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Eradication of p53-Mutated Head and Neck Squamous Cell Carcinoma Xenografts Using Nonviral p53 Gene Therapy and Photochemical Internalization

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Photochemical internalization (PCI) technology has been used for PEI-mediated p53 gene transfer in mice bearing head and neck squamous cell carcinoma (HNSCC) xenografts. Using luciferase as a reporter gene, PCI led to a 20-fold increase in transgene expression 48 h after transfection and sustained transgene expression for 7 days. Therefore, iterative p53 gene transfer was performed by means of a weekly single injection of PEIGlu4/p53 complexes alone or with PCI for 5 (group A) or 7 (group B) weeks. The efficiency of p53 gene therapy was evaluated by following tumor growth and expression of P53-related downstream proteins (P21, MDM2, Bcl2, Bax). Apoptosis induction was evidenced through caspase-3 activation and PARP cleavage. Using PCI, tumor growth inhibition was observed in all transfected animals. Further, successful tumor cure was achieved in 17% (group A) and 83% (group B) of animals. PCI-mediated p53 gene transfer led to higher P53 protein expression that was correlated with induction of Bax and P21 proapoptotic proteins, repression of Bcl2 as well as activation of caspase-3, and cleavage of PARP. The present study demonstrates that PCI enhances the *in vivo* efficiency of PEI-mediated p53 gene transfer and can be proposed for p53 gene therapy in HNSCC.

Key Words: p53, transfection, gene therapy, PCI, polyethylenimine, HNSCC

INTRODUCTION

In normal cells, wild-type (wt) P53 acts as a powerful transcription factor that binds as many as 300 different promoter sequences within the genome [1]. The p53 gene controls the expression of several effector genes, including p21, mdm2, Bax, and Bcl2 [2]. Indeed, in normal cells, the transcriptional activity of the P53 protein is highly regulated and exposure to a wide variety of stress signals leads to enhancement of the basal activity of P53 [1] that can broadly alter patterns of specific gene expression leading to cell cycle arrest and/or apoptosis. Mutations of the p53 gene are common genetic alterations found in many human malignancies. In head and neck cancer, mutant p53 status is found in nearly 50% of the cases and is associated with poor prognosis [3]. In this context, reversion of carcinogenesis using p53 gene transfer has been investigated. Enhanced apoptosis induction and tumor regression has been evidenced in head and neck squamous cell carcinoma (HNSCC) preclinical tumor models [4,5] and encouraging results have been reported in clinical trials with induction of significant tumor regression after gene transfer [6–8]. In most studies, viral vectors have been used but alternative approaches with nonviral carriers have been proposed. Despite their low transfection efficiency, nonviral vectors are interesting due to their ability to circumvent the immune response occurring against viral proteins, which limits iterative administration [9]. Polyethylenimine (PEI) derivatives are

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polycationic molecules [10] forming stable ionic complexes with plasmid DNA. With excess PEI, DNA is compacted into positively charged particles interacting with anionic syndecans at the cell membrane, thereby facilitating the uptake of the DNA by endocytosis [11]. Thus, PEI–DNA complexes escape from the lysosome and eventually enter the nucleus [12]. PEI derivatives can be of linear (22 kDa) or branched (25 kDa) topologies, which both have proven efficient for gene transfer *in vitro* and *in vivo* [13,14]. Our previous results showed that the tetraglucosylated (Glcα4Glcα4Glcα6Glc) derivative of linear PEI (PEIGlu4) led to higher transfection efficiency *in vitro* [15] and *in vivo* [16]. In the past 5 years, over a hundred publications have appeared dealing with *in vivo* gene delivery with PEI for uses as diverse as intramuscular vaccination [17], delivery to the intestine [18] or pancreas [19], or adventitial gene delivery to rabbit carotid artery [20]. No acute toxicity has been reported for PEI derivatives following various routes of administration [14,21,22]. Success has promoted a number of preclinical studies moving toward therapy, with the large majority dealing with cancer.

PEI gene transfer efficiency can be enhanced by using targeting vectors bearing ligands such as sugar residues [23,24], RGD peptides [25], proteins [26], or gold nanoparticles [27].

As another procedure for improving PEI-mediated transfection efficiency, photochemical internalization (PCI) has been extensively investigated. PCI is based on light-induced intracytoplasmic release of macromolecules such as DNA from endocytotic vesicles of cells previously incubated with a photosensitizer. PCI protocols are similar to those used for photodynamic therapy of HNSCC [28]. In the case of PCI, the photosensitizer and the PEI both enter cells by endocytosis, suggesting that they can be combined for efficient p53 gene transfer. PCI has been described as significantly enhancing the *in vitro* transfection efficiency of both adenoviral and nonviral vectors [29–31]. We previously showed that PCI combined with PEIGlu4-mediated p53 gene transfer in p53-mutated cell lines enhanced the transfection rate and led to higher apoptosis and cell death induction [32]. Long-lasting and time-delayed transgene expression was evidenced when PCI was combined with PEIGlu4 in head and neck carcinoma cells [33].

Considering these results, we now investigate the effects of PCI on PEIGlu4-mediated wt p53 gene transfer in mice bearing mutant p53 HNSCC xenografts, using a weekly intratumoral injection schedule that could be easily translated into clinical investigation.

Results and Discussion

p53 Mutation Analysis

Direct sequencing after PCR demonstrated that the xenografted tumor bears a homozygous mutation of exon 6, codon 193 (CAT:CTT), resulting in a His-to-Leu substitution (Fig. 1).

Optimization of the Light Dose for PCI

Forty-eight hours after photosensitization with 50 μg of AlPcS2a, we injected PEIGlu4/pLuc-CMV complexes into the tumor. Then 6 h later, we exposed the tumor area to either 135 or 90 J/cm^2 light dose once a week for 5 consecutive weeks. The macroscopic aspect of the tumors showed extended necrosis when the tumors were treated with 135 J/cm^2 (the necrotic zone was already visible 24 h after illumination) (Fig. 2A). In contrast, only a minor skin reaction without any necrosis was evidenced in the tumors that received 90 J/cm^2 (Fig. 2B). Furthermore, while we observed a significant decrease in the tumor volumes, i.e., a photodynamic effect, with 135 J/cm^2 PCI, the 90 J/cm^2 light dose did not induce any significant tumor regression in mice transfected with the therapeutically irrelevant luciferase gene (Fig. 2C).

Quantitative Luciferase Transgene Expression Kinetics

From the above data, we evaluated the PCI-induced effects on the luciferase transgene expression with the light dose of 90 J/cm^2 (Fig. 3). Luciferase, with a half-life of 3 h in animal cells, does not accumulate and is an ideal reporter of transfection kinetics. In the absence of PCI, the luciferase expression level was maximal 24 h after transfection and progressively decreased and was no longer significant (<100,000 RLU) after 72 h. While PCI did not have any significant effect on the transgene expression level 24 h after the transfection, it increased very significantly ($P < 0.001$) and prolonged the transgene expression level from 48 to 168 h posttransfection. With PCI, the maximum of luciferase expression was achieved 48 h after transfection, with a 100-fold increase in comparison with the expression measured with PEIGlu4/pCMV-Luc alone at the same time point and a 20-fold increase in comparison to the maximum levels measured with the PEIGlu4/pCMV-Luc alone (at 24 h). Twenty-four hours after transfection, luciferase expression was lower when PCI was used compared to PEIGlu4 alone. As already proposed [34], this should be related either to the effect of PCI on the dynamics of the cell microtubules, which are known to be affected by photochemical damage, or to the delayed gene expression caused by cell cycle inhibition following photochemical treatment.

With PCI, the luciferase expression level was still significant (>100,000 RLU) 7 days after transfection. We observed no significant toxicity in terms of weight loss (<10%) or mortality in any case.

These data were in agreement and extended those previously reported [15,16], suggesting that the PCI

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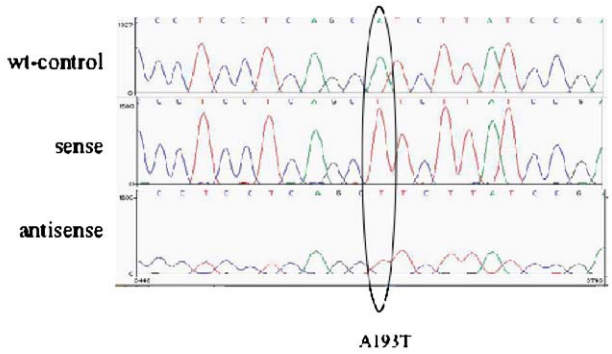

FIG. 1. Mutation analysis of the tumor xenograft. p53 mutations were characterized by direct sequencing after PCR amplification. Results achieved demonstrate that the tumor xenograft bears a homozygous mutation of exon 6, codon 193 (CAT:CTT).

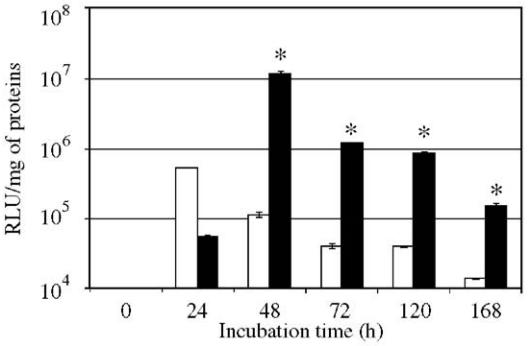

FIG. 3. Quantitative luciferase activity kinetics. Tumors were removed from sacrificed animals 24, 48, 72, 96, 120, or 168 h after injection of PEIGlu4/pLuc alone (white bars) or in combination with PCI (black bars). Tumors were rapidly frozen in liquid nitrogen and assayed for luciferase activity. Results are mean values of three measurements ± SD. *Significant ($P < 0.01$ Student test) variation between tumor transfected with and without PCI.

protocol can potentiate the therapeutic outcome of p53 gene transfer.

Tumor Growth Inhibition after Iterative wt p53 Gene Transfer

For tumor growth inhibition experiments, we performed the p53 gene transfer using a single injection of PEIGlu4/wt-p53 complexes, once a week, alone or in combination with PCI. We applied this protocol for 5 (group A) or 7 (group B) consecutive weeks. We observed no differences in tumor growth between control mice and mice treated with irrelevant PEI/DNA complexes alone or combined with PCI (Fig. 4).

Moreover, we observed no significant toxicity as evidenced by mortality, weight loss, or inflammation using the single iterative weekly injection protocol. The animals were successfully protected from the light and the heat emitted by the lamp using the aluminum foil. Most mice recovered without sequelae except in 2 cases (of a total of 115 mice, i.e., <2%) in which the animals died within 24 h. Death was not related to

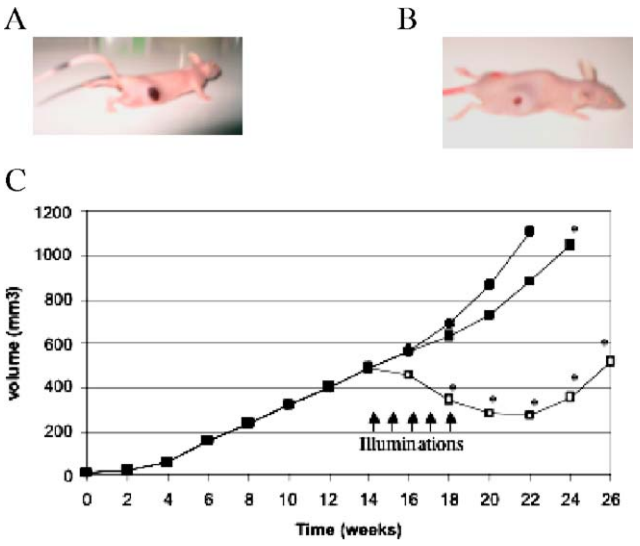

FIG. 2. Optimization of the PCI experimental conditions. PCI was carried out on anesthetized mice bearing 500-mm^3 tumors as a weekly single treatment repeated five times. The mice were photosensitized with 50 μg of $AlPcS_{2a}$ and kept in the dark for 48 h before being transfected by PEIGlu4/pCMV-Luc complexes and illuminated with either 90 (black square) or 135 J/cm^2 (open square) red light. (A and B) The macroscopic analysis was performed 24 h after the first illumination and the tumor size was measured once a week. (C) Tumor size of PCI-treated animals was compared to that of control mice that received saline solution instead of photosensitizer (black circle); $n = 6$ mice per group. *Significant ($P < 0.05$, Student test) variation between control and PCI-treated mice. Error bars were omitted for clarity and remained within 10% of the mean value.

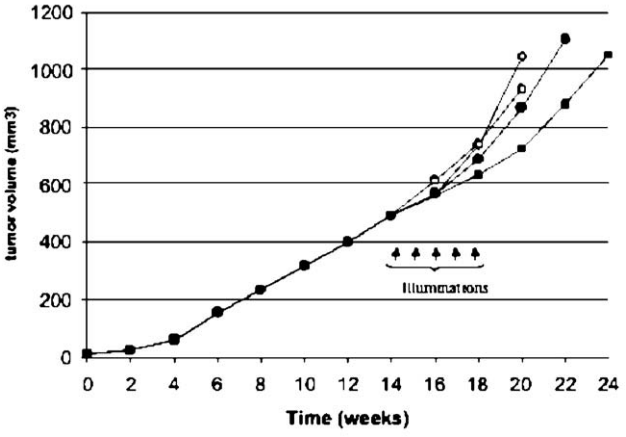

FIG. 4. Influence of PCI and irrelevant gene transfer on the evolution of the tumor. The tumor size was determined in untreated (black circle) and $AlPcS_{2a}$ no-light (open square), $AlPcS_{2A}$ + 90 J/cm^2 (black square), and PEIGlu4/pCMVLuc + PCI (open circle) treated mice. Transfection was performed by single weekly injection of PEIGlu4/pCMVLuc with PCI and was repeated for 5 consecutive weeks. Results are mean values of six independent determinations. Error bars are omitted for clarity and were always below 10% of the mean.

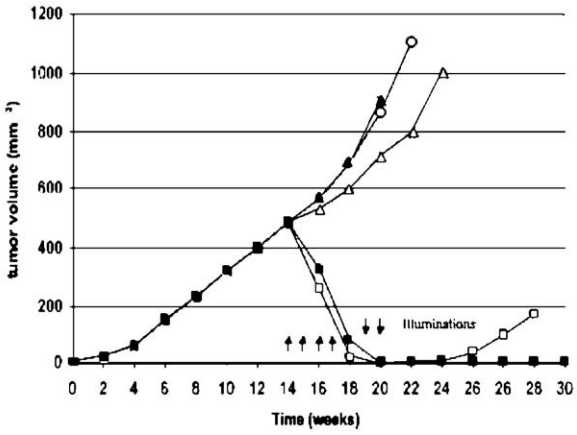

FIG. 5. Tumor growth inhibition after wt p53 gene transfer. Human head and neck squamous cell carcinoma specimens taken from surgical resections (10 mm^3) were subcutaneously xenografted into nude mice. Once the tumor volume reached 500 ± 20 mm^3, mice were transfected using p53/PEIGlu4 complexes alone (triangles) or in combination with PCI (squares), once a week for 5 (open symbols) or 7 (close symbols) weeks. The tumor growth was followed over 10 additional weeks. Control mice hosted tumors injected with saline solution (open circles). Results are mean values of six independent determinations. Error bars are omitted for clarity and were below 10% of the mean.

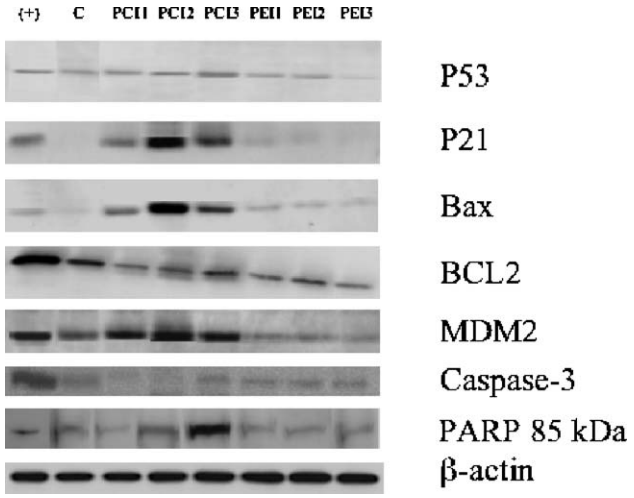

FIG. 6. Western blot analysis of P53 target proteins. Tumors were removed from untreated mice (C) or after one, two, or three transfections using p53/PEIGlu4 alone (PEI1, PEI2, PEI3) or combined with PCI (PCI1, PCI2, PCI3). Western blot analysis of P53, P21, Bax, Bcl2, MDM2, caspase-3, and 85-kDa cleaved PARP proteins was then performed. β-Actin was used as an internal control.

iteration of transfection or PCI since it eventually could occur during the first injection of photosensitizer or of PEI/DNA. This absence of toxicity suggests a good tolerance after repeated p53 gene therapy by PCI. This could be of great importance for the clinical use of this approach.

Fig. 5 shows that an iterative weekly single injection of wt p53 gene transfer without PCI did not influence tumor growth whatever the number of injections (groups A and B). When we used PCI to enhance wt p53 gene transfer, we observed a dramatic tumor regression in all the transfected animals (12/12). Indeed, 1 week after the last wt p53 gene transfer using PCI, total tumor regression was achieved in 67% (4/6) of group A mice and in 100% (6/6) of group B mice. Three weeks after the last transfection, tumor relapse was observed in 75% (3/4) of tumor-free group A mice and in 17% (1/6) of tumor-free group B mice. Finally, successful tumor cure was achieved in 17% (1/6) of group A mice and in 83% (5/6) of group B mice that were still tumor free 8 weeks after the last transfection.

Compared to our previous results achieved with PEIGlu4 without PCI [35], the present data demonstrate that using PCI for gene transfer allows us to reduce the number of transgene injections (once versus four times a week) and to achieve tumor eradication and not only tumor control. Compared to data reported in the literature with viral vectors, the present results are very promising since only tumor regression, not tumor eradication, has ever been reported, even when gene transfer was combined with conventional therapies [36,37].

Analysis of p53 Effector Protein Expression and Apoptosis Molecular Markers after wt-p53 Gene Transfer

We performed p53 gene transfer as a weekly single injection of PEIGlu4/p53 complexes alone or combined

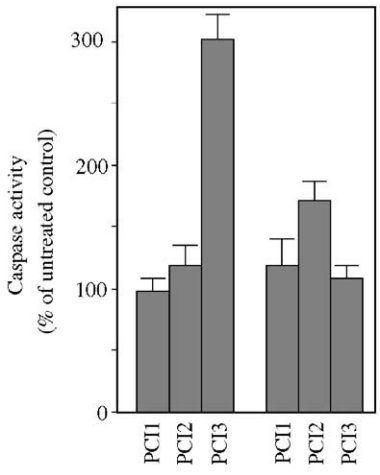

FIG. 7. Variation in caspase-3 activity after wt p53 gene transfer. Protein extracts from tumors removed after one, two, or three transfections using p53/PEIGlu4 alone (PEI1, PEI2, PEI3) or combined with PCI (PCI1, PCI2, PCI3) were analyzed for caspase-3 activity using DEVD substrate cleavage assay. Results are expressed as percentage caspase activity relative to untreated controls.

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with PCI, and we repeated this protocol one, two, or three times. One week after the last transfection, we sacrificed the mice and extracted the tumors for Western blot analysis of P53, P21, MDM2, Bax, Bcl2, caspase-3, and 85-kDa cleaved PARP proteins and caspase-3 activity. As shown in Fig. 6, PCI further increased the level of P53 and further enhanced the MDM2 protein level. These data were fully correlated with the increased induction of P21 and Bax proapoptotic proteins and the further inhibition of the Bcl2 antiapoptotic protein as well as the induction of caspase-3 protein expression after p53 gene transfer by PCI. At the other hand, caspase-3 activity was increased and correlated with PARP cleavage (Fig. 7). The relative protein induction or inhibition was more significant after two gene transfer procedures by PCI. No difference was seen after either two or three p53 gene transfers by PCI except for caspase-3 activity and PARP cleavage, which were further enhanced after three treatments. These data showed that the use of PCI for PEIGlu4-mediated p53 gene transfer leads to synergistic induction and/or inhibition of the p53 effector proteins and leads to higher activation of caspase-3 and cleavage of PARP.

We found a basal level of the 85-kDa fragment of cleaved PARP in untreated (data not shown) and control tumors, probably due to the tumor hypoxia. This was consistent with constitutive expression and activity of caspase-3 in control tumors.

The data suggest a key role for p53 in the antitumoral effect that seems to occur via apoptosis. PARP cleavage can be activated by reactive oxygen species [38]. Even if PCI is able to generate reactive oxygen species that could be capable of inducing PARP cleavage, under our experimental conditions, the apoptosis seemed to be associated with the p53 because no significant variation was observed in the 85-kDa cleaved PARP fragment between untreated tumors and tumors treated by PCI with irrelevant gene (data not shown).

PCI for PEIGlu4-mediated p53 gene transfer leads to synergistic induction and inhibition of the p53 target genes controlling cell cycle kinetics.

Altogether, these data showed that p53-dependent apoptosis is implicated in the tumor regression since proapoptotic proteins (P21, Bax) were up-regulated while the Bcl-2 antiapoptotic protein was inhibited.

CONCLUSION

In conclusion, the present results demonstrate that PCI enhances the *in vivo* efficiency of PEIGlu4-mediated gene transfer and could be proposed as a potent delivery system for p53 gene therapy in HNSCC. Referring to the protocols that are used in clinical trials of photodynamic therapy in head and neck cancers [28], PCI should be safely usable in patients. The combination of p53 nonviral gene therapy with PCI is an innovative therapeutic approach that could be proposed either alone or associated with conventional chemo- and radiotherapies, since the p53 status has direct clinical implications regarding response to classical cancer therapies [36,37]. This strategy has already been proposed using viral vectors but the use of PCI PEIGlu4-mediated p53 gene transfer could overcome the problem of immune response encountered with the iterative use of adenovirus. All these considerations favor ongoing experimentation aiming at transferring the use of PCI for intratumoral gene transfer into the clinics.

MATERIALS AND METHODS

Polyethylenimine derivatives and plasmids. PEIGlu4 was obtained from Polyplus-Transfection SAS (Illkirch, France) as a 100 mM stock solution. PEIGlu4 was diluted in a 5% glucose solution.

The plasmids used were pNCNeo (Dr. J.-M. Heard, Pasteur Institute, Paris, France) and pCMV-Luc (Dr. Patrick Erbacher, Polyplus-Transfection) encoding respectively the P53 protein and luciferase under the control of the cytomegalovirus promoter. Plasmids were purified from *Escherichia coli* using Maxiprep columns (Marligen Biosciences, Inc., USA).

Photosensitizer and light source for photochemical internalization. Disulfonated aluminum phthalocyanine ($AlPcS_{2a}$) was kindly provided by PCI Biotech AS (Oslo, Norway). AlPcS2a was dissolved in 0.1 M NaOH and thereafter diluted in phosphate-buffered saline, pH 7.5, to a final concentration of 5 mg/ml. The photosensitizer was light-protected and stored at −20°C prior to use.

A CureLight broadband light source (PCI Biotech AS, Oslo, Norway) was used for *in vivo* illumination of the tumors. CureLight broadband is a 150-W halogen lamp (Xenophot, 64640 HLX EFR) with adjustable light diameter/focus 30–55 mm and with filters selecting light in the 570–670 nm spectral band. The device was calibrated before each illumination using a power meter provided with the lamp.

Tumor xenografts. Anonymous tumor material was derived from an untreated patient undergoing surgical resection of a mouth floor tumor and was pathologically classified as head and neck squamous cell carcinoma bearing mutant p53 status as determined using immunohistochemistry with DO-7 monoclonal antibody (Dako, Trappes, France). p53 mutations were characterized by direct sequencing of the PCR products according to the procedure reported by Franco *et al.* [40].

All animal experiments were performed in compliance with the French Animal Procedure Act (from May 2001). Six-week-old female athymic Swiss *nu/nu* mice (Harlan, Gannat, France), weighing 18–22 g, were used. Mice were housed in plastic cages under standard conditions (25°C, 50% relative humidity, 12 h light/dark cycles) and provided with water and food *ad libitum*. Human tumor specimens (10 mm^3) were implanted subcutaneously in the right flank of the anesthetized (isoflurane, Forene; Abbott Laboratories, Rungis, France) mice.

Tumors were used for experiments or maintained by sequential passage in nude mice. All tumor material used in this study was taken from passage 5 to 10 of the original human tumor xenografts. Within approximately 12 weeks, tumor volume reached 500 mm^3. Tumor volumes were calculated [41] as $4\pi/3 \times (\text{length}/2 \times \text{width}/2 \times \text{depth}/2)$.

Tumor growth was followed by measuring once a week the tumor dimensions using a 0–150 mm electronic digital caliper (Omni-Tech, France). When the tumors were treated (i.e., in the case of transfection or photosensitization), the measurements were performed before starting the treatments so that the initial volume was taken. The doubling time of the xenografts was 1.8 days ± 0.3 (standard deviation, $n = 150$). Animals were randomly distributed in the different groups. For tumor resection or for ethical purpose, when the tumor volume reached 1200 mm^3, mice were sacrificed using isoflurane inhalation.

Transfection procedure. Briefly, 7.5 μl of 100 mM PEIGlu4 derivative were vortex mixed with 50 μg of the desired plasmid in a final volume of 100 μl

of 5% glucose solution. The glucosylated PEI/DNA mixture (*N/P* ratio = 5) was left for 15 min at room temperature before being injected intratumorally in mice. All experiments were carried out in experimental groups containing at least six mice bearing 500 mm^3 tumors. Control groups contained animals bearing untransfected tumors and animals treated with irrelevant PEIGlu4/DNA complexes. The intratumoral injections were carried out on animals anesthetized using isoflurane inhalation. In all experiments, the weight of the mice was recorded.

Optimization of the PCI experimental conditions. PCI was carried out on anesthetized mice bearing 500-mm^3 tumors. For PCI treatment, the selected mice were photosensitized by intratumoral administration of 50 μg of $AlPcS_{2a}$ and kept in the dark for 48 h according to Selbo *et al.* [42]. PCI-generated cytotoxicity was evaluated by macroscopic analysis of tumor necrosis, and light dose conditions that did not induce any necrosis were selected. The mice were transfected by intratumoral administration of PEIGlu4/pLuc-CMV complexes and allowed to stay for 6 additional hours in the dark for homogeneous plasmid diffusion. Finally, the animals were covered with aluminum foil except above the tumor area, for which a hole in the foil with a diameter 2 mm larger than the tumor was made, and the tumors were photoirradiated. Illuminated mice were then kept protected from light for 48 h, after which the animals were reintroduced into normal light/dark conditions until the next photosensitization. When PCI was not applied, the animals were only transfected by intratumoral injection of PEIGlu4/DNA complexes. However, to have comparable experimentation procedures, control mice were injected with saline solution instead of photosensitizer 48 h before the transfection and kept in the dark exactly as often as the PCI-treated mice.

Influence of PCI on transgene expression. Tumors were removed 24, 48, 72, 120, 144, or 196 h after transfection with PEIGlu4/pCMV-Luc complexes alone or combined with PCI and the tumors were rapidly frozen in liquid nitrogen. The influence of PCI on the transgene expression was determined using a luciferase activity assay as already described [35]. Results were expressed using the MokriWin software, as light units integrated over 10 s per milligram of proteins using the bicinchoninic acid assay (Bio-Rad, Marnes La Coquette, France). All data were corrected for luciferase background measured in untreated control tumors. No modification in luciferase background was found in tumors treated with PEIGlu4/p53 complexes, considered as irrelevant for these experiments (data not shown).

Tumor growth inhibition experiments. To analyze the influence of the gene transfer procedure on the tumor growth, tumors were transfected once a week with either PEIGlu4/p53 complexes alone or in combination with PCI for 5 (group A) or 7 (group B) consecutive weeks. The therapeutic effect was evaluated by weekly measurement of the tumor volume. The negative controls consisted in mice injected with saline solution and mice transfected using irrelevant PEIGlu4/pCMV-Luc complexes combined or not with PCI. Tumor cure was defined as the absence of tumor relapse for 8 weeks after tumor complete regression.

Analysis of p53 downstream effectors and apoptosis induction. CCL17 and CAL27 human head and neck carcinoma cell lines exposed to 5-fluorouracil at the IC_{50} for 72 h were used as control for P53, P21, MDM2, and caspase-3 (CCL17) and Bax and Bcl2 (CAL27) protein analysis according to previous experiments [39]. Both cell lines were cultured in phenol-red-free RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS) and 1% antibiotics penicillin–streptomycin mixture, at 37°C in a 5% CO_2 atmosphere. All culture reagents were from Life Technologies (Eragny, France), except the FCS, which was purchased from Dutscher (Brumath, France).

P21, MDM2, Bax, Bcl2, and caspase-3 were analyzed as P53-downstream effector proteins; caspase-3 activity and PARP cleavage were assessed as apoptosis induction markers. Western blots were performed as already reported [43] with slight modifications. β-Actin was used an internal control. Monoclonal antibodies used were M7001 (clone DO7; Dako), sc-817 (Santa Cruz, TebuBio, Le Perray en Yvelines, France), sc-5304 (Santa Cruz), A3533 (Dako), M0887 (clone 124; Dako), CPP32 (Santa Cruz), 552596 (Pharmingen, Becton–Dickinson, Le Pont de Claix, France), and sc-1616 (Santa Cruz) for P53, P21, MDM2, Bax, Bcl2, caspase-3, cleaved-PARP (Asp214), and β-actin, respectively. Briefly, after thawing, tumor samples were transferred into 10-ml plastic tubes containing 2 ml precooled ice-cooled lysis buffer (Promega, Charbonnières, France) supplemented with a protease inhibitor cocktail (1 ml/20 g tissue; Sigma–Aldrich, St Quentin Fallavier, France). The proteins were separated in SDS–polyacrylamide gels (5–10%) and transferred to polyvinylidene difluoride membrane (Bio-Rad) at 200 mA using a Transblot 3D (Bio-Rad). Nonspecific binding was blocked with 5% milk (P53, cleaved PARP, and Bcl2) or 1% bovine serum albumin + 1% milk (P21, MDM2, caspase-3, and Bax) in phosphate-buffered saline/0.1% Tween 20 for 1 h at room temperature. Immunodetection of the proteins was performed using specific primary monoclonal antibodies (dilution 1:200) overnight at 4°C. Subsequently, membranes were washed and incubated with horseradish peroxidase-secondary polyclonal antibodies (Santa Cruz). After complete washes immunoreactive proteins were visualized with ECL reagent (Amersham Biosciences, Orsay, France) according to the manufacturer's recommendations.

Caspase-3 activity was measured in the protein extracts as already described in [44]. Results were expressed as activity value relative to untreated controls.

Statistical analysis of data. Unless otherwise indicated, all experiments were performed in triplicate and results presented as mean values ± standard deviation of three replicated independent experiments. Non-parametric Mann–Whitney test was employed to determine the statistical significance with a limit set to $P < 0.05$ using Statview 5.0 software (SAS Institute, Inc., USA).

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