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Advances in Brief

Photochemical Internalization: A Novel Technology for Delivery of Macromolecules into Cytosol[1]

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Abstract

The therapeutic usefulness of macromolecules, such as in gene therapy, is often limited by an inefficient transfer of the macromolecule to the cytosol and a lack of tissue-specific targeting. The possibility of photochemically releasing macromolecules from endosomes and lysosomes into the cytosol was examined. Endocytosed macromolecules and photosensitizer were exposed to light and intracellular localization and the expression of macomolecules in the cytosol was analyzed. This novel technology, named photochemical internalization (PCI), was found to efficiently deliver type I ribosome-inactivating proteins, horseradish peroxidase, a $p21^{ras}$-derived peptide, and a plasmid encoding green fluorescent protein into cytosol in a light-dependent manner. The results presented here show that PCI can induce efficient light-directed delivery of macromolecules into the cytosol, indicating that PCI may have a variety of useful applications for site-specific drug delivery, *e.g.*, in gene therapy, vaccination, and cancer treatment.

Introduction

It is widely acknowledged that macromolecules have great potential as therapeutic agents (1–3). Despite a wealth of data from *in vitro* and animal systems (see Ref. 4) and many ongoing clinical studies, thus far there are few reports on the successful *in vivo* use of macromolecules in human therapies. As pointed out by Verma and Somia (2), the ability to direct the action of macromolecules to specific cells, organs, or tissues would be of great benefit for many kinds of therapies. In gene therapy, the delivery of the nucleic acid into the correct intracellular compartment constitutes a major technological challenge for the efficient employment of nucleic acid therapies. For example, a great problem in standard liposome transfection methods is that a very large fraction of the nucleic acids taken into the cell is retained in intracellular vesicles resembling endocytic vesicles and is unable to reach the intracellular compartment, where nucleic acids can accomplish their biological activity (5). This may also occur in other therapies in which macromolecules are the active substance (6). A number of photosensitizers, including $TPPS_4$,[3] $TPPS_{2a}$, and $AlPcS_{2a}$ used in the present study, localize primarily to the endosomes and lysosomes of cells (7). In combination with exposure to light, these photosensitizers induce the formation of reactive oxygen species, primarily singlet oxygen (8). Singlet oxygen has a very short lifetime and a short range of action (10–20 nm; Ref. 9). Light activation of photosensitizers located in the membranes of endosomes and lysosomes may therefore destroy the membranes, whereas the contents of the organelles remain intact (10). Thus, we have explored the potential use of such photosensitizers to rupture endosomes and lysosomes and thereby deliver endocytosed macromolecules into the cytosol (Fig. 1). The method has been named PCI. In this report, it is demonstrated that PCI can be used for the site-specific delivery of a variety of therapeutic molecules.

Materials and Methods

Irradiation. Two different light sources were used to treat the cells, both of which consisted of a bank of four fluorescent tubes. Cells treated with $TPPS_4$, $TPPS_{2a}$, and 3-THPP (Porphyrin Products, Logan, UT) were exposed to blue light (model 3026; Appl. Photophysics, London, United Kingdom; light intensity reaching the cells, 1.5 mW/cm^2), whereas cells treated with ZnPc (CPG 55847; provided by Ciba-Geigy, Basel, Switzerland), BPD-MA (QLT), and $AlPcS_{2a}$ (Porphyrin Products) were exposed to red light (Philips TL 20W/09) filtered through a Cinemoid 35 filter (light intensity reaching the cells, 1.35 mW/cm^2).

Fluorescence Microscopy. The cells were analyzed by fluorescence microscopy as described previously (11). For analysis of fluorescein-labeled molecules, the microscope was equipped with a 450–490 nm excitation filter, a 510 nm dichroic beam splitter, and a 510–540 nm band pass emission filter.

Preparation of Plasmid-pLys Complexes and Treatment of Cells. Plasmid-pLys complexes (charge ratio, 1.7) were prepared by gently mixing 5 μg of plasmid (pEGFP-N1; Clontech Laboratories, Inc., Palo Alto, CA) in 75 μl of HBS [20 mM HEPES (pH 7.4)] with 5.3 μg of pLys (MW 20700; Sigma, St. Louis, MO) in 75 μl of HBS. The solutions were incubated for 30 min at room temperature, diluted with culture medium, and added to the cells.

THX cells were incubated with 20 μg/ml $AlPcS_{2a}$ for 18 h at 37°C, washed, and incubated in sensitizer-free medium for 3 h before incubation with plasmid-pLys complexes for 2 h. The pEGFP-N1/pLys-treated THX cells were washed once and incubated for 2 h in culture medium without additions before exposure to light. The cells were incubated at 37°C for 2 days, subcultured, and incubated for an additional 5 days before analysis of GFP expression by flow cytometry.

HCT-116 cells were incubated with 20 μg/ml $AlPcS_{2a}$ for 18 h, washed, and transfected with plasmid-pLys complexes for 6 h before light exposure in plasmid-free medium. After 40 h of incubation at 37°C, the GFP expression was studied by microscopy.

Flow Cytometry Analysis. The cells were trypsinized, centrifuged, resuspended in 400 μl of culture medium, and filtered through a 50-μm mesh nylon filter. The cells were then analyzed in a FACStar plus flow cytometer (Becton Dickinson). GFP was measured through a 510–530 nm filter after excitation with an argon laser (200 mW) tuned on 488 nm. $AlPcS_{2a}$ was measured through a 650 nm longpass filter after excitation with a krypton laser (50 mW) tuned on 351–356 nm. Cell doublets were discriminated from single cells by gating on the pulse width of the GFP fluorescence signal. The data were analyzed with PC Lysys II software (Becton Dickinson).

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[3] The abbreviations used are: $TPPS_4$, tetra(4-sulfonatophenyl)porphine; pLys, poly(L-lysine); $TPPS_{2a}$, meso-tetraphenylporphine with two sulfonate groups on adjacent phenyl rings; $AlPcS_{2a}$, aluminum phthalocyanine with two sulfonate groups on adjacent rings; 3-THPP, (3-hydroxyphenyl)porphine; ZnPc, liposome Zn phthalocyanine; BPD-MA, liposome benzoporphyrin derivative monoacid; PCI, photochemical internalization; GFP, green fluorescent protein; HRP, horseradish peroxidase; RIP, ribosome-inactivating protein.

Preparation of Fluorescein-Peptide and Treatment of Cells. The fluorescein-labeled Val^{12}-$p21^{ras}$-peptide (residues 5–21) was kindly synthesized and provided by Alan Cuthbertson (Nycomed Amersham).

BL2-G-E6 cells were incubated with 30 μg/ml of the fluorescein-labeled $p21^{ras}$-derived peptide for 18 h, followed by 20 μg/ml $AlPcS_{2a}$ for 18 h and 1 h in drug-free medium before exposure to red light.

Results and Discussion

To document both the relocation and biological effect of macromolecules after PCI, NHIK 3025 cells were treated with the type I RIP gelonin in combination with $TPPS_{2a}$ and light (Fig. 2). Type I RIPs have the same ability as type II RIPs to inactivate ribosomes, but in contrast to type II RIPs, they do not bind to cells and are relatively nontoxic to intact cells (12). Type I RIPs are internalized by fluid phase endocytosis and transported to lysosomes, where these toxins are degraded (13), whereas type II RIPs can be transported retrogradely from endosomes through the Golgi apparatus and endoplasmic reticulum to the cytosol, where these toxins exert their cytotoxic action (14). We found that under conditions in which each treatment alone exerted minor effects, cells became extremely sensitive to a combination of gelonin, $TPPS_{2a}$, and light as measured by protein synthesis (Fig. 2*a*) and colony-forming ability (Fig. 2*c*). As shown, treatment with 10 μg/ml gelonin and a photochemical dose that separately reduced protein synthesis 3- and 1.5-fold, respectively, reduced the protein synthesis more than 300-fold when the two treatment modalities were combined (Fig. 2*a*). The effect of the combined treatment was clearly dependent on the light dose (Fig. 2*b*).

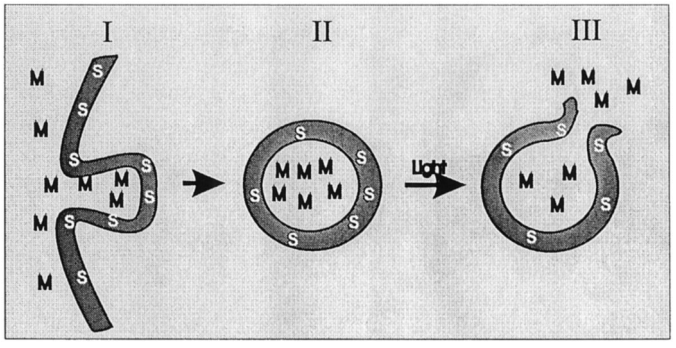

Fig. 1. Transport of molecules to the cytosol by PCI. *I*, the photosensitizer (*S*) and the molecules of choice (*M*) are endocytosed. *II*, both substances end up in the same vesicles. *III*, upon exposure to light, the membranes of the vesicles rupture, and the contents are released into cytosol.

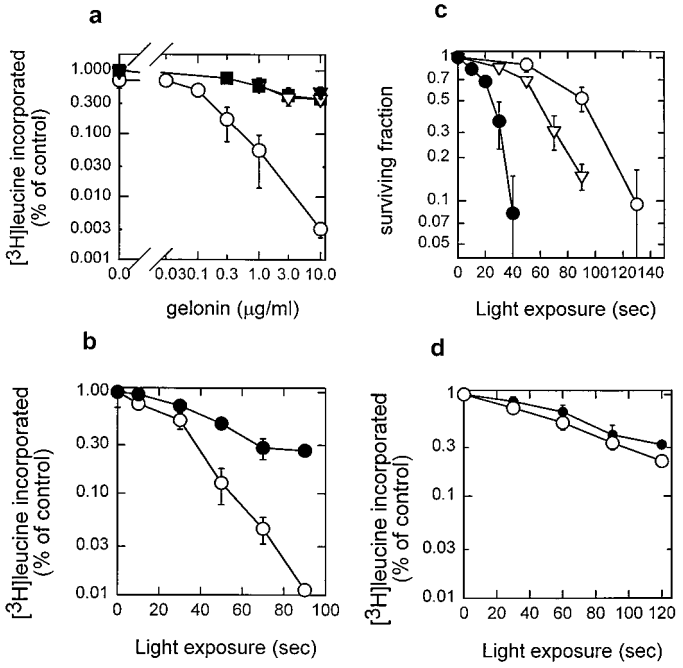

Fig. 2. Cytotoxic effect of combining photochemical and gelonin treatment in NHIK 3025 cells. The cells were treated with 3.2 μg/ml $TPPS_{2a}$ (*a-c*) or 0.2 μg/ml liposome-bound ZnPc (*d*) and the indicated concentration of gelonin for 18 h, followed by 1 h in drug-free medium before exposure to light. Protein synthesis was measured as described previously (24). *a*, protein synthesis after treatment with gelonin alone (■), gelonin and 50 s of light (▼), gelonin and $TPPS_{2a}$ (▽), and gelonin and $TPPS_{2a}$ in combination with 50 s of light (○). *b*, protein synthesis after treatment with $TPPS_{2a}$ and light in the absence (●) or presence of 0.2 μg/ml gelonin (○). *c*, colony-forming ability of cells treated with $TPPS_{2a}$ and light in the absence (○) or presence of 0.2 μg/ml (▽) or 2 μg/ml (●) gelonin. *d*, protein synthesis in cells treated with ZnPc and light in the absence (●) or presence of 1 μg/ml gelonin (○).

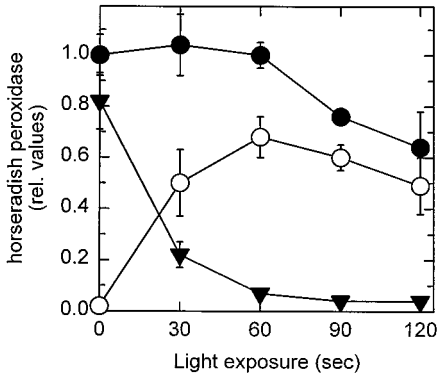

Fig. 3. Ability of PCI to deliver HRP into the cytosol. NHIK 3025 cells were treated with 3.2 μg/ml $TPPS_{2a}$ and 1 mg/ml HRP for 18 h. The medium was then replaced with drug-free medium before exposure to the indicated light doses. HRP activity (25) was measured in intact cells (○) and in cytosol (●) separated from cytosol-free cell corpses (▼) by electropermeabilization and a density centrifugation technique (10).

PCI-induced delivery of gelonin into the cytosol was studied in 16 cell lines of various origins (15). In all cell lines, photoactivated $TPPS_{2a}$, $TPPS_4$, and $AlPcS_{2a}$ were found to potentiate the toxicity of gelonin. However, in the human osteosarcoma cell line OHS, the potentiation was only observed in the presence of NH_4Cl or chloroquine. Four different photosensitizers (ZnPc, BPD-MA, 3-THPP, and $chlorine_6$) found to be mainly nonlysosomally located (7, 16) were not able to potentiate the effect of gelonin on protein synthesis as illustrated by experiments with ZnPc (Fig. 2*d*).

To further explore the versatility of PCI, two other type I RIPs, saporin and agrostin, were also subjected to PCI and found to induce results similar to those obtained with gelonin (15).

By using HRP, we could demonstrate that PCI actually induces the release of a large fraction (>60%) of endocytosed HRP (Fig. 3).

Mutant oncogene peptides hold great promise as cancer vaccines acting as antigens in the stimulation of cytotoxic T-lymphocytes (17). To induce a direct activation of $CD8^+$ T cells, the peptides need to enter the cytosol of the antigen-presenting cells (18). Thus, the success of the treatment is dependent on the efficient delivery of peptides into cytosol. To evaluate PCI for cytosolic delivery of cancer-specific peptides, a fluorescein-labeled $p21^{ras}$ peptide encompassing residues 5–21 and containing a Val^{12} mutation (G12V) was used (19). In BL2-6-E6 mouse fibroblasts, the ras peptide colocalized well with $AlPcS_{2a}$, indicating endocytic uptake of the peptide (Fig. 4). After a 4-min exposure to light, the fluorescein-labeled ras peptide and $AlPcS_{2a}$ were found to be located diffusely in the cytoplasm. Similar effects were not observed in cells exposed to the fluorescein-labeled ras peptide and light only (data not shown).

Gene therapy, including antisense and ribozyme technology, is a new and promising treatment modality that has thus far been ham-

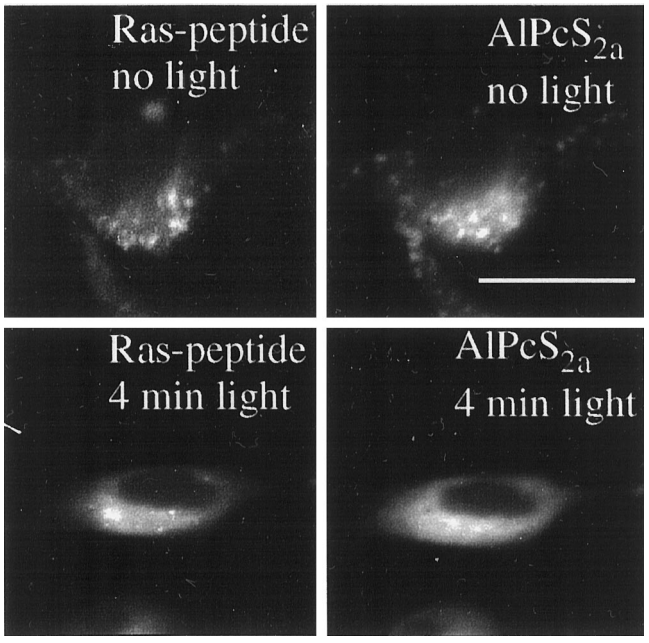

Fig. 4. Photochemically induced relocalization of a peptide. BL2-G-E6 cells were incubated with a fluorescein-labeled $p21^{ras}$-derived 5-21, Val^{12} peptide and $AlPcS_{2a}$ as described in "Materials and Methods." The cells were examined for fluorescein-peptide and $AlPcS_{2a}$ localization by fluorescence microscopy before (*top panels*) and 30 min after (*bottom panels*) a 4-min exposure to red light. *Bar*, 20 μm.

pered by the lack of safe, specific, and efficient delivery systems (2). To show that PCI can also be used to enhance the delivery of functional genes, THX cells were transfected with a pLys-complex of a plasmid (pEGFP-N1) coding for GFP. The expression of GFP was analyzed by flow cytometry (Fig. 5, *a* and *b*) and fluorescence microscopy (data not shown). As can be seen from Fig. 5*a*, treatment with $AlPcS_{2a}$ and light led to a strong increase in the percentage of the cells expressing GFP. The fraction of the cells that was positive for this reporter molecule increased from 1% at no light treatment to 50% after a 5-min light exposure. GFP expression was not enhanced by light in cells treated with pEGFP-pLys in the absence of a photosensitizer. A complex of an irrelevant plasmid (encoding heme oxygenase) and pLys did not induce green fluorescence when combined with $AlPcS_{2a}$ and light (data not shown). Consequently, in a light-directed manner, PCI can substantially increase the efficiency of transfection of a functional gene to THX cells. Similar results were obtained using $TPPS_{2a}$ as a photosensitizer and BHK-21 and HCT-116 as target cells (data not shown). The essentially nonlysosomally located sensitizer 3-THPP induced only a minor increase in GFP expression (Fig. 5*b*). PCI of pEGFP-N1 not complexed with pLys did not induce the expression of GFP (data not shown).

The presented technology is efficient as a delivery tool for a large variety of macromolecules, and it is likely that PCI can also be used for the delivery of other macromolecules as well as small molecules that are unable to enter the cytosol alone. The contribution from the different vesicular compartments probably depends on the rate of degradation of the macromolecule of choice and the treatment conditions. The photosensitizers used in the present study are taken up by endocytosis, but it is likely that lysosomotropic weak base photosensitizers (7) can also be used in PCI. In conclusion, PCI seems to fulfill several criteria for being an ideal drug delivery tool for gene therapeutics as well as for other purposes: (*a*) it has no restrictions on the size of the molecule to be delivered, as long as the molecule can be endocytosed by the target cell; (*b*) it is not dependent on cell proliferation; (*c*) it is site specific in that only areas exposed to light are affected; and (*d*) it is not oncogenic (20). In addition, PCI may potentially be combined with other principles for generating site- or tissue-specific drug action, such as targeting by the use of specific ligands for cell surface structures, using regulatory gene elements that confer tissue specificity (21), or the use of disease-specific drugs, opening a possibility of obtaining substantial synergistic effects in the specificity of drugs for target cells. In cancer gene therapy, the goal of targeting the main bulk of neoplastic cells may be reached by taking advantage of the bystander effect in several molecules, such as thymidine kinase/ganciclovir or VP22 fusion proteins (22). It should be emphasized that photosensitizers similar to those being used in this study have been approved for treatment of esophageal and lung cancer and are under clinical investigation for treatment of a large variety of other neoplastic lesions, including lesions in the bladder, gastrointestinal tractus, and brain, as well as mesothelioma, basal cell carcinoma, and the treatment of several other diseases (23).

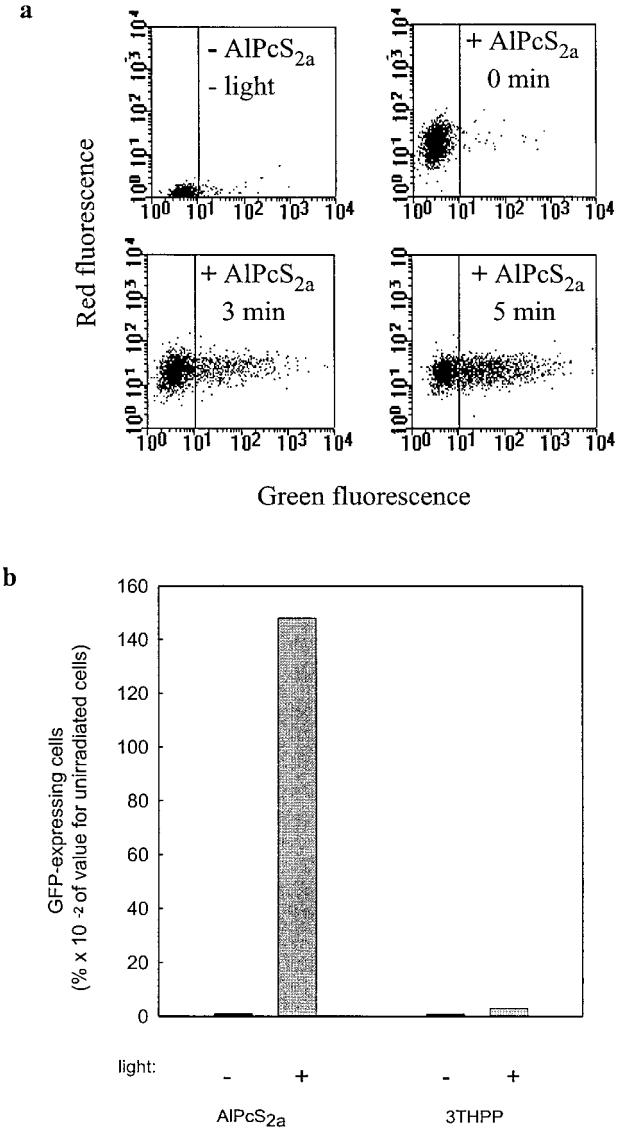

Fig. 5. Photochemically induced expression of GFP. *a*, expression of GFP in THX cells treated with pEGFP-N1-pLys complex in the absence of $AlPcS_{2a}$ and light or in the presence of $AlPcS_{2a}$ followed by exposure to light as indicated on the figure. The cells were analyzed by flow cytometry, reckoning the cells on the right side of the drawn *line* as positive for GFP expression. *b*, expression of GFP in THX cells treated for 18 h with a photosensitizer (20 μg/ml $AlPcS_{2a}$ or 0.25 μg/ml 3-THPP) followed by a 6-h transfection with pEGF-N1-pLys complex and exposure to light inactivating 50% of the cells. GFP expression was analyzed by flow cytometry as described in *a*.

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