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An improved rearranged Human Papillomavirus Type 16 E7 DNA vaccine candidate (HPV-16 E7SH) induces an E7 wildtype-specific T cell response

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Abstract

A new and very promising approach in vaccine development is the application of naked DNA. In comparison to conventional vaccines it offers several advantages, especially if there is a need for the development of low cost vaccines. Infection with high-risk human papillomaviruses (hr-HPVs) is the major risk factor for the development of cervical cancer (cc), the third most common cancer in women worldwide. The HPV E7 oncogene is constitutively expressed in HPV-infected cells and represents an excellent target for immune therapy of HPV-related disease. Therefore, we chose the HPV-16 E7 as model antigen in the development of a therapeutic DNA vaccine candidate. For safety reasons the use of a transforming gene like the HPV-16 E7 for DNA vaccination is not feasible in humans. In consequence we have generated an artificial ("shuffled") HPV-16 E7-gene (HPV-16 E7SH), containing all putative cytotoxic T-lymphocyte (CTLs) epitopes and exhibiting high safety features. Here, we show the induction of a strong E7-wildtype (E7WT) directed cellular and humoral immune response including tumor protection and regression after in vivo immunization in the murine system. Moreover, the vaccine candidate demonstrated immunogenicity in humans, demonstrated by priming of antigen-specific T cells in vitro. Importantly, the artificial HPV-gene has completely lost its transforming properties as measured in soft agar transformation assays. These results may be of importance for the development of vaccines based on oncogenes or oncoproteins.

Keywords: Gene shuffling; Cervical cancer; Immunotherapy; Tumor regression; In vitro immunization; T cells

1. Introduction

A naked DNA vaccine provides remarkable advantages in comparison to protein- or peptide-based vaccines making it of potential interest also for the use in Third World countries [1]. Its production costs are relatively low and predictable (M. Schleef, PlasmidFactory, Bielefeld, Germany, personal communication). Furthermore, DNA is stable, does not require refrigeration for storage and can easily be modified. With respect to prime-boost regimen naked DNA lacks unwanted immune reactions against other components of the vaccine as is observed in case of vector based-vaccines [2].

Abbreviations: APCs, antigen presenting cells; B-LCLs, B-lymphoblastic cell line; cc, cervical cancer; CIN, cervical intraepithelial neoplasia; CpG, unmethylated cytidine phosphate guanosine motifs; CS, calf serum; CT, cardiotoxin; CTLs, cytotoxic T lymphocytes; C-X-X-C, Zn binding motifs; DCs, dendritic cells; E7SH, rearranged ("shuffled"=SH) gene of the E7WT; E7WT, E7-wildtype gene/antigen of Human Papillomavirus Type 16; HLA, human leukocyte antigen; hr-HPVs, high-risk human papillomaviruses; i.m., intramuscularly; nt, nucleotides; PBMC, peripheral blood mononuclear cell; pRB, retinoblastoma protein; s.c., subcutaneously; Th, T helper cells; VP22, herpes simplex virus VP22

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In this study, we have selected cc as a model for the development of a safe therapeutic DNA vaccine candidate. Worldwide approximately 370,000 cases of cc are being diagnosed each year and almost 200,000 deaths are attributed to this disease [3]. In Third World countries cc is one of the major cause of cancer-related deaths. About 80% of women dying from this disease originate from low-budget countries where screening programs for early detection and the medical infrastructure for treatment are not available. In contrast in the more developed world the mortality was reduced (by 70% in the US) during the last 50 years as a consequence of cytological screening programs [4]. Treatment of cc patients by surgery, radiotherapy or chemotherapy results in a significant loss of quality of life. Even when optimal treatment is available about 40% of all cc patients die of this disease [5]. Therefore, the development of an effective and save therapeutic vaccine is needed.

A necessary event for the development of premalignancies like cervical intraepithelial neoplasia (CIN) and cc is infection by hr-HPVs [6]. So far over 120 HPV types are identified [7], 18 of which were found to be associated with cc [8]. HPV-16 is responsible for about 50% of the cases [9]. Due to the fact, that the oncoprotein E7 of the hr-HPVs is exclusively and consistently expressed by HPV-infected tumor cells [10] it represents a specific target for an immune therapy directed against cc and its premalignant dysplasia. The E7, however, is an oncoprotein with transforming activity by interfering with the cell cycle control. The E7 alters cell growth regulation by inactivating the pRB (retinoblastoma) tumor suppressor protein [11,12] and contains two metal-binding motifs (C-X-X-C) [13,14].

For safety reasons a functional oncogene cannot be applied to humans. Therefore, efforts were made to inactivate the oncogenic properties of the HPV-16 E7. Some investigators have introduced point mutations into the sites of the E7-oncogene that are associated with transforming potential [15,16], whereas others have used HLA- (human leukocyte antigen) restricted singular epitopes [17,18].

These approaches, however, may lead to an unwanted loss of naturally occurring epitopes that is potentially associated with a decrease in vaccine efficacy. Our aim was to supply all potential naturally occurring T cell epitopes, covering the broad range of MHC restriction. In consequence, prior knowledge of the patient's HLA-haplotype is not required. This is especially important in the outbred human population. In addition, a more potent immune response may be induced, involving all occurring HLA-restriction elements in the vaccine. We had generated in a "proof-of-principle" study an artificial HPV-16 E7 gene (HPV-16 E7SH) of the first generation [19]. In this study we showed that an oncoprotein with a rearranged primary sequence still induces E7WT-specific CTLs in mice but is devoid of transforming properties. We had taken advantage of the earlier finding in our laboratory that fusion with the VP22 gene of Herpes Simplex Virus Type 1 strongly enhances the CTL response in mice [20]. In order to translate the therapeutic DNA vaccine candidate into a clinical trial we decided to further improve its safety features. For this reason we did not fuse any heterologous genes. We enhanced immunogenicity by placing a Kozak-sequence [21] in front of the gene [22] and chose the plasmid-vector pTH [23] applicable to humans [26]. More importantly we redesigned the E7 itself. The sequence was taken apart at exactly at the positions that are critical for transforming properties of the protein (pRB-binding site, C-X-X-C motifs) and reassembled in a "shuffled" order as "core" gene. This sequence was codon optimized to humans (almost identical to mice). The original junctions destroyed by the dissection were added as an "appendix" with non-codon optimized sequence to minimize recombination events reconstituting the wildtype sequences (see also Fig. 1).

Tumor protection and regression experiments will give a first impression on immunogenicity and effectivity of tumor vaccines. They do not fully reflect, however, the responses induced in humans. "In vitro immunization" of human lymphocytes by antigen-loaded dendritic cells (DCs) may be used as a model [24]. Loading of DCs by DNA transfection is a suitable technique [25] and specific T cell priming verifies the potential immunogenicity of the DNA vaccine candidate.

Here, we show that the HPV-16 E7SH DNA vaccine candidate of the second generation induces specific immunity in vivo in mice and after in vitro immunization of human lymphocytes and, therefore, holds promise for a safe therapeutic HPV-vaccine.

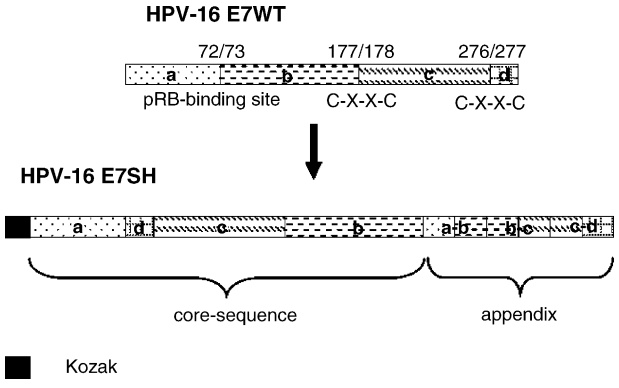

Fig. 1. Map of the artificial HPV-16 E7SH gene. The HPV-16 E7 wildtype gene (E7WT, above) was dissected at the positions corresponding to the pRB binding site (nt 72/37) and between the two C-X-X-C motifs (nt 177/178 and nt 276/277). The resulting four fragments a, b, c and d were rearranged ("shuffled") forming the core element with the sequence a, d, c, b. To avoid loss of putative CTL epitopes at the junctions a-b, b-c and c-d, these sequences (3×27 nt $= 3 \times 9$ amino acids) were added as an appendix forming the complete HPV-16 E7SH gene. To minimize the potential risk of "back-to-wildtype recombination" the codons of the core element were optimized for expression in humans according to the Kazusa codon usage database (www.kazusa.or.jp/codon/). A Kozak sequence was added in front of the gene to enhance translation.

2. Materials and methods

2.1. Generation of the recombinant vectors

The HPV-16 E7SH gene was engineered by three consecutive PCR reactions as described earlier [22]. The following overlapping primers were used: 1st PCR reaction (the Kozak sequence is underlined): 5'GC GGC CGC GGA TCC CCC GGG AAG CTT CCC GCC GCC ACC ATG CAC GGC GAC ACC CCC ACC CTG CAC GAG TAC ATG (16E7SH/1), 5′GCA CTT GGG TTT CTG GCT GCA GAT GCA GTA CAG GTC GGT GGT CTC GGG CTG CAG GTC CAG CAT GTA CTC GTG CAG GGT GGG (16E7SH/2), 5′TGC AGC CAG AAA CCC AAG TGC GAC AGC ACC CTG CGG CTG TGC GTG CAG AGC ACC CAC GTG GAC ATC CGG ACC CTG GAG GAC (16E7SH/3), 5′CTC GCT GCT GTC GTT CAG CTG CTC GTA GGG GCA CAC GAT GCC CAG GGT GCC CAT CAG CAG GTC CTC CAG GGT CCG GAT GTC (16E7SH/4); 2nd PCR reaction: 5'CAG CTG AAC GAC AGC AGC GAG GAG GAG GAT GAG ATC GAC GGC CCC GCC GGC CAG GCT GAG CCC GAC CGG GCC CAC TAC AAC (16E7SH/5), 5′ATT TAA TTG CTC ATA ACA GTA GAG ATC AGT TGT CTC TGG TTG GCA GCA GAA GGT CAC GAT GTT GTA GTG GGC CCG GTC GGG (16E7SH/6), 5′TAC TGT TAT GAG CAA TTA AAT GAC AGC TCA GAG CAT TAC AAT ATT GTA ACC TTT TGT TGC AAG TGT GAC TCT ACG CTT CGG (16E7SH/7), 5′GAA TTC CTC GAG TCT AGA CTG CAG TTA TGG TTT CTG AGA ACA GAT GGG GCA CAC AAT TCC TAG TGT GCC CAT GCA CAA CCG AAG CGT AGA GTC ACA CTT (16E7SH/8); 3rd PCR reaction: the resulting fragments A (1st PCR reaction) and B (2nd PCR reaction) were fused in a further PCR step using the primers 16E7SH/1 and 16E7SH/8.

The resulting gene product was cloned via 5′*Hin*dIII and 3′*Xba*I into the immunization vector pTHamp [23], which has already been used in a version without any antibiotic resistance gene (pTHr) in humans [26].

The pTHamp-E7SH construct was sequenced by Medigenomix (Martinsried, Germany) using the following primers: 5′CAT GGG TCT TTT CTG CAG TC (forward), 5′CAG CGA GCT CTA GCA TTT AG (reverse).

Generation of the HPV-16 E7SH-recombinant pBabe-puro plasmid: the E7SH gene was excised via *Hin*dIII/*Xba*I endonucleases from the pTHamp-E7SH construct (see above). The gene was cloned after mung bean enzyme treatment (resulting in blunt ends) into the *Eco*RI digested and mung bean enzyme prepared retroviral expression vector pBabe-puro [27]. The pBabe-puro E7SH construct was sequenced by Medigenomix (Martinsried, Germany) using the following primers: 5′GCC TCG ATC CTC CCT TTA TCC (forward), 5′CCC TAA CTG ACA CAC ATT CCA CAG GGT CGA (reverse). The pBabe-puro E6 and pBabe-puro E7WT constructs were a kind gift of Dr. M. Tommasino (IARC, Lyon, France).

2.2. Northern blot and RT-PCR analysis

RNA from transfected dendritic cells (DCs) was isolated by Trizol (Invitogen, Carlsbad, California, USA) according to the user manual. Northern hybridisation was performed with the gene of HPV-16 E7WT. The probe was labeled with the random primed labeling kit (Roche, Mannheim, Germany). Hybridisation was done at 42 °C for 48 h and afterwards washed 3 times at 68 °C for 30 min with 2 × SSC, 0.1% SDS.

Primers for RT-PCR were: reverse transcription: 5′AAG CAG TGG TAA CAA CGC AGA GTA CTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TAG CAG CT, PCR forward primer WT: 5′CC AAG CTT TTA GGG TTT CTG GCT GCA, forward primer SH: CGG GAT CCC CAC GGC GAC ACC CCC ACC and reverse primer: 5′CG GGA TCC CAC GGC GAC ACC CCC ACC. AmpliTaq Gold polymerase (Applied Biosystems, Foster City, Canada) was used and DNA amplified by 10 min 95 °C (1 cycle), 15 s 95 °C, 30 s 55 °C (25 cycles), 1 min 72 °C.

2.3. Western blot analysis

E7 protein expression in nucleofected DCs was detected by Western blot. DCs (5×10^5) were lysed 24 h after transfection of pTHamp-E7WT or pTHamp-E7SH by boiling for 10 min in SDS sample buffer and directly separated by SDS-PAGE. Lysed CaSki cells were used as a positive control. The proteins were transferred to Immobilon-P transfer-membrane (Millipore, Bedford, USA) by a Trans-blot semi-dry transfer cell (BioRad, München, Germany). To detect E7 proteins an anti-HPV-16 E7WT polyclonal goat antiserum (gift from W. Zwerschke, Innsbruck, Austria) was used followed by an anti-goat-HRP secondary antibody (Dianova, Hamburg, Germany).

2.4. Retroviral expression of the HPV-16 gene

BOSC23 cells (5×10^6) were transfected with 10 μg recombinant pBabe-puro vector by $CaCl_2$ precipitation method (including the addition of 25 μM Chloroquine in order to enhance transfection efficacy) and retrovirus-containing supernatant was harvested 48 h later. NIH3T3 cells (5×10^5) were infected with 5 ml freshly harvested retroviral supernatant (+5 μg/μl Polybrene, in order to enhance infection efficacy) and selection was started two days later by adding 2 μg/ml puromycin (Sigma, Deisenhofen, Germany).

2.5. Soft agar transformation assays

NIH3T3 cells infected with recombinant pBabe-puro retrovirus were selected with 2 μg/ml puromycin for 2 days and 2.5×10^4 cells were subsequently resuspended in 10 ml warm (40 °C) DMEM/10% calf serum (CS) containing 1% melted agar and 1 μg/ml puromycin. Of this

suspension 2 ml (5×10^3 cells) were poured onto a precast base layer consisting of 5 ml DMEM/10% CS with 1% soft agar and 1 μg/ml puromycin in 50 mm Petri dishes preparing three soft agar cultures in parallel for each infection. Soft agar cultures were incubated at 37 °C/5% CO_2 adding 0.5 ml complete DMEM/10% CS without puromycin once per week. After four weeks all foci located within a 9 cm^2 field of the soft agar plate were counted.

2.6. DNA vaccination

Female C57BL/6 mice (WiGa-Charles River, Hamburg, Germany) were kept under SPF isolation conditions and standard diet at the animal facilities of the German Cancer Research Center Heidelberg, Germany. Six- to eight-week-old mice were pretreated as indicated by injection of 50 μl of 10 μM cardiotoxin (CT) (Latoxan, Rosans, France) into each *musculus tibialis anterior* 5–6 days prior to DNA vaccination. For vaccination, 50 μl of plasmid DNA (1 μg/μl in PBS) was injected into each muscle (100 μg DNA per animal). Ten to twelve days after vaccination animals were sacrificed and spleens were isolated. All manipulations on live animals were performed under Isofluran anesthesia (CuraMed Pharma, Karlsruhe, Germany).

2.7. Cell lines and culture conditions

All cell lines used in the murine part were of C57BL/6 origin ($H2^b$ context). RMA cells [28], RMA-E7 transfectants [29], C3 tumor cells [30] and splenocytes were cultured as published elsewhere [22].

The packaging cell line BOSC23 [31] was cultured in DMEM with 10% FCS, 2 mM of glutamine, 100 U/ml of penicillin and 100 μg/ml of streptomycin. NIH3T3 cells were obtained from ATCC and were cultured in the same medium except that 10% CS was added.

Human T cells were isolated from non-plastic adherent peripheral blood mononuclear cells (PBMC) by binding to sheep red blood cells and density gradient centrifugation. T cells were cultured in RPMI 1640 supplemented with 10% heat-inactivated pooled human AB plasma in U-bottom 96-well plates at a density of $1–2 \times 10^5$/well. T cells were stimulated three times once per week with plasmid DNA-nucleofected or peptide pulsed autologous irradiated DCs at a ratio from 10:1 to 20:1. With the second stimulation 100 U/ml IL-2 (Boehringer, Mannheim, Germany) and IL-7 (Promo Cell, Heidelberg, Germany) were added. B-LCLs used were autologous EBV-immortalized cell lines of each donor investigated. The cell lines were individually generated by incubating donor B cells with infectious EBV-containing culture supernatant of B95-8 cells (ATCC No. CRL-1612). T2 cells (ATCC No. CRL-1992) are defective in endogenous antigen presentation. T2 cells display only empty HLA-A2 molecules on their cell surface that can exogenously be loaded with peptides.

2.8. Generation of human DCs

Human monocyte-derived DCs were generated from buffy coats of HLA-A2-positive healthy donors basically as described by Romani et al. [32]. PBMCs were isolated by Ficoll-Hypaque density-gradient centrifugation. PBMCs were washed four times with PBS and resuspended in CellGro DCs medium (Cell Genix, Freiburg, Germany). PBMC (5×10^7) were transferred into 75 cm^2 culture dishes and incubated for 2 h. Non-adherent PBMCs were removed by gently washing the culture dishes with PBS. Adherent monocytes were cultured in 5 ml of CellGro medium containing IL-4 (Immunotools, Friesoythe, Germany) and GM-CSF (Immunex, Seattle, USA) each at 1000 U/ml for 6 or 7 days. Afterwards DCs were harvested and stored frozen or matured with a cocktail of cytokines: 10 ng/ml IL-1β (R&D Systems, Minneapolis, MN, USA), 1000 U/ml IL-6 (Promocell, Heidelberg, Germany), 10 ng/ml TNF-α (R&D Systems), 1 μg/ml PGE_2 (Pharmacia, Erlangen, Germany) for 24 h [33].

2.9. Antigen loading of human DCs

Mature DCs were nucleofected by Amaxa Nucleofector technology and the Amaxa Nucleofection Kit for human DCs (Amaxa, Köln, Germany, Cat. No. VPA-1004, the composition of the kit is not disclosed by the company) using an optimized protocol.

Briefly, DNA (5 μg) was applied for nucleofection of 2×10^6 DCs in 100 μl of reagent and program U-7 was used. For peptide pulsing immature DCs were washed two times and resuspended in serumfree RPMI 1640 (Invitrogen, Paisley, UK) and 10 μg/ml peptide was added. After 4 h the peptide pulsed DCs were washed two times. After loading DCs were cultured for 24 h in 1 ml of CellGro medium containing IL-4, GM-CSF, and maturation cytokine cocktail. DCs were irradiated (60 Gy) and washed 3 times before stimulation of autologous T cell cultures. Peptides used for restimulation were pools of either HLA-A* 0201 restricted minimal epitopes $E7_{11–20}$ (YMLDLQPETT) $E7_{82–90}$ (LLMGTLGIV), $E7_{86–93}$ (TLGIVCPI) or peptides of about 35 aa in length spanning the complete E7 sequence: (p1) MHGDTPLHEYMLDLQPETT DLYCYEQLNDSSEEE; (p2) LCYEQLNDSSEEEDEIDG-PAGQAEPDRAHNIVT; p3) GQAEPDRAHNIVTFCCK-CDSTLRLCVQSTHVDIR; (p4) TLRLCVQSTHVDIR-TLEDLL MGTLGIVCPICSOKP. Control peptide was HPV-16 $E6_{28–36}$ (TIHDILECV).

2.10. In vitro restimulation of murine CTL lines

$1–2 \times 10^7$ spleen cells (pretreated with ACT lysis buffer [17 mM Tris/HCl, 160 mM NH_4Cl, pH 7.2] to deplete erythrocytes) were co-cultured with 2×10^6 irradiated (100 Gy) RMA (controls) or RMA-E7 cells in 25 cm^2 culture flasks in αMEM. First in vitro restimulations were performed at the day of the spleenectomy and were weekly repeated up to four

times. Four to five days after the first in vitro restimulation the spleen cell cultures were seeded in a serial dilution into 24-well plates (6 wells per flask) in 2 ml of αMEM + medium per well.

Starting with the second in vitro restimulation additionally to the RMA/RMA-E7 cells (1×10^5 per well) or T helper (Th) epitope $E7_{48–54}$ [34], freshly isolated (ACT lysis buffer pretreated) splenocytes (5×10^6 per well) were added as feeder cells (irradiated by 100 Gy) as a source of the co-stimulating B.7 molecule. Cultures were incubated at 37 °C and 7.5% CO_2 in a humidified incubator.

2.11. IFN-γ Elispot assays

Murine IFN-γ Elispot assays were performed ex vivo and 5 or 6 days after each in vitro restimulation as described elsewhere [22].

Spots were quantitated with an Elispot reader (Autoimmune Diagnostics AID, Strassberg, Germany). Spots of the negative-control wells were substracted from spots of the test samples ($E7_{49–57}$ peptide stimulated). An animal was scored positive when the number of IFN-γ secreting cells was at least 100% above the highest number of IFN-γ secreting cells of the control animal (empty vector recipients).

Human IFN-γ Elispot assays were performed 3 weeks after the last restimulation. A 96-well plate (MultiScreen HA, Millipore Eschborn, Germany) was coated over night with monoclonal anti-human IFN-γ antibody 1D1k (10 μg/ml) in PBS (Mabtech, Hamburg, Germany) and blocked with RPMI, 0.4% human serum albumin (DRK Blutspendedienst, Germany). T cells (5×10^4) were pre-incubated for 4 h with DCs (5×10^3) in a V-bottom 96-well plate and afterwards seeded onto MultiScreen plates. After 20 h cells were washed off with PBS/Tween 20 (0.05%). The plate was treated for 2 h with monoclonal antibody 7-B6-biotin (Mabtech) at 2 μg/ml in PBS/BSA (0.5%) followed by six washes with PBS/Tween 20. Streptavidin-alkaline phosphatase antibody (Sigma–Aldrich, Deisenhofen, Germany) was incubated at 50 ng/ml in PBS for 2 h. The plate was washed three times with PBS/Tween 20 and three times with PBS alone. The staining reaction was developed with BCIP/NBT (Sigma) for 10–20 min and plates rinsed in tap water. Counting of Elispot plates was done with an Elispot Reader equipped with KS Elispot Software version 4.3.56 (Zeiss, Halbergmoos, Germany).

2.12. ^{51}Cr release assays

^{51}Cr release assays was performed 5–6 days after an in vitro restimulation of murine spleen cells as described elsewhere [22]. An animal was scored positive when the specific lysis of the specific target (RMA-E7 cells) was at least 10% above the lysis of the control target (RMA cells).

2.13. Flowcytometric cytotoxicity assay

T cell lines stimulated with human DCs were restimulated twice with specific HPV-16 E7WT 9mer peptide-pulsed autologous irradiated B-lymphoblastic cell lines (B-LCLs). The assay was performed as published earlier [35]. Briefly, T2 cells were labeled with CFSE (2.5 μM in 2 ml PBS for 8 min), washed two times and loaded overnight with 9mer peptide pools (10 μg/ml) and β_2-microglobulin (3 μg/ml) in serum free medium and used as target cells. Cold target inhibition was done with K562 cells to block NK activity. Effector: target: cold target ratio was 10:1:10. First, T cells (2×10^5/well) were incubated in a 96-well V-bottom plate with the same number of "cold target" unlabeled K562 for 30 min. Afterwards 2×10^4 target cells were added followed by incubation for 4 h at 37 °C. Cells were stained with propidium iodide (1 μg/ml) and lysed target cells were determined by counting FL1/FL3 double positive cells on a FACS Calibur (Becton Dickinson, Heidelberg, Germany). Basal lysis was determined by counting propidium iodide positive target cells in samples without effector cells. Specific lysis was calculated according to the formula: specific lysis (%) = dead cells in sample (%) − dead cells in control (%).

2.14. Tumor protection studies

Six- to eight-week-old C57BL/6 mice were immunized twice (days 0, 14) under isofluran anesthesia with 50 μg DNA in PBS in each *musculus tibialis anterior* after pretreatment with CT, respectively (100 μg DNA per animal, see DNA vaccination). Sixteen days after the boost-vaccination the animals were challenged subcutaneously in the shaved right flank with 5×10^5 C3 cells (in 100 μl PBS). The tumor size was measured every 2–4 days with the aid of a ruler. The experimental endpoint was 35 days after the first appearance of the tumor, when the animal was sacrificed. Tumor sizes on the animals within a group were calculated as arithmetic means with standard error of the means (S.E.Ms.). In the tumor protection experiment an individual was indicated as "protected", if the tumor area at the endpoint of the experiment was within the "0–25 mm^2" field.

2.15. Tumor regression studies

C57BL/6 mice received 5×10^5 C3 cells in 100 μl of PBS subcutaneously in the right shaved flank. When small tumors were palpable in all animals (days 5–18) the first DNA-injection (recombinant or empty plasmid) was applied intramuscularly (i.m.) in both *musculus tibialis anterior* five days after CT-pretreatment (see tumor protection studies). The boost-vaccination was performed 12–15 days later, again after CT-pretreatment. Tumor sizes (measured with a ruler) were determined every 2–4 days until mice had to be sacrificed. Tumor sizes of the mice within a group were calculated as arithmetic means with standard error of the means (S.E.Ms.). In the tumor regression experiments an individ-

ual was indicated as a "regressor", when the tumor area at the endpoint of each experiment was within the "0–25 mm^2" field.

2.16. ELISA

Murine E7-specific serum antibodies were detected with a capture ELISA essentially as described earlier [22,36]. The absorbance in wells with GST as antigen defined the background reactivity of a serum, which was then subtracted from the absorbance with the GST HPV-16 E7WT proteins to calculate the specific reactivity of a serum against the antigen HPV-16 E7. All measurements were performed in duplicates including a positive control serum on each 96-well plate. An animal was scored positive when the OD_{450} was at least 100% above the OD_{450} mean in the control group.

3. Results

The aim of this study was to generate a therapeutic HPV-16 E7 DNA-vaccine candidate, which is safe for the use in humans. To this end, we constructed a rearranged HPV-16 E7 gene of the second generation (designated HPV-16 E7SH), which was – in comparison to the first generation – modified in a more educated way resulting in higher safety features. Here, we have investigated the potential of the new artificial gene to induce an immune response, which is directed against the E7WT antigen in vivo (in the murine model) and in vitro (transfection of human DCs). Furthermore, we wanted to verify our hypothesis that the 16 E7SH gene of the second generation has lost the transforming properties of the 16 E7WT oncogene.

3.1. Generation of the HPV-16 E7SH gene

In order to rearrange ("shuffle" = SH) the HPV-16 E7 gene and, thereby knock out the transforming properties, we dissected the E7WT gene within the three transformation-related regions (between nt 72/73, 177/178, 276/277). The first is associated with pRB-binding [37]; the other two (containing C-X-X-C motifs) are associated with protein stability [13,14]. The four resulting fragments a, b, c, and d were rearranged into the so-called "core element" with the fragment sequence a, d, c, b. In order to maintain all potential naturally occurring CTL-epitopes located at the junctions (9 aa [=27 nt] in each direction, per junction $2 \times 27 = 54$ nt) between the fragments a, b, c and d, were fused as an "appendix" to the core element. The $H2^b$-restricted CTL-epitope $_{49}$RAHYNIVTF$_{57}$ [30] was retained within the core element as well as the HLA-A2 restricted epitopes [38] $_{11}$YMLDLQPETT$_{20}$ and $_{82}$LLMGTLGIV$_{90}$, whereas the third known HLA-A2 restricted epitope ($_{86}$TLGIVCPI$_{93}$) has been destroyed in the core element by the dissection at nt 276/277, but was rescued and is now located within the appendix. The structure of the E7SH gene: a, d, c, b, a-b, b-c, c-d is shown in Fig. 1. The gene was generated by PCR using 8 overlapping long primers covering the whole 16 E7SH sequence in 3 reactions (see materials and methods). In addition the Kozak-sequence was placed in front of the gene to enhance mRNA translation [21,22].

3.2. The E7SH gene induces E7WT-specific CTL responses and E7-specific antibody induction in vivo

To address whether the rearranged E7SH gene is able to induce a cellular immune response directed against the E7WT antigen, we immunized in 5 independently performed experiments C57BL/6 mice once with 100 μg of the pTHamp-E7WT or with the corresponding pTHamp-E7SH vector. Ten of the twelve E7WT-injected and all 22 mice that received E7SH reacted to peptide $E7_{49–57}$ stimulation and scored positive in IFN-γ Elispot assays. Control animals received empty vector in PBS ($n = 20$, all scored negative). Fig. 2a shows one experiment where already ex vivo a strong activation of $E7_{49–57}$-WT specific splenocytes was shown by IFN-γ Elispot assay. Both the E7WT (0.09 ± 0.01) and the E7SH (0.11 ± 0.03) group reacted, whereas the control animals (empty vector) showed about 10fold less INF-γ secretion (0.01 ± 0.004). To determine, if the specifically activated splenocytes had cytolytic activity, we performed ^{51}Cr-release assays. After two rounds of in vitro restimulation strong specific cytolytic activity against the E7WT-expressing RMA-E7 transfectants was shown in two independent experiments. In these experiments all E7WT ($n = 8$) and E7SH ($n = 8$) vaccinated animals displayed strong E7WT-specific lysis of the RMA-E7 target cells (Fig. 2b shows one representative experiment). The mean of specific lysis of the RMA-E7 cells in the two experiments was comparable in the E7SH-group (41 and 35%) and the E7WT immunized animals (32 and 40%). The splenocytes of the control mice (empty vector) did not show any E7-specific lysis, demonstrating that the E7-specificity shown in vitro was actually primed in vivo by the DNA vaccine candidate.

Some investigators found a stronger immune response after a second DNA application [39]. Therefore, we immunized in two experiments a total of 10 mice twice (prime: day 0, boost: day 13 or 21) with the pTHamp-E7SH construct, from which all scored positive in IFN-γ Elispot assay (CTL epitope $E7_{49–57}$), whereas none of the control animals ($n = 10$) reacted positively (Table 1). In contrast to McShane et al. [39], we were not able to show an enhancement of the cellular immune response (portion of IFN-γ secreting splenocytes) after a second DNA injection (data not shown) in comparison to mice immunized once (Fig. 2). Moreover, in one of the two prime-boost experiments, we immunized the mice ($n = 5$) without CT pretreatment (see also tumor regression experiments). Neither difference in the percentage of IFN-γ secreting cells nor in the positively evaluated animals was observed between the plus versus minus CT groups (Table 1).

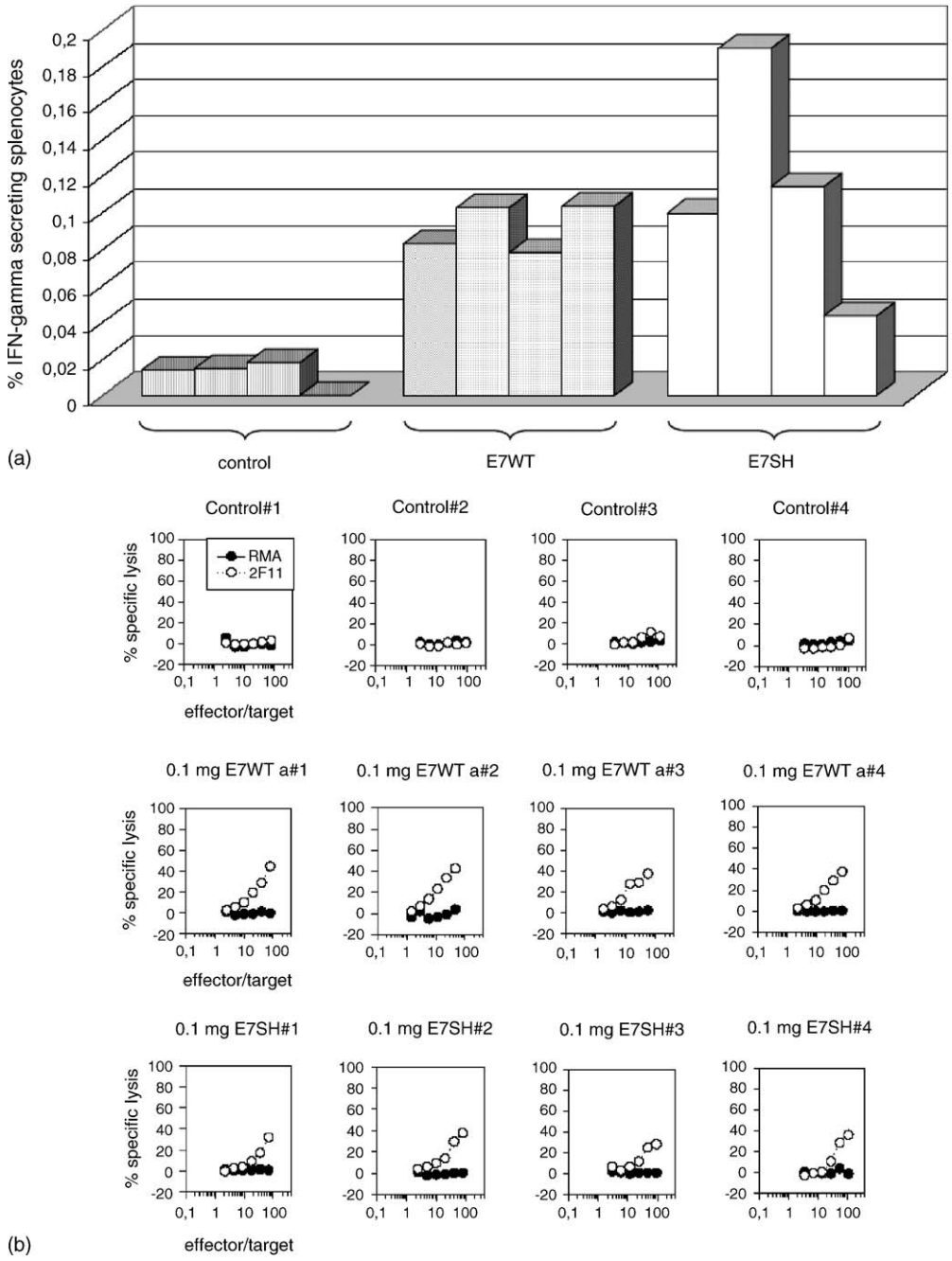

Fig. 2. CTL responses in C57BL/6 mice after DNA immunization. Four mice per group were immunized once i.m. with 50 μg empty plasmid or with pTHamp vector encoding E7WT or E7SH in each *musculus tibialis anterior*. (a) ex vivo IFN-γ Elispot assay. Each bar represents the number of activated T cells from an individual animal. (b) ^{51}Cr-release assay after two rounds of in vitro restimulations using either syngeneic parental RMA (black circles) or E7WT-expressing RMA-E7 tranfectants (2F11 cells, white circles). One representative of two assays is shown.

Because there is evidence that particularly the CD4+ cells are critical to mount a successful immune response against tumor tissue [40], we divided the splenocyte cultures of one prime-boost experiment (no CT-pretreatment) and restimulated them either with a CTL epitope (RMA-E7) or with a Th epitope (soluble). Indeed, 5/5 animals reacted positively by IFN-γ Elispot assay (Th epitope $E7_{48–54}$) after the first in vitro restimulation, suggesting that CD4+ Th cells are also primed after E7SH DNA vaccination (Table 1).

In the context of the prime-boost immunized mice an E7WT reactive humoral immune response was found by ELISA in 10/10 animals (data not shown in detail, for summary see Table 1).

Together, these results show, that the E7SH gene of the second generation has per se – without the fused VP22 gene – the potential to induce an E7WT directed humoral immune response and a strong in vivo CTL response directed against the E7-specific immunodominant CTL epi-

Table 1
Summary of 5 independently performed immunization experiments in C57BL/6 mice

E7-DNA immunization	IFN-γ Elispot assay		^{51}Chrom-release-assay	Tumor-protection	Tumor-regression	Serum IgG
E7WT	10/12 ($E7_{49–57}$)		8/8	n.d.	n.d.	n.d.
E7SH	22/22 ($E7_{49–57}$)		8/8	n.d.	n.d.	n.d.
E7SH/E7SH (prime-boost)	$E7_{49–57}$ 10/10[a]	$E7_{48–54}$ 5/5[a]	n.d.	7/9	31/40[b]	10/10[c]

The table gives the numbers of positively evaluated animals in all performed IFN-γ Elispot assays, ^{51}Cr-release assays, tumor-experiments (protection and regression) and the serum IgG ELISA. The endpoint of the tumor protection experiment ($n = 1$) was 35 days after the first appearance of tumors, the animals of the regression experiments ($n = 4$) was sacrificed 32–48 days after the application of the C3 tumor cells, before first tumors reached sizes of 400 mm^2; n.d., not done.

[a] 5 individuals in the $E7_{49–57}$ restimulated group and all in the $E7_{48–54}$ restimulated group were CT-untreated.

[b] 20 mice were CT-untreated, of which 15 were scored as "regressors".

[c] 5 animals were CT-untreated.

tope $E7_{49–57}$ (Elispot assay). Induced T cells are cytolytic towards E7WT-expressing target cells (^{51}Cr-release assay) and react to the E7-specific Th epitope ($E7_{48–54}$, Elispot assay). Moreover, the potential of the E7SH gene to induce a cellular immune response in vivo is comparable to the original wildtype-gene.

3.3. E7SH-DNA immunization mediates tumor protection

Next we wanted to determine whether the E7SH gene was able to protect animals from an outgrowth of E7-expressing syngeneic tumors. Nine animals received twice (days 1 and 11) E7SH DNA, whereas the controls ($n = 10$) received the empty vector. Sixteen days after the boost-vaccination the animals were challenged with a tumorigenic dose of syngeneic E7WT-expressing C3 cells. After 35 days all animals of the control group had developed tumors with a size range of 100–400 mm^2. In the E7SH-treated group 7 animals had tumors between 0 and 25 mm^2 and therefore showed a clear protection from tumor growth (Table 1). The remaining two had tumors of 100–199 and 300–400 mm^2, respectively (mean of the treated group: 69 ± 44 mm^2), whereas the average tumor size within the control group was significantly higher (202 ± 21 mm^2, data not shown in detail).

3.4. The E7SH gene induces tumor regression

The aim of a therapeutic tumor vaccine is the induction of an effective immune response eradicating established tumors. Therefore, we next investigated if vaccination with the E7SH gene induces a cellular immune response able to control established E7-expressing tumor cells in vivo. In 4 tumor regression experiments a total of 80 animals were transplanted with a tumorigenic dose of syngeneic C3-tumor cells (day 0). When the tumors had reached a mean size of 25 mm^2 at days 5–18, 40 animals were primed with 100 μg of the E7SH-encoding plasmid, while control mice ($n = 40$) received empty pTHamp vector. About two weeks after the first immunization a booster vaccination (days 13–34) was given and further 12–27 days later the experiments were terminated when the tumor size in the first control mice reached 400 mm^2. The therapeutic effect was comparable in all 4 tumor regression experiments. Taken together, at days 32–48, 31/40 animals of the E7SH immunized group were classified as tumor-regressors (tumor sizes 0–25 mm^2) and in only 1/40 individuals a large tumor (300–400 mm^2) was detected. In contrast 31/40 mice of the control group had developed a tumor size between 26 and 400 mm^2 (300–400 mm^2: 20/40) (Fig. 3 shows one representative experiment of 4 performed; for summary see Table 1). These data clearly indicate that the E7SH gene of the second generation induces an effective cellular immune response that is able to control even established tumors.

Taking into consideration that our aim is the application of the therapeutic DNA-vaccine candidate in human beings, we also immunized the animals in two tumor regression experiments ($n = 20$ E7SH-encoding vector, $n = 20$ empty vector) omitting CT pretreatment. Within the E7SH vaccinated mice in the CT-treated cohort 16/20 animals and in the CT-untreated cohort 15/20 mice were defined as "tumor-regressors" (0–25 mm^2 tumor-size at the endpoint of the

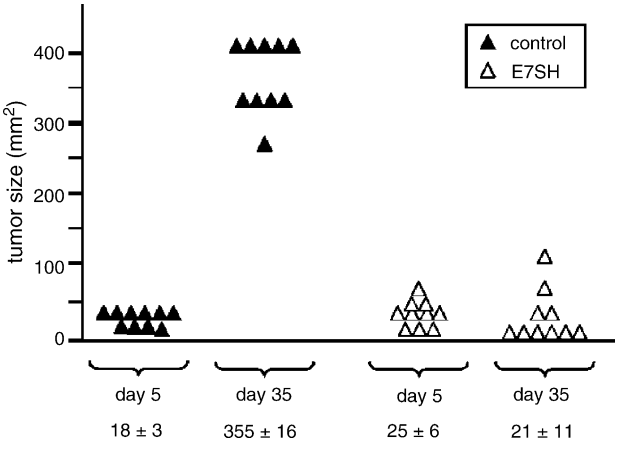

Fig. 3. Growth of C3 tumors in C57BL/6 mice after i.m. immunization with the E7SH gene. Mice received tumor cells and were immunized twice at days 5 and 21 with empty vector (black triangles) or with the E7SH-encoding pTHamp construct (white triangles). Data gives the surface tumor size at days 5 (first immunization) and 35. The mean ± S.E.M. at days 5 and 35 is shown. Because of the tumor size in the control group the experiment was terminated at day 35.

experiments). From these data we conclude that a CT pretreatment is not necessary to induce tumor-regression in this mouse model. Moreover, due to the fact that CT probably will never be allowed for use in humans, this finding has important implications in view of a clinical trial.

To understand if the E7SH-immunization is able to induce long lasting protective effects we re-challenged tumor free mice that had eradicated the tumors after E7SH DNA vaccination. Eight tumor regressors (tumor size = 0 mm^2) from two experiments (with CT pretreatment) were re-challenged with C3 tumor cells into the left flank (days 70 or 90, respectively, after first application of tumor cells). Forty-one or thirty-four days post re-challenge the two naive control animals of each re-challenge experiment had developed a tumor of 100 mm^2 in size. Six of eight of the tumor regressors remained without any palpable tumors. Two animals of the re-challenged groups, however, developed tumors with a size of 100 mm^2. Taken together, these findings indicate the induction of a HPV-16 E7-specific memory in some of the tumor regressors.

3.5. HPV-16 E7SH transduced human DCs induce specific CTL responses to E7WT in vitro

As a first approach to transfer the E7SH gene of the second generation to the human system, we used an in vitro immunization protocol. Human monocyte-derived DCs of 4 donors were nucleofected with the plasmids pTHamp-E7WT and pTHamp-E7SH, respectively, or a control plasmid pCMV-EGFP. Blood donors were overall healthy individuals and buffy coats were anonymously obtained from the blood bank Suhl, Germany. For ethical and regulatory reasons, there was no information available on sex or previous or current HPV infection. However, given the high rate of infection with HPV, it cannot be excluded that they have had contact with the virus before.

Expression of the target antigen was verified by Northern blot analysis, RT-PCR, and Western blot analysis of transduced DCs. In all assays specific expression of the E7WT and E7SH was detected (Fig. 4a–c). In order to prove efficient processing and presentation of the recombinant antigen, we

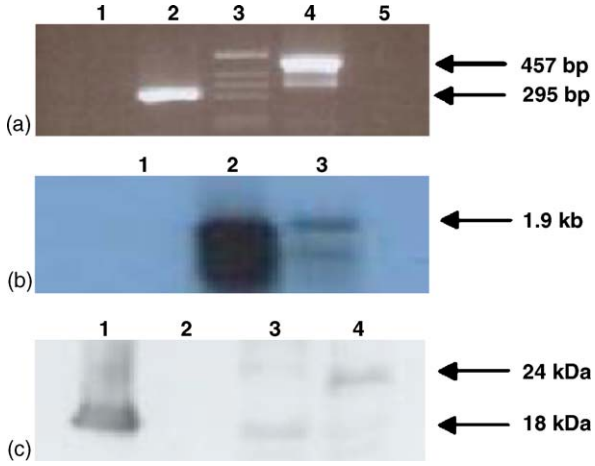

Fig. 4. Transcription and translation of nucleofected genes in human DCs. (a) RT-PCR analysis of nucleofected dendritic cells. Transfection of: (1) pCMV-EGFP amplified with E7WT primers; (2) pTHamp-E7WT amplified with E7WT primers; (3) Standard; (4) pTHamp-E7SH amplified with E7SH primers; (5) pCMV-EGFP probed with E7SH primers). (b) Northern blot hybridisation of nucleofected DCs. (1) pCMV-EGFP; (2) pTHamp-E7WT; (3) pTHamp-16 E7SH. (c) Western blot analysis of (1) CaSki cells, and nucleofected DCs (2) pCMV-EGFP; (3) pTHamp-E7WT; (4) pTHamp-E7SH. Representative results of one of two independent experiments are shown.

generated T cell lines specific for the E7WT antigen by three stimulations with 35mer peptide-pulsed autologous DCs. The T cell lines ($n = 4$) were then restimulated once with DNA vaccine-nucleofected autologous DCs. Specific IFN-γ secretion was detected by Elispot in 3 of 4 independently generated T cell lines (Table 2). To show that DNA vaccine-transduced DCs can also prime resting naive T cells the experiment was repeated inversely. T cells were three times stimulated with DNA-transduced DCs and the resulting T cell lines tested by restimulation with peptide-pulsed DCs. In parallel priming with peptide-loaded DCs was performed as a positive control. E7-specific T cells were induced by peptide-loaded DCs from all 4 donors. DNA-nucleofected DCs primed 3 of 4 donors. As shown in Fig. 5a the frequency of specific T cells was between 0.25 and 0.4% as calculated from Elispot analysis

Table 2
Specific INF-γ release of E7WT-specific T cell lines upon restimulation with E7SH nucleofected DCs

	Restimulation with HPV-16 E7WT 35 mer peptides pulsed DC			Restimulation with pTHamp-E7SH transfected DC		
	Number of spots/5 × 10^4 T cells		p	Number of spots/5 × 10^4 T cells		p
	Control	Specific reaction		Control	Specific reaction	
Donor 1	301 (±9)	>600[a]	<0.01	198 (±155)	353 (±30)	<0.12
Donor 2	402 (±27)	>600[a]	<0.01	275 (±2)	427 (±6)	<0.01
Donor 3	319 (±14)	>600[a]	<0.01	386 (±9)	445 (±0.7)	<0.01
Donor 4	247 (±40)	>600[a]	<0.01	91 (±4)	>600[a]	<0.01

T cell lines were stimulated three times with HPV-16 E7WT 35 mer peptide pool. For Elispot assay cell lines were restimulated once with autologous DCs pulsed with 35 mer peptide pool or nucleofected with pTHamp-E7SH. As controls DCs were pulsed with control peptide or transfected with CMV-EGFP. Number of spots (mean of duplicates) are shown and p-values were calculated by Student's t-test. Reactions were regarded as statistically significant when $p < 0.05$.

[a] Spot number was above resolution of the Elispot reader.

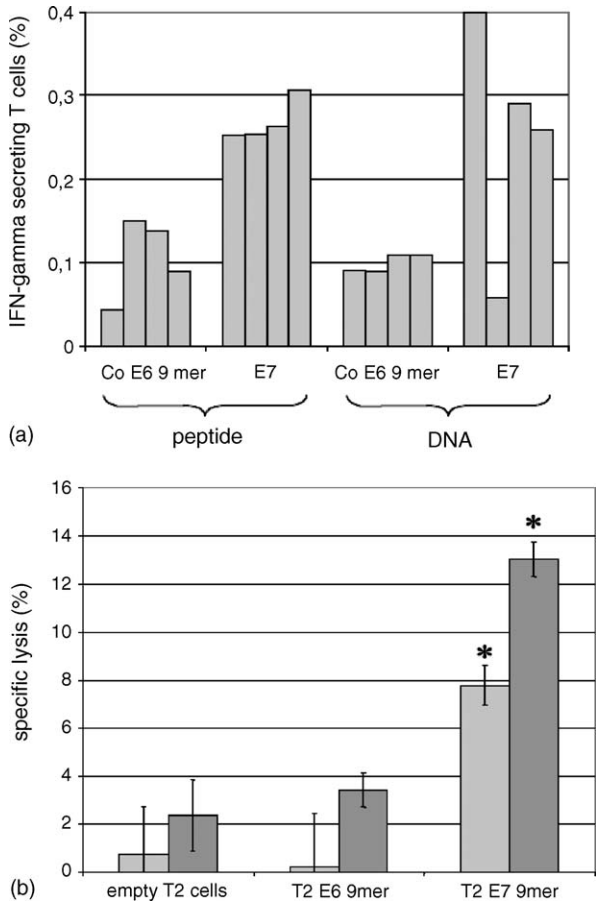

Fig. 5. CTL response of human T cells against HPV-16 E7 after priming with pTHamp-E7SH transduced or 35mer peptides-loaded DCs. Human T cells were stimulated three times with pTHamp-E7SH or HPV-16 E7WT 35 mer peptides. (a) INFγ Elispot was performed three weeks after the third restimulation. Percent of INFγ-secreting T cells of individual donors is shown. HPV-16 E7WT 35 mer peptide or pTHamp-E7SH primed T cell lines were restimulated with control peptide (HPV-16 $E6_{28–36}$) or with HPV-16 E7WT 35 mer peptides. (b) Flow-cytometric cytotoxicity assay of a representative donor after two additional restimulation rounds with 9 mer peptides. T2 cells were used as target cells and were pulsed with HPV-16 $E6_{28–36}$ 9 mer control peptide or HPV-16 E7WT 9 mer peptide pool before they were added to the peptide primed T cell line (light bars) or DNA primed T cell line (dark bars). The *p*-value were calculated by Student's *t*-test ($^{*}P < 0.02$).

and was comparable between peptide and DNA induced T cell lines. Interestingly, T cell lines primed with peptide-loaded DCs had 60–80% of CD4 cells whereas DNA-loaded DCs induced 50–70% CD8 T cells ($p = 0.035$, data not shown).

To enhance the proportion of specific CTL in the T cell lines for a functional cytotoxicity assay, we restimulated the responding T cell lines twice with autologous B-LCL pulsed with HLA-A2 restricted 9mer epitopes of the E7WT. To exclude de novo priming antigen inexperienced T cell lines of the same donors were restimulated in parallel (not shown). All T cell lines generated with 35 mer peptide-pool or with DNA-transduced DCs had significant and specific lytic capacity (Fig. 5b). These data demonstrate that the pTHamp-E7SH DNA-loaded DCs, as expected, do prime T cells recognizing epitopes derived from the E7WT antigen.

3.6. The synthetic E7SH gene has lost measurable oncogenic properties in soft agar transformation assays

It was shown that E7-transformed cells are capable of growing in an anchorage independent manner in soft agar resulting in the formation of foci [41]. To verify our hypothesis that the rearranged oncogene has lost its transformation properties we infected murine fibroblasts (NIH3T3) with recombinant ecotropic retroviruses encoding the E7WT gene or the E7SH gene. Indeed, the HPV-16 E7WT gene alone is sufficient to induce "transformation" in immortalized rodent fibroblast [42], whereas the co-transfection of the HPV-16 E6 and E7WT genes leads to a more effective transformation [37,43]. To take advantage of this enhancement in assay sensitivity we decided to co-infect the cells with a corresponding HPV-16 E6 recombinant retrovirus. A set of triplicates/transfection were performed and the following transfections were performed: E7WT ± E6, E7SH ± E6. Infections with empty vector served as negative controls (see Table 2). Foci (>8 cells) were counted after 4 weeks.

As a result of two experiments we found that the E7WT gene transforms NIH3T3 cells very efficiently (17.7 and 13.6% transformed cells, respectively). The co-transfection together with the E6 gene enhanced the transformation capacity as expected (21.6 and 17.9%). In contrast, in both E7SH and E6/E7SH infected cultures, the percentage of transformed cells was low (0.4 and 0.6% or 0.2 and 0.8%) and comparable to the negative controls (empty vector: 0.3 and 0.2%). In summary, the synthetic E7SH gene of the second generation failed to induce an anchorage independent growth in mouse fibroblasts after retroviral infection, indicating the loss of its transforming properties, while the E7WT gene induces foci formation (see Table 3).

Table 3
Soft-agar-transformation assays: results of two independent infection sets

Experiment	Plasmid-combination			Average foci ±S.E.D.	Transformed cells (%)
	E6	E7WT	E7SH		
A	−	−	−	7 ± 4	0.3
	−	+	−	407 ± 109	17.7
	−	−	+	9 ± 3	0.4
	+	+	−	497 ± 127	21.6
	+	−	+	5 ± 5	0.2
B	−	−	−	4 ± 4	0.2
	−	+	−	312 ± 64	13.6
	−	−	+	13 ± 2	0.6
	+	+	−	412 ± 160	17.9
	+	−	+	9 ± 4	0.8

Triplicates of each transfection (empty vector, E7WT, E7SH, E7SH + E6, E7WT + E6) were performed in each experiment (A, B), respectively. After 4 weeks all foci located within a 9 cm^2 field of the soft agar plate were counted. Data given are the mean of foci numbers of the triplicates ± standard deviation. The transformation efficiency was calculated as the percentage of foci number (right panel).

4. Discussion

In this study we have demonstrated that a rearranged oncogene is able to induce a cellular immune response directed against the wildtype antigen in mice and potentially in humans, but lacks measurable transforming properties as shown by soft agar transformation assays after retroviral infection of murine fibroblasts. For our DNA-immunization experiments we selected the HPV-16 E7 oncogene that is a candidate for a therapeutic vaccine. It is permanently and exclusively expressed in cc as well as in its precursors (CIN) and represents, therefore, a specific target for immune therapy. In fact, the Human Papillomavirus Type 16 alone causes about 50% of all cc on a global scale. An attractive approach for adjuvant therapy may be the development of a HPV-specific vaccine.

Vaccines for HPV therapy in clinical trials were mostly peptide and protein- or vector-based (for review see 1). These vaccination strategies show potential disadvantages, e.g. the HLA-restriction in the case of peptide application or the fact that immunization with soluble protein induces a Th2-biased response lacking efficient anti-tumor efficacy. When viral or bacterial vectors are used, the induction of neutralizing antibodies hampers potentially the clinical efficacy and, moreover, the use of these vectors bears the risk of potential pathogenicity, especially in immunocompromised patients [2]. In contrast, the application of naked DNA combines advantages like low costs (predictable production cost, no need of cold chain) and safety in patients. In contrast to peptide vaccination no prior knowledge of the patients HLA-make-up is necessary, because the complete coding sequence of the gene is applied.

Indeed, the application of DNA was shown to induce both antigen-specific cellular and humoral immune responses by several investigators [44]. Following injection of plasmids, expression of the encoded antigen was reported to last for up to 19 months [45]. Professional APCs are either transfected directly and express the antigen themselves [46] or they take up the protein-antigen by phagocytosis after expression and secretion or lysis by other cells, e.g. myoblasts [47].

Because the HPV-16 E7WT gene shows transforming activity it is not applicable as vaccine in humans. Different efforts were undertaken to knock out these properties [15–18], but all of them are potentially associated with the loss of naturally occurring epitopes and consequently, they may compromise broad effectiveness in the population.

In a first approach we showed in a "proof-of-principle"study that vaccination with a rearranged HPV-16 E7WT gene (first generation E7SH) was able to induce E7WT-specific CTLs without the loss of naturally occurring CTL epitopes [19]. In this construct the immunodominant $H2^b$-restricted epitope $_{49}$RAHNIVTF$_{57}$ was unaltered. However, one of the three known HLA-A2 restricted epitopes, $_{86}$TLGIVCPI$_{93}$, had been lost in the core element by the dissection process. It was only rescued by the addition of the appendix underlining the potential benefit of our construct.

Because the HPV gene of the proof-of-principle study has induced only a moderate CTL-response, we enhanced its immunogenicity by the fusion to the Herpes Simplex Virus Type 1 derived VP22 gene [20]. With regard to our aim to transfer the therapeutic DNA vaccine candidate to clinical trials we renounced of fusing any virally derived gene in our current approach. Indeed, by the use of the pTHamp immunization vector and the Kozak-sequence in front of the gene, the HPV-16 E7SH gene of the second generation is highly immunogenic in mice and man. More importantly, the synthetic gene of this study was modified in a more educated way than the previously published shuffled gene where we dissected the segments without considering biologically relevant functions. Here, we selectively dissected the E7WT-gene within the three sites associated with transforming properties (pRB-binding site, Cys-X-X-Cys motifs). To face the potential concern that shuffled oncogenes could revert to the wildtype by recombination between the core and the repetitive sequences in the appendix, we used codon-optimization within the core element only, producing different nucleotide sequences in both regions.

In view of clinical application, our aim was also to avoid CT in the in vivo immunization experiments [49]. CT is obtained from the snake *Naja nigricollis* and causes inflammation within the muscle [50,51], which should recruit immune cells. Attracted antigen presenting cells (APC) are expected to present the DNA-encoded antigen. Due to its potential toxic effects CT will probably never be allowed for use in humans. Therefore, we decided to inject the HPV-16 E7SH gene without any adjuvants in the murine model. Here, we show, that CT pretreatment is neither necessary for CTL-induction nor for tumor-regression.

Different routes of DNA administration have been investigated, e.g. intradermal (i.d.) and i.m. applications. It is well known that the route of application has a great impact on the quality of the immune response, whereby the i.m. application favours a cellular immune response [48], with obvious advantages for cancer therapy. Since we intend to develop our vaccine candidate for clinical application we decided to investigate the i.m. vaccination route in the animal model only.

A critical point in DNA immunization studies is the choice of the vector backbone in the pre-clinical phase. Most vectors are only available with the ampicilline-resistance gene and are therefore obsolete for use in humans. Since only traces of residual β-lactam antibiotic could trigger an anaphylactic shock syndrome in predisposed patients [52]. Furthermore, the ampicilline-resistance gene could be taken up by physiologically occurring bacteria after plasmid-application [52].

The vector used in our study is also available in a version, which is propagated without any antibiotic resistance gene [52] and has already been applied to humans [26]. One further advantage is the presence of unmethylated cytidine phosphate guanosine motifs (CpG-motifs), capable of inducing both maturation and acitvation of professional APCs [53]. It was also shown, that unmethylated CpG motifs within the plas-

mid backbone are critical to induce cytolytic T cells to secrete IFN-γ [54] and stimulate professional APCs to induce Th1 cytokines [55]. Therefore, immunestimulating DNA should be very useful in the design of an antitumor vaccine.

Another concern regarding the safety of DNA vaccines, is integration of plasmid DNA leading to an induction of oncogenes or inactivation of tumor suppressor genes. In mouse experiments, however, it was shown that even under the most unfavourable conditions the mutation rate is undetectable, i.e. at least 3000 times below the frequency of spontaneous mutations [56]. In clinical trials mostly HIV genes are being tested as antigens. No severe side effects or induction of DNA-specific antibodies were reported [57]. In contrast, a humoral immune response after DNA immunization was found in a mouse model [58]. The number of anti-DNA IgG secreting B cells increased by two to three fold shortly after vaccination but no symptoms of autoimmunity were detected [58]. Thereby, the risk of autoimmune reactions following the application of a HPV-16 E7 DNA vaccine candidate is considered to be very low since no homology between the viral and cellular proteins is known.

DNA vaccination is well tolerated in practice as is shown by the first HPV-directed therapeutic DNA vaccine trial: Zycos announced the completion of a phase 2b clinical trial. The vaccine ("ZYC101a") consists of several minigenes encapsidated into Zycos' proprietary microparticles that code for HPV-specific peptides. The packaging is assumed to facilitate the transport of the DNA to the APCs and lymphoid tissues. One hundred and sixty-one patients with biopsy-proven high-grade cervical dysplasia were included into this randomized, double blind, and placebo-controlled trial. The vaccine was reported to be safe and well tolerated, but regression of the lesions was statistically significant only in women below 25 years of age with 70% versus 23% in the placebo group [59].

In a first attempt to test for immunogenicity of the E7SH gene in the human system, we have used DNA-transduced autologous DCs as immunogens in "in vitro immunization" protocols. Expression in DCs was verified and processing and presentation tested by functional T cell stimulation assays. Since priming of naive T cells was found the vaccine candidate holds promise for induction of an active immune response also in humans.

Cytolytic T cells were induced reacting to HLA-A* 0201 restricted E7 epitopes. We have not analyzed in detail the epitope-specific response in the human T cell lines. A broadly applicable vaccine should not be HLA-restricted to a single transplantation antigen. We have, therefore, tested the T cell response in human T cell lines to the complete antigen by stimulation with transfected or long peptide pool-pulsed DCs representing all possible epitopes. This is important, because HLA types other than A* 0201 will contribute to rejection of HPV-positive cells. We found significant Th1 responses also in HLA-A* 0201 negative blood donors and in cervical cancer patients after induction with autologous DC pulsed with recombinant E7 protein [24].

The efficiency of an immune response induced in humans upon passive uptake of the DNA by DCs ex vivo remains to be investigated. Also the potential alteration of the cellular physiology by the artificial protein needs further attention. It is interesting to note, that priming by peptide-loaded DCs induced a bias for CD4 T cells, while DNA-transfected DCs rather supported outgrowth of CD8 T cells. This might be explained by the route of antigen presentation favouring the exogenous MHC class II pathway in the case of long peptides, or the cytosolic MHC class I pathway by the DNA vaccine. Another explanation would be the twist towards a Th1 response by an adjuvant effect of CpG motifs in the DNA construct and consequently preferential outgrowth of CTLs. These CD8 T cells had lytic activity and a high frequency in the T cell lines.

Taken together, our newly developed vaccine candidate combines several advantages, i.e. the preservation of all putative CTL-epitopes and therefore applicability to all patients independently of their HLA-make-up. Additionally several safety features were introduced like: (i) no need of additional genes to enhance immunity; (ii) specific dissection of function-associated sites of the wildtype gene; and (iii) codon-optimization of the core element only. Indeed, the construct is ready for a first clinical trial in the near future and is considered for further development of an HPV-specific vaccine in humans.

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