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1075 Kinetics of Photofrin II in Perifocal Brain Edema
Experimental Study

AUTHOR(S): Stummer, Walter, M.D.; Götz, Claudia, M.D.; Hassan, Ahmad, M.D.; Heimann, Axel, D.V.M.; Kempski, Oliver, M.D., Ph.D.

Department of Neurosurgery, Ludwig-Maximilians University (WS, CG, AHa), Munich, Germany; Institute for Neurosurgical Pathophysiology, Johannes Gutenberg University (AHe, OK), Mainz, Germany

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ABSTRACT: PHOTODYNAMIC THERAPY IS under intense investigation as a possible adjuvant for the treatment of malignant tumors of the central nervous system. It relies on the fact that photosensitizers are selectively taken up or retained by malignant tissue. However, most brain tumors are accompanied by substantial vasogenic edema as a consequence of blood-brain barrier disruption within the tumor, leading to extravasation and propagation of plasma constituents into the surrounding brain tissue. Systemically administered photosensitizers may enter healthy tissue together with the edema fluid, possibly leading to sensitization of tissues outside the tumor. To test this hypothesis, vasogenic edema was induced by cold injury to the cortex in rats. The edema thus obtained is highly reproducible and very similar to tumor-associated edema. Just after injury induction, Photofrin II (PF-II), a commonly used photosensitizing agent, was administered at a dose of 5 mg per kilogram of body weight along with fluorescein isothiocyanate (FITC)-labeled albumin to mark edema advancement. After 1, 4, 12, or 24 hours, the brains were removed and frozen, and cryosections were studied with high-sensitivity video fluorescence microscopy for edema extravasation within the lesion and propagation of PF-II into the surrounding gray matter. PF-II advanced with edema along the corpus callosum underlying the cortex to a distance of 5 mm from the lesion after 4 hours. With the exception of the lesion, PF-II fluorescence returned to baseline after 24 hours, indicating subsequent washout. Propagation was comparable to the spreading of FITC-marked albumin. The authors conclude that photosensitizers spread with edema, an observation that may be pertinent to a number of questions concerning photodynamic therapy of cerebral tumors.

KEY WORDS: Blood-brain barrier; Brain edema; Drug delivery; Photodynamic therapy; Photofrin II

Recent advances in surgery, radiation therapy, and chemotherapy notwithstanding, the prognosis for patients suffering from glioma, the most common cerebral tumor, still remains poor. Inevitable local recurrence of the tumor suggests that more aggressive local adjuvant therapy could be beneficial [2].

Currently, photodynamic therapy (PDT) is under investigation as a possible adjuvant for glioma therapy after radical debulking of the tumor mass [18,27,29,32,36,42]. This mode of therapy relies on the fact that certain photosensitizers, applied intravenously or directly, are selectively taken up or retained by malignant tumors. Irradiation with light of an appropriate wavelength activates the photosensitizer and leads to rapid tumor necrosis [14]. Hematoporphyrin derivative, obtained by acidification of hematoporphyrin, and Photofrin II (PF-II) (Photomedica Inc., Raritan, NY), a more purified form of hematoporphyrin derivative, are the most commonly used photosensitizers, and have been reviewed extensively [19]. PF-II is currently the leading photosensitizer undergoing clinical investigations.

Nevertheless, it is known that in tumor-associated edema, which is of the vasogenic type, serum proteins and edematous fluid leak through the walls of tumor vessels into the extracellular space of the surrounding brain tissue. Hematoporphyrin and its derivatives are bound to serum albumin and lipoproteins [31,39]. Thus, a spreading of porphyrins with tumor-associated edema becomes conceivable. However, the general opinion related to this matter seems to be that porphyrins remain selectively localized in cerebral lesions that produce vasogenic edema. In this context, the work of Wise and Taxdal [43] is frequently cited [7,20,27,32,36]. It is surprising that no further research has been committed to the possibility of porphyrin propagation with peritumoral edema, although such a phenomenon would be of utmost importance. On the one hand, tumor-free areas might be affected by photodynamic therapy via transport of the sensitizer together with edema fluid into adjacent tissues. On the other hand, tumor cells spreading outside the tumor mass might be reached by PDT through edema-associated sensitizer transport.

The present study was designed to assess the kinetics of PF-II within vasogenic edema in the cold lesion model [22]. This model was chosen because edema production is well standardized and resembles tumor-associated edema in many respects [8]. The absence of tumor cells allows correlation of PF-II-fluorescence and edema production without interference from accumulation of the tumor-associated sensitizer.

MATERIALS AND METHODS

Cold injury and specimen preparation

Thirty-six male, adult Wistar rats (weight, 300 ± 20 g) were anesthetized with 3.6% chloral hydrate (1.2 ml per 100 g body weight). After cannulation of the right external jugular vein, the rats were fixed in a stereotactic holder. A longitudinal incision was made in the scalp and the left parietal bone was exposed for a craniotomy of 4 mm in diameter, 4 mm dorsal and 3 mm lateral to the bregma. Cold injury was induced by applying a copper stamp cooled to -68°C with a mixture of dry ice and acetone to the exposed dura with the aid of a micromanipulator for exactly 15 seconds. Bovine albumin marked with fluorescein

isothiocyanate (FITC; Sigma, Deisenhofen, Germany) in a 2.5% saline solution was injected at a dose of 0.6 ml per 100 g body weight over 3 minutes immediately before trauma was induced. During and within 30 seconds after trauma, PF-II was administered at a dose of 5 mg per kilogram body weight. The venous catheter was then removed and the scalp incision carefully sutured.

Animals were killed at intervals of 1, 4, 12, and 24 hours after trauma (six at each interval). An additional six animals were killed at 4 hours after trauma, of which three had PF-II only and three FITC-labeled albumin only. A control group of six animals without trauma or intravenous application of test compounds was killed for the evaluation of autofluorescence.

The animals were killed by intracardiac perfusion with freshly prepared, phosphate-buffered 2% paraformaldehyde solution. The brains were then removed and immediately frozen in methylbutane cooled to -80°C. Serial coronal 20-μm sections were prepared with a cryostat (Leitz, Wetzlar, Germany) at -20°C and mounted on standard laboratory slide glass.

Digital video fluorescence macroscopy

PF-II fluorescent images (see *Fig. 1* spectra) of the frozen sections were viewed through a Wild-Leitz macroscope (Wetzlar, Germany) using an interference BP 405 excitation filter (bandwidth 9.2 nm; SMT, Seefeld, Germany), an RSP 455 nm beamsplitter (Wild-Leitz), and an LP 460 barrier filter (Wild-Leitz). FITC-linked fluorescence (see *Fig. 1* for spectra) was selectively obtained with a BP 450-490 excitation filter (Seefeld), an RSP 510 beamsplitter (Wild-Leitz), and an LP 515 barrier filter (Wild-Leitz). Both filter combinations were mounted on Leitz filter cubes in a Wild-Leitz Ploemopack connected to the macroscope. An HBO/100 W/2 mercury lamp (Osram, Munich, Germany) was used as light source.

Fluorescent images were digitized on-line with a 512 × 512 pixel matrix of a Kontron Electronics image analysis processor (Eching, Germany) via a silicon-intensified target tube camera (Hamamatsu TV Co. Ltd., Hamamatsu, Japan). Camera gain, offset, and sensitivity were kept constant throughout all measurements.

High-sensitivity fluorescence detection demanded a number of image-processing procedures to correct for illumination errors leading to fluorescence distortion, camera offset, and slide autofluorescence. Pixel by pixel subtraction of an imaged empty slide from the fluorescence image compensated for camera offset and slide autofluorescence. A fluorescing reference image that was uniform and reproducible (the frosted end of a standard laboratory slide) was used for correction of illumination errors and long-term differences in illumination intensity. Correction was accomplished by pixelwise division of fluorescence and reference images, so that the specimen fluorescence became expressible in units of the reference fluorescence. Short-term fluctuations in illumination intensity were accounted for by obtaining one reference and one empty slide image immediately preceding the digitalization of every fluorescence image. An additional on-line integration procedure consisting of pixel by pixel addition over six camera cycles reduced background noise and enhanced contrast in a controllable way. Integration was also implemented for the empty slide and reference images before subtraction or division, so that the net fluorescence, expressed in units of the reference fluorescence, remained the same. To avoid PF-II bleaching, PF-II fluorescence was measured first in every specimen, and FITC fluorescence immediately thereafter by changing to the appropriate filter combination.

By means of attenuation of the illumination intensity of a control specimen with neutral density filters of defined transmission, the correlation with the gray matter value was found to be $r = 0.999$, which verifies the imaging procedure described above. The corrected fluorescence images were evaluated by electronically superimposing a standardized screen of windows, each window measuring 0.625 mm × 0.625 mm, over the area of the lesion. Additional windows were placed over the corpus callosum in the midline (0.75 mm × 0.5 mm) and over the ipsilateral thalamus (1.3 × 1.5 mm) (*Fig. 2*). The mean gray value was calculated for each window.

RESULTS

Specificity of Photofrin II and fluoroscein isothiocyanate fluorescence detection

To ascertain that PF-II fluorescence did not interact with FITC fluorescence, three animals were given only PF-II and another three only FITC-labeled albumin at the time of trauma induction. In both groups, the fluorescence detected was specific for the substance that was administered. When viewed with the alternate filter combination, fluorescence did not exceed the values for autofluorescence registered in the control group without trauma or intravenous compounds, which was below 3% of the maximal fluorescence encountered in these experiments for filter combinations specific for both FITC and PF-II (data not shown).

Distribution of Photofrin II fluorescence and fluoroscein isothiocyanate fluorescence

Qualitative evaluation of the fluorescence images demonstrated marked PF-II fluorescence inside the lesion at all points of time studied after the induction of cold injury, leading to clear demarcation of the lesion. The fluorescence did not remain confined to the lesion, however, and was detectable in the neighboring gray matter as early as 1 hour after the lesion, although the intensity was considerably lower. At 4 hours, PF-II fluorescence was present in the corpus callosum and, advancing in this structure, reached the midline at a distance of approximately 5 mm from the lesion (*Fig. 3*). At 12 hours, the distribution of PF-II remained essentially the same as at 4 hours, but had lost intensity, whereas at 24 hours, fluorescence was mainly restricted to the lesion. Furthermore, marked fluorescence was detected

within the choroid plexus of the lateral and third ventricles at all times after trauma. There was no difference to the eye in the qualitative distribution of FITC-linked fluorescence and PF-II fluorescence.

Figure 4 demonstrates the distribution of PF-II fluorescence within and in the vicinity of the lesion. Maximal fluorescence signals of PF-II were obtained within the lesion at all times. After 1, 4, and 12 hours, however, significant elevations of fluorescence were detectable up to 1.6 mm beneath the brain surface, or 1.3 mm beneath the lesion, demonstrating propagation of PF-II into adjacent brain tissue.

For comparison of PF-II and FITC fluorescence, all data were expressed as the percentage of the respective fluorescence in the lesion after 1 hour, because this was considered the closest possible reference for both substances. A further simplification was achieved by averaging the values of all windows with a similar distance from the lesion. *Figure 5* demonstrates the data thus obtained. Within the lesion, both PF-II and FITC fluorescence show equivalent courses, with the greatest fluorescence being found after 1 hour, declining slightly after 4 hours, and then greatly to less than 50% after 24 hours. At a distance of zero to 1.3 mm from the lesion, the course was similar, although FITC fluorescence was marginally higher than PF-II fluorescence. At 1.3 to 2.6 mm away from the lesion, the relative FITC fluorescence was elevated at all times after trauma with respect to control values, reaching a maximum after 12 hours and being significantly higher than the PF-II linked fluorescence after 4, 12, and 24 hours. On the other hand, PF-II fluorescence reached a maximum after 4 hours and was statistically higher than the control after 1, 4, and 12 hours, but not at 24 hours after trauma.

In the midline corpus callosum, at a distance of 5 mm from the lesion, there was no increase in PF-II- or FITC-related fluorescence 1 hour after trauma. After 4 hours, both were significantly elevated, and FITC fluorescence was almost double the fluorescence detected for PF-II. Both substances peaked after 12 hours, the PF-II fluorescence returning to baseline values after 24 hours, whereas FITC fluorescence still remained significantly elevated with respect to both control and PF-II fluorescence.

In the ipsilateral thalamus, which was located approximately 8 mm away from the lesion, a significant increase in FITC fluorescence was noted after 4 hours, peaking at 12 hours and returning to baseline at 24 hours. No increase in PF-II fluorescence was registered in this region.

DISCUSSION

Kinetics of Photofrin II extravasation

The present study demonstrates that PF-II was extravasated within a cortical lesion and advanced into adjacent tissue in a manner similar to FITC-labeled albumin. After 4 hours, PF-II was detectable as far as 5 mm away from the lesion. The greater the distance to the lesion, the less PF-II could be registered as compared with FITC-labeled albumin. Unlike PF-II fluorescence, appreciable amounts of FITC-linked fluorescence were found in the ipsilateral thalamus, 8 mm from the lesion. Contrary to the findings of Wise and Taxdal [43], these observations show that PF-II advances with edema, and may lead to sensitization of tissue surrounding a lesion that produces vasogenic edema. However, this sensitization may only be temporary, as our experimental evidence suggests that PF-II is consequently washed out or metabolized faster than FITC-labeled albumin. Such kinetics may be explained by the short half-life of PF-II in serum, which has been reported to be 4 hours [33], so that serum leaking from the lesion after this time may contain relatively little of the compound. The longer half-life of FITC-labeled albumin [23], next to the higher doses in which it was administered, may in turn explain the differences encountered between PF-II and FITC fluorescence. Like PF-II, FITC binds avidly to serum albumin and has previously been used as an edema tracer [23]. Its fluorescent characteristics allowed selective detection and did not interfere with PF-II fluorescence (see *Fig. 1*).

Vasogenic edema induced by cold lesion: A model for tumor edema

The cold lesion model chosen for this investigation has been employed before to study the pathophysiology [24,38,40,41] and radiological evaluation [3,11] of peritumoral edema, because it allows the induction of highly reproducible vasogenic edema similar to tumor-associated edema [8]. The use of an animal tumor model, on the other hand, seemed less suitable for this purpose, due to the high variability of tumor growth and edema formation and the retention of PF-II in tumor cells.

In tumor-associated edema, junctional abnormalities and fenestrae within the tumor [12] lead to increased permeability of the blood-brain barrier within the tumor. Consequently, serum proteins and edematous fluid leak into the extracellular space of the tumor and the surrounding brain tissue, particularly the white matter between the myelinated axons of the long fiber tracts [1,16,44]. In the cold-induced injury model, leakage is similarly restricted to the lesion area [24], and the most obvious mechanism is extravasation of fluid through ruptured endothelial cells [30]. Fluid then spreads with bulk flow into the adjacent white matter [5,37]. Basically, there are no major differences in the composition of both types of vasogenic edema derived by experimental brain tumor models or by the cold lesion with regard to water, protein, and electrolyte content [8]. Thus, it seems warranted to assume that in cerebral tumors, PF-II is likewise extravasated, and transverses edematous tissue adjacent to the tumor. The marked fluorescence of PF-II encountered within the choroid plexus at all times after trauma has been described previously [21] and is probably the consequence of the incomplete blood-brain barrier in this structure, rather than a phenomenon related to edema.

Photosensitizer uptake and retention

It was of interest that PF-II fluorescence was

highest in the primary lesion at 1 hour, and remained significantly elevated compared with surrounding edematous tissue at all times. This indicates semiselective uptake or retention within the lesion, albeit this observation was not specific for PF-II. The course of FITC-related fluorescence was exactly identical (*Fig. 5*). However, malignant tumors of the central nervous system may behave differently with respect to uptake or retention characteristics of sensitizing dyes. Selective uptake or retention has been demonstrated in vivo, in vitro, and clinically in neurosurgically relevant malignant tissue [17,21,25,35]. In the present study, fluorescence was greatest in the first hour after PF-II administration in contrast to studies with various experimental tumors, which reported maximal uptake at 6 hours [21] and 24 hours [6,15].

Extravasation of sensitizers and propagation with cerebral edema may help to explain some of the dispute related to the question of photosensitizer accumulation in normal brain tissue. Several authors have reported sensitization of normal brain and consequent necrosis after PDT treatment [4,9,13] while others have not [19]. The surgical procedures employed in some of these studies may lead to a traumatic breakdown of the blood-brain barrier, causing sensitizer extravasation and sensitization of normal tissue. Craniectomy by itself may suffice to disrupt the blood-brain barrier [34].

Conclusions for clinical application of photodynamic therapy

The spreading of photosensitizers with edema may be pertinent to the concept of photodynamic therapy of neurosurgical tumors. First, temporary sensitization of peritumoral tissue should be considered when planning therapy, to ensure that photosensitizer concentrations adjacent to the tumor are minimal. This may be of importance when treating tumors situated in sensitive structures such as the brain stem. Especially when higher doses of photosensitizer are administered, circulation times may be longer, leading to extravasation of more substance that may spread further than the 5 mm observed in the present study. Second, adjuvant radiotherapy after tumor excision and PDT [28] could have considerable side-effects in brain regions transversed by edema, given that the surgical procedure alone probably induces significant edema. Radiation therapy has been shown to enhance the effect of PDT [26]. Likewise, the time interval between surgical debulking and subsequent PDT should be as short as possible, to anticipate spreading of photosensitizers with edema induced by the surgical trauma and unwanted side-effects.

On the other hand, brain adjacent to tumor may contain infiltrative "nests" of tumor cells in areas with a relatively intact blood-brain barrier [44], which are probably responsible for local recurrences after tumor excision [2]. These have in part been held responsible [7,19,28,36] for the somewhat disappointing clinical studies employing PDT [19,36]. Therefore, extravasation of photosensitizers, advancement of these substances with edema through the brain adjacent to the tumor, and consequent washout may be beneficial for the concept of PDT, under the assumption that malignant cells of the central nervous system selectively accumulate or retain photosensitizers. Also, the multiple treatments necessary in any course of radio- and chemotherapy must be considered. Christensen et al. [10] have reported marked differences in survival of a human carcinoma cell line in vitro dependent on the different cell cycle stages. Therefore, repeated treatments may be necessary to kill cells that are less susceptible during the first treatment. Extravasation in inflamed, edematous tissue as a consequence of forgoing PDT may allow more sensitizer to enter tissue and to accumulate in malignant cells outside the primary blood-brain barrier leakage.

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Reprint requests: Prof. Oliver Kempski, Institute for Neurosurgical Pathophysiology, Johannes Gutenberg University Mainz, Langenbeckstraβe 1, 55101 Mainz 1, Germany.

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COMMENTS

The authors have attempted to measure the spread of the tumor photosensitizer Photofrin II into cerebral edema. This has particular clinical importance, as the use of a photosensitizer in cerebral edema may render edematous brain susceptible to phototherapy changes such as increased cerebral edema and even necrosis. It is essential that photodynamic therapy affect the "nests" of tumor cells situated in the "brain-adjacent-to-tumor" region, as it is these cells that are usually responsible for the recurrence of tumor after conventional therapies. It is probable that photosensitizers reach these cells located within the peritumoral edema as a result of the local alteration in the blood-brain barrier.

The authors have presented a good study, although the methodology utilized to study cerebral edema does have important differences from the clinical situation. The investigations do emphasize the previous experimental results demonstrating that dosimetry of both the sensitizer and light and the timing of light administration are critical to achieving maximal killing of tumor cells and to minimizing the effects of photodynamic therapy on the adjacent brain [(1)].

Andrew H. Kaye
Victoria, Australia

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The authors used a cold-induced injury to the rat cortex to assess the fluorescence distribution at 1, 4, 12, or 24 hours of Photofrin II (PF-II), the most commonly used photosensitizing agent for photodynamic therapy, and fluorescein isothiocyanate (FITC)-labeled albumin, an agent used to assess cerebral edema after a brain insult. The digital video fluorescence microscopy technique did require on-line correction; nevertheless, the results of their investigations are consistent with the observations of others [(1)].

The investigators found the following: a) that PF-II fluorescence was marked within the cold lesion at all observation times; b) that PF-II extravasates into perilesional brain edema in a manner similar to FITC-labeled albumin, although the PF-II fluorescence returned to baseline earlier than did the FITC; c) that significant PF-II fluorescence was detected up to 5 mm from the lesion, whereas FITC fluorescence was detected at 8 mm; and d) that as the distance from the lesion increased, the amount of PF-II present decreased, as did the the ratio of PF-II to FITC-labeled albumin.

The presence of photosensitizer in a perilesional location is essential to the practice of cavitary photodynamic therapy of malignant brain tumors. It is well recognized that malignant astrocytic tumors extend into the neuropil, well beyond the grossly resected tumor; even apparently totally resected metastatic brain tumors have clusters of "satellite" tumor nests remote from the main resectable mass. The application of cavitary photodynamic therapy might reasonably be considered to be an atraumatic, but not necessarily selective, extension of tumor cell killing beyond the margin of tumor resection.

The authors suggest that the perilesional sensitization is temporary, and thus, that treatment planning should be undertaken to ensure that the concentration of photosensitizer adjacent to the tumor

is minimal when treating tumors situated in sensitive areas of the brain. Such a high degree of selectivity of tissue killing is presently being sought. Because there appears to be a threshold of photosensitizer concentration below which the photodynamic effect will not occur, regardless of the light dose, selectivity will be possible as long as there is a difference in the concentration of photosensitizer in the tissue to be destroyed and the tissue to be preserved. However, consistent selective preservation of normal brain tissue will require techniques to measure clinical photosensitizer concentrations accurately in vivo.

There has been some controversy as to the presence of porphyrin photosensitizer in normal brain. The work of Dereski et al. [2] has shown that normal, nonlesional rat brain undergoes an increase in vascular permeability and infraction at energy densities as low as 70 J/cm^2 with a dose of PF-II of 12.5 mg/kg administered 48 hours before photodynamic therapy. Control animals revealed this effect not to be due to craniectomy; furthermore, these investigators showed in preliminary work that the infraction effect of photodynamic therapy in their rat model can be made to occur through intact skull at the same energy and photosensitizer doses. These findings suggest a high degree of sensitivity of normal brain to photodynamic therapy.

The level of selectivity presently achieved in photodynamic therapy of brain tumors is the consequence of many factors. The limited and differential light penetration of tumor and brain is an important determinant of selectivity. Using intraoperative measurements in the clinical setting, we have previously shown that the penetration depth of 630-nm light in vivo is approximately 1.5 mm for normal brain, 2.5 mm for peritumoral infiltrated brain, and 3.0 mm for glioblastoma [3,4]. It is important to note that the penetration depth is that distance in millimeters wherein light energy falls off by 37% (1/e); tumor cell killing may occur over a number of penetration depths, depending on the energy of the incident light and the concentration of photosensitizer.

The use of photodynamic therapy in very highly sensitive brain structures such as the brain stem must await considerable improvement in selectivity of effect.

Paul J. Muller
Toronto, Ontario, Canada

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2. Dereski MO, Chopp M, Chen Q, Hetzel F: Normal brain tissue response to photodynamic therapy: Histology, vascular permeability and specific gravity. **Photochem Photobiol** 50:653-657, 1989.

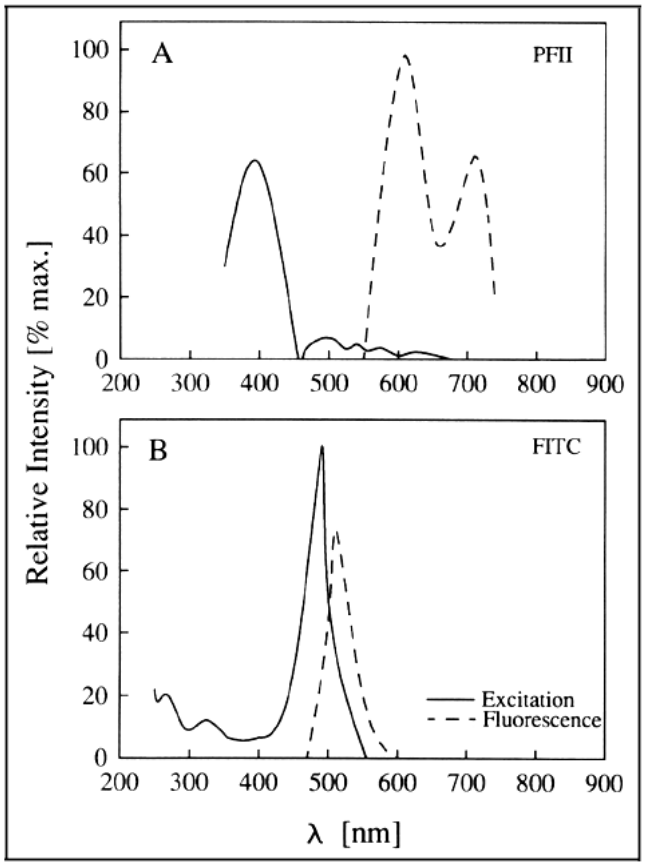

Figure 1. Excitation and fluorescence spectra of Photofrin II (*PF-II*) (*A*) and fluorescein isothiocyanate (*FITC*) (*B*). Selective detection of fluorescence was achieved by filtering light with a BP 405 nm filter (bandwidth 9.2 nm) for PF-II and a BP 450-490 filter for FITC.

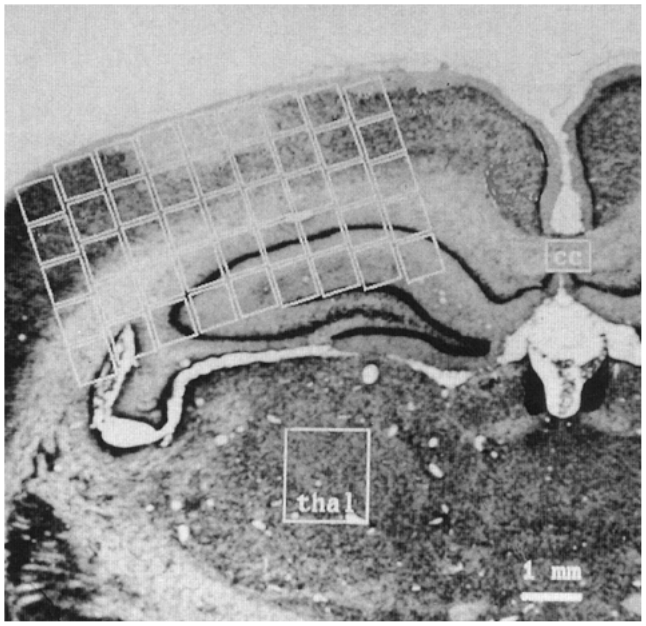

Figure 2. Microscopic image of brain slice stained with cresyl violet with an overlay of *windows* for assessment of fluorescence distribution. Windows were placed in a reproducible manner by centering a vertical column of five windows (0.625×0.625 mm each) on the lesion, aligning with the cortex surface. Four more columns were added on both sides, also aligning with the cortex surface. Windows lateral to the third ventricle measured 1.3×1.5 mm, and in the midline corpus callosum 0.75×0.5 mm. The lesion is discernible by lacking neurons.

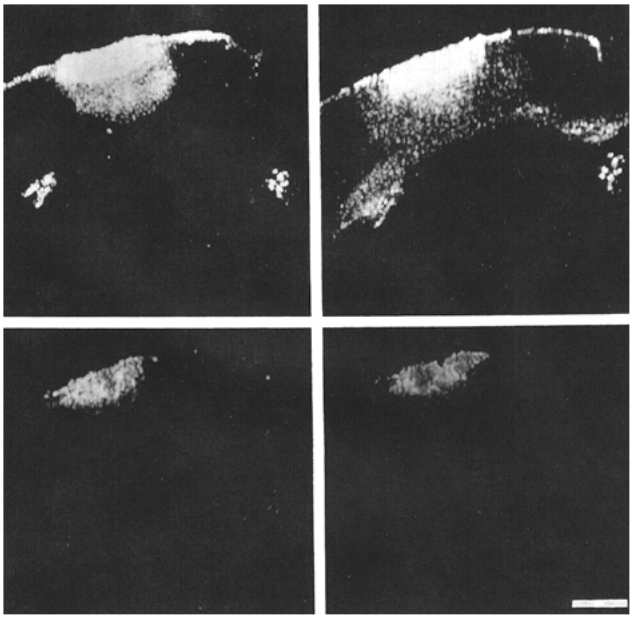

Figure 3. Gray-scale images of Photofrin II fluorescence in brain slices through trauma at 1 hour (*top left*), 4 hours (*top right*), 12 hours (*bottom left*), and 24 hours (*bottom right*) after trauma. Note Photofrin II propagation into perifocal gray matter, advancing into and along the corpus callosum. Note also transient accumulation of PF-II in the choroid plexus of the lateral and third ventricles (*scale bar*, 2 mm).

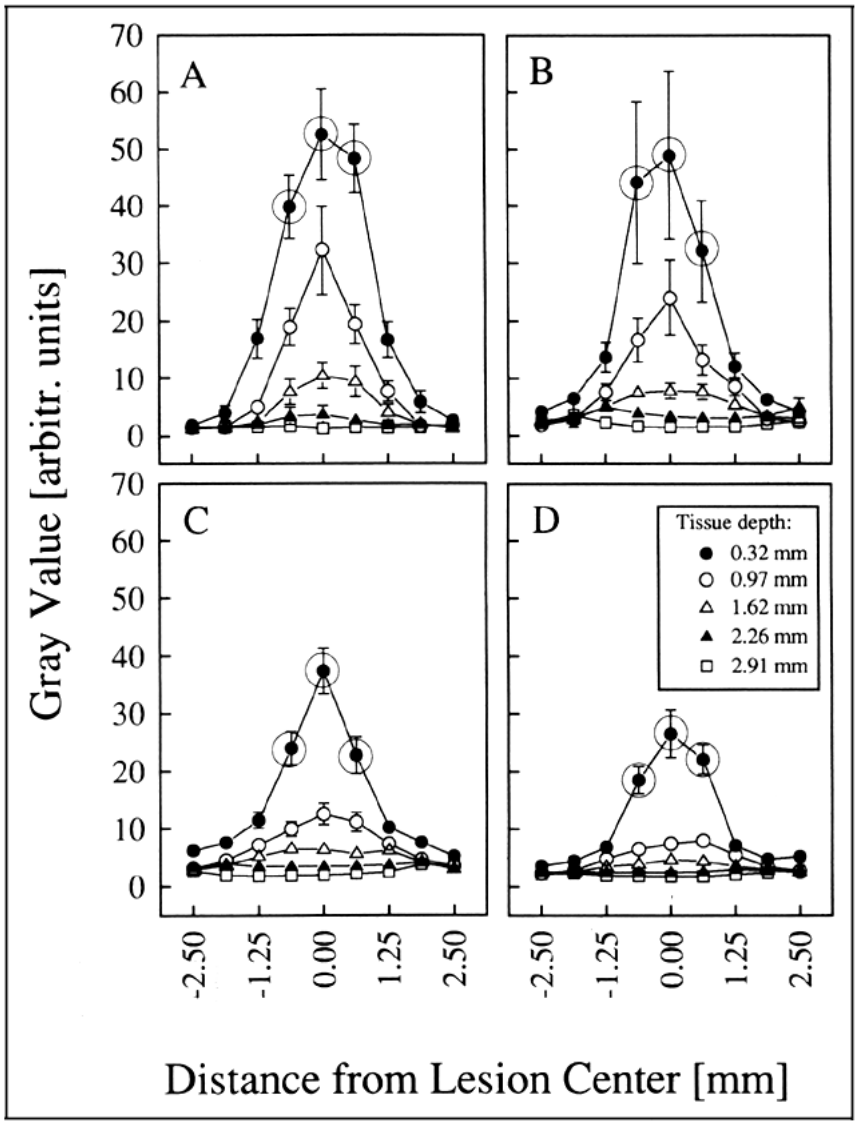

Figure 4. Quantitative distribution of Photofrin II fluorescence after 1 hour (*A*), 4 hours (*B*), 12 hours (*C*), and 24 hours (*D*) after trauma. Each value represents one window of the overlay (see *Fig. 2*). Values obtained at the same depth with respect to the cortex surface are connected by curves. Data from the lesion itself are circled. All data are mean values plus or minus the standard error.

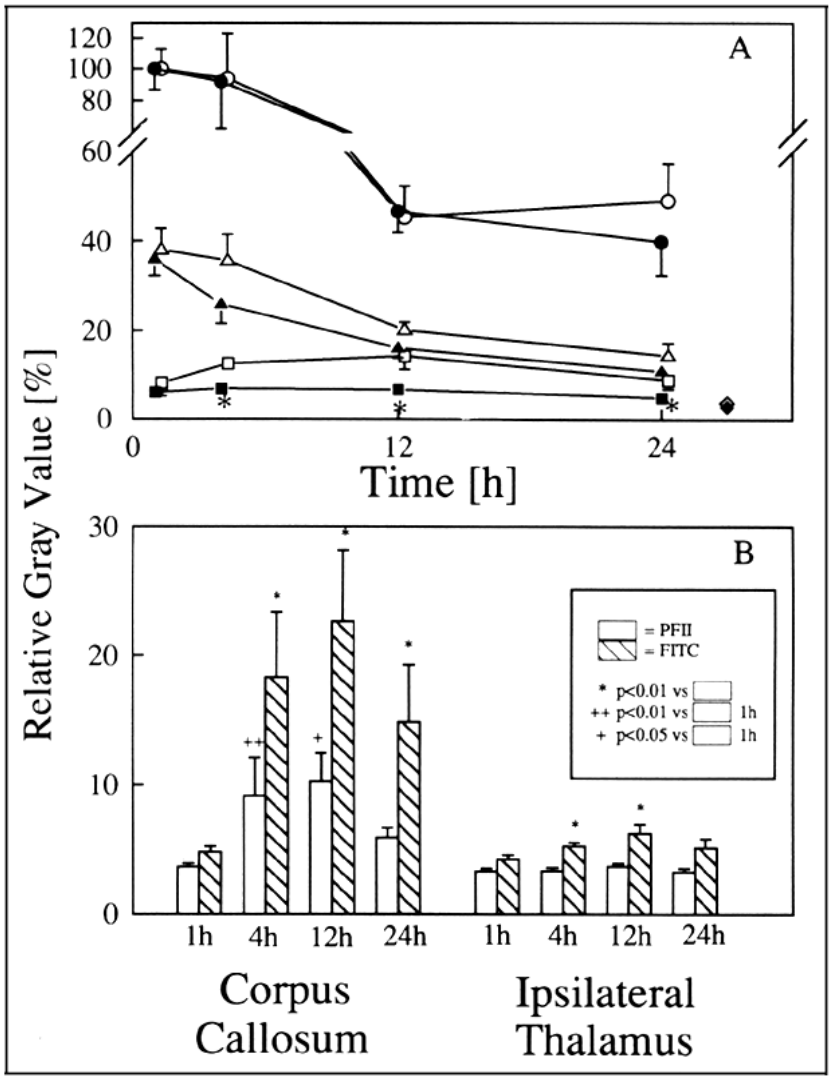

Figure 5. Course of Photofrin II (*PF-II*) and fluorescein isothiocyanate (*FITC*) fluorescence within and at different distances from the lesion. *A*, measurements from the lesion and immediate vicinity: *filled symbols*, PF-II; *open symbols*, FITC; *circles*, measurements directly over the lesion; *triangles*, measurements 0 to 1.3 mm from the lesion center; *squares*, measurements 1.3 to 2.6 mm from the lesion center; *rhomboids*, measurements in animals without lesion; *asterisk*, statistically significant differences as compared with FITC ($P < 0.05$, U-test). *B*, measurements from the corpus callosum (5 mm from the lesion) and ipsilateral thalamus (8 mm from the lesion); note the peak of fluorescence dependent on the distance from the lesion. All data are summarized as mean values plus or minus the standard error.