Insulin-mediated Intracellular Targeting Enhances the Photodynamic Activity of Chlorin e₆

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ABSTRACT

Photodynamic therapy has been applied quite extensively over the last few years, whereby the activation of photosensitizers by light causes the production of reactive oxygen species such as singlet oxygen, which is cytotoxic. The goal of this study was the enhancement of the photodynamic activity of photosensitizers through their delivery to specific, sensitive intracellular compartments of target cells. We synthesized a BSA-insulin-chlorin e₆ conjugate that bound specifically to the insulin receptors (EC₅₀ 1 nm) of the human hepatoma cell line PLC/PRF/5 and could be internalized by receptor-mediated endocytosis. Photodynamic activity, as assessed by various tests, indicated EC₅₀ at about 100 times lower concentrations of conjugate compared to free chlorin e₆ itself; and lower doses of irradiation were necessary to activate the conjugate compared to free chlorin e₆. Inhibition of endocytosis of the conjugate abrogated the enhanced photodynamic activity of the conjugate above that of free chlorin e₆. Endocytosis and subsequent localization around and in the cell nucleus of the BSA-insulin-chlorin e₆ conjugate could be visualized using both FITC-labeled conjugate and 2',7'-dichlorofluorescin diacetate, a fluorescent indicator of the production of active oxygen species due to chlorin e₆ activation. It was concluded that photodynamic activity of the conjugate is higher than that of free chlorin e₆ through its receptor-mediated delivery into sensitive intracellular compartments.

INTRODUCTION

Over the last few years, a number of photosensitizers have been used in cancer and other therapies. Upon photo-irradiation, photosensitizers generate active oxygen species which are cytotoxic. Porphyrins (1) are the most efficient among the better-known photosensitizers, which include psoralens (2, 3), phthalocyanines (4), naphthalocyanines (5), acridine orange (6, 7), and merocyanine 540 (8). In terms of their use in photodynamic therapy, porphyrins represent a compromise between physical, chemical, and biological properties, including a unique absorption spectrum with intense lines in the visible range, low toxicity for the organism, and the ability to accumulate in tumor tissues to a greater degree than in normal tissues (9). However, normal cells are also able to accumulate porphyrins, and porphyrins are only excreted very slowly from the body; therefore, patients administered these substances i.v. have posttherapeutic complications in the form of skin burns after direct exposure to sunlight (10). Clearly, major difficulties with respect to photodynamic therapy relate to increasing the specificity of the uptake of photosensitizers, in particular, target cells, and thereby to reduce the concentration of porphyrins necessary to be administered to patients.

Singlet oxygen comprises 80% of all of the active oxygen species generated upon photoactivation of porphyrins. Injury initiated by singlet oxygen is localized very near the site of its production so that its effectiveness is 0.1 μm at most; consequently, the effect of photosensitizers is integrally dependent on their site of cellular accumulation (11). It has been shown that chlorin e₆ (12) and a mixture of hematoporphyrin derivatives (13) localize in cellular membranes, including the plasma and mitochondrial membranes. The plasma membrane, where lipid peroxidation is initiated, has been suggested to be a sensitive target of porphyrin action (14).

Photodynamic activity can be enhanced concurrently with a decrease in the concentration of photosensitizers necessary to be administered by using porphyrin derivatives which localize at specific subcellular sites other than the plasma membrane. The chlorin e₆ derivative mono-L-aspartyl chlorin e₆, for example, has higher photodynamic activity than chlorin e₆, presumably through its ability to be endocytosed by the cell and enter lysosomes (15). Like chlorin e₆, however, mono-L-aspartyl chlorin e₆ accumulates in both tumor and in normal cells (16).

Enhancement of the specificity of photodynamic action may be achieved by coupling photosensitizers to an antibody or ligand specific to surface antigens/receptors on the target cells. Conjugates of chlorin e₆ with monoclonal antibody to tumor cells have been proposed (17–19), which may enhance selectivity of porphyrin photodynamic action by binding specifically to the surface of tumor cells. The cellular target of this conjugate is the plasma membrane (17, 18). Intracellular compartments, such as the nucleus and the lysosome (15), are potentially much more sensitive targets, as demonstrated by their sensitivity on exposure to radiation, for example (20).

Enhancement of photodynamic effects and a decrease in the concentration of a drug administered may be accomplished by using internalizable ligands as an alternative to monoclonal antibodies to confer cell type selectivity. Endocytosis of photosensitizer conjugates may lead to their accumulation in lysosomes or penetration to the nucleus, according to the ligand used. In addition, multiple molecules of photosensitizers may be incorporated within a single conjugate molecule, which amplifies the local effect of photosensitization within sensitive intracellular compartments. We have previously described conjugates of chlorin e₆ coupled to con A or insulin, both of which are specific for high affinity receptors of particular cell types and internalized by receptor-dependent processes (21, 22). Whereas insulin is transported to the nucleus subsequent to endocytosis (23, 24), con A is targeted to the Golgi as well as lysosomes. Both conjugates displayed photodynamic activities significantly above that of chlorin e₆ alone, while in the case of the con A-conjugate, internalization was shown to be necessary for photodynamic activity (22).

In this study, we analyze the photodynamic action of BSA-insulin-chlorin e₆ conjugate, its dependence on internalization, and the intracellular distribution of the conjugate in cells of the PLC/PRF/5 human hepatoma line. The photodynamic activity of the conjugate is superior to that of free chlorin e₆, which appears to be directly attributable to its ability to be internalized and transported to near and within the cell nucleus.

MATERIALS AND METHODS

Synthesis of Conjugates. Chlorin e₆ was isolated from nettle (Urtica dioica L.) leaves (21, 22) using a modification of the method of Fischer (25).

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1 To whom requests for reprints should be addressed.
2 The abbreviation used is: PAGE, polyacrylamide gel electrophoresis.
BSA was conjugated to chlorin $e_6$ using cyclohexyl-3(2-morpholinoethyl)carbodiimide meth-4-toluene sulfonate (Serva; Ref. 26). Insulin was then coupled to the diazoylated BSA-chlorin $e_6$ conjugate by means of glutaraldehyde (Merck; Ref. 27), followed by dialysis (M, 50,000 cutoff). The BSA-insulin-chlorin $e_6$ (1:13:16) conjugate was analyzed using TLC on Silica gel G (Merck) in an ethanol-acetic acid (95:5) mixture (28). Analysis showed that over 95% of chlorin $e_6$ was covalently bound to BSA; analysis of the conjugate by SDS-PAGE indicated the absence of free insulin in the conjugate. To obtain insulin conjugate labeled with FITC (Sigma Chemical Co.; BSA-insulin-FITC; 1:4:10) FITC-labeled BSA was initially prepared (29), and then insulin was coupled to the BSA-FITC using glutaraldehyde as above.

**Iodination of Conjugate of BSA-Insulin-Chlorin $e_6$ and Insulin.** Insulin and conjugate were $^{125}$I-iodinated using iodogen (Pierce; Ref. 30). The specific activities of insulin and conjugate were 461 and 2235 Ci/mmole, respectively.

**Cell Culture.** Human hepatoma PLC/PRF5 cells were cultured at 37°C in a humidified incubator with 5% CO$_2$ atmosphere in RPMI 1640 (Serva) containing 10% heat-inactivated FCS as described previously (22).

$^{125}$I-labeled Insulin Binding. PLC/PRF5 cells were grown in 12- or 24-well plates (Flow) to subconfluence (~2 days) and then washed in serum-free medium containing 2 mg/ml BSA. Competition of insulin binding was measured by incubating cells with $^{125}$I-insulin (0.4 nM; 461 Ci/mmole) at 4°C for 18 h in the presence of BSA-insulin-chlorin $e_6$ conjugate (0.06–60 nM) in RPMI 1640 with 2 mg/ml BSA. The cells were then washed in Hank's solution containing 2 mg/ml BSA, and the cells were removed using Versen solution and then counted on a MiniGamma 1275 gamma counter (Pharmacia LKB).

$^{125}$I-labeled BSA-Insulin-Chlorin $e_6$ Binding and Endocytosis. PLC/PRF5 cells were grown in 12- or 24-well plates to subconfluence (~2 days) and then incubated for 1 or 18 h at 37°C with $^{125}$I-labeled conjugate (0.07–2.3 nM) in the absence or presence of unlabeled insulin in RPMI 1640 containing 2 mg/ml BSA. Subsequent to incubation with conjugate, cell surface-bound conjugate was removed by incubation of the cells with 1 m citrate buffer (pH 3) for 15 min at 4°C, and endocytosed conjugate was determined by resuspending the cells using Versen solution prior to gamma counting. Nonspecific binding accounted for about 40% of total $^{125}$I-labeled conjugate binding and endocytosis.

**Preparation of Cell Extracts.** Preparation of cell extracts to estimate $^{125}$I-labeled conjugate after internalization was performed by washing the cells with 1 m citrate buffer (pH 3) to remove surface-bound conjugate, prior to resuspension using Versen solution. The cell suspension was freeze-thawed three times prior to SDS-PAGE.

**Electrophoresis.** Molecular weight estimations of conjugates, as well as assessment of the intactness of the $^{125}$I-labeled BSA-insulin-chlorin conjugate before and after internalization by PLC/PRF5 cells, were performed by analysis using SDS-PAGE according to Laemmli (31). The gel was then sliced, and the slices (0.5 cm) were gamma counted.

**Irradiation of Cells.** Twenty-four h after plating, the cells were washed in RPMI 1640 containing BSA (2 mg/ml)-10 mM HEPES (pH 7.2) without sodium bicarbonate and incubated with various concentrations of conjugates (in the presence or absence of insulin) or chlorin $e_6$ in the same medium. For viability measurements, the cells were then washed with Hank's solution without phenol red prior to irradiation with a 150 W halogen lamp (0.4 kW/m²) to activate the chlorin $e_6$ moieties.

**Determination of Cell Survival.** For estimation of the cell survival rate using the colony formation test, PLC/PRF5 cells were placed in serum-containing medium after treatment and irradiation and cultured for 10 days, after which the number of colonies was counted. Determination of the survival rate by trypan blue exclusion was carried out follows. One h after irradiation, 0.1% trypan blue (Serva) in Hank's solution was added to the cells, and the percentage of cells which had lost the capacity to exclude the dye counted (32).

**Visualization of Conjugates.** After incubation with the BSA-insulin-FITC conjugate at 37°C, cells were washed in Hank's solution, 10 mM NH$_4$Cl was added, and cells were incubated for 10 min at 37°C. NH$_4$Cl reduces intracellular acidification and hence decreases the reduction in quantum fluorescent yield of fluorescein at low pH (29, 33, 34). FITC fluorescence was excited with a xenon lamp fitted with a 490-nm interference filter. To visualize the site of chlorin $e_6$ photoactivation due to the conjugate, 2',7'-dichlorofluorescin diacetate (35), an indicator of active oxygen species, was used. 2',7'-Dichlorofluorescin diacetate penetrates into cells and is deacetylated by intracellular esterases at 37°C; the active oxygen species generated by chlorin $e_6$ activation react with 2',7'-dichlorofluorescin to yield the fluorescent derivative 2',7'-dichlorofluorescin (35); therefore, 2',7'-dichlorofluorescin diacetate can be used as an indicator of the sites of generation of active oxygen species produced upon chlorin $e_6$ photoactivation. Cells were washed in Hank's solution without phenol red after incubation with the BSA-insulin-chlorin $e_6$ conjugate, and 2 μM 2',7'-dichlorofluorescin diacetate was added. The cells were then washed in Hank's solution and irradiated for 2–8 min under the microscope using a helium-neon laser LG-66 (632 nm, 150 μW; Riazan, Russia). Using the prism, the laser beam was rotated through 90° to focus on the cells from below using the microscope condenser. The device used for visualization, recording, and processing of cell images consisted of a universal microscope Axioplan (Zeiss), a hybrid visual converter (GP-3; MELZ, Moscow, Russia), and an image analyzer IBAS (Kontron). Pairs of images (cell image and background; 256 frames) were successively recorded for each group of cells and linearly intensified and analyzed after background subtraction (29, 36).

**RESULTS**

**Binding and Endocytosis of the BSA-Insulin-Chlorin $e_6$ Conjugate by PLC/PRF5 Cells.** We set out to synthesize a BSA-insulin-chlorin $e_6$ conjugate (1:13:16) in order to deliver chlorin $e_6$ into particular target cells via insulin receptors. After coupling BSA to chlorin $e_6$ and insulin, a 66:34 mixture of monomers and dimers of the conjugate was obtained. The mean molecular mass of the $^{125}$I-labeled conjugate was 258 kilodaltons, as determined by SDS-polyacrylamide (4.5%) gel electrophoresis (data not shown). PLC/PRF5 cells have specific receptors for insulin, the number of specific binding sites at 4°C being 168,000 per cell, with a dissociation constant ($K_d$) of 4.1 μM (21). Binding of $^{125}$I-labeled insulin to PLC/PRF5 cells at 4°C was measured in the presence of increasing concentrations of unlabeled BSA-insulin-chlorin $e_6$ conjugate (Fig. 1). Results indicated that the conjugate bound specifically to the insulin receptor with high affinity (EC$_{50}$, 1.0 nM; Table 1).

After the incubation of PLC/PRF5 cells with $^{125}$I-labeled BSA-insulin-chlorin $e_6$ for 1 or 18 h at 37°C, 3000 molecules remained surface bound per cell (Fig. 2A), in close agreement with comparable measurements for $^{125}$I-labeled insulin (22), with 19,000 molecules internalized per cell. After 18 h incubation, the amount of specifically endocytosed conjugate increased to 44,000 (Fig. 2B), while surface-

![Fig. 1. Competition of $^{125}$I-labeled insulin binding to PLC/PRF5 cells with unlabeled BSA-insulin-chlorin $e_6$ conjugate. Cells (2 x 10$^5$ cells/well) were incubated with 0.4 nM $^{125}$I-labeled insulin in the presence of increasing concentrations of unlabeled BSA-insulin-chlorin $e_6$ conjugate as indicated for 18 h at 4°C. The cells were then washed in Hank's solution containing 2 mg/ml BSA and removed using Versen solution prior to $\gamma$-counting. Results represent the mean (bars, ± SE) of triplicate wells.](image-url)
bound conjugate remained the same (~3,000 molecules/cell). SDS-PAGE analysis of extracts prepared from cells after 18 h incubation at 37°C indicated that more than 80% of cellular 125I-labeled conjugate was of high molecular weight and, hence, in the native form (results not shown). The results clearly indicated that the BSA-insulin-chlorin e6 conjugate binds specifically to insulin receptors and is subsequently endocytosed by PLC/PRF/5 cells. The results for binding and internalization of the BSA-insulin-chlorin e6 conjugate (1:13:16) are compared to those for insulin in Table 1.

Intracellular Distribution of Conjugates. To study the subcellular distribution of internalized insulin conjugates within the PLC/PRF/5 cells, a BSA-insulin-FITC conjugate (1:4:10) was derived. After incubation of PLC/PRF/5 cells with BSA-insulin-FITC conjugate for 4 h, fluorescence was detected around and also within the nucleus (Fig. 3A), which was further increased in the perinuclear area after 18 h (Fig. 3C). Excess free insulin inhibited accumulation of the BSA-insulin-FITC conjugate in the cells (Fig. 3E), indicating that the endocytosis was insulin receptor specific. These results were compared to those for the distribution of the sites of photo-induced damage by the BSA-insulin-chlorin e6 conjugate using 2',7'-dichlorofluorescin diacetate (see “Materials and Methods”) as an indicator of the sites of generation of active oxygen species produced upon chlorin e6 photosactivation. 2',7'-Dichlorofluorescin diacetate penetrates into cells and, upon intracellular deacetylation, reacts with the active oxygen species generated by chlorin e6 activation to yield the fluorescent derivative 2',7'-dichlorofluorescein. Cells incubated with the BSA-insulin-chlorin e6 conjugate at 37°C for 4 h, then incubated with 2',7'-dichlorofluorescin diacetate and laser photo-activated, exhibited enhancement of fluorescence, indicating the production of active oxygen species; no fluorescence is observed upon long wavelength photoactivation of cells incubated with 2',7'-dichlorofluorescin diacetate alone. Fig. 4 shows fluorescence before (Fig. 4, A and C) and after (Fig. 4, B and D) 8 min of laser irradiation of the cells. An increase of fluorescence, attributable to the production of 2',7'-dichlorofluorescein due to chlorin e6 activation, was evident within and around the nucleus (Fig. 4B). Free insulin inhibited the accumulation of the BSA-insulin-chlorin e6 conjugate in the cells, resulting in reduced fluorescence upon laser irradiation (Fig. 4D). It was concluded that the subcellular distribution of the BSA-insulin-FITC conjugate and the sites of production of active oxygen species with the BSA-insulin-chlorin e6 conjugate were coincident.

Photodynamic Action of the Conjugate BSA-Insulin-Chlorin e6. The photodynamic activity of the BSA-insulin-chlorin e6 conjugate was compared to that of chlorin e6 and indicated that the photody-namic action of the conjugate when incubated with the cells at 37°C was higher than that of chlorin e6, as indicated by the estimation of cell viability using the trypan blue exclusion test. Excess free insulin inhibited the photodynamic action of the conjugate, demonstrating that the photodynamic effects of the conjugate were insulin receptor dependent. At 4°C, where there is membrane receptor binding but no endocytosis, the photodynamic action of the conjugate and chlorin e6 were essentially the same (Fig. 5). Clearly, the enhanced photodynamic activity of the conjugate was directly attributable to its ability to be efficiently internalized. The EC50 for the conjugate and chlorin e6 by this test were 27 and 3000 nM, respectively (Table 2).

Table 1: Insulin receptor binding properties for the 125I-labeled BSA-insulin-chlorin e6 (1:13:16) conjugate compared to those for 125I-labeled insulin in PLC/PRF/5 cells

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Insulin (A)</th>
<th>Conjugate (B)</th>
<th>B/A</th>
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</thead>
<tbody>
<tr>
<td>$K_d$ (nM)</td>
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<td>1.0 (EC50)</td>
<td>0.27</td>
</tr>
<tr>
<td>$B_{max}$ (molecules/cell)$^a$</td>
<td>12000</td>
<td>12000</td>
<td>1.0</td>
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<tr>
<td>Surface bound (molecules/cell)$^b$</td>
<td>9500</td>
<td>3000</td>
<td>0.32</td>
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<tr>
<td>Internalization (molecules/cell)$^b$</td>
<td>19000</td>
<td>19000</td>
<td>1.0</td>
</tr>
</tbody>
</table>

$^a$ $B_{max}$ is the number of binding sites/cell obtained from Scatchard analysis. This value is much lower than that measured at 4°C (see “Results”), due largely to cooperative binding effects (Ref. 37).

$^b$ Cells were incubated for 1 h at 37°C with 2.3 nM 125I-labeled insulin or 125I-labeled conjugate, prior to analysis for internalization and binding as described in “Materials and Methods” (see “Results” and Figs. 1 and 2). Surface binding is the ligand removed by incubation 15 min at 4°C with 1 m citrate buffer (pH 3), and internalization is what remains cell-associated after incubation.

Colonies formation was also used to assess relative photodynamic activity (Fig. 6), yielding EC50s of 2.6 and 320 nM, respectively, for the conjugate and chlorin e6. This EC50 was lower than that estimated using the trypan blue exclusion test (Table 2). The quantitatively different results obtained by the two tests are the direct result of the fact that the trypan blue exclusion test is dependent upon plasma membrane injury alone, whereas the colony formation test indicates the capacity of cells to divide (32).

The dose dependence of irradiation also demonstrated a more pronounced photodynamic effect of the conjugate (Fig. 7); the D97 (the irradiation dose resulting in 37% survival of cells) for chlorin e6

![Fig. 2. Surface binding and endocytosis of 125I-labeled BSA-insulin-chlorin e6 by PLC/PRF/5 cells. Cells (2 × 10^6 cells/well) were incubated with the indicated concentrations of 125I-labeled BSA-insulin-chlorin e6 conjugate with or without a 1000-fold excess of unlabeled insulin to determine specific binding and endocytosis. After 1 (A) or 18 h (B) at 37°C, the conjugate bound to the cell surface was removed (A) by incubation (15 min at 4°C) with 1 m citrate buffer (pH 3) in order to measure surface-bound conjugate (A), subsequent to which the cells were removed using Versene solution and γ-counted to measure endocytosed conjugate (B). Results are the mean (bars, ± SE) of triplicate wells.](image-url)
was 95 kJ/m², compared to a value of 26 kJ/m² for the conjugate at the same concentration of chlorin e₆, indicating a 3–4-fold higher efficiency of conjugated chlorin e₆ in terms of photodynamic activity. Comparative photodynamic activities for chlorin e₆ and the BSA-insulin-chlorin e₆ (1:13:16) conjugate are summarized in Table 2.

**DISCUSSION**

Our experiments indicate that our photosensitizing BSA-insulin-chlorin e₆ conjugate bound specifically to the insulin receptors of hepatoma PLC/PRF/5 cells and is subsequently internalized and transported to near and within the cell nucleus. Endocytosis and subsequent localization of the BSA-insulin-chlorin e₆ conjugate in and around the cell nucleus could be visualized using both FITC-labeled conjugate and 2',7'-dichlorofluorescein diacetate, a fluorescent indicator of the production of active oxygen species due to chlorin e₆ activation. Our conjugate exhibited significantly higher photodynamic activity than free chlorin e₆, as estimated by the two tests for cell viability. Interestingly and importantly, the inhibition of endocytosis of the conjugate abrogated the enhanced photodynamic activity of the conjugate above that of free chlorin e₆; the photodynamic action of our conjugate incubated with PLC/PRF/5 cells at 0°C, where no endocytosis occurs, was markedly lower than at 37°C, although PLC/PRF/5 cells possess higher numbers of insulin-binding sites on the cell surface at 4°C compared to at 37°C. Clearly, the conjugate is plasma membrane localized after incubation at 4°C, similar to the normal cellular site of accumulation of free chlorin e₆, resulting in the observed similar photodynamic activities of the conjugate in the absence of internalization and free chlorin e₆ (Fig. 5). The observed increase in photodynamic efficiency of BSA-insulin-chlorin e₆ conjugate (when incubated with cells at 37°C) in comparison with free chlorin e₆ thus appears to be the direct result of the cellular entry of the conjugate through the insulin moiety, which, subsequent to receptor binding and internalization, targets the conjugate to intracellular compartments, such as endosomes and lysosomes, and ultimately the

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**Fig. 3. Intracellular distribution of BSA-insulin-FITC conjugate in PLC/PRF/5 cells.** After incubation with 6.4 nM conjugate for 4 h (A and B) or 18 h (C-F) at 37°C, the cells were washed in Hanks’ solution and treated with 10 mM NH₄Cl for 10 min at 37°C, prior to visualization of cellular fluorescence using video-intensified microscopy. In the case of the cells in (E) and (F), incubation was in the presence of a 1000-fold excess of insulin. A, C, and E, fluorescent illumination; B, D, and F, phase-contrast pictures of the same field of cells as in A, C, and E, respectively. n, nucleus. Bar, 10 μm.

**Fig. 4. Intracellular distribution of the production of active oxygen species after cell irradiation detected by the production of 2,7'-dichlorofluorescein.** The BSA-insulin-chlorin e₆ conjugate (3.7 nM) was incubated for 4 h at 37°C without (A and B) and with (C and D) a 1000-fold excess of insulin. After incubation, the cells were washed in Hanks’ solution, incubated with 2',7'-dichlorofluorescein diacetate for 5 min at 37°C, and then washed in Hanks’ solution and irradiated. Cells were visualized by video-intensified microscopy. A and C, before irradiation by laser; B and D, 8 min after laser irradiation (at 632 nm). n, nucleus. Bar, 10 μm.
INTERNALIZABLE CHLORIN e6 CONJUGATE

Stained cells / well

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Chlorin</th>
<th>Conjugate</th>
<th>Insulin</th>
</tr>
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<tbody>
<tr>
<td>37</td>
<td>+</td>
<td>+</td>
<td>-</td>
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<td>4</td>
<td>-</td>
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<td>+</td>
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</table>

Fig. 5. Temperature- and insulin receptor-dependence of the enhanced photodynamic activity of the BSA-insulin-chlorin e6 conjugate above that of chlorin e6. Cells were incubated with equivalent concentrations of chlorin (36.5 nM conjugate and 1 µM chlorin e6) for 18 h at the indicated temperature in the presence or absence of excess free insulin as indicated, prior to irradiation using 120 kJ/m². Cell viability was estimated by the trypan blue exclusion test (see "Materials and Methods"). Results are the mean (bars, ± SE) of triplicate wells.

Table 2. Summary of the photodynamic properties of BSA-insulin-chlorin e6 (1:13:16) conjugate compared to chlorin e6

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Free chlorin (A)</th>
<th>Conjugate (B)</th>
<th>Chlorin residue (C)</th>
<th>B/A</th>
<th>C/A</th>
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<tr>
<td>EC50 (nm)</td>
<td>3000</td>
<td>27</td>
<td>734</td>
<td>0.009</td>
<td>0.25</td>
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<tr>
<td>EC50 (nm)</td>
<td>330</td>
<td>2.6</td>
<td>71</td>
<td>0.008</td>
<td>0.22</td>
</tr>
</tbody>
</table>

* Concentration of chlorin e6 residue in conjugate.
* Ratio calculated per chlorin e6 residue, rather than the concentration of conjugate.
* Trypan blue test.
* Colony formation test.

nucleus (23, 24, 38). It is concluded that the photodynamic activity of the conjugate is higher than that of free chlorin e6 through its receptor-mediated delivery into sensitive intracellular compartments. In a previous study, we described a con A-chlorin e6 conjugate (22), where con A provided an interaction with cell receptors on the target cells and also served as intracellular carrier of chlorin e6. Whereas insulin is transported to the nucleus subsequent to endocytosis (23, 24), con A is targeted to the Golgi as well as lysosomes (22). Similar to the results here for insulin conjugates, we were able to demonstrate that internalization was necessary for enhanced photodynamic activity on the part of the con A-chlorin e6 conjugate (22).

A number of authors have used chlorin e6 conjugated to monoclonal antibodies specific for tumor cells. At saturating concentrations (100 nm) of the conjugate (Ref. 39; chlorin e6 residue concentration was 680 nm), it was able to kill one-half of the cells at a light dose of 15 J/cm² and to kill 100% cells at 50 J/cm². Another conjugate (17) at saturating concentration was able to kill one-half of the cells at 30 J/cm² and 100% cells at 58 J/cm². Our conjugate, BSA-insulin-chlorin e6, demonstrated a more pronounced photodynamic effect; at a saturating concentration (18 nm; 500 nm chlorin e6 residue concentration), the conjugate was able to kill one-half of the cells at 2.4 J/cm² and to kill ~100% of the cells at 9.6 J/cm².

Chlorin e6 conjugated to microspheres (13) possessed higher photodynamic activity than free chlorin e6, but the concentrations of chlorin e6 for these conjugates showing photosensitizing activity were much higher (EC50, 430 nm) than those in the conjugate BSA-insulin-chlorin e6 in this study. Based on the results presented here and in our previous study (22), this is almost certainly due to the fact that the antibody conjugates interact solely with the cell surface; in the case of the microsphere-conjugates, cellular entry is via phagocytosis and subsequent location in phagosomes, which are presumably not very sensitive organelles in terms of photosensitization and the contents of which neither efficiently enter other membrane vesicle transport systems of the cell (e.g., lysosomes) nor efficiently exit into the cytoplasm prior to transport to the nucleus.

The results here represent the basis of a new approach to photodynamic therapy based on high specificity for particular cell types. Insulin was chosen as the ligand in this study in order to test whether a polypeptide ligand could confer both cell-type specificity and re-
conceptor-mediated endocytosis on a photosensitizing conjugate which retains high photoactivating activity. The fact that several nonhepatoma cancer cell types possess insulin receptors (40, 41) means that insulin conjugates themselves might be useful in treating cancers other than hepatomas, but their direct applicability in cancer therapy is probably limited; certain hepatoma cell lines, HTC cells for example, do not possess very high numbers of insulin receptors. Importantly, however, the conjugate approach described here is clearly applicable to a wide variety of ligands and cancer cell types, e.g., ligands such as insulin-like growth factors (in a wide variety of cancers, including neuroblastomas and osteosarcomas), nerve growth factor (in the case of neuroblastomas or gliomas), or melanocyte-stimulating hormone (in the case of melanomas), could clearly be used conjugated to BSA-chlorin e₆ to target chlorin e₆ to sensitive intracellular compartments of requisite tumor cell types in identical fashion.

Internalizable conjugates specific for the cell type, and which are also capable of targeting photosensitizers to particular intracellular sites, help to diminish the concentrations of photosensitizers necessary for full effect. Such conjugates, exemplified by the BSA-insulin-chlorin e₆ described here, consist of modular components: (a) a photosensitizer; (b) a ligand specific to the desired target cell that provides internalization through endocytosis and/or intracellular transport to the most sensitive subcellular organelles, such as the lysosome and nucleus; and (c) a carrier, a neutral molecule enabling coupling of the ligand to the photosensitizer. In our case, insulin plays a dual role, conferring cell type specificity as well as targeted transport to selected intracellular compartments. Such conjugates of photosensitizers able to be internalized by receptor-mediated endocytosis and accumulated by directed intracellular transport are superior to others since they contribute to achieving a "triple selectivity" of photosensitization, determined by: (a) the type of cells with which conjugates of photosensitizers interact and by which they are taken up; (b) the particular subcellular compartment in which these conjugates accumulate; and (c), although not the scope of this study, by localized photo-irradiation (e.g., through the use of optical fibers, etc.). All this enhances the efficiency and specificity of photodynamic action coincident with a marked decrease in the active concentrations of photosensitizers which need to be administered. This should ultimately assist in achieving the long-term goal of being able to precisely kill tumor (or other diseased) cells without damaging normal tissue.

REFERENCES


3 D. A. Jans, unpublished data.