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Photochemically enhanced gene transfection increases the cytotoxicity of the herpes simplex virus thymidine kinase gene combined with ganciclovir

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Tumor targeting is an important issue in cancer gene therapy. We have developed a gene transfection method, based on light-inducible photochemical internalization (PCI) of a transgene, to improve gene delivery and expression selectively in illuminated areas, for example, in tumors. In the present work, we demonstrate that PCI improved the nonviral vector polyethylenimine (PEI)-mediated transfection of a therapeutic gene, the 'suicide' gene encoding herpes simplex virus thymidine kinase (HSVtk). In U87MG glioblastoma cells *in vitro,* the photochemical treatment stimulated expression of the HSVtk transgene, and, consequently, enhanced cell killing by the subsequent treatment with the prodrug ganciclovir (GCV). When relatively low doses of DNA (1 μg/ml) and the PEI vector (N/P 4) were used, HSVtk gene transfection followed by the GCV treatment did not have an effect on cell survival unless the photochemical treatment was performed, which potentiated the cytotoxicity to 90%. These findings indicate that photochemical transfection allows: (i) selective enhancement in gene expression and gene-mediated biological effects (cell killing by the Hsvtk/GCV approach) in response to illumination; (ii) the use of low, suboptimal for the nonviral transfection methods without PCI, doses of both DNA and the vector, which may be relevant and advantageous for therapeutic gene transfer *in vivo*.
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The development of methods for obtaining the effect of drugs selectively in the diseased area is of great importance for many cancer therapy approaches. In cancer gene therapy, both targeted delivery of a therapeutic gene and targeted gene expression are being employed to achieve gene-mediated effects specifically in tumors.[1–3] Recently, we have developed a technology, generally named photochemical internalization (PCI), for the specific light-directed delivery of different macromolecules, including proteins and genes, into the cytosol of target cells.[4,5] The method is based on photochemical principles also used in photodynamic therapy of cancer (PDT)—a localized cancer treatment involving tumor-localizing photosensitizers followed by focused light activation.[6] In the PCI technology, photosensitizers localizing in endocytic vesicles are used. When such photosensitizers are activated by light, induced photochemical reactions rupture the membranes of endocytic vesicles so that the vesicular constituents are released into the cytosol of the illuminated cells.[7] In general, endosome-disruptive strategies are of great importance for realizing the full potential of macromolecular drugs. Most macromolecules, including gene/vector complexes, are taken into the cell via endocytosis and often stay trapped in the endocytic vesicles being unable to reach therapeutic targets in other places in the cell.[8] PCI provides a possibility to liberate such macromolecules in response to illumination thereby potentiating the activity of the molecules at specific (i.e. illuminated) sites.

The potential of the PCI technology has been demonstrated *in vivo* in an animal model delivering the protein toxin gelonin in a study where complete regression of subcutaneous tumors was seen in PCI-treated animals.[9] Photochemical delivery of genes with either viral or nonviral vectors has so far only been demonstrated *in vitro*, using only reporter genes like enhanced green fluorescence protein (eGFP) and beta-galactosidase.[7,10,11]

To examine the PCI method on a therapeutically relevant gene and to establish a nonviral vector-based system for future *in vivo* applications, we have established

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an *in vitro* model using a transgene encoding herpes simplex virus thymidine kinase (HSVtk) followed by the treatment with the prodrug ganciclovir (GCV). The HSVtk/GCV approach is known as 'suicide' gene therapy, where the product of the HSVtk gene phosphorylates the nontoxic prodrug GCV, and the phosphorylated form of GCV then causes the death of HSVtk-expressing cells.[12] Moreover, the toxic metabolite can induce a killing effect also on neighboring cells not expressing HSVtk, a so-called bystander effect,[13] which enhances the therapeutic potential of the HSVtk/GCV approach, but also strengthens the requirement for controlling where in the body the gene is delivered and expressed.

The HSVtk gene has already progressed into clinical trials for various types of tumors, mostly for glioblastomas.[14,15] However, only marginal clinical benefit has been observed so far, mainly due to difficulties in achieving therapeutic levels of gene transfer and expression in tumor cells.[14,16]

Most of the studies on the HSVtk/GCV system, including reported clinical trials, employed viruses as vectors for HSVtk delivery,[15,17,18] while only a few studies used nonviral vectors,[19–21] which, in general, are safer than viruses.

In the present study, we used the nonviral vector polyethylenimine (PEI), which has been shown to be a promising alternative to viral vectors in applications *in vivo* and a suitable backbone for coupling to targeting ligands.[22–24] We employed PCI to improve and target PEI-mediated delivery of the HSVtk gene. In this work, we show light-dependent expression of the HSVtk gene and demonstrate a light-potentiated effect of the HSVtk/GCV approach on glioblastoma U87MG cells *in vitro*. The possibility to modulate the delivery, expression and, consequently, the biological effect of a therapeutic transgene offered by the light-dependent PCI technology could be a valuable supplement for the suicide gene therapy as well as for other approaches used in cancer gene therapy.

Materials and methods

Reagents and cell culture

The plasmids pEGFP-N1 (encoding eGFP; 4. 7kb) was purchased from Clontech Laboratories, Inc. (Palo Alto, CA), and pNGVL1-TK (encoding HSVtk; 5.1kb) was obtained from National Gene Vector Laboratory, University of Michigan (MI). Ganciclovir was from Roche (Basel, Switzerland). The photosensitizer aluminum phthalocyanine disulfonated on adjacent rings ($AlPcS_{2a}$) was from Frontier Scientific (Logan, UT) and polyethylenimine 22 kDa (ExGen 500) was from MBI Fermentas (Lithuania).

The human glioblastoma U87MG cells (ATCC HTB-14) were cultured in DMEM medium containing 4.5 g/L glucose; human colon carcinoma HCT 116 cells (ATCC CCL-247) were cultured in RPMI-1640 medium. Both media were supplemented with 10% fetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin and 2 mM glutamine (all from Bio Whittaker, Walkersville, MD). The cells were cultured at 37°C in 5% CO_2 atmosphere.

Preparation of plasmid DNA complexes with polyethylenimine

Plasmid DNA/PEI complexes at N/P ratio 4 (N/P = molar ratio of PEI nitrogen to DNA phosphate) were prepared by mixing plasmid and PEI solutions prepared separately: 5 μg of plasmid DNA and 11 μl of commercial PEI solution (10 μM in PEI) was each diluted in 50 μl 20 mM HEPES buffer containing 5% glucose, vortexed and incubated at room temperature for 10 minutes. The PEI solution was added to DNA solution, vortexed 10 seconds and incubated for 10 minutes at room temperature. The mixture was diluted with culture medium to 5 ml or to 1 ml to get transfection solutions containing 1 or 5 μg/ml DNA, respectively, and 500 μl was applied to each well in 12-well plates.

Transfection with DNA/PEI complexes

For transfection with the eGFP gene, $70–75 \times 10^3$ U87MG or HCT 116 cells were seeded out into each well in 12-well plates. For transfection with the HSVtk gene in the cytotoxicity studies 25×10^3 cells were seeded out into each well in 24-well plates. At 6 hours after plating, the photosensitizer $AlPcS_{2a}$ was added to a final concentration of 10 μg/ml. After overnight incubation, the cells were washed three times with culture medium and incubated in 500 μl $AlPcS_{2a}$-free medium containing DNA/PEI complex for 4 hours. Treated cells were washed once with medium, and after the addition of 1 ml of culture medium the cells were exposed to red light. Illumination was performed as described previously,[25] briefly, from a bench of four light tubes (Philips TLD 18W/79) and a long pass filter with a cutoff at 550–600 nm (the light intensity reaching the cells was 13.5 W/m^2). The illumination time was chosen empirically for each cell line. The cells were incubated at 37°C before further analysis. All the procedures after the addition of $AlPcS_{2a}$ were performed in the darkness, apart from the illumination step where indicated. The control cells contained the photosensitizer and were treated identically, except that they were not exposed to light.

Flow cytometry analysis

For the detection of eGFP, 2 days after the treatment with the pEGFP-N1/PEI complex the cells were trypsinized and resuspended in 400 μl of culture medium and filtered through a 50 μm mesh filter. After the addition of 1 μg/ml propidium iodide (PI) (to stain the dead cells), the cells were analyzed by a FACS-Calibur flow cytometer (Becton Dickinson, San Jose, CA). For each sample 10,000 events were collected. eGFP fluorescence was measured through a 510–530 nm filter after excitation with an argon laser (15 mW, 488 nm). Dead cells were discriminated from viable cells by gating on the PI fluorescence signal. Cell doublets were discriminated from

single cells by gating on the pulse width of the side scatter signal.

Cytotoxicity assay

The day after treatment with the pNGVL1-TK/PEI complex GCV was added (to a final concentration 25 μg/ml for U87MG cells and 10 μg/ml for HCT 116). Cells were incubated in the presence of GCV for 4 days changing the medium containing GCV every day. The cell survival was then evaluated by measuring the inhibition of protein synthesis (assayed for [3 H]leucine incorporation into proteins) as previously described.[26]

Western blot analysis for HSV-TK protein

At various time points (24, 44 and 96 hours) after treatment with the pNGVL1-TK/PEI complex (with and without illumination), the cells were harvested and lysed in lysis-buffer (125 mM Tris/HCl, 4% SDS, pH 6.8 containing 10 μl/ml protease inhibitor cocktail (Sigma, St Louis, MO)) for at least 30 minutes on ice. The cell lysate (50–70 μl) was incubated with benzonase (1 μl) (Merck, Darnstadt, Germany) for 10 minutes on ice to shear DNA. Total protein was measured using the DC protein assay (BioRad, Hercular, CA), and 30 μg of protein for each sample was boiled for 5 minutes together with 3 × sample buffer and applied on 12.5% SDS PAGE gel (BioRad). Equal loading among the samples was confirmed by Coomassie blue staining. Proteins were electrotransfered on PVDF membrane (Amersham Bioscience, Buckinghamshire, UK) by wet transfer overnight at 25 V. The nonspecific binding to the membrane was blocked using 5% dry milk in TTBS buffer (10 mM Tris-HCl pH 7.5, 100 mM NaCl, 0.1% Tween 20) for 1 hour at room temperature. The membrane was incubated with the primary antibody, rabbit polyclonal anti-HSV-TK (obtained from Dr W Summers at Yale University, New Haven, CT) at a dilution of 1:10,000, followed by incubation with the secondary antibody, goat anti-rabbit conjugated to horseradish peroxidase (Amersham Pharmacia Biotech, Little Chalfont, UK) at a dilution 1:20,000. The membrane was then incubated for 5 minutes in chemiluminescence (ECL Plus) reagent (Amersham Biosciences, Buckinghamshire, UK) and visualised with Storm 840 (Molecular Dynamics, Sunnyvale, CA). The intensity of the bands was quantified by the ImageQuant 5.0 program (Molelcular Dynamics).

Gap junctional intercellular communication (GJIC) assay

Cells ability to perform GJIC was assessed by dye-spreading assay using the fluorescent dye Lucifer yellow (LY) (MW 443; Sigma, St Louis,. MO).[27] The cells (200×10^3) were seeded out into 3 cm dishes and 2–3 days later LY (10% wt/vol solution in water) was microinjected into single cells by a glass microcapillary using Eppendorf 5242 pressure control unit coupled to a Narishige micromanipulator mounted on a Nikon Diaphot 300 microscope (Tokyo, Japan). Then the cells were fixed in 2% formaldehyde in PBS, and 5–8 minutes after microinjection the fluorescence micrographs were recorded using a standard FITC-filter block on Nikon E800 microscope. Parallel phase contrast images were taken. The dye transfer was evaluated by overlapping the fluorescence and phase contrast images of the cells using the AnalySIS PRO 3.00 software (Soft Imaging System GmbH).

Statistical analysis

The difference between the transfection efficiencies, that is, percentage of eGFP-positive cells obtained by transfection without or with PCI were tested for significance by paired or unpaired *t*-tests whenever the normal distribution and equal variance criteria were fulfilled.

For the statistical analysis of the killing effect of different treatments presented in Figure 4, we calculated −ln of survival after each treatment, that is, $y(D) = -\ln S(D)$, where S is a relative survival after a light dose D.[28] For each light dose, that is, 4-minutes light, 5-minutes light and no light, we compared the effect of the following treatments: photochemical treatment alone (y_{phot}), HSVtk/GCV approach alone ($y_{\mathrm{HSVtk/GCV}}$), combined treatment (photochemical + HSVtk/GCV approach) (y_{comb}) and single treatments (y_{HSVtk} or y_{GCV}). The killing effects were compared calculating the difference between–ln S values. The purely additive effect of the HSVtk/GCV approach and photochemical treatment is then supposed to be

$$\begin{aligned} y_{\mathrm{add}}(D) &= y_{\mathrm{HSVtk/GCV}}(D) + y_{\mathrm{phot}}(D) \\ &= -(\ln S_{\mathrm{HSVtk/GCV}}(D) + \ln S_{\mathrm{phot}}(D)). \end{aligned}$$

The extra effect obtained by combining these two treatments is

$$\Delta y(D) = y_{comb} - y_{add}.$$

When Δy is zero, there is no extra effect obtained by the combining the treatments (i.e. no synergism, no antagonism), and positive Δy indicates synergy, that is, potentiation of the total killing effect when the photochemical and the HSVtk/GCV approaches were combined.

Results

Photochemical transfection using PEI as a gene transfer vector

Previous studies[29,30] have shown that PCI may enhance gene transfection mediated by several cationic polypeptide- or lipid-based gene vectors. In order to further develop PCI for therapeutic utilization, that is, the delivery of a therapeutic gene, a cationic polymer 22 kDa PEI was chosen as a carrier of transfecting DNA, since PEI is one of the most promising and effective gene transfection vectors for *in vivo* use. To evaluate PEI as a gene vector in combination with PCI, a plasmid encoding the reporter eGFP gene was selected,

and the efficiency of transfection was evaluated by measuring the percentage of eGFP-expressing cells. Different concentrations of DNA/PEI complexes were tested at a ratio PEI to DNA of N/P = 4 with the aim of finding the conditions, giving the highest difference in transfection efficiency obtained by PEI-mediated transfection alone or in combination with PCI. Under these conditions, one could expect obtaining specific, light-dependent gene expression, which would be advantageous for targeted tumor therapy.

In glioblastoma U87MG cells, increasing the concentration of DNA from 1 μg DNA up to 5 μg DNA per ml of transfection medium, the efficiency achieved by the PEI-mediated transfection also increased significantly, from $0.25 \pm 0.028\%$ ($n = 5$) to $21 \pm 4.7\%$ ($n = 5$) eGFP-expressing cells ($P \leq .01$ as found by unpaired t-tests) (Fig 1a). Thus, a five-fold increase in the amount of both the transfecting DNA and PEI led to more than 80-fold increase in transfection efficiency, indicating the contribution not only from the increased amount of the transgene, but possibly also a "threshold" effect of PEI. Light exposure enhanced the fraction of transfected cells to 5.6 ± 0.7 and $27.6 \pm 4.3\%$ eGFP-positive cells for 1 and 5 μg/ml DNA, respectively (Fig 1a) ($P = .002$ and .034, respectively, as found by paired t-tests).

For comparison we also used another cell line, human colon carcinoma HCT 116, established in our laboratory earlier as a model for PCI-based transfection using other nonviral and viral vectors.[11,30] HCT 116 cells also demonstrated the significant PCI-dependent increase in transfection efficiency (Fig 1b), and the level of enhancement was similar to the enhancement observed for U87MG.

Figure 2 shows the fold-increase (FI) in transfection efficiency in U87MG cells induced by PCI in respect to the PEI-based transfection alone using DNA doses in the range 1–5 μg/ml. As can be seen, the effect of PCI was the highest (FI varied between 13 and 33 with an average of 23.3 ± 4.0) in the cases where the PEI-mediated transfection alone was very inefficient (<0.5%), that is, at low DNA/PEI doses. In the case of the higher dose of DNA and, consequently, when higher efficiency was obtained by the PEI-mediated transfection alone, the fold increase due to PCI was only 1.43 ± 0.19, but still statistically significant.

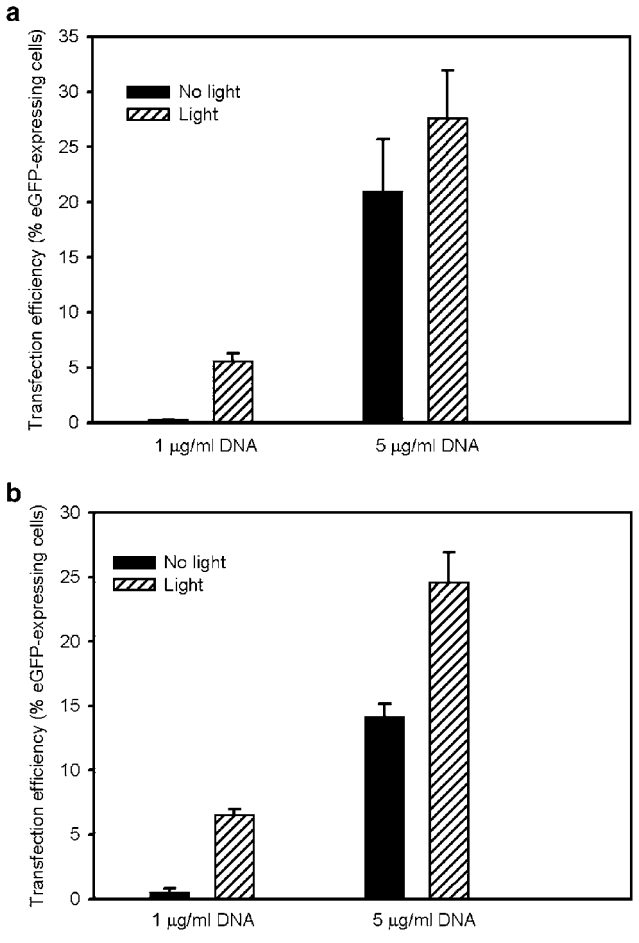

Figure 1 Effect of PCI on PEI-mediated transfection in (**a**) U87MG and (**b**) HCT 116 cells. The cells were preincubated with the photosensitizer $AlPcS_{2a}$ and treated with the pEGFP-N1 plasmid (1 or 5 μg/ml) complexed to PEI (N/P = 4) for 4 hours followed by the exposure to light for 5 minutes (U87MG cells (**a**) or 5 minutes 20 seconds (HCT 116 cells (**b**). Control cells were treated with $AlPcS_{2a}$ and pEGFP-N1/PEI, but not exposed to light. After 2 days, the cells were analyzed for eGFP-expression by flow cytometry. Error bars are SE of five parallels (**a**) and SE of 2–6 parallels (**b**).

Photochemical transfection of the HSVtk gene: effect on the expression of TK protein

The efficacy of transfection with the therapeutically relevant HSVtk gene, using PEI alone or in combination

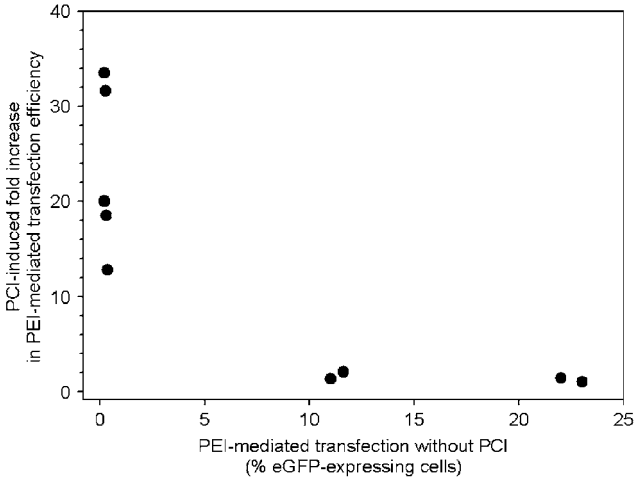

Figure 2 PCI-induced fold increase in transfection efficiency as a function of the efficiency achieved by PEI-mediated transfection alone. U87MG cells were preincubated with $AlPcS_{2a}$ and treated with the pEGFP-N1 plasmid (concentration range from 1 to 5 μg/ml) complexed to PEI (N/P = 4) for 4 hours and exposed to light for 5 minutes. The cells transfected by the help of PEI were not exposed to light. After 2 days the cells were analyzed for eGFP-expression by flow cytometry. The fold increase between PEI-mediated transfection alone (abscissa) and PCI-dependent transfection was calculated and plotted on the ordinate. Each dot means the result from one sample.

with PCI, was compared by measuring the level of the expressed TK protein. A polyclonal anti-HSV-TK antibody was used to visualize the ≈44 kDa TK protein on Western blots. As could be seen in Figure 3a, cells transfected with the high dose (5 μg/ml) DNA and without PCI expressed clearly detectable level of HSV-TK, which was further increased by light. On the contrary, there was no detectable TK expression in cells that were treated with the low dose (1 μg/ml) DNA and not exposed to light (Fig 3a, 0-minute light). However, low dose of DNA when combined with light resulted in clearly detectable and light-dose dependent amount of TK protein (Fig 3a, 4- and 5-minute light).

To compare the level of the expressed TK protein at different time points after the transfer of the low-dose HSVtk gene, cell lysates were prepared at 24, 44 and 96 hours after the treatment, and analyzed by Western blotting. The intensity of the TK bands was calculated and is presented on an arbitrary scale in Figure 3b. Nonilluminated cells did not show any TK expression up to 96 hours, while in photochemically treated cells TK protein was notable at all time points with the highest relative level at 44 hours after PCI of HSVtk gene.

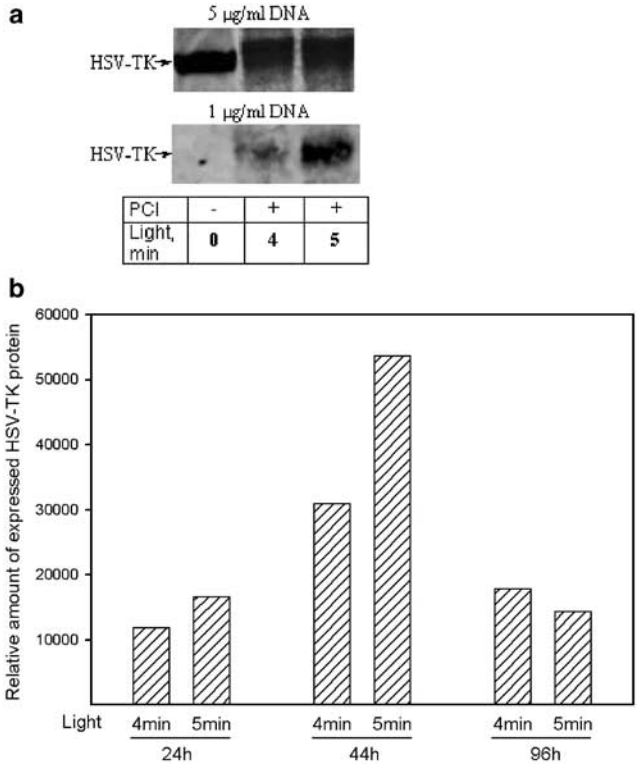

Figure 3 PCI-induced expression of HSV-TK protein in U87MG cells. The cells were preincubated with $AlPcS_{2a}$ and treated with the pNGVL1-TK plasmid (5 μg/ml i.e. high DNA dose (**a**) or 1 μg/ml, i.e. low DNA dose (**a**, **b**)) complexed to PEI (N/P = 4) for 4 hours followed by the exposure to light for 4 or 5 minutes as indicated in the figure. Control cells were treated with $AlPcS_{2a}$ followed by the pNGVL1-TK/PEI complex, but not exposed to light. The cells were harvested at 44 hours (**a**) or 24, 44 and 96 hours (**b**) after illumination and analyzed for the expression of HSV-TK protein with a polyclonal rabbit anti-HSV-TK antibody. (**b**) The intensity of each band on the Western blots was determined and expressed on an arbitrary scale showing the relative level of expressed TK protein at 24, 44 and 96 hours after the treatment with 1 μg/ml pNGVL1-TK/PEI and PCI (there was no detectable TK protein in the samples transfected by the help of PEI without PCI).

Photochemical transfection of the HSVtk gene increases the cytotoxic effect when combined with GCV treatment

Although the level of light-induced expression obtained with the low DNA dose was poorer than in the case of the high DNA doses without light (Fig 3a), the low dose of DNA/vector was chosen for the further studies of the cytotoxic effect of the HSVtk transgene. Under these conditions, the highest PCI-induced fold increase in expression of the transgenes, both eGFP (Fig 2) and HSVtk (Fig 3a, low DNA dose), was observed. Therefore, one could expect the highest treatment specificity in a clinical situation.

To evaluate the potential of PCI-enhanced expression of HSVtk gene for the "suicide" HSVtk/GCV approach, cell survival was measured. Photosensitizer-pretreated U87MG glioblastoma cells were incubated with HSVtk plasmid/PEI with or without illumination, followed by the treatment with GCV for 4 days before cell survival was evaluated. As was shown by Western blotting, the light-induced expression of the TK protein lasted for at

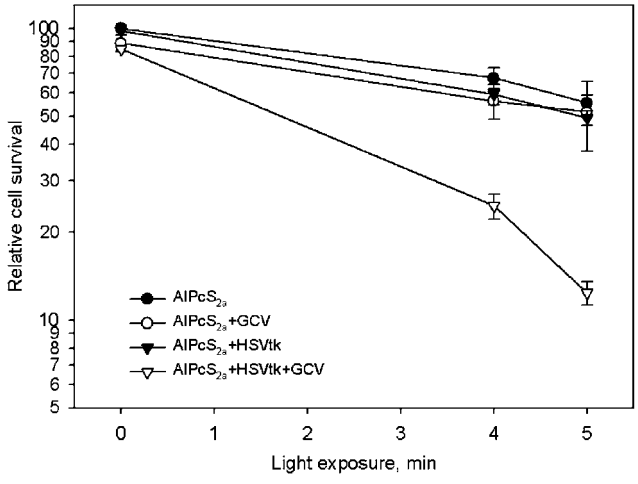

Figure 4 PCI-induced potentiation of the cytotoxic effect of the transfection with HSVtk gene followed by the treatment with GCV. U87MG cells were pretreated with the photosensitizer $AlPcS_{2a}$ followed by incubation with the pNGVL1-TK/PEI complex (1 μg/ml plasmid) for 4 hours. Then the cells were exposed to light for 4 or 5 minutes, or not exposed to light (0 minute). The next day medium containing 25 μg/ml GCV was added and the cells were incubated for 4 days changing the GCV-containing medium every day. Cell survival was evaluated measuring protein synthesis and expressed as relative to survival of the cells that were treated only with $AlPcS_{2a}$ and not illuminated. The cells that were exposed only to photochemical treatment were incubated with $AlPcS_{2a}$ followed by light. The other controls were incubated with $AlPcS_{2a}$ and pNGVL1-TK/PEI, alternatively, with $AlPcS_{2a}$ and GCV. Error bars represent SE of 3–7 parallels.

least 4 days (Fig 3b, 96-hour sample). The cell survival after the photochemical transfection of HSVtk gene followed by GCV treatment was compared with the cell survival after the photochemical treatment alone. When the cells were treated with 1 μg/ml HSVtk plasmid and no light was applied (i.e. without PCI), the HSVtk/GCV approach had no significant effect on cell survival (Fig 4, 0-minute light). This corresponds to the data from Western blot analysis (Fig 3), where no expression of the TK protein was observed, and TK enzyme is necessary for the phosphorylation of GCV to induce the toxicity.

However, transfection with 1 μg/ml HSVtk plasmid in combination with light (i.e. PCI-mediated transfection), which increased the percentage of transfected cells to $\sim$6% after 5 minute illumination (Fig 1a), resulted in only 12.4 ± 1.1% surviving cells, that is, the treatment induced 87% cell killing after incubation with GCV (Fig 4). The cell survival in this case was reduced approximately five times as compared to the photochemical treatment alone, which left $\sim$55% of the viable cells. Photochemical transfection with the irrelevant pEGFP-N1 plasmid followed by GCV resulted in cell survival identical to the photochemical treatment alone (data not shown). This indicates that the plasmid DNA not containing HSVtk gene does not induce an unspecific effect on cell survival, and the light-enhanced killing effect of the HSVtk/GCV approach shown in Figure 4 is mediated by the product of HSVtk gene.

In order to evaluate the synergy of the combined treatment (photochemical + HSVtk/GCV approach), we calculated $-\ln$ of relative survival for different treatments and compared the killing efficiency of these treatments as described in Materials and methods under ''Statistical analysis''. The difference between the killing induced by the combined treatment (photochemical + HSVtk/GCV approach) and the killing observed by only the photochemical treatment was 1.01 ± 0.24 ($P \approx .01$) and 1.50 ± 0.22 ($P < .01$) for 4- and 5-minute light, respectively. This indicates that the killing effect observed after the photochemical transfection of the HSVtk gene and GCV treatment was significantly higher than the effect of the photochemical treatment alone, indicating the photochemical potentiation of the HSVtk/GCV approach. We calculated Δy, that is, the measured killing effect of the combined treatment (photochemical + HSVtk/GCV) compared to the additive effect of photochemical treatment and the HSVtk/GCV approach when used separately. Δy was found to be 0.85 ± 0.24 ($P \approx .05$) for 4-minute light and highly significant, that is, 1.34 ± 0.22 ($P < .01$) for 5-minute light. The positive Δy value indicates synergy, that is, the appearance of an extra killing effect when the HSVtk/GCV approach was applied in combination with photochemical treatment.

The photochemical treatment in combination with single regimes, either HSVtk alone or GCV alone, had no an additional effect on cell survival as compared to the photochemical treatment alone (Fig 4), indicating that neither TK alone nor GCV alone was toxic to the cells at the doses used.

For comparison, we also tested HCT 116 cells. Although a similar light-dependent increase in transfection with the reporter eGFP gene was registered for both cell lines (Fig 1a, b), the cytotoxicity results obtained after the transfer of HSVtk gene to HCT 116 cells were different as compared to U87MG. The 1 μg/ml dose of HSVtk plasmid was too low to cause any significant effect with or without PCI (data not shown), therefore a five times higher dose of HSVtk gene was employed for the HCT 116 cells. Under these conditions, the HSVtk/GCV approach alone without light induced $\sim$22% toxicity, photochemical treatment alone yielded $\sim$20% cytotoxicity, and the combination of these two treatments resulted in $\sim$34% killed cells (Fig 5). As can be seen in Figure 5, the killing effect of a combined treatment was not as obvious as in U87MG cells. The Δy value for 5 minutes 20 seconds light was -0.045 ± 0.069 and not significantly different from zero, indicating that a purely additive, nonsynergistic effect was obtained when the photochemical and the HSVtk/GCV treatments were combined.

GJIC

To examine whether cell–cell communication could be responsible for the enhanced killing efficiency (synergy) observed in U87MG cells after PCI of HSVtk gene followed by GCV, GJIC was assessed and compared to GJIC in HCT 116 cells. The fluorescent dye LY was injected into a single cell, and the spreading of the dye to the neighboring cells was observed. LY cannot penetrate

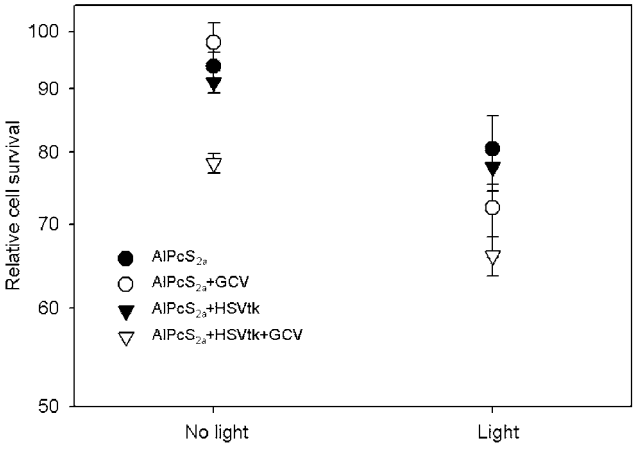

Figure 5 Effect of HSVtk/GCV treatment on the survival of HCT 116 cells. HCT cells were pretreated with the photosensitizer $AlPcS_{2a}$ followed by the incubation with the pNGVL1-TK/PEI complex (5 μg/ml plasmid) for 4 hours. Then the cells were exposed to light for 5 minutes 20 seconds, or not exposed to light. The next day medium containing 10 μg/ml GCV was added and the cells were incubated for 4 days changing GCV-containing medium every day. Cell survival was evaluated measuring protein synthesis and expressed as relative to survival of the cells that were treated only with $AlPcS_{2a}$ and not illuminated. The cells exposed only to photochemical treatment were incubated with $AlPcS_{2a}$ followed by light. The other controls were incubated with $AlPcS_{2a}$ and pNGVL1-TK/PEI, alternatively, with $AlPcS_{2a}$ and GCV plus/minus light. Error bars represent SE of 2–4 parallels.

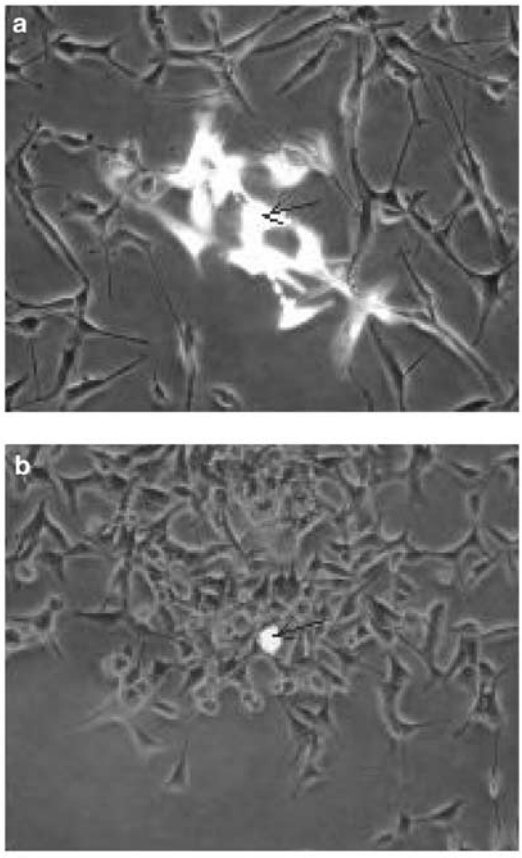

Figure 6 GJIC in (**a**) U87MG and (**b**) HCT 116 cells. LY was microinjected into a single cell (pointed by an arrow) from a cell monolayer, and 5–8 minutes after microinjection the cells were observed for the dye spreading by the microscope using an objective with $\times 20$ magnification.

the cellular membrane, but can transfer to the adjacent cells through gap junctions. The appearance of many fluorescing cells indicates good GJIC allowing dye spreading. As can be seen in Figure 6a, relatively many U87MG cells became fluorescing, although only one cell was injected with the dye, indicating good GJIC in this cell line. In contrast, in HCT 116 cells the dye was not transferred to the other cells since only one cell was fluorescing (Fig 6b), indicating low GJIC in HCT 116 cells.

Discussion

In the present study, we demonstrate the feasibility of using a photosensitizer and light, that is, photochemical treatment, to modulate the delivery, expression and, consequently, the biological effect of a therapeutically relevant cytotoxic gene delivered by the nonviral vector PEI. We employed the HSVtk gene as an example of a therapeutic gene to investigate *in vitro* if the PCI technology could potentiate and target the effect of the HSVtk/GCV approach used in cancer gene therapy.

The current study with the HSVtk gene is based on the results obtained with the reporter eGFP gene. These results showed that the impact of PCI enhancing transgene expression is highest under conditions, where PEI-mediated gene delivery without PCI gives low, if any, gene transfection. Thus, when we used a relatively low dose of DNA (1 μg/ml) and a modest amount of PEI, there were hardly any cells expressing the transgene unless PCI was performed. At this DNA/PEI dose, PCI increased the percentage of cells expressing the transgene 20–30 times. Using the same low dose of a plasmid containing HSVtk gene, we also demonstrated PCI-enhanced transfection in U87MG cells: the HSV-TK protein was detected only in the cells that were exposed to photochemical treatment. These observations might have several important advantages for cancer gene therapy: (1) PCI could induce expression of the cytotoxic transgene at the transgene doses too low to give the expression using the transfection methods without PCI. This might indicate PCI to be a highly targeted treatment, which would allow the transgene-mediated effects to be obtained specifically in illuminated cells, leaving the surrounding cells unaffected. (2) Usually, it is difficult to obtain high amounts of transfecting DNA in the target tissues, especially using nonviral vectors. The amount of the therapeutic DNA that reaches the final target might be very low, for example, due to bad tissue penetration, improper cellular delivery and/or intracellular transport and degradation of the delivered DNA.[31] In this respect, the PCI-potentiated transfection at the low "suboptimal" DNA doses might be very important for *in vivo* gene delivery. (3) Photochemical transfection can make it possible to use lower amount of gene vector than what would be possible without PCI. Gene vectors containing PEI are among the most promising nonviral vectors suggested for *in vivo* use.[24,32] However, as it was shown by Chollet et al,[33] toxicity is a major concern when using systemically delivered PEI particles, and the PEI toxicity is inherent to the PEI mode of action. PEI works as an endosmolytic agent and releases DNA from the endocytic vesicles, but to act as an efficient gene delivery vector some critical concentration of PEI must be achieved inside the vesicles. The data presented in Figure 1 also suggested the necessity of higher amounts of PEI for the efficiency of PEI-mediated (without PCI) transfection: 70–80-fold increase in transfection efficiency was achieved by only a five-fold increase in the DNA dose. This possibly indicates the contribution from the increased amount of PEI at the higher DNA doses, likely via improved endosomal release. It also indicates that the release from endocytic vesicles, even when PEI is used as a gene carrier, is one of the limiting steps in gene transfer, especially at suboptimal DNA/PEI doses. Therefore, the possibility of using PCI to reduce the dose and, consequently, the nonspecific toxicity related to PEI might be of practical value.

Although the illumination significantly enhanced the efficiency of transfection with a low DNA dose, the final percentage of cells expressing a transgene was still less than 10%. However, such low, but controlled

light-dependent gene expression might be sufficient in many approaches used in cancer therapy. Thus, in the present *in vitro* study on glioblastoma cells, we demonstrate that the effectiveness of gene therapy approach based on the HSVtk transgene and prodrug GCV may be significantly and selectively improved when the transgene was delivery by the help of photochemical transfection. Transfection using a relatively low dose (1 μg/ml) of HSVtk plasmid and PEI as a carrier followed by GCV treatment had no effect on cell viability, while photochemical transfection followed by GCV resulted in $\sim$90% killing of glioblastoma cells, which was significantly higher than the killing induced by the photochemical treatment alone. This extra killing effect obtained by combining the photochemical and the HSVtk/GCV approaches probably is the result of photochemically induced HSVtk expression. Analysis of Western blots (Fig 3a) confirmed that when the low DNA dose was used the photochemical stimulus was necessary to confer observable HSVtk expression, and in the case of the high DNA dose, photochemical treatment significantly enhanced expression of HSVtk. As we suggested earlier, the enhancement of transgene expression most likely occurs because of a more proper intracellular routing, that is, photochemically induced endosomal release of the transgene, so that the gene has a better chance to be expressed.[29]

The absence of a direct correlation between the low proportion of cells expressing a transgene and the high proportion of killed cells after PCI with the low DNA dose can be explained assuming that a bystander effect plays an essential role: the toxic effects, mediated by the PCI-enhanced level of the expressed TK protein, could be spread to the neighboring nontransfected cells. As has been shown by several investigators, as few as 5–10% of cells expressing the HSVtk gene might be enough for pronounced killing of HSV-TK-negative tumor cells and reduction of tumor burden if a bystander effect is involved.[13,34] Gliomas usually demonstrate intercellular communication (GJIC), and GJIC seems to be important for a bystander effect.[35] The glioblastoma cells used in the present work also demonstrated good GJIC (Fig 6a), and probably therefore show high sensitivity to GCV treatment after photochemical transfection of the HSVtk gene (Fig 4). In contrast, the colorectal carcinoma HCT 116 cells, although being transfected to the same level by PCI-transfection (Fig 1b), do not perform GJIC (Fig 6b), and most likely therefore PCI of HSVtk gene followed by GCV results in much lower cell kill.

To achieve a therapeutic response against cancer, the extension of the toxic effects to the tumor cells not expressing a transgene is an important issue especially for treatments based on nonviral vectors. Several approaches have been suggested to improve the spreading of the killing effect. One possibility is, for example, to increase the GJIC level, for example, via transfecting a connexin gene involved in intercellular communication.[36,37] An alternative way is to fuse, for example, a suicide gene with a gene encoding a protein having the ability of intercellular trafficking like HIV-1 Tat or VP22 peptides of Hsv-1.[38,39] These peptides provide translocating ability to the fused suicide protein. However, none of these principles on their own are selective to tumors, and therefore the combination of light-dependent transfection and strategies for favoring bystander killing could be a very interesting approach for both potentiating and targeting gene therapy-mediated killing of tumor cells. In general, it is likely that the future direction in cancer therapy lies in approaches combining various concepts, and PCI-based transfection could be combined with other strategies suggested for tumor targeting, thus enhancing the level of targeting. For example, PCI could be employed for improving the efficiency of gene vectors coupled to targeting ligands that assure attachment of the gene vector to specific cells via ligand-receptor interaction.[40] Work is in progress in our laboratory where PEI coupled to ligands for tumor-associated receptors are employed in combination with PCI.[41] Another interesting possibility for PCI in targeted gene therapy would be the delivery of genes with inducible promoters responding to external stimuli. For example, the photochemical treatment in itself is an effective inducer of oxidative stress and can function as a molecular switch for the selective expression of genes as was shown by Luna et al.[42,43] In this case, the photochemical treatment would control gene expression in a dual mode—firstly, via the improvement of gene delivery (induction of endosomal release) and secondly, via the initiation of gene expression.

The combined treatment PDT and the suicide gene therapy might be an interesting approach in cancer therapy, to destroy cancer cells that were not efficiently killed by PDT alone. Although cytotoxic effects induced by PDT have already been clinically tested for various tumor types,[6] the effective tumor depth that photochemical treatment reaches is usually restricted due to limited light penetration. However, for the induction of the suicide HSVtk/GCV approach, lower light doses are needed than for the induction of the efficient killing by PDT, as demonstrated in Figure 4. Therefore, photochemically enhanced transfection of the HSVtk gene might help to affect deeper tumor layers, which were not affected by PDT alone.

The possibility to deliver light by means of fiber optic devices allows reaching tumors at different sites within a body.[6] In addition, a photosensitizer, the other component necessary to perform PCI, preferentially localizes in tumors,[6] which is beneficial for obtaining and targeting PCI effects. All this, together with the earlier documented PCI *in vivo* delivering proteins,[9] suggests that technically it should be possible to perform photochemical gene transfection *in vivo*.

The present study was designed to prove the principle that PCI-based gene transfection using the nonviral vector PEI can function with a therapeutically relevant gene, and such combined treatment might find a therapeutic utility in cancer therapy as discussed above. On the basis of here presented study, stimulated by previous works on PCI *in vitro*,[10,29] we have initiated *in vivo* PCI studies to target a transgene/PEI complexes to tumors.

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