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Research paper

Protective efficacy of a plasmid DNA vaccine against transgene-specific tumors by Th1 cellular immune responses after intradermal injection

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ABSTRACT

With DNA vaccines, it is important to monitor the movement of transfectants and to overcome immune deviations. We used a pCMV-LacZ plasmid (expressing β-galactosidase) and a pcDNA-hNIS plasmid (expressing the human sodium/iodide symporter [hNIS] gene) as non-secreted visual-imaging markers. Transfectants carrying the hNIS or LacZ gene migrated to peripheral lymphoid tissues. hNIS-expressing cells were observed specifically in the LNs and spleen. Anti-β-galactosidase was detected in LacZ DNA immunized mice after boosting twice, suggestive of Th2 humoral immune responses. Antibody isotyping defined the humoral immune response. A dominant IgG2a type occurred in hNIS-immunized mice in ELISAs. IgG2a/IgG1 ratios increased after hNIS DNA vaccination. High levels of INF-γ–secreting cells were identified in ELISpot and increased IFN-γ levels were found in cytokine ELISAs. Tumor growth decreased in hNIS DNA-immunized mice. In conclusion, humoral immune responses switched to the Th1 cellular immune response, even though we administered plasmid DNA by intra dermal injection.

1. Introduction

DNA vaccines can induce both cellular and humoral immunity and have been considered as an attractive immunization strategy to protect the host against various infections, cancers, and autoimmune diseases [1–3]. As with other reporter genes, the LacZ gene has been studied in the context of long-term imaging for gene therapy experiments [4–6]. When a DNA vaccine is injected into a target site and internalized by antigen presenting cells (APCs) without being degraded, strong immune responses can result in the host [7]. The fate of DNA vaccines may be evaluated by determining their effective distributions or expression levels in the host following injection [8]. Indeed, following injection with DNA vaccines encoding β-galactosidase (LacZ) revealed detectable LacZ expression at the injection site and in other organs. Such biodistribution analysis has predominantly been confirmed after sacrificing experimental animals and conducting gene-expression analysis in their organs with molecular approaches, such as *in situ* hybridization, immunohistochemistry, or reverse transcriptase-polymerase chain reaction (RT-PCR) experiments. However, *in vivo* real-time monitoring of gene expression is required to study gene expression following DNA vaccination in living animals.

Successful vaccination using DNA encoding the human sodium/iodide symporter (hNIS) gene would be beneficial for 2 reasons, i.e., targeted immunotherapy against NIS-expressing cancer cells and as an innovative and objective tool for evaluating the efficacy of a vaccine using scintigraphic imaging [7,9–12]. Therefore, we evaluated the use of an *in vivo*-imaging method based on the optical imaging reporter gene, hNIS. The hNIS protein emits γ-rays in the presence of

Abbreviations: Th, T helper; i.d., intradermal; i.p., intraperitoneal; s.c., subcutaneous; Ig, immunoglobulin; ELISA, enzyme linked sorbent assay; ELISpot, enzyme-linked immunospot; INF, interferon; IL, interleukin; DNA, deoxyribonucleic acid; RNA, ribonucleic acid; mRNA, messenger RNA; APC, antigen presenting cell; DC, dendritic cell; CTL, cytotoxic T lymphocytes; hNIS, human sodium/iodide symporter; MHC, major histocompatibility complex; GFP, green fluorescence protein; CD, cluster of differentiation; FBS, fetal bovine serum; RPMI 1640, Roswell Park Memorial Institute 1640; DMEM, Dulbecco's Modified Eagle's medium; PBS, phosphate buffered saline; HBSS, Hank's balanced salt solution; TE, Tris-ethylenediaminetetraacetic acid buffer; dLNs, draining lymph nodes; MLN, mesenteric LNs, PCR, polymerase chain reaction; RT-PCR, reverse transcriptase-polymerase chain reaction; HRP, horseradish peroxidase; TMB, tetramethylbenzidine; BCIP/NBT, 5-bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium; CPM, counter per minute; EGFP, enhanced green fluorescence protein; ROI, regions of interest

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Technetium-99 m (Tc99m). Several gene-expression imaging methods developed for detecting injected 'reporter' genes in animals and humans require the use of tissues to be obtained after death. However, methods employing hNIS as a reporter gene in conjunction with ^{124}I, ^{125}I, ^{131}I, or Tc99m have enabled the visualization of various biochemical processes in the tissues of living subjects [7].

We choose LacZ and NIS as reporter genes to trace immune responses following DNA immunization because neither of the encoded proteins is secreted from cells. LacZ is a cytoplasmic protein and NIS is a transmembrane protein. These proteins can be loaded onto the major histocompatibility complex (MHC) class I after translation, in addition to MHC class II after APC engulfment. Hence, these 2 genes together can stimulate both Th1 and Th2 immune responses *in vivo* and skew immune deviation [13].

Modulating the tumor microenvironment is imperative for success in cancer immunotherapy [14]. However, due to the complexity of the tumor microenvironment, immunotherapeutic approaches may not be successful. To overcome these impediments, many groups have evaluated the hNIS protein as a specialized active iodide transporter [15,16]. Expression of the NIS protein causes cancer cells to accumulate therapeutic radionucleotides; thus, NIS gene transfer offers a possible radionucleotide gene therapy [17,18]. Hence, the phenotypic modulation of cancer cells and the subsequent modification of antitumor immunity by NIS radioiodine gene therapy have not been adequately evaluated.

In this study, we aimed to induce Th1 responses after intradermal (i.d.) injection of plasmid DNA, which predominantly induces humoral responses. We also monitored the distribution and duration of hind leg LacZ or NIS gene expression after i.d. administration of a naked DNA vaccine in the upper dorsum [19,20], using an *in vivo* imaging system. We used the NIS or LacZ plasmids to detect immune responses to either transgene as a model for testing the capacity of inducing specific Th1 immune responses in the context of a dominant pre-existing Th2 immune profile. We used non-secreted transgenes to exclude the uptake of secretory proteins. We found that anti-LacZ humoral immune responses and specific anti-hNIS Th1 immune responses were induced by repeated immunizations to skew immune deviation [13]. In conclusion, our data showed that i.d. DNA injection, which predominantly results in Th2 responses, could induce Th1 cellular immune responses after repeated i.d. injections of DNA expressing intracellular transgenes.

2. Materials and methods

2.1. Plasmid DNA vectors

The hNIS-expressing vector, pcDNA3.1-FL-hNIS (pcDNA-hNIS), was kindly provided by Dr. S. Jhiang (Ohio State University, OH, USA) and β-galactosidase-expressing pCMVβ and green fluorescence protein (GFP)-expressing pEGFP vectors were purchased from Clontech (CA, USA). hNIS gene expression was controlled using the cytomegalovirus (CMV) promoter, and expression of the neomycin-resistance gene was driven by the simian virus 40 promoter. Plasmids were amplified in *Escherichia coli* DH5α bacteria, and large-scale plasmid preparations were performed using endotoxin-free Giga Prep columns (Qiagen, CA, USA).

2.2. In vitro expression of vectors

2.2.1. Detecting expression of the pEGFP plasmid in CT26 cells

Six-well tissue culture plates were used for the transfection experiments. The day before transfection, CT26 cells growing in 75-cm^2 flasks were trypsinized, and 10% was added to 18 ml Medium 199 (Hyclone, USA) supplemented with 10% fetal bovine serum (FBS); 3 ml each of this cell suspension was seeded into individual wells of a 6-well plate. The Transfast™ reagent (Promega, USA) was used to transfect the cells, according to the manufacturer's instructions. Briefly, 2 ml of Transfast™ reagent was mixed with 1 mg of pEGFP plasmid, incubated at room temperature for 15 min, and added to the cells. Six hours later, the DNA–transfection reagent mixture was replaced with Medium 199. At 48 h post-transfection, fluorescence was observed using a FACSAria flow cytometer (BD Bioscience, San Jose, CA, USA).

2.2.2. β-gal staining for CT26 cells after transfection

LacZ DNA transfections were performed as described above for the pEGFP plasmid transfections. After transfection, CT26 cells were washed 3 times for 5 min at room temperature. The cells were then fixed (2% formaldehyde, 0.2% glutaraldehyde in PBS, pH 7.6–7.8) for 5 min at room temperature, rinsed with PBS, mixed with substrate solution (1 mg/ml X-Gal-substrate, Sigma–Aldrich, Deisenhofen, Germany), and incubation at 37 °C for 6 h. The transfection efficiency was determined visually.

2.2.3. Detecting expression of the NIS plasmid in CT26 cells

NIS DNA transfections were performed as described above for the pEGFP plasmid transfections. Following transfection, the ability of cells to internalize 99mTc or ^{131}I was determined as previously described [14]. CT26 cells (5×10^4) were plated in 24-well plates and cultured with Dulbecco's Modified Eagle's medium (DMEM) containing 10% FBS for 24 h. ^{125}I uptake was determined by incubating cells with 500 μl of Hank's balanced salt solution (HBSS; Gibco BRL. Co.) containing 3.7 kBq of carrier-free ^{125}I and 10 μM sodium iodide (NaI) at 37 °C for 30 min to yield a specific activity of 740 MBq/mmol (20 mCi/mmol). Following incubation, the cells were quickly washed twice with HBSS and detached using 500 μl of trypsin. Radioactivity was measured using a gamma counter (Cobra II, Packard; Perkin Elmer).

2.3. Immunization

Specific pathogen-free 6-week-old female BALB/c mice were obtained from Japan SLC, Inc (Japan, Shizuoka Prefecture) and handled under specific pathogen-free conditions according to the guidelines issued by the Seoul National University Animal Research Committee. To monitor *in vivo* plasmid trafficking, mice were injected i.d. into the thigh with 100 μg of pCMV-LacZ or pcDNA-hNIS suspended in endotoxin free Tris-ethylenediaminetetraacetic acid buffer (50 μl; TE; Qiagen), using a 30-G insulin syringe (Becton Dickinson, NJ, USA). To identify tumor-protective or antigen (Ag)-specific cellular immune responses, mice were immunized 3 times at 2-week intervals in the hind leg with hNIS DNA or in the dorsal skin with LacZ DNA. The mice were anesthetized by intraperitoneal (i.p.) injection with 0.3 ml of a 1:1:9 solution of rompun, (Parke Davis, Germany), ketamine (Bayer, Germany), and saline (RKS).

2.4. PCR amplification of the LacZ and hNIS plasmids

PCR primers were designed to amplify the LacZ and NIS transgenes. LacZ gene was detected in the dorsal skin. The distributions of the LacZ and hNIS genes were detected in various organs (such as the draining lymph nodes (dLNs), non-dLNs, spleen, muscles, liver, and heart) by PCR using the following gene-specific primers:

LacZ (forward: 5′-TTCACTGGCCGTCGTTTTACAACGTCGTGA-3′ and reverse: 5′-ATGTGAGCGAGTAACAACCCGTCGGATTCT-3′) and NIS (forward: 5′-AGATGAGCTGACACGGAACAG-3′ and reverse: 5′-CTGGG GAAAAGTGGGAAAAAGAG-3′). LacZ and NIS DNA levels were normalized to the β-actin gene, which was amplified using a forward (5′-CTGTGCTATCCCTGTACGCC-3′) and reverse (5′-ATGTGACAGCTCC CCACACA-3′) primer. DNA was amplified in 50-μl reactions containing 5 μl of PCR buffer, 50 nM of each deoxynucleotide, 5 nM each of forward and reverse primers, and 1 U Taq DNA polymerase. Standard PCR conditions comprised 94 °C denaturation for 5 min, followed by 35 cycles of denaturation at 94 °C for 45 s, annealing at 63 °C for 45 s, and extension at 72 °C for 45 s. The final extension step was performed at