Cell cycle dependency of Porphyrin uptake in glioma cell line

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Summary
We used 2 color analysis system to assess the porphyrin uptake and DNA content in 4 established cell lines of glioma employing flow cytometry (FCM). The FCM study revealed porphyrin uptake in all cells, regardless of the phase of the cell cycle they were in. However those in G0/G1 phase showed moderate uptake of porphyrin and those in the G2/M phase showed a higher uptake. These results indicated the advantage of using porphyrin as the carrier of tumor targeting agents as a therapeutic strategy for malignant tumors.
Key words
brain tumor, cell cycle, porphyrin, flow cytometry

Introduction

Porphyrin is selectively uptaken by tumor cells[1]. Because of this property, porphyrin has been used as a tumor targeting agent in photodynamic therapy[1]. Some researches using porphyrin as boron carrier for boron neutron capture therapy(BNCT) are in progress[2-5].

The radiosensitivity of tumor cells is an important factor in radiation therapy of malignant tumors. Quiescent cell populations are more resistant to gamma ray radiation than all tumor cells in vivo[6]. Rapidly proliferating tumors contain a large G2/M cell population which is highly radiosensitive in vivo. The quiescent cell population and G0/G1 phase cells are radioresistant in vivo. To achieve cure of the tumor, the target of radiotherapy is the G2/M phase population which is not sufficient to control the tumor; thus the G0/G1 phase population should also be controlled. In this study, we investigated porphyrin uptake in each cell cycle using a double staining technique for porphyrin and DNA content and 2-dimensional flow cytometry analysis.

Materials and Method

Cell preparation.

A-172, U-251, 9L, ONS cell lines were cultured for several days in RPMI 1640 medium(Gibco, New York, NY) or Earle's minimum essential medium(MEM) solution(Gibco, New York, NY). A-172 and U-251 are human glioblastoma cell lines. ONS is human medulloblastoma cell line. 9L is rat gliosarcoma cell line. They were

maintained at 37°C in an atmosphere of 5% CO_2 in air. Cells were grown in petri dishes and detached with 0.25% trypsin solution. The cells were washed with phosphate buffered saline (PBS). Each cell suspension were centrifuged and pellets including about $1x10^6$ cells were cultured in RPMI 1640 medium with the porphyrin ATX-S10-Na (13,17-bis(1-carboxypropionyl) carbamoylethyl-8-ethenyl-2-hydroxy-3-hydroxyiminoethylidene-2,7,12, 18- tetramethyl-porphyrin sodium, Toyo Hakka Co. Ltd., Okayama, Japan) at the concentration of 25μg/ml of RPMI 1640 solution for 2 hours at 37˚C in an atmosphere of 5% CO_2 in air. The molecular formula of ATX-S10-Na is $C_{42}H_{41}N_7O_{12}Na_4$, and its molecular weight is 927.79. Each cell suspension was centrifuged and the pellets were fixed with ice-cooled 70% ethanol and stored at 4˚C for several hours. Fixed cells were then stained with chromomycin A3 at the concentration of 20μg/ml for 1 hour at 4˚C. The cells were centrifuged and each pellet was suspended in PBS.

Analysis of the cells by fluorescent microscopy and flow cytometry.

The uptake of porphyrin and staining of DNA with chromomycin A3 were confirmed by fluorescent microscopy (Axiophot, Carl Zeiss Co., Ltd., Oberkochen, Germany). Staining of DNA with chromomycin A3 was observed as green fluorescence using a band pass filter of 515-565nm. The uptake of porphyrin was observed as red fluorescence using a band pass filter of more than 590nm.

Cell suspensions were filtered through a mesh of 2mm pore (Becton Dickinson, Sanjose, CA) to remove debris or clusters of cells. The $1x10^5$ cells of each cell lines was analyzed with flow cytometry. The flow cytometric data acquisition was conducted using FACSort (Becton Dickinson). The activated wave length of chromomycin A3 is 400-460nm(peak:420nm). The fluorescent wave length of chromomycin A3 is 490-610nm(peak:560nm). The activated wave length of ATX-S10 is 400-417nm and the peak fluorescent wave length of ATX-S10 is 677nm[7]. DNA content stained with chromomycin A3 was measured with FL2 range. The fluorescence of ATX-S10 was measured with FL3 range. Data were analyzed with Lysis 2 software. The cells in G0/G1 and G2/M phase were identified from the histogram of FL2-height.

Results

Fluorescent microscopy

All cell lines stained with chromomycin A3(Fig. 1) and showed porphyrin ATX-S10 uptake(Fig. 2). Porphyrin ATX-S10 seemed to be uptaken into both the cytoplasm and nucleus.

Flow cytometry

Figures 3-6 show the dot plot of the relation between FL2 and FL3(upper right), histogram of FL2-height(lower right) and histogram of FL3-height(upper left) of A-172, U-251, 9L and ONS cell lines. The dot plot of the relation between FL2 and FL3 showed two clusters of cells. The vertical axis is FL2 fluorescence, the horizontal axis is FL3 fluorescence. The FL2-height histogram of all cells revealed double peaks corresponding to cells in the G0/G1 and G2/M phase. The horizontal axis is FL2 fluorescence and vertical axis is cell number. The FL3-height histogram of all cells showed a single peak. The vertical axis is FL3 fluorescence and horizontal axis is cell number. The mean FL3-height of cells in the G2/M phase was higher than that of cells in the G0/G1 phase. This phenomenon was observed in all the cell lines in investigated in this study.

Discussion

Treatment of malignant tumors requires a multimodality therapy involving surgery, radiation, chemotherapy and immumotherapy. Malignant brain tumors are the most radioresistant ones. Surgery combined with radiotherapy and/or chemotherapy results in a mean survival of 1 year for malignant glioma[8]. BNCT is a bimodal radiation therapy using alpha particles induced by neutron capture reaction. Thermal neutrons have little effect on normal brain tissue, however, tumor tissue containing a boron compound is selectively damaged by alpha particles[9].

For an effective BNCT, boron uptake into tumor cells is essential. In the research of BNCT, boron uptake has been investigated in relation to whole tumor tissue. However the boron uptake and radiosensitivity of each cell line probably depends on the phase of the cell cycle. So the effect of BNCT should be examined for each cell line in different phase of the cell cycle. Borocaptate sodium(BSH) and Boronophenyl

alanine(BPA) are 2 major boron compounds used in BNCT. BSH is selectively uptaken by tumor tissue and does not cross the intact blood brain barrier[10]. The uptake of BSH by tumor cells is not related to the cell cycle. BPA, on the other hand, showed selective uptake by actively dividing cells[11]. The tumor control effect of BNCT using BPA was better than that using BSH[12]. This difference might be due to the more uptake of BPA by G2/M phase cells. However, BNCT using BPA does not include therapy to quiescent cell population. Cancer therapy requires cytotoxic or cytocidal effects not only on proliferating G2/M cells but also on G0/G1 cells which may enter the active cell cycle. After selective death of cells in cells cycle, quiescent cells enter the proliferating phase, which is called recruitment[13]. BNCT is being performed as non-fractionated radiation therapy. Hence, the target of BNCT should include all cells at rest and in cell cycle. Our data demonstrated the cell cycle independent property of porphyrin uptake by tumor cells. The cells in G0/G1 phase showed moderate uptake of porphyrin and the cells in G2/M phase showed more uptake of porphyrin than the cells in G0/G1 phase. The uptake of porphyrin by each cell was almost proportional to the DNA content in each cell. These results support the therapeutic strategy of BNCT using porphyrin compounds. The targets of BNCT using porphyrin compounds are cells at rest and cells undergoing cell division. On BNCT using porphyrin compounds a more lethal effect is expected for cells in the G2/M phase.

There are very few reports on porphyrin uptake by tumor cells in relation to cell cycle. Fukuda reported the kinetics of porphyrin accumulation in cell lines after exposure to delta aminolevulinic acid (ALA), the natural precursor of tetrapyrrole biosynthesis[14]. The intracellular porphyrin content of cells in the G2/M phase was slightly higher than that of cells in the G0/G1 or S phase in this study. There is little obvious cell cycle dependent variation in the synthesis of porphyrin from ALA. They reported the small differences in intracellular porphyrin content may be attributed to a slight reduction in the rate of loss of porphyrins in cells in the G2/M phase. Fiedorowicz et al. reported the effect of photodynamic therapy with some porphyrin on human leukemic cell lines[15]. The photosensitivity of cells was almost the same regardless of the phase of they were in, but the cells in the G2/M phase

were slightly more sensitive than cells on other phases of the cell cycle. These results support those of our FCM study.

As for the mechanism of porphyrin uptake, the low density lipoprotein(LDL) receptor-mediated pathway was reported to be the carrier of porphyrin into cells[16]. Tumor cells were shown to have enhanced LDL receptor activity and large amounts of LDL receptor on their each cell surface[16,17]. The amount of LDL receptor in tumor cells in each phase of the cell cycle should be investigated in future studies. In our results, the cell lines showed some different pattern of FACS in each cell line. This may be resulted from the different amounts of LDL receptor on each cell line. And the percentage of G2/M phase is different to each cell line. This may be influenced by cell condition or malignancy of each cell line. This phenomenon that the porphyrin uptake of cells in the G2/M phase is higher than that of cells in the G0/G1 phase was uniformly kept in the cell lines in investigated in this study.

In summary, in case of bimodal therapies using porphyrin, photo-dynamic therapy and neutron capture therapy, the uptake of the drug by the cells should be considered in relation to the cell cycle. Future studies of neutron capture therapy should be directed to observe the cytocidal effect of porphyrin compounds on tumor cells.

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Legend of figures

Fig. 1 The photograph of the fluorescent microscopy. The green fluorescence indicate DNA stained with chromomycin A3.

Fig. 2 The photograph of the fluorescent microscopy. The red fluorescence indicate the porphyrin uptake into tumor cells.

Fig. 3 The flow cytometry data of A-172 cell line. The upper left is the dot plot of the relation between FL2 and FL3, the upper right is the histogram of FL2-height and the lower left is the histogram of FL3-height.

Fig. 4 The flow cytometry data of U-251 cell line.

Fig. 5 The flow cytometry data of 9L cell line.

Fig. 6 The flow cytometry data of ONS cell line.