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Experimental study on photodynamic diagnosis of cancer mediated by chemiluminescence probe

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Abstract **A novel method of photodynamic diagnosis of cancer mediated by chemiluminescence probe is presented. The mechanism for photodynamic therapy involves singlet oxygen ($^1O_2$) generated by energy transfer from photosensitizers. $^1O_2$ can react with 3,7-dihydro-6-{4-{2-(*N*′-(5-fluoresceinyl)thioureido)-ethoxy}phenyl}-2-methylimidazo{1,2-a}pyrazin-3-one sodium salt (FCLA), which is a *Cypridina* luciferin analog and a specific chemiluminescence probe for detecting $^1O_2$ and superoxide (O_2^-). The reaction of FCLA and $^1O_2$ can give emission with peak wavelength at about 532 nm. In the present study, FCLA was chosen as an optical reporter of $^1O_2$ produced from the photosensitization reaction of hematoporphyrin derivative in model solution and in nude mice with transplanted mammary cancer. Photosensitized chemiluminescence from the reaction of FCLA with $^1O_2$ was detected by a highly sensitive Intensified Charge-Coupled Device detector. The chemiluminescence was markedly inhibited by the addition of 10 mmol/l sodium azide (NaN_3) to the model solution and minor effects were observed at the addition of 10 μmol/l superoxide dismutase, 20 mmol/l mannitol and 100 μg/ml catalase, respectively, thus indicating that $^1O_2$ generation from photosensitization reaction mainly results in light emission. Experiments in vivo with tumor-bearing mice showed a clear chemiluminescence image of tumor. The study suggests that this novel method may be applicable to the diagnosis of superficial tumors.** **© 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.**

Key words: Photodynamic diagnosis; Singlet oxygen; *Cypridina* luciferin analog; Hematoporphyrin derivative; Chemiluminescence

1. Introduction

Photodynamic therapy (PDT) is an evolving modality for the treatment of cancers. This therapy involves the selective uptake and retention of a photosensitizer in a tumor, followed by irradiation with light of a specific wavelength, thereby initiating tumor necrosis through formation of the reactive oxygen species (ROS) such as singlet oxygen ($^1O_2$) and free radicals in the irradiated tissues [1,2]. Research has demonstrated that the destroying effect is mainly due to the formation of $^1O_2$ (type II PDT process) resulting from the interaction of a light-excited photosensitizer with molecular oxygen (triplet ground state) [3].

In recent years, the diagnosis of early-stage tumor attracts more and more attention. Photodynamic diagnosis (PDD) is a well-known method for tumor localization, which is based on the accumulation of photosensitizers in a tumor during PDT. The common PDD is a kind of fluorescence diagnosis, which detects the fluorescence of photosensitizers excited with their strongest absorption wavelength. However, the diagnostic accuracy is only about 70% because the main absorption wavelength of photosensitizers generally used is in the ultraviolet region. During conventional PDD, using so short excitation light leads to some serious problems. Firstly, this is the disturbance of autofluorescence from normal tissues, since in biological tissues there are many kinds of chromophores which can be excited by the short wavelength light to emit strong fluorescence disorderly, which can greatly disturb the detection of fluorescence from photosensitizers. Secondly, ultraviolet irradiation may produce damage in biological tissues. Thirdly, the poor penetration ability of short wavelength excitation light makes it impossible for diagnosing deeper tumors.

During the PDT process the absorption of photon energy results in generation of $^1O_2$. It is clear that $^1O_2$ plays an important role in PDT. Considering the limitations of conventional PDD and the key role of $^1O_2$ in PDT, a novel PDD method mediated by chemiluminescence probe was put forward and studied by experiments in this report. The method utilizes a photosensitizer as locator of tumors and a chemiluminescence probe as a reporter of $^1O_2$. Thus a tumor can be shown through detecting the two-dimensional image of the photosensitized chemiluminescence emitted from the reaction of the chemiluminescence probe with $^1O_2$ using a highly sensitive Intensified Charge-Coupled Device (ICCD) detector.

In the present study, hematoporphyrin derivative (HpD) was used as a photosensitizer [4] and 3,7-dihydro-6-{4-{2-(*N*′-(5-fluoresceinyl)thioureido)ethoxy}phenyl}-2-methylimidazo{1,2-a}pyrazin-3-one sodium salt (FCLA) was used as a chemiluminescence probe of $^1O_2$. FCLA, recently developed by Goto and co-workers, is characterized by emitting light at the longest wavelength (532 nm) among the chemiluminescent substances known up to now [5–7]. Conventional photosensitized reactions of HpD excited by a high-pressure mercury lamp equipped with a 550 nm long-pass filter were performed in vitro and in vivo. Experimental results showed that $^1O_2$ generated from photosensitized reaction can be recorded and imaged in a model system and in tumor-bearing nude mice.

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2. Materials and methods

2.1. Reagents

FCLA, purchased from Tokyo Kasei Kogyo Co., was dissolved in double-distilled water and stored at −20°C until needed. Photosensitizers of HpD (100 mg/20 ml), produced in Beijing Institute of Pharmaceutical Industry, were diluted in 0.01 mol/l phosphate buffer saline (PBS, pH 7.4) solution before experiments. Cu–Zn superoxide dismutase (SOD, from bovine erythrocytes) and human serum albumin (HSA; 96%) were obtained from Sigma Chemical Co. Deuterium oxide (D_2O, 99.9 atom % D, from Aldrich Chemical Co.) was stored under nitrogen until needed. Sodium azide (NaN_3), mannitol, catalase and other chemicals were all made in China.

2.2. Experimental setup

The experimental setup (shown in Fig. 1) used to record the two-dimensional image of the photosensitized chemiluminescence was based upon an ICCD detector (model: ICCD-576-s/1, from Princeton Instruments, USA), which was combined with a photographic lens of large aperture (Nikon 50 mm, $f=1.4$) and a detector controller (ST-130 controller, from Princeton Instruments, USA). The detection system was a specially designed gated detection. A 50 W high-pressure mercury lamp (from Bio-Rad) in conjunction with a 550 nm long-pass filter (OG 550, from Coherent Co., USA) was used as the irradiation source for photosensitization reactions.

All chemiluminescence measurements were carried out at 25°C. The specimen, on a tunable stage, was placed in a light-tight box. Through the photographic lens the chemiluminescence emitted from the specimen was collected and imaged onto the sensitive photocathode of the ICCD detector in appropriate exposure time. The photocathode was cooled to −40°C to reduce thermal noise. The spectral response of the detector was 400–950 nm. The chemiluminescence imaged on the photocathode by the lens generates photoelectrons, which were multiplied by the microchannel plate of the ICCD detector. The two-dimensional images of the chemiluminescence were displayed in a 576×384 pixel (picture element) format and processed with WinView software in a computer. The average counts per unit area were calculated in the specimen regions after subtraction of the background counts.

2.3. Preparation of tumor model

Female nude mice with a body weight of about 25 g were used in this study. A saline suspension of 1×10^7 disaggregated Bcap-37 human breast cancer cells was inoculated subcutaneously into the right shoulder of the mice, which caused a tumor to grow at a superficial place. The photosensitized chemiluminescence measurements were performed 2 weeks after inoculation.

2.4. Measurement of photosensitized chemiluminescence mediated by FCLA in model solution

A photosensitizer in PBS solution containing serum albumin such as bovine serum albumin and HSA appears to be a good model for PDT in vivo [8]. In our experiments, a standard quartz cuvette ($1\times1\times4$ cm) was used for measurements of photosensitized chemiluminescence in vitro. Components of the model solution were prepared just before use. The standard reaction mixture contained 20 μmol/ml HpD, 5 μmol/l HSA, 2 μmol/l FCLA and 0.01 mol/l PBS at pH 7.4 in a total volume of 1 ml. The model solution on the stage was irradiated uniformly for 20 s with a power density of 0.2 W/cm^2. Then the photosensitized chemiluminescence was recorded immediately after the irradiation was stopped. As contrast, the chemiluminescence was also detected when either HpD or FCLA was omitted from the model solution. To confirm the origin of photosensitized chemiluminescence, various ROS quenchers and D_2O were added to the model solution before irradiation and the effects of quenchers and D_2O on chemiluminescence intensity were recorded and compared. In order to prevent the quenchers added from photosensitized destruction during irradiation, the amounts of quenchers were increased in experiments. The quenchers used included 10 mmol/l NaN_3, 10 μmol/l SOD, 20 mmol/l mannitol and 100 μg/ml catalase.

2.5. Measurement of photosensitized chemiluminescence mediated by FCLA in tumor-bearing nude mouse

When the tumor reached approximately 1000 mm^3 in volume after 2 weeks of inoculation, the nude mouse was injected with 200 μg HpD in 0.9% sodium chloride systemically through a tail vein. After a period of 24 h to allow localization of HpD in the tumor, the mouse was given a superficial injection of 0.5 ml of 5 μM FCLA into the tumor and at the same time, as a contrast, the equivalent amount of FCLA was injected subcutaneously in the intact nape of the mouse. Then after a period of 60 min for FCLA to diffuse, the mouse was anaesthetized with sodium pentobarbital (50 mg/kg, i.p.). Afterwards the mouse was placed on the black stage in the light-tight box.

The mouse in the light-tight box was irradiated uniformly for 60 s with a power density of 0.4 W/cm^2 to allow a photosensitization reaction to occur in vivo. Photosensitized chemiluminescence mediated by FCLA was recorded immediately after the irradiation was stopped.

3. Results

3.1. Measurement of photosensitized chemiluminescence mediated by FCLA in model solution

In experiments, we observed that the model solution containing HpD, HSA and FCLA in PBS showed considerable light emission immediately after the irradiation was stopped and the light emission intensity attenuated rapidly with time, which could last for about 180 s. The photosensitization chemiluminescence images of the model solution recorded for the first 30 s after irradiation are given in Fig. 2. In contrast, the images with minor chemiluminescence intensity are

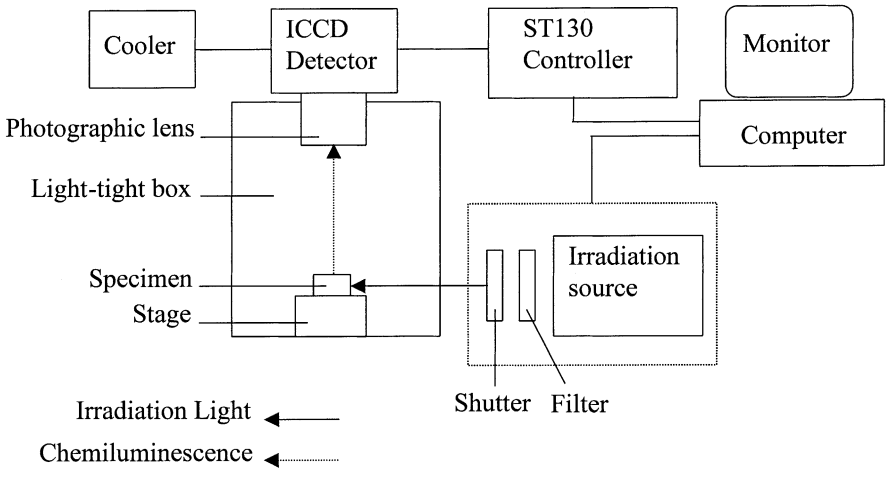

Fig. 1. The schematic setup of the two-dimensional photosensitized chemiluminescence imaging system.

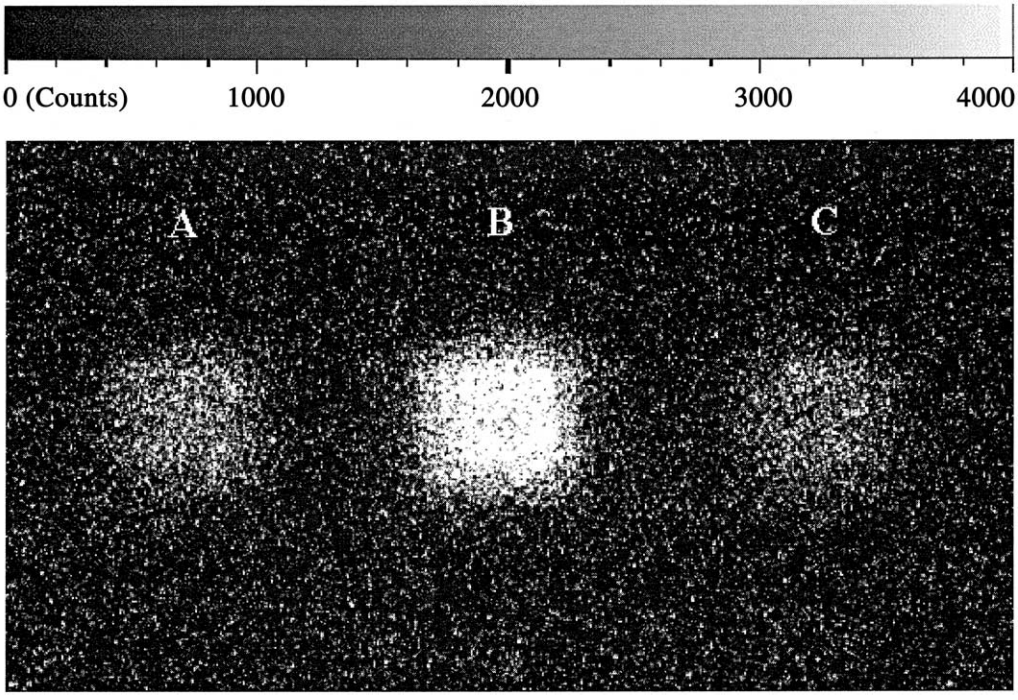

Fig. 2. Images of photosensitized chemiluminescence mediated by FCLA in model solution (exposure time is 30 s). A: Light emission from PBS (10 mmol/l, pH 7.4) with HSA (5 μmol/l), HpD (20 μmol/l) and visible light (>550 nm, 20 s), in the absence of FCLA. B: Light emission from PBS (10 mmol/l, pH 7.4) with HSA (5 μmol/l), HpD (20 μmol/l), FCLA (2 μmol/l) and visible light (>550 nm, 20 s). C: Light emission from PBS (10 mmol/l, pH 7.4) with HSA (5 μmol/l), FCLA (2 μmol/l) and visible light (>550 nm, 20 s), in the absence of HpD.

also recorded when either HpD or FCLA was omitted from the model solution prior to the performance of irradiation (results are shown in Fig. 2). The comparison of chemiluminescence intensity in Fig. 2 is given in Fig. 3. Fig. 4 shows the time course of photosensitized chemiluminescence.

3.2. *Effects of quenchers and D_2O on photosensitized chemiluminescence mediated by FCLA in model solution*

FCLA can selectively react with both $^1O_2$ and O_2^- and give chemiluminescence with a wavelength of 532 nm [7]. $^1O_2$ and O_2^- can be eliminated by NaN_3 and SOD in appropriate amounts, respectively. In order to confirm the effect of $^1O_2$ on the photosensitized chemiluminescence mediated by FCLA, several quenchers and D_2O were tested in our experiments (results are given in Fig. 5). We observed that the addition of the O_2^- scavenger SOD, the H_2O_2 scavenger catalase and $^{\bullet}OH$ scavenger mannitol to the above model solution prior to the performance of irradiation did not cause a very significant decrease (decreased by 23.5%, 12.8% and 8.7%, respectively, compared with control) in the integral chemiluminescence intensity of the first 30 s after irradiation was stopped. However, the integral chemiluminescence intensity was markedly inhibited (about 69.5%) by NaN_3, which is an effective quencher of $^1O_2$. Considering the excellent selectivity of FCLA to $^1O_2$ and O_2^-, the results suggested that the photosensitized reaction of HpD mostly elicits the formation of $^1O_2$ but not of O_2^-. (Because of the selectivity of FCLA, in our experiments the generation of $^{\bullet}OH$ and H_2O_2 in photosensitization action of HpD cannot be known well.) The photosensitization reaction of HpD by the substitution of D_2O for H_2O is often used as evidence that $^1O_2$ is involved, since the lifetime of $^1O_2$ is approximately 10 times longer in D_2O than in H_2O, thus allowing it more time to exert effects on targets

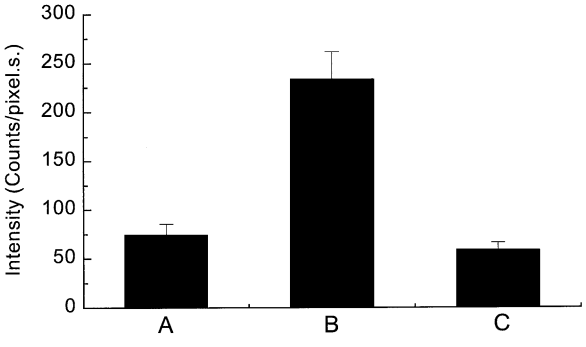

Fig. 3. Comparison of photosensitized chemiluminescence intensity of samples in Fig. 2 with histogram. Data are presented as mean ± S.D. of at least three separate experiments.

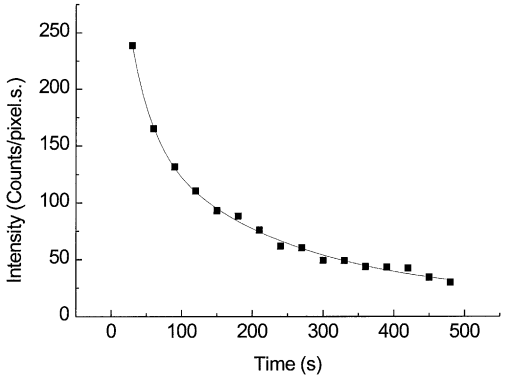

Fig. 4. Time course of photosensitized chemiluminescence in model solution with PBS (10 mmol/l, pH 7.4), HSA (5 μmol/l), HpD (20 μmol/l), FCLA (2 μmol/l) and visible light (>550 nm, 20 s).

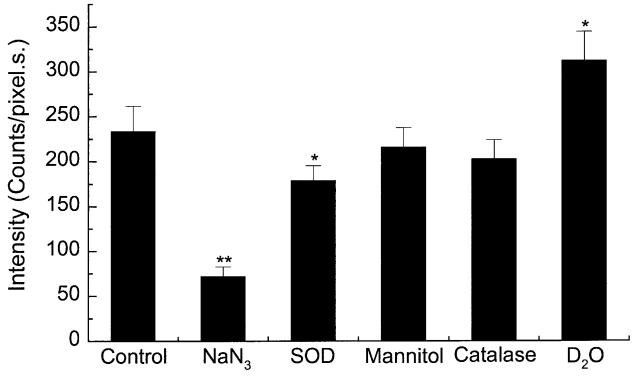

Fig. 5. Effects of quenchers and D_2O on photosensitized chemiluminescence mediated by FCLA in model solution. The quenchers and D_2O (99.9 atom % D) were added before irradiation with visible light (>550 nm, 20 s). Quenchers used are the $^1O_2$ quencher NaN_3 (10 mmol/l), the O_2^- scavenger SOD (10 μmol/l), the $^{\bullet}OH$ scavenger mannitol (20 mmol/l) and the H_2O_2 scavenger catalase (100 μg/ml). Data are presented as mean ± S.D. of at least three separate experiments. $^*P < 0.05$ in comparison with control; $^{**}P < 0.01$ in comparison with control.

[9]. The result of photosensitized chemiluminescence in D_2O is also shown in Fig. 5.

3.3. *Imaging of photosensitized chemiluminescence mediated by FCLA in tumor-bearing nude mouse*

Fig. 6 shows the images obtained from a photosensitized tumor-bearing nude mouse. The image of (A) in Fig. 6 was obtained on illuminating the mouse with dim white light. This was done for the purpose of positioning the mouse and the tumor. The tumor was located in the right shoulder of the mouse. The photosensitized chemiluminescence image recorded for the first 60 s after irradiation is shown as (B) in Fig. 6. The luminescent area in (B) of Fig. 6 corresponds to the tumor region. No clear chemiluminescence was observed around the intact nape and other regions of the mouse.

4. Discussion

Since it was discovered that porphyrin-based photosensitive dyes preferentially accumulated in malignant tissues, HpD has undergone extensive experimental and clinical research as a photosensitizer for PDT of tumors [10–12].

The photodynamic effect is a result of three primary processes. First, the ground state of the sensitizer (S_0) is optically excited to produce the excited singlet state ($^1S_1$). Population from this excited state is transferred by intersystem crossing (a radiationless transition) to the lowest triplet state ($^3S_1$) of the sensitizer. The triplet state photosensitizer may exert damaging effects through two main mechanisms, of type I and type II. In a type I reaction, electron transfer from the triplet sensitizer produces free radicals. In a type II reaction, collisional energy transfer from the triplet sensitizer to ground-state molecular oxygen ($^3O_2$) produces highly reactive $^1O_2$ and returns the sensitizer to its ground state:

$$S_0 + h\nu \rightarrow {}^1S_1$$

$$^1S_1 \rightarrow {}^3S_1$$

$$^3S_1 + RH \rightarrow \text{free radicals (type I, electron transfer)}$$

$$^3S_1 + {}^3O_2 \rightarrow S_0 + {}^1O_2 \text{ (type II, energy transfer)}$$

Free radicals and $^1O_2$ produced as results of type I and type II mechanisms can react readily with many biological targets and destroy a wide variety of cells.

For many years attention has concentrated on the type II production of $^1O_2$, which has frequently been suggested to be the major intermediate for photodynamic actions both in vitro

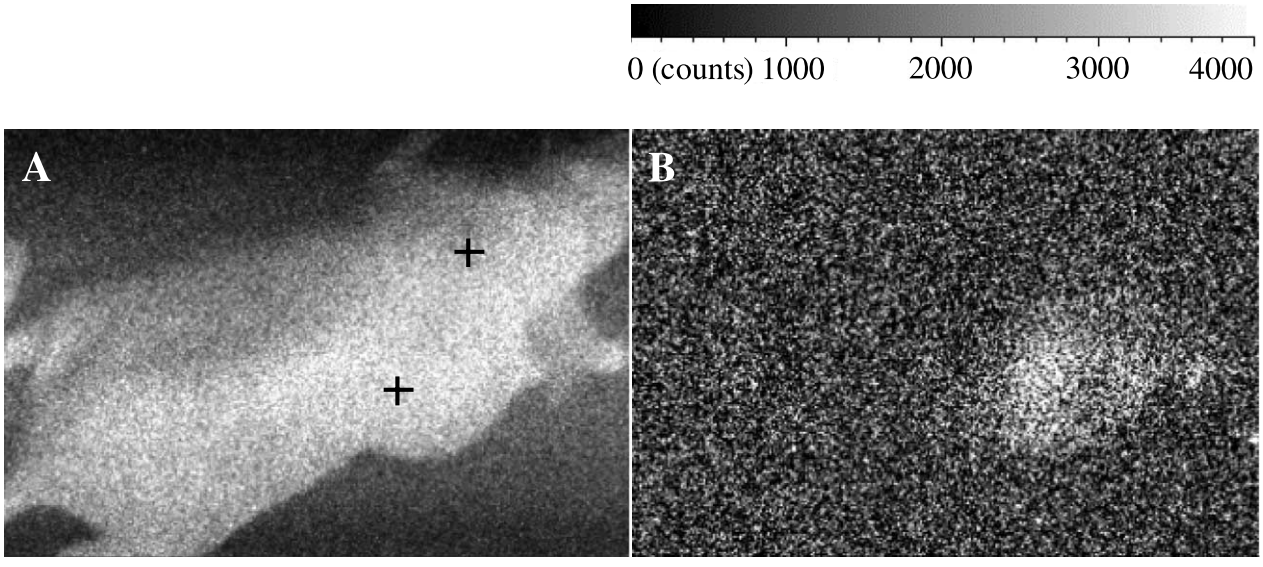

Fig. 6. Image of photosensitized chemiluminescence mediated by FCLA in tumor-bearing nude mouse. A: Image of nude mouse obtained on illumination with dim white light. The tumor can be observed in the right shoulder. Two black crosses indicate the position where FCLA was administered. B: Photosensitized chemiluminescence image from the tumor-bearing nude mouse. The bright area corresponds to the tumor region. No clear light emission was recorded over the nape. Exposure time is 60 s immediately after irradiation with visible light (>550 nm, 60 s).

and in vivo [13,14]. In this study, the results of photosensitized chemiluminescence mediated by FCLA demonstrated the $^1O_2$ formation from photosensitization reaction of HpD, and the effects of azide inhibition and deuterium enhancement further verified $^1O_2$ generation. Our study also shows the O_2^- generation from the photosensitization reaction but the amounts are minor and of no significance. The production of H_2O_2 and $^{\bullet}OH$ cannot be known well in our experiments because of the selectivity of FCLA to $^1O_2$ and O_2^-.

As a sensitive chemiluminescence probe, FCLA can selectively react with $^1O_2$ and O_2^- to give emission at 532 nm [15]:

$$\mathrm{FCLA} + {}^1\mathrm{O}_2 \text{ or } \mathrm{O}_2^- \rightarrow \mathrm{FCLA{=}O}^*$$

$$\rightarrow \mathrm{FCLA{=}O} + h\nu\ (532\ \mathrm{nm})$$

Combination of the $^1O_2$ production from the photosensitization reaction and the FCLA reaction with $^1O_2$ makes it possible to observe photosensitized chemiluminescence mediated by FCLA in vitro and in vivo. In the model system, very faint emission was observed when either HpD or FCLA was omitted. The emission of the model solution without FCLA may directly come from direct de-excitation of $^1O_2$ or reaction of excited photosensitizers with HSA, and emission without HpD may be from the slow auto-oxygenation of FCLA by dissolved oxygen [16].

In a tumor-bearing nude mouse, due to the accumulation of HpD in malignant tissues, a clear photosensitization chemiluminescence image of the tumor was obtained. The appreciable light emission of this FCLA-mediated chemiluminescence makes it possible to apply this method to the diagnosis of cancer. Although this novel method can only be adapted to the detection of emission from superficial tumors at present, because the emission wavelength of FCLA (532 nm) is too short to penetrate biological tissue, it should be pointed out that the autofluorescence can be avoided completely with this method.

Data indicate that in vivo during the exponential growth of tumors, native free radicals accumulate and reach a relatively high steady state [17]. So in the course of photosensitization in vivo a competition occurs between $^1O_2$-mediated effects and the interactions between the triplet photosensitizer and native free radicals formed in tumor cells. These facts might interfere with the method and might be one of the reasons why the chemiluminescence intensity recorded in vivo is much lower than in solution. However, the fact of oxygen existing in the tumors makes it possible to produce $^1O_2$ in vivo during photosensitization though its amount is less than in solution. Also, the high efficiency and selectivity of FCLA trapping $^1O_2$ enable us to observe the $^1O_2$ generation in vivo with the FCLA-mediated chemiluminescence.

Since the ICCD used is very sensitive to light and in order to avoid the disturbance of scattered light, fluorescence and phosphorescence, we did not record the FCLA-mediated chemiluminescence at the same time as the irradiation but recorded it immediately after the excitation was shut down.

The present results indicate that $^1O_2$ production in photosensitization reaction of HpD can be detected by FCLA-mediated chemiluminescence, which is named photosensitized chemiluminescence in this report. Utilizing the photosensitized chemiluminescence method to detect tumors in nude mice, a clear emission image was obtained. With this novel method, longer wavelength irradiation light (>550 nm) is converted to shorter wavelength chemiluminescence (532 nm), so the disturbance of autofluorescence can be avoided completely.

5. Conclusions

This study demonstrates that the main product from the photosensitization reaction of HpD is $^1O_2$ but not O_2^-. The $^1O_2$ produced from photosensitized reaction can be detected with the chemiluminescence probe of FCLA. In vivo photosensitized chemiluminescence imaging was performed with a tumor-bearing nude mouse and a clear tumor image was obtained. The photosensitized chemiluminescence mediated by FCLA can be used as a novel PDD method of tumors and has potential applications in clinics for tumor diagnosis.

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