Chronic Hypoxia as a Factor of Enhanced Autofluorescence of Endogenous Porphyrins in Soft Biological Tissues

K.S. Litvinova, D.A. Rogatkin*, O.A. Bychenkov, V.I. Shumskiy

Moscow Regional Research and Clinical Institute "MONIKI" named after M.F. Vladimirskiy, Shepkina str. 61/2, Moscow, 129110, RF.

ABSTRACT

This report, as a continuation of 8-years research on the problem of noninvasive clinical fluorescence diagnostics efficiency, discusses a hypothesis of influence of a chronic hypoxia state in soft alive tissues on the intensity of a laser-induced endogenous porphyrins' autofluorescence in a red region of optical spectra. Earlier this hypothesis was proposed on the basis of analysis of fluorescence activity for erosive-ulcerative impairments of the upper part of a gastrointestinal tract (*SPIE Proc., vol. 4613, 2002. - p.286-294*). Today the hypothesis additionally is confirmed by means of observation after patients with another illness and by means of analysis of some well-known literature data. An authors' methodology of clinical trails to verify the hypothesis using an up-to-date noninvasive fluorescence diagnostic technique is presented as well. Both theoretical reasons and all new clinical data show that the chronic hypoxia state can be one of the major factors of appearance of a large and abnormal laser-induced autofluorescent signal from biotissues in the spectrum range 600-800 nm, which is associated with abnormally high accumulation of endogenous porphyrins in the tissues. So, the noninvasive autofluorescent diagnostic technique could be a powerful tool to estimate *in vivo* a chronic hypoxia condition in soft biotissues. For that purpose a classification of chronic hypoxia levels versus *in vivo* autofluorescence contrast coefficients in tissues is proposed as well.

Keywords: Fluorescence Spectroscopy, Tissue, Laser, Porphyrin, Chronic Hypoxia.

1. INTRODUCTION

The laser noninvasive fluorescent diagnostics is now widely studied and applied *in vivo* in different areas of medicine such as an oncology, dermatology, etc. [1]. The most interesting application of that is the noninvasive optical detection of diseases, especially cancer, with the use of a laser induced fluorescence of endogenous biological fluorophores [2, 3], what frequently is called an *autofluorescent diagnostics* (AFD) [4]. One of the typical endogenous fluorophores as well as one of the easily detected fluorophores *in vivo* is a porphyrin and its derivations (protoporphyrin IX, for example [1]). Porphyrin has a well-known, strong and specific double-peak of a "red" *autofluorescence* (AF) with emission wavelength maxima at 630 and 690 nm [5], what made it possible to detect a porphyrin's fluorescence of necrotic tumors even in 1924 [10, 11]. After that, it was estimated that not only a number of cancerous tissues have an enhanced and specific "red" AF, but also different tissues with different inflammatory and purulent processes have frequently an enhanced "red" AF as well [5]. During 1996-2006 a lot of research groups all over the World using the modern laser noninvasive spectroscopic equipment have demonstrated a potential of *in vivo* noninvasive AFD to detect different diseases, precancerous and cancerous changes in tissues [1-8]. However, up to now in many clinical cases the biological and/or biophysical reasons to differ between normal and abnormal (diseased) tissues by means of analysis of their AF spectra, especially of porphyrin's AF spectra, are not well understood yet [1]. It is frequently supposed that an abnormal (enhanced) accumulation of porphyrins in tissues can be a consequence of enhanced proliferation of cells and of enhanced production of porphyrins in cells, especially in cancerous tissues [5, 12, 13], consequence of a microbes' metabolism [5, 9], of a decreasing of a hydrogen parameter pH in tissues [2, 3, 15], or of enhanced absorption by diseased tissues the porphyrins from a local bloodstream [1, 12, 13].

Recently on the basis of analysis of AF activity for erosive-ulcerative impairments of the upper part of a gastrointestinal tract it was proposed that the frequently observed *in vivo* enhanced AF of endogenous porphyrins in diseased tissues is a simple consequence of a chronic hypoxia condition (state) in the tissues [16]. In the present short our analytical and clinical study the hypothesis of a chronic hypoxia is additionally confirmed by means of observation after patients with another illness and by means of the analysis of main well-known literature data. The correct understanding

* Dr. Dmitrii A. Rogatkin: rogatkin@monikiweb.ru ; phone/fax +7-495-681-89-84 ; http://page-nii-r2.narod.ru

of mechanisms of enhanced porphyrin's AF in tissues is important, in that number, for the problem of medical interpretation of AF diagnostic data, especially in a case of development of a system software for a real laser noninvasive AFD equipment, what demands a clear understanding what a result of the AFD must be indicated on a computer monitor's screen as a medical (third level) result of diagnostics [17]. From the other hand, in the light of this problem, the medical accuracy and reliability of AFD data are very important as well. So, this paper discuses a medical interpretation as well as accuracy and reliability of *in vivo* laser AFD results on the basis of the chronic hypoxia hypothesis.

2. MATERIALS AND METHODS

We have reanalyzed our previous results on clinical AFD when we used *in vivo* registration of porphyrin's spectra in normal and different diseased tissues in three areas of medicine – oncology [18, 19], gastroenterology [16, 20] and a medicine of occupational (professional) diseases [21]. In the most of these clinical trials we have used modified medical spectroscopic equipment "LESA-01 Biospec" (Biospec Co., RF) with an optical fibre probe [16]. The distance between laser source fiber and light receiving fibers was around 0.5 mm. He-Ne laser (10 mW, 632 nm) was used as a source of laser radiation for excitation of porphyrins' AF. The sensitivity of a photodetector unit of our spectroscopic system was around 10^{-11} W with a signal to noise ratio not less than 10:1. The typical *in vivo* registered spectra of porphyrins' AF from normal and cancerous tissues are shown in Fig.1. The backscattered laser line in all registered spectra was reduced in ~1000 times by a special step-selective optical filter. In all our study a registered AF phenomenon was quantitatively expressed through a *modified coefficient of fluorescent contrast* (K_f) calculated by the formula [22]:

$$K_f = 1 + (I_f \cdot \beta - I_{bs}) / (I_f \cdot \beta + I_{bs}), \qquad (1)$$

where: I_f is an amplitude of a registered signal in the maximum of the AF spectrum (see Fig. 1), I_l – is an amplitude of the registered signal in a backscattered laser radiation line, β - is the reducing filter coefficient ($\beta \approx 10^3$).

In our clinical study in oncology we have examined around 150 patients with different skin and head and neck cancers before radiotherapy course, during and after that and around 50 healthy volunteers [18, 19]. For each healthy volunteer normal AF spectra were collected from the different skin and oral cavity areas. For each examined oncologic patient AF spectra were collected from the centre of the visible area of a presented cancer, from 3-5 points around the visible centre of the tumour and, additionally, from the normal (intact) soft tissue next to the cancerous area. The aim of this study included the keeping of initial (before treatment) AF data and the keeping of dynamics of that during radiotherapy course to find out a correlation between initial porphyrins' AF spectra and an efficacy of the next radiotherapy procedures. But in the light of the present problem we have reanalysed all these data on the subject of the looking for a correlation between AF spectra and a chronic hypoxia state in tissues.

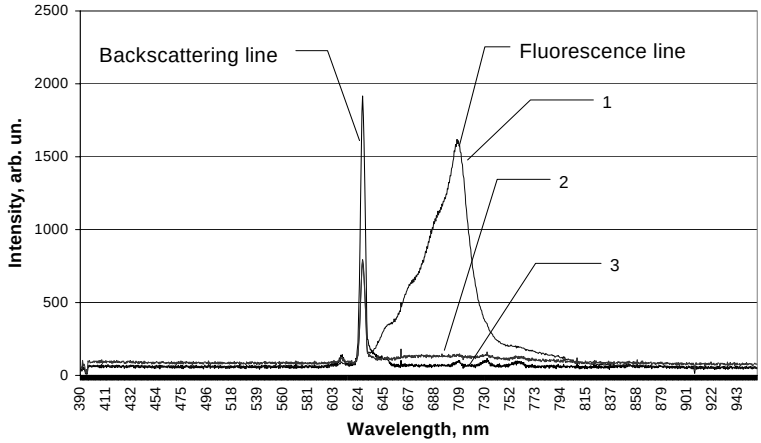

Fig. 1. Typical registered AF spectra in our research.
1- visible center of an oral mucosa tumor; 2 – surrounding intact tissue;
3 - standard non-alive and non-fluorescent scatter.

The research in a gastroenterology included the keeping of initial AF porphyrin's spectra before the low-level laser therapy and observation after dynamics of that during the therapy course for the patients with different erosive-ulcerative impairments (EUI) of the upper part of the gastrointestinal tract (UPGT) [16, 20]. All diagnostic procedures were performed with the use of conventional endoscopic equipment and technique. We have tried to find out a correlation between initial porphyrins' AF spectra and an efficacy of the next laser therapy course. To eradicate the Helicobacter Pylori a modern routine of anti-ulcerous drugs were used as well. 192 patients were presented in this study with a history of progressive UPGT EUI from 2 months up to 1 year. By ulcer localization all patients were classified into: gastric ulcer – 97 cases (50.5%), duodenal ulcer – 95 cases (49.5%). In 81 cases (42.2%) ulcer was combined with gastric and duodenal erosion, 4 patients showed a combination of gastric and esophageal ulcers. 145 (78.0%) patients were known to have ulcers for over 5 years. The ulcer size varied from 5 to 15 mm in the majority of cases.

Our clinical research in the area of medicine of occupational diseases included the aim to study AF porphyrins' spectra in a finger skin for patients with the so-called professional "vibration disease" (in English literature - "*white fingers syndrome*") – the occupational disease caused by a long local vibration of hands [21]. Different vascular disorders such as angiodystonic or angiospastic syndrome, predominantly on fingers, together with lesions of distal ends of the peripheral nerves of upper extremities play a leader's part among clinical features of the vibration disease (VD) caused by the local vibration. It is suggested that pathogenesis of the trophic disturbances under the VD is, first of all, associated with persistent troubles of blood microcirculation in fingers what is very important for the oxygen transport into that. We have observed 78 male in-patients aged 38-60 years (mean age 47.8±1.9) with VD of different stages. A control group in the study has consisted of 12 men aged 35-60 years (mean age 42.1±2.6) without any cardiovascular disorders.

3. SUMMARY RESULTS AND DISCUSSION

As we have reported previously [18, 19], for our oncologic patients we have observed some objective and strong differences in AF spectra for different kinds of tumors, different stages of tumor growth and different stages of radiotherapy courses, what could be explained by differences in concentration of porphyrin's molecules in the tested tissues. But that time really we couldn't synonymously find the correspondence between differences in viewed AF porphyrins' spectra and varieties in localization, morphological forms, stages of cancer or other peculiarities. Very diverse differences in AF spectra were observed both for different forms and stages of a cancer and for the same ones. Moreover, in a number of clinical cases (around 30%) there was not any visible AF signal for strong mucosa oral tumors before treatment and during that. A number of patients (around 40%) had enhanced initial AF spectra not from all surface of tumour, but only from 1-2 points around its center. During the radiotherapy treatment a lot of patients had an enhanced AF after 2-3 days of ionizing irradiation, what could be explained by appearance of necrotic zones in tumour with a high concentration of porphyrins after the γ-ray daily exposure. But the same reaction in 50-60% cases we have observed immediately after the single γ-ray exposure only. The rest 40-50% cases showed direct opposite results - a decrease of AF intensity after the daily γ-ray exposure. Moreover, the response of the tissue for every patient in meaning of AFD data was not predictable from one day to another. It was negative or positive without any visible correlation with previous results, stage of the cancer, etc. In a total we have estimated that a mean initial K_f value for all oncologic patients with the enhanced initial AF spectra from cancerous tissues was around $K_f = 0.65 \pm 0.15$ while the healthy volunteers had a mean value $K_f < 0.1$.

For the patients with progressive UPGT EUI we have estimated [16, 20] that the mean initial K_f value was on a level of $K_f = 0.30 \pm 0.07$. And the less initial AF intensity was observed the more effective laser therapy course was marked for these patients. Taking K_f as a function of time (per days of a treatment) we could saw that practically all our patients with positive dynamics of the cure showed an equalization between ulcer's AF K_f values and intact mucosa K_f values on a level of $K_f = 0.03\text{-}0.09$ by the end of the treatment course.

The studying of endogenous porphyrin's AF spectra of affected hand's tissues in patients with different stages of VD showed a long-term rise in the fluorescence contrast coefficient K_f as well [21]. In stage 2 VD patients with expressed trophic disorders in hand finger skin the mean K_f value was $K_f = 0.23 \pm 0.09$. In the initial stage VD (stage 1 VD) patients coefficient K_f was also increased but less significantly - $K_f = 0.17 \pm 0.08$. In a control group the same index didn't exceed $K_f = 0.08 \pm 0.05$.

Total summarized results of our previous researches are presented in a table 1.

Table 1.

Total results in our previous studies.

Case of observation	Average value of initial K_f	Chronic hypoxia in tissue
Normal skin	0.07±0.04	Absence
Normal mucosa	0.06±0.03	Absence
Stage III-IV cancer	0.65±0.15	Strong
UPGT EUI	0.30±0.07	Middle
Stage 2 VD	0.23±0.09	Middle
Stage 1 VD	0.17±0.08	Low

What can unite all these experimental data? If we suppose that the enhanced accumulation of porphyrins in tissues is a biochemical consequence of enhanced cells' proliferation, then we can't explain our results in a gastroenterology and a medicine of occupational diseases, because, for example, it is well known that there is no any enhanced cell's proliferation in the hand fingers in a case of VD. Also, there is no any enhanced cell's proliferation in a case of patients with UPGT EUI. Otherwise, all our patients with enhanced initial cell's proliferation and, accordingly, with enhanced initial K_f values in the EUI area had to show a better therapeutic outcome than others. But we saw a directly opposite result [20]. Our results can't be explained by a microbe etiology as well – in the cases of VD there is no any one in a general case. In our opinion, only one phenomenon in tissues can explain all our mentioned above results without any contradictions – a hypoxia. In all these cases a chronic hypoxia is one of the principal and total factors, which could exist in tissues under all of mentioned above diseases. It is known, for example, that in accordance with a general radiotherapy experience the malignant tissues and tumours often have a state of a strong chronic hypoxia and so they are frequently resistant toward ionising radiation. What's more – frequently a chronic hypoxia is observed not per a total volume of malignant tumours, but in the local fields of that only (different fractions of hypoxic cells in tumors [23, 24]) that exactly we had seen in our experiments. Chronic hypoxia frequently presents under a white fingers syndrome as well as under a different progressive UPGT EUI. Exactly the chronic hypoxia can has a strong influence on porphyrins' metabolism in all of these mentioned cases from the theoretical point of view [13].

Thus, in our opinion, endogenous porphyrins in tissues are a good fluorescent metabolic indicator like others well-known fluorescent metabolic indicators – a reduced nicotinamide adenine dinucleotide (NADH) and oxidized flavins adenine dinucleotide (FAD) [1]. So, now we can additionally suggest a classification of levels of a chronic hypoxia condition, which can be detected with the use of AFD, versus our numeric contrast coefficient K_f:

K_f<0.1 – the absence of a visible chronic hypoxia;
K_f=0.1-0.2 – an initial (light) stage of hypoxia;
K_f=0.2-0.4 – a middle stage of chronic hypoxia;
K_f>0.4 – a heavy stage of chronic hypoxia.

It is interesting to note, that all these obtained results allow us now to explain some previous unexplained results in oncology, which were found out long before this paper was written. For example, 10 years ago we have reported of frequently observed *in vivo* enhanced AF spectra in oncologic patients from theirs intact, surrounding tumor tissues [22]. That time we were working with III-IV stages of cancers and the increased coefficient K_f for intact tissues could be a simple consequence of a total intoxication of the organism on terminal stages of a cancer causing the ischemia and hypoxia in all of patient's tissues. Changeable character of AF, being observed during a chemo-radiotherapy course [18], could indicate different destructive processes in a microcirculation system caused by the ionizing radiation. General decreasing of K_f by the end of the radiotherapy course [18, 19] could be a consequence of a total destruction of vessels in a tumor if porphyrins are absorbed by cells from a bloodstream, or could be a consequence of a new angiogenesis and reduction of hypoxia status of cells in a tumor by the end of the therapy. In any case we can mark now a visible correlation between blood microcirculation processes and a porphyrins AF in a tested tissue.

Additionally, 10 years ago it have been estimated that a relative random error of AF measurements was on a level of 3-5% for a non-alive fluorescent and scattering media and on a level around 30-40% for cancerous tissues [22]. We can ask now: Is 30-40% dramatically for the correct medical interpretation of AFD data? And can answer now: On suggested classification the value of K_f is changed in two times or more from stage to stage, so an error of 30-40% is not very significant for the medical interpretation of AFD data with the use of the K_f criterion.

4. CONCLUSION

Summarizing all our results we have to certify that all our clinical data allows us to propose a possibility to establish a direct correlation between levels of chronic hypoxia in a tested tissue and levels of a porphyrin's coefficient K_f measured by *in vivo* AFD technique. From the view point of the so-called "third level" of AFD systems' software [17] (medical level), which is more discussible today, it means that software calculation algorithms have to content a direct interpretational procedure to translate the calculated coefficient K_f into a clear medical information – the probability of a chronic hypoxia state and levels of it in a tested tissue. It must be additionally noted, that we didn't carry out any special research to study the phenomenon of porphyrins accumulation in tissues as it is, and/or to find out any biochemical (biophysical) reasons of increased porphyrin accumulation in tissues or cells. We have examined our patients to understand better the diagnostic possibilities of *in vivo* AFD in clinical practice and have reanalyzed different clinical situations only. But our results gave us an additional reason to propose an existence of a correlation between viewed AF spectra of porphyrins and a chronic hypoxia in tissues. It, of course, doesn't exclude any other and detailed biochemical reasons of porphyrin accumulation in cells, for example, a low (an acidic) pH in cells [1, 12, 15], although it can be proposed easily that the chronic hypoxia (ischemia) in tissues could be one of the initial and principal factors for the changes in pH of the medium as well [22].

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