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Construction of a triple modified p53 containing DNA vaccine to enhance processing and presentation of the p53 antigen

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ABSTRACT

More effective and less toxic treatments are urgently needed in the treatment of patients with cancer. The tumour suppressor protein p53 is a tumour-associated antigen that could serve that purpose when applied in an immunologic approval to cancer. It is mutated in ~50% of the tumours resulting in p53 *overexpression*, which can serve as target for therapy. To improve the immunisation results in patients with p53 overexpression tumours we constructed a DNA vaccine that could lead to improved processing and presentation of p53 peptides in the MHC-class I. We constructed a triple modified p53 fusion protein containing DNA vaccine by (1) addition of a xeno-antigen (mouse or rat p53 fragment), (2) potentiation of intra-cytoplasmatic accumulation of p53 by deleting the nuclear signalling part, (3) improving the processing to peptides of p53 by addition of ubiquitin. *In-vitro* experiments confirmed correct construction of the DNA vaccine. Preliminary testing in normal and HLA-A2 mice of this triple modified p53 containing DNA construct meant for human application showed a trend towards a superior immunogenicity.

1. Introduction

For many decades active immune therapy has been considered to have enough promise as a treatment of established cancer, to warrant fundamental and clinical studies into its applicability. Although many of these clinical studies show some tumour responses in some patients, the overall results have underscored the fundamental problems of immune therapy rather than adding to its being given a place in the oncological armamentarium.

Central in the concept of active immune therapy is the choice of an antigen. The window of activity of any vaccine is determined by tolerance and tumour specificity and it has become clear that tumour antigens that are unmodified in structure or way of presentation, are unlikely to be effective. Among the many antigens that have been investigated for active immune therapy the p53 antigen stands out as an interesting example. If mutated antigens are used absolute tumour specificity can be obtained, but even with wild type p53 some specificity is possible. This is caused by the abundance of the long-lived mutated protein in the cytoplasm of most tumour cells as compared to the nuclear localisation of the short-lived wild type protein in normal cells. In addition to being expressed in over 50% of tumours, this expression will take place throughout the cell cycle of individual cancer cells. The probability that a window of opportunity exists is supported by experimental and clinical observations: Preclinical studies using mouse models and an *ex-vivo* human culture model have shown that induction of an anti-p53 CTL response selectively killed tumour cells and spared normal cells [1–9].

Of importance is also the observation that no absolute tolerance to p53 is maintained in humans as both p53-specific T-cells and antibodies have been shown to be present in cancer patients [1–3]. To improve the immunisation results in patients with p53 over expression tumours, we constructed a DNA vaccine that should lead to the expression of a considerably modified p53 protein. Goal of the modification was ultimately enhancement of presentation of p53 peptides in the MHC-class I. This was to be brought about by directing the protein towards the cytoplasm rather than the nucleus. Once trapped in the cytoplasm processing by the proteasome system could be facilitated by the direct presence of ubiquitin. Finally we have considered the advisability of the presence of a xenogeneic antigen that escapes tolerance by definition. We have therefore constructed a triple modified DNA vaccine that expresses a p53 fusion protein that should be directed to the cytoplasm as it lacks its nuclear expression signal. Moreover, it should be readily ubiquitinated as it ends with the ubiquitin sequence. For a xenogeneic signal expression the human p53 sequence starts with a rat or mouse sequence. Despite the steady rise in the number of vaccination studies, no single route of administration had demonstrated its superiority so far. We have chosen for intramuscular (i.m.) DNA immunisation [10,11]. Important advantages of this approach are:

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stable and prolonged expression of protein synthesis, possibility of upregulation of MHC-class I expression after DNA vaccination in muscle cells [12], minimal risk of integration, simple GMP manufacturing procedure and ease of administration to the patient. To increase the transfection efficiency after intramuscular injection of the plasmid DNA we used electroporation, a technique that may be superior compared to ultrasound [13,14].

Summarising, immunotherapy is an alternative potential tool in the treatment of cancer. P53 is an ideal target, however methods to improve the anti-tumour response against an antigen are needed. Here we describe *in-vitro* and *in-vivo* results of a triple modified p53 DNA vaccine designed for enhanced processing and presentation of the p53 peptides in the MHC-class I in concurrence with a xenogeneic co-stimulatory antigen.

2. Materials and methods

2.1. DNA constructs

For the construction of triple modified plasmids individual fragments of mouse p53 (AA 1-53), rat p53 (AA 1-58), human p53 (AA 57-308) and the first repeating sequence of ubiquitin were cloned in TOPO blunt vectors (Invitrogen, Breda, the Netherlands). The plasmid and DNA fragments were cut with various restriction enzymes including *BsmHI*, *SpeI*, *EcoRI and BsiWI.* The individual fragments were subsequently combined in the desired sequence to form triple combined fragments in TOPO blunt vectors. Finally the obtained triple combined fragments were inserted in an eukaryotic PUC 118 expression vector, resulting in the plasmids mhp53ubiquitin, mhp53stop, rhp53ubiquitin, and the rhp53stop.

2.2. Cell lines and mice

The p53 negative human osteosarcoma cell line Saos-2 (HTB-85) and EL4 cells (TIB39) were obtained from the American Tissue Culture Collection (ATCC, LGC Promochem, Europe). The p53 positive cell line A2780-m273 is described by Sleijfer et al. [15].

Eight to twelve-weeks-old female C57BL/6 mice were purchased from Harlan Netherlands BV (Horst, the Netherlands). Transgenic HLA-A2 mice (C57BL/6 origin mice transgenic for A2/K^b as described by Heukamp et al. [16] were a kind gift of Dr. F. Ossendorp (Department of Immunohematology and Blood Transfusion, Leiden University Medical Center, Leiden, the Netherlands). All mice were housed at the Central Animal Facility at the University of Groningen under conventional conditions.

2.3. Transfection of Saos-2 and treatment with the proteasome inhibitor

Saos-2 cells were transfected with our constructs (2.5–3 μg DNA/10^6 cells) using Fugene 6^{TM} transfection reagents (Roche Biochemicals, Woerden, the Netherlands). Inhibition of the proteasome was performed by co-incubation of transfected cells for 0, 4 or 20 h with 10 μM of the proteasome inhibitor MG132 (Biomol, Exeter, United Kingdom). Cells were harvested three days after transfection.

2.4. Detection of p53 protein by SDS-polyacrylamide gel electrophoresis followed by Western blotting

After transfection and/or treatment with MG132, Saos-2 cells were washed twice with ice-cold PBS, lysed with standard SDS-sample buffer containing 5% beta-mercapto-ethanol and boiled for 5 min. Protein levels were determined using the Bradford protein assay. Total cell lysates were size fractionated on a 7.5% SDS-polyacrylamide gel electrophoresis (Biorad, Veenendaal, the Netherlands) and transferred on to activated polyvinylidene difluoride membranes (Millipore, Etten-Leur, the Netherlands). The antibody p53PAB240 (SantaCruz Biotechnologies, Santa Cruz, USA) was used to detect the hp53 products followed by a secondary rabbit anti-mouse antibody labelled with horseradish peroxidise (DAKO, Glostrup, Denmark). Chemiluminescence was employed for detection using the BM Chemiluminescence blotting kit (Roche Biochemicals, Mannheim, Germany).

2.5. Immunocytochemical detection of p53 expression

Immunocytochemical staining of p53 was performed on air dried, acetone/methanol (1:1) fixed cytospins of Saos-2 cells using a 3-step indirect immunoperoxidase staining protocol. The antibody p53PAB240 (SantaCruz Biotechnologies, Santa Cruz, USA) was used to detect the hp53 followed by a secondary biotine-labelled rabbit anti-mouse antibody in combination with a third anti-biotin horseradish peroxidase (HRP)-labelled streptavidin (DAKO, Glostrup, Denmark). HRP activity was visualised with amino-ethyl-carbazole (AEC).

2.6. Immunisation of mice

Plasmids were grown in large quantities in *E. coli*, purified using an Endo Free Plasmid Giga Kit (Qiagen Chatsworth, CA) and dissolved in 0.9% NaCl at a concentration of 2 g/l. Female C57Bl/6 mice or C57Bl/6 transgenic A2K^b mice, 8–12-weeks-old, were anesthetised by breathing halothane (5% induction, 2% maintenance) in $N0_2/0_2$ (70%/30%) supplied over a cylindrical ventilation cap held over the head. Vaccination was performed by i.m. injection of 50 μl in each hind limb. After the i.m. injection the muscles at the injections site were electroporated (BTX 830; 200 V/cm, 20 ms, 8 pulses; VWR International, Roden, the Netherlands) using 5 mm size pads. Vaccinated mice were sacrificed 3 weeks after the second immunisation (week 6) and spleens were removed aseptically.

2.7. ELISPOT assay

Specific spleen cell IFN-gamma secretion was assayed by ELISPOT. Briefly, 96-wells plate for ELISA (high affinity, Greiner Bio-One, Alphen a/d Rijn, the Netherlands) were incubated with 50 μl of capture-purified anti-mouse IFN-gamma in 0.1 M Na_2HPO_4 buffer pH 9.0 (5 μg/ml; BD Biosciences, Breda, the Netherlands) overnight at 4 °C. Plates were washed four times with PBS + 0.02% Tween-20 and then blocked with 4% BSA in Iscoves modified DME medium (IMDM, Invitrogen, Breda, the Netherlands) supplemented with penicillin, streptomycin, and 5×10^{-5} M 2-mercapto-ethanol for minimal 1 h at 37 °C. Spleen lymphocytes were added at a known concentration in the same medium in the presence of p53 containing EL4 cells (EL4 cells transfected with the plasmid containing the desired epitope) and plates were incubated for 20–24 h at 37 °C and 5% CO_2. Non-transfected control EL4 cells were used as negative (non-specific) controls. Stimulation with PMA or ionomycin were used as positive control. Plates were washed with 100 μl water 4 °C to lysate the cells and in addition washed with PBS 0.02% Tween 20 followed by incubation with 50 μl of biotinylated rat anti-mouse IFN-gamma (0.5 μg/ml; BD Biosciences, Breda, the Netherlands) for 1 h at 37 °C. After additional washes, plates were incubated with alkaline phosphatase-labelled streptavidin (1:1000; BD Biosciences, Breda, the Netherlands) for 1 h at 37 °C and 5% CO_2. After three times washing with PBS 0.02%/Tween-20 and two times washing with PBS, spleen cell IFN-gamma secretion was visualised by staining with BCIP (Sigma–Aldrich, Zwijdrecht, the Netherlands) in agarose. Spots were counted by reversed light microscope. After correction for non-specific spots, p53-specific