Intracellular scFvs against the viral E6 oncoprotein provoke apoptosis in human papillomavirus-positive cancer cells

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ABSTRACT

The E6 protein of Human papillomavirus type 16 (16E6) is involved in the tumorigenesis of human cervical cells by targeting numerous cellular proteins. We have designed a strategy for neutralizing 16E6 based on the intracellular expression of single-chain Fv antibodies (scFvs) specific to 16E6. Recombinant adenovirus vectors were constructed to allow expression of two 16E6-binding scFvs and one 16E6-non-binding scFv in HPV16-positive and -negative cells. Expression of the scFvs provoked two types of effects: i) inhibition of proliferation of all cell lines tested, this aspecific toxicity being likely due to the aggregation of unfolded scFvs. ii) apoptosis observed only in HPV16-positive cervical cancer cell lines after expression of 16E6-binding scFvs, this specific effect being proportional to the intracellular solubility of the scFvs. These data demonstrate the feasibility of intracellular immunization with anti-16E6 scFvs and highlight the importance of the solubility of the intracellular antibodies.

INTRODUCTION

Cervical cancer is the second most common cause of cancer fatality in women worldwide. More than 99% of these cancers are linked to viral infection with high-risk human papillomaviruses (HPVs) [1]. Epidemiological studies have demonstrated that HPV16 is the most prevalent type corresponding to more than 50% of HPVs detected in cervical cancers [2]. The oncogenic effect of these viruses is principally due to the expression of two early viral proteins, E6 and E7, which play a crucial role in immortalization and transformation of the host cells by interfering with cellular proteins involved in the regulation of the cell cycle [3]. The 16E6 is a small zinc-binding protein, the solution structure of which has revealed a novel zinc-binding fold [4]. The stimulation of the ubiquitin-mediated degradation of the p53 tumor suppressor protein via interaction with the cellular E6AP ubiquitin-protein ligase was the first described activity of the high-risk HPV E6 proteins associated with carcinogenesis [5]. However, in addition to E6AP and p53, many cellular targets of high-risk HPV E6 protein have been identified [6, 7] and it appears that some 16E6-induced cell-transformations in tissue culture and *in vivo* are independent of p53 degradation [8-10].

The 16E6 protein is sufficient to induce immortalization of human mammary epithelial cells [11] and continued expression of this protein is required for the maintenance of the transformed phenotype of HPV16-positive cervical cancer cells [12]. Therefore, 16E6 represents an ideal target for therapeutic strategies of HPV16-positive cervical carcinoma and different approaches such as antisense RNA [13-16], ribozymes [17], siRNA [18-20], peptide aptamers [21] and intracellular antibodies [22] have been used to down regulate or inactivate E6 in HPV-positive cervical cancer cells. The decrease of E6 activity observed in HPV-transformed cells led to inhibition of cellular proliferation [13, 15-17] or apoptosis [14, 18, 19, 21, 22]. These findings confirmed the suitability of targeting the E6 protein as a possible therapeutic approach for carcinoma of the cervix.

Materials and methods

Cell lines. The following cell lines were used in this study: SiHa, CaSki (both derived from a HPV16 integrated human cervical carcinoma), HeLa (derived from a HPV18 integrated human cervical adenocarcinoma), A549 (derived from a human epithelial lung carcinoma). All the above cells were cultured in monolayer cultures in Dulbecco's modified Eagle's medium (DMEM) supplemented with 2 mM L-glutamine, 10% (v/v) foetal calf serum, 50 µg/ml penicillin and 50 µg/ml streptomycine (Invitrogen).

Expression of scFvs and dot-blot assays. The scFvs fused to the B10 tag were expressed in the periplasm of *E. coli* XL1-Blue as described [23]. Equivalent amounts (10 µL) of periplasmic extracts from bacteria transformed with scFv-expressing plasmids were loaded on SDS-PAGE and analyzed by Western blot using the B10 monoclonal antibody and peroxydase-labeled goat anti mouse IgG. Nitrocellulose strips carrying dots (50 ng) of either the Maltose Binding Protein (MBP) or 16E6 fused to MBP (MBP-E6) were incubated with 2 mL of periplasmic extracts. Binding of scFv to 16E6 was revealed using the B10 monoclonal antibody and peroxydase-labeled goat anti mouse IgG.

Recombinant adenoviruses. Coding sequences of 1F4-L177P, 1F4 and 1F4-P41L scFvs fused to a B10 tag [24] under the control of the CMV promoter were inserted in replacement of the E3 region into the adenovirus 5 genome using homologous recombination in *E. coli* as described [25], Fig. 2A). The recombinant adenoviruses were produced and purified after transfection of 293 cells [25].

Immunofluorescence analysis. Twenty-four hours after transduction with recombinant adenoviruses at a MOI of 100 plaque-forming-units (pfu)/cell, cells were fixed and analysed by immunofluorescence as previously described [26]. The scFvs were revealed by the B10 monoclonal antibody and by a FITC-conjugated anti-mouse secondary antibody. The nuclei of the cells were stained with Hoechst 33258 (Molecular Probes).

Western blot analysis. Twenty-four hours transduction with recombinant adenoviruses at a MOI of 100 pfu/cell, cells were detached with a rubber policeman and incubated 30 min on ice in lysis buffer (150 mM NaCl, 20 mM Tris/HCl pH8.0, 2 mM DTT, 1% NP-40 containing protease inhibitor mix "Complete™" from Roche Applied Science). After a 30-min centrifugation at 12 000 rpm, cellular extracts were fractionated into soluble (supernatant) and insoluble (pellet) forms. Equivalent amounts of soluble or insoluble extracts were analyzed by SDS/PAGE 12% and Western blotting. The scFvs were revealed with the B10 monoclonal antibody and peroxydase-labeled goat anti mouse IgG. The percentages of soluble and insoluble scFvs were quantified using the Quantity-One software (Bio-Rad).

Methylthiazolyldiphenyl-tetrazolium bromide (MTT) assays. To measure proliferation, cells were seeded into 96-well plates one day before transduction. At 50% confluency, cells were transduced with recombinant adenoviruses at a MOI of 100 pfu/cell. After 12 h, 24 h or 48 h of transduction, cells were washed with PBS and incubated at 37°C with 100 µL of a 500 µg/ml MTT solution in media. Two hours later, the blue formazan crystals were dissolved by adding 100µL of MTT stop solution (64% isopropanol, 32% butanol-2, 40 mM HCl) and the absorbance at 595nm was measured.

Apoptosis. Cells were stained with annexin V-FITC (Annexin V-FLUOS Staining kit, Roche Diagnostics) and propidium iodide according to the manufacturer instructions 24h or 48h after transduction by recombinant adenoviruses at a MOI of 100 pfu/cell. Cells were analysed on a FACScalibur (BD-Biosciences) using a dual filter for FITC and propidium iodide. In this test, live, apoptotic and necrotic cells are distinguished on the basis of annexin V-FITC binding and PI exclusion: live cells are not stained with any of the reagents, apoptotic cells are positive for annexin V binding and negative for PI whereas necrotic cells are stained with both reagents

RESULTS AND DISCUSSION

scFvs are small functional antibody fragments consisting of the heavy and light chain variable (V_H and V_L) segments held together by a flexible linker that possess the binding and specificity of the whole antibody [27]. During the antibody production, the formation of intra- and inter-chain disulfide bonds inside the endoplasmic reticulum allows the stabilization of each immunoglobulin domain and the assembly of heavy and light chains. The intracellular immunization strategy [28] aimed at neutralizing an antigen inside cells with intracellular scFvs (intrabodies) [29] requires the expression of functional scFvs in the cytoplasm of the target cells. However, the reducing environment of this compartment does not allow the formation of intra-domain disulfide bonds within the V_H and V_L [30-32]. As a result the main challenge of intracellular immunization remains the correct folding and stability of scFvs expressed in the cytoplasm. In previous reports, we described monoclonal and single-chain antibodies against 16E6 [23, 26, 33, 34] and we evaluated here the potential of 16E6-specific intrabodies for intracellular immunization of HPV-positive cancer cells using recombinant adenoviruses. We previously reported the construction of 1F4 scFv which binds to the N-terminus of 16E6 [23]. To test its capacity to function as an intrabody, we investigated its solubility and activity when expressed in the cytoplasm of *E. coli* [34] or COS cells [35]. We found that a strong expression of 1F4 in the cytoplasm of *E. coli* or COS cells resulted in nearly 100% of non-folded, aggregated and insoluble scFv [35]. This aggregation was toxic for the transfected COS cells and resulted in aspecific cell death. To overcome this problem, a random mutagenesis of 1F4 was performed in an attempt to isolate scFvs that would be more stable when expressed in the cytoplasm. This allowed us to isolate two scFvs each presenting one point mutation, i.e. 1F4-L177P [34] and 1F4-P41L (EW, unpublished results). The 1F4-L177P (L47P in V_L) had lost the capacity to bind to E6 and still aggregated when expressed in the cytoplasm whereas 1F4-P41L (P41L in V_H) still bound to E6 (Fig. 1B) and had an increased solubility after cytoplasmic expression (EW, unpublished results). The three scFvs were expressed in *E. coli* and we confirmed, using a dot-blot assay, that 1F4 and 1F4-

P41L did bind to 16E6 whereas 1F4-L177P did not (Fig. 1B).

As plasmid transfection efficiency can be very variable from one human cell line to another, we chose to use a vector derived from adenovirus type 5, known for its very high gene transfer efficacy. Coding sequences of 1F4-L177P, 1F4 and 1F4-P41L were inserted into the adenovirus type 5 genome to obtain three recombinant viruses, namely Ad1F4-L177P, Ad1F4 and Ad1F4-P41L. To assess the intracellular immunization potential of the 16E6-specific scFvs we employed two HPV16-transformed cell lines, SiHa and CaSki, and two control cell lines, A549 and HeLa. HeLa cells are transformed by HPV18 and express 18E7 and 18E6, 18E6 is not recognized by the scFvs reported here [23]. The four different cell lines were transduced with the scFv-expressing vectors (Ad1F4-L177P, Ad1F4 and Ad1F4-P41L) and analyzed 24 hours later by immunofluorescence. The use of a MOI of 100 pfu/cell allowed expression of the scFv in nearly 100% of the cells (Fig. 2B). In these conditions, we observed a lower level of scFv-production per cell than in COS cells transfected with scFv-expressing plasmid [35]. A higher MOI gave a stronger expression of the scFvs resulting in a high aspecific toxicity and a lower MOI resulted in a weaker percentage of cells transduced (data not shown).

After validating the high gene transfer efficacy of the scFv-encoding adenoviruses, we determined the solubility of each 16E6-intrabody by Western blotting of cellular extracts (Fig. 2C). Although the majority of the scFvs were present into the insoluble fraction of cellular extracts, the relative proportion of soluble intrabodies was higher in this study (5-10%; Fig. 2C) than in previous results with transfected COS cells (<1%) [35]. This global higher solubility of scFvs was due to a lower expression in the human cell lines after transduction with recombinant adenoviruses at a MOI of 100 pfu/cell than in COS cells after plasmid transfection (data not shown). The intracellular solubility of 1F4-P41L (9.9%) was nearly twice as high as the solubility of 1F4-L177P (5.2%) and 1F4 (5.6%).

We next investigated the effects of the expression of the anti-16E6 intrabodies on the proliferation of HPV16-positive cancer cell lines using a methylthiazolyldiphenyl-tetrazolium bromide (MTT) assay (Fig. 3A). In all cases, there was a strong inhibition of proliferation of the cell lines tested after expression of the scFvs, even with the negative control scFv (1F4-L177P) and negative control cell lines (HeLa and A549, Fig. 3A). This was very likely due to the toxic effect of the aggregation of unfolded intrabodies even though not as strong as was observed earlier with transfected COS cells [35]. However in addition to this non-specific effect we observed a specific effect namely that the intracellular expression of E6-binding scFvs (1F4 and 1F4-P41L) provoked cell death in HPV-positive cells (SiHa and CaSki) whereas the 1F4-L177P did not. Interestingly, the increased cytosolic solubility of the 1F4-P41L intrabody correlated with a more pronounced specific cell death in HPV16-positive cervical cells.

To further analyze the observed cell death, cells were stained with annexin V-FITC/propidium iodide (PI) and examined by flow cytometry (Fig. 3A). HeLa, A549, SiHa and CaSki cells were analyzed 24h or 48h after transduction by the scFv-expressing adenoviruses. Intracellular expression of the three scFvs in HPV16-negative cell lines led to a very small increase of apoptotic cells when compared to non-transduced cells (1-3% at 48h, Fig. 3B). Similarly the negative control intrabody, 1F4-L177P, did not enhance apoptotic cell rates significantly. In contrast, transduction of 1F4 and 1F4-P41L increased apoptosis specifically in HPV16-transformed cells (up to 13.9% with 1F4-P41L in CaSki), this effect being proportional to the intracellular solubility of the intrabody. Of note, the proportion of apoptotic cells induced by the 1F4-P41L was 9.5 fold higher in the HPV16-positive CaSki cells than in HeLa cells. Our results corroborate previous reports showing that targeting E6 with either antisense RNA [14], peptide aptamer [21], siRNA [18] or transfection of intrabodies [22] provokes apoptosis in HPV16-transformed cells. However the apoptosis induced by the 1F4-P41L intrabody in SiHa or CaSki cells was not strictly correlated with an increase in p53 protein level as assayed by immunofluorescence or Western blot (data not shown), the precise pathway involved in

the apoptosis that we observed remains to be investigated.

This work demonstrates the feasibility of intracellular immunization with anti-16E6 scFvs for specific killing of cervical cancer cells. Our study was greatly facilitated by the use of adenovirus as a gene transfer and expression vector. This vector not only allowed the expression of the scFv uniformly in all the cells transduced *in vitro* (Fig. 2B) but also provides a useful vector for expression of scFv *in vivo* for instance for preclinical studies in animal models. The importance of intracellular solubility of intrabodies is shown by the fact that increased cytosolic solubility of the 1F4-P41L intrabody was correlated with increased specific cell death in HPV16-positive cervical cells. It may therefore be worthwhile to further improve the intracellular solubility of anti-E6 scFvs by using protein-engineering approaches [36-39] since this could lead to the development of scFvs with improved therapeutic potential against cervical carcinoma.

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Fig. 1. Expression of scFvs in *E. coli* and detection of 16E6. (A) Equivalent amounts (10 µL) of periplasmic extracts from bacteria transformed with plasmids expressing either 1F4-L177P, 1F4 or 1F4-P41L were loaded on SDS-PAGE and analyzed by Western blot using the B10 antibody and HRP-anti mouse antibody. (B) *E. coli* expressed scFvs were tested for binding to 16E6. Periplasmic extracts (2 mL) were incubated on dot-blots carrying the Maltose Binding Protein (MBP) or 16E6 fused to MBP (MBP-E6). The minus (-) symbol refers to control performed with periplasmic extracts from XL1-Blue transformed with the empty expression plasmid. Bound scFvs were detected with the B10 antibody and HRP-anti mouse antibody.

Fig. 2. Expression of scFvs 1F4-L177P, 1F4 and 1F4-P41L. **(**A) Schematic representation of the recombinant adenoviral genomes. (B) Immunofluorescence detection of the expressed scFvs. Twenty-four hours after transduction with Ad1F4-L177P, Ad1F4 or Ad1F4-P41L at a MOI of 100, cells were fixed and analysed by immunofluorescence as described in the materials and methods. Non-transduced cells (nt) served as a negative control. (C) Solubility of expressed scFvs in HeLa, A549, SiHa and CaSki cells. Equivalent amounts of soluble or insoluble extracts were analyzed by Western blotting. The percentages of soluble and insoluble scFvs were quantified by VersaDoc (Bio-Rad). The average of solubility of each scFv from 3 independent experiments for the four cell lines. Error bars are standard deviations.

Fig. 3: Effects of the anti-16E6 scFv in HPV16-transformed cell lines. (A) The proliferation of HeLa, A549, SiHa and CaSki cells were measured using the MTT assay 12h, 24h or 48 h of transduction with either Ad1F4-L177P, Ad1F4, Ad1F4-P41L or medium only (non-transduced : nt). The absorbance of formazan products at 595nm reflect the number and viability of the cells. (B) Example of apoptosis analysis, 48h after transduction. Apoptotic and necrotic cells were distinguished on the basis of annexin V-FITC reactivity and PI exclusion. Live, non-apoptotic cells are not stained with any of the reagents (lower left hand quadrant). Apoptotic cells exhibited intense green (FITC) and low or intermediate red (PI) fluorescence (lower right hand quadrant, early and late stages of

apoptosis, respectively). Necrotic cells are positive with both reagents and exhibit strong green and red fluorescence (upper right hand quadrant). (C) The mean values, calculated from five independent experiments, of apoptotic cells (annexin-V-FITC positive, IP-negative) are represented. The negative control values (percentages of apoptotic cells in non-transduced cells) were subtracted from the indicated percentages. Error bars are standard deviations.