Fluorescence Studies of Hematoporphyrin Derivative in Normal and Malignant Rat Tissue

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ABSTRACT

Laser-induced fluorescence in rat tissue was studied during the uptake and clearing period of i.v.-injected hematoporphyrin derivative. A malignant rat tumor and normal tissue of 20 different kinds from the tumor-bearing animals were investigated. A pulsed nitrogen laser (337 nm) was used in conjunction with an optical multichannel analyzer system, in which the whole fluorescence light distribution was captured for each laser pulse. Several of the organs exhibited an initial and a delayed intensity peak in the characteristic hematoporphyrin derivative laser-induced fluorescence intensity (630 nm) that might be interpreted as due to intracellular transformations of different chemical components of the hematoporphyrin derivative preparation. By dividing the background-free 630-nm signal by the blue fluorescence intensity, a dimensionless quantity is obtained that could have many advantages in practical endoscopic laser-induced fluorescence work. This ratio was also shown to exhibit a larger contrast between tumor and surrounding tissue. The ratio between the two red fluorescence peaks was also found to be useful for discriminating tumor from normal tissue. A combination of the two ratios was shown to be particularly valuable for tumor discrimination.

INTRODUCTION

HPD is known to localize at a higher level in malignant tumor tissues than in normal tissue (1–7). HPD also possesses the property of emitting a characteristic dual-peaked red fluorescence distribution when excited in the Soret band, peaking at 405 nm. These two properties of HPD form the basis for early tumor detection, which can be particularly valuable in endoscopic investigations. Thus, after previous HPD administration, bronchial (8–18) as well as bladder (19–22) tumors have been shown to exhibit characteristic fluorescence when excited with ultraviolet or violet light.

The utilization of an optimum function of fluorescence spectral intensities for enhancing the contrast between malignant and normal tissue utilizing the full spectral signature of the HPD fluorescence was emphasized in a previous paper (23). This paper presents extensive new LIF results for normal and tumor rat tissue. The investigation was performed on animals that had been given injections 2 h, 4 h, 8 h, 1 day, 3 days, and 4 days prior to the fluorescence measurement. The inclusion of both short and long intervals allowed a spectral study during both the HPD uptake and clearing phases in normal and tumor tissue. Besides 15 different tissues that were briefly studied earlier (23), organs which have a particular interest in endoscopic applications were included, such as the inner walls of the urinary bladder, esophagus, stomach, trachea, and large intestine.

MATERIALS AND METHODS

Animals and Tumors. Inbred female Wistar/Furth (WF) rats weighing approximately 250 g were used. A s.c. tumor on the outside of the right hind leg had been induced by local inoculation with syngeneic tumor cells prepared from a colon adenocarcinoma (DMH-W49) (24). The original colon adenocarcinoma was chemically induced by s.c. injection of 1,2-dimethylhydrazine and was passed in vivo. At the time of animal sacrifice the tumors were 10–20 mm in diameter. At this size some of them showed visible necrotic parts.

HPD Administration. The animals were given injections i.v. (left vena femoralis) at a level of 5 mg/kg of body weight with HPD solution (0.5 mg/ml; Photofrin I solution, Lot 8411 K46; Photofrin Medical, Inc., Cheektowaga, NY), diluted 10 times in physiological saline. The Photofrin solution was kept frozen in darkness until time of use.

Laser-induced Fluorescence. The set-up for laser-induced fluorescence measurements has already been described (23). A nitrogen laser, emitting at 337.1 nm, was used as a convenient excitation source. On the short-wavelength side of the main HPD absorption band, however, this wavelength quite efficiently excites HPD and also provides a smooth blue-colored tissue fluorescence that, as we will see, can also be taken advantage of. The laser had a pulse duration of about 3 ns and a repetition rate of 16 Hz. An interferent filter at 340 nm was used in the beam to eliminate unwanted plasma lines from the laser. The light impinging on the target had been focused to a diameter of about 3 mm and had a peak power of about 8 kW. The target tissue was pressed from below towards a metal ring covered with a thin fluorescence-free quartz window. Fluorescence light from the irradiated tissue was reflected horizontally by a flat first-surface aluminum mirror. The reflected light was then focused with a d = 10 cm, f = 15 cm quartz lens on the 0.2-mm entrance slit of an optical multichannel analyzer system (Tracor Northern IDARSS). An intensified and gated diode-array detector (TN-1223-4IG) was used to capture the entire near-UV-visible-near-IR spectrum at a spectrometer dispersion of 24 nm/mm. The signals from the diodes were read, and the resulting full spectrum could be displayed on a screen. The diode array had a background level which was subtracted in every spectrum. The spectral response of the system could be adjusted to the flat case using an experimentally determined correction function that was stored in the minicomputer of the system. The spectra together with proper identification were transferred to a floppy disc. The spectra could then be read on an X-Y recorder. Manual data reduction was used in the present work.

Fluorescence Measurements. The measurements were performed in 2 different series, one containing 15 animals and the other one 14 animals. In the first series groups of 3 rats were given injections 2 h, 4 h, 8 h, and 1 day prior to the fluorescence measurements. In the other series 3 rats were given injections 1 day before, 6 rats 2 days, and 2 rats 4 days before the measurements. Besides the animals to which HPD had been administered, each series contained 3 control rats that did not receive any HPD. The measurements in the two series were performed without any alteration in the experimental set-up or in the procedure of handling the animals. In order to be able to relate measured fluorescence intensities for different organs on different occasions it is important to use a stable fluorescence standard. A 3-mm-thick layer of Rhodamine 6 G dye in water solution (70 μg/liter) kept in a small nonfluorescent aluminium cup was used as a fluorescence standard. The fluorescence intensities measured in the experiments are all expressed in this standard unit.

Fluorescence spectra of the different organs were recorded by accumulating data for 80 laser pulses. The background signal was also recorded for 80 laser pulses. Recordings from the fluorescence standard were regularly taken. Spectra for heart, lung, abdominal wall, liver, kidney, spleen, stomach, and small and large intestine were recorded in situ with the abdomen of the sacrificed animals cut open. Spectra for the skin of the foot and tail were measured without any prior shaving.
procedure. The surface of the tumor was investigated after removal of
the skin above the tumor using a scalpel. Two different kinds of spectra
from the tumor surface were recorded, one from the inside of the skin
covering the tumor, the other one from the tumor capsule. Finally, the
tumor was cut open, and fluorescence spectra from the inner part of
the tumor mass were taken. Spectra from the muscle fascia of the
healthy hind leg at the same location as the tumor site were also
recorded.

RESULTS

In Fig. 1, a fluorescence spectrum from the tumor capsule of
a rat given an injection 8 h prior to the sacrifice is shown. The
characteristic HPD signature with a dual-peaked distribution
in the red spectral region can be clearly seen. In this figure
some characteristic signal intensities are indicated by $A$, $B$, $C$, and
$D$. $A$ and $C$ are the background-free intensities at 630 and
675 nm, respectively, and $D$ indicates the background level at
600 nm. Finally, $B$ is the intensity of the blue normal tissue
fluorescence peak at about 470 nm. The height of the first HPD
peak ($A$) above a smoothly decreasing background, expressed
in terms of the fluorescence standard unit, has been evaluated
for assessing the HPD presence in the tissue.

We have also measured $A$ in the same way in the spectra for
the rats not given injections in order to evaluate the naturally
occurring red fluorescence. In Figs. 2 and 3 the values of the
signal $A$, with standard deviations, are given as a function of the
time after HPD injection. In Fig. 2 information on the tumor and the surface of different organs is represented. In Fig.
3 corresponding data for the inner walls of six organs which in the
human case would be endoscopically accessible are given. In
each diagram the signal intensity for animals investigated at
various times after injection is plotted together with the signal
intensity (dashed line) not related to HPD obtained from the
control animals. In general, each point represents measure-
ments on 3–6 rats. As can be seen, the background is low for
many of the organs. However, stomach, large intestine, and
most of the inner walls have higher background values. The
skin of the foot and tail has very high backgrounds correspon-
ding to a prominent red peak, as discussed before (23). The
HPD-related intensity exceeding the background intensity dif-
fers widely in the different organs and is normally stronger than
the background value. However, for the skin samples, except
for the skin inside covering the tumor, the background level
dominates strongly, and HPD injection does not make a very
significant contribution. For the other organs quite a clear fall-
off in the background-free HPD fluorescence intensity can be
seen over the studied period of time. For the malignant tissue,
both inside and outside the tumor, a much higher level than for
the surrounding muscle is obtained, and a significant fall-off
occurs not earlier than 72–96 h post-HPD injection. For the
other tissues the significant fall-off starts earlier, and a typical
half-life for the signal is about 48 h.

As has been pointed out by us (23, 25) and others (26), it is, in
practical diagnostic work, advantageous to monitor a dimen-
sionless quantity that is related to the HPD uptake. The sim-
plest such function is a ratio such as $A/B$, where the background-
free HPD signal is divided by the natural blue tissue fluores-
cence intensity. Since the quantity is dimensionless, it is not
sensitive to spatial or temporal variations in the illumination.
For the same reason, the signal is influenced neither by the
surface topography nor by the distance from the sample to the
measurement equipment, which would be very valuable in en-
doscopic work. Thus, it is also interesting to consider the data
for $A/B$ for the different tissues investigated. In Figs. 4 and 5
the $A/B$ values are given, corresponding to the ones presented
for $A$ in Figs. 2 and 3. Again, each point is presented with its
standard deviation. The value for the reference animals not
given injections is also given. As already noted in our previous
papers (23, 25), contrast between tumor and surrounding muscle
is seen to increase in an $A/B$ representation (Fig. 4, left).
This is due to a decrease in the level of blue fluorescence for
tumor compared to muscle. Advantage was taken of this con-
trast enhancement, particularly in our imaging LIF measure-
ments on tumors on a muscle background (25). The situation
is illustrated in Fig. 6, in which the results of point measure-
ments of LIF levels in eight positions along a line starting in
muscle and extending into a tumor are given. For each of the
points a small LIF spectrum is displayed, and the evaluated
signal levels are plotted. The importance of measuring the
signal at 630 nm background free is illustrated. The blue
fluorescence intensity strongly decreases in the tumor. Because
of its high value in the muscle region the total 630-nm signal,
$A'$, including background, is as high as in muscle as in tumor
tissue. In the $A/B$ ratio a strongly enhanced contrast is obtained.

In a comparison of the $A/B$ and $A$ levels for tumor and other
tissues we note from Figs. 2 to 5 that tumor gains in significan-
cence over stomach, intestine, skin tissues, and several of the inner
walls in an $A/B$ representation, whereas it decreases in signifi-
cance for lung, liver, kidney, and spleen. It would be very
advantageous to be able to discriminate tumor tissue over all
other tissues based on its spectral characteristics. As we can
see from Figs. 2 to 5 this is not generally possible in the $A$
and $A/B$ representation.

A property that has not been utilized so far is the internal
structure of the red light distribution in the 610- to 700-nm
region. One way to characterize this structure is to form the
dimensionless ratio $C/A$. In Fig. 7 the $C/A$ data for organs with
a high $A$ or $A/B$ value are given. In order to increase the
statistical significance the values have been grouped into three
time intervals. We note that the highest value of the $C/A$ ratio
is obtained for tumor. We also observe that the value decreases
with time, which indicates a chemical transformation. In our
previous work the signal-to-noise ratio did not allow this trend
to be observed. At a time delay of 3 days the discrimination
between tumor tissue and other tissues is optimum for the $C/A$
function.

DISCUSSION

In this paper extensive results on the temporal dependence
of the red LIF at 630 nm from tissue, following HPD admi-

![Fig. 1. LIF spectrum from the tumor capsule of a rat that had been given an injection of HPD (5 mg/kg) 8 h earlier. The fluorescence intensities $A$, $B$, $C$, and $D$ that were evaluated and utilized in this study are indicated. This curve has not been spectrally corrected.](attachment:fig1.png)
The fluorescence properties of HPD (Hematoporphyrin Derivative) were studied in malignant tumors and various normal tissues of rats. The fluorescence signal was measured at different times after HPD injection, and the data were normalized to correct for different detector sensitivities. The fluorescence signal was found to vary significantly among different organ types and over time. For instance, the signal was close to a maximum 2 hours after injection for most organs, with a reduction to half of the maximum value typically following after 2 days. Exceptions to this behavior were observed for the abdominal and bladder walls, which showed a complete disappearance of the signal within less than 24 hours. For the malignant tumor tissue, substantial signal fall-off began only 3 days postinjection, related to the HPD retention phenomenon. Similar observations were made for normal tissues, with an initial time behavior showing a signal minimum 4-8 hours postinjection, followed by a fast increase to or beyond the early value. This behavior is attributed to the initial distribution of HPD within the tissue and the subsequent intracellular transformation of its components. The HPD fluoroscence intensity reflects the sum of the intensity from different components and is affected by the nature of the drug, its monomers, dimers, oligomers, and large aggregates. The temporal changes of the measured fluorescence intensities are important for the assessment of tissue HPD concentrations, which is crucial for photodynamic therapy.
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Fig. 4. Data for the A/B ratio shown for the organs considered in Fig. 2 (points). Bars, SD. The value of the ratio for rats not given injections is indicated by a dashed line. (Data are not spectrally corrected since evaluation of Level B was performed at the blue signal maximum, the wavelength of which varies between 452 and 477 nm for different organs. After spectral correction the local blue maximum frequently disappears. However, the spectral correction to the A/B ratios is less than 10% in all cases except for stomach, for which the corrected value is 16% lower.

As already discussed, there are many practical advantages in monitoring a dimensionless quantity related to the presence of HPD. We have presented data for the A/B ratio, in which the background-free HPD peak has been divided by the tissue blue fluorescence. Thus, the shape of the spectrum is measured rather than a specific signal intensity. In a multicolor LIF imaging system as described in Ref. 25 (one-dimensional imaging) and Ref. 34 (2-dimensional imaging), this allows the use of threshold values that are independent of parameters such as illumination nonuniformities, surface topography, and object distance, parameters that are frequently uncontrollable in clinical praxis. Influences of wavelength-independent, common attenuations of the light are then also reduced.

For the studied tumor type on a muscle background it was found that the blue fluorescence decreases in the tumor while the red intensity increases. Thus, contrast enhancement is also gained in A/B monitoring. It would be very interesting to investigate to what extent this property also pertains to other tumor types in different organs. By careful comparison between the A and A/B data, we note that the statistical fluctuations are larger in the latter representation. This means that the level of blue fluorescence varies more between different animals than the HPD uptake. However, in practical tumor screening a particular individual is considered, and the observed fluctuation may not be very detrimental.

As we have seen, a high A/B ratio alone is not sufficient as a
The optimum choice of the discriminator levels $c_1$ and $c_2$ must be relative intensities of the two red peaks (C/A) are a further consideration for the tissues presently investigated by requiring $A/B > c_1$ and $C/A > c_2$, where $c_1$ and $c_2$ are constants. The use of such criteria can be illustrated in a figure such as the one shown in Fig. 8. The threshold values $c_1$ and $c_2$ should be optimized according to the signal-to-noise ratio available. The values of the constants $c_1$ and $c_2$ are of the order of 0.1 and 0.5, respectively. By successively increasing the values it can be ascertained that tissues still fulfilling the double criterion really correspond to tumors. On the other hand, certain tumors might be overlooked. By lowering the values of $c_1$ and $c_2$ sufficiently it is ascertained that all tumors are detected. However, an increasing number of normal tissue samples will tend to be included with the true tumors. Depending on the particular application, the optimum choice of the discriminator levels $c_1$ and $c_2$ must be made. Clearly, our conclusions should not be extrapolated too far beyond the scope of the actually performed investigation on rats, but the results suggest that a combination of spectroscopic accept/reject criteria based on dimensionless quantities could also prove valuable for clinical applications. In a computerized multicolor LIF 2-dimensional imaging system, adapted, e.g., to a bronchoscope or a cystoscope and displaying only areas fulfilling an acceptance criterion, the areas "surviving" interactive increments of both the $c_1$ and $c_2$ thresholds longest might be the most suitable places for obtaining biopsy specimens.

While difficult to use for quantitative HPD assessment, the LIF technique has the great practical advantage over chemical extraction methods or radiolabeling techniques of being non-intrusive and free from extra biological strain. It provides possibilities for real-time discrimination of tumor from normal tissue. Fluorescence monitoring instrumentation has been constructed (9, 11, 17, 22, 26), and encouraging results have been obtained in clinical work (12, 13, 15-18, 21, 23, 24). It is likely that the techniques can be further refined. In particular, by using optimization processing of all the spectroscopic information as attempted in this paper, it might be possible to detect smaller tumors and/or substantially reduce the HPD dose needed for diagnostic investigations. With low systemic HPD levels the skin photosensitization problem, presently calling for about 1 month of reduced ambient light exposure to patients, should be considerably reduced, enabling a more widespread use of HPD LIF techniques for early tumor detection.

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