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ЭФФЕКТИВНОСТЬ ФОРМИРОВАНИЯ ПРОТИВООПУХОЛЕВЫХ ИММУННЫХ ОТВЕТОВ В СИСТЕМЕ ПРОФИЛАКТИЧЕСКОЙ ВАКЦИНАЦИИ МЫШЕЙ ТЕСТИКУЛЯРНЫМИ АНТИГЕНАМИ

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Резюме. Появление злокачественной опухоли ассоциируется с нарушением механизмов пролиферации, дифференцировки, способности к апоптозу. Этих изменений недостаточно для того, чтобы иммунная система распознала и уничтожила мутировавшие клетки. Причина этого явления – слабая иммуногенность опухолево-ассоциированных антигенов (ТАА). Противоопухолевая вакцинация является наиболее эффективным специфическим методом как профилактики рецидива заболевания, так и терапевтическим инструментом лечения в онкологии. Одним из главных условий эффективности противоопухолевой иммунотерапии является повышение иммуногенности опухолевых клеток. Иммунизация моно- или олиго-ТАА-производными пептидами не обеспечивает общее подавление развития опухоли и даже создает благоприятные условия для селективного роста отдельных клонов опухолевых клеток, не имеющих общих АГ с вакцинальными клетками. Ксеногенные АГ обладают высокой иммуногенностью и эффективны в разрушении иммунной толерантности к человеческим аналогам, представленным на опухолевых клетках. В нашей работе мы использовали тестикулярные АГ барана в качестве источника ксеногенных ТАА. Яички овец содержат большой набор ТАА. Экспериментальные мыши были иммунизированы липосомальной тестикулярной вакциной, полученной из яичек барана. Через месяц после вакцинации мышам подкожно имплантировали опухолевые клетки карциномы LLC. Обнаружено, что продолжительность жизни мышей опытной группы была в 2 раза выше по сравнению с сингенным контролем, при этом у 20% из них опухоль не развилась вообще. В спленоцитах мышей, у которых не было опухолей, определяли Т-регуляторные клетки и Т-клетки памяти. Мы обнаружили достоверное снижение как наивных Т-регуляторных ($CD4^+CD25^+$), так и активированных ($CD4^+CD25^+FoxP3^+$), а также Т-памяти ($CD4^+CD44^+$), в том числе популяцию центральных Т-памяти ($CD4^+CD44^+CD62L^+$) в селезенке предварительно иммунизированных мышей по сравнению с лимфоцитами мышей, полученных из интактной селезенки. Исследование содержания IFNγ и IL-10 в супернатантах мышиных спленоцитов, полученных от вакцинированных мышей без

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опухолей, показало достоверное снижение количества IL-10, но не IFNγ. На основании полученных результатов мы полагаем, что иммунизация ксеногенными опухолевыми АГ может привести к формированию эффективного противоопухолевого ответа, направленного на опухолеассоциированные АГ, имеющиеся на собственной опухоли.

Ключевые слова: противоопухолевые вакцины, карцинома легких Льюиса (LLC), опухолевые антигены, тестикулярные антигены, иммунотерапия, иммунный ответ

EFFICIENCY OF THE FORMATION OF ANTITUMOR IMMUNE RESPONSES IN THE SYSTEM OF PREVENTIVE VACCINATION OF MICE WITH TESTICULAR ANTIGENS

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Abstract. Appearance of a malignant tumor is associated with impaired mechanisms of proliferation, differentiation, apoptosis ability. However, these changes are not enough for immune system to recognize and destroy mutated cells. Weak immunogenicity of tumor-associated antigens (TAA) and the insufficiency of co-stimulating molecules on the surface of tumor cells is a reason for this phenomenon Since biochemical processes of tumor cells and healthy tissue cells are identical, therefore creation of effective chemotherapeutic drugs is limited not by selectivity of their action. So antitumor vaccination is the most effective specific method for both preventing recurrence of a disease and a therapeutic treatment tool in oncology. Xenogeneic proteins are highly immunogenic and effective in breaking immune tolerance to human analogs. In our work, we used sheep testicular AG as a source xenogenic TAAs. Sheep testicles contain a large set of TAAs. Experimental mice were immunized with type liposomal testicular vaccine from sheep, one month after vaccination, to induce tumor growth, cells of carcinoma LLC were implanted in mouse. The life expectancy of the experimental group of mice was 2 times higher compared to the syngenetic control and 20% of them did not develop the tumor at all. In the spleen of mice who did not have tumors after pre-vaccination sheep liposomal testicular AG, T-regulatory cells and T-memory cells were measured. We found a credible decrease in both naive Treg ($CD4^+CD25^+$), activated ($CD4^+CD25^+FoxP3^+$) and both T-memory ($CD4^+CD44^+$) and central memory ($CD4^+CD44^+CD62L^+$) in spleen pre-vaccination mice with compared to the contral intact spleen. Content of IFNγ and IL-10 in supernatants of mouse slenocytes derived from vaccinated mice with no tumors was investigated and showed a reliable decrease in the amount of IL-10, but not IFNγ. We believe that immunization with xenogenic tumor AGs can lead to the formation of a protective antitumor response.

Keywords: cancer vaccines, Lewis lung carcinoma (LLC), tumor-specific antigens, testis antigens, immunotherapy, immune response

Introduction

Conventional anti-cancer treatment is mainly based on surgical operation, radiological therapy and chemotherapy. During treatment, the chemotherapy-resistant cells survive and obviously acquire selective growth advantages over drug-susceptible cells. Accumulation of drug-resistant cancer cells over time usually converts into less efficient chemotherapy as compared to the previous treatment cycles. However, tumor cells can be differentiated from normal cells and carry surface immunogenic markers. The latter (tumor-associated antigens (TAA)) are targeted by the immune system [7]. TAAs include: oncogene products, mutated mucins, viral antigens, fetal antigens, and cell differentiated antigens. Activation of naive T-cells occurs due to TAA recognition coupled to MHC molecules, lymphocyte T-cell receptor and non-antigen-specific costimulatory signal. Co-stimulatory molecules such as B7-1/CD80, B7-2/CD86, CD40 are expressed on professional APCs, but are usually downmodulated on tumor cells. There are mechanisms for evading immune surveillance of malignant cells, which plays a key role in tumorigenesis. Thus, transformed cells survive and produce clones.

The immune system consists of both innate and adaptive arms to prevent oncogenesis.

Over the last few years, different types of cancer vaccines have been developed, including cell-based vaccines (i.e. DC-based Sipuleucel), protein/peptide vaccines, tumor cell-based vaccines, viral/bacterial-based vaccines and gene-based vaccines, including RNA and DNA vaccines [10]. However, clinical ap-

plication of cancer vaccines were not very effective due to the fact that most TAAS are presented by non-mutated weakly immunogenic proteins. Furthermore, immunizations with mono- or oligo-TAA-derived peptides often fail to control the overall tumor development, and even creates favorable conditions for selective growth of the particular tumor cell clones that lack vaccine Ags [4]. Therefore, compared to individual TAA-derived peptides, multi-antigen-based vaccine can elicit strong polyclonal immune responses to different TAAs. Autologous tumor cell vaccines are typically combined with an immunostimulatory adjuvant (e.g., *Bacillus Calmette-Gu rin*, BCG). Allogeneic tumor cell vaccines are typically prepared from two or three established human tumour cell lines [3]. Finally, to our knowledge, xenogeneic cell-based vaccines could constitute the most effective approach to stimulate antitumor immune responses in clinical cancer settings [11, 12]. This opinion is substantiated by observations that xenogeneic proteins are highly immunogenic and effective in breaking immune tolerance to human analogs. In addition, a wide variety of animal tumor cell lines are available, which could be exploited to design vaccines with maximal antigenic overlap with target tumors in order to elicit strong polyclonal tumor-specific immune responses.

Vaccination of mice with a TAA xenogeneic tumors induces a specific humoral and $CD8^+$T-cell-response against TAA tumors in mice [8]. Vaccination of tumor-bearing rats with antigens (CSH-275) from human colon adenocarcinoma showed prolonged survival time compared to control group. An effective antitumor immunity was also revealed in an experimental model of B-cell lymphoma using the xenogeneic cell vaccine "trioma". Immunotherapy of mouse solid tumors with xenogeneic cell vaccine consisting of endothelial cells has revealed a significant protection against tumor growth, regression of prevalent tumors and extended survival rate of tumor-bearing mice. Similar results were obtained while using a xenogeneic cell vaccine for the treatment of B16 melanoma in mice. The use of xenogeneic homologous proteins, in some mouse solid and hematopoietic tumors, mounts an effective protective and therapeutic antitumor immunity [14].

In our study, we used sheep testicular AG as a source of xenogenic TAAs. Sheep testicles contain a large set of TAAs [1, 2] and can be used to induce a prominent antitumor immune response.

Materials and methods

Experimental animals (mice)

The experiments were carried out on adult (4-5 months) specific pathogenic-free C57BL/6 (B6, H-2b) mice obtained from the nursery of laboratory animals "Rassvet", Siberian Branch of the Russian Academy of Medical Sciences (Tomsk). Animals (males weighing 15-20 grams) were housed per 10 mice/cage, under normal conditions, with a 12-hour light regime, drinking water ad libitum, on granulated diet.

Tumor cell line

Tumor cell line of murine Lewis lung carcinoma LLC was obtained from the Cancer Research Center of the Russian Academy of Medical Sciences (Moscow).

Vaccine preparation

The sheep testicles were homogenized to further isolate cells followed by two washouts with RPMI-1640 medium by centrifugation at 1500 rpm for 30 min. After the final washout, the cells were resuspended in physiological saline and to count cell number in hemocytometer. The viability of tumor cells usually ranged within 90-100% of the total cell number. The cell concentration was adjusted to 50 million/ml. To obtain a first and the second type liposome vaccine, the antigenic material was freeze-dried and placed in 50 ml vials at the required weight equivalents. The method for obtaining liposomal vaccine contains know-how approach.

Experiment model

Experimental mice were immunized with type liposomal testicular vaccine obtained from sheep testicles 3 times at weekly intervals. 3 groups of mice were randomized for different routes of administration as well as vaccine types: control (without vaccination), liposomal testicular vaccine (LTV) and syngeneic testicular vaccine (STV). Vaccines were dried, and diluted in 0.9% NaCl, in a total volume of 400 μl/mouse for inoculation.

One month after vaccination, of Lewis lung carcinoma LLC (200 thousand cells per mouse) cells were implanted under the skin of the anterior abdominal wall of mice to induce tumor growth. On days 7-9 after inoculation, tumor growth was noticeable in all animals.

Survival assessment

Mice (n = 10) inoculated with LLC Lewis lung carcinoma tumor cells were placed in cage after conducting a course of vaccination to assess host survival. The survival rate was 50% compared to the control group. The data were depicted in plots.

Measurement of serum cytokines

Concentration of IFNγ and IL-10 were assessed by ELISA in the mouse sera, which were stored at -180C until use. Enzyme-linked immunosorbent assay was performed on an ELISA processor "Berthold technologies Tristar LB 941'' at a wavelength of 450 nm. Assays were performed by using commercial ELISA kits (manufactured by Tonbo Biosciences, International, Inc.).

Assessment of cell subpopulations by flow cytometry

Percentage of splenic Treg and T-memory cell subsets was measured by staining with anti-CD4-FITC, CD8-FITC, CD44-PE, CD62-APC, CD4-APC, CD25-FITC, FOXP3-PE (BioLegend, BD Bioscience) anti-

bodies on BD FACSCanto II flow cytometer according to the manufacturer's instructions.

Statistical data analysis

Data (mean values added with appropriate standard deviations) were reproduced in at least 3 independent experiments. Statistical data processing was carried out by using the nonparametric Mann–Whitney test as well as Kaplan–Mayer criterion.

Results and discussion

The experiments were carried out as preventive vaccination. The mice were initially immunized by xenogeneic (relative to mice) liposomal testicular AG (the main experimental group) and mouse syngeneic testicular AG (syngeneic control) (see Materials and Methods). One month after vaccination, the mice were vaccinated with the LLC tumor, the control group of mice was not previously vaccinated, and only tumor cells were injected. Next, we assessed the duration of mouse lifespan that was found in 50% of control and syngeneic control mice was 20 and 20-25 days, respectively. The mice in the experimental group lived by 2-fold longer, about 41-43 days, and 20% developed no tumors (Figure 1). Splenocytes from tumorfree mice after LTV pre-vaccination were assessed for quantity of T-regulatory cells and T-memory cells (Table 1). We found markedly decreased percentage both of naive Treg ($CD4^+CD25^+$) and activated ($CD4^+CD25^+FoxP3^+$) in spleen of pre-vaccinated mice compared to intact animals: from 12.84±0,77 to 5.85±0.47 in the $CD4^+CD25^+$ population and from 0.95±0.17 to 0.33±0.07 among $CD4^+CD25^+FoxP3^+$ subset. Similar dynamics of such parameters were found in memory T-cells. We found a significant decrease in both splenic T-memory ($CD4^+CD44^+$) and central memory ($CD4^+CD44^+CD62L^+$) cells after pre-vaccination compared to intact mice: from 68.81±3.71 to 31.89±1.46 in the $CD4^+CD44^+$ population and from 4.35±0.59 to 0.7±0.06 in the $CD4^+CD25^+CD62L^+$ population.

In order to determine the importance of contribution by regulatory and memory T-cells in formation of anticancer immunity, we immunized intact mice with LTV and xenogeneic splenocytes. In the spleen of vaccinated mice T-regulatory cells and T-memory cells were measured.

We did not observe any prominent changes in the number of $CD4^+CD25^+$, $CD4^+CD25^+FoxP3^+$, $CD4^+CD44^+$, $CD4^+CD44^+CD62L^+$ while comparing control mice after vaccination with LTV and xenogeneic splenocytes.

Thus, we found no direct effect of vaccination on reducing number of regulatory and memory T-cells in mice. Level of IFNγ and IL-10 in supernatants of mouse slenocytes isolated from vaccinated tumor-free mice was investigated and showed a marked decrease in IL-10, but not IFNγ level (Table 2).

In our study, we showed that the formation of an effective anticancer response is associated with a significant reduction in the Treg-cell number in the spleen that was confirmed by numerous research data. Studies performed in several experimental models have demonstrated that Tregs depletion with

TABLE 1. CONTENTS OF TREG AND TEM IN SPLENOCYTES OF MICE IMMUNIZED WITH SHEEP LIPOSOMAL TESTICULAR VACCINES

Contents of Treg and Tem in splenocytes of mice immunized with Sheep Liposomal Testicular vaccines, which did not have tumors after implantation of LLC carcinoma (n = 10, p ≤ 0.01)				
	$CD4^+CD25^+$	$CD4^+CD25^+FoxP3^+$	$CD4^+CD44^+$	$CD4^+CD44^+CD62L^+$
1. Liposomal testicular AG	5.85±0.47	0.33±0.07	31.89±1.46	0.700±0.068
2. Intact group	12.84±0.77	0.95±0.17	68.81±3.71	4.35±0.59
Contents of Treg and Tmem in splenocytes of mice immunized with Liposomal Testicular vaccines				
	$CD4^+CD25^+$	$CD4^+CD25^+FoxP3^+$	$CD4^+CD44^+$	$CD4^+CD44^+CD62L^+$
1. Liposomal testicular AG	10.17±1.03	1.13±0.19	65.84±2.05	4.57±0.52
2. Sheep xenogeneic splenocytes	16.22±0.24	0.73±0.11	73.46±2.06	5.35±0.31
3. Intact group	12.84±0.77	0.95±0.17	68.81±3.71	4.35±0.59

TABLE 2. CONCENTRATION OF IFNγ AND IL-10 IN MOUSE SPLENOCYTE SUPERNATANTS

Concentration of IFNγ and IL-10 in mouse splenocyte supernatants in the prophylactic variant of immunization of mice with testicular AG of a ram with no tumor growth (n = 5)		
Cytokines	Testicular vaccine	Control group
IL-10	18.610±0.514**	25.90±2.41
IFNγ	141.70±39.34	205.70±28.08

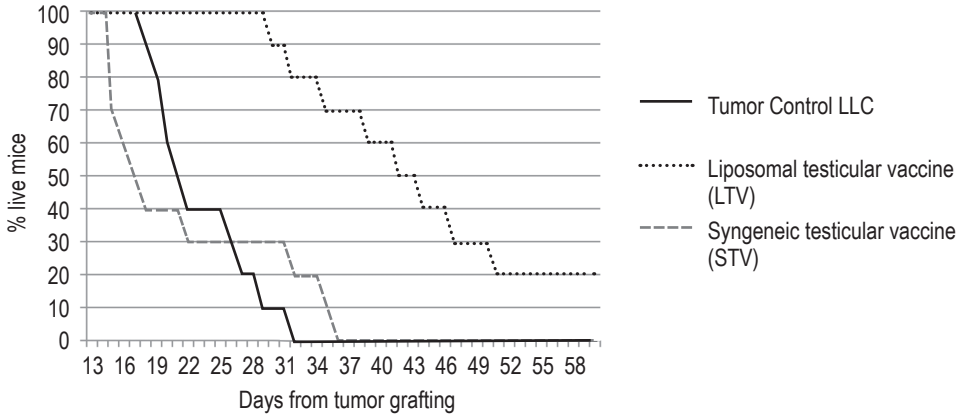

Figure 1. Survival of LLC tumor-bearing mice with prophylactic vaccination

anti-CD25 mAbs enhanced development of anti-tumour immunity followed by tumour rejection [6, 9]. Previous preclinical studies were shown to reduce Treg number and enhance Ag-specific immune responses by IL-2/diphtheria toxin fusion protein Denleukin diftitox [15]. Various studies reported that activated Tregs express high levels of immune checkpoint molecules, including CTLA-4 and PD-1 [5], thereby allowing to consider them as a promising target for Ab-based immune-therapeutics treatment. Indeed, anti-PD-1 mAb is currently used against different types of cancer (lung, colon, melanoma, renal cell carcinoma) due to its ability to reduce immunosuppressive Tregs activity [13].

Hence, our study showed that owing to xenogenic vaccination it is possible to achieve 100% efficiency in establishing anticancer reactions, manifested by massive inoculation of tumor cells to vaccinated mice resulting in no overt tumor disease.

Based on the data obtained, we believe that immunization with xenogenic tumor AGs can lead to formation of protective antitumor response targeting own tumor-associated AGs. In addition, significant changes were recorded in some immune parameters in tumor-free mice compared to immunized control animals. Further studies on immunity parameters are required, which will allow us to confidently determine significant immune links underlying formation of prominent antitumor protection.

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