healthy subjects they did not alter.

Generally, an increase in K_m values signifies that the same enzyme-substrate reaction velocity requires a lower substrate concentration. As mentioned, in our measurement system, K_m is an apparent parameter, calculated from the fluorescence intensity measurements of fluorescein accumulated by living cells *in situ*. In contrast to direct enzyme-substrate interaction *in vitro* when the concentrations of reagents are known, under the present experimental conditions the actual intracellular substrate concentrations remained unknown. We operated only with defined external substrate concentrations. The conversion of substrate concentrations from the extracellular to the intracellular levels depends, primarily, on the active and passive mechanisms of the cell membrane permeability to the substrate, *i.e.*, on the cell membrane condition. Unlike in healthy donor

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cells, in cells derived from BC patients only, a rapid activation of PBMCs by unfractionized tumor tissue can lead to changes in the cell membrane status and the corresponding decrease in the effective intracellular substrate concentration necessary for the maximal enzyme-substrate interaction.

The K_m changes after incubation of the peripheral cells with tumor tissue may be alternatively explained by changes in the intracellular enzyme status, namely, enzyme concentration, conformation, or affinity for the substrate, which may be relevant to carcinogenesis.

Both assumptions can explain, to some extent, the obtained results. There is a need for additional investigations of the mechanisms involved in early activation of circulating blood cells by tumor tissue exposure and of the biopathogenic mediators of such activation.

The possible connection of the total enzymatic activity of esterases in PBMC (assessed in our study via fluorescein accumulation) and BC pathogenesis can be reinforced by the evidence of an indirect relationship between total intracellular esterases activity and chemical protein carbamoylation. The modifications of the total activity of esterases in tumor cells, as well as in the peripheral mononuclear cells, may be related to impairment of protein carbamoylation and the consequent changes in the processes of cellular growth and proliferation (36–38). It seems possible that carbamoylation-related alterations of macromolecular synthesis in carcinogenesis may lead to the perturbed state of tumor-associated cells and circulating lymphocytes, as well as to a change in receptor expression, suppression of PBMC immune response, and, finally, to the detrimental effects of tumor on the regulatory systems (10, 32, 35, 39, 40). Additionally, the contravention of carbamoylation process may result, for example, in a change of the cytostatic drug toxic effects (41, 42).

Interestingly, the K_m changes in breast and colon cancer patients had the same tendencies, which may be evidence common for all solid tumor mechanisms of protein synthesis change, and of the enzymatic reactions independent of the cancer type. The above results make such a conclusion logical and allow us to suggest the present methodology as an additional technique for determining the PBMC immune response, not only in BC, but also in various other types of solid cancer.

The evaluation of the relation between general biochemical characteristics and extensive clinical parameters indicates that in patients with a better clinical-pathological status (lymph node-negative tumors, hormone-receptor preservation, and the absence of Her-2/*neu* protein overexpression), PBMC exposure to tumor tissue leads to a sharp increase in the K_m level as compared with patients with a poorer prognosis. Our observations may present evidence of step-by-step alterations of biochemical processes during cancer development with a partial preservation of normal function in BC patients who exhibit better clinical characteristics.

In connection to a major aspect of this work concerning the character of biochemical reactions in cells incubated with autologous or homologous tissue, it is important to note that the present initial results pose numerous questions regarding the nature and mechanisms of interaction between peripheral lymphocytes and tumor tissues of various origin. The obtained results open a wide field for research concerned with the role of intracellular esterases in lymphocyte activation processes, as well as with specificity of esterase intracellular activity in normal physiological conditions and pathology.

Our results indicate that significant differences are obtained even within statistically small groups, which speaks in favor of our concept. This may present evidence of at least a tendency of increase in K_m values after tumor tissue exposure in cancer patients. Finally, based on the results obtained from 61 participants, the following conclusions are suggested.

Firstly, on exposure to tumor tissue, the peripheral blood cell enzymatic reactions in BC patients are markedly different from enzymatic reactions in healthy individuals, based on the classic signs K_m and V_{max}.

Secondly, higher K_m values of tumor tissue-activated PBMCs are associated with a better prognostic status of BC patients.

Finally, the basic characteristics, K_m and V_{max}, of the enzymatic reactions of live peripheral white blood mononuclear cells can serve as an additional criterion for monitoring and prognosis of BC.

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