DOI 10.1007/s10517-017-3934-4

Effect of *Acorus calamus L.* Polysaccharide on CD274 and CD326 Expression by Lewis Lung Carcinoma Cells in Mice

K. A. Lopatina[1,4], E. A. Safonova[1], K. V. Nevskaya[2], M. N. Stakheeva[3], A. M. Gur'ev[2], E. P. Zueva[1], T. G. Razina[1], E. N. Amosova[1], S. G. Krylov[1], and M. V. Belousov[2]

Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 164, No. 7, pp. 119-122, July, 2017
Original article submitted January 18, 2017

Tumor cells can maintain their growth via immunosuppression and escape from host antitumor immunity by controlling the PD-1/PD-L1 system. Expression of PD-L1 (CD274) is an inhibitory signal for T cells, while the increase in CD326 expression in the tumor tissue correlates with metastasis development. The experimental preparation on the basis of α(1,2)-L-rhamno-α(1,4)-D-galactopyranosyluronan from *Acorus calamus L.* produces an antitumor effect: it reduces tumor node size and the number and area of metastases after transplantation of Lewis lung carcinoma. Using flow cytometry, we demonstrated a decrease in the population of tumor cells expressing surface CD274 (PD-L1) and CD326 antigens after 20-day course of α(1,2)-L-rhamno-α(1,4)-D-galactopyranosyluronan.

Key Words: *polysaccharides; PD-L1; CD274; CD326; tumor*

The development of modern drug requires deep understanding of the fundamental molecular pathogenetic mechanisms of various diseases. The strategy aimed at reduction of the number of immunosuppressive cells in the tumor and normalization of antitumor immunity is promising for the therapy of tumors. It is known that tumor cells and microenvironment express CD274 (PD-L1) and CD326 (EpCAM) stronger than other tissues. Hyperexpression of PD-L1 (CD274) in the tumor correlates with metastasis development in the lymph nodes and is considered as one of the mechanisms of immunological tolerance. EpCAM is involved in cell signaling, migration, proliferation, and differentiation. High EpCAM expression in the tumor is an indicator of aggressiveness and high metastatic potential [9-11]. Some PD-L1 inhibitors are approved for the use in clinical practice [5] and detection and evaluation of CD326 expression in various tumors is the basis for the development of diagnostic criteria and predictions of tumor progression [11].

To date, preclinical testing of an injection form of a preparation on the basis of α(1,2)-L-rhamno-α(1,4)-D-galactopyranosyluronan from *Acorus calamus* L. is completed; this preparation increased the efficiency of chemotherapy. Experiments demonstrated that this drug produced an indirect antitumor effect due to lymph node cell activation. In addition, the polysaccharides of sweet flag rootstocks suppressed the development of the Th2-dependent response and in parallel stimulated Th1 reactions [1,7].

The aim of this study was the search for possible molecular targets of the immunomodulatory effect of sweet flag polysaccharides under conditions of tumor growth: analysis of the effect of α(1,2)-L-rhamno-α(1,4)-D-galactopyranosyluronan from *Acorus calamus* L. on Lewis lung carcinoma (LLC) cell population expressing surface CD274 (PD-L1) and CD326 antigens in C57Bl/6 mice.

[1]Laboratory of Oncopharmacology, E. D. Goldberg Research Institute of Pharmacology and Regenerative Medicine, Tomsk National Research Medical Center; [2]Department of Pharmaceutical Analysis, Centre for Introduction of Technologies, Siberian State Medical University, Ministry of Health of the Russian Federation; [3]Laboratory of Molecular Oncology and Immunology, Research Institute of Oncology, Tomsk National Research Medical Center; [4]Laboratory of Phytochemistry of Siberian Botanical Garden of National Research Tomsk State University, Tomsk, Russia. ***Address for correspondence:*** k.lopatina@pharmso.ru. K. A. Lopatina

MATERIALS AND METHODS

The experimental batch of formulation of α(1,2)-L-rhamno-α(1,4)-D-galactopyranosyluronan from the sweet flag rootstock (*Acorus calamus L.*) represented a 1% sterile isotonic solution for injections (hereinafter, the preparation) manufactured in the Center for Introduction of Technologies of National Research Tomsk State University (series 011212 for preclinical research). Isolation and standardization of α(1,2)-L-rhamno-α(1,4)-D-galactopyranosyluronan from the sweet flag rootstock was carried out using dynamic extraction, ethanol precipitation, ion exchange chromatography, filtration through semipermeable membrane, freeze drying, chromato-mass-spectrometry and MR spectroscopy [7].

The study was carried out on mature 2-3-month-old female C57Bl/6 mice (n=34) weighing 19-26 g (conventional 1st category animals; certificate of the Research Centre for Biomedical Technologies No. 188-05). The mice were kept according to regulations adopted by the European Convention for Protection of Vertebrates used for Experiments and Other Scientific Purposes (Strasbourg, 1986). Design of the experiments was approved by the Ethical Committee of E. D. Goldberg Research Institute of Pharmacology and Regenerative Medicine.

Transplantation of solid LLC was carried out using tumor tissue homogenate in sterile physiological solution. Donor animals were sacrificed by cervical dislocation, tumor specimens without necrotic areas were excised, squeezed through a shredder press, and injected to mice intramuscularly into the left hind paw (5×10^6 tumor cells in 0.1 ml; counted under a microscope).

The drug was administered daily in a dose of 50 mg/kg starting from day 2 of tumor development for 20 days. The dosage and drug administration route were selected in accordance with previous data demonstrating preparation efficacy in the dose range of 20-50 mg/kg and equivalence of intravenous and intraperitoneal administration for mice [1,4]. The control group received physiological saline in an equivalent volume according to the same scheme. On day 11, cytofluorometry was performed. The number and area of metastases in the lungs of animals were determined on days 11 and 21. On days 11, 14, 16, 18 and 21, the tumor node was measured.

Effectiveness criteria for the antitumor effect of the drug were following: tumor volume was calculated by the formula:

$$(a\times b^2)/2,$$

where a and b are tumor node length and width. The percent of tumor growth inhibition (TGI) was calculated by the formula:

$$(V_1-V_2)\times100/V_1,$$

where V_1 and V_2 are tumor volume in the control and experiment, respectively [6].

On day 11 of tumor development, the mice were sacrificed, the tumor capsule was sterilely opened, tumor specimens free from necrotic tissue were excised, mechanically minced, homogenized, and filtered through 70-μ nylon filters. The cells were washed with Hanks solution (Sigma-Aldrich) and brought to a concentration of 10^6/ml.

FITC anti-mouse CD326 (Ep-CAM) antibodies and PE/Cy7 anti-mouse CD274 (B7-H1, PD-L1) antibodies (BioLegend) were added to the prepared samples according to manufacturer's protocol. A separate cell suspension was prepared and treated with isotypic FITC Rat IgG2a and PE/Cy7 Rat IgG2b antibodies (BioLegend). The samples were incubated on ice in the darkness for 15-20 min. Then, 2 ml Hanks solution was added, the samples were centrifuged at 350g for 5 min, and the supernatant was removed. The procedure was repeated twice. After washing, the samples were resuspended in 1 ml BD FACSFlow Sheath Fluid (Becton Dickinson). Analysis was carried out on a FACSCanto II flow cytofluorimeter (Becton Dickinson).

For each parameter, the arithmetic mean and standard errors of the mean were calculated. The differences between the groups were verified using the nonparametric Mann—Whitney U test. The differences were considered significant at $p\leq0.05$. This significance test was selected due to small sample size (number of animals in the groups was 5-12) and experimental design (independent groups). In addition, small sample size limited the possibility of testing the hypothesis on normal data distribution, therefore, we used the nonparametric Mann—Whitney test for assessment of the differences between the means of the studied parameters between the groups [2,3].

RESULTS

On day 11 of LLC development, the tumor node reached visible size and constituted ~1000.9 mm^3 in the control group (Fig. 1). In the preparation-treated group, the tumor volume was lower (800.6 mm^3). At this term, number and area of metastases in the lungs were significantly lower (by 2.8 and 8 times, respectively). By day 14, TGI reached 25.1%, significant differences in the primary tumor volume persisted on day 16 and 18 (13.9 and 14.8% TGI respectively; Fig. 1, Table 1).

At the end of the experiment (on day 21 of LLC development), the tumor node volume significantly decreased by 15.9% in comparison with the control. At these terms, the number of metastases in the preparation-treated group was significantly lower by 1.3

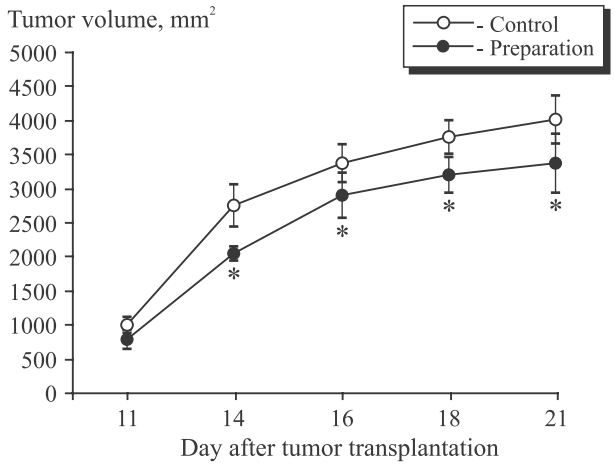

Fig. 1. Tumor growth dynamics in mice with LLC after course treatment with α(1,2)-L-rhamno-α(1,4)-D-galactopyranosyluronan from *Acorus calamus* L. *$p \le 0.05$ in comparison with the control.

times, and the area of metastases was by 1.4 times smaller than in the control (Table 1).

To elucidate possible mechanisms of these phenomena, cell subpopulations of the tumor node were analyzed. On day 12, the proportion of $CD326^+$ tumor cells in control LLC samples was 8.3% (8300.0±514.8 of $CD326^+$ cells). In the preparation-treated animals, this indicator was significantly lower — 7320.0±521.5 $CD326^+$ cells (Table 2).

PD-L1 (CD274) is expressed by tumor microenvironment cells, in particular by T and B cells, NK cells, dendritic and endothelial cells and monocytes. When PD-L1 binds to PD-1, T cells receive an inhibitory signal and their proliferation and cytokine production decrease [8-10,12]. According to our data, expression of the CD274 marker, tumor volume and metastasis-specifying parameters in non-treated animals were higher than those in the drug-treated animals. In the control, the number of cells expressing PD-L1 (CD274) in the tumor suspension was 28.5%. In the drug-treated animals, the population of $CD274^+$ cells was significantly smaller (by 1.4 times) than in the control (Table 2).

Tumor growth decrease and inhibition of metastasis growth in animals, treated with the sweet flag polysaccharide were followed by a decrease in the population of $CD326^+CD274^+$ cells in comparison with the control.

Thus, course administration of α(1,2)-L-rhamno-α(1,4)-D-galactopyranosyluronan from *Acorus calamus* L. in a dose of 50 mg/kg intraperitoneally produced antitumor and antimetastatic effects starting from day 11 after tumor transplantation, which was accompanied by a decrease in the number of $CD326^+$, $CD274^+$, and $CD326^+CD274^+$ cells in the tumor node.

TABLE 1. TGI and Metastasizing in C57Bl/6 Mice with LLC Treated Intraperitoneally with α(1,2)-L-Rhamno-α(1,4)-D-Galactopyranosyluronan from *Acorus calamus* L. (*n*=12; *M*±*m*)

Day after tumor transplantation	TGI, %	Number of metastases per mouse		Area of metastases, mm²	
		control	preparation	control	preparation
11	20.0	7.6±2.3	2.7±1.3*	2.4±1.8	0.3±0.7*
14	25.1	—	—	—	—
16	13.9				
18	14.8				
21	15.9	46.5±4.9	35.1±10.2*	124.4±21.3	90.2±13.3*

Note. *$p \le 0.01$ in comparison with the control, "—" not measured.

TABLE 2. Effect of α(1,2)-L-Rhamno-α(1,4)-D-Galactopyranosyluronan from *Acorus calamus* L. on Cell Subpopulations in LLC Tumor Node in C57Bl/6 Mice (*n*=5; *M*±*m*)

Group	Abs. cell number		
	$CD326^+$	$CD274^+$	$CD326^+CD274^+$
Control	8300.0±514.8	28520.0±1943.5	4030.0±816.7
Preparation	7320.0±521.5*	20540.0±1628.8*	3396.0±385.5*

Note. Total number of events in each sample was 100,000. *$p \le 0.05$ in comparison with the control.

REFERENCES

1. Zueva EP, Lopatina KA, Razina TG, Gur'ev AM. Polysaccharides in Oncology. Tomsk, 2010. Russian.
2. Lakin GF. Biometry. Moscow, 1990. Russian.
3. Lemeshko BYu, Lemeshko SB. Power and robustness of criteria used to verify the homogeneity of means. Measurement Techniques. 2008;51(9):950-959.
4. Lopatina KA, Razina TG, Zueva EP, Krylova SG, Guryev AM, Amosova EN, Rybalkina OY, Safonova EA, Efimova LA, Belousov MV. Preclinical studies of α(1,2)-L-RAMNO-α(1,4)-Dgalactopiranoziluronan from rhizomes Acorus calamus L. in cancer experiment. Sib. Onkol. Zh. 2015;(1):59-63. Russian.
5. Moiseenko VM, Volkov NM. The most important events in oncology in 2014: immunotherapy of malignant neoplasms. Prakt. Onkol. 2015;16(1):6-12. Russian.
6. Treshchalina EM, Zhukova OS, Gerasimova GK, Andronova NV, Garin AM. Methodical recommendations on preclinical evaluation of antitumor activity of drugs. Manual for Preclinical Studies of Drugs. Part I. Mironov AN, ed. Moscow, 2013. P. 642-656. Russian.
7. Belska NV, Guriev AM, Danilets MG, Trophimova ES, Uchasova EG, Ligatcheva AA, Belousov MV, Agaphonov VI, Golovchenko VG, Yusubov MS, Belsky YP. Water-soluble polysaccharide obtained from Acorus calamus L. classically activates macrophages and stimulates Th1 response. Int. Immunopharmacol. 2010;10(8):933-942.
8. Brahmer JR, Tykodi SS, Chow LQ, Hwu WJ, Topalian SL, Hwu P, Drake CG, Camacho LH, Kauh J, Odunsi K, Pitot HC, Hamid O, Bhatia S, Martins R, Eaton K, Chen S, Salay TM, Alaparthy S, Grosso JF, Korman AJ, Parker SM, Agrawal S, Goldberg SM, Pardoll DM, Gupta A, Wigginton JM. Safety and activity of anti-PD-L1 antibody in patients with advanced cancer. N. Engl. J. Med. 2012;366(26):2455-2465.
9. Fuse H, Tomihara K, Heshiki W, Yamazaki M, Akyu-Takei R, Tachinami H, Furukawa K, Sakurai K, Rouwan M, Noguchi M. Enhanced expression of PD-L1 in oral squamous cell carcinoma-derived CD11b+Gr-1+cells and its contribution to immunosuppressive activity. Oral Oncol. 2016;59:20-29.
10. Patel SP, Kurzrock R. PD-L1 Expression as a predictive biomarker in cancer immunotherapy. Mol. Cancer Ther. 2015;14(4):847-856.
11. Patriarca C, Macchi RM, Marschner AK, Mellstedt H. Epithelial cell adhesion molecule expression (CD326) in cancer: a short review. Cancer Treat. Rev. 2012;38(1):68-75.
12. Zou W, Wolchok JD, Chen L. PD-L1 (B7-H1) and PD-1 pathway blockade for cancer therapy: mechanisms, response biomarkers, and combinations. Sci. Transl. Med. 2016;8(328):328rv4. doi: 10.1126/scitranslmed.aad7118.