Stimulation and Inhibition of 5-ALA induced PpIX-fluorescence in the diagnosis of Fibrosarcoma cultivated on the CAM using glucose versus Ethanol as modulating agents

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Abstract

The fluorescence properties of biological tissues have been considered as intrinsic parameters to discriminate diseased from normal conditions. In vivo fluorescence diagnosis of cancer is based on special fluorescent dyes and their properties of tumour selective retention. The experimental in vivo model of the chorioallantoic membrane (CAM) of chicken embryos was used for cultivating a murine tumourous system consisting of the SSK II fibrosarcoma. Proto porphyrine (PpIX) synthesis in CAM inoculated tissues as well as in native CAM was induced by 5-ALA. The modulation effects of several biochemicals on the 5-ALA induced PpIX production were tested. The fibrosarcoma cells have not revealed autofluorescence with distinctively higher signal intensities than the substrate tissue. Fibrosarcoma cells are clearly distinguished by higher xenofluorescence intensities compared to the CAM tissue in the background. 5-ALA induced xenofluorescence intensity in fibrosarcoma was significantly enhanced by glucose and inhibited by ethanol. It can be concluded that some chemical agents can modulate the intensity of 5-ALA induced xenofluorescence through their modulation the enzymatic cell activity and these can be used for improvement by varying both the diagnostic and the therapeutic effectiveness of the photosensitizers in its application in the photo therapy process.

Keywords

5-ALA, PpIX fluorescence, fibrosarcoma, CAM, Modulating agents

Introduction

The fluorescence properties of biological tissues have been considered as intrinsic parameters to discriminate diseased from normal conditions. A detailed analysis of autofluorescence is supposed to provide information on the characteristics of a given tissue [(1)]. Extrinsic fluorescent markers are agents which are known to interact with the normal native cellular enviroment [(2)]. Fluorescence of these injected agents such as 5-ALA induced PpIX has been developed as a method for detection of carcinoma in situ and other early stage tumours[(1,2)]. The experimental in vivo model of the chorioallantoic membrane (CAM) of chicken embryos was used for cultivating a murine tumourous system consisting of the SSK II fibrosarcoma.

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Materials and methods

Cell lines and culture conditions

The murine fibrosarcoma cell line SSK II [from Kummermehr H.] was cultured under sterile conditions in DMEM (ICN-Flow) supplemented with 10% fetal calf serum (FKS; Seromed-Biochrom), 2% glutamine (ICN-Flow) and 2% penicillin/streptomycin (ICN-Flow). Cell cultures were incubated in a 97% humidified atmosphere with 7,5% CO2 at 37 ºC. Confluent

monolayers were used for subcultivating. Cells were harvested after detaching with trypsin/ EDTA, centrifuged at 1000g and recultured at appropiate cell densities.

Tissue growth on the chorio-allantoic membrane (CAM)

The harvested cells were resuspended in DMEM containing 2% Ultroser (Gibco-BRL) instead of FKS. Cells were inoculated at cell densities of 2x106/20 µl culture medium into silicon O-rings (inner diameter: mm) put onto the CAM´s of opened eggs after 4-5d of incubation. Inoculated eggs were covered with tape and incubated for further 3-4d (37.8 ºC, 97% humidity). The incubated eggs entered the experiments after the inoculated tissues had reached macroscopically three dimensional stages of growth (Fig. 1).

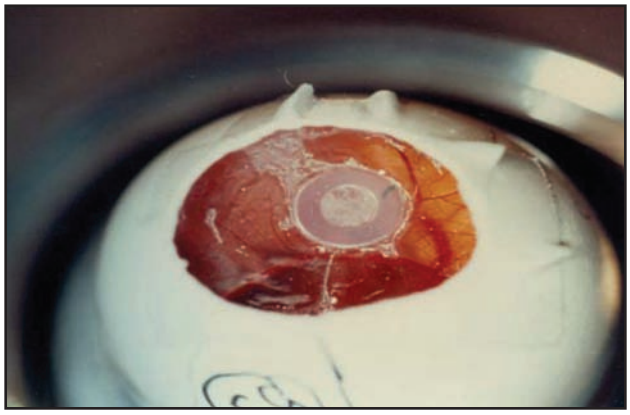

Fig. 1: Fibrosarcoma cultivated on CAM

Modulation of PpIX fluorescence

In a first step PpIX synthesis in CAM inoculated tissues as well as in native CAM was induced by 5-ALA. Furthermore, the modulation effects of several biochemicals on the 5-ALA induced PpIX production were tested. The biochemicals used and their known effects on cellular metabolism are listed in table 1 along with the concentrations employed. All substances were solved in PBS and administrated onto the CAM in 1ml portions. Preparations were done under dimmed lighting conditions. The prepared eggs were covered with aluminum foil and incubated for 3-4h.

Fluorescence measurements

Autofluorescences and xenofluorescences were excited with a HBO-X 100 W lamp (Carl Zeiss) using a band pass filter $\lambda = 405 \pm 5$ nm. Fluorescence emissions were detected by an ICCD camera (C2400-87; HAMAMATSU) using a long pass filter with a cut-off wavelength $\lambda = 630$ nm. The intensities were visualized by real time digital image processing (Argus 10; HAMAMATSU) and analysed on a "false colour" scale. The scheme of the experimental set-up is shown in Fig. 2.

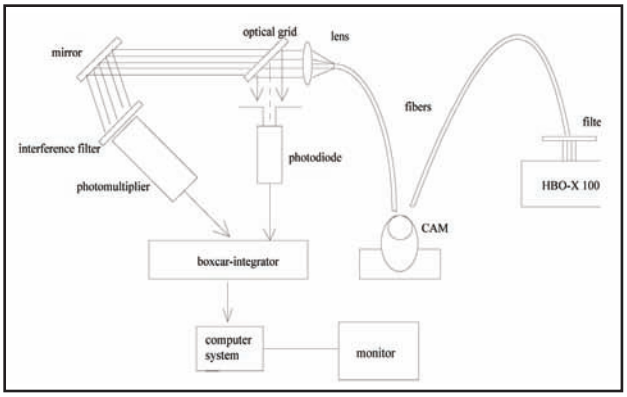

Fig. 2: Experimental set-up of the fluorescence measurements

Results

Studies on the PpIX-autofluorescence and 5-ALA induced PpIX-xenofluorescence of native CAM tissue were performed in order to facilitate the intensitiy scaling of the non-induced and the 5-ALA induced protoporphyrin fluorescence of the inoculated cells later on. The autofluorescence score was estimated from ten measurments.

Autofluorescenece

The mean CAM autofluorescence score was 2 ± 0.67. The fibrosarcoma cells SSk II analysed 3 days after inoculation on CAM of 5 – 6 days incubated eggs, did not reveal

Biochemical	Concentration	Metabolic effect
5-ALA	0,4 mM	Induces Pp IX synthesis
Glucose	250µM	Elevation of the energy-metabolism
Ethanol	0,004 %	Inhibition of 5-ALA-synthetase, utilisation of $NADH+H^+$

Table 1: Biochemicals used for the modulation studies on the PpIX- fluorescence

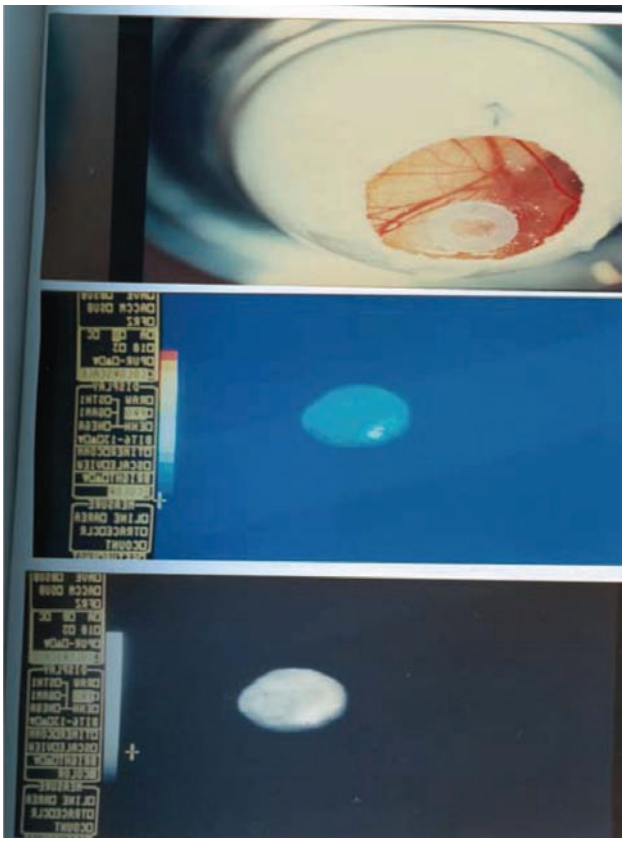

Fig. 3: Autofluorescence of SSK II cells on CAM, A. overview picture; B. coloured scale (average 2.1 a. u.) C. grey scale

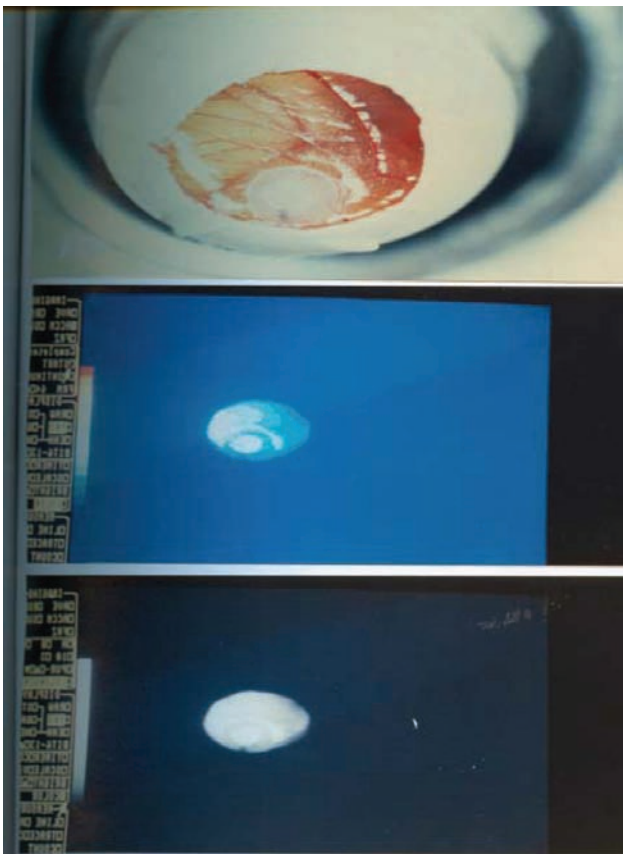

Fig. 4: Xenofluorescence of SSK II cells on CAM. A. overview picture. B. coloured scale (average 10 a. u.) C. grey scale

distinctively autofluorescence signal intensities than the substrate tissue (Fig. 3). The mean autofluorescence of the inoculated SSK II was scaled by a two- grade (SD. ± 0.94).

5-ALA induced Xenofluorescenece

Incubating the CAM tissue with 1 ml of 0.4 mmolar 5-ALA solution for 3 hours resulted in strongly intensefied xenofluorescence signals. Xenofluoresceneces of the CAM was scaled aside the yolk by the average value of 5 (SD. ± 0.94). Incubating the SSK cells cultivated on CAM with 1 ml of 0.4 mmolar 5-ALA solution for 3 hours resulted in high protoporphyrin IX xenofluorescence signals. SSK II cells are clearly distinguished by higher xenofluorescence intensities compared to the CAM tissue in the background. Fluorescence intensities of the (SSK II)-xenotissues illustrated in Fig. 4 was scaled by the average value of 8 (SD. ± 1.33).

Modulation of 5-ALA induced xenofluorescence of fibrosarcoma

Incubating the SSK II cells cultivated on CAM tissue with 1 ml of 0.4 mmolar 5-ALA solution plus 1 m glucose with concentration of 250 μM resulted in increasing the fluorescence intensity of fibrosarcoma tissues with a mean score of 12.6 (SD. ± 0.67) (Fig. 5). 5-ALA induced xenofluorescence intensity in the SSK II fibrosarcoma was enhanced by addition of glucose. Inhibition of xenofluorescence intensities were recorded after addition of ethanol (0.004%) to 5-ALA and incubating with the investigated tissues for three hours. Mean xenofluorescence score of 0.8 (SD. ± 0.53) (Fig. 6). Simultaneous treatment with ethanol completely inhibited xenofluorescences by 5-ALA. Table 2, shows the all results of auto and xenofluorescence with their standered deviations.

Discussion

From the main pre-conditions for curative treatment of cancer is the removal of as much abnormally transformed tissue as possible at the earliest stage of progression [3,4,6,7]. Biological tissues exhibit fluorescence emission that cover almost all the visible spectral range under

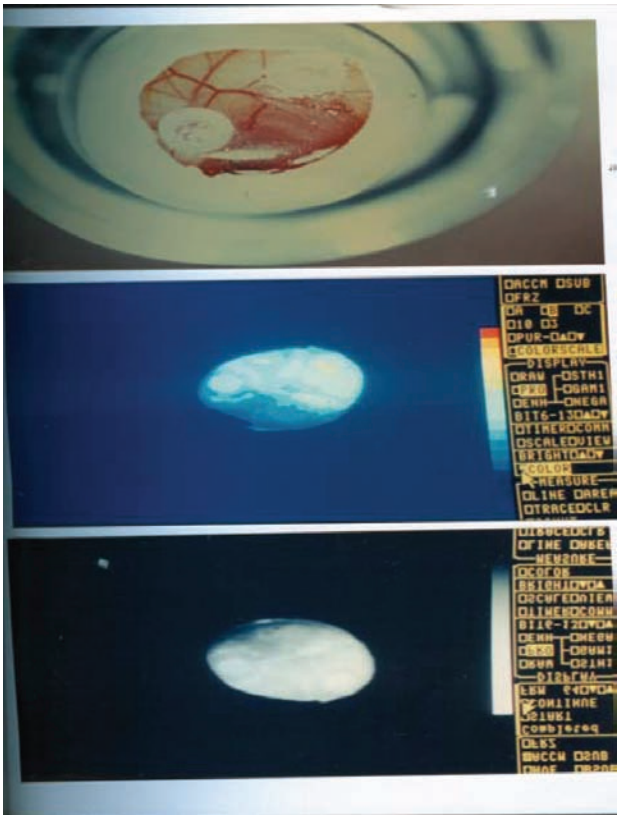

Fig. 5: Xenofluorescence of SSK II cells on CAM ALA + Glucose; A. overview picture B. coloured scale (average 11.6 a. u.) C. grey scale

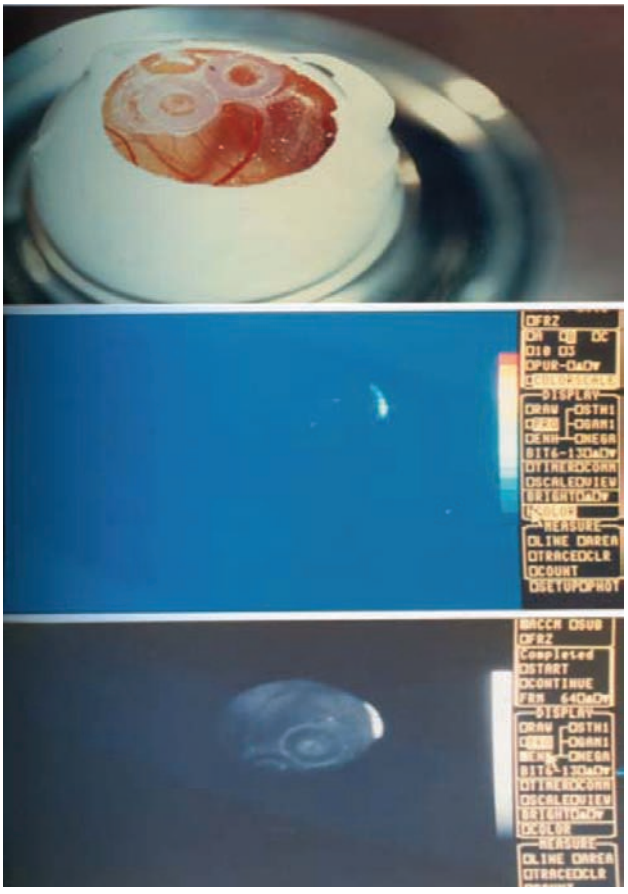

Fig. 6: Xenofluorescence of SSK II cells on CAM ALA + Ethanol; A. overview picture; B. coloured scale (average 0.8 a. u.) C. grey scale

CAM autofluorescence	2 ± 0.67
CAM xenofluorescence (+ ALA)	5 ± 0.94
Fibrosarcoma autofluorescence	2 ± 0.94
Fibrosarcoma xenofluorescence (+ ALA)	8 ± 1.33
Fibrosarcoma xenofluorescence (+ALA + Glucose)	12.6 ± 0.67
Fibrosarcoma xenofluorescence (+ ALA + Ethanol)	0.8 ± 0.53

Table 2: The mean SSK II auto- and xenofluorescence with effects of modulations

suitable excitation conditions. The fluorescence properties of these tissues have been considered as intrinsic parameters to discriminate diseased from normal tissues [1, 8,9,10]. Detailed analysis of autofluorescence and xenofluorescence (induced fluorescence) is supposed to provide information on the characteristics of a given tissue, since most of the biological components responsible for fluorescence emissions are either related to structural aspects of tissue or involved in metabolic and functional processes of cells. In vivo fluorescence diagnosis of cancer is based on special fluorescent dyes and their properties of tumour selective retention [4, 11, 12, 13].

5-ALA, as a physiological substrate of the heme synthesis, is reformed in eucaryotic cells into protoporphyrin IX (PP IX) on the inner matrix of the mitochondrial membranes catalyzed by the enzyme ferrochelatase [5]. If the capacity of this enzyme overstressed with exogenous application 5-ALA, Pp IX accumulates at high concentrations and therefore PP IX- fluorescence emissions are intensified. Pp IX preferentially accumulates in neoplastic tissues, because of a functional defect of the ferrochelatase enzyme [6].

Exogenous administration of 5-ALA, an early precursor in haem synthesis, induces accumulation of endogenous photoactive

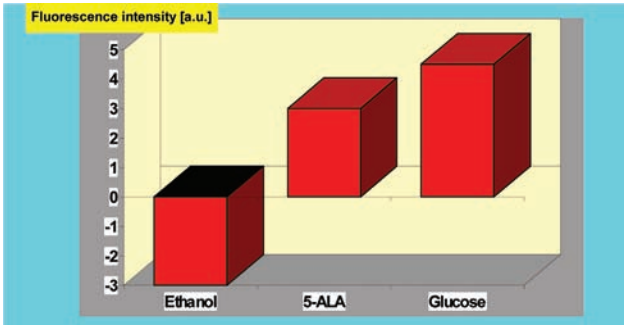

Fig. 7: Modulation of 5-ALA induced xenofluorescence of SSK II tissues inoculated onto the CAM (The modulated fluorescence intensities [a.u.] illustrated where obtained by subtracting the average intensity measured for CAM tissue after incubation with 5-ALA).

porphyrins, in particular protoporphyrin IX [3,14, 15, 16]. Although autofluorescence is simple and available, the use of exogenous agents offers a mean enhance contrast. Therefore, the future will probably make increasing use of exogenous fluorophores or metabolic inducers of fluorophores. Fluorescence of injected extrinsic fluorescent agents such as 5-ALA has been developed as a method for detection of carcinoma in-situ and other early stages of tumours. The experimental in-vivo model of chorioallantoic membrane (CAM) of chicken embryos was used for cultivating a murine tumour tissue system consisting of the SSK II fibrosarcoma. This in vivo model of CAM was empolyed for studying the autofluorescence and xenofluorescence of Pp IX characterestics of fibrosarcoma cell lines. Furthermore, the effects of both glucose and ethanol on the xenofluorescence intensities of the fibrosarcoma tissues were investigated.

After administration of 5-ALA to the CAM inoculated tissues the fibrosarcoma cell line SSK II tumours exhibited higher fluorescence intensities in comparison to its autofluorescence. The fluorescence intensities of fibrosarcoma cell line were increased after incubation with glucose, while ethanol drastically decreased xenofluorescence intensities [17,18,19]. Addition of an energy elevator e.g glucose increased differentially the fluorescence intensity of the abnomal tissues. Scince the electron transport chain in the tumour cells is very likely to be reduced, the energy and reducing- equivalants producing processes cannot be down regulated as much as in normal tissue cells when glucose is supplemented. On the other hand, the addition of ethanol, which acts as 5-ALA-synthetase inhibitor- drastically decreased the Pp IX fluorescence intensities in fibrosarcomatous tissues. It has been reported that certain steroids and large number of chemicals and drugs increase porphyrin production rates in animal and in chicken embryo liver cells. The effect of glucose on drug promoted induction of porphyrin synthesis was studied in chick embryo liver cell culture with or without addition of exogenous 5-ALA. Induction of 5-ALA synsthetase was abolished by glucose [7]. Another study investigated the synergism of glucose administration combined with phototherapy reported that such combination therapy produced a higher percentage of animal cured than using phototherapy alone. This therapeutic approach required lower light doses for the treatment of cancer [8].

So, it can be concluded that some chemical agents can modulate the intensity of 5-ALA induced xenofluorescence through their modulation of the enzymatic cell activity and these can be used for improving both the diagnostic and the therapeutic effectiveness of the photosensitizers in its application in the phototherapy process.

Acknowledgements

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