ISSN 0026-2617, Microbiology, 2010, Vol. 79, No. 4, pp. 435–438. © Pleiades Publishing, Ltd., 2010.
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EXPERIMENTAL ARTICLES

Inhibition of Urokinase Synthesis in a Tumor Cell Culture by the Lipid Fraction from the Spores of the Anaerobic Bacterium *Clostridium butyricum*

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Received September 10, 2009

Abstract—The inhibitory action of the lipid fraction of the extract from the spores of the anaerobic bacterium *Clostridium butyricum* on urokinase synthesis by the *CEM* line human leukemia lymphoid cells, the *HBL* line human breast cancer cells, and the CLL Lewis lung carcinoma cells in vitro was established.

Key words: bacterial spore lipid fraction, urokinase-type plasminogen activator, type IV collagen, antitumor activity.

DOI: 10.1134/S002626171004003X

In the process of differentiation of bacterial vegetative cells into dormant forms, the character of their lipid metabolism drastically changes. For example, it was established that lipid content in bacterial cysts is twice as high as in vegetative cells [1]. This reason alone is sufficient to arose interest in lipids of anabiotic forms. It was noted that the phenolic lipid content increased drastically when dormant forms were formed: for instance, 5-alkylresorcinols and 6-alkylpyrones substituted for phospholipids in the cytoplasmic membrane of *Azotobacter vinelandii* cells in the process of cyst formation [2].

Amphiphilic lipids with a benzene ring in their structure, i.e., phenolic lipids and resorcinolic lipids, have variegated biological activity. The universal character of their properties results from their effect on the rate of peroxide lipid oxidation and on the cell membrane composition and fluidity [3, 4].

The literature data indicate that phenolic lipids initiate the formation of anabiotic forms in bacteria due to the inhibition of metabolic processes [5–7] and of the activity of enzymes, including proteases. Proteases are known to be involved in the processes of cell division, cell motility, etc.

Along with their bacteriostatic activity, the phenolic lipids isolated from different natural sources reveal a high antitumor activity. Thus, the phenolic lipid bilobol (5-*n*-pentadec–8-enylresorcinol) isolated from *Ginkgko biloba* completely inhibited the growth of murine sarcoma S180 cells over 4 days of use [8].

It was shown earlier that the extract from *Clostridium butyricum* spores had a bacteriostatic effect on the vegetative cells of the producer culture. The lipid fraction of the *C. butyricum* spore extract preparation inhibited autolysis of the cell walls of the producer bacteria by 87%, whereas the initial spore extract preparation inhibited autolysis of the cell walls of the producer bacteria only by 30% [9].

It was found that the lipid fraction of the *C. butyricum* spore extract preparation had an antitumor effect on the CBRB-Rb breast cancer murine model. After five injections, the recipient mice showed a significant improvement in the dynamics of survival compared to the control animals [10].

It is considered that the main feature of the malignant phenotype is its close relation to the urokinase-type plasminogen activator and the proteolysis imbalance contributing to the invasion of exogenous matrix, the basal membrane [11, 12].

The aim of this work was to study in vitro the influence of the lipid fraction of bacterial spores on the synthesis of the urokinase-type plasminogen activator by the cells of malignant *CEM* line human leukemia, *HBL* line human breast cancer, and *CLL* Lewis lung carcinoma.

MATERIALS AND METHODS

The bacterial culture of *Clostridium butyricum* strain 35/11 was obtained from the Department of the Physiology of Spore-Forming Bacteria of the Institute of Microbiology, Russian Academy of Sciences. The spores of the anaerobic bacteria *C. butyricum* were obtained according to the method described in [13]. The method for obtaining the spore extracts was described earlier [14]. The lipids were isolated from the extracts with a mixture of chloroform and metha-

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Table 1. Effect of the preparation of the *C. butyricum* spore lipid fraction on urokinase synthesis by tumor cells

Preparation concentration in the tumor cell cultivation medium (μg/ml)	Cell fluorescence (*mean*)		
	CEM	*HBL*	*CLL*
20	6.2	6.5	6.3
40	5.3	7.1	6.0
200	5.9	7.2	6.4
0 (control)	11.6	8.9	18.6

nol according to the conventional technique [15, 16] and dried in vacuum. The preparation of the *C. butyricum* lipid fraction was prepared according to the method described earlier [17]. The sterile preparation was introduced into the cultivation medium at the final concentrations of 0.02, 0.04, and 0.2 mg/ml. The cells of the lines *CEM, HBL*, and *CLL* were cultivated under the standard conditions for 48 h at 37°C in the atmosphere of 5% CO_2 on RPMI-1640 medium with glutamine (*Gibco*) with the addition of 7% embryonic serum.

Urokinase synthesis on the cell surface was assessed with the immunochemical method [18] using fluorescein isothiocyanate (FITC)-labeled rabbit antibodies against human urokinase (*f-RAH Ura*) kindly provided by Imtek (Moscow, Russia).

The cells were removed from the support with 0.2% EDTA in physiological saline, washed by centrifugation, and resuspended in 2 ml of phosphate buffer. The aliquots of the cell suspension were incubated (30 min at 18–20°C) with *f-RAH Ura* antibodies (20 μg/ml); the cells were then washed twice with 0.2% bovine serum albumin in phosphate buffer. The washed cells were resuspended in 0.5 ml of phosphate buffer, and the fluorescence was measured on an Epics Elite flow-type cytofluorimeter (Coulter Electronics). The fluorescence mean value (for at least 5000 cells) was expressed in *mean* conventional units (Multi Graph, IMMUNO, Coulter Electronics). According to the method of determination, a sample of 2000 cells is considered to be representative. In parallel, the cells grown under the standard cultivation conditions, without introducing the spore lipid fraction preparation, were treated according to the same method (control).

Immunochemical determination of type IV collagen on the cell surface was carried out according to the method described in [18]. The cells were removed from the support with 0.2% EDTA in saline, washed by centrifugation, and resuspended in 2 ml of phosphate buffer. The aliquots of the cell suspension were incubated (30 min at 18–20°C) with rabbit antibodies against human collagen (*RIH C44*, Imtek, Russia) at a concentration of 20 μg/ml; the cells were then washed twice with 0.2% bovine serum albumin in phosphate buffer and incubated with FITC-labeled goat antibodies against rabbit immunoglobulins (*f-GARI*, Imtek, Russia) for 30 min at 18–20°C. The cells were then washed with BSA solution. The washed cells were suspended in 0.5 ml of phosphate buffer, and the fluorescence was measured on an Epics Elite flow-type cytofluorimeter (Coulter Electronics). The fluorescence mean (for at least 5000 cells) was assessed in *means* conventional units (Multi Graph, IMMUNO, Coulter Electronics). For comparison, determination of collagen on the surface of the cells cultivated under the standard conditions, without introducing the lipid fraction preparation, was carried out (control).

RESULTS AND DISCUSSION

The results of the study of the effect of the preparation of *C. butyricum* spore lipid fraction on the synthesis of the urokinase-type plasminogen activator by tumor cells in model cell systems are presented in Table 1.

The results indicate that the spore lipid fraction preparation inhibits urokinase synthesis by tumor cells. Under the experimental conditions, the level of inhibition of urokinase expression in the cells of different lines varied, probably due to the differences in their physiological characteristics. For example, according to the *mean* value, urokinase synthesis by the *CLL* cells cultivated in the presence of 0.04 mg/ml of the preparation in the medium decreased from 18.6 conventional units in the control to 6.0 conventional units, i.e., more than threefold. The urokinase synthesis by the *HBL* cells decreased almost 1.5-fold.

Binding of the urokinase plasminogen activator to its receptor triggers proteolytic cascade resulting in plasminogen converting to plasmin. Due to its proteolytic activity, plasmin degrades the extracellular matrix and the basal membrane components; it also activates other metalloproteinases. The electron microscopic studies showed local dissolution of the basal membrane at the sites of contact with invading cells [11, 12]. The basal membrane is a resilient structure that serves as a mechanical barrier precluding the spread of malignant cells and the formation of second-order tumors. Type IV collagen is the main structural protein of the basal membrane [11, 12].

The authors therefore investigated the effect of the spore lipid fraction preparation on the expression of type IV collagen by tumor cells in vitro. The results are presented in Table 2.

These results show that the amount of collagen on the cell surface increased in the presence of the spore lipid fraction preparation in the tumor cell cultivation medium. According to the fluorescence value, the *CEM* and *HBL* line cells increase the amount of collagen from 0.96 and 1.80 conventional units, respectively, in the control to 3.99 and 3.60 conventional units, respectively, when grown in the presence of 0.2 mg/ml of the spore lipid fraction preparation.

According to the literature data, an increase in the amount of type IV collagen on the surface of tumor cells may result not only from the modulation of the synthesis of collagen itself but also from the inhibition of the plasminogen activator synthesis [11, 12].

We suggest that the most probable factor of the biological activity of the spore lipid fraction is a lipid of phenolic nature, butyl benzoate, with a substitute in the *para* position [10]. Phenolic lipids are present in numerous organisms. Their biological activity, physiological role, and involvement in the regulation of metabolic processes have been revealed, but remain poorly understood [4, 8]. It may be suggested that phenolic lipids play a significant role in the general regulatory mechanisms of both prokaryotic and eukaryotic cells, contributing, among other things, to the process of carcinogenesis.

Antitumor activity is one of the very interesting characteristics of phenolic lipids. These substances differ completely from the classical antitumor drugs. Their primary target appears to be the cytoplasmic membrane. They are not mutagens and do not possess immunosuppressive properties. Another interesting aspect of the use of phenolic lipids is their involvement at the molecular level in signal transduction (transmission) from the basal membrane to the nucleus. By changing the physicochemical state of the membrane, phenolic lipids are likely to change the conformation of membrane-bound receptor molecules. The G proteins associated with the membrane receptors, in turn, activate adenylate cyclase and increase the level of intracellular cAMP, which interacts with proteinase A as a secondary intermediary, determining the synthesis of cell proteins [19]. Such a relation was reliably established, for example, for such lipid esters as alkyl lysophospholipids [20].

The system of the urokinase-type plasminogen activator, including the plasminogen activator proper, its related receptor, and two specific inhibitors of the activator, is central in many biological processes, including fibrinolysis, inflammation, atherosclerotic plaque formation, angiogenesis, and metastasizing [21]. In certain malignant types of cancer, a positive correlation between the presence of one or more members of the plasminogen activator system and the negative prognosis was noted. On the contrary, the inhibition of expression of these components leads to decreased invasion and metastasizing of many tumors [22]. The treatment aimed at inhibiting the functions of the urokinase plasminogen activator and its receptor, including the specific inhibitors of plasminogen activator, antagonist peptides, the monoclonal antibodies capable of preventing the binding of the activator to its receptor, and the gene therapy technologies inhibiting urokinase and/or its receptor expression have an antitumor effect [23]. These approaches, although promising, require confirmation in experiments on mammals and require further serious studies. At present, only one example of a synthetic inhibitor of the urokinase plasminogen activator system is known, PS-341, which is presently undergoing clinical trials [20–22].

Table 2. Effect of the preparation of the *C. butyricum* spore lipid fraction on the synthesis of type IV collagen by tumor cells

Preparation concentration in the tumor cell cultivation medium (μg/ml)	Cell fluorescence (*mean*)		
	CEM	*HBL*	*CLL*
20	2.49	2.50	2.90
40	3.48	3.20	3.60
200	3.99	3.60	4.90
0 (control)	0.96	1.80	2.00

In this work, the inhibitory action of the *C. butyricum* spore lipid fraction on the plasminogen activator system has been established for the first time using cell models.

According to the literature data [3] and the results of our studies [9], the spore lipid fraction preparation has low toxicity, which distinguishes it favorably, as the preparations of natural origin in general, from the preparations of chemical synthesis and makes it potentially promising for medicine.

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