AUTOFLUORESCENCE OF TRANSPLANTABLE HEPATOMA A22 (MH–A22): PROSPECTS OF TUMOR TISSUE OPTICAL BIOPSY

Mindaugas Tamosiunas1,*, Julija Makaryceva1, Jurate Labanauskiene2, Saulius Bagdonas1, Ceslava Aleksandraviciene2, Janina Didziapetriene2, Laima Griciute2, Ricardas Rotomskis1,2,*

1Vilnius University, Laser Research Center, Vilnius 10222, Lithuania
2Institute of Oncology, Vilnius University, Vilnius LT-08660, Lithuania

Aim: Autofluorescence of experimental tumor (hepatoma A22 (MH–A22)) was employed to discriminate the optical differences between necrotic and non-necrotic tumor, hemorrhagic tumor and healthy tissue. Methods: The experiment was performed ex vivo using the transplantable tumor from the right haunch of hybrid mice (C57Bl/CBA). Blue LED light (λem. = 405 nm) was applied for autofluorescence excitation and fibre optics based spectrophotometer was used for spectra detection. Results: We observed that necrotic tumor tissue is characterized by the absence of endogenous porphyrins fluorescence, and registered spectra do not possess differences in the red spectral region (600–710 nm) in comparison with normalized autofluorescence spectra of muscle. Moreover, only certain segments of non-necrotic tumor bear the fluorescence of endogenous porphyrins. Conclusions: Based on the experimental results, we suggest that the absence of long-waved fluorescence differences between necrotic tumor tissue and healthy tissue, e.g. muscle can impede the demarcation between healthy and tumor tissue. The uneven distribution of endogenous porphyrins in non-necrotic tumor tissue as well as the absence of endogenous porphyrins fluorescence in the small experimental tumors complicates the localization of cancerous tissue based on the autofluorescence registration.

Key Words: autofluorescence, optical biopsy, tumor diagnostics, necrosis, fluorescence imaging, proliferating cell nuclear antigen.

When the biological tissue is exposed to selected UV or short–wavelength visible light, the fluorescence appears from native chromophores present in the tissue. As reviewed by Richards–Kortum and Sevc–Muraca [1], the major molecules, which contribute to the autofluorescence signal in biological tissue, have been identified as tryptophan (emission at 350 nm), tyrosine (300 nm), phenylalanine (280 nm), chromophores in elastin and collagen (400 nm), pyridoxine metabolites (420 nm), flavins (520–540 nm), nicotinamide adenine dinucleotide (440–450 nm), lipo–pigments (430–460 nm, 540–640 nm) and porphyrins (600–700 nm).

Several studies have been performed to define the potential of autofluorescence for cancer diagnosis. Alfano et al [2] was the first to observe different spectral profiles of endogenous fluorophores in normal tissues of rat (kidney, prostate) and mouse (bladder) and cancerous tissues. Clinical results, obtained by Yang et al [3] indicated that the fluorescence of endogenous porphyrins could be used for the detection of tumor. The consistency of 89% between autofluorescence and traditional biopsy data was obtained in preliminary diagnosis of buccal carcinoma [3].

The origin of endogenous porphyrins appearance in cancerous tissue has been discussed in literature. A. Policard first reported the red fluorescence of necrotic tumors in 1924 (see review [4]). F. Ronchese [5] proposed that ulceration is essential for the production of the red fluorescence in human cutaneous squamous cell carcinoma. Ghadially et al [6] found bright red fluorescence limited to the exterior of ulcerated tumors, and attributed it to endogenous porphyrins. Several possible hypotheses on the protoporphyrin appearance in tumor tissue have been suggested: it is a product derived from microbes; it was produced by the tumor tissue or was taken and accumulated in tumor from the blood stream [7]. The fluorescence of endogenous porphyrins in tumors of oral cavity has been attributed to the presence of several different species of bacteria inhabiting ulcerated and necrotic tissues [8]. Many bacterial organisms that inhabit necrotic tissue are able to produce red fluorescing porphyrins under the presence of exogenous 5–aminolevulinic acid (5–ALA) [9]. Betz et al [10] states that red fluorescence of endogenous porphyrins localizes on the bacterial coating of the necrotic parts of some oral neoplasms after 5–ALA administration.

Supposedly, the enhanced fluorescence of endogenous porphyrins in cancerous tissue (mostly in tumors of non oral cavity) is the consequence of the tumor–specific metabolic alterations [11, 12], results from the porphyrin retention in tumors [13] which can be caused by tumor hypervascularity [14]. The viable part of the brain tumor (astrocytoma) localized in white matter possesses fluorescence peaks assumed to originate from endogenous porphyrins [15]. However, typi–
Fluorescence spectroscopy is a promising technique for the real time and non-invasive diagnosis of tumors and could fill the gap between the primary examination of tumor and the invasive biopsy. Point spectroscopy, a technique also known as optical biopsy, involves registration of the fluorescence spectrum arising from a single point in tissue. The aim of this study was to examine the autofluorescence spectra of experimental tumor (hepatoma A22 (MH-A22)) and to relate the spectral properties of particular tumor areas to the state of the tissue of malignant tumor. Autofluorescence imaging and the analysis of spectral data collected from cancerous and normal tissues ex vivo provide information on the fluorescence properties and distribution of endogenous chromophores in non–homogenous tissues.

The fluorescence imaging of tumor tissue was performed using image analysis system (Hamamatsu) equipped with a microscope (Zoom Master, Prior, England), CCD camera (C5405, Hamamatsu, Japan), a RGB monitor (PVM–145Q, Sony, Japan) and image processor (Argus 20, Hamamatsu, Japan) connected to a personal computer (Fig. 2). A specimen of malignant tissue was placed in a microscope focus plane and could fill the gap between the primary examination of tumor and the invasive biopsy. Point spectroscopy, a technique also known as optical biopsy, involves measurement of the fluorescence spectrum arising from a single point in tissue. The aim of this study was to examine the autofluorescence spectra of experimental tumor (hepatoma A22 (MH-A22)) and to relate the spectral properties of particular tumor areas to the state of the tissue of malignant tumor. Autofluorescence imaging and the analysis of spectral data collected from cancerous and normal tissues ex vivo provide information on the fluorescence properties and distribution of endogenous chromophores in non–homogenous tissues.
mal Facility of Immunology Institute of Lithuanian Academy of Sciences. Hepatoma A22 (MH–A22) was maintained and passaged in Oncology Institute, Vilnius University. Approximately $1 \cdot 10^5$ cells were inoculated subcutaneously to the right haunch of the mice. Tumor growth was documented regularly by measurements with Vernier calliper in three orthogonal dimensions. Experiment started 7 days after inoculation of malignant cells, when the tumor volume reached 0.07 cm$^3$. Further measurements (6 mice) were performed 9 days, 12 days, 14 days, 16 days and 19 days after inoculation of malignant cells (up to 1 cm$^3$ of tumor volume). Control specimens of healthy tissue (muscle) were prepared from the same mice. The muscle samples from 2 healthy mice were used for control autofluorescence measurements.

Mice have been sacrificed under the guidelines of Good Laboratory Practice. Tumors were excised from mice and were divided into 2–5 slices (about 3 mm thickness), depending on a size of tumor. Specimens of tumor tissue were placed between two glasses. The delineation of non-necrotic, necrotic and hemorrhagic areas of tumor was performed by microscopy studies on tumor tissue specimens. Point measurements of tumor and muscle autofluorescence as well as imaging of the examined specimens were performed. All specimens were kept on ice during the experiment (up to 4 h after tissue excision).

The expression of the cell proliferation associated marker, proliferating cell nuclear antigen (PCNA), was evaluated by immunohistochemical analysis of the specimens of tumor tissue. The samples of tumor tissue were fixed in 4% neutral formalin for three days, then frozen to $–20^\circ$C and cut with freezing microtome into slices of 4–5 µm width. The immunohistochemical reaction was performed by the method of streptavidin–biotin peroxidation, using the monoclonal antibodies (Mo AK PC10, DAKO AS, Denmark) to reveal the proliferating cell nuclear antigen.

The experiments on animals and animal husbandry were carried out in compliance with national and European regulations and were approved by the Lithuanian Animal Care and Use Committee.

RESULTS

Autofluorescence spectra ex vivo were recorded on 22 specimens of tumor tissue and 6 specimens of healthy tissue. The evaluation of tumor morphological structure indicated that the areas of non–necrotic and necrotic tumor are present in specimens of large tumors. Tumor necrosis was not detected in the specimens of the small tumors. Hemorrhagic sites occurred both in tumors and in muscle specimens as a result of tissue resection. We have analysed 90 autofluorescence spectra of non–necrotic areas in tumors, 60 spectra of necrotic tumor areas, 33 spectra of hemorrhagic areas and 25 of healthy tissue (muscle) from tumor–bearing mice. The mean fluorescence spectra of malignant and normal tissues ex vivo are presented in Fig. 3. All spectra of cancerous and healthy tissues exhibit broad and poorly structured emission bands in 490–800 nm spectral region under excitation at 405 nm. However, significant spectral modifications were detected depending on the state of tissue.

Autofluorescence spectra of healthy tissue differ from tumor tissue both in spectral shape and intensity. The mean autofluorescence of healthy tissue (muscle) is characterized by the major fluorescence band centered at 560 nm (curve 4, Fig. 3). Autofluorescence of the muscle is more intense comparing to that of tumor tissue; however some muscle spectra exhibit reduced autofluorescence intensity in 490–800 nm spectral region. Spectra of areas of non–necrotic and necrotic tumor tissue (curves 1–3, Fig. 3) are characterised by several bands in 490–710 nm spectral region and lower autofluorescence intensity overall comparing with healthy muscle tissue.

In order to compensate for variation in fluorescence intensity and to distinguish spectral differences, the mean autofluorescence spectra have been normalized to the same intensity at 725 nm (Fig. 4, 5). No additional differences in spectral shape were revealed after normalization of these data to the total integrated area under the spectral curve (data not shown).
The spectra in Fig. 3 and Fig. 4 (curves 1, 2) indicate that two different spectral types were detected in specimens of non-necrotic tumor. Significant differences in autofluorescence shape occur in 610–710 nm spectral region. A narrow emission band near 633 nm and a broad emission band around 700 nm (spectrum 2 in Fig. 3, 4) are characteristic for limited areas of non-necrotic tumors being measured starting from the 14th day after the inoculation of tumor cells (large tumors). The second type of autofluorescence spectra of non-necrotic tumors (spectrum 1 in Fig. 3, 4) lacked those bands and had similar shape with spectra from necrotic tumor and healthy tissue in 610–710 nm spectral region. Such spectra were detected both in the areas of large non-necrotic tumors and in all tumors being measured up to the 14th day after the inoculation of tumor cells (small tumors).

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Distinct variations in shape of spectra from non–necrotic, necrotic and hemorrhagic areas of tumor and also healthy tissue (muscle) occur in 490–600 nm spectral region (Fig. 4). Both types of the mean spectra of non–necrotic tumor exhibit lower relative fluorescence at 560 nm in comparison with muscle fluorescence. The shape of mean autofluorescence spectrum of necrotic tumor tissue (spectrum 3 in Fig. 4, spectrum 1 in Fig. 5) significantly differs from those measured in non–necrotic tumor and muscle in 490–600 nm spectral region. It is characterized by reduced fluorescence intensity in this spectral region with dips around 536 and 577 nm.

Autofluorescence spectra in hemorrhagic areas of tumor tissue, similarly to those of necrotic tumor tissue, are characterized by dips in 500–600 nm spectral region (Fig. 5). The blood absorbance spectrum is presented (Fig. 5, curve 3) for comparative purposes. The absorbance peaks at 536 and 577 nm belong to hemoglobin.

Additional examination of small and large tumors ex vivo was performed using image analysis system equipped with Argus 20 (Hamamatsu) image processor. The morphology of the tumor tissue was preliminary studied under the white light microscope. Microscopy analysis of the large tumor specimens under white light illumination served for the visual delineation of the areas of non–necrotic and necrotic tumor as well as hemorrhagic areas of tumor (Fig. 6, a). The white–light images were compared with autofluorescence images obtained under UV light illumination (Fig. 6, b). Red autofluorescence was detected only in the areas of non–necrotic tumor when a long band pass filter (T<sub>620</sub> = 80%) was placed before CCD camera. However, some areas of non–necrotic tumor exhibited no red autofluorescence and by this were undistinguishable from necrotic and hemorrhagic areas of examined tumors. Typical autofluorescence patterns obtained after the imaging of the specimens of small tumors exhibited no red autofluorescence at all (data not shown).

The histological analysis of tumor tissue evidenced that the areas of proliferating and necrotic cells naturally occur in the large tumors (Fig. 7). The majority of proliferating tumor cells were distinguished as having an intensive expression of proliferating cell nuclear antigen (PCNA). This expression (up to 98% of positive PCNA–cells on the average) can be visualized by the product of the chromogen immunohistochemical reaction (oxidized DAB precipitate) appearing in the cells, which varies in colour intensity (from light brown to dark brown). PCNA negative cells were coloured by hematoxyllyn (light grey/ blue colour), the cytoplasm was stained with 0.1% solution of eosin (from light rose till violet rose colour). The cells of proliferating tumor are enlarged, there are only very small spots of necrosis. The necrotic sites are located near the zones of intense cell proliferation, and
areas of tumor (Fig. 5, spectrum 1). Diminished autofluorescence of necrotic sites in tumor exhibit the same dips around 542 and 577 nm. This can be explained by the presence of a large amount of blood clots in necrotic tissue. The relative decrease of fluorescence intensity in the middle-waved spectral region theoretically could be employed for the identification of necrotic tumor, although it could be difficult to distinguish between the necrotic tumor tissue and the hemorrhagic (cancerous or healthy) tissue. In such a case, the middle-waved autofluorescence could not provide useful information while performing the spectroscopic examination of tissues in a resection area. In fact, that complicates some aspects of autofluorescence application in diagnostics of malignant tissues especially when it is important to ensure that no malignant tissue is left in contact with healthy tissue after tumor surgery.

The enhanced amounts of endogenous porphyrins naturally occur in some tumors localized e.g. in colon and oral cavity. We state that the optical biopsy of necrotic tumor tissue highlight the absence of the fluorescence bands of endogenous porphyrins (Fig. 3, 4). Our results are in good agreement with results obtained by Anderson–Engels et al [15] showing that autofluorescence of endogenous porphyrins was not detected in necrotic areas of brain tumor. We would like to point out that normalized autofluorescence spectra of necrotic tumor are identical to the normalized spectra of healthy tissue (muscle) in 600–710 nm spectral region. The spectroscopic identification or imaging of the tumor tissue considering only the long-waved autofluorescence becomes inaccurate, as the necrotic places of tumor tissue are spectroscopically attributed to non-cancerous tissue.

The uneven distribution of endogenous porphyrins and significant variations of their fluorescence intensity characterize the autofluorescence of non–necrotic tissue of large tumors (Fig. 4). Our results are in agreement with data reported in [10, 21, 23] that only limited areas of some sections of tumors exhibit fluorescence bands of endogenous porphyrins. Two different types of autofluorescence spectra occur in non–necrotic areas of large tumors (Fig. 4). Type I autofluorescence spectra are characterized by the presence of endogenous porphyrin fluorescence. The fluorescence spectrum with peaks at 633 and 700 nm is interpreted as belonging to endogenous protoporphyrin IX [12]. Type I spectra were obtained in the areas of non–necrotic, non–hemorrhagic tissue of the large tumors, starting from 14th day after the inoculation of tumor cells. Type I spectra were not detected in small tumors. Type II autofluorescence spectra (containing no endogenous PpIX fluorescence bands) were detected in small tumors and in some non–necrotic areas of large tumors. These data imply that the concentration of endogenous porphyrins in tumor tissue (hepatoma A22(MH–A22)) depends on tumor growth rate. When the tumors are small, no endogenous PpIX fluorescence occurs; it can be detected only in the areas of large non–necrotic tumors. The presence of endogenous porphyrins in non–necrotic areas
of the large tumors can possibly be related to the altered metabolism of the cancerous tissue. However, the relationship between the appearance of endogenous porphyrins near the necrotic areas and presence of some porphyrin-producing bacteria in large hepatoma A22 (MH–A22) tumors could not be excluded. Our data state that the measurements of endogenous porphyrin autofluorescence alone are not useful for the detection of hepatoma A22 (MH–A22) tumor, especially at the early growth stage.

Obtained data on fluorescence imaging of tumors are in good agreement with the spectral data. PpIX fluorescence rapidly bleaches within the tissue [15, 26] so the experimental conditions, which minimize the effect of illumination on tumor specimen, were selected. The fluorescence images of specimens of tumor tissue recorded starting from 620 nm revealed an uneven distribution of low intensity autofluorescence, attributed to protoporphyrin IX. To our knowledge, the direct relationship between the enhanced long–waved autofluorescence and microscopic morphology of tissue or cellular structures is not observed [21]. The area of enhanced long–waved autofluorescence does not completely correspond to the area of non–necrotic tumor tissue (Fig. 6, b), as some non–necrotic areas of large tumors as well as small tumors (data not shown) were characterized by the absence of endogenous protoporphyrin IX fluorescence. We note that the demarcation line based only on the measurements of endogenous porphyrin autofluorescence intensity can provide misleading data for diagnosis of cancerous tissue: the hemorrhagic and necrotic areas of tumor and some areas of non–necrotic tumor tissues could not be distinguished from a non–malignant tissue by processing the imaging of tumor in the red spectral region.

In conclusion, the present study demonstrates that the shape variations in tumor autofluorescence spectra in 500–710 nm cannot be always related with the state of the tumor tissue.

Our data show that some areas of tumor tissue accumulate higher amounts of endogenous porphyrins, which could be clearly distinguished by red fluorescence, both spectroscopically and by visualization. However, the autofluorescence of endogenous porphyrins alone could not delineate the margins of the tumor. The tumor area possessing enhanced red autofluorescence was found to be smaller than the total area of non–necrotic tissue in tumor specimens. Using 600–710 nm spectral region analysis it was not possible to distinguish early staged hepatoma A22 (MH–A22) tumors from the healthy tissues. Autofluorescence of healthy tissue (muscle) was found to be slightly higher than that of tumor in 550–600 nm spectral region.

Endogenous porphyrins were not detected in necrotic and hemorrhagic areas of the large tumors. Those areas can be distinguished spectroscopically by the decreased green–yellow autofluorescence in 500–600 nm spectral range. However, the similar spectral pattern may characterize the hemorrhagic tissue located near the surgical borders of a tumor. Thus, the better spectroscopic discrimination between cancerous and healthy tissues can probably be achieved combining autofluorescence data obtained in several spectral regions, particularly, extending the range of fluorescence detection towards the blue spectral region.

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REFERENCES

АУТОФЛУОРЕСЦЕНЦИЯ ПЕРЕВИВНОЙ ГЕПАТОМЫ А22 (МН–А22): ПЕРСПЕКТИВЫ ОПТИЧЕСКОЙ БИОПСИИ ОПУХОЛЕВОЙ ТКАНИ

Цель: аутофлуоресценцию трансплантированной опухоли (гепатома 22 (МН–22)) применили для выявления оптических особенностей опухоли с участками некроза и без таковых, опухоли с участками кровоизлияний и здоровой мышечной ткани. Методы: в экспериментах in vivo были использованы ткани опухолей, принятых в правое бедро мышей (С57BI/CBA). Для возбуждения аутофлуоресценции применяли полупроводниковый диод (λ = 405 нм). Спектрометр, оснащенный волоконно-оптическими светодиодами, использовали для регистрации спектров аутофлуоресценции исследуемых тканей. Результаты: в спектрах некротизированных участков опухоли отсутствуют полосы флуоресценции, характерные для эндогенных порфиринов; в красной области спектра (600–710 нм) не выявлены различия между нормированными спектрами некротизированных участков опухоли и здоровой ткани. Флуоресценция эндогенных порфиринов отсутствовала также в опухолях малого размера и наблюдалась только в некоторых сегментах опухолей большего размера без признаков некроза. Выводы: на основе экспериментальных данных мы полагаем, что отсутствие различий в аутофлуоресценции в красной области спектра между некротизированными участниками опухоли и здоровой мышечной тканью может обусловливать неточную деградацию границ опухолевой ткани при проведении оптической биопсии. Неравномерное содержание эндогенных порфиринов в участках опухоли без признаков некроза, а также отсутствие флуоресценции эндогенных порфиринов в опухолях малого размера свидетельствуют о том, что для установления границ опухоли недостаточно руководствоваться одной лишь флуоресценцией эндогенных порфиринов.

Ключевые слова: аутофлуоресценция, оптическая биопсия, онкодиагностика, некроз, флуоресценция, PCNA.