Photofrin Uptake by Murine Macrophages

Mladen Korbelik, Gorazd Krosl, and David John Chaplin

Cancer Imaging [M. K., G. K.] and Medical Biophysics Unit [D. J. C.], British Columbia Cancer Research Centre, Vancouver, British Columbia, Canada V5Z 1L3

ABSTRACT

The uptake of Photofrin by murine peritoneal macrophages in vivo and in vitro was examined. Cellular Photofrin content was measured either by performing a fluorometric assay or by using 14C-labeled drug. For comparison, the uptake of Photofrin by murine SCCVII tumor cells (squamous cell carcinoma) was also examined under the same conditions. The data demonstrate that macrophages have a much greater capacity for Photofrin uptake than SCCVII tumor cells. Photofrin contents at 24 h after drug administration (25 mg/kg) measured 420 ± 90 (SD), 74 ± 15, and 15 ± 2 μg/mg of cell protein for peritoneal macrophages, tumor-associated macrophages, and SCCVII tumor cells, respectively. Factors that modify macrophage activity also influence the uptake of the drug by macrophages. The results support the assumption that Photofrin uptake by macrophages is dominated by phagocytosis of highly aggregated components of the drug. In vivo accumulated Photofrin material in peritoneal macrophages, tumor-associated macrophages, and tumor cells has shown very similar in vitro clearance from all three cell types. Only 20–30% of Photofrin was lost from the cells during the initial 24 h, mainly between 1 and 4 h of clearance incubation.

INTRODUCTION

One of the most important properties of Photofrin which makes it a clinically successful photosensitizer used in PDT3 of neoplastic disease is its selective localization in tumors (1, 2). The underlying mechanism for tumor localization of Photofrin and its distribution within the tumor tissue is, however, still being investigated.

In recent years increasing evidence has been generated on the accumulation of Photofrin and other photosensitizers in macrophages that infiltrate tumors. One of the earliest observations was published by Bugelski et al. (3). They have found particularly high levels of hematoporphyrin derivative (a somewhat less purified preparation of Photofrin) located in macrophages scattered throughout a mouse tumor. Chan et al. (4) have used flow cytometric analysis and cell sorting to study the content of photosensitizer chloroaluminum sulfonated phthalocyanine in cellular fractions of a mouse colorectal carcinoma. They have separated tumor-derived populations of high and low sensitizer content and identified macrophages among the cells with high drug content. Henderson and Bellnier (5) have suggested, based on their experience, that macrophages exhibit extremely high affinity for Photofrin accumulation. Henderson and Donovan (6) have demonstrated PDT-induced release of prostaglandin E2 from mouse peritoneal macrophages treated with Photofrin and light in vitro.

However, the uptake and retention of photosensitizers by macrophages have thus far not been characterized in any detail.

In this work, we have examined Photofrin uptake by murine peritoneal macrophages in vitro and in vivo. We have also compared the uptake and retention of Photofrin in macrophages and tumor cells.

MATERIALS AND METHODS

Chemicals. Photofrin was kindly provided by Quadralogic Technologies Phototherapeutic, Inc. (Vancouver, BC, Canada). [14C]Hematoporphyrin (which is identical to Photofrin), 4 μCi/ml (1 Ci/mol), was purchased from Abraham Biotechnology, Inc. (Fair Lawn, NJ). Heme (porphyrin) was purchased from Sigma Chemical Co. (St. Louis, MO). The concentrations of LDL and HDL cited include both lipid and protein weight components. ScintiGest, a tissue solubilizer manufactured by Fisher Scientific Co. (Fair Lawn, NJ), consists of cetyltrimethylammonium bromide (70%), methyl alcohol (20%), and potassium hydroxide (9.9%). Cytochalasin B and a Protein Assay Kit (Lowry method) were also purchased from Sigma.

Harvesting Macrophages and Photofrin Exposure in Vitro. Thio-glycollate-elicited peritoneal macrophages were collected from 9–11-week-old male C3H mice. The mice received one i.p. injection of 0.5 ml of thio-glycollate broth (Difco Laboratories, Detroit, MI) 3 days before the macrophage collection. After sacrifice, the peritoneum was washed with 5 ml of cold PBS. The lavages from three to five mice were pooled, and cells were pelleted by centrifugation and resuspended in Eagle’s MEM with 10% FBS (all by Gibco, Burlington, Ontario, Canada) at a concentration of 1–2 × 106 nucleated cells/ml. The cells were transferred into 60-mm plastic Petri dishes (Falcon 3002; Becton Dickinson and Company, Lincoln Park, NJ) and incubated for 2 h (except when noted) in complete growth medium at 37°C. Nonadhering cells were then washed away, and Photofrin was added to the samples at a given concentration. The growth medium with 1% FBS was used during the exposure to Photofrin, unless stated otherwise. The exact number of cells per sample was determined by subtracting the number of nonattached nucleated cells from the total of nucleated cells originally plated. The macrophage preparations used in this study contained 90–95% macrophages, as judged by the expression of nonspecific esterase activity.

Tumor cells used for comparison of Photofrin uptake in vitro were obtained from murine SCCVII squamous cell carcinoma. The cells were maintained in vitro for 3–4 weeks before being used in the experiments. Eagle’s MEM with 10% FBS was also used for cultivation of these cells. Cellular protein contents of macrophages and tumor cells were determined using the Lowry method.

In experiments testing the effects of individual plasma proteins, macrophages were exposed to Photofrin (40 μg/ml) in the presence of LDL, HDL (both at 0.2 mg/ml), or albumin (5 mg/ml) or in protein-free medium. Before the cell exposure, the Photofrin-protein mixture was first incubated for 30 min at 37°C, to allow for optimal interactions of Photofrin with the proteins. For Photofrin clearance incubation, macrophages were washed free of the drug and plasma protein and further incubated in complete growth medium with 10% FBS for an additional 24 h. In all cell culture experiments the pH was maintained at 7.2–7.4. To prevent photodestruction of Photofrin, all procedures were performed in subdued light, and the Petri dishes were wrapped in aluminum foil.

Photofrin Uptake in Vivo and Its Clearance in Vitro. The animals were sacrificed at different times after receiving Photofrin (25 mg/kg i.v.), and peritoneal macrophages were harvested and transferred into Petri dishes as described above. After a brief incubation in vitro with...
no Photofrin (15 min), which enabled macrophage attachment to the substrate and removal (washing away) of nonadherent cells, the samples were taken for determination of Photofrin content from the cell extracts using a fluorometric assay (see below). To study the effect of cytochalasin B on Photofrin uptake in vivo by peritoneal macrophages, the animals were given 15 mg/kg of this drug i.p. dissolved in a total volume of 0.06 ml of dimethyl sulfoxide, immediately before Photofrin administration. The same volume of dimethyl sulfoxide alone, given i.p., had no effect on Photofrin uptake.

Cellular retention of Photofrin was examined using cells collected 24 h after in vivo administration of the drug (25 mg/kg i.v.). After sacrifice, peritoneal macrophages were harvested from the animals and transferred into Petri dishes, as described above. The clearance incubation was performed in complete growth medium with 10% FBS, with no drug present. At the indicated times, nonadherent cells were washed away, macrophages were collected, and their Photofrin content was determined from the cell extracts by a fluorometric assay (see below).

Photofrin clearance from TAM and tumor cells was determined using the cells obtained from female C3H mice (9-11 weeks old) bearing s.c. implanted SCCVII tumor (500–800 mg). Implantation and maintenance of this tumor have been described in detail elsewhere (7).

The animals had received Photofrin (25 mg/kg i.v.) 24 h earlier. The tumors were excised from the sacrificed animals, and single-cell suspensions were obtained by an enzymatic digestion procedure, as described previously (7). Separation of TAM and tumor cells from these cell suspensions was achieved by a differential cell attachment procedure. The cells suspended in Eagle's MEM with 10% FBS were first plated into 100-mm plastic Petri dishes (Falcon 3003) at a concentration of 5–6 x 10^6 cells/dish and left for 5 min at 37°C (CO₂ incubator). The nonattached cells were then removed using two additional washings with 5 ml of medium. The cells that remained attached to the substrate were almost exclusively macrophages, as confirmed by positive staining for nonspecific esterase and by cell morphology. The nonattached cells were transferred into a second 100-mm Petri dish and incubated for 10 min at 37°C. The cells that remained nonattached after that time were mostly tumor cells and nonadhering host cells. They were transferred into a third Petri dish in which they were incubated for the duration of Photofrin clearance. Before collection of these cells for Photofrin content determination, nonadherent cells were washed away; the remaining attached cells represented a highly purified population of tumor cells. Over 90% of these cells were identified as tumor cells, as judged by the absence of positively stained cells for nonspecific esterase and by morphological examination.

Determination of Photofrin Content by Fluorometric Assay. This method has been described in detail earlier (8). For harvesting, the cells were rinsed with cold PBS and detached from the Petri dish using a rubber policeman. Following centrifugation of the cell suspension in PBS, the cell pellet was resuspended in 3 ml of fresh PBS and transferred into the cuvettes for fluorescence spectroscopy. After the measurement, the cell suspension was transferred into a separate tube to which ScintiGest was added at a final dilution of 1:10 (v/v). The samples with ScintiGest were left overnight at 60°C to facilitate the process of solubilization of cell proteins and hydrolysis of Photofrin aggregates. This renders cell extracts in which optimal fluorescence of different Photofrin components can be registered and thus gives the reading that reflects total cellular Photofrin content. The fluorescence of Photofrin in live cells is strongly dominated by highly fluorescing components (porphyrin monomers), while it does not reflect the concentration of highly aggregated and less-fluorescing species of this drug (9). Photofrin fluorescence intensity in samples was determined at the maxima for excitation and emission, using a Farrand Optical System 3 scanning spectrofluorometer. Appropriate controls were used to subtract all the fluorescence not originating from Photofrin in cells. In the experiments in which the measurement of Photofrin fluorescence in live cells was omitted, the cells were treated with ScintiGest immediately after harvesting. The quantitative determination of Photofrin concentration in the cell extracts was achieved using standard solutions of known drug concentration.

Determination of Photofrin Content by Radiolabeled Drug. Cells were exposed to [14C]polyhematoporphyrin under the same in vitro conditions as for the other Photofrin uptake experiments. The cell extracts in ScintiGest were also prepared in the same way as for the fluorometric assay. Before radioactivity measurement, the cell extracts were neutralized with glacial acetic acid. H₂O₂ (30%) was then added (0.5 ml/1.5 ml cell extract), followed by a scintillation fluid (ScintiVerse Bio-HP; Fisher Scientific Co.). Radioactivity was measured in an LKB 1214 liquid scintillation spectrometer. The concentration of the photosensitizer in the samples was determined using a calibration curve prepared from radioactivity counts obtained with the ScintiGest-treated solutions of known concentrations of radiolabeled drug.

RESULTS

The uptake of Photofrin by murine peritoneal macrophages and SCCVII tumor cells under in vitro conditions is shown in Fig. 1. Before exposure to Photofrin, macrophages harvested from the peritoneum were cultured for 2 h in the growth medium supplemented with 10% FBS. The nonattached cells were then washed away, and the macrophages were exposed to Photofrin in the medium with 1% FBS. The SCCVII tumor cells were exposed to Photofrin under the same conditions as the macrophages. The results for Photofrin uptake by these two
types of cells, based on measurement of the drug fluorescence in live cells (Fig. 1a), show much better accumulation of the drug by tumor cells than by macrophages. The uptake curves for both cell types show a saturation reached after the initial 2–3 h of exposure to the drug. However, quite different results were obtained when Photofrin fluorescence in the same cells was measured in the cell extracts (Fig. 1b). The uptake capacity by macrophages was in this case clearly much superior to that seen with tumor cells. The shape of the curves remained basically unchanged.

Another reliable method for assessment of cellular Photofrin content, measurement of radioactivity from 14C-labeled drug, was also used for the uptake study. In this case, exposure to [14C]polyhematoporphyrin was fixed at 24 h, with different drug concentrations tested. The results were expressed on a per cell protein basis. Macrophages are much smaller than tumor cells. Determination of cellular protein content based on the Lowry method showed that tumor cells have 3 times more cellular protein than macrophages (278 versus 92 pg protein/cell, respectively). The data demonstrate that macrophages, compared to tumor cells, have a much greater capacity for photosensitizer uptake (Fig. 2).

The effects of the presence of nonadhering cells from peritoneal exudate on the sensitizer uptake by peritoneal macrophages are shown in Table 1. Two h after the initiation of in vitro culture, nonadherent cells were washed away in one group of samples, while in the other samples these cells were not removed. All the samples were then incubated for 24 h in complete growth medium and exposed to 14C-labeled photosensitizer. The effect of the presence of nonadhering cells during in vitro cultivation of macrophages and during the exposure to the sensitizer was examined when there was no serum present during the exposure to the drug. The presence of the nonadhering cells was obviously stimulating the sensitizer uptake by macrophages, which was 40–50% greater than in the samples where these cells were washed away shortly after the initiation of in vitro culture.

In further experiments, the effects of serum on the sensitizer uptake by macrophages were tested in the presence of nonadherent cells. In the same experiments SCCVII tumor cells were also examined. The results (Fig. 3) show that 1% FBS already strongly inhibited the sensitizer uptake by both types of cells, and further increase in serum concentration exhibited very limited additional inhibitory effect. This effect of serum was more pronounced with macrophages than with tumor cells (a reduction to approximately 1/10, and to 1/4–1/5 of the sensitizer level seen in serum-free medium with the former and the latter cell type, respectively). This is best seen by following the ratio (macrophages/tumor cells) of the sensitizer levels on a per cell protein basis (Fig. 3, inset). The ratio value, which in the absence of serum was 11.0 ± 1.4 (SD), already was almost halved with 1% serum present. With higher serum concentrations, the ratio has declined even further, although much less prominently.

In Fig. 4 the effects of interaction of Photofrin with the individual plasma proteins on the uptake and subsequent retention of Photofrin by peritoneal macrophages in vitro are shown. The inhibition of Photofrin uptake compared to protein-free samples was obtained with all the proteins, with the greatest effect shown by albumin. The ensuing retention of the drug in macrophages was not affected to any greater degree by the interaction with plasma proteins during the Photofrin uptake period, although the samples with LDL and albumin showed...
somewhat inferior Photofrin retention. As the net result of these differences in Photofrin uptake and retention in peritoneal macrophages, the levels of the drug that remained in the cells were the lowest in albumin and LDL samples. In HDL samples the remaining Photofrin content was also markedly lower than in protein-free samples.

The uptake of Photofrin by peritoneal macrophages can also be followed in vivo. In this case the drug was administered i.v. to the mice at different times before the animals were sacrificed, peritoneal macrophages were harvested, and Photofrin content was determined in the cell extracts by fluorometric assay. The data (Table 2) show that Photofrin accumulates faster during the initial hours following drug administration, with subsequent slower additional increase in Photofrin levels. It took 20 h to double the cellular level reached 4 h after administration of the drug. In the same table, the results for Photofrin measurement in macrophages obtained from mice which received cytochalasin B immediately before Photofrin are also shown. The uptake of the drug by peritoneal macrophages in vivo was evidently inhibited by cytochalasin B.

Retention of Photofrin in peritoneal macrophages, tumor-associated macrophages, and SCCVII tumor cells under in vitro conditions following the uptake of the drug in vivo is also shown (Fig. 5). The clearance curves for the three cell types are remarkably similar. They show some loss of Photofrin material from cells between the 1st and 4th h of clearance incubation, and very limited further clearance between 4 and 24 h of incubation. All three cell types retained 70-80% of Photofrin after 24 h of clearance incubation. The initial levels (zero time clearance) of Photofrin per µg of cell protein in peritoneal macrophages and TAM were 420 ± 90 and 74 ± 13 ng, respectively, and 15 ± 2 ng in tumor cells. Calculated per cell protein, the levels of Photofrin in TAM were approximately 15 times higher than in tumor cells. Retention of Photofrin accumulated in vivo in SCCVII tumor cells is also shown (Fig. 5b). In contrast to the other three clearance curves shown in Fig. 5, the rate of Photofrin loss from the cells was in this case evidently greater during the initial hours of clearance incubation.

**DISCUSSION**

Many fast-growing animal and human tumors have broken the barrier of host immunosurveillance, but they still contain large populations of TAM (10-13). One of the important elements involved in malignant progression in many solid tumors is the attraction of macrophages and their subsequent subversion to a supportive factor in tumor growth (11). Macrophages possess uniquely powerful capacities of secretion (of biologically potent agents), versatility of action, and endocytic internalization (14, 15). These cells are, however, highly dependent on external factors to trigger and direct their action.

It was suggested by Jori (16) that porphyrins, phthalocyanines, and other photosensitizers that can remain in highly aggregated form in vivo are phagocytized to a large degree by
TAM. Circulating monocytes (and other fixed phagocytes) that phagocytize these drug aggregates in the bloodstream may infiltrate later tumor tissue. In our recent work, we have shown that most Photofrin found in the murine SCCVII tumor is in fact contained in TAM.

The evidence presented in this work demonstrates that macrophages have Photofrin uptake capacity vastly superior to that of SCCVII tumor cells. In addition, it is also shown that factors that modify macrophage activity also influence Photofrin uptake by these cells. Cytochalasin B, a known inhibitor of phagocytosis (17), reduced in vivo uptake of Photofrin by peritoneal macrophages (Table 2). In contrast, the presence of non-adherent nucleated cells from peritoneal lavage (mainly lymphocytes) induces macrophage activation (18) and stimulates Photofrin uptake by macrophages (Table 1). Measurement of Photofrin fluorescence in live cells, which registers almost exclusively highly fluorescing monomerized porphyrin, has not detected most Photofrin material taken up by macrophages (Fig. 1). All of these results support the assumption that Photofrin uptake by macrophages is dominated by phagocytic internalization of highly aggregated drug component.

The interaction of Photofrin with human plasma proteins and complete serum inhibits the uptake of this drug by macrophages, as it does with tumor and other cells (8, 19). However, the presence of serum inhibits the photosensitizer uptake in macrophages to a greater extent than it does with tumor cells (Fig. 3). The above can be interpreted as a consequence of disaggregation of Photofrin material induced by serum and its components (8). This disaggregation of the drug affects its uptake by macrophages more (fewer aggregates to be phagocytosed) than its uptake by tumor cells.

The ensuing retention of Photofrin material is less affected in macrophages by Photofrin-plasma protein interaction (Fig. 4) than it is in tumor cells (8). In the experiments with individual plasma proteins (Fig. 4), macrophages have presumably still phagocytosed the same type of drug material, i.e., the remaining Photofrin aggregates. Tumor cells in such circumstances, however, take up altered drug material with more poorly retaining Photofrin species, released upon interaction with plasma proteins.

Both peritoneal macrophages and TAM have shown very good retention of Photofrin accumulated in vivo, and similar retention qualities were exhibited by tumor cells (Fig. 5). In these experiments it was not possible to determine cellular Photofrin contents immediately after collecting cells from the mice, because of the time needed for cell separation (15–20 min for peritoneal macrophages, more than 1 h for TAM and tumor cells). However, since the clearance of Photofrin from these cells in vitro is very limited, there is no indication that there was a significant loss of the drug from the cells during the isolation procedure.

The clearance of Photofrin from peritoneal macrophages (Fig. 4), tumor cells (Fig. 5b), and other cells (8) following the uptake of the drug in vitro is much greater than for Photofrin accumulated in the cells in vivo. In the former case, the clearance curves are characterized by an initial phase of rapid Photofrin clearance during the initial 1–3 hours of the clearance incubation, followed by a phase of further limited loss of the drug from the cells (8, 20). It can be assumed, therefore, that in the cells collected after accumulation of Photofrin in vivo the initial phase of fast Photofrin clearance has mostly been completed. The involvement of TAM in the accumulation of Photofrin and other photosensitizers in tumors with prominent macrophage content necessitates careful reexamination of clinical ramifications.

It has already been shown that Photofrin-based PDT results in a release by macrophages of tumor necrosis factor (21), which is known to inactivate tumor cells (22). Release of a variety of bioactive substances from TAM induced by PDT, by either their destruction or activation, can also have profound effects on tumor cells, tumor vasculature, and other host cells.

REFERENCES

Photofrin Uptake by Murine Macrophages

Mladen Korbelik, Gorazd Krosl and David John Chaplin


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/51/9/2251

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.