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1．研究者氏名 金朝暉

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研究テーマ 実験皮膚腫瘍に対する光力学療法と超音波力学的療法の併用効果の研究

2．本年度の研究業績

(1) 学会・研究会等においての口頭発表 (有) ・ 無 (学会名・内容)

1. 色素性母斑の一種か？ 第14回日本皮膚病理組織学会，1998.7，東京．

2. 新しい腫瘍親和性物質を利用した診断法．第19回日本レーザー医学会大会，1998.9，東京．

3. 光力学的癌治療での活性酸素生成とアポトーシス誘導の相関について．第57回日本癌学会総会，1998.9，横浜．

(2) 学会誌等に発表した論文 (有) ・ 無 (雑誌名・論文名)

Porphyrins，7(2-3)，145-149，1998：Intracellular distribution of 5-aminolevulinic acid (5-ALA) produced protoporphyrin-IX (Pp-IX) in a cultivated cell and the 5-ALA-based photodynamic therapy (PDT) of squamous cell carcinoma (SCC).

3．今後の研究計画

新しい光増感剤の外用剤を開発し，より腫瘍親和性に富み，外来でも治療可能な簡易で有効な光力学的治療法を確立する．

それと共に光力学的診断法を各皮膚疾患に対して試行し確立する．

4．研究指導者の意見

金朝暉氏の行っている光力学療法は皮膚癌の治療法として手術療法に匹敵する有力な手段となりうることが期待されている．そのためより安全でより確実な方法を得るための基礎的な研究が続けられている．今後の成果がおおいに期待できる

研究指導者氏名　熊切正信

5．研究報告

別紙形式を参考に、報告本文4000字以上で報告して下さい（枚数自由・ワープロ使用）

タイトル・要旨等は日本語で、KEY WORDS以下は日本語或いは英語で記入して下さい。

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研究テーマ

実験皮膚腫瘍に対する光力学療法と超音波力学的療法の併用効果の研究

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要旨

光増感剤とレーザー光照射による光力学療法(photodynamic therapy, PDT)は、表在性皮膚腫瘍に対して有効な治療法となりうることが報告されている。しかし、浸潤性皮膚腫瘍には PDT の治療効果は少ない。近年超音波増感剤と超音波照射による癌治療の試みが提唱され、超音波力学的療法(sonodynamic therapy, SDT)とよばれている。そこで今回、扁平上皮癌組織を C3H マウスの背部に移植した実験動物腫瘍を用いて、PDT と SDT のそれぞれ単独および併用療法とを施行し、比較検討した。その結果、単独療法群と比べ併用療法群では腫瘍の退縮が顕著であり、延命効果が認められた。組織学的に深部の腫瘍組織の壊死が確認された。PDT と SDT の併用療法は有効な皮膚癌治療法の一つとなりうると考えられる。

KEY WORDS

photodynamic therapy; sonodynamic therapy; cancer therapy

研究報告

INTRODUCTION AND PURPOSE

Photodynamic therapy (PDT) is a non-thermal and non-invasive tumor treatment modality, which involves administration of a tumor-localizing photosensitizer and subsequent irradiation of the tumor with visible light (usually laser light). This process results in excitation of the sensitizer molecules to generate active oxygen species, especially singlet oxygen, which are responsible for tumor necrosis. The major advantage of PDT is the selective effect on tumor

tissues while with minimum destruction of normal tissues because of the preferential accumulation of the photosensitizer into tumors and local irradiation of a low output power of light. In addition, it usually has a cosmetic outcome (Svanberg et al, 1994; Harth et al, 1998) since the healing after PDT is mainly a process of regeneration rather than scarring (Barr et al, 1987). So far, PDT has been increasingly used for skin cancers including Bowen's disease, basal cell carcinoma and squamous cell carcinoma (SCC) (Dougherty et al, 1978; Dahlman et al, 1983; Kennedy et al, 1990; Cairnduff et al, 1994; Svanberg et al, 1994; Baas et al, 1996; Stables et al, 1997). However, successful application of it has been limited to superficial lesions (Cairnduff et al, 1994; Svanberg et al, 1994; Regula et al, 1995; Peng et al, 1995; Gossner et al, 1998). Light penetration may be an important factor influencing the depth of PDT-induced tumor necrosis. Unfortunately, when laser beam passes into the deeper tissue, the light attenuation is unavoidable. To overcome this problem interstitial fibers can be used, but with a technical difficulty of placing the fiber to achieve an equal irradiation throughout the tumor. Another approach to killing the tumor cells in the deeper level is to use higher light fluence rate because of a fluence rate dependency of the laser penetration into tissues. However, when it was practically applied, a decreased PDT effect was observed, possibly due to the oxygen depletion (Foster et al, 1991). Besides modifying PDT itself, the combined use of PDT with other forms of treatments such as hyperthermia, radiotherapy and chemotherapy has been explored (Creekmore and Zaharko, 1983; Waldow and Dougherty, 1984; Nahabedian et al, 1988; Matsumoto et al, 1990; Prinsze et al, 1992). Though synergistic effects were reported, such combinations could also potentiate the damage of normal tissues. Furthermore, the results are still preliminary.

Unlike laser light, ultrasound has a good tissue penetrating ability, reaching the deeper areas while maintaining its energy. It has been used in the treatment of cancer, with the results varying greatly (Kremkau, 1979). Using a combination of ultrasound and a chemical for tumor control is a relatively new modality which is referred to as "sonodynamic therapy" (SDT) (Umemura et al, 1989, 1992). Such a combination could induce a synergistic cytotoxic effect both in cell culture and experimental tumor studies (Tachibana et al, 1993; Umemura et al, 1993; Yumita et al, 1990, 1996; Miyoshi et al, 1997; Uchida et al, 1997). The typical chemicals used in SDT are photosensitizers, and also called sonodynamic sensitizer (sonosensitizer). Yumita and colleagues (1990, 1996) reported that the growth of implanted tumors in mice could be significantly inhibited by SDT with a photosensitizer (hematoporphyrin (Hp) or a gallium porphyrin analogue (ATX-70)), while Hp or ATX-70 alone had no inhibitory effect and ultrasound alone showed a small inhibitory effect. From the above, it was thought that an efficient destruction of tumor tissues in the deeper areas might be achieved by combining SDT with PDT because of the better tissue penetrating ability of ultrasound. Using murine leukemia L1210 cells incubated with mesoporphyrin, an agent having both photodynamic and sonodynamic activity (Kessel 1977; Kessel et al, 1994), Kessel and coworkers (1995) examined the interactions between PDT and SDT. They found an enhanced cytotoxic effect by a combined use of the two modalities. However, there have not yet been any reports concerning such a combination in vivo.

Using transplanted SCC in C3H/He mice, this study was designed to investigate the combined effect of PDT and SDT on tumor growth, survival of mice, and depth of induced tumor necrosis, aiming at exploration of a new modality for nodular tumors, which show poor responses to the conventional PDT. In order to determine whether the combined effect depends on the type of sensitizer used, two different chemicals, PH-1126 (a pheoforbide derivative) and ATX-70 (a gallium porphyrin analogue) were used, respectively. The former drug has been developed in Japan as a new potent photosensitizer (Saito et al, 1996), and the latter one, initially being described as a photodynamic sensitizer (Nakajima et al, 1989), is so far most often used as a strong sonosensitizer (Umemura et al, 1992; Kessel et al, 1994). Moreover, our preliminary studies (unpublished data) showed that both of the two agents were active not only for PDT and also for SDT, using the same tumor model as in the present study.

MATERIALS AND METHODS

Sensitizers

Pheoforbide Derivative

PH-1126 (molecular weight = 666) was obtained as a powder from Hamari Chemical Ltd. (Osaka, Japan). It was mixed with same weight of DL-tartaric acid powder ([CH (OH) $COOH]_2$ = 150.09, Nacalai Tesque, Inc., Kyoto, Japan), and dissolved in 5% dextrose aqueous solution (for iv-injection, Otsuka Seiyaku Ltd., Naruto, Japan) by grinding the powder of these two agents to form a tartaric acid salt of PH-1126 at a concentration of 1 mg/ml as a stock solution.

Gallium-Porphyrin Analogue

ATX-70 (7,12-bis (1-decyloxyethyl)-3,8,13,17-tetramethyl-porphyrin-2,18-bispropyonylasparginic acid gallium (III) salt; purity> 95%) was a generous gift from Dr. Isao Sakata, Toyo Hakka Kogyo Ltd., Okayama, Japan. It was dissolved in physiological saline at a concentration of 1 mg/ml as a stock solution.

PH-1126 and ATX-70 were stored in the dark at - 4 °C and each stock solution was made just before administration.

Animals and Tumors

Male and female C3H/He mice (Charles River, Osaka, Japan) were used in this study. They were housed at room temperature with a 12 hr light/dark cycle and allowed free access to water and food. The tumor throughout was a squamous cell carcinoma (SCC), which developed spontaneously in a C3H/He mouse (Urano et al, 1976), and was maintained by serial subcutaneous passage in isologous C3H/He mice. Nonnecrotic tumor material for implantation was obtained by sterile dissection of tumors from donor mice and was cut into small fragments (about 1 mm^3). Single tumor fragment was transplanted subcutaneously into the right flank of recipient C3H/He mice (6-8 week old, weighing 20-25 g). Mice were allocated for treatment when their tumors reached 7-10 mm in a diameter. At this size, tumor was well vascularized and spontaneous necrosis was minimal. Before irradiation the skin overlying the tumor and surrounding area was closely shaved with electric clippers and depilatory, and tumors with overlying skin being pigmented were excluded from the study. Irradiations were performed under intraperitoneal (i.p.) anesthesia (pentobarbital 65 mg/kg body weight).

Laser Light Delivery Systems

Two types of laser systems were utilized, a pulsed (50 Hz) YAG-Optical-Parametric-Oscillator (OPO) laser system and a Krypton ion laser system. The OPO laser system (Ishikawajima-Harima Heavy Industries Co., Ltd. [IHI], Tokyo, Japan) is newly developed with an easily tuning wavelength range of 620-670 nm. It was tuned to emit light irradiation at 650 nm for the SCCs at the optimum accumulation time (36 hr) (Ishihara et al, 1991) after administration of PH-1126. The Krypton ion laser system (Model LI-2530A, Toshiba Ltd., Yokohama, Japan) was used as the light source at 575 nm for irradiation of the SCCs at the optimum accumulation time (24 hr) (Yumita et al, 1996) after ATX-70 injection. The wavelengths of 650 nm and 575 nm correspond to the maximum light absorption peak of PH-1126 and ATX-70, respectively. Light from these two types of laser was delivered via a single optical fiber and the output end of the fiber was positioned to focus the laser irradiation into a uniform light spot with 1.0-cm diameter. The total light dose is expressed as joules/square centimeter (J/cm^2).

Ultrasound Irradiation System

The ultrasound irradiation system consists of four parts, including generator, amplifier, oscilloscope and transducer. Sine waves were generated by the generator (Model MG442A, Anritsu Electric, Tokyo) at a frequency of 1.0 MHz, 37 ± 1W/50 Ω, and 44 ± 1Volts, and amplified by the power amplifier (Model 240L, ENI, INC, Rochester, NY, USA). Driven by the sine waves, the transducer (supplied from Hitachi Central Research Laboratory, Tokyo, Japan), 12 mm in diameter, delivered ultrasound at an output acoustic power of 0.51 W/cm^2. The oscilloscope (V-252, 20 MHz, Hitachi, Japan) could monitor the driving waves during ultrasound irradiation. Figure 1 is an illustration of tumor-bearing mice being exposed to ultrasound irradiation by placing the transducer in contact with the skin overlying the tumor while with the base of tumor being fixed on a steel platform. During practical treatments, ultrasound gel was used to serve as contact medium between the transducer and the tumors, and the aluminum matching layer of the transducer was cooled by circulating water to keep the transducer and the tumors temperature below the hyperthermal level (< 42 °C).

Treatment Protocol

The combination effect of PDT and SDT was investigated using PH-1126 or ATX-70 as a sensitizer. In the case of PH-1126, the tumor-bearing mice were divided into four groups, which were classified by treatment modality as: control (n = 5), PDT alone (n = 7), SDT alone (n = 6), and PDT+SDT (n = 8). The control mice received neither PH-1126 injection nor irradiation of laser light or ultrasound. The other three groups of mice were i.p. injected with PH-1126 (5 mg/kg) and, 36 hr later, were exposed to laser light of 650 nm (PDT), ultrasound (SDT), or laser light immediately followed by ultrasound (PDT+SDT), respectively. The total light dose of 44 J/ was used for both PDT alone and the combination therapy.

For ATX-70, similar treatment protocols were applied with group size of 5-6 mice. At 24 hr before irradiation, ATX-70 was given i.p. to mice at a dose of 5 mg/kg. The laser light was delivered at a total dose of 88 J/ cm^2 of 575 nm.

After treatments, the mice were housed in a room with subdued lighting, and the macroscopic changes of tumors were observed and photographed.

Evaluation of Tumor Growth and Survival of Mice

Following PDT and/or SDT, each tumor was measured daily by means of a caliper in the first week and afterwards every other day until day 20 post irradiation. By assuming a hemiellipsoidal structure for the tumor nodule, individual tumor volumes (V) were calculated using the following formula: V (cm^3) = (π/6) L X W X H (L = length, W = width, H = height) (Mukhtar et al, 1991; Peng and Moan, 1995). The survivals of each group of mice were recorded until day 120 post irradiation, and the survival time was defined as the time from the irradiation day to death.

Histological Studies

For microscopic observation of the depth of necrosis, we sampled tumors 72 hr after irradiation, which was considered to be the time of maximum necrosis, based on the macroscopic findings (described in the results) and tumor growth curves (Figs. 3A and 4A). Using PH-1126 or ATX-70, the combination therapy and the more efficient one of single treatments (PDT with PH-1126 or SDT with ATX-70) were repeated again on additional tumor-bearing mice. Untreated tumors were also sampled as control. The removed tumors were fixed in 10% formalin solution, processed for routine paraffin embedding, cut in sections (5 μm thick), and stained with hematoxylin-eosin.

Statistical Analysis

Statistics were performed using the StatView 4. 11 software (Abacus Concepts Inc., Berkely, CA). Tumor volumes and survivals of mice in the different experiment groups were compared, respectively. Tumor volumes were presented as the mean ± SD (SD = standard deviation), and the survivals of mice were shown as the mean survival day (MSD) ± SD. Data were analyzed by a nonparametric ANOVA, and followed by a Fisher's multiple comparisons test for tumor growth study, or by a generalized Wilcoxon test for survival study. Statistical significance was achieved at $P \leq 0.05$.

RESULTS

Macroscopic Findings

Before treatment tumors looked healthy as shown in Fig. 2A, and immediately after illumination the treated area did not show noticeable changes or exhibited little edema (Fig. 2B). Afterwards, different degrees of edema and necrosis were the two main changes generally found in all treated tumors. The edema was present in tumor and surrounding skin within the illumination field, being most pronounced 12 hr after treatment and resolved over the subsequent 36 hr. The necrosis was restricted to tumor area, being observed 24 hr after irradiation and reaching its maximum degree at 72 hr post-irradiation. The most severe tumor necrosis was found in the combination of PDT and SDT, especially with PH-1126, with four of total eight tumors being replaced by a necrotic slough or an ulcer (Fig. 2C). In any single treatment groups, no tumor was completely replaced eradicated, and the general findings consisted of a dense necrosis crust or an ulcer on the surface of tumors.

The normal skin distant from the irradiation area did not show any obvious changes (Figs. 2C), and symptoms of systemic toxicity were not observed in all treated mice.

Effects of Combination of PDT and SDT with ATX-70 on Tumor Growth and Survival of Mice

Figure 1A shows the growth curves of the tumors after PDT and/or SDT with ATX-70. There were no statistical differences between tumor volumes of any of the experimental groups (Control, PDT, SDT and PDT+SDT) before treatments. Statistical analysis was performed on the data obtained from day 3, at which time the tumor necrosis was maximum. At all time points from day 3 to 20 post-irradiation, significant differences ($P < 0.05$-0.0001) in tumor volume were obtained between any two of the four groups. Twenty days after treatment, the mean tumor volume of the PDT+SDT group, PDT group, SDT group and control group were 0.141 (SD 0.021), 1.339 (SD 0.111), 0.427 (SD 0.071), and 1.835 (SD 0.199) cm^3, respectively. Based on the above, the tumor growth inhibition ratios were 92.3, 76.7 and 27% for the PDT+SDT, SDT and PDT groups, respectively, demonstrating the antitumor efficiency in the following order: PDT+SDT >SDT > PDT. However, the combined effect of PDT and SDT is not shown to be greater than the arithmetic sum of the effects of PDT alone and SDT alone (Fig. 3A). Figure 1B shows the survival curves of the treated mice. Mean survival was significantly prolonged in the group treated with PDT+SDT (MSD ± SD = 105.8 ± 21.1 days), as compared with that of the control group ($P = 0.044$, MSD ± SD = 77.8 ± 12.5 days). Compared to control, SDT alone led to a minor survival prolongation ($P > 0.05$, MSD ± SD = 99.0 ± 24.5 days), while PDT alone did not lead to a survival prolongation ($P > 0.05$, MSD ± SD = 80.3 ± 13.7 days). Significant difference in mean survival time was also not obtained between PDT+SDT and either single therapy. As shown in Fig. 3B, none of control and single PDT groups of mice could survive to day 120, and 40% of single SDT group of mice (n= 5) remained alive at this time. However, the

combination therapy increased the 120-day survival to 60% of five mice.

Effects of Combination of PDT and SDT with PH-1126 on Tumor Growth and Survival of Mice

Figure 2A shows the growth curves of the tumors after PDT and/or SDT with PH-1126. There were no statistical differences between tumor volumes of any of the experimental groups (Control, PDT, SDT and PDT+SDT) before treatments. For the same reason as in the case of ATX-70, statistical analysis was performed on the data obtained from day 3. At all time points from day 3 to 20 post-irradiation, significant differences ($P < 0.001$-0.0001) in tumor volume were obtained between any two of the four groups. On day 20 after treatment, the mean tumor volume of the PDT+SDT group, PDT group, SDT group and control group were 0.041 (SD 0.042), 0.454 (SD 0.112), 1.068 (SD 0.141), and 1.859 (SD 0.082) cm^3, respectively. Based on the above, the tumor growth inhibition ratios were 97.8, 75.6 and 42.5% for the PDT+SDT, PDT and SDT groups, respectively, demonstrating the antitumor efficiency in the following order: PDT+SDT >PDT > SDT. However, it is also shown that the combined effect of PDT and SDT was not greater than the arithmetic sum of the effects of PDT alone and SDT alone (Fig. 4A). Figure 2B shows the survival curves of the treated mice. The mean survival time of PDT+SDT group (117.6 ± 6.8 days) was significantly greater than either that of control (81 ± 15.7 days, $P = 0.002$), that of PDT alone group (93.6 ± 21.9 days, $P = 0.019$), or that of SDT alone group (81 ± 18.1 days, $P = 0.003$). Compared to control, either single therapy could not enhance the survival of mice ($P > 0.05$). As shown in Fig. 4B, none of control and single SDT groups of mice could survive to day 120, and only 28% of single PDT group of mice (n= 7) were still alive at this time. However, the combination therapy increased the 120-day survival to 88% of eight mice.

Effects of PH-1126 Dose on Tumor Growth and Survival of Mice

We previously investigated the effects of single PDT using 10 mg/kg PH-1126 on tumor growth and survival of mice using the same animal tumor model (unpublished data). As an aid to comparison of PH-1126 dose effects, the data using 10 mg/kg PH-1126 were plotted in Figure 4A and B, respectively. Except for the drug dose, the light irradiation conditions were same as in the present experiment. Before irradiation, there were no statistical differences between tumor volumes of the experiment groups. Twenty days after treatment, mean tumor volume of the PDT (10 mg/kg PH-1126) group was 74.4% lower (0.116 ± 0.012 cm^3) than in PDT (5 mg/kg PH-1126) group (0.454 ± 0.112 cm^3, $P < 0.05$), but 64.6% higher than PDT+SDT (5 mg/kg PH-1126) group (0.041 ± 0.042 cm^3, $P > 0.05$). The data in Figure 4B indicate that within 72 hr after light irradiation, 5 of 8 mice (62.5%) treated with 10 mg/kg PH-1126 died, while no death was found in mice after single PDT or the combined use of it with SDT using 5 mg/kg PH-1126.

Histological Alterations

All tumors were sampled 72 hr post irradiation. Control slides showed the usual tumor architecture without obvious necrosis, with the tumor blood vessels being intact (Figs. 5A and a). In the tumors after single PDT with PH-1126, the superficial parts became completely necrosis with obvious hemorrhage and thrombi (Fig. 5B), while the base did not show obvious necrosis, though hemorrhage and dilated vessels were obvious (Fig. 5b). The tumors after single SDT with ATX-70 showed similar changes, but with lesser vascular changes compared with PH-1126 (not shown). In the tumors after the combination therapy with PH-1126, the major portion of tumors showed necrosis extending from the surface to its base with hemorrhage (Fig. 5C), but in the base less amounts of viable tumor cells still existed with neighboring vessels showing hemorrhage and organization (Fig. 5c). Being same as PH-1126, the combination therapy with ATX-70 also showed similar phenomenon but with no obvious microsculature alterations (not shown).

By comparing macroscopic and microscopic changes 72 hr after irradiation, it was found that though some tumors were determined to disappear with the naked eye or became

nonpalpable, actually undamaged tumor tissues still remained.

DISCUSSION

When a combination cancer therapy is designed, besides the aim of obtaining additive or synergistic antitumor effect, the selectivity of the effect must be considered. In respect of this point, the combination of PDT and SDT is therefore very attractive since both of them have relatively high tumor selectivity by using a tumor-localizing sensitizer and a limited area of laser light or ultrasound irradiation. In addition, using one agent as a common sensitizer for PDT and SDT is also a characteristic of this combination. As we know, PDT is a process of activation of a sensitizer by laser light, while SDT is initiated by the interaction of a sensitizer and ultrasound (Yumita et al, 1990; Umemura et al, 1992). Before laser light irradiation, a tumor-localizing sensitizer is in the ground state. On absorption of light, it can be brought to an excited triplet state. But this triplet species have an extremely short lifetime (millisecond range), and decay quickly back to the ground state (Van Hillegersberg et al, 1994). Hence, after exposure to laser light, the sensitizer maybe just experience the photochemical modifications which do not influence its other properties such as sonodynamic activity.

In the results, the combination of PDT and SDT, using either ATX-70 or PH-1126 as a sensitizer, was found to yield a greater antitumor effect than PDT alone and SDT alone. However, it was also found that the combined effect of PDT and SDT is not greater than the arithmetic sum of the two individual effects, using either of the two agents (Figs. 3A and 4A). Based on the above, it is suggested that additive effects could be obtained by the combined use of PDT and SDT, not depending on the type of the sensitizer, strong PDT or SDT agent. Considering treatment mechanisms, the present result has an inclination of a similar mechanism of action for PDT and SDT, which seems to contrast with the in vitro study by Kessel' group. By comparing the modes of photodynamic versus sonodynamic cytotoxity with murine leukemia L1210 cells in culture, they suggested clearly different mechanisms involved in the two modalities (Kessel et al, 1995, 1996). However, in vitro study can not involve tumor microvasculture, which has been reported to be an important target of in vivo PDT (Nelson et al, 1988). Our histological studies also suggest that PH-1126 and ATX-70 maybe have different actions on tumor vessels, possibly due to their distributions in tumor. Taking into account the tumor-killing agents, it is generally believed that photodynamic cell damage occurs primarily as a result of singlet oxygen formation via photosensitized oxidation (Valenzeno, 1987; Foote, 1991; Henderson and Dougherty, 1992), while cytotoxic agent involved in SDT remains to be established. Miyoshi and coworkers (1995) reported hydroxide radicals rather than singlet oxygen could be detected during sonodynamic action by using ATX-70 in aqueous solutions exposed to ultrasound with electron paramagnetic resonance (EPR) method. It seems that different cytotoxic agents are involved in PDT and SDT, however, this different radical from singlet oxygen can also be explained to be just intermediates during sonochemical reaction.

Although a better understanding of action mechanisms is very helpful for optimization of PDT parameters, the key to getting a cancer cure using this modality lies in matching the depth of necrosis to the depth of tumor (Fan et al, 1997). However, with the increasing depth of tumor, the red light used for PDT unavoidably gradually attenuates, so when reaching the deeper layer, it is not sufficient to induce a photochemical reaction with a sensitizer beyond the threshold for PDT necrosis. The microscopic results showed that the PDT-induced necrosis was largely superficial, though the base of tumors sensitized with PH-1126 demonstrated obvious vascular damage (Figs. 6A and B). A unique advantage of SDT is its potentiality for deep-seated lesions since the ultrasound used has a good tissue penetrating ability. When combined with SDT, as shown in the present study (Fig. 7B), the treatment-induced necrosis involved even the base of the tumors. This phenomenon may be explained by that the subsequent SDT could compensate

for the decreased PDT effect on the deeper tissues. By microscopic observation, it was also hoped that a relatively homogeneous effect of SDT on tumor tissues could be shown because ultrasound can maintain its energy into deep area. Unexpectedly, the most observable result of SDT is a superficial necrosis, which is beyond our explanation. Different from this result, other researchers reported a significant necrosis in the major part of the tumor tissue after the ATX-70-based SDT using a Colon 26 tumor implanted in the mouse kidney (Yumita et al, 1997). In their experiment, 500 kHz and 1MHz ultrasound were combined, and delivered in a progressive wave mode.

Analyzing the tumor growth curves combined with the microscopic alterations, it is implied that the tumor necrosis and shrinkage reflect the acute effects of PDT and/or SDT, thereafter, tumor cell proliferation may be affected by a delayed effect. This after action can be clearly demonstrated by the growth curve of the tumors treated with the combination of PDT and SDT using ATX-70 (Fig. 3B). Within 5 days after irradiation, the mean tumor size gradually decreased, and then followed by the tumors remaining at this size for about 4 days, indicating a period of stasis in tumor cell proliferation.

As a confirmatory evidence for the local treatment effect, the survival study was designed. The tumor models used in the present experiment are SCCs transplanted in mice. After tumor transplantation, the tumor-bearing mice without treatment usually survive for 3 months before dying. The death was considered to be as a result of an extensive local tumor because autopsy did not show any obvious metastasis. This study shows that the combination therapy using either ATX-70 or PH-1126 could significantly prolong the survival of mice, demonstrating that strong inhibition of tumor growth can also result in a survival advantage. The improved survival of mice also corroborates the enhanced therapeutic effect by the combination of PDT and SDT. In addition, it is shown that the combined use of PDT and SDT did not increase the systemic toxicity.

By means of the combination of PDT and SDT, we also hoped to use a relatively low sensitizer dose while without reducing the antitumor efficiency. In our previous experiments using the same mouse tumor model (unpublished data), PDT with 10 mg/kg PH-1126 and 88 J/cm^2 laser light resulted in 100% mortality of mice. Reducing the light dose to 44 J/cm^2 could result in good tumor control, but still caused mortality with a percentage of 62.5% (Figs. 4A and B). The dose of PH-1126 (10 mg/kg) was thought to be high enough to produce systemic toxicity in the form of acute lethality. So, in the present study we selected a dose of 5 mg/kg of PH-1126. Comparing the effect of PH-1126 dose (10 versus 5 mg/kg), it was found that using lower dose could avoid mortality but resulted in a significantly reduced PDT efficiency. It suggests that the efficiency of PH-1126-based PDT is strongly dependent upon PH-1126 dose. The lower the dose used, the lower the PDT efficacy achieved. Figure 4A also shows that when combined with SDT, PDT with 5 mg/kg PH-1126 has a slightly but not significantly stronger inhibition effect on tumor growth than PDT alone using 10 mg/kg PH-1126. From the above, an important implication is that when SDT is combined with PDT, lesser amounts of sensitizer could be used while without decreasing the treatment effect. This finding has considerable clinical value because at present the major side effect of PDT in clinical practice is a drug dose-related skin photosensitivity which usually lasts for at least 4-6 weeks, and restricts patients from outdoor activity (Dougherty et al, 1990; Ris et al, 1992).

In the present study, the doses of sensitizer (PH-1126 and ATX-70), laser light and ultrasound were chosen based on our preliminary experiment results as well as other previous studies (Saito et al, 1996; Yumita et al, 1996), not producing any apparent signs of toxicity while achieving a comparable antitumor efficiency. In addition, the doses of laser light for PH-1126 (44 J/cm^2) and ATX-70 (88 J/cm^2) were used low enough that thermal effect could be precluded (Suzuki et al, 1987), though it has been reported that hyperthermia may potentiate PDT (Kinsey et al, 1983; Glassberg, 1991). The reason for undesired thermal effect is that until the actions of PDT and SDT are more fully understood, it is better to restricts studies to situations in which only these two modalities are investigated at a time.

In conclusion, with a mouse SCC model, PDT and SDT exhibited an additive mode of interaction, using either PH-1126 or ATX-70 as a sensitizer. In spite of not achieving a synergistic effect, for nodular tumors the two modalities should be combined because such a combination could increase the depth of tumor necrosis as well as without increasing destruction of normal tissue. When combined with SDT, PDT could allow a low dose of sensitizer, thus decreasing the risk of generalized skin photosensitivity. However, based on the tumor growth curves and microscopic studies, a complete tumor cure could not be achieved in this experiment. This failure suggests that the treatment parameters used here are not optimum, or that the mouse SCC may be a resistant tumor model. For residual tumor cells, PDT and/or SDT can be repeated because such treatment does not cause cumulative toxicity.

Finally, although an argon-pumped dye laser is the system most often used in PDT, but it is clinically not easy to handle, and its optical parts need continued replacement and maintenance. Thus, there is a need for new laser system to be more suitable for clinical practice. In the current study, we used a newly developed OPO laser system, which is tunable between 620-670 nm, as a light source for PH-1126, and found it is simple and easy to manipulate. The present PDT results indicate the potentiality of the OPO laser system as a candidate for the argon-dye laser system. In addition, our group originally used the ultrasound transducer, and the present SDT results demonstrate its value for further study. ,

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