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ORIGINAL ARTICLE

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Long-term tumor growth suppression in mice immunized with naked DNA of the human tumor antigen mucin (MUC1)

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Abstract Naked DNA is an attractive tool for vaccination studies. We have studied naked DNA vaccination against the human tumor antigen mucin, encoded by the gene *MUC1*. C57/BL6 mice were immunized twice, on day 1 and day 10, with plasmid pCI-MUC1, intramuscularly. Five days after the last immunization tumor challenge experiments were performed using the tumor cell line MC38, expressing human MUC1. In 85% of mice immunized with the mucin plasmid tumor growth inhibition was observed, whereas control mice developed tumors. Re-tumor challenge after three months revealed no tumor growth in mice immunized with the mucin plasmid. These encouraging results, showing long-term protection against tumor growth, indicate the potential usefulness of naked DNA vaccination for clinical immunotherapy.

Keywords Naked DNA · Mucin (MUC1) · Vaccine · Tumor re-challenge

Introduction

Currently, several vaccination approaches for tumor therapy are being investigated [24]. Some groups have used dendritic cells (DC) – the most potent antigen-presenting cells – as vaccines and have shown that DC pulsed with immunogenic peptides, proteins, tumor cell lysates or tumor cell-dendritic cell hybrids can induce primary T cell responses [21, 5, 15]. However, such cellular-based vaccines have some limitations. In particular, as they have to be manufactured for every patient individually, they are both cost- and work-intensive. However, for vaccination in clinical trials, methods that are easy to handle are preferred, so there is a need for the generation of simple, novel vaccines. The use of naked DNA as a vaccine would circumvent such limitations, but it is only applicable if the antigen is known and encoded by cDNA [6]. Recently, naked DNA has been used as a vaccine against viral diseases [23], e.g. against HIV [16], hepatitis B [26] and measles [7], or against malaria [19]. These studies have been successful in animal models, but the detailed mechanisms have not been fully clarified so far. Furthermore, the optimal way of application and boosting has not yet been investigated. Another important aspect is how long the immune response is maintained. Compared to infectious diseases, tumor applications of naked DNA have only rarely been studied. It has been used to immunize against the idiotypic determinants of B cell neoplasia [1], and against epitopes of the carcinoembryonic antigen (CEA) [25].

Several human tumor-associated antigens recognized by cytotoxic T cells (CTL) have been identified during the last couple of years [2]. CEA [3] and mucin [8] are examples of such tumor-associated antigens. Several vaccine approaches using CEA, expressed preferentially on colon carcinoma cells, have been performed [4].

In this study, we used mucin, encoded by the gene *MUC1*, as a tumor antigen expressed on human breast, pancreatic, colon and ovarian cancer cells. CTL against mucin can be detected in cancer patients [14]. The use of mucin-based vaccines in cancer therapy is currently intensely studied [20], but it is still unclear which approach is efficient. Here, we wanted to investigate if vaccination using naked mucin cDNA as a vaccine leads to long-term tumor growth inhibition in a mouse model system.

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Material and methods

Vector construction

The cDNA of human MUC1, containing 22 tandem repeat sequences from pDKOF (kindly provided by O.J. Finn, Pittsburgh)

and the complete cDNA of the human CEA, were cloned into the multiple cloning site of the plasmid pCI (Promega). DNA was prepared using JET star Maxi Prep columns and verified by restriction analysis.

Expression of the recombinant proteins on transiently transfected 293 cells was demonstrated by FACS analysis and Western blot analysis, using BC-2 (Medac) and D14-HD11 [13] monoclonal antibodies.

Cell lines

The murine colon adenocarcinoma cell line MC38 and its derivatives, MC38-CEA [18] and MC38-MUC1, were a kind gift from Dr. J. Schlom, NIH, USA. Both clones have been developed by stable transduction of MC38 cells with retroviral vectors encoding the cDNA of CEA or MUC1, respectively. These cell lines were maintained in DMEM with 10% fetal bovine serum, 2 mM glutamine, 100 U/ml penicillin and 100 μg streptomycin (Gibco/BRL).

FACS analysis

The expression of the tumor antigens mucin and CEA on MC38-MUC1 or MC38-CEA, respectively, was verified by FACScan analysis (Becton Dickinson, Bedford, Mass., USA). Cells were stained with the mAb (anti-MUC1) BC2 and the mAb (anti-CEA) D14-HD11 according to standard protocols. CEA-expressing MC38 cells or transfected 293 cells were used as negative controls to determine MUC1 expression and vice versa.

Immunoblotting

Cells were harvested 24 h after transfection. Equal aliquots were electrophoresed on a 7.5% sodium dodecyl sulfate gel. After electroblotting onto nitrocellulose and blocking (TBST containing 5% FCS and 3% dried milk), membranes were stained via incubation with BC-2 (1:60) or D14-HD11 (1:250) at 4 °C overnight and then a secondary antibody (goat anti-mouse alkaline-phosphatase-conjugated), according to the manufacturer's instructions. Molecular masses were determined by comparing with protein standards (BioRad).

Immunization and tumor challenge in mice

C57/BL6 mice were purchased from Charles River Wiga GmbH (Sulzfeld, Germany). They were held in accordance with the guidelines of the German Animal Protection Law and with permission of the responsible authority. Mice were immunized intramuscularly into the hind leg with 50 μg DNA, encoding either MUC1 or CEA, in PBS on day 1 and 10. In the mucin group, 11 of the 17 mice were co-injected with 50 ng recombinant murine granulocyte macrophage colony-stimulating factor (rmGMCSF) (PromoCell), along with the plasmid. Five days after the last immunization, 10^5 tumor cells (MC38-MUC1 or MC38) or 10^6 tumor cells (MC38-CEA) were injected subcutaneously into the right flank. A dose of 10^5 cells (MC38, MC38-MUC1) or 10^6 cells (MC38-CEA) was chosen since in previous studies all mice in these groups ($n = 5$) developed palpable tumors at the site of injection within approximately 10 days. Tumor diameters were measured twice per week with a caliper and tumor volumes were calculated according to $(A \times B^2)/2$, where A represents the largest and B the smallest diameter. Mice were sacrificed when the tumor volume exceeded 10% of body weight (1 cm^3). Appropriate controls of mice vaccinated with PBS, or equivalent amounts of mock-DNA (pCI vector), or mice challenged with MUC1- or CEA-negative tumors, respectively, were performed as described in Figs. 2, 3, and 4. Protected mice of the pCI-MUC1-immunized group were re-challenged with 10^5 MC38-MUC1 cells after 90 days.

ELISA

To detect antibodies specific for mucin, 96-well plates (NUNC Maxisorb) were coated overnight at 4 °C with 10 μg/μl of a 60mer synthetic peptide [$(PDTRPAPGSTAPPAHGVTSA)_3$, Biosyntan GmbH, Berlin] in carbonate buffer pH 9.6. The plates where washed 5 times with PBS containing 0.05% Tween 20 and blocked with PBS containing 0.05%Tween 20 and 5% FCS for 1 h. Serial dilutions of mouse sera from tail bleeds (taken on day 19 after tumor challenge from mice challenged with MUC1-negative tumors) were added per well and incubated at room temperature for 1 h. Antibody BC2 was used as a positive control. After washing the plates 5 times, total immunoglobulin was detected using polyclonal peroxidase-coupled horse-anti-mouse Ig. Plates were developed with o-phenylenediamine according to standard conditions and absorbance was measured at 405 nm in an ELISA reader.

Cytotoxicity

Splenocytes of mice immunized with pCI-MUC1 or PBS (not challenged with tumor cells) and killed 7 days after the final vaccination were cultured for 5 days in the presence of human IL2 (40 U/ml) and antigen (2 μg/ml 60mer MUC1 peptide or irradiated MC38-MUC1 cells) and a standard 4-h chromium-release assay was performed. MC38-MUC1 and MC38 target cells were labeled for 60 min with 80μCi ^{51}Cr in 0.5 ml RPMI at 37 °C, 5% CO_2.

Results

Mucin and CEA expression after transfection of 293 cells

Both MUC1 and CEA were expressed on the cell surface of 293 cells after transient transfection with pCI-MUC1 or pCI-CEA, respectively (Fig. 1A). In a Western blot mucin appears as several bands, ranging from 120 to >200 kDa (Fig. 1B). It has been previously shown that the lower molecular weight range refers to early intermediate non-glycosylated forms of mucin produced during biosynthesis, the lower band refers to the protein after cleavage of a 20-amino-acids signal peptide. The fully glycosylated protein shows an apparent mass of >200 kDa [12]. Since both CEA and MUC1 are extensively glycosylated, the same phenomenon is likely to be responsible for the broad lane that represents the CEA molecule, ranging from ~85 to >200 kDa (Fig. 1B).

Tumor protection induced by pCI-MUC1 DNA immunization

After inoculation of MC38-MUC1 tumor cells, no tumor growth was observed in 85% ($n = 11$) of mucin immunized mice, even 150 days after tumor challenge (Fig. 2) ($P < 0.001$). Co-injection of rmGM-CSF did not influence the obtained survival rates. In mice immunized with the CEA plasmid, only 2 out of 6 mice developed a tumor after injection of CEA-expressing tumor cells (Fig. 3) ($P = 0.0195$). In the negative control groups (mice immunized with mock-DNA or PBS) all animals developed tumors and were sacrificed when the tumor volume reached 1 cm^3, after 28–40 days. Re-challenge of

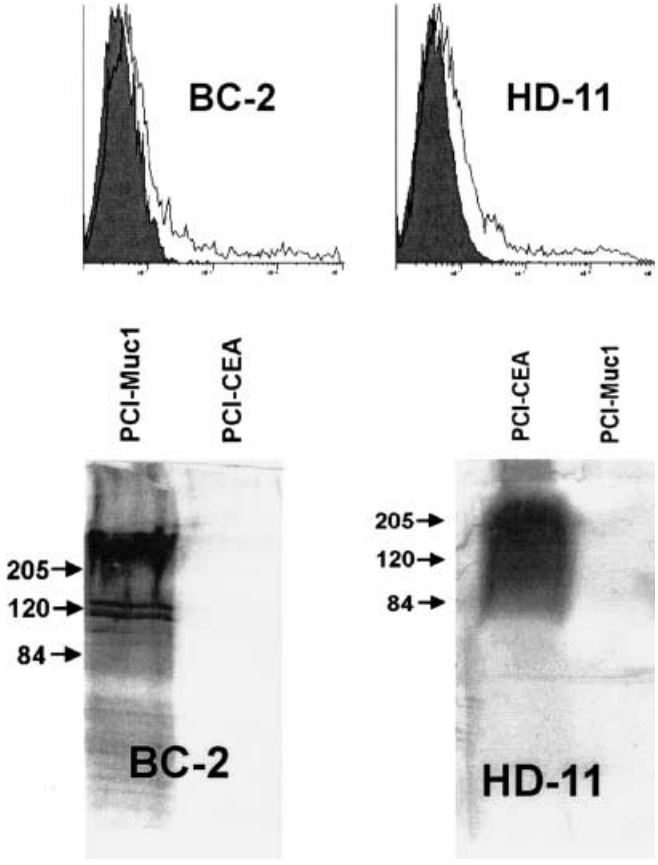


Fig. 1 (**A**) FACS analysis and (**B**) Western blot analysis of 293 cells transiently transfected with pCI-MUC1 (*left*) or pCI-CEA (*right*). As a mock control, equal amounts of pCI-CEA-transfected cells were used for BC-2 (anti-MUC1) staining or pCI-MUC1-transfected cells for HD11 (anti-CEA) staining, respectively

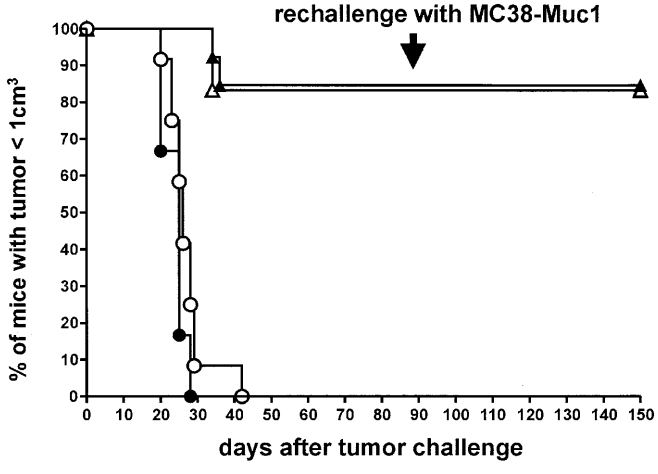


Fig. 2 Survival of mice after immunization and challenge with 10^5 MC38-MUC1 tumor cells. Groups of C57/BL6 mice were immunized intramuscularly with 50 µg of pCI-MUC1 (*open triangles*, $n=6$), pCI-MUC1 plus 50 ng of rmGMCSF (*closed triangles*, $n=11$), pCI-CEA (*closed circles*, $n=6$) or PBS (*open circles*, $n=11$). Tumor re-challenge was performed with MC38-MUC1 cells on day 90. Mice where sacrificed when the tumor volume reached 1 cm^3

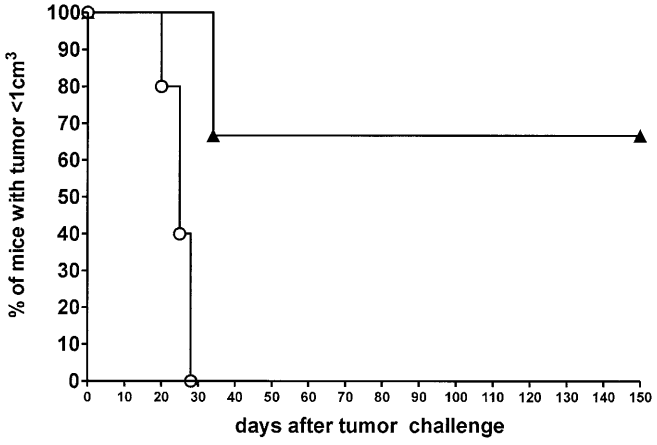


Fig. 3 Survival of mice after immunization and challenge with 10^6 MC38-CEA tumor cells. Mice where vaccinated with pCI-CEA (*closed triangles*, $n=6$) or PBS (*open circles*, $n=5$) and sacrificed when tumor volume reached 1 cm^3

six protected mice from the mucin group with the same number of tumor cells after 3 months did not lead to any tumor growth (Fig. 2). To further define the specificity of the protection, mice immunized with pCI-MUC1 or mock-DNA were challenged with either wild-type or mucin-expressing MC38 cells (Fig. 4). Vaccination with pCI-MUC1-protected mice from MC38-MUC1 tumor growth, but did not protect mice from growth of mucin-negative MC38 tumors. Immunization using mock-DNA (pCI) did not protect mice from MC38-MUC1 tumor growth.

Mucin specific immune responses

Without in vitro re-stimulation, splenocytes isolated from mucin-immunized mice (not challenged with tumor

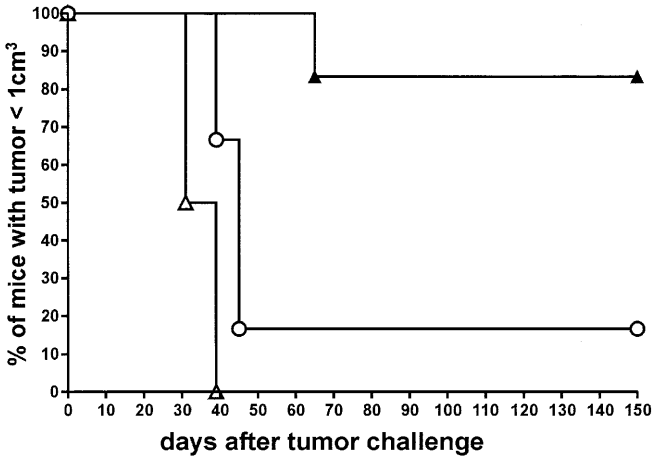


Fig. 4 Survival of mice ($n=6$) vaccinated with pCI-MUC1 and challenged with either 10^5 MC38-MUC1 cells (*closed triangles*) or 10^5 mucin-negative MC38 cells (*open triangles*). A third group of mice was vaccinated with pCI (mock-DNA) and challenged with 10^5 MC38-MUC1 cells (*open circles*). Mice were sacrificed when tumor volume reached 1 cm^3

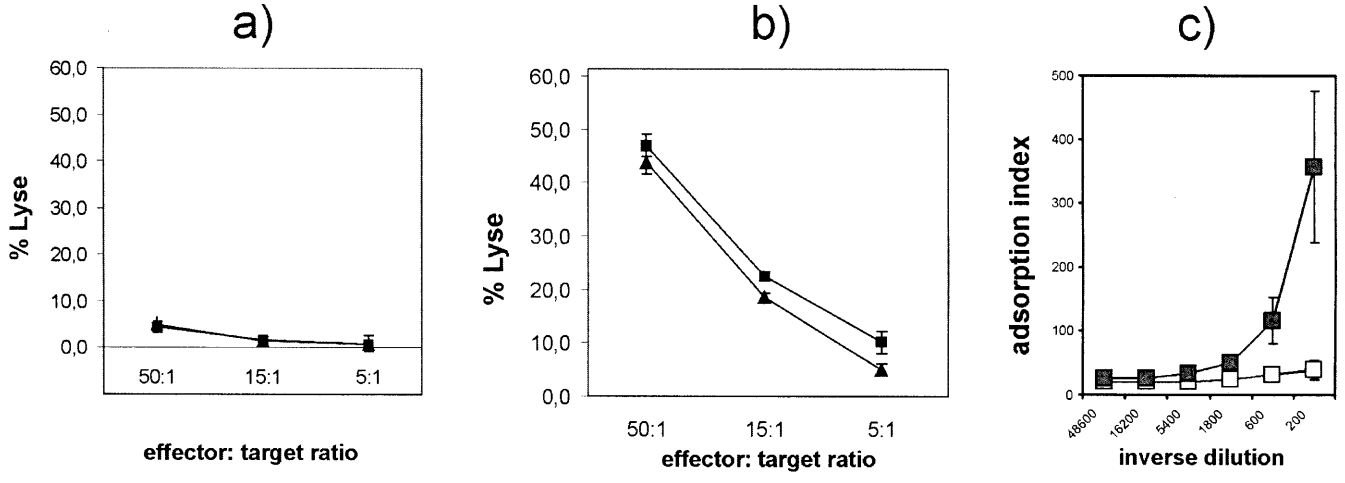
cells) 7 days after the final vaccination were not able to lyse MC38-MUC1 (Fig. 5A). After 5-day re-stimulation with synthetic tandem repeat 60mer, lysis was detectable, but was not significantly specific for mucin (Fig. 5B). Non-mucin-expressing MC38 cells were lysed at a similar range (Fig. 5A, B). The DNA-vaccination did induce detectable, but low, antibody titers against epitopes located within the tandem repeats of the mucin molecule, as shown by ELISA (Fig. 5C). Humoral responses where evaluated in mice immunized with pCI-MUC1 and challenged with mucin-negative tumors, since the injection of MC38-MUC1 tumor cells by itself already induced a similar antibody response against mucin.

Discussion

We present a simple vaccination protocol that confers protection against MUC1-positive tumors. Recently, different mucin vaccines based on dendritic cells have been evaluated. For example, Gong et al. [10] have successfully used dendritic cells infected with an adenovirus that encodes the *MUC1* cDNA in mice as a vaccine. Furthermore, they have induced an immune response using fusions of mucin-expressing human autologous or allogeneic dendritic cells in vitro [9]. Cellular-based vaccines are both cost- and work-intensive, since they have to be manufactured for every patient individually. Non-cellular-based vaccines could overcome these limitations. Naked DNA is one example of a non-cellular-based vaccine [17]. There is only one previous study using naked mucin cDNA as a vaccine. Graham et al. [11] developed a *MUC1*-based vaccine that conferred protection in 78% of all experimental groups and 43% protection in control groups after three applications of 100 μg DNA encoding a *MUC1* cDNA in Balb/C mice. These promising results were achieved for 60 days [11]. In comparison, we achieved 85% protection from mucin-expressing tumors with only two injections of 50 μg plasmid. All mock-vaccinated mice developed tumors, with the exception of one experiment, in which 5 out of 6 mice developed tumors. The better outcome of the pCI-based DNA vaccine may be due to features of the vector, differences in the tumor model or the capacity of different mouse strains to present mucin epitopes. Since there was no information about the duration of the anti-tumor response after vaccination, we wanted to evaluate in this study for how long such an immunization protects from tumor challenge. Therefore, we performed a tumor re-challenge after 90 days. Interestingly, we could show that this re-challenge did not result in tumor growth in any of the protected mice until day 150. This tumor protection may correlate with the presence of CTL.

However, we were not able to detect CTL specific for mucin without re-stimulation. A 5-day in vitro re-stimulation in the presence of synthetic mucin tandem repeat peptide and IL-2 did not selectively lead to the expansion of mucin-specific CTL, because mucin-negative MC38 tumor cells were lysed to a similar degree. The specificity of the protection was verified by performing a tumor challenge of mucin-immunized mice with MC38-MUC1 cells or mucin negative MC38 cells. Vaccination with naked DNA (pCI-MUC1) protected mice specifically from MC38-MUC1 tumor growth, but not from mucin-negative MC38 tumors. Antibodies binding the tandem repeat peptide used for re-stimulation could be detected, however, at low titers. There is evidence that additional application of rmGMCSF could enhance the immune response [22]. However, without rmGMCSF an almost complete tumor-growth inhibition was achieved already, and we were not able to detect any further improvement with GMCSF.

Fig. 5 Cytotoxicity assay and humoral response in mice vaccinated with pCI-MUC1 (not challenged with MUC1 expressing tumor cells). Lysis of MC38-MUC1 (*closed squares*) or MC38 target cells (*closed triangles*) by splenocytes from mice sacrificed 7 days after the final vaccination prior to (**A**) and after (**B**) in vitro re-stimulation with the 60mer mucin peptide. **C** ELISA assay to determine antibodies directed against mucin tandem repeat epitopes in pCI-MUC1-vaccinated mice (*closed squares*) and in mock-vaccinated mice (*open squares*). The data represent mean values of 6 individually tested sera



It remains to be determined whether the pCI-based naked DNA vaccine can compete with virus- or dendritic cell-based vaccination strategies in a mouse model, where MUC1 is a self-antigen and tolerance has to be overcome. The data presented suggest an immunotherapeutic strategy for long-term tumor protection using naked-DNA vaccination.

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