Competitive Uptake of Porphyrin and LDL via the LDL Receptor in Glioma Cell Lines: Flow Cytometric Analysis

Yasushi Shibata M.D., Ph.D.[a], Akira Matsumura M.D., Ph.D.[a],
Fumiyo Yoshida B.S.[b], Tetsuya Yamamoto M.D.[a],
Kei Nakai M.D.[a], Tadao Nose M.D., Ph.D.[a],
Isao Sakata Ph.D.[c], Susumu Nakajima M.D., Ph.D.[d]

[a]Department of Neurosurgery, [b]College of Medical Technology,
University of Tsukuba, Tsukuba, Ibaraki 305-8575, Japan,
[c]Photochemical Co., LTD., Okayama 719-03, Japan,
[d]Department of Operative Surgery, Asahikawa Medical College,
Asahikawa, Hokkaido 078, Japan

Address for correspondence:
Yasushi Shibata, M.D., Ph.D.,
Department of Neurosurgery, Institute of Clinical Medicine,
University of Tsukuba,
Tsukuba, Ibaraki 305-8575, Japan
Telephone: +81-298-53-3220
Fax: +81-298-53-3214
E-mail: y-shibata@amy.hi-ho.ne.jp

Address for reprint requests:
Akira Matsumura, M.D., Ph.D.,
Department of Neurosurgery, Institute of Clinical Medicine,
University of Tsukuba,
Tsukuba, Ibaraki 305-8575, Japan

Summary

We examined the simultaneous uptake of porphyrin and low-density lipoprotein (LDL) by 4 established cell lines of glioma and normal fibroblasts using flow cytometry (FCM). The results indicated porphyrin and LDL showed competitive conjugation with the LDL receptor. These results support the theory of the porphyrin uptake via the LDL receptor.

Key words

Brain tumor, LDL, porphyrin, flow cytometry

Introduction

Porphyrin is selectively uptaken by tumor cells [1,2]. Because of this property, porphyrin has been used as a tumor-targeting agent in photodynamic therapy [1,2]. Some researches using porphyrin as boron carrier for boron neutron capture therapy (BNCT) are in progress [3-8]. Porphyrin is believed to be uptaken via the low-density lipoprotein (LDL) receptor [2,9,10], however the mechanism of uptake of porphyrin is not completely understood. Tumor cells, neoplastic astrocytes in particular highly express the LDL receptor [11]. We examined simultaneous porphyrin and LDL uptake into 4 established cell lines of glioma and normal fibroblast using flow cytometry (FCM) and analyzed the mode of conjugation of porphyrin and LDL with the LDL receptor.

Materials and Method

Cell preparation

A-172, U-251, 9L, ONS, NB-1 cell lines were cultured for several days in RPMI 1640 medium (Gibco, New York, NY, USA) or Earle's minimum essential medium (MEM) solution (Gibco). A-172 and U-251 are human glioblastoma cell lines. 9L is a rat gliosarcoma cell line. ONS is a human medulloblastoma cell line. NB-1 is a rat fibroblast cell line. They were maintained at 37^{o} 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 was centrifuged and pellets of about $1x10^6$ cells were resuspended in RPMI 1640 medium or Earle's MEM solution and cultured

with fluorescent-labeled LDL or fluorescent porphyrin. In the case of LDL staining, the cells were stained with fluorescent labeled LDL, that is, acetylated LDL labeled with 1,1'-dioctadecyl-3,3,3',3'-tetramethylindo-carbocyanine perchlorate (Dil-Ac-LDL, Biomedical Technologies, Stoughton, MA, USA) at the concentration of 25μg/ml in RPMI 1640 for 2 hours at 37˚C in an atmosphere of 5% CO_2 in air. In that of porphyrin staining, the cells were stained with ATX-S10·Na, (Photochemical Co., LTD., Okayama, Japan) at the concentration of 25μg/ml in RPMI 1640 for 2 hours at 37˚C in an atmosphere of 5% CO_2 in air. The chemical name of ATX-S10·Na is 13,17-bis (1-carboxypropionyl) carbamoylethyl-8-ethenyl-2-hydroxy-3-hydroxy iminoethylidene-2, 7,12, 18- tetramethyl-porphyrin sodium salt; its molecular formula is $C_{42}H_{41}N_7O_{12}Na_4$, and its molecular weight is 927.79 [4].

LDL receptor stability against trypsin

We investigated whether LDL receptors are changed with trypsin solution. One group of cells was detached with 0.25% trypsin solution. Another group of cells was mechanically detached without trypsin solution. Each cell were washed with PBS and centrifuged. Each pellets were resuspended in RPMI 1640 medium and cultured with Dil-Ac-LDL at the concentration of 25 g/ml in RPMI 1640 for 2 hours at 37˚C in an atmosphere of 5% CO_2 in air. Each cell suspension was centrifuged and the pellets were fixed with ice-cooled 70% ethanol over night.

Staining with LDL and porphyrin

Cells were stained according to different protocols as follows. Cells in Group 1(sequential LDL-porphyrin) were first stained with Dil-Ac-LDL at the concentration of 25μg/ml in RPMI 1640 for 2 hours at 37˚C in an atmosphere of 5% CO_2 in air and subsequently stained with ATX-S10-Na at the concentration of 25μg/ml in RPMI 1640 for 2 hours at 37˚C in an atmosphere of 5% CO_2 in air. Cells in Group 2(sequential porphyrin-LDL) were first stained with ATX-S10-Na at the concentration of 25μg/ml in RPMI 1640 for 2 hours at 37˚C in an atmosphere of 5% CO_2 in air and then stained with Dil-Ac-LDL at the concentration of 25μg/ml in RPMI 1640 for 2 hours at 37˚C in an atmosphere of 5% CO_2 in air.

Cells in Group 3(simultaneous) were simultaneously stained with Dil-Ac-LDL and ATX-S in RPMI 1640 for 2 hours at 37˚C in an

atmosphere of 5% CO_2 in air. The concentration of Dil-Ac-LDL was 25μg/ml and that of ATX-S10 was gradually increased from 0.5 to 50 μg/ml. Each cell suspension was centrifuged and the pellets were fixed with ice-cooled 70% ethanol over night and then stored at 4˚C.

Inhibition of LDL receptor with monoclonal antibody

Cells were incubated with anti-human LDL receptor mouse monoclonal antibody (Progen biotechnik GmbH, Heidelberg, Germany) at the concentration of 5 ug/ml in RPMI 1640 for 1 hour at 37˚C in an atmosphere of 5% CO_2 in air. Each cell were washed with PBS and centrifuged. Each pellets were resuspended in RPMI 1640 medium and cultured with Dil-Ac-LDL or ATX-S10 at the concentration of 25 g/ml in RPMI 1640 for 2 hours at 37˚C in an atmosphere of 5% CO_2 in air. Each cell suspension was centrifuged and the pellets were fixed with ice-cooled 70% ethanol over night and then stored at 4˚C.

Analysis of the cells by flow cytometry

Cell suspensions were filtered through a mesh with 2mm pores (Becton Dickinson, San Jose, CA, USA) to remove debris or clusters of cells. The $1x10^5$ cells of each cell line were analyzed by flow cytometry. The flow cytometric data acquisition was conducted using FACSCalibur (Becton Dickinson). The peak-activated wavelength of Dil-Ac-LDL is 420nm and its peak fluorescent wavelength is 560nm. The activated wavelength of ATX-S10 is 400-417nm and its peak fluorescent wavelength is 677nm[4,9]. DNA content stained with Dil-Ac-LDL was measured within the FL2 range. The fluorescence of ATX-S10 was measured within the FL3 range. Data were analyzed with Cell Quest software (Becton Dickinson).

Results

LDL receptor stability against trypsin

Figure 1 show the histogram of FL2 of A-172 (upper) and U-251 (lower) cells. The results of the cells detached using trypsin solution are left side and the results of the cells mechanically detached are right side. The peaks of FL-2 histogram were almost same. The histogram using trypsin solution, however, shows sharper peak. And cells were completely detached by using trypsin solution. So we used trypsin solution to detach cells in following experiments.

Staining with LDL and porphyrin

Figures 2-6 show the dot plot of the relation between FL2 and FL3 (upper), histogram of FL2-height (middle) and histogram of FL3-height (lower) of A-172 and 9L cell lines. The vertical axis of the dot plot of the relation between FL2 and FL3 is FL3 fluorescence; the horizontal axis is FL2 fluorescence. The horizontal axis of the histograms corresponds to FL2 or FL3 fluorescence and the vertical axis corresponds to cell number. The porphyrin and LDL uptakes by tumor and normal fibroblast cells showed almost no difference among cell lines. Cells stained for LDL demonstrated fluorescence only in FL2 (Fig. 2). Cells stained for porphyrin demonstrated fluorescence only in FL3 (Fig. 3). Cells in group 1 (sequential LDL-porphyrin) showed fluorescence only in FL2 (Fig. 4). Cells in group 2 (sequential porphyrin-LDL) showed fluorescence only in FL3 (Fig. 5). These results mean the first compound was uptaken by tumor cells whereas the secondary compound was not. Cells in group 3 (simultaneous) showed fluorescence in both FL2 and FL3. Sequential changes in porphyrin concentration in the medium were associated with sequential changes in LDL fluorescence (Fig. 6).

Inhibition of LDL receptor with monoclonal antibody

Figure 7 shows FL2 histogram of A-172 (upper) and U-251 (lower) cells incubated with Dil-Ac-LDL. The results of cells treated with anti-LDL receptor monoclonal antibody are in right side and those without the treatment is in left side. The peak fluorescence of A-172 and U-251 with anti-LDL monoclonal antibody was almost same as those without anti-LDL monoclonal antibody. The FL2 fluorescence was not suppressed by the anti-LDL monoclonal antibody

Figure 8 shows FL3 histogram of A-172 (upper) and U-251 (lower) cells incubated with ATX-S10. The results of cells treated with anti-LDL receptor monoclonal antibody are in right side and those without the treatment is in left side. The peak fluorescence of A-172 and U-251 with anti-LDL monoclonal antibody was almost same as those without anti-LDL monoclonal antibody. The FL3 fluorescence was not suppressed by the anti-LDL monoclonal antibody.

Discussion

Rapidly proliferating tumor cells show high cholesterol uptake via

LDL receptor-mediated endocytosis and have been shown to exhibit enhanced LDL receptor activity and large amounts of LDL receptors on the surface of each cell [9,11,12-14]. As for the mechanism of porphyrin uptake, the low-density lipoprotein (LDL) receptor-mediated pathway was reported to be the carrier of porphyrin into the cells [9,10,12].

The anti-LDL receptor monoclonal antibody could not block the uptake both of Dil-Ac-LDL and porphyrin. This result means that both porphyrin (ATX-S10) and Dil-Ac-LDL have competitive receptor, which differ from the LDL receptor blocked by the present study. The antibody we used recognized one epitope of human LDL receptor. Further study should be performed to determine the exact receptor epitope of both porphyrin and Dil-Ac-LDL. Because this antibody did not block Dil-Ac-LDL uptake into the glioma cells, it is understandable that this antibody could not block the porphyrin uptake into the glioma cells. If the monoclonal antibody completely blocked the LDL receptor, the uptake of porphyrin would be investigated more in detail.

Our results demonstrated a competitive conjugation of porphyrin and LDL with the same LDL receptor on tumor cells. Variability of LDL and porphyrin uptake by different types of tumor and normal cell lines was not recognized in our study. These results indicate that the LDL receptor can be easily saturated with porphyrin or LDL in vitro studies. Porphyrin uptake by tumor cells was influenced with the concentration of concomitant LDL. So to improve porphyrin uptake by tumor cells, suppression of LDL concentration in target tumors or upregulation of the LDL receptor in the tumor cells may be effective as a therapeutic strategy. Local control of LDL concentration in the tumor may be difficult in vivo. We will investigate the possibility of modifying the LDL concentration in tumor tissue in future studies.

Acknowledgments

This research was financially supported by the Fund-in-Trust for Cancer Research from the Governor of IBARAKI Prefecture, Japan. We thank Ms. Kazuko Morita for excellent assistance in the experiments.

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Legends for figures

Fig. 1 A-172 (upper) and U-251 (lower) cells stained for LDL showed peak fluorescence in FL2. The results of cells using trypsin solution (left) were almost same as the results of cells without trypsin (right).

Fig. 2 A-172 cells stained for LDL demonstrated fluorescence only in FL2. The upper figure is the dot plot of the relation between FL2 and FL3, the middle one is the histogram of FL2-height and the lower one is the histogram of FL3-height.

Fig. 3 A-172 cells stained for porphyrin demonstrated fluorescence only in FL3.

Fig. 4 A-172 cells stained for LDL (left) demonstrated fluorescence only in FL2, and these stained for porphyrin (middle) showed fluorescence only in FL3. The cells in Group 1 (LDL-porphyrin, right) showed fluorescence only in FL2, almost same as those stained with porphyrin only.

Fig. 5 9L cells stained for LDL (left) demonstrated fluorescence only in FL2, and these stained for porphyrin (middle) showed fluorescence only in FL3. These cells in Group 2(porphyrin-LDL, right) showed fluorescence only in FL3, almost same as those stained with LDL only.

Fig. 6 A-172 cells in Group 3 showed fluorescence in both FL2 and FL3. Continuous changes in porphyrin concentration in the medium were associated with continuous changes in LDL fluorescence. The

concentrations of LDL and porphyrin were described in the figure.

Fig. 7 A-172 (upper) and U-251 (lower) cells stained for LDL showed peak fluorescence in FL2. The results of cells with anti-LDL receptor monoclonal antibody (right) were almost same as the results of cells without the antibody (left).

Fig. 8 A-172 (upper) and U-251 (lower) cells stained for porphyrin showed peak fluorescence in FL3. The results of cells with anti-LDL receptor monoclonal antibody (right) were almost same as the results of cells without the antibody (left).