Absorption and elimination of Photofrin II in human immortalization esophageal epithelial cell line SHEE and its malignant transformation cell line SHEEC

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[Abstract] Background and Objective: In photodynamic therapy (PDT), the mechanism of photosensitizer uptake in cancerous tissue has yet to be elucidated. This study investigated the affinity of tumor cells to the photosensitizer Photofrin II. Methods: A UV-Vis spectrophotometer was used to measure the absorption spectra of various cell-culture media. The fluorescence spectrum of Photofrin II was determined by spectrofluorometer. Photofrin II absorption and elimination were detected in the immortalized human esophageal epithelial cell line SHEE and its malignant transformation cell line SHEEC. Results: The maximum excitation wavelength of fluorescence for Photofrin II was (395.0 ± 0.5) nm, and the maximum emission wavelength was (634.1 ± 0.5) nm. The laser used had a wavelength of 630 nm and could permeate various types of cell-culture media. Absorption and elimination of Photofrin II showed no significant differences between SHEE and SHEEC at the same concentration and time. Absorption of Photofrin II in SHEE and SHEEC increased with the increase in concentration and duration, plateauing at a concentration of 30 μg/mL for 150 min. The content of Photofrin II in SHEE and SHEEC showed a slight change after 15–30 min and diminished rapidly after 30 min. Conclusions: High photosensitizer concentration in tumor tissue may have no correlation to its affinity to tumor cells.

Key words: esophageal neoplasm, cell line, SHEE, SHEEC, photodynamic therapy, photofrin II

In the last 20 years, photodynamic therapy (PDT) has been developed as a novel therapy for malignant diseases. Its unique advantages include the targeted killing of local cancer cells without affecting surrounding normal tissue, limited complications, simple procedure, and repeatable application.1 PDT relies on the highly selective retention of photosensitizers in tumor tissue rather than in the surrounding normal tissue. The mechanism of photosensitizer concentration in tumor cells is not completely understood: it may concern malnutrition in tumor cells, a lack of porphyrin materials, high proliferative rates of tumor cells, abnormal lymph drainage, leakage in blood vessels, or the specific affinity between photosensitizers and molecular markers in newly grown cells (referred to as affinity for tumor cells) involving the high affinity between some organelles, including chondriosome and lysosome, and the photosensitizers.2 Additionally, vascular endothelial growth factors excreted by tumor cells have a role in concentrating photosensitizers. As most porphyrin materials concentrated in tumor blood vessels and the PDT based on them initially induce damage to blood vessels, it is hypothesized that the specific affinity between photosensitizers and tumor blood vessels (referred to as affinity for tumor vessels) may also cause the concentration of photosensitizers in tumor tissue.3

The human immortalized esophageal epithelial cell line SHEE retains characteristics of primary squamous epithelial cells, while its homologous malignant transformed esophageal cancer cell line SHEEC has characteristics of tumors. SHEEC is advantageous in research on whether tumor cells have better affinity for photosensitizers than normal cells because it completely avoids the effects of a possible affinity for blood vessels, and this has not been reported in the literature. Photofrin II is the only photosensitizer approved for tumor therapy in clinical
practice by the United States Food and Drug Administration (FDA). Compared with the first-generation hematoporphyrin derivatives (HPDs), it has higher tumor photosensitizer activity and less toxicity, which renders it suitable for research on the uptake and excretion of photosensitizers.

To identify the characteristics of uptake and elimination of Photofrin II in SHEE and SHEEC, our research used fluorescence spectrophotometry to measure both the uptake of Photofrin II cultured at different concentration levels for different time periods and the uptake of Photofrin II incubated at the same concentration at sequential time points. Our research explored whether cancerous cells have a higher affinity for photosensitizers than normal cells, to provide an experimental basis for choosing the optimum sensitizer dosage and the optimum drug-light interval in photodynamic experiments in vitro.

Materials and Methods

Materials

The SHEE an SHEEC lines were established and cultured in consecutive passes by Zeng Yi of the National Institute for Viral Disease Control and Prevention, Chinese Center for Disease Control and Prevention, and Shen Zhongying of the Medical College of Shantou University. SHEE retained the phenotype of primary epithelial cells, such as single-layer growth and anchor-dependent cell aggregation without colony formation in soft agar or tumor formation after transplantation. From a biological perspective, SHEE was similar to the primary cell line and retained its proliferation ability and differentiation potential. The SHEEC line was established through SHEE induced by 12-O-tetradecanoyl-phorbol-13-acetate (TPA).

Reagents and preparations

Photofrin II at 75 mg/vial was purchased from Sinclair Pharmaceuticals (Godalming, Surrey, United Kingdom) and stored in the dark at 4°C. Fetal bovine serum was purchased from Invitrogen (Carlsbad, California, United States). The trypsin and M199 medium were purchased from Hyclone (Logan, Utah, United States).

The phosphate buffered saline (PBS) preservative (10×) was prepared and stored at ambient temperature for future use. The PBS solution was prepared, sealed, and stored at 4°C. The cell-freezing medium was instantly prepared. The antibiotic solution (100×) was prepared and packaged with 5 mL in each set, and sealed and preserved at -20°C. The M199 medium was prepared and packaged with 200 mL in each set, and preserved at -20°C for future use. The M199 complete medium was prepared, sealed, and stored at 4°C. The trypsin was prepared and packaged with 1 mL in each vial, and stored at -20°C to be diluted with PBS at a ratio of 1:4 before use.

Equipment

The spectrofluorometer (F-3010) was purchased from Hitachi (Hitachi, Japan). The UV-spectrophotometer (UV-2201) was purchased from Shimadzu (Tokyo, Japan). The CO2 incubator (MCO-175) and the low-temperature refrigerator (MDF-330) were purchased from Sanyo (Tokyo, Japan). The inverted microscope (TE2000-U) was purchased from Nikon (Tokyo, Japan). The blood cell counter (XBK-25) was purchased from the Medical Optical Instruments Factory of Shanghai Medical Instruments Co., Ltd. (Shanghai, China). The culture bottles and cell-culture plates were purchased from Falcon (France).

Experiments on the uptake of Photofrin II

Measurement of the excitation and radiation spectra of Photofrin II

Photofrin II was diluted to 30 μg/mL with the fetal bovine serum-free M199 medium and was automatically scanned using the F-3010 spectrofluorometer. The wavelength was set between 200 nm and 800 nm with a sensitivity of ±0.1 nm and a narrow slit of 3.3 nm. The wavelengths of maximum excitation and maximum radiation of Photofrin II were measured.

Measurement of the uptake spectra of the culture media

The M199 medium, the M199 complete medium, the PBS solution, and the residual culture medium following cell culturing were randomly sampled to measure the light transmittance using the UV2210 UV-spectrophotometer at wavelengths between 220 nm and 800 nm. The actual light dose received by the targeted cells was calculated according to the following formula: the actual light dose = the light dose from the light system × the light transmittance of the culture medium.

Testing the uptake of Photofrin II in SHEE and SHEEC

The SHEE and SHEEC lines were cultured in the M199 medium containing 10% fetal bovine serum at 37°C and incubated with 5% CO2. SHEE and SHEEC in the logarithmic phase were selected, digested by 0.25% trypsin, diluted to 1.0 × 106/mL, and injected into the 6-well cell-culture plate with 1 mL in each well. The cell-culture plate was placed in the incubator with 5% CO2 at 37°C for 24 h. SHEE and SHEEC were randomized into 43 groups, with 3 parallel wells for each group, and were rinsed with PBS 3 times. In the 13 uptake concentration groups, SHEE and SHEEC were cultured with Photofrin II prepared with fetal bovine serum-free M199 medium at concentrations of 0, 5, 10, 15, 20, 30, 40, 50, 60, 70, 80, 90, and 100 μg/mL, and placed in the incubator with 5% CO2 at 37°C for 120 min. The cell-culture plates were rinsed with PBS 3 times. SHEE and SHEEC were digested with trypsin and pyrolyzed in hypotonic solution. An amount of 0.5 mL of 0.25% trypsin was added to each well, and the plates were placed in the incubator with 5% CO2 at 37°C. After tri-distilled water was added, the cells were exposed to continuous air circulation until no complete cells were visualized under the microscope. The Photofrin II fluorescence in SHEE and SHEEC was detected using the spectrofluorometer. In the 3 sets of the other 30 uptake time groups, SHEE and SHEEC were cultured with Photofrin II prepared with fetal bovine serum-free M199 serum medium at concentrations of 30, 15, and 10 μg/mL, respectively (in regard to the values in the uptake concentration groups). The cells in each set were placed in the incubator with 5% CO2 at 37°C for 0, 15, 30, 60, 90, 120, 150, 180, 210, and 240 min, respectively. The cell-culture plates were rinsed with PBS 3 times. SHEE and SHEEC were digested with trypsin and pyrolyzed in a hypotonic solution. An amount of 0.5 mL of 0.25% trypsin was added to each well, and the plates were placed in the incubator with 5% CO2 at 37°C. After 0.5 mL of tri-distilled water was added, the cells were exposed to continuous air circulation...
Measurement of fluorescence intensity of the cell samples. Values of fluorescence intensity of the treated samples were measured using the F-3010 spectrofluorometer. As parameters, the excitation wavelength was set to 395 nm, the radiation wavelength to 634.1 nm, the sensitivity to ± 0.1 nm, and the narrow slit to 3.3 nm. The maximum fluorescence values were read on the screen. The mean value for each of the 3 wells was interpreted as the amount of Photofrin II in SHEE and SHEEC.

Elimination of Photofrin II

SHEE and SHEEC were cultured according to the experimental steps described in testing Photofrin II uptake. SHEE and SHEEC in the logarithmic phase were selected, digested by 0.25% trypsin, diluted to 1.0 × 10⁵/mL, and injected into the 6-well cell-culture plate with 1 mL in each well. The cell-culture plate was placed in the incubator with 5% CO₂ at 37°C for 24 h. With 3 parallel wells for each group, SHEE and SHEEC were randomly divided into 21 groups and rinsed with PBS 3 times. SHEE and SHEEC were cultured with Photofrin II prepared with fetal bovine serum-free M199 medium at concentrations of 10 μg/mL, 15 μg/mL, and 30 μg/mL, and placed in the incubator with 5% CO₂ at 37°C for 150 minutes. The M199 medium with Photofrin II was removed and the cells were rinsed with PBS 3 times. SHEE and SHEEC were then cultured with Photofrin II prepared with fetal bovine serum-free M199 serum medium for 0, 15, 30, 45, 60, 90, and 120 min, respectively, and were digested with trypsin and pyrolyzed in a hypotonic solution. A total of 0.5 mL of 0.25% trypsin was added to each well, which were then placed in the incubator with 5% CO₂ at 37°C. After 0.5 mL of tri-distilled water was added, the cells were exposed to continuous air circulation until no complete cells were visualized under the microscope. The Photofrin II fluorescence in SHEE and SHEEC was detected using a spectrofluorometer according to the experimental steps described in measuring fluorescence intensity.

Results

Excitation and radiation wavelengths of fluorescence for Photofrin II

At wavelengths of 229 nm, 280 nm, and 395 nm, fluorescence could be excited from Photofrin II. The maximum excitation wavelength was (395 ± 0.5) nm and the emission wavelength was (634.1 ± 0.5) nm (Fig. 1).

Uptake spectra of the culture media

The light transmittance was measured between 220 nm and 800 nm using the UV-2201 UV-spectrophotometer. The manual scanning results are displayed in Figure 2. The light transmittance of the M199 medium, the M199 complete medium, the PBS solution, and the residual culture medium at 600 nm was 100%.

According to the above-mentioned results, light with an excitation wavelength of 630 nm required for Photofrin II could thoroughly pass through the culture media.

Uptake spectra of Photofrin II in SHEE and SHEEC

The uptake concentration group. The fluorescence values after SHEE and SHEEC were cultured with Photofrin II at different concentrations for 120 minutes (Figs. 3 and 4) are detailed in Table 1. Statistical analysis of the uptake of Photofrin II in SHEE and SHEEC showed no significant difference after culturing for 120 min (F= 2.137, P=0.169). The uptake of Photofrin II in SHEE and SHEEC was concentration-dependent, increasing with the concentration (F=16.578, P<0.001), and reaching a plateau concentration at 30 μg/mL. There was a significant difference between the 30 μg/mL group and the groups with concentrations equal to or lower than 20 μg/mL (P<0.001, P<0.001, P=0.008, and P=0.049, respectively), while no significant difference was observed between the 30 μg/mL group and the other groups with high concentrations (all P>0.05).

The uptake time group. When the concentration of Photofrin II...
was set to 10, 15, or 30 μg/mL, the time points of the uptake peak of Photofrin II in SHEE and SHEEC are displayed in Table 2. There was no significant difference between the uptake of Photofrin II in SHEE and SHEEC (P>0.05). As time went on, the uptake of Photofrin II in SHEE and SHEEC increased significantly (P<0.001) and plateaued after 150 min. The fluorescence values of Photofrin II in SHEE and SHEEC after cell culture for less than 150 min and more than 150 min were significantly different (P<0.01), while there was no significant difference at different time points after cell culture for more than 150 min (P>0.05).

**Elimination spectra of Photofrin II in SHEE and SHEEC**

When SHEE and SHEEC were incubated with Photofrin II at 3 concentrations for 150 min, the culture medium was replaced by a Photofrin II-free M199 culture medium. The detection results are shown in Table 3. No significant difference was observed regarding the elimination of Photofrin II from SHEE and SHEEC (P>0.05). The content of Photofrin II in the 2 groups decreased and the content of Photofrin II in SHEE and SHEEC was maintained at a high level within 30 min at concentrations of 10 μg/mL and 15 μg/mL. There was no significant difference between different time points after cell culture for less than 30 min (P>0.05).

<table>
<thead>
<tr>
<th>Concentration of photofrin II (μg/mL)</th>
<th>SHEE cells</th>
<th>SHEEC cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1.519±0.089</td>
<td>1.467±0.061</td>
</tr>
<tr>
<td>5</td>
<td>4.378±0.019</td>
<td>4.247±0.272</td>
</tr>
<tr>
<td>10</td>
<td>6.777±0.099</td>
<td>6.815±0.146</td>
</tr>
<tr>
<td>15</td>
<td>8.838±0.096</td>
<td>6.955±0.008</td>
</tr>
<tr>
<td>20</td>
<td>10.811±0.072</td>
<td>8.519±0.409</td>
</tr>
<tr>
<td>30</td>
<td>10.449±0.045</td>
<td>10.405±0.334</td>
</tr>
<tr>
<td>40</td>
<td>9.641±0.057</td>
<td>11.207±0.090</td>
</tr>
<tr>
<td>50</td>
<td>10.651±0.182</td>
<td>9.739±0.109</td>
</tr>
<tr>
<td>60</td>
<td>12.323±0.133</td>
<td>9.899±0.106</td>
</tr>
<tr>
<td>70</td>
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<td>10.359±0.152</td>
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<td>80</td>
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<td>13.370±0.070</td>
</tr>
<tr>
<td>90</td>
<td>12.641±0.018</td>
<td>11.015±0.173</td>
</tr>
<tr>
<td>100</td>
<td>12.339±0.137</td>
<td>12.086±0.029</td>
</tr>
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</table>

Table 1  The fluorescence intensity of SHEE and SHEEC cells after 120 min of incubation with different concentrations of photosensitizer photofrin II
binding to tumor cells was 5:1. PDT damaged microvessels, tumor blood vessels and instead concentrated on the affinity for statistically significant difference between SHEE and SHEEC in homologous SHEE and SHEEC, and found that there was no characteristic of uptake and elimination of Photofrin concentration of $3 \times 10^{10}$ and the elimination of Photofrin from SHEE and SHEEC increased rapidly after cell culture for more than 30 min. At concentrations of 30 $\mu$g/mL, there was no significant difference between the time points after cell culture for 15 min or less ($P > 0.05$) and the elimination of Photofrin from SHEE and SHEEC increased rapidly after cell culture for more than 15 min.

### Discussion

The current research detected and compared the characteristics of uptake and elimination of Photofrin regarding homologous SHEE and SHEEC, and found that there was no statistically significant difference between SHEE and SHEEC in the uptake or elimination of Photofrin.

The current research excluded the influence of the affinity for tumor blood vessels and instead concentrated on the affinity for tumor cells. The results indicated that the selective uptake of photosensitizers in tumor tissue was not correlated with the affinity for tumor cells but for the affinity for blood vessels, which is consistent with the report by Bugelski et al.5 In their research, hematoporphyrin derivative (HpD) closely bound to the vascular matrix in 2 groups of established cancers and sarcomas and the ratio of HpD binding to the vascular matrix as opposed to those binding to tumor cells was 5 : 1. PDT damaged microvessels, which resulted in local necrosis and cell death, as well as detachment and necrosis of tissue for tumor therapy. Svanberg et al.10 explained that 1) the tumor blood vessels were distributed unevenly and were more affected by PDT than normal tissue, 2) PDT induced excretion of blood clotting factors including thromboxane from surrounding tumor tissue so the tumor blood vessels shrank and the blood cells concentrated, and 3) the blood flow within the tumor was slower than in normal tissue. Following the local or systemic application of photosensitizers, ectodermal tumors were affected. Some studies have found that hydrophilic photosensitizers, delivered by albumin and serum proteins, tend to concentrate in the tumor parenchyma and tumor blood vessels, damage the tumor blood vessels, cut off the supply of oxygen and nutrients to tumors, and kill tumor cells.15-13

As photosensitizers and laser-generation systems develop, PDT has become popular in cancer treatment.14-16 Most researchers currently agree that PDT is highly selective for tumor tissue with little damage to normal tissue.17 PDT has 2 aspects of selectivity: laser radiation and photosensitizer uptake. The selective uptake of photosensitizers in cancerous tissue is the basis for PDT’s use. When the photosensitizers are injected into the body, the concentration of the photosensitizers in tumor tissue is higher than in surrounding normal tissue.18 The current research found that the selective uptake of sensitizers might not be correlated with the affinity for tumor cells, which provides a basis for theoretical research on PDT.
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However, there were limitations in the current research. Though the SHEE cell line retained the characteristics of primary squamous epithelial cells, it is not really a normal epithelial cell, and the SHEEC cell line is not a real tumor cell even if it maintained the characteristics of tumors. Consequently, the current research could only guide future investigations and the conclusion that the concentration of photosensitizers in cancerous tissue was not correlated with the affinity for tumor cells and should be verified in-depth research.

In the current research, the uptake of Photofrin II in SHEE and SHEEC was concentration-dependent and plateaued at a concentration of 30 μg/mL. The uptake of Photofrin II increased with time, but reached its peak after cell culture for 150 min. When SHEE and SHEEC were cultured with Photofrin II for 150 min, the culture medium changed to a Photofrin II-free M199 medium and the elimination of Photofrin II in SHEE and SHEEC was observed. The content of Photofrin II did not change within 15 min after the M199 medium was used and decreased rapidly after cell culture for more than 15 min. It was suggested that SHEE and SHEEC should be radiated in vitro within 15 min after cell culture with Photofrin II-free M199, following cell culture with Photofrin II at concentrations below 30 μg/mL for less than 150 min.

References