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Enhanced photodynamic effects using fractionated laser light

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Abstract

Photodynamic eradication of tumour cells depends on the presence of a photosensitizer and light delivery to the cells. The present study investigated the influence of fractionated light (on–off mode) on cell killing as documented by a colony-forming assay. Photosensitizers were *m*-THPC (ethanol soluble, Foscan®) and *m*-THPC-MD (water soluble, both from Scotia Pharmaceuticals, Guildford, UK). Fractionated laser light at a wavelength of 652 nm with a light duration of 0.05 s was more effective than continuous illumination at the same power density for both photosensitizers. We propose that fractionated laser light is more toxic due to short phases of recovery during the dark intervals, probably resulting in more singlet oxygen under these conditions. By use of Foscan®, for example, and fractionated laser light, a similar effect is expected for the treatment of solid tumours. In this case we expect improvements in photodynamic therapy (PDT) for patients by lowering the concentrations of photosensitizer and/or by reducing the applied light dose.

Keywords: MCF-7 cells; Colony-forming assay; Photodynamic therapy; Photosensitizers; Chlorin; Laser light; Continuous; Fractionated

1. Introduction

Since the Federal Food and Drug Administration of the USA gave its approval to photodynamic therapy (PDT) as an alternative form of therapy against cancer in 1995, PDT has increasingly been used as a routine clinical application. PDT works using a photosensitizer, which can be applied topically or systemically and is non-toxic to the patient. Toxicity develops only after exposure to light of a defined wavelength and only in the irradiated area. Most work has been done using haematoporphyrin derivatives (HPD) [1–4], but recently improved photosensitizers have been developed. They are already more effective at lower concentrations, and represent pure substances (ZnPC [5–8] or *m*THPC [9–15], for example). Most of these findings were established with continuous delivery of laser light. PDT and effects by using light other than continuously showed partial improvement [16], while others did not see an advantage of fractionation laser light exposure compared to continuous exposures [17–19]. Andreoni et al. [20] irradiated cell cultures after incubation with HPD at 631 nm under continuous exposure and at 337.1 nm using pulsed exposures. The latter method had a more pronounced effect on cell toxicity. The aim of this study was to investigate the effect of both continuous and fractionated light at 652 nm under comparable and well-controlled conditions. We investigated two second-generation photosensitizers (derivatives of chlorin, see Ref. [21] for a review of second-generation photosensitizers) for alternative light application. We established conditions for sub-lethal cell killing by homogeneous light application. Using fractionated laser light we could demonstrate a considerable enhancement of the PDT effect in vitro. A series of preclinical and clinical consequences for PDT may arise because of these findings and these will be discussed.

2. Materials and methods

2.1. Cells

MCF-7, an oestrogen-dependent human breast carcinoma cell line, was used for our studies. Cells were cultivated in Opti-MEM medium (Gibco, Basel, Switzerland) supplemented with 10% foetal calf serum, 25 IU ml^{-1} penicillin and 25 mg ml^{-1} streptomycin. The cells were incubated in a gas incubator at 37°C, 5% CO_2 atmosphere and 100% humidity. After seven to eight days in culture the cells reached confluence and exhibited the features of contact-inhibited monolayers. The monolayers were washed once with Hank's solution (Balanced salts HBSS, Gibco, Basel, Switzerland)

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and dissociated with trypsin EDTA in 25 cm^2 dishes (Falcon, Becton Dickinson UK, Basel, Switzerland) for 3 min at 37°C, 5% CO_2 and 100% humidity. For the colony-forming test, 500 cells were seeded into Petri dishes of 6 cm diameter and incubated 24 h later with either photosensitizer prior to laser irradiation. After light exposure the cells were washed and supplied with fresh medium. 10 days after light exposure the cells were fixed and stained according to the May–Grünwald–Giemsa (azur–eosine–methylene blue) procedure and survival was determined on the basis of a colony-formation assay. For each experiment, five Petri dishes were used and colonies of at least 50 cells were counted under a dissection microscope. The number of colonies was expressed as a fraction of the untreated control.

2.2. Photosensitizer

For these experiments we used two photosensitizers from the second generation, namely ethanol-soluble *meta*-tetrahydroxyphenylchlorin (*m*THPC, Foscan®) and its methoxy-PEG2000 water-soluble derivative (*m*THPC-MD) both from Scotia Pharmaceuticals Ltd., Guildford, UK.

2.3. Laser system

We used a laser system (argon ion laser, Coherent Innova 310, pumping a standing-wave dye laser, Coherent 599, GMP, Renens, Switzerland) which was connected to the experimental PDT laboratory via an optical glass fibre. Petri dishes were mechanically suspended and irradiated from the bottom to avoid light modifications that may originate due to medium covering the cells. Laser light was modified by an interconnected shutter remote control (Uniblitz T132) allowing light fractions in the range of milliseconds. The on and off times were equal for both modes. The fluence rate was 8.80 W m^{-2} for all illuminations.

2.4. Experimental approach

Experimental groups consisted of control cultures (without treatment), Petri dishes containing photosensitizer in the medium but not irradiated, cultures that were irradiated with laser light but without photosensitizer, and finally the test group with the same concentration of the photosensitizer (incubated for 24 h) and with laser irradiation at a wavelength of 652 nm, which interacts with *m*THPC. Sub-lethal doses for MCF-7 cells were determined in order to assess more quantitatively the effects of fractionated laser light. 10 days after irradiation all cultures were chemically fixed and stained according to the Giemsa–May–Grünwald procedure.

2.5. Statistical methods

Each experiment consisted of five Petri dishes. The counted colonies were statistically analysed by the Mann–Whitney U-test. Each experiment was repeated four times with each photosensitizer.

3. Results

3.1. Preceding conditions for the experiments

Based on previous results we established conditions for sub-lethal killing of the cells [22]. These conditions were essential in order to establish significant differences between the two methods of illumination. MCF-7 cultures were incubated in medium containing 2.0 μg ml^{-1} of the photosensitizer *m*THPC for 24 h. Dark toxicity was tested in parallel and was not found to be significantly enhanced for *m*THPC (Fig. 1). The effect of laser light alone was tested as well prior to the experiments. Although not significantly different, a tendency of enhanced cell killing was observed when fractionated light doses of 2.7 J cm^{-2} were applied (data not shown).

3.2. Experiments with mTHPC

Both continuous and fractionated laser light (duration of illumination time 0.05 s, followed by a dark phase of the same length) were given for 5 or 2 min. Compared to the untreated controls, both fractionated and continuous light of a duration of 5 min induced 100% cell killing (data not shown). A difference was observed when illuminating for 2 min (Fig. 1). In all experiments, the total illumination time of both continuous and fractionated light applications was identical. Here the fractionated form had a significantly higher effect. The same effect, but less prominent, was observed with shorter light durations (Table 1).

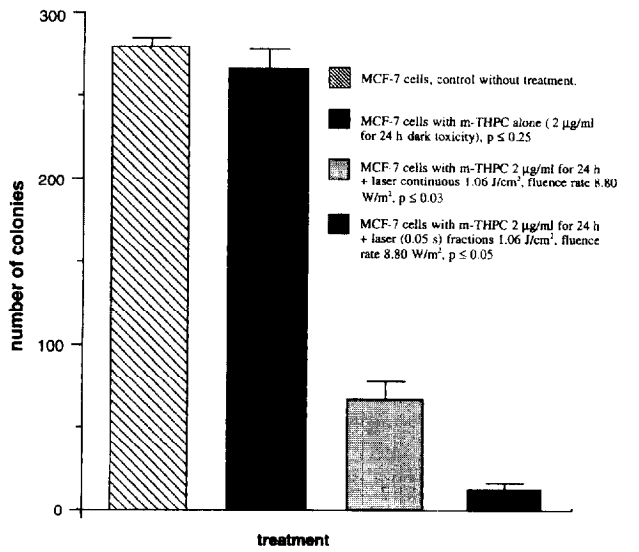

Fig. 1. Effects of continuous and fractionated laser irradiation on MCF-7 cells using *m*-THPC as photosensitizer. An untreated control was used for each experiment. Each experiment was performed four times under the same conditions.

Table 1
Survival of MCF-7 cells after continuous and fractionated illumination

Treatment	Average value	Survival (%)	p
Control	275 ± 6	100	
m-THPC 2 μg ml^{-1} (4 h) + laser 2 min contin. (1.06 J cm^{-2})	88 ± 10	32	≤0.03
m-THPC 2 μg ml^{-1} (4 h) + laser fract. 0.05 s (1.06 J cm^{-2})	60 ± 4	21.8	≤0.05
m-THPC 2 μg ml^{-1} (4 h) + laser fract. 30 s (2 min) (1.06 J cm^{-2})	70 ± 13	25.4	≤0.03
m-THPC-MD 2 μg ml^{-1} (4 h) + laser 5 min contin. (2.7 J cm^{-2})	209 ± 4	75.9	≤0.03
m-THPC-MD 2 μg ml^{-1} (4 h) + laser fract. 0.05 s (5 min) (2.7 J cm^{-2})	180 ± 6	65.4	≤0.03

3.3. Experiments with mTHPC-MD

Investigations with *m*THPC-MD were carried out under analogous conditions. A short incubation time of 4 h with this photosensitizer showed more pronounced effects with fractionated laser light than continuous light delivery (Table 1). Following prolonged incubation time (24 h) and illumination for 2 min with either continuous or fractionated light, the PDT effect was more pronounced and very similar results were obtained to those from experiments with *m*THPC (Fig. 2).

4. Discussion

Fractionated energy sources or energy pulses are known from biological experiments and from radio-oncological treatments. Tumours are treated more effectively with fractionated X-ray irradiation rather than with single-dose treatments [23]. Concerning our experiments with fractionated light application during PDT, we made use of previous experiments describing the biological effects of the two photosensitizers in vitro [22,24]. In fact a slightly higher dark toxicity of *m*THPC-MD compared to mTHPC was found in the experiments here. A longer incubation time with *m*THPC-MD in vitro was needed compared with *m*THPC. For example, when the incubation time with *m*THPC-MD was increased up to 24 h, the PDT effect under given conditions was similar. This could be caused by the altered nature of the chlorin molecule, namely, its water solubility, due to the attached polyethyleneglycol chain. Basic mechanisms for this behaviour, however, are not yet understood.

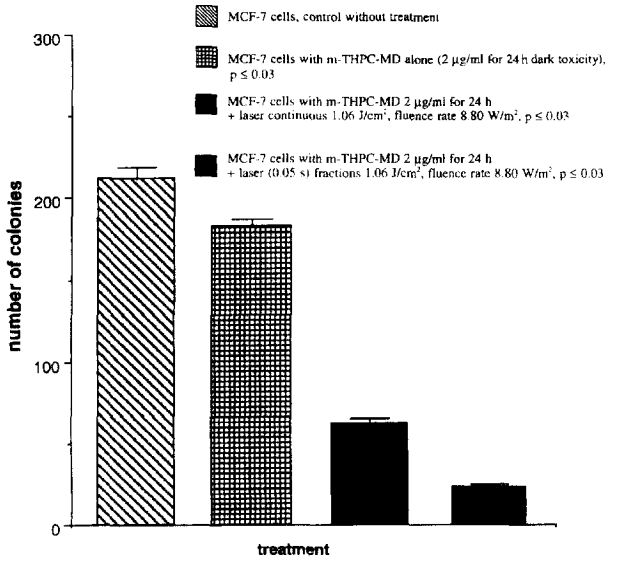

Fig. 2. Effects of continuous and fractionated laser irradiation on MCF-7 cells using *m*-THPC-MD as photosensitizer. An untreated control was used for each experiment. Each experiment was performed four times under the same conditions.

Using sub-lethal conditions in vitro we demonstrated that fractionated light (on–off cycles) was significantly more efficient in cell killing compared to continuous light applied at the same power density (Figs. 1 and 2). With both kinds of photosensitizers, *m*THPC and *m*THPC-MD, 0.05 s fractions have been shown to improve PDT significantly. Even light fractions of a duration as long as 30 s were more effective than continuous light application, but those of 0.05 s duration seemed to be best. These findings are in agreement with the observation of Andreoni et al. [20], although their pulsed lasers had a wavelength different from that of continuous light. We are far from understanding the basic mechanism for the enhancement of PDT activity through light fractionation. One possible explanation may be the partial recovery of targeted cells between light pulses. During exposure this short recovery time may lead to a more toxic effect due to greater production of singlet oxygen and/or free radicals. Photochemically generated singlet oxygen, however, is the main component responsible for cytotoxicity induced by PDT [25]. This will be a matter for further investigations, including studies with cell organelles such as the mitochondria. We recently found that mitochondria from normal rat liver were heavily affected under PDT conditions and using *m*THPC [26]. It may well be that fractionation protocols will be developed that are even more effective in enhancing a given PDT effect. In a theoretical study related to vascularized tissues, optimal fractionation times ranged between 30 and 60 s and a strong relationship with the distance between capillaries and cells was claimed [27]. The fact, however, that PDT conditions in vitro are significantly improved due to shorter fractions of light is very promising for future clinical applications in PDT. This might be especially true for Foscan®, which is already in clinical use. If similar improvements can be found in vascularized solid tumours in animal

models or during clinical treatment, advantages are obvious. First, a potential reduction in the amount of photosensitizer per kilogram body weight may be possible. This will be important because lower concentrations will enable the treatment to be used for more patients, as higher concentrations may be associated with additional health problems. The effects of a range of conditions of light delivery including fractionation or otherwise modulated light need to be established in greater detail. The mechano-electronic laser-light shutter systems applied in this study are limited and new kinds of laser sources for PDT are needed. A promising combination may consist of a powerful diode laser system, delivering computer-controlled laser fractions.

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