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Dendritic cells transfected with a polyepitope DNA construct stimulate an antitumor cytotoxic response in various tumor diseases.

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Abstract

Dendritic cells (DCs) loaded with tumor-associated antigens (TAA) are known to be the important agents in antitumor response realization and still figure in lots of treatment schemes in cancer immunotherapy research. Here, we evaluated a cell-based protocol involving the use of original DNA constructs encoding the wide range of TAA epitopes expressed on different epithelial cancers. The constructs were transfected into ex-vivo-generated DCs of cancer patients (breast cancer, colorectal cancer, and non-small cell lung cancer). Direct cytotoxicity assay of effector cells, activated with the transfected DCs, showed a significant increase in the cytotoxicity against autologous tumor cells. The use of DNA-constructs encoding a large number of TAA's for the *in vitro* DC loading to activate the T cell response could be a reliable and unified approach for immunotherapy and relapse prevention in patients with epithelial cancers.

Keywords: Dendritic cells; Polyepitope DNA construct, Cytotoxicity; T cells, Epithelial cancers.

Declaration

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Conflicts of interest

The authors have no conflicts of interest to declare.

Availability of data and material

The datasets used are available from the corresponding author on reasonable request.

Code availability

Not applicable

Authors contributions

Conceptualization, S.S. and A.M.; formal analysis, V.K.; investigation, E.K., J.S., J.L., I.O., J.K., V.K.; project administration, S.S. and V.K.; resources A.K., N.K., V.K., S.S., A.S., A.V.; supervision S.S.; validation, E.K., J.S., J.L., I.O., J.K.; visualization, V.K., J.S., J.K., E.K. and I.O.; writing—original draft preparation, E.K. and V.K., M.K.; writing—review and editing, S.S., M.K. and V.K.; data curation, V.K.; funding acquisition, S.S. All authors have read and agreed to the published version of the manuscript.

Introduction

New technologies in cancer immunotherapy have made significant strides with the emergence of approaches such as blocking checkpoint molecules [16, 17] and genetically modifying effector cells [18, 19]. Nevertheless, overcoming the limitations appearing and reduction of the cost of such technologies is still to be done, and the study of all possible approaches to the induction of an immune response does not lose its relevance. One of the important aspects of efficient immune response formation is proper antigen presentation to enable endogenous antigen-specific cytotoxic T cell response [1, 2]. DCs play a key role in activation of the antitumor immunity [3]. The functional activity of DCs was shown to be significantly reduced in patients with cancers [14, 15]. In cancer, the ability of DCs to capture tumor antigens and present them to T cells, as well as to mount an effective cellular response, is impaired [4]. The main reason is considered to be the impairment of DC maturation process [3] as well as the T cell activation mechanisms [4].

Transferring the whole process of the endogenous immune response activation *ex vivo*, outside the immunosuppressive tumor influence, is shown to be feasible in obtaining T-cells efficient for tumor elimination when administered to patients as part of combination therapy [20, 13]. Herewith, a number of studies have confirmed the effectiveness of DC antigen loading using

DNA constructs [5-8]. In this regard, we are still interested in finding the right combination of these approaches to produce the optimized and unified protocol available for wide clinical use for immunotherapy in cancer patients.

Tumor-associated antigens are usually presented in a wide range of epithelial tumors, and each tumor can express a wide range of known antigens. Previously we showed the efficiency of in vitro generation antitumor immune response with the use of DCs transfected with DNA constructs encoding epitopes of particular tumor-associated antigen determinants [12, 16]. In the current study, we optimized the approach and tested the hypothesis about the efficiency of using genetic constructs encoding a wide range of TAA's for the activation of T cell cytotoxicity against autologous tumor cells from tumors of different localization

Materials and methods

The study object

Heparinized venous blood and tumor samples obtained from 9 patients with colorectal cancer (CRC), 13 patients with non-small cell lung cancer (NSCLC), and 18 breast cancer (BC) patients receiving treatment at the City Clinical Hospital No. 1 (Novosibirsk) and Novosibirsk Regional Oncology Center were used in the study. The inclusion criterion was no history of surgery, chemotherapy and/or radiation therapy. Adenocarcinomas of the large intestine, lung or breast were histologically verified in all patients in accordance with the pathology. Voluntary informed consent was obtained from all patients. This study was approved by the local Research Institute of Fundamental and Clinical Immunology (RIFCI) ethics committee.

All subjects gave their informed consent for inclusion before they participated in the study. The study was conducted in accordance with the Declaration of Helsinki, and the study was approved by the local Research Institute of Fundamental and Clinical Immunology (RIFCI) ethics committee.

Polyepitope DNA constructs

Original DNA vaccine constructs were developed using the corresponding artificial genes based on the pmax plasmid [7]. In particular, the following designs were created: The pmax-CTL1 construct containing epitopes from MAGE-A10, NY-ESO-1 and MUC-1; the pmax-CTL2

construct containing epitopes from MAGE-A3, PRAME, EpCAM and MUC-1; the pmax-CTL3 construct containing epitopes from EpCAM, CEA, GuanylylCyclase C and 5T4; the pmax-CTL4 construct containing epitopes from Legumain, VEGFR-1, VEGFR-2, FAP and Fos-related antigen-1; the pmax-CTL5 construct containing epitopes from Brachyury, SOX2, Snail1 and Snail2; the pmax-PolyTh construct containing epitopes from HER2, hTERT, p53, WT1, NY-ESO-1, VEGFR-2, survivin, and MAGE-A3. For DC transfection, a mixture of an equimolar amount of all DNA constructs was used, thereby providing a wide range of antigenic information.

Effective antigenic determinants recognized by cytotoxic T lymphocytes were analyzed using specialized software (more than 500 epitopes were used) [7]. Fragments containing epitopes capable of binding to the largest number of allomorphs of human MHC class II molecules (HLA-DR) were selected when designing the DNA constructs. The fact that amino acid residues flanking the epitope in the protein (the target antigen) can be important for interaction with the corresponding T cell receptor was taken into account during fragment selection. Prediction of cytotoxic T cell epitopes was carried out for the allelic variant of HLA-A*0201 class I molecules.

Generation of mature dendritic cells

Mononuclear cells (MNCs) were isolated from peripheral blood of patients with colorectal cancer, breast cancer, and NSCLC using the standard method of Ficoll-Urografin density gradient centrifugation ($\rho = 1.077$). The obtained MNCs were then incubated for 30 min in 5% CO₂ at 37°C in order to isolate cells with increased adhesion ability. Non-adherent MNCs were isolated with the medium, precipitated by centrifugation, and cultured in a 75 cm² culture flask (TPP, Switzerland) to be further used at a concentration of 2×10^6 cells/mL in complete RPMI-1640 medium containing 10% FCS (Hyclone, Austria), 2 mM *L*-glutamine (Biolot, St. Petersburg, Russia), 10 mM HEPES buffer (Sigma, USA), 5×10^{-4} M 2-mercaptoethanol (Sigma, USA), 40 µg/mL gentamicin (KRKA, Slovenia), and 200 U/mL benzylpenicillin (Biosintez, Penza, Russia) prior to seeding. The adherent fraction was cultured in a 48-well plate (Greinerbio-one, Germany) at a concentration of 1×10^6 cells/mL in 0.5 mL of complete RPMI-1640 medium supplemented with 50 ng/mL rhGM-CSF and 100 ng/mL rhIL-4 (Peprotech, USA) for 96 h to obtain immature DCs. In order to obtain mature DCs, rhTNF- α (25 ng/mL)

(PeproTech, USA) was added to the culture of immature DCs, and the cells were incubated for 24 h. In the resulting DCs, the expression of maturation and differentiation markers was assessed by flow cytometry using antibodies labeled with various fluorochromes (CD11c-PE-Cy7, CD83-APC, and HLA-DR-FITC) (BioLegend, USA).

Magnetic transfection and evaluation of the efficiency of dendritic cell transfection

Magnetic transfection of mature DCs was performed in a volume of 0.5 mL in a 48-well plate. Plasmids pmax (the control plasmid) and pmax-MQ/UB-CTL 1/8 (the target plasmid) were used for transfection. Magnetic transfection was carried out using Promokine reagents (Germany); the procedure was performed according to the manufacturer's protocol. Plasmids were dissolved in DMEM medium (the State Research Center of Virology and Biotechnology "Vector") in separate tubes; MATra-A reagent was added in the ratio of 0.3 µg of plasmid per 0.3 µl of reagent, and the mixture was incubated at room temperature for 20 min. In parallel, DCs were precipitated by centrifugation at 1,200 rpm for 5 min, and RPMI-1640 medium was replaced with 250 µl of DMEM medium. Next, the plasmid-MATra-A complex was added to the cells (25 µl per well); the plate was placed on a magnetic stand for 15 min. The medium was replaced after transfection: the DMEM medium was removed, and 300 µl of complete RPMI-1640 medium was added. Transfected cells were incubated overnight in 5% CO₂ at 37°C.

Co-culturing of dendritic cells and mononuclear cells

The obtained DCs transfected with pmax and pmax-MQ/UB-CTL 1/8 plasmids were co-cultured with the fraction of non-adherent MNCs (at a concentration of 1×10^6 cells/mL) for 120 hrs to prime specific antigens (at a 1:10 DCs : MNCs ratio). Non-adherent MNCs cultured under the same conditions, as well as cells cultured in the presence of DCs not transfected with plasmids (MNCs+DC(0) group), were used as the control.

Generation of autologous tumor cells

Tumor cells were obtained by cold trypsinization of the tumor samples obtained from the patients during planned surgical intervention. A tumor sample was divided into small fragments in RPMI-1640 medium containing 80 µg/mL gentamicin, 400 U/mL benzylpenicillin, and 5

μg/mL amphotericin B (PanEco, Moscow, Russia), placed in trypsin (Biolot, St. Petersburg, Russia), minced with scissors, and left overnight at +4°C. To inhibit trypsin, RPMI-1640 medium containing 10% FBS was added and mixed thoroughly; the homogeneous suspension was precipitated (1,000 rpm, 10 min), and the cell count was calculated in a Goryaev chamber. Cells were frozen in FCS with 10% DMSO and stored at -70°C. Cells were thawed and cultured in complete RPMI-1640 medium 24 hrs prior to the cytotoxicity test.

Evaluation of the cytotoxic activity of mononuclear cells against tumor cells

The cytotoxic activity was analyzed by assessing the level of lactate dehydrogenase (LDH) in a conditioned medium in the co-culture of the DCs + MNCs cell population and autologous tumor cells of colorectal cancer, breast cancer, and NSCLC. The procedure was carried out according to the kit manufacturer's instructions (Promega, USA). After co-culturing of the non-adherent fraction of MNCs and DCs transfected with plasmids, as well as culturing of the cells of the control groups for 120 hrs, the cell suspension was washed, and the resulting cells were seeded at a cell concentration of 1×10^6 cells/mL into round-bottom plates (TPP, Switzerland) (well volume, 50 μL) containing pre-thawed autologous tumor cells at a 10:1 ratio and incubated for 16–18 hrs. Cell seeding and the experimental protocol were carried out in accordance with the instructions for the “CytoTox 96 Non-Radioactive Cytotoxicity Assay” kit (Promega, USA). Optical density (OD) was measured on an Anthos 2020 spectrophotometer (Biochrom, UK) at a single wavelength (490 nm). The cytotoxic effect was calculated according to the formula proposed by the kit manufacturer and expressed as a percentage: % cytotoxicity = $((\text{OD}(\text{experimental lysis}) - \text{OD}(\text{spontaneous lysis of effector cells}) - \text{OD}(\text{spontaneous lysis of tumor cells})) / (\text{OD}(\text{maximum lysis of tumor cells}) - \text{OD}(\text{spontaneous lysis of tumor cells}))) \times 100\%$.

Statistical data analysis

Statistical data analysis was carried out using the GraphPad Prism software. Normality of the sample distribution was assessed using the Kolmogorov–Smirnov test. Non-parametric Friedman test was used for statistical verification in case of non-normal distribution, corrections for multiple comparisons were made. The differences were considered to be significant at error probability $p < 0.05$. Data are presented as median ± standard deviation. The number of

individuals per group is indicated by n in figure captions.

Results

Generation of mature dendritic cells and effector cells

Mature DCs of patients with colorectal cancer, breast cancer, and NSCLC with a more than 80% content of CD11c⁺HLA-DR⁺ cells (Fig. 1A) and a more than 40% content of CD83⁺ cells (Fig. 1B) were obtained *in vitro* using the protocol described above. The unified protocol for generation of DCs was used for CRC-, BC- and NSCLC-derived cells, and it was found to be effective for CRC- and BC-derived dendritic cell maturation. At the same time, in case of NSCLC-derived cells, absence of significant increase in the number of CD83⁺ cells was shown.

Fig. 1 The relative count of CD11c⁺HLA-DR⁺ cells in the monocyte-derived cell culture (A) and the relative count of CD83⁺ cells in the CD11c⁺HLA-DR⁺ monocyte-derived cell culture (B) from patients with colorectal cancer (n=9), breast cancer (n=18), and NSCLC (n=13); Immature DC – cell culture derived from patients' monocytes by culturing with IL-4 and GM-CSF for 4 days; mature DC – cell culture derived from patients' monocytes by culturing consistently with IL-4 and GM-CSF for 4 days and then with TNF- α for 2 days. The protocol for obtaining mature DCs included culturing with IL-4 (100 ng/mL) and GM-CSF (50 ng/mL) for 4 days, addition of a maturation stimulus TNF (25 ng/mL) followed by incubation for 2 days. Data are presented as the median $\pm$ standard deviation. Arrows indicate differences at $p < 0.05$.

The described cell-based protocol allows generation of mature DCs derived from peripheral blood monocytes of patients with colorectal cancer, breast cancer, and NSCLC. The resulting

DCs are characterized by high expression levels of the CD11c⁺HLA-DR⁺ and CD83⁺ markers.

The next stage involved magnetic transfection of the obtained DCs with an original polyepitope DNA construct. An average efficiency of the DNA transfection of DCs was about 70% (the Flow-FISH method).

After the transfected DCs had been co-cultured with the autologous MNCs obtained from cancer patients, final studies were conducted to evaluate the efficacy of the developed cell-based vaccine, which consisted of antigen-activated MNCs (effector cells) and mature DCs transfected with a polyepitope DNA construct. The efficiency of mounting of a specific cytotoxic immune response in the co-culture of MNCs and DCs was evaluated using the cytotoxicity assay against autologous tumor cells.

Evaluation of the cytotoxic activity of mononuclear cells against tumor cells

The LDH-releasing cytotoxicity assay showed that cytotoxicity of MNCs activated by DCs transfected with a polyepitopic DNA construct reached its maximum values (51.2%, 57.2%, and 67.4% for patients with BC, NSCLC, and CRC, respectively) when co-cultured with mature antigen-activated DCs transfected with the original DNA construct (Fig. 2A, B, C).

Fig. 2 Cytotoxic response in a co-culture of MNCs from breast cancer patients (n=18) (A), from patients with NSCLC (n=13) (B), from patients with colorectal cancer (n=9) (C) and

autologous DCs transfected with a DNA construct encoding the epitopes of tumor-associated antigens to autologous tumor cells (n=18). MNCs – mononuclear cells; MNCs+DCs(0) – mononuclear cells cultured in the presence of dendritic cells not transfected with a DNA construct; MNCs+DCs(C) – mononuclear cells cultured in the presence of dendritic cells transfected with the control plasmid; MNCs+DCs(T) – mononuclear cells cultured in the presence of dendritic cells transfected with a target plasmid (DNA construct). Data are presented as the median $\pm$ standard deviation. Arrows indicate statistically significant intergroup differences ($p \leq 0.05$).

Discussion

The existing DC-based therapeutic approaches are aimed at eliciting the antigen-specific antitumor immune response. For this purpose, various sources of antigenic substrates and/or methods of delivery to antigen-presenting cells (dendritic cells) are used. In the current study, we used DNA constructs encoding the epitopes of the most common tumor-associated antigens characteristic of epithelial cancers (in particular, CRC, BC, and NSCLC).

By using a unified protocol for generation of DCs with common dendritic cell maturation factors, we obtained cell cultures containing more than 80% of CD11c⁺HLA-DR⁺ cells and more than 55% CD83⁺ cells (except for DCs derived from NSCLC patients, which showed a lower CD83 expression level (47%)). The absence of significant increase in the relative number of CD83⁺ DCs in case of NSCLC-derived cells, for our opinion, characterizes the differences in immune response depending on the type of cancer: in particular, the ability of DCs to mature could differ in tumors of the same histological origin (epithelial cells) but located in different organs and thus having different cell and tissue microenvironments. A number of authors reported that NSCLC progression is characterized by severe immunosuppression and impaired ability to induce maturation of antigen-presenting cells (dendritic cells) [9, 10, 11]. The authors attribute immunosuppression in this type of cancer to the expression of immunosuppressive molecules (B7-H3, IL-10, and TGF- β) by DCs in the tumor microenvironment, which ultimately leads to suppression of the immune response [11]. For this reason, the sensitivity of DC precursors to growth factors is reduced in NSCLC and does not lead to a high expression level of maturation and co-stimulatory molecules.

Direct cytotoxicity assay against autologous tumor cells as target cells is proven to be an

objective approach for *in vitro* assessing the effectiveness of the immune response generated [8]. In our studies, high antitumor cytotoxic activity of effector cells after co-culturing with antigen-loaded DCs was assessed on day 5. The tumor cell death rate was more than 51%, significantly exceeding the average values for the control group (MNCs). It was shown that the DNA construct encoding the epitopes of the major tumor-associated antigens of various epithelial cancers can equally enhance the direct cytotoxic immune response against tumor cells of epithelial origin and various localization (in particular BC, CRC, and NSCLC). Previously, we used a similar DC transfection protocol, but using a DNA construct encoding HER2/neu epitopes in order to generate an antigen-specific immune response. The increased population of cytotoxic cells specific for HER2/neu epitopes was shown after co-culturing antigen-specific DCs derived from breast cancer patients with MNCs [12]. In terms of cytotoxicity generated, the results of current research showed comparable efficiency levels comparing with the HER2/neu protocol, thus we can state that the use of DNA constructs encoding the wide range of TAA epitopes can also be efficient in stimulation of antitumor T-cell response, but the wide range of TAA epitopes enables the use of such approach in treatment of more cancer types. We also previously tested a protocol for generation of antigen-primed DCs in breast cancer patients *in vivo* [13]. There we used autologous tumor cell lysate as an antigen source and showed the increase in the count of CD8⁺ cells, which exhibit strong antitumor cytotoxic activity [13]. In this regard, we see that autologous tumor lysate can also be a viable option for DC antigen loading, but here we should take into account that lysate requires the preparation of tumor material for every patient separately, and in lots of cases, tumor tissues are not available. At the same time, the use of DNA constructs enables the unification of antigen-loading protocol for different patients and even tumor types, and it could be a reliable solution in situations tumor material is not available.

Data availability

The datasets generated during the current study are available from the corresponding author on request.

Conflict of interests

The authors declare no competing interests.

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