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Effect of double-stranded DNA on maturation of dendritic cells *in vitro*

Ekaterina A. Alyamkina [a], Olga Y. Leplina [b], Ludmila V. Sakhno [b], Elena R. Chernykh [b], Alexandr A. Ostanin [b], Yaroslav R. Efremov [c], Alexandr G. Shilov [c], Anastasia S. Proskurina [c], Konstantin E. Orishchenko [c], Evgenia V. Dolgova [a], Vladimir A. Rogachev [c], Valeriy P. Nikolin [a,c], Nelly A. Popova [a,c], Stanislav N. Zagrebelniy [a], Sergey S. Bogachev [c,*], Mikhail A. Shurdov [d]

[a] Novosibirsk State University, Novosibirsk, Russia
[b] Institute of Clinical Immunology, Siberian Branch, Russian Academy of Medical Sciences, Novosibirsk, Russia
[c] Institute of Cytology and Genetics, Siberian Branch, Russian Academy of Sciences, Novosibirsk, Russia
[d] LLC Panagen, Gorno-Altaisk, Russia

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ABSTRACT

A preparation of human genomic fragmented double-stranded DNA (dsDNA) was used as maturation stimulus in cultures of human dendritic cells (DCs) generated in compliance with the interferon protocol. Culturing of the DCs in medium with 5 μg/ml of the DNA preparation was associated with a decrease in the relative proportion of CD14 + cells and an increase in that of CD83 + cells. These changes are markers of DC maturation. The efficiency with which the DNA preparation was able to elicit DC maturation was commensurate with that of lypopolysaccharide from bacterial cell, the standard inducer of DC maturation. Generated *ex vivo*, matured in the presence of the human DNA preparation, pulsed with tumor antigens mouse DCs were used as a vaccine in biological tests for its antitumor activity. The experimental results demonstrate that reinfusion of mature pulsed with tumor antigens DCs cause a statistically significant suppression of tumor graft growth.

1. Introduction

The progressive course of diseases, such as viral hepatitis, tuberculosis, malignancies, have been thought to be largely due to impairment of the immune system, to a defect of the antigen specific response, in particular. The specificities of DCs, their ability to present antigen and to activate T-lymphocytes allows to incriminate their functional impairment as a likely cause why immune defense mechanisms can fail [1].

DCs possess the unique ability to prime naive T-lymphocytes and to induce the immune response to various bacterial, viral, and tumor antigens. There is ample reason for considering DC-based vaccines as promising approaches to treatment of chronic infectious and oncological diseases [2,3]. Thus, clinical trials of these vaccines showed that they are well tolerated, and in 10–30% of patients with melanomas, prostate cancer, kidney cancer, lymphomas and other malignancies tumor regressed [4–8].

In basic and applied medicine, DCs are traditionally generated by culturing peripheral blood monocytes with GM-CSF and IL-4 for 5–7 days (immature DCs) followed by their stimulation to full maturation for 24–48 h, using different factors for this purpose (mature DCs) [9,10]. Semi-mature DCs (those that have not reached full maturity) can be generated faster by culturing blood monocytes in the presence of GM-CSF and IFN-α [11,12]. IL-4 → IFN-α substitution appears to be a more physiological system, because, as known, IFN-α is a member of the "first wave" cytokines, arising earliest in response to many pathogenic stimuli. Importantly, IFN-α induced DCs (IFN-DCs) in this case express markers of both myeloid and plasmacytoid cells; they are able to capture antigen; they migrate more rapidly owing to the expression of CC-chemokin receptor R7 (CCR7); they remain stable in the absence of cytokines and are capable of activating Th1-response [13–15]. Yet another advantage of IFN-DCs is that they produce IFN-α that has direct antiviral and antitumor activities, also stimulates cellular and humoral immunity [11].

Lypopolysaccharide from bacterial cell (LPS) is renowned as a powerful inducer of final DNA maturation. It should be stipulated that bacterial DNA is endowed with a broad spectrum of stimulatory actions on different populations of immunocompetent cells (T- and B-lymphocytes, natural killer cells, monocytes) [16–22]. Consequently, potential capacity of exogenous DNA to exert stimulatory influence on DC maturation and differentiation cannot be ruled out.

It is established that dsDNA, irrespective of origin, form, and nucleotide sequence, can activate cellular and humoral immune response. Moreover, this effect is accomplished independently of the

* Corresponding author. Address: 10 Lavrentieva ave, 630090, Novosibirsk, Russia.
E-mail address: gorbi@bionet.nsc.ru (S.S. Bogachev).

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TLR9 immunomodulation pathway. Various inducer forms of DNA were identified; it also became clearer how they might affect functional activity of immunocompetent cells. It also became known that dsDNA in the form of nucleosomes [23,24], purified mammalian, bacterial, and viral genomic DNAs [25,26], CpG-motif free dsDNA [26,27] cause maturation and enhance activities of DCs and macrophages. The associated changes have been well described as a characteristic activation of synthesis of co-stimulatory molecules (MHC I and II, CD40, CD54, CD86, CD83) [23,25,27–30], cytokines (INF α and β, TNF-α, IL-6, IL-8, IL-12p40/70) [23,25,26], nitroxide (NO) [30]. There is, at this juncture, no clear explanation how this activation proceeds. However, it has been suggested that it may be primarily due to the aberrant presence of the right-handed B-form dsDNA in the cell and that the activity of TANK-binding kinase-1, TBK1, may be crucial for it [31–33].

We have previously evaluated the ability of a preparation of human genomic fragmented dsDNA to induce allostimulatory activity of human DCs *ex vivo* [34]. It proved that this dsDNA preparation as a maturation stimulus caused an induction of allostimulatory activity of IFN-DCs, with the response being comparable or even stronger than the one LPS elicited. The reverse effects of the dsDNA preparation and LPS in certain cases are of interest. LPS was without marked effect, whereas the dsDNA preparation was inductive to a considerable allostimulatory activity of IFN-DCs and vice versa. This prompted us the idea that the effects of the maturation stimuli may be implemented via different pathways. This may have implications for development of more efficient DC vaccines because two or more additively active inducers would boost functional potentialities of DCs.

Here, we compare the effect of a preparation of exogenous DNA and LPS on IFN-DC phenotype in man and mouse. We also demonstrate that injections of an exogenously generated DC vaccine caused a considerable retardation of growth of grafted tumor in mice.

2. Methods

2.1. DNA preparation

Human DNA preparation was isolated from the placentas of healthy women using a phenol-free method. It was fragmented in an ultrasonic disintegrator at a frequency of 22 kHz to obtain a mixture of DNA fragments with a size 200–6000 bp. The human DNA was a pharmacopeian preparation «Panagen» (Registration certificate Medical Drugs of Russia No. 004429/08 of 09.06.2008). This preparation does not contain steroid hormones and RNA. It gives negative PCR results for hepatitis B virus DNA, hepatitis C virus RNA, HIV DNA, HIV RNA. The DNA preparation does not contain polysaccharides; it is also endotoxin-free.

2.2. Generation of human DCs

Mononuclear cells (MNCs) were obtained from heparinized venous blood in ficoll-verografin density gradient. DCs were obtained by culturing the adherent MNC fraction in 24-well plates (Nunc, Denmark) for 5 days in RPMI-1640 medium (Sigma–Aldrich, USA) supplemented with 0.3 mg/ml L-glutamine, 5 mM HEPES buffer, 100 μg/ml gentamicin, and 5% inactivated serum (blood group AB) in the presence of 1000 U/ml GM-CSF (Schering-Plough, Switzerland) and 1000 U/ml INF-α (Roche, Switzerland). LPS *Escherichia coli* (Sigma–Aldrich, USA) was used as specific maturation stimulus; it was added at a concentration 10 μg/ml 24 h before DC culturing ended. Human genomic DNA was added as specific maturation stimuli at 1, 5, or 25 μg/ml 24 h before termination of DC culturing. DCs generated without maturation stimulus served as negative control.

Phenotypes of DCs were analyzed on a FACSCalibur flow cytometer (BD Biosciences) using FITC or PE-labeled monoclonal antibodies – CD83, CD14, CD25 (BD Biosciences).

2.3. Animals

Three-month old CBA/Lac mice that were bred at the animal facility of the Institute of Cytology and Genetics (the Siberian Branch of the Russian Academy of Sciences) were used in experiments. Mice in groups of 10 were housed in plastic cages. They had free access to food and water. All experiments were performed in accordance with protocols approved by the Animal Care and Use Committee of the Institute of Cytology and Genetics.

2.4. Generation of mouse DCs

Bone marrow derived mononuclear cells were prepared from CBA/Lac mice in ficoll-verografin density gradient (1088 g/ml). Mononuclear cells were cultured in the presence of GM-CSF (80 ng/ml) and IL-4 (20 ng/ml) (BD Biosciences) for 6 days. Maturation inducers, either DNA (25 μg/ml) or TNF-α (20 ng/ml), were added on day 6 of culturing. Cells were primed at the same time with specific antigen, Ehrlich's tumor homogenate.

The extent to which cells were mature was analyzed on day 7. Monoclonal antibodies to surface maturity markers CD34-PE-Cy7 (Santa Cruz), CD80-FITC (BD Biosciences), and CD86-APC (BD Biosciences) were used for this purpose. The obtained results were analyzed on a flow cytometer FACSAria (BD Biosciences).

2.5. Mouse immunization

Generated DCs, matured in the presence of antigen, were injected subcutaneously to mice ($n = 5$). Immunization protocol was 10^6 cells, first immunization, 10^5 cells, second and third immunizations. Each immunization was done 14 days after the preceding. 10^5 cells of Ehrlich's tumor were grafted 14 days after the last mouse immunization. Mice ($n = 9$) not subjected to pretreatment, with Ehrlich's tumor grafted also in the amount 10^5 cells, served as controls.

3. Results

3.1. Effect of human genomic fragmented dsDNA on maturation of human DCs

Our previous study showed that IFN-DCs generated from blood monocytes from healthy donors is distinguished by its heterogeneity obviously due to relative proportion of non-mature, semi-mature and mature DCs [35]. This variability was manifested not only at the phenotypic but also at the functional (allostimulatory activity in mixed lymphocyte culture) levels. Our study of tuberculosis patients demonstrated a characteristic differentiation retardation of IFN-DCs observed as a decrease in the relative proportion of mature CD83 + DCs, also as an attenuation of allostimulatory activity in mixed lymphocyte culture [36,37]. The DC dysfunction we established in tuberculosis patients may have resulted from altered properties of the blood monocytes from which DCs were generated *in vitro*. In contrast to monocytes from healthy donors, those from tuberculosis patients were characterized by a weaker expression of CD86 and HLA-DR, a twofold increase in subpopulation of CD14+/CD16+, FasL+, and IL-10 + monocytes, also by a rise in the production of IL-10 and IL-6 in response to stimulation with endotoxin [38,39].

Our data and those of [40–42] clearly indicate that the functional (allostimulatory) activity of DC vaccines and, hence, their