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DNA vaccine against human papillomavirus type 16: Modifications of the E6 oncogene

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ABSTRACT

Since its discovery, DNA vaccination has become an effective strategy for the development of vaccines against cancer including cervical carcinoma (CC). The formation of CC is associated with human papillomavirus (HPV) infection. Viral E6 and E7 oncoproteins are suitable targets for therapeutic vaccination. To adapt the HPV16 E6 oncogene for DNA immunisation, we performed several modifications. First we fused the E6 gene with the 5′ or 3′-terminus of the *Escherichia coli* β-glucuronidase (GUS) gene and showed enhanced immunogenicity of the 3′ fusion (GUS.E6). Then, as the E6 oncogene contains two alternative introns that result in the production of truncated forms of the E6 protein, we abolished the 5′ splice site in the E6 gene. This modification completely eliminated the expression of the truncated E6 transcripts and thus increased the production of the full-length E6 protein. At the same time, it moderately reduced the immunogenicity of the modified non-fused (E6cc) or fused (GUS.E6cc) genes, probably as a consequence of the substitution in the immunodominant E6 epitope following the abolishment of the splice site. Furthermore, we reduced the oncogenicity of the E6 protein by two point mutations (E6GT) that, together, prevented E6-mediated p53 degradation. Finally, we constructed the GUS.E6GT gene characterized by enhanced safety and immunogenicity when compared with the wild-type E6 gene.

1. Introduction

Persistent infection with human papillomaviruses (HPV) is the main etiological factor in cervical cancer, the second most common cancer in women worldwide. Oncogenic high-risk (HR) HPV genotypes 16 and 18 are responsible for approximately 70% of all cervical cancers. Recently, two prophylactic vaccines based on virus-like particles (VLPs) produced by recombinant technology and protecting against infection with HPV16 and HPV18 have been licensed [1]. However, the development of therapeutic vaccines is still a topical problem, as preventive vaccination is of limited use and cannot cope with current HPV infection [2]. Since the viral oncoproteins E6 and E7 that are constitutively produced in all HPV-infected cells and that contribute to the transformation of epithelial skin or mucosal cells are also necessary for the maintenance of the transformed state [3], they became promising targets for the development of the therapeutic HPV vaccines.

DNA vaccines represent a potential form of antigen-specific immunotherapy of tumours because they can induce cytotoxic T-lymphocyte (CTL) response [4]. However, the low efficacy of DNA immunisation hampered its clinical use. Several strategies enhancing immunogenicity of the DNA vaccines have been developed including the modification of an antigen-encoding gene [5]. For clinical use of DNA vaccines, their safety must also be carefully considered. In our previous studies, we focused on the modification of the HPV16 E7 oncogene. To reduce its transformation potential, we altered it by point mutations resulting in the substitution of three amino acids in the pRb-binding site of the E7 protein [6]. Furthermore, to enhance its immunogenicity, we fused the modified E7GGG gene with sequences encoding sorting signals of lysosome-associated membrane protein 1 (LAMP-1), *Escherichia coli* β-glucuronidase (GUS) or mouse heat shock protein 70 (Hsp70) and demonstrated a superior antitumour effect of the E7GGG.GUS chimeric construct [7–9]. As the E7 oncoprotein is a relatively small protein (98 amino acids) with a limited number of epitopes [10] and immunity against the E6 oncoprotein (158 amino acids) is more readily induced in HPV16-infected people and is probably more important for the elimination of infected cells [11,12], E6 should also be included in the therapeutic HPV vaccines.

The HPV16 E6 oncoprotein is a multifunctional protein with several cellular targets. The first identified target, and apparently the most relevant, is the p53 tumour suppressor protein that can promote cell cycle arrest or apoptosis in infected cells. To overcome this obstacle, the E6 protein abrogates the functions of the p53 protein by inducing its degradation through the ubiquitin-proteasome pathway. The cellular E6AP ubiquitin ligase that binds both E6 and p53 plays a critical role in this process. Furthermore, the E6 oncoprotein can inhibit p53 activity independently of inducing

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its degradation [13]. Mutations that affect E6 binding with p53 or α-helix partners, including E6AP, have been shown to reduce E6 oncogenicity in *in vivo* assays [14–16].

In the HPV16 E6 open reading frame (ORF), one 5′ (donor) splice site and two 3′ splice (acceptor) sites have been identified. Alternative splicing results in the production of E6*I and E6*II transcripts from which truncated E6*I and E6*II proteins are transcribed. The E6*I transcript is the most abundant E6 mRNA in cervical cancer, premalignant lesions and cancer-derived cell lines [17–19].

In this study, we modified the E6 oncogene by fusion with GUS and by mutagenesis aiming at abolition of alternative splicing in the E6 gene or at reduction of the ability of the E6 protein to induce p53 degradation. The immunogenicity of the constructs was evaluated in mice after DNA immunisation by a gene gun.

2. Materials and methods

2.1. Animals

Female C57BL/6 mice (H-2^b; Charles River, Germany), 6–8-week-old, were used in the immunisation experiments. All animals were maintained under standard conditions and in accordance with the guidelines for the proper treatment of laboratory animals at the Center for Experimental Biomodels, Charles University, Prague.

2.2. Cell lines

Human embryonic kidney 293T cells transducted with simian virus 40 (SV40) large T antigen [20] were supplied by courtesy of J. A. Kleinschmidt, DKFZ, Heidelberg, Germany. TC-1 cells (kindly provided by T.-C. Wu, John Hopkins University, Baltimore, MD) were prepared by transformation of C57BL/6 mouse primary lung cells with the HPV16 E6/E7 oncogenes and human activated *H-ras* [21]. Saos-2 cells (obtained from DSMZ, Braunschweig, Germany) were established from primary human osteogenic sarcoma [22]. All cells were grown in Dulbecco's modified Eagle's medium (D-MEM; PAA Laboratories, Linz, Austria) supplemented with 10% foetal calf serum (FCS; PAA Laboratories), 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin.

2.3. Plasmids

The construction of the plasmids pBSC and pBSC/E7GGG.GUS was described previously [6,8]. The plasmid pEA16E6 (kindly provided by F. Momburg, DKFZ, Heidelberg, Germany) contains the HPV16 E6 oncogene that was excised with *Eco*RI and *Bam*HI and cloned into pBK-CMV (Stratagene, La Jolla, CA) between the *Eco*RI and the *Bam*HI sites generating the pBK/E6 plasmid. The plasmid pBSC/E6 was constructed by excision of the E6 gene from pBK/E6 with *Sal*I and *Xho*I and its ligation into the *Xho*I restriction site of the pBSC plasmid. The plasmid pBSC/GUS [8] was modified by mutagenesis to generate the pBSC/GUS-STOP vector with the abolished termination codon of GUS and the *Hind*III site added [23].

For the generation of the pBSC/E6.GUS plasmid, the E6 gene was amplified from pBK/E6 using primers 5′-CTGACCCGGGGCCG-CCATGCACCAAAAGAGAACTG-3′ (forward) and 5′-CTAGCCCGGG-CCCAGCTGGGTTTCTCTACGT-3′ (reverse). The PCR product was digested with the *Xma*I restriction enzyme (underlined sequences in primers) and cloned into pBSC/E7GGG.GUS between the *Xma*I sites thus replacing E7GGG. To construct pBSC/GUS.E6 plasmid, the DNA fragment encoding E6 was amplified from pBSC/E6 using primers 5′-CACAAGCTTTGATGCACCAAAAGAGAACTGC-3′ (forward) and 5′-CACAAGCTTTTACAGCTGGGTTTCTCTACG-3′ (reverse), digested with *Hind*III (underlined sequences in primers) and ligated into the *Hind*III site of the pBSC/GUS-STOP plasmid.

The pBSC/EGUS plasmid with an eliminated initiation codon of the GUS gene was prepared previously [23]. The pBSC/E6.EGUS plasmid was constructed as follows: the E6 gene was excised from the pBSC/E6.GUS plasmid with the *Xma*I restriction enzyme and subsequently ligated into the *Xma*I site of pBSC/EGUS.

The E6 gene was mutated with the Altered Sites® II Mammalian Mutagenesis System (Promega, Madison, WI). The 5′ splicing site was abolished by two point mutations introduced with the oligonucleotide 5′-AAGTCATATAGCTCGCGTCGCAGTA-3′ (underlined nucleotides represent the mutations) resulting in the E6cc gene (Fig. 1). This mutation was also introduced into the E6.GUS fusion gene.

Substitutions C70G and/or I135T were created using the oligonucleotides 5′-CATTTATCACCTACAGCATA-3′ and 5′-CCGACCCCTTGTATTATGGA-3′, respectively. The resultant genes with one or both mutations were designated E6G, E6T and E6GT (Fig. 1).

The mutated genes were cloned between the *Eco*RI sites of the pBSC plasmid. The modified GUS.E6 fusion genes were prepared by the amplification of the mutated E6 genes from the pBSC/E6cc and pBSC/E6GT plasmids and their subsequent introduction into the *Hind*III site of pBSC/GUS-STOP.

The accuracy of all modified genes was confirmed by DNA sequencing.

The p53 expressing plasmid, pcDNA3.1wtp53, was kindly provided by M. Brazdova (Institute of Biophysics, Brno, Czech Republic). pTR-UF2 is a green fluorescent protein (GFP) expression plasmid [24].

The plasmids were propagated in *E. coli* XL1-blue strain cultured in Luria Broth Medium with 100 μg/ml of ampicillin added and purified with the Qiagen Plasmid Maxi Kit (Qiagen, Hilden, Germany).

2.4. Reverse transcription PCR

293T cells (7×10^5) were grown on 6-cm plates and transfected by modified calcium phosphate precipitation in HEPES-buffered saline solution [25] with 6 μg of plasmids. Total RNA was extracted from transfected cells after 2 days of incubation by the RNeasy Kit (Qiagen) following the manufacturer's instructions. Two μg of RNA were incubated with 2 U of RNase-Free DNase (Promega) in a 20-μl reaction volume containing 1× reaction buffer (Promega) at 37 °C for 30 min. DNase was stopped with 2 μl of DNase stop solution (10 min at 65 °C; Promega). Reverse transcription was performed with 11 μl of DNase-treated RNA in a 20-μl reaction volume containing 1.25 μM oligo(dT) primer, 0.5 mM of each dNTP, 1× reaction buffer (Promega) and 200 U of M-MLV reverse transcriptase (Promega). PCR amplification of E6 cDNA was performed with HotStarTaq DNA polymerase (Qiagen) and Q-solution (Qiagen) using E6-derived primers 5′-GCAAGCAACAGTTACTGCGA-3′ (forward) and 5′-GCTGGGTTTCTCTACGTGTT-3′ (reverse). The lengths of the expected products were 357, 174, and 57 bp. The reaction was performed in a PTC-200 thermal cycler (MJ Research, Watertown, MA): activation of the polymerase at 95 °C for 15 min; 40 cycles of denaturation at 94 °C for 1 min, annealing at 60 °C for 1 min, and elongation at 72 °C for 1 min; incubation at 72 °C for 5 min. Amplification of β-actin served as an internal control [7]. The amplified products were separated in a 3% agarose gel, stained with ethidium bromide, photographed under UV light and analysed with ScanPack 3.0 software (Biometra, Göttingen, Germany) for quantification of the PCR products.

2.5. Immunoblotting staining of E6-containing proteins

293T cells were transfected with 6 μg of the appropriate plasmids and after 2 days, the cells were collected and lysed on