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The hemagglutinin–neuraminidase gene of Newcastle Disease Virus: A powerful molecular adjuvant for DNA anti-tumor vaccination

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ABSTRACT

Plasmid-encoded DNA vaccine is a novel and potentially powerful tool for cancer therapy. Since the strength of immune responses induced by DNA vaccine is usually rather low, a major goal in DNA vaccine development is to enhance vaccine-induced immunity. In this study, we investigated an approach based on the use of a viral surface protein with pleiotropic function as a potential immune enhancer. To this end, we prepared bicistronic DNA plasmids encoding the hemagglutinin–neuraminidase (HN) protein of Newcastle Disease Virus in addition to a tumor target antigen. We demonstrate a higher tumor antigen-specific T cell-mediated immune response and a lower humoral response upon vaccination with a bicistronic DNA plasmid with incorporated HN gene. In a prophylactic immunization tumor model with the surrogate tumor antigen beta-galactosidase (β-gal) and in a therapeutic immunization tumor model with the xenogeneic tumor antigen human Epithelial Cell Adhesion Molecule (hEpCAM), HN gene incorporation into the DNA vaccine led to better survival and tumor regression in mice. There was also cross protection in the therapeutic tumor model against a second challenge by the parental mouse mammary carcinoma cells in mice vaccinated with the bicistronic plasmids.

This is the first report describing the HN protein as an immunomodulator for enhanced antigen-specific T cell responses via DNA plasmids. The results show that co-expression of HN with a tumor target antigen through bicistronic vectors ensures precise temporal and spatial co-delivery to direct anti-tumor immune responses preferentially towards Th1.

1. Introduction

Anti-cancer vaccination aims at inducing or augmentation of anti-tumor immune responses with the final goal to eradicate tumor cells and to induce long-term immunological memory allowing long-term immune surveillance against emerging cancer cells [1]. Strong immune responses towards tumor associated antigens (TAAs) are mandatory since many of them represent overexpressed self-antigens shared by cancer and normal cells [2] and are thus poor immunogens due to immune tolerance mechanisms. Plasmid DNA (pDNA) encoding an immune target antigen has raised much interest in the field of vaccinology. This represents a simple and elegant approach for inducing endogenous synthesis of the encoded target protein and inducing efficient CD4 and CD8 T-lymphocyte responses. Compared to viral vectors, DNA plasmids raise less severe regulatory and manufacturing problems [3,4]. They are less immunogenic by themselves and no problems exist with pre-existing or induced neutralizing antibodies. However, the actual levels of gene expression by DNA vectors are much lower than those achieved with viral vectors. Previous studies have shown that DNA vaccines are capable of eliciting cell-mediated immunity and protective immune responses in mice [5–7]. However, results of clinical studies revealed a relatively low potency of DNA vaccines to induce strong immune responses in large animals and in humans. To improve the efficacy of such vaccines, various strategies have been developed by trying to improve gene delivery [8,9], to optimize administration routes [10–12] or to enhance immunogenicity of the antigen encoded by the pDNA [13] by adding adjuvant signals for the induction of adaptive immune responses. One approach to enhance both the magnitude and duration of the induced immune responses to DNA-encoded antigens is to insert genes coding for molecular adjuvants within the expression vector [14,15]. The nature of an adjuvant is very important since it can determine the particular type of immune response, which may be skewed towards Th1 and cytotoxic T cell (CTL) responses or Th2 and

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antibody responses [16]. Cytokines (such as IL-12 and IL-2) and co-stimulatory molecules (such as B7-1 and B7-2) have all been used to strengthen Th1 T cell-mediated immune responses against the expressed antigens [17].

In the present study, we included a viral protein as new possible genetic adjuvant in DNA vaccines to modulate the immune response induced against the encoded specific target antigen. The viral protein evaluated here is derived from Newcastle Disease Virus (NDV) which is an avian paramyxovirus with a single-strand RNA of negative polarity. This genome codes mainly for six genes in the order (from 3′ to 5′) of nucleoprotein (NP), phosphoprotein (P), matrix protein (M), fusion protein (F), hemagglutinin–neuraminidase (HN) and large polymerase protein (L) (3′-NP-P-M-F-HN-L-5′) [18]. The reason we selected a gene from NDV is that this virus has very interesting anti-neoplastic and immune stimulatory properties [19,20]. Promising results were obtained from clinical studies which were performed in different tumor entities in which we used autologous irradiated tumor cells modified by infection with NDV as vaccines [21,22]. In addition, this virus has a long history demonstrating a high safety profile in humans. One of the surface proteins of this virus, the HN is a 74-kDa membrane type II glycoprotein which protrudes from the viral envelope or from the membrane of infected cells and expresses on its extracellular domain both hemagglutinin (HA) and neuraminidase (NA) activity. This protein plays a key role in mediating the attachment of the virus to host cell receptors containing terminal sialic acids. Due to its agglutinating properties, this molecule has been shown to play a role in facilitating the contact and attachment between infected cells and other cells. To investigate the possibility of using HN as an adjuvant for anti-tumor DNA vaccination, we inserted two genes coding for a target antigen and for the HN protein into a bicistronic vector. We then assessed the efficacy of such constructs to vaccinate against the encoded antigen. As tumor targets, we selected two different foreign antigens with different cellular localizations: one cytoplasmic (bacterial beta-galactosidase (β-gal)) and one transmembrane protein (the human Epithelial Cell Adhesion Molecule (hEpCAM)). The β-gal is a bacterial protein which is coded by the lacZ gene and can be used as a surrogate tumor antigen. The hEpCAM molecule, also known as GA733-2, KSA, 17-1A Ag, is a cell surface glycoprotein expressed on some normal and over-expressed on many neoplastic epithelial cells as, for example, colorectal carcinomas [23,24]. It was shown to be a suitable target for active immunotherapy against carcinomas. The results to be shown demonstrate that a cellular immune response against a target antigen induced by a DNA vaccine can be significantly improved by co-immunization with the HN gene of NDV. A bicistronic plasmid vector co-expressing a tumor antigen and the HN protein appears as a promising tool to modulate the polarity of immune responses against TAAs and to enhance, in prophylactic and therapeutic settings, the efficacy of DNA vaccines against tumors.

2. Materials and methods

2.1. DNA expression vectors

The plasmid pCMV-lacZ (pCMV SPORT-βgal) was purchased from Invitrogen (Karlsruhe, Germany). The bicistronic vector pTandem1 with an IRES sequence has been obtained from Novagen, Darmstadt, Germany. The HN sequence was generated by PCR (Primer Up – GCTCAGCCCCTTATGGCCAGCTGGCAGCGTAAG and Primer Down – GTTTAAACAACATGGACCGCGCAGTTAGCC) from pBK-SFV-HN [25] and cloned into pTandem1-HN. The IRES-HN sequence was generated by PCR (Primer Up – CCGGGATCCACTAGTGGTTATTTTCCACC and Primer Down – CGGCATGCGTCGACTATGGCCAGCTGGCAGCGTAAG) from pTandem1-HN and cloned into pCMV-lacZ to construct pCMV-lacZ-IRES-HN. pSPORT vector was cloned from pCMV-lacZ by cutting out the lacZ sequence and re-ligation of the plasmid backbone. It is used as the control pVector for lacZ gene based DNA immunization. A plasmid pSPORT6-EpCAM encoding 1.5-kb of the human EpCAM gene (extracellular and transmembrane domain) was kindly provided by Dr. Frank Momburg (DKFZ, German Cancer Research Center, Heidelberg, Germany). The hEpCAM gene was enzyme-digested from pSPORT6-EpCAM and cloned into pTandem1 to construct pCMV-hEpCAM. This sequence was cloned into pTandem1-HN to construct pCMV-hEpCAM-IRES-HN. The pTandem1 plasmid was used as a control pVector for hEpCAM gene based DNA immunization. *Escherichia coli* strain Top10 was transformed with each plasmid and then grown in Luria-Bertani medium (Difco, Detroit, USA). Large-scale of the plasmids was prepared by Endo-free Qiagen plasmid-Giga kits (Qiagen, Hilden, Germany) according to the manufacturer's instructions. DNA was then suspended in sterile Endo-free water and stored in aliquots at −20 °C for subsequent use in immunization. For *in vivo* injections, the DNA was diluted and adjusted to 0.5 mg/mL or 1 mg/mL in Endo-free PBS (PromoCell, Heidelberg, Germany) before use.

2.2. Cell lines and transfection

The different cell lines used in this study were obtained from the tumor cell bank of the German Cancer Research Center (DKFZ, Heidelberg, Germany). They were cultured at 37 °C and 5% CO_2. All cell culture media were supplemented with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, 10 mM HEPES, 100 U/ml penicillin and 100 μg/mL streptomycin (all purchased from Gibco Invitrogen, Karlsruhe, Germany). Baby hamster kidney (BHK) cells (BHK21; American Type Culture Collection, Rockville, MD, USA) were maintained in modified eagle medium containing 0.1 mM non-essential amino acids, 1.0 mM sodium pyruvate and other supplements (as indicated above). The mastocytoma cell line P815 and its lacZ-transfected variant P13.1 were cultivated in RPMI-1640 medium with supplements (as indicated above) and β-mercaptoethanol at a final concentration of 50 μM. ESb and the β-gal-expressing transfectoma (ESb-lacZ) were maintained in DMEM with supplements. The medium for lacZ-transfected cells was supplemented with 200 μg/mL G418 to maintain stable lacZ gene expression. DA3, a mammary carcinoma cell line, and DA3-EpCAM (DA3 transfected with human EpCAM gene) were cultivated in RPMI-1640 medium complemented with supplements (as indicated above) and with β-mercaptoethanol (50 μM). The purified plasmids were transfected into BHK21 cells using the jetPEI reagent according to the manufacturer's instructions (Polyplus, Illkirch, France).

2.3. Antibodies and flow cytometry

The following antibodies: HN.B (specific for the HN protein of NDV, produced from hybridoma cell lines kindly provided by R. Iorio (Department of Molecular Genetics and Microbiology, University of Massachusetts Medical School, Worcester, USA)), HEA125 (specific for hEpCAM, kindly provided by Dr Gerhard Moldenhauer, DKFZ, Heidelberg, Germany) and the second antibody PE labeled goat anti-mouse IgM+G+A (SouthernBiotech, Eching, Germany) were used in this study. Propidium iodide (PI, 1 μg/mL) was added 10 min before FACS acquisition to exclude dead cells. Viable cells (5×10^4 to 5×10^5) were acquired with the CellQuest software on a FACSCalibur (BD Biosciences, Heidelberg, Germany). FlowJo software (Tree Star, San Carlon, CA) was used to analyze FACS data.