Optical imaging in photodynamic therapy: mechanisms and applications

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ABSTRACT

Molecular excitation of photosensitizing agents provides reactive excited states, which can initiate chemical reactions, but it can also lead to molecular relaxation *via* radiative photophysical processes, providing the basis for fluorescence diagnostics. The best-known example of the former is Photodynamic Therapy (PDT), which is now approved for the treatment of a number of neoplastic and non-neoplastic pathologies. Although the concept of the use of photodynamic agents in diagnostics is as old as their use in therapy, the focused development of this aspect has been relatively recent. Typically, photodynamic agents have high triplet yields and relatively long triplet lifetimes (microsecond range), which allows them to interact and destroy molecular targets near them either directly or indirectly by producing other toxic molecular species. Associated with a high triplet yield is the fortunate attribute of most PDT agents in having low but finite fluorescence quantum yields. Fluorescence from these molecules may be used not only for diagnostics of disease *de novo* but also for guided surgery, PDT dosimetry and therapeutic monitoring. Other uses of fluorescence in PDT (not necessarily from the PDT agents) include the development of technologies that allow tracking of cells during treatment *in vivo*, studies of sub-cellular localization of molecules for mechanistic studies and photosensitizer tracking for specific targeting. An overview of studies on these aspects from different laboratories will be presented.

Keywords: Photodynamic therapy, optical imaging, photo-immunotargeted therapies, treatment monitoring.

INTRODUCTION

Molecules that absorb light and emit fluorescence can be useful probes for diagnostic applications and for the study of basic cellular processes[1]. If these same molecules can, from their activated states, also initiate photochemistry so as to destroy cells/tissues locally they can serve the dual purpose of being diagnostic and therapeutic agents. Photodynamic therapy (PDT), an emerging therapeutic modality, incorporates such molecular probes called photosensitizers (PS). Since most PS are fluorescent, drug localization can be determined by macroscopic and microscopic fluorescence imaging. These agents achieve selective targeting by two complementary paths: biological-chemical targeting enhances localization/accumulation of the PS at target sites, and optical targeting confines light delivery to target tissues. Once the PS is located at the site of interest, it is activated by the appropriate wavelength of light to its first excited state (S_1). The PS can then follow various paths for energy dissipation: it can return to its ground state (S_0) with the emission of fluorescence, which, with appropriate imaging and/or spectroscopic technology, can be used for diagnostic purposes. Alternatively the activated PS in its S_1 state can undergo intersystem crossing to another electronically excited state at a slightly lower energy level, called the first triplet state T_1. In this state it is more efficient at initiating photochemically induced species due to the increased lifetime of the triplet state relative to other excited states. Figure 1 depicts the different pathways of molecular excited states. The best-known biomedical application of triplet state-induced photochemistry is PDT, where the production of singlet oxygen is largely thought to be the dominant mechanism of tissue damage. If the triplet state is not quenched by interaction with other molecules, it will generally relax back to its S_0 state with the emission of phosphorescence in the process, which, although more difficult to detect, may also be useful for diagnostic purposes.

The first part of this review will focus on novel ways to target PS to tumor tissues; while in the second part we'll discuss the advances made in using aminolevulinic acid (ALA) for therapy, diagnosis, and guided surgery. Finally, we will present a new method for monitoring tumor cells *in vivo* during treatment.

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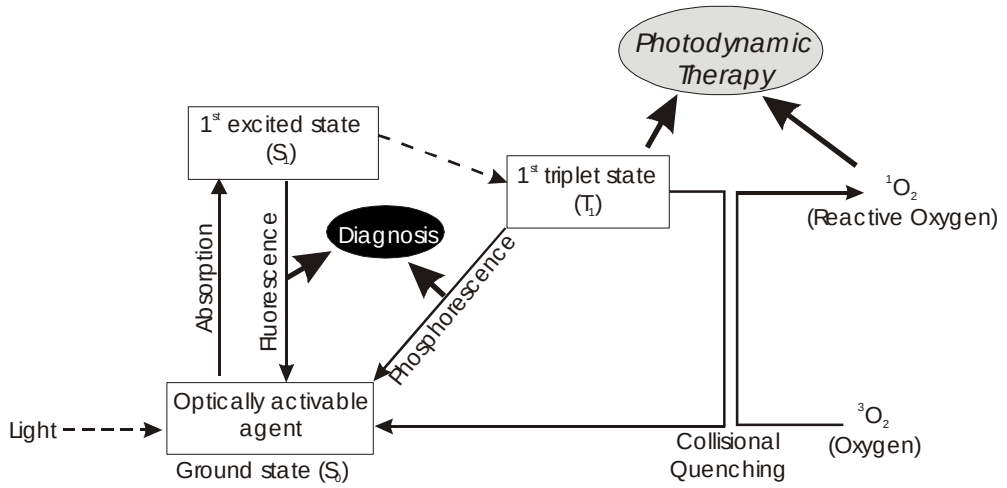

Figure 1: A simplified energy level diagram for the photoexcitation of a PS. Absorption of a photon results in the excitation of the absorbing molecule from the ground singlet state (S_0), to the first excited singlet state (S_1). Fluorescence, which can be used for diagnostic purposes, comes during de-excitation from S_1, back to the ground state S_0. Alternatively, the first triple state (T_1) is generated after intersystem crossing from S_1. Phosphorescence is emitted when the molecule relax back to S_0 from T_1. T_1 can initiate photochemical reactions directly, giving rise to reactive free radicals, or transfer its energy to the ground state oxygen molecules ($^3O_2$) to give rise to excited singlet state oxygen molecules ($^1O_2$) (adapted from[2]).

1. TARGETED OPTICAL IMAGING AND PDT

An important determinant of successful PDT is the localization of the PS in neoplastic tissue. Therefore, more precise drug targeting is desirable in order to ensure success but also to reduce toxicity to uninvolved tissues and organs in complex sites, such as the abdominal cavity. The use of molecular delivery systems that recognize specific molecular markers are highly desirable and under active investigation. It is important to note that contrary to conventional targeted chemotherapy where the drug has to be released from the carrier moiety to elicit a response; this is not a prerequisite when carrier molecules are used for delivery of PS in PDT. Furthermore, the requirements for specificity of the delivery molecule are less stringent in PDT. This is due to the dual selectivity of the treatment. As long as the delivery agent has preferential (not necessarily exclusive) affinity for the target tissue, improved selective phototoxicity is expected. Thus, carrier-mediated PDT offers the possibility of using non-tumor specific targeting molecules, providing a greater repertoire of usable compounds. However, the problems associated with the use of large molecules, such as complicated synthesis, transport barriers, and potential systemic toxicity, are similar for PS conjugates and for other conjugates.

a) PS localization in tumors

Based on two therapeutically relevant compartments of malignant tumors, PS are often classified as cellular or vascular, a reference to the primary site of action under the conditions studied. However, the transport of the PS, *in vivo*, is a dynamic process. The timing of the light exposure is very important. The appropriate choice of delay time after PS administration may allow targeting different compartments within the tissue (e.g., vascular and extravascular) using the very same PS. The concentration of agents varies between the blood, interstitial space, and tumor parenchymal cells and with time. Hydrophilic molecules usually remain in circulation until excreted, while hydrophobic compounds leak rapidly out of the vessels and are retained in tumor tissue[3]. This dynamic process results in a preferential accumulation of PS in tumors, and early clearance from the blood after injection.

The reason for the preferential accumulation of PS in tumors compared to normal tissue is not clearly understood but several properties of tumor tissue may contribute to this selective accumulation. It may be a result of the greater proliferative rates of neoplastic cells, poorer lymphatic drainage, leaky vasculature, or more specific interaction between the PS and marker molecules on neoplastic cells (for example elevated numbers of low-density lipoprotein receptors[4]). Furthermore, tumor stroma contains a high amount of collagen, shown to bind porphyrins[5], and a high amount of lipid, that has a high affinity for lipophilic dyes[3]. Cationic PS are suggested to be cellularly localized after a short intravascular phase and thus act at the tumor cell level. These compounds accumulate in tumor cells because of

the much steeper electrical potential across the mitochondrial membrane of tumor cells compared to normal cells[6,7]. The best developed of the series are the benzophenothiazine dyes, and animal studies using these dyes show high cure rates following light activation[8,9].

b) Photo-immunotargeted therapies (PIT)

Tumor targeting with antibodies is based on the concept that molecular markers are present on tumor cells, and the ability to obtain specific monoclonal antibodies (MAb) that recognize these markers. It is thought that neoplastic transformation generates new and specific antigens not present on normal cells. However, this is not the case with all tumors, and MAb with a high level of specificity for tumor markers are extremely rare. Photo-immunotargeting has therefore many advantages over standard immunotherapies because it combines two therapeutic principles. The molecule recognized by the MAb doesn't have to be expressed exclusively on neoplastic tissues and the MAb doesn't need to have an intrinsic effector function. This means, the MAb does not need to initiate on its own a reaction that would lead to tumor destruction. However, photoimmunotargeting requires conjugates with high PS to MAb ratios, which makes the synthesis complicated. PS can be linked chemically to the MAbs directly or they can be linked via polymers[10,11]. The chemical reactions that are necessary for the synthesis and the resulting structural alterations of the molecules' environments may lead to a loss of activity of the MAb (reduced affinity), the PS (reduced singlet oxygen yield upon irradiation) or both. In an ideal scenario the PS and antibody activities will be preserved, while at the same time allowing maximal PS incorporation[12].

The real utility of PIT may lie in its use as a detection modality and therapeutic response monitoring methodology. With this as a focus, we have tested an anti-EGFR MAb coupled to either the near infrared fluorescent dye Cy5.5 for detection or to the photochemically active dye, chlorin (ce_6) for therapy of premalignancy in the hamster cheek pouch carcinogenesis model[13]. Head and neck cancers including oral cancer and precancer overexpress EGFR. Furthermore, EGFR overexpression was also detected in the chemically induced malignant transformation leading to carcinoma in the hamster cheek pouch model[14]. These findings suggested that the overexpression of EGFR might be used as a marker for early diagnosis and treatment of oral precancer. Targeting the EGFR with antibody-delivered photoactive molecules may destroy the EGFR overexpressing cells, while the normal, low expression levels of EGFR in the healthy tissue do not lead to enough PS concentration in the normal mucosa. Targeted PDT thus will cause the premalignant lesion to regress. The MAb bound fluorescent dye may also allow us to monitor the progress of the treatment.

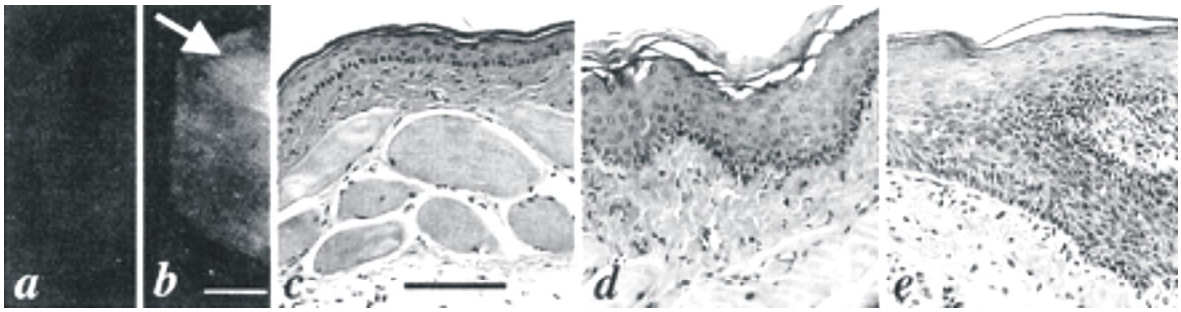

Figure 2: Fluorescence images of normal (a) and carcinogen-treated (b) hamster cheek pouches, 6 days after the injection of the EGFR-Cy5.5 conjugated Mab. (Bar, 5 mm). An area of increased fluorescence can be seen within the carcinogen treated cheek pouch (arrow). H&E histology shows (c) normal mucosal epithelium for the carcinogen-untreated cheek pouch (bar, 100 μm), (d) mild dysplasia for the carcinogen-treated cheek pouch, and (e) moderate dysplasia for the hot spot[13].

Figure 2 shows a representative image from an animal with a papillary tumor measuring 5 mm in diameter. The normal cheek pouch has no visible fluorescence (figure 2.a), whereas the tumor-bearing pouch has a clearly delineated tumor in the fluorescence image (figure 2.b) after systemic injection of anti-EGFR-Cy5.5 MAb conjugate[13]. Cy5.5 has several advantages over fluorescein, a dye commonly used for diagnosis in animals and humans: it has good solubility, high fluorescence quantum yield, and a longer emission wavelength (emits at 702 nm after 675 nm excitation). This leads to increased sensitivity of detection of premalignant lesions because fluorescence from molecules localized

deeper into tissue can be detected, and background autofluorescence is minimized because the endogenous fluorophores present in tissue do not absorb at 675 nm.
We have also conjugated EGFR-MAb to the PS ce_6. Following PDT with this conjugate we found that the overexpression of EGFR in carcinogen treated hamsters was significantly reduced to background levels compared to non-illuminated areas. No difference between illuminated and dark areas was seen in the normal cheek pouch. These results demonstrate the potential for development of immunotargeted photodiagnosis as a diagnostic tool and as a method of monitoring response to therapy.

c) Other methods for targeting PS

The purpose of the brief discussion below is primarily to inform the reader of other approaches to selective photoactivation. The examples are primarily therapeutic but there is no reason for not exploiting these same strategies for imaging and diagnostic purposes. Alternative ways of targeting PS to a specific cell population need to take advantage of certain properties of these cells, which either distinguish them from other cell or tissue types, or differentiate malignant from normal cells. The following approach that was recently used by Zhang[15] is based on altered sugar metabolism in cancer cells. They demonstrated that the PS pyropheophorbide 2-deoxyglucosamide (pyro-2DG) selectively accumulated in tumors. Rapidly growing tumors are able to maintain high glucose catabolic rate by upregulation of hexokinase. This enzyme phosphorylates glucose to glucose-6-phosphate, which is then retained in the cell[16]. Pyro-2DG is taken up by cells and becomes a substrate for hexokinase and the chemically altered PS is retained in the cells. Since cancer cells upregulate this enzyme, more of the PS is retained in tumor cells than in normal cells. This strategy of PS administration and subsequent photoactivation caused efficient mitochondrial damage.

Another approach exploits the biological changes associated with T-cell activation. Activated T-cells play an important role in many inflammatory diseases and malignancies of lymphocytes (lymphomas). The P-glycoprotein, product of the *MDR1* gene, plays an important role in multidrug resistance of cancer cells by extruding cytotoxic chemicals[17]. However, upon activation of T cells with mitogens, inactivation of the P-glycoprotein has been reported[18]. A recent study[19] used this characteristic to specifically target activated T cells with the PS TH9402. This study showed that only activated T cells retained the PS and photoactivation resulted in their selective depletion. This targeting strategy could be potentially used to monitor and treat graft-versus-host disease or other diseases mediated by activated T cells.

Macrophage targeting is currently being developed for the detection and treatment of vulnerable plaque, a non-cancer pathology. Using chlorin(e6) conjugated to maleylated albumin, Hamblin[20] and coworkers were able to specifically target macrophages in the vulnerable plaques in a rabbit model of atherosclerosis. Figure 3 shows aortic ring segments taken from an uninjected atherosclerotic rabbit (Figure 3, column 1), a conjugate injected normal rabbit (figure 3, column 2) and a conjugate injected atherosclerotic rabbit (figure 3, column 3). The uninjected atherosclerotic sections show typical autofluorescence. The conjugate-injected aortic section from a normal rabbit shows a small amount of fluorescence, while the conjugate-injected atherosclerotic rabbit shows a large amount of fluorescence in the thick plaque. These data suggest that macrophage targeting could be used for the detection/treatment of vulnerable plaques.

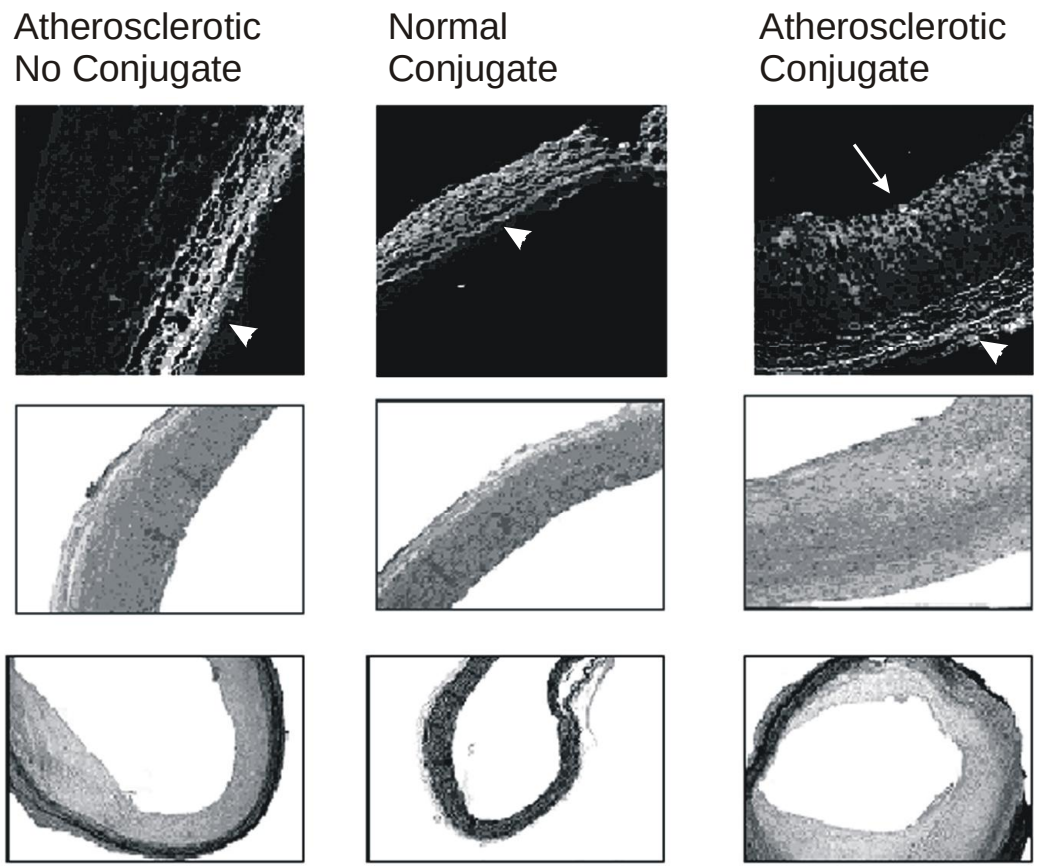

Figure 3: Rings taken from the aortic segments from an uninjected atherosclerotic rabbit (column 1), a conjugate-injected rabbit (column 2) and a conjugate-injectected atherosclerotic rabbit (column 3) were snap frozen and 10 μm cryosections cut. Adjacent sections were examined with confocal laser scanning fluorescence microscopy (row 1) and stained with hematoxylin and eosin (row 2), and with Verhoeff's elastic lamina stain (row 3). Arrow head shows typical autofluorescence. Arrow indicates area of targeted macrophage. Images courtesy of Micheal R. Hamblin, PhD[20].

To enhance the delivery of PS several groups have conjugated PS to small molecules such as growth factors and hormones. Akhlynina[21] conjugated ce_6 to insulin and demonstrated specific receptor mediated internalization and phototoxicity 100x higher than that obtained with free ce_6. Gijsens[22] used the epidermal growth factor (EGF) conjugated to ce_6 to target EGFR positive tumors. This conjugate was highly phototoxic. Transferrin conjugated to ce_6 was also used to kill mammary adenocarcinoma cells in culture[23]. To improve the selectivity of PpIX toward cancerous cells Rahimipour[24] conjugated PpIX to a gonadotropin-releasing hormone.

2. OPTICAL IMAGING WITH AMINOLEVULINIC ACID

a) Aminolevulinic acid (ALA)

A relatively novel way of PS targeting is the use of a prodrug approach, where the molecule is not a pre-synthesized PS, but is converted into a PS *in situ*. This is the case with aminolevulinic acid (ALA) or its esters. Every cell requires heme enzymes for a number of functions including ATP formation. Therefore each cell has the capacity to synthesize heme. Under physiological conditions heme production in non-erythroid cells is regulated by the expression levels of the enzyme ALA-synthase (ALA-S), which is under a negative feedback control by free heme. ALA-S provides ALA, the first committed precursor of heme formation. When exogenous ALA is added, this rate-limiting step is bypassed and downstream metabolites are synthesized in excess. The last step in this pathway involves the conversion of PpIX, a PS, to heme (a non-photosensitizing species). When exogenous ALA is added, ferrochelatase, the enzyme

responsible for the insertion of iron into PpIX, becomes rate limiting. It is unable to utilize the excess PpIX that is formed. PpIX therefore accumulates in the cells and renders them photosensitive[25]. When esters of ALA are used, these have to be first hydrolyzed before ALA can enter the heme pathway.

b) Diagnosis using ALA

Along with photosensitivity comes fluorescence, and thus the use of ALA-induced PpIX has gained much interest in diagnostic applications. Exogenous ALA-dependent PpIX fluorescence is being developed for the detection of early malignancies, carcinomas *in situ*, and certain cancer precursor lesions. The detection of localized pathologies is based on increased PpIX formation in diseased as compared to normal surrounding tissue. The reason for this selectivity is not entirely clear and several components may contribute to a different extent depending on the organ and tissue that is probed. In some epithelial tissues such as the epidermis, the disturbance of the physiological barrier function in lesional skin accounts for increased ALA penetration (figure 4).

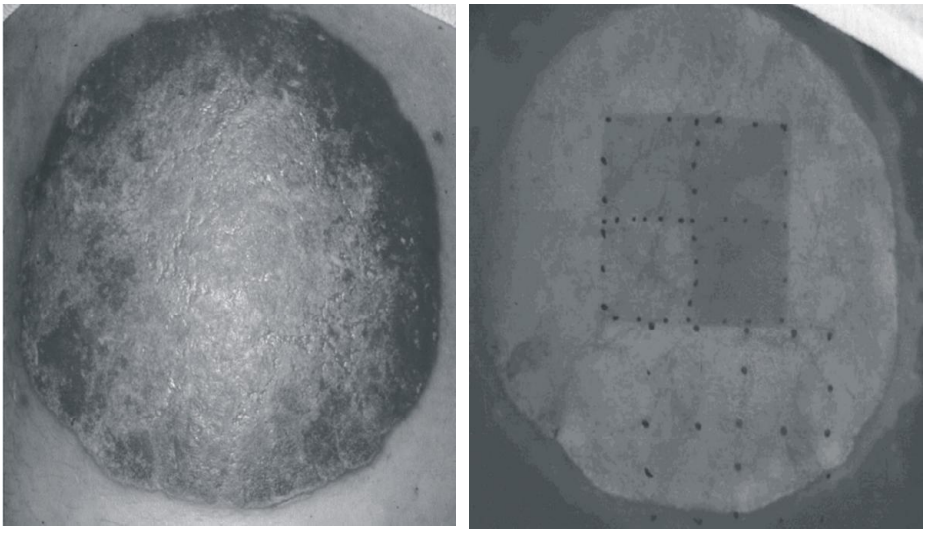

Figure 4: ALA-induced porphyrin fluorescence in psoriatic skin. In psoriasis, inflammation and hyperproliferation lead to thickened, bright red skin areas (plaques), covered with large scales (left panel). The same plaque shows red fluorescence after treatment with topical ALA (right panel, pale colored area). The psoriatic skin allows more efficient uptake of ALA due to a disturbed epidermal barrier function. The squares in the center of the plaque were irradiated clockwise with increasing doses of blue light, resulting in increased photobleaching and thus reduction of the fluorescence intensity (darker area at center of panel).

The use of ALA for diagnosis was first tested in accessible organs including the skin[26,27], the bladder[28], oral cavity[29], lungs[30], and the upper and lower gastrointestinal tract[31]. The hollow organs can be reasonably conveniently viewed with optical devices and PpIX fluorescence is used to identify pathologies that are less or not at all visible with conventional white light-based inspection. For example, in urology ALA-induced PpIX fluorescence is used for the early diagnosis of urothelial dysplasia and carcinoma using a modified cystoscope[32]. Patients have their bladder filled with an ALA solution. After 4-6 hours, the physician first performs cystoscopy under white light illumination. Then, filtered blue light is used to excite the red porphyrin fluorescence, which is observed through the cystoscope.

c) Guided surgery using ALA

ALA-PpIX has been shown to be useful not only for detecting premalignant or malignant tissue, but also for the detection of tumor margins. During surgery for primary brain tumors, the surgical challenge is to remove the malignant tissue as radically as possible, while preserving as much of the healthy brain tissue as possible. The finger-like extensions of these so-called gliomas infiltrate the healthy tissue and thus it is often difficult to distinguish the tumor margins. If the patient ingests ALA prior to surgery, the tumor-associated disruption of the blood-brain-barrier results in increased ALA delivery to tumor areas and consequently higher localized PpIX concentrations. Under a modified operating microscope blue light induced PpIX fluorescence is then used to guide the targeted resection of the fluorescent malignant tissue with careful sparing of nonfluorescent normal brain. This strategy has been employed

successfully in clinical studies and has resulted in prolonged patient survival, which is a sensitive measure of efficient surgery in brain tumors[33].

3. *IN VIVO* CYTOMETRY FOR CELL TRACKING

Flow cytometry has been used extensively for the characterization and quantification of circulating cells in animals and humans. In conventional flow cytometry, cell samples are extracted and analyzed outside the body. This technique is highly sensitive and specific, as it can utilize a significant number of sophisticated fluorescent probes developed to label very specific cellular features[34]. However, isolation of frequent blood samples in small animals, such as mice, is restricted by the limited total blood volume of the animal. Thus, acquisition of information on a dynamic process, which involves rapid changes, requires sacrificing a significant number of animals and limits observation of such processes within a single subject. In addition, sample isolation and processing may introduce artifacts that obstruct or modify the characterization of the events of interest.

To address this issue an *in vivo* flow cytometer with the capability to detect and count circulating fluorescently labeled cells in live animals, without the need to extract a blood sample, was developed[35]. Thus, we can monitor continuously the number of circulating cells of interest, in their native environment, without affecting the physiology of the animal. Figure 5 shows the depletion kinetics of circulating prostate cancer cells with different metastatic potential in mice and in rats.

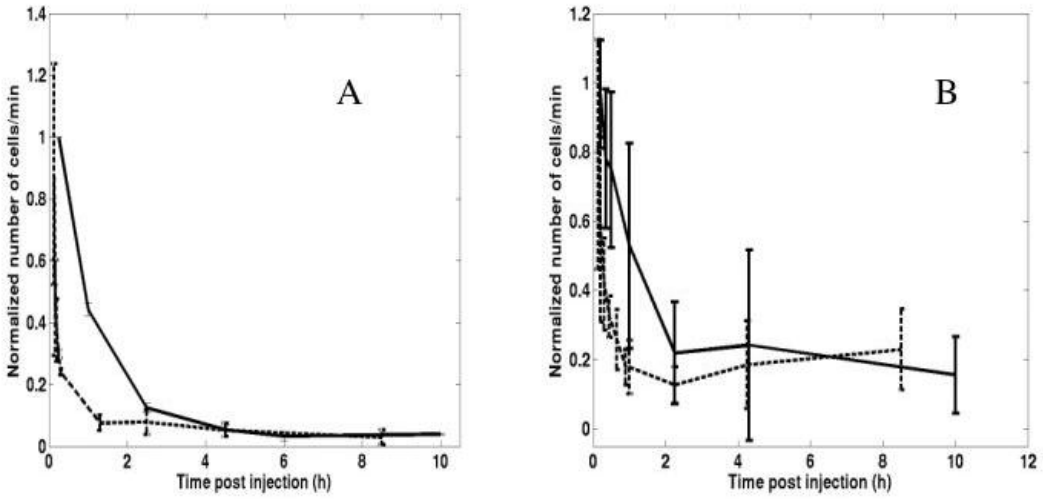

Figure 5: Depletion kinetics of fluorescently labeled LNCaP (solid lines) and MLL (dashed lines) prostate cancer cells in SCID mice (A) and Copenhagen rats (B).

We find that both cell lines become depleted from the circulation very rapidly. Especially in the case of SCIDs, we observed very small variations from animal to animal, yielding very consistent rates of depletion for circulating prostate cancer cells. Interestingly, in both mice and rats the more highly metastatic MLL cells become depleted at a faster rate than the LNCaP cells. We do not find any statistical differences in the rate of depletion of the same cell line in different hosts. However, we have noticed in the rat studies that following a significant rapid depletion, a percentage of cells reappears and then become re-depleted at a slow rate over the course of days. Tumor cell shedding and an enhancement in metastatic potential is a problem that we plan to study in the context of subcurative tumor treatment with therapies such as photodynamic therapy. This technology will allow us to track cells during treatment *in vivo*.

CONCLUSION

This review of photodiagnosis and phototherapy is a summary of specific aspects of optical targeting. It is important for the reader to be aware that this is a very brief summary of literature and concepts in a rapidly emerging field. With the advent of new molecular probes and light delivery and light capturing techniques, the field of optical treatment and diagnosis is rapidly evolving, to the benefit of the patient, as new clinical applications are developed to improve patient care. Many of the PS described throughout this chapter are potentially useful for both diagnosis and therapy. The fluorescence produced by these compounds may be exploited for several purposes: the identification and delineation of malignant tissues, the quantification of PS at the tumor site, and potentially the monitoring of oxygen and PS consumption during therapeutic light exposure. In an optimal scenario targeted delivery identifies diseased tissue and in the same procedure treatment occurs. A targeting molecule (MAb, or peptidse based) would deliver a PS specifically to tumors and would be used for diagnosis and treatment. The progressive improvement of remote imaging devices and molecular targeting strategies make this an attractive aim of preclinical and clinical development.

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