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Cellular Uptake of Hematoporphyrin Derivative in KK-47 Bladder Cancer Cells

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Summary. The fluorescence emission spectra and degree of fluorescence polarization of hematoporphyrin derivative (HpD) have been investigated using HpD-containing KK-47 cells, PBS and cetyl trimethyl ammonium chloride (CTAC) micellar solutions. The fluorescence emission bands in the HpD-containing cell suspension were red-shifted and broadened as compared to those in the PBS solution. The degree of the polarization in the PBS and CTAC micellar solutions did not change with increasing incubation time, but in the cell suspension it increased temporarily and then decreased 4 h after incubation. These results suggest that HpD monomers and dimers may bind weakly to the outer cell membrane, and then slowly distribute throughout the intracellular loci in strong-binding form. In addition, the cellular uptake and/or binding loci of HpD were considered to be the mitochondria and nuclear membrane by subcellular fractionation and fluorescence microscopic studies.

Key words: Hematoporphyrin derivative, Cellular uptake, Human bladder cancer cell line, Subcellular fractionation.

Introduction

Hematoporphyrin derivative (HpD) has been introduced to the photoinactivation of malignant tumors as a photosensitizer by Dougherty and his coworkers [1], due to the fact that injected HpD tends to accumulate in neoplastic tissue to a higher degree than in normal tissue [2]. In our clinic, this photoinactivation has been adopted for the management of bladder tumors. The affinity of HpD for malignant cells has been looked upon as being related to the clinical effect of the photoradiation therapy. Accordingly, to elucidate the clinical benefits of photoradiation therapy, HpD-uptake and HpD-localization at the cellular and subcellular level are fundamentally important subjects. A fluorescence microscopic study in vitro [3] has demonstrated that the localizing site of HpD in malignant cells is the mitochondria. However, the intracellular binding site of HpD has not yet been established by such biochemical techniques as subcellular fractionation.

In the present study, the cellular uptake and/or binding of HpD were optically studied using a bladder cancer cell line, KK-47. To study the intermolecular combination of cell-binding HpD, HpD-containing PBS and CTAC micellar solutions were adopted as a contrast based on the fact that the micelle exerts a monomerizing effect on HpD molecules dissolved in dimers or aggregate form. Furthermore, the intracellular distribution of HpD was investigated using differential centrifugation of cell homogenates and a fluorescence microscope.

Materials and Methods

Reagents. Clinically applicable HpD supplied by Dr. T. J. Dougherty (Roswell Park Memorial Institute, Buffalo, N.Y.). PBS (pH 7.4) was prepared according to Dulbeco's formula. Ham's F12 medium (Nissui Pharmaceutical Co. Tokyo) and calf serum (Igaku Seibutsugaku Kenkyusho, Nagoya) were used for cell cultivation. Sucrose (Wako Pure Chemical LTD, Tokyo) was used for subcellular fractionation. Phenol reagent (Wako Pure Chemical LTD, Tokyo) was used for the quantitative estimation of proteins in subcellular fractions. Bovine serum albumin (BSA; Wako Pure Chemical LTD, Tokyo) and cetyl trimethyl ammonium chloride (CTAC; Wako Pure Chemical LTD, Tokyo) were dissolved in the PBS at concentrations of 0.1 M and 3%, respectively.

Cell Cultivation. KK-47 cells were derived from a human bladder carcinoma. Their biological and histological properties have been reported in previous papers [4–6]. The cells at the 130th passage which had been grown in a log-growth phase in sealed culture bottles were freed from the glass by gentle trypsinization and redispersed as a monocellular suspension in Ham's F12 medium supplemented with 20% calf serum and aminobenzyl penicillin 50 μg/ml, at a concentration of 10^5 cells/ml. Tissue culture dishes, 6 x 1.5 cm (Falcon, Co., Cal., USA) containing 5 ml of the cell suspension were incubated in 5% CO_2 in air at 37 °C for 48 h and then washed with serum-free Ham's F12 medium twice. HpD was added to the dishes at a final concentration of 200 μg/ml for 3 h at 37 °C. The dishes

Subcellular fractionation

Cells (3.2×10^7 cells)
|
Homogenized in 0.25M sucrose
|-----(1=total homogenate)
Centrifuged at 700Xg for 10min
|-----(2=nuclear fraction)
Sup
|
Centrifuged at 10000Xg for 20min
|-----(3=mitochondrial fraction)
Sup
|
Centrifuged at 105000Xg for 1hr
(Hitachi RPS-50 rotor)
|-----(4=microsomal fraction)
Sup-----(5=supernatant fraction)

Fig. 1. Procedure conditions for subcellular fractionation of KK-47 cells. See the Materials and Methods section in the text

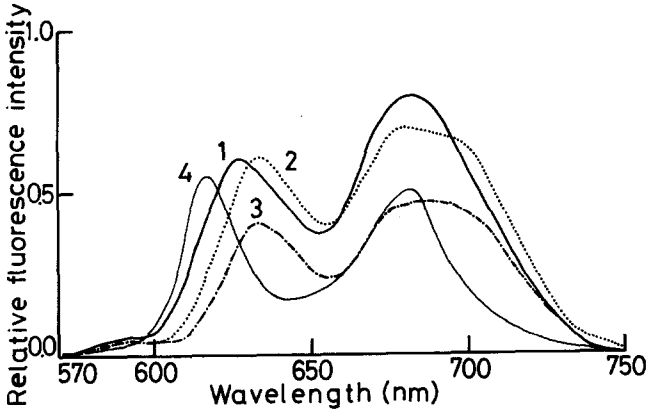

Fig. 2. Fluorescence spectra of HpD in bladder cancer KK-47 cells were exposed to 200 μg/ml HpD for 3 h, then washed with the serum-free Ham's F12 medium to remove free-HpD. Curves 1, 2 and 3 are KK-47 cell suspensions incubated for 0, 2 and 24 h, respectively; the concentration of HpD incorporated in KK-47 cells was estimated to be 1.2 μg/ml from the calibration curve; the fluorescence intensity vs. the concentration of HpD in PBS. Curve 4 is PBS contained 0.6 μg/ml HpD

were washed with the serum-free medium to remove free-HpD in the culture medium and cultivation was continued for varying periods. The cells were harvested from the dishes using a policeman and resuspended in the serum-free medium at the concentration of the original cell suspension. The resulting cell suspension was subjected to the following optical measurements. As a control, PBS containing 0.6 μg/ml HpD was used.

Subcellular Fractionation. Cell homogenates in isotonic sucrose were separated by differential centrifugation into nuclear, mitochondrial, microsomal and supernatant fractions according to the experimental schedule shown in Fig. 1. The relative content of HpD was expressed in terms of relative fluorescence intensity: exciting wavelength, 398 nm; monitoring wavelength, 630 nm. Proteins in each of the fractions were quantitatively determined using Lowry's method [7].

Optical Investigation. The fluorescence spectra and fluorescence polarization of HpD were determined using a fluorescence spectrophotometer (Hitachi, model MPF-4). The fluorescence spectra obtained were corrected by two standard phosphors; 4-dimethylamino-4'-nitrostilbene and N,N-dimethyl-m-nitroaniline. Fluorescence photomicrographs of HpD-containing KK-47 cells were taken by a microphotometer (Leitz, model MPV-2).

Results

The fluorescence spectra of HpD in the KK-47 cell suspension are shown in Fig. 2. At 0 h incubation, immediately after washing out of free-HpD in the medium with the serum-free Ham's F12 medium, the fluorescence bands appeared at 627 nm and 682 nm (Curve 1). After 2 h incubation, both bands red-shifted by 7 nm and the latter band was broadened as indicated by Curve 2. The fluorescence intensity decreased after a 24 h incubation (Curve 3), but the band shape and position were unchanged during a subsequent 2 h-incubation.

The degree of fluorescence polarization (p) was calculated according to the following equation [8]:

$$p = \frac{I_{||} - I_{\perp}(I_{\perp}/I_{||})}{I_{||} + I_{\perp}(I_{\perp}/I_{||})} \quad (1)$$

where $I_{||}$ and $I_{\perp}$ are the fluorescence intensities observed through a polarizer oriented parallel and perpendicular to the plane of polarization of the excitation beam. As shown in Fig. 3, the degrees of polarization in the PBS and 0.1 M CTAC micellar solution were 0.007 and 0.022, respectively, and were unchanged with increasing incubation time except the initial, very short incubation time in the micellar solution as indicated by Curves 1 and 2. The degree of polarization in the cells (Curve 3), rapidly increased from 0.020 to 0.11 during an incubation period of 2 h and then decreased gradually. As indicated by Curve 4, when HpD (0.6 μg/ml) was dissolved in 3% BSA solution at 37 °C, a similar rapid increase and subsequent decrease were observed. These results of the polarization study suggest the occurrence of initial binding of HpD to BSA and the cells, and the subsequent ultimate strong binding of HpD into BSA and the cells.

Figure 4 is a fluorescence photomicrograph of HpD-containing KK-47 cells after a 6 h incubation time resulting in strong binding of HpD in the cells. The cells show a bright red-fluorescence of intracellular HpD excited by blue light; wavelength, 350 nm to 410 nm. The nucleus was found to be free of fluorescence, and was well-demarcated with red fluorescence of the cytoplasm. This picture indicates that HpD may distribute throughout the cytoplasm, cross

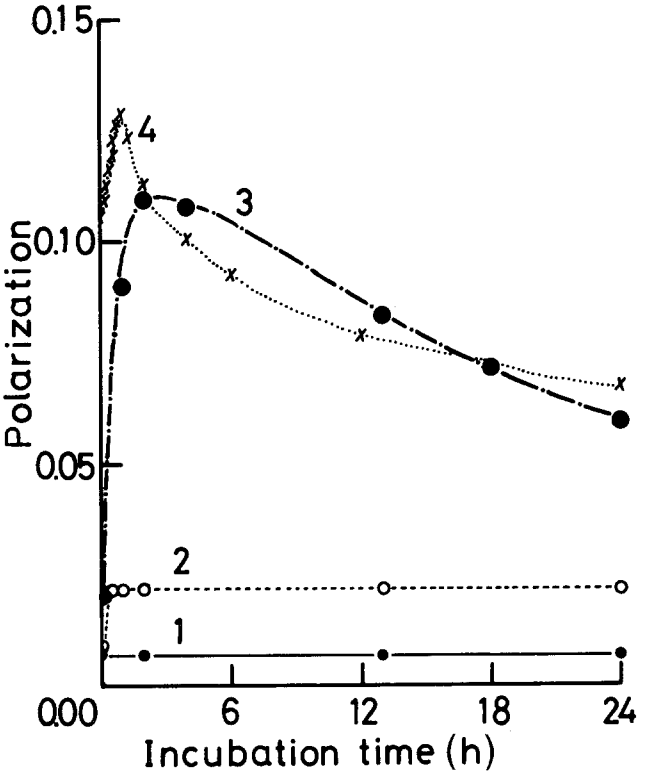

Fig. 3. Changes in the degree of polarization of HpD with increasing incubation time in PBS and KK-47 cell suspension at 37°C. Curve 1, PBS (pH 7.4); Curve 2, 0.1 M CTAC micellar solution; Curve 3, KK-47 cell (10^5 cells/ml) suspension; Curve 4, 3% BSA solution; concentration of HpD in Curves 1, 2 and 4 were 0.6 μg/ml; concentration of HpD in Curve 3, 1.2 μg/ml; excited with a monomer band of HpD (398 nm)

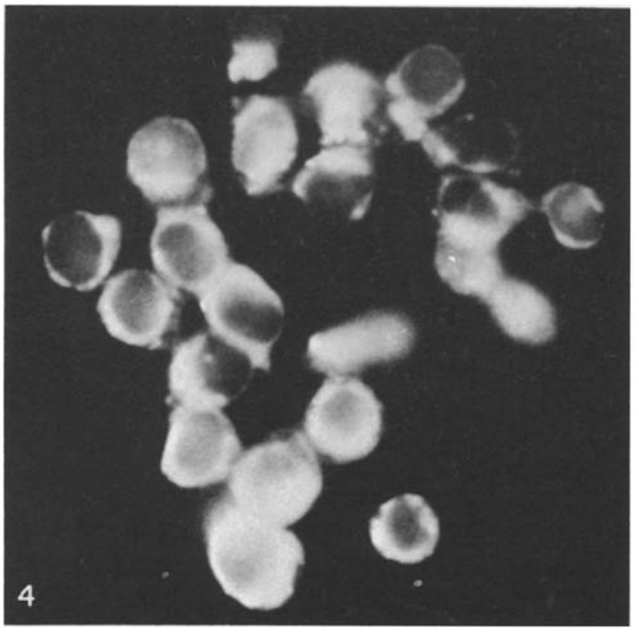

Fig. 4. Fluorescence photomicrograph (x250) of HpD-containing KK-47 cells. The cells (6.4 x 10^4 cells/ml) were exposed under the same conditions as those of Fig. 1 and incubated for 6 h

Table 1. Relative content per unit protein

Cell fraction	HpD[a]	Protein[b]	HpD / Protein
Nuclear	0.541	0.098	11.0
Mitochondrial	0.633	0.060	21.1
Microsomal	0.126	0.052	4.85
Supernatant	0.007	0.062	0.23

[a] fluorescence intensity
[b] mg/3.2 x 10^7 cells

the cell membrane and may not be incorporated in the nucleus.

The relative content of HpD per mg protein of the 4 fractions is listed in Table 1. The HpD content of the mitochondrial and nuclear fractions was greater as compared with that of the other fractions. However, because there was no fluorescence in the nucleus as shown in Fig. 4, the HpD content in the nucleus fraction may depend on the presence of a HpD-bound nuclear membrane.

Discussion

Two key results are presented in this paper: (I) the cellular HpD-uptake mode by the KK-47 cells was suggested by comparing the changes of the fluorescence polarization in the PBS and CTAC micellar solutions (Fig. 2); (II) the HpD-incorporating site of the cells is found mainly to be the mitochondria and nuclear membrane based on the subcellular fractionation method (Table 1).

Dougherty et al. [9] have reported that the cellular uptake in vitro of HpD was evidenced by a rapid, weak binding followed by a slow phase of strong binding. A similar HpD-uptake mode to their report was suggested by our optical investigations. It has been said that the fluorescence emission spectrum and degree of fluorescence polarization are changed by the environment; e.g., viscosity, polarity and so on, around the phosphor bound by the cells. The fluorescence emission peaks may be red-shifted because the excited singlet state of HpD is stabilized by binding the cells. The degree of fluorescence polarization may increase at a high viscosity environment because that degree reflects the ease of vibration of the side-chains in the HpD molecule. Accordingly, it was considered from Fig. 2 and Curve 3 in Fig. 3 that the HpD molecules may rapidly bind to the cell membrane at the initial time and then incorporate slowly with the intracellular stable binding site after 2 h incubation time. When HpD molecules were incorporated into BSA (Curve 4), the changes of the polarization coincided with the results obtained in the cells (Curve 3), and supported the presence of HpD molecules strongly bound by the cellular protein.

Berns et al. [3] showed a phase-contrast micrograph and fluorescence micrograph of myocardial cells treated with

HpD (25 μg/ml) for 1 h, and concluded that the intracellular binding site of HpD was the mitochondria. However, from our fluorescence photomicrograph and subcellular fractionation studies, it was confirmed that HpD was bound not only by the mitochondria but also by the nuclear membrane.

In conclusion, the optical results obtained suggest that HpD monomers and dimers may bind weakly to the outer cell membrane, and then may slowly distribute into the intracellular sites in strong binding form. In addition, from the results obtained by differential centrifugation and fluorescence microphotograph, the intracellular binding sites of HpD were considered to be the motochondria and the nuclear membrane.

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