High expression of GADD-45α and VEGF induced tumor recurrence via upregulation of IL-2 after photodynamic therapy using NPe6

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Abstract. NPe6 is a novel second-generation photosensitizer used for photodynamic therapy (PDT). PDT using NPe6 and diode laser (664 nm) induces cell death, inflammatory reactions, immunological responses and damage to the microvasculature. In this study, we evaluated the influence of the immunological responses and of enhanced angiogenesis on the anti-tumor effect of NPe6-PDT using cytokine-over-expressing Lewis lung carcinoma (LLC), LLC-IL-2 cells both *in vitro* and *in vivo*. We showed by DNA microarray analysis *in vitro* that IL-2 and GADD-45α (growth arrest and DNA damage 45 alpha) mRNA expressions were induced by 3 h after NPe6-PDT applied at a dose killing 90% of the cells (LD_{90}). IL-2-overexpressing cells (LLC/IL-2 cells) were resistant to the loss of clonogenicity as compared to the parental LLC cells *in vitro*. Furthermore, in female C57BL/6 mice, NPe6-PDT produced a cure rate of 66.7% in LLC tumors, whereas the cure rate was only 16.6% in LLC/IL-2 tumors, and overexpression of IL-2 caused failure of NPe6-PDT, with tumor recurrence, *in vivo*. These results suggest that IL-2 expression may play an unfavorable role in attenuation of the antitumor effect of NPe6-PDT. It has been reported that the expression of vascular endothelial growth factor (VEGF), in particular, may cause tumor recurrence after PDT and exert unfavorable effect in relation to attenuate the anti-tumor activity of PDT. Results of immunohistochemical analysis of LLC/IL-2 tumors have revealed that the expressions of GADD-45α and VEGF are induced in these tumors after PDT, and in particular, 12 h after PDT, the expression levels were much higher as compared with those in the LLC tumors. The results of our studies using *in vitro* and *in vivo* models suggest that the cell death caused by PDT was inhibited by induction of GADD-45α expression and that tumor recurrence was promoted by the enhancement of VEGF expression mediated by IL-2 upregulation. Therefore, it is speculated that the use of an IL-2 inhibitor may improve the efficacy of NPe6-PDT.

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Introduction

Photodynamic therapy (PDT), one of the treatment modalities for cancer, uses a photosensitizer and laser irradiation to induce the production of reactive oxygen species in cancer cells (1-4). PDT is widely used as a treatment option for solid cancers and some non-cancerous diseases. The first health agency approval of PDT using Photofrin®, most commonly employed photosensitizer, was obtained in Canada in 1993 for the treatment of bladder cancer. Subsequently, approval of photofrin was also obtained in the Netherlands and France for the treatment of advanced lung cancers, and in Germany and Japan for the treatment of early stage lung cancer (5,6). In order to enhance the efficacy of PDT and expand its clinical applications, a variety of second-generation photosensitizers are now evaluated as to their efficacy in cancer therapy (7).

We conducted a phase II clinical study to investigate the anti-tumor effect and safety of a second-generation photo-sensitizer, mono-L-aspartic chlorine e6 (NPe6, talaporfin sodium, Laserphyrin®) in patients with centrally located early stage lung cancers. The study demonstrated excellent anti-tumor effects and safety, especially a low incidence of skin photosensitivity, of the therapy (8). The Japanese Government approved NPe6 using a diode laser for early-stage lung cancer and the number of candidates for PDT is rapidly increasing. We have encountered cases of local recurrence after complete response (CR) in lung cancers

treated by NPe6-PDT, and therefore, consider it very important to elucidate the precise mechanisms underlying the effects of PDT using NPe6 (9,10).

It has been reported that PDT induces direct tumor cell kill as well as indirect effects on the tumor microenvironment (3). PDT rapidly induces apoptosis, inflammatory reactions, tumor-specific and/or -non-specific immune reactions and damage of the microvasculature of the tumor bed. Sitink *et al* reported that the microvasculature damage induced by PDT is readily observable histologically and is associated with a significant decrease of the blood flow and severe hypoxia in the tumor (11). Ferrario *et al* reported that reduction in vascular perfusion associated with PDT-mediated injury of the microvasculature produced tumor tissue hypoxia, which, in turn, induced vascular endothelial growth factor (VEGF) expression via activation of the hypoxia-inducible factor-1 (HIF-1) transcription factor (12).

Recently, we demonstrated using VEGF-overexpressing cells (SBC-3/VEGF) that PDT with ATX-s10 (Na), a novel second-generation photosensitizer, prevent tumor recurrence despite induction of VEGF and promotion of tumor angiogenesis (13). However, the relationship between the anticancer potency of PDT and the expressions of cytokines, such as VEGF, is still controversial.

We have previously reported that PDT using NPe6 induced expressions of certain kinds of cytokines, e.g., IL-2, IL-6, IL-12 and TNF-α, in tumors (14). However, the immunological mechanisms underlying PDT have never been clarified and it remains unknown whether induction of cytokine expressions are involved in the anti-tumor effect of PDT (15,16).

In this study, in order to elucidate the precise mechanisms underlying the effects of NPe6-PDT, we evaluated, both *in vitro* and *in vivo*, the role of cytokine expressions in the anti-tumor effects of PDT using cytokine-overexpressing cells and also examined whether an increase in the expression of cytokines induced by PDT could lead to tumor recurrence.

Materials and methods

Cell lines. Lewis lung carcinoma (LLC) cells derived from a spontaneous carcinoma of the lung in a C57BL/6 mouse (17) were used. We named IL-2-transfected LLC cells as LLC-IL-2 (18). Similarly, IL-6-transfected LLC cells were named as LLC-IL-6 (19). LLC, LLC-IL-2 and LLC-IL-6 cells were cultured in Iscoves's medium (IBL, Fujioka, Japan) containing 20% fetal bovine serum (FBS) at 37˚C in humidified air containing 5% CO_2. The human lung cancer cell line, SBC-3 was originally established at the Department of Medicine, Okayama University School of Medicine (Okayama, Japan). SBC-3 cells were cultured in RPMI-1640 containing 10% FBS (20,21).

Photosensitizer. Mono-L-aspartyl chlorine e6 (talaporfin sodium, Laserphyrin, NPe6), a dark blue-green, water soluble compound, was provided by Meiji Seika Kaisha, Ltd., Kanagawa, Japan (6-8,14). NPe6 has a major absorption band at 664 nm and a molecular weight of 799.69.

Laser unit. A new high-power red laser diode system (Matsushita Electric Industrial Co., Ltd. Osaka, Japan), potentially useful for photodynamic therapy (PDT) with NPe6, was employed (6-8,14). This system has a power output of 10-500 mW/cm^2 at the fiber tip. The irradiation mode is continuous-wave (CW), and the delivered energy can be adjusted from 1-1,000 J/cm^2. In our study, the diode laser wavelength was adjusted to 664 nm to match the absorption bands of NPe6 (14).

cDNA microarray analysis. Gene expressions in the lung cancer cells, namely, SBC-3 cells before and 3 h after PDT, were analyzed using a cDNA microarray (13). PDT was applied to the SBC-3 cells at the LD_{90} (10.0 μg/ml NPe6, 10J/cm^2 laser irradiation) dose and the mRNAs were extracted and purified using the total RNA kit (Qiagen, Hilden, Gemany). RNA was reverse-transcribed in the presence of [α-^{32}P]-dATP using the Atlas Pure Total RNA labeling system (Clontech, Palo Alto, CA, USA). We used a commercially available cDNA microarray, the Atlas Human 1.2 Array (Clontech), which contains an array of 1176 cancer-related gene fragments. Information about genes on the microarray can be obtained from the URL; http://atlas info.Clontech.com. Hybridization of the [^{32}P]-labeled cDNA with the array membrane was performed according the manufacturer's instructions. The membrane was studied by an imaging analyzer (BAS2000; Fuji Photo Film, Tokyo, Japan) and the hybridization signals were quantified by the ArrayGauge version 1.2 software. Gene expressions were quantified as the tumor-to-normal fluorescence ratio (T:N ratio). The genes were judged as being overexpressed when the ration was >2.0 and as underexpressed when the ratio was <0.5 (22).

Clonogenic cell survival. Cells were collected from the monolayer with trypsin immediately after NPe-6-PDT. Aliquots of the cells were seeded into 25 cm^2 flasks in amounts sufficient to yield 50-150 colonies. After incubation for 10-14 days, the cells were stained with 0.1% crystal violet in 20% ethanol, and colonies containing at ≥50 cells were counted (13,23,24). The plating efficiency of the untreated cells was 30-40%.

Animals and tumor model. Female C57BL/6 mice were entered into the study at 5 weeks of age. A total of 1x10^7 LLC and LLC-IL-2 cells in a volume of 0.1 ml were injected s.c. into the right hind flank of the experimental mice. The tumor volume was measured twice a week using a pair of Vernier calipers (11-13). Seven to ten days after the transplantation, tumors measuring over 100 mm^3 were used for the PDT and surgical experiments.

In vivo treatment protocols. LLC cells and LLC/IL-2 cells were harvested during the exponential growth phase. Cells were washed twice in Hank's solution (Invitrogen, Carlsbad, CA, USA) and 10^7 cells were inoculated subcutaneously into the right thigh of C57BL/6 mice (11-13). The transplanted tumors were treated by NPe6-PDT when they reached 6-7 mm in diameter. Two hours after NPe6 (5 mg/kg) was administered intravenously; the tumors were irradiated with a 664-nm laser at the dose of 100 J/cm^2. The laser spot size was 14 mm. The power output at the fiber tip was 154 mW. The irradiation

Table I. Gene expression in SBC-3 cells following NPe6-PDT.

	Genbank no.	Up	Down
c-jun proto-oncogene; transcription factor AP-1	J04111	6.7	
Extracelluar signal-regulated kinase 3 (ERK3); MAP kinase 3	X80692	2.1	
Cyclin-dependent kinase inhibitor 1C; p57-KIP2	U22398	2.2	
rho6 protein	Y07923	4.4	
CDC-like kinase 1 (CLK1)	L29222	8.8	
Growth arrest and DNA-damage-inducible protein 153 (GADD153)	S40706	5.2	
Growth arrest and DNA-damage-inducible protein (GADD45)	M60974	6.2	
DNA-binding protein CPBP	U44975	4.7	
Integrin alpha E precursor (ITGAE); mucosal lymphocyte-1 antigen; hml-1 antigen; CD103 antigen	L25851	6.4	
Purine-rich single-strand DNA-binding protein alpha (PURA)	M96684	3.5	
Caspase-8 precursor	U60520	3.3	
Nerve growth factor-inducible PC4 homolog	Y10313	2.6	
Interleukin-2	A14844	2.8	
Interleukin-6	X04602	2.6	
Amphiregulin (AR)	M30704	2.3	
Endothelial plasminogen activator inhibitor-1 precursor	X04429	2.8	
Macrophage inhibitory cytokine 1 (MIC1)	AF019770	2.3	
Serine/threonine-protein kinase PLK1 (STPK13)	U01038		2.3
Insulin-like growth factor binding protein 4 precursor (IGF-binding protein 4; IGFBP4; IBP4)	M62403		2.8
Leukemia inhibitory factor precursor (LIF)	X13967		2.7
Tumor necrosis factor receptor 1 (TNFR1); tumor necrosis factor binding protein 1 (TBP1); CD120A antigen	M33294		2.4
DNA repair protein XRCC9	U70310		3.1
rac-alpha serine/threonine kinase; akt1	M63167		2.5
Tublin gamma subunit	M61764		2.7
Antigen KI-67	X6550		2.2
CD9 antigen	M38690		2.3

time was 16 min 40 sec. After the NPe6-PDT session, the mice were monitored for tumor recurrence 3 times a week for 49 days. Tumor volumes were calculated using the following formula: Tumor volume = LD2/2 (L, long diameter; D, short diameter). Tumor volumes >400 mm^3 were recorded and judged as representing recurrences (11-13,25).

Immunohistochemical analysis. The LLC or LLC/IL-2 tumors in mice were collected at various time-points before and after the NPe6-PDT (before PDT, and 12 and 24 h after PDT), and fixed in 1% formaldehyde (24). We performed immunohistochemical analysis of these samples with IL-2 rabbit polyclonal antibody (sc-7896; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), VEGF rabbit polyclonal antibody (ab2992; abcam®, CA, USA), and GADD-45α rabbit polyclonal antibody (sc-792; Santa Cruz Biotechnology, Inc.).

Results

Induction of IL-2 by NPe6-PDT. The results of the microarray analysis revealed that 17 genes were overexpressed (T:N ratio >2.0) and 9 genes were underexpressed in the tumor tissue following NPe6-PDT at the LD_{90} dose (7.5 μg/ml NPe6, 10 J/cm^2 laser irradiation) (13). In regard to the cell cycle-related genes, the mRNAs of MAP kinase 3, $p57^{KIP2}$ and CLK1 genes were induced by 3 h after the PDT (Table I). DNA damage-inducible genes (GADD-45, GADD-153) were also highly overexpressed after PDT. GADD-153 was originally identified as a growth arrest and DNA damage-inducible gene (26), and Wong *et al* reported that it was highly overexpressed following photofrin-PDT (27). It has been reported that GADD-45 strongly induced IL-2 expression in the peripheral T cells (28). As shown in Table I, NPe6-PDT induced the expression of IL-2, IL-6 mRNAs, consistent with

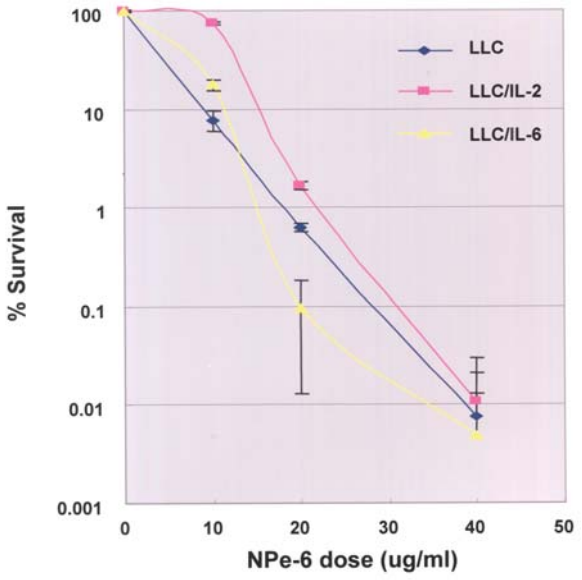

Figure 1. Loss of clonogenicity of LLC cells, LLC/IL-2 cells and LLC/IL-6 cells as a result of NPe6-PDT. Exponentially growing cultures of each cell line were treated with 10, 20 or 40 μg/ml of NPe6 for 3 h, and then irradiated with 664-nm laser light at the dose of 10 J/cm^2. Immediately after the PDT, the cells were trypsinized, collected, diluted and plated. The data from the PDT-treated cells were normalized to the plating efficiency of untreated cells of the same cell line. Each datum is the mean ± standard deviation of the results from three independent experiments.

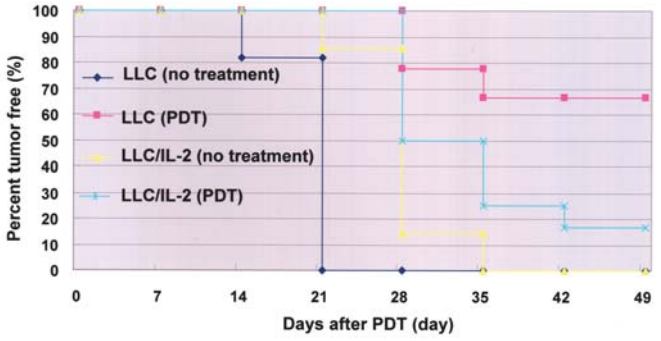

Figure 3. Mice transplanted with LLC or LLC/IL-2 tumors measuring 5-7 mm in diameter were treated by NPe6-PDT (5 mg/kg, i.v.) and laser irradiation (100 J/cm^2). Tumor response was monitored over a 35-day evaluation period. The tumor volume was calculated using the following formula; $V=LD^2$. L, the longest diameter; D, the shortest diameter. Tumor volumes >400 mm^3 were recorded as representing recurrences. The tumor volume of the LLC/IL-2 tumors increased >400 mm^3 by 49 days after the PDT. Untreated LLC tumors (solid diamonds, N=10) and LLC tumors treated by PDT (solid squares, N=10), untreated LLC/IL-2 tumors (solid triangles, N=10), and LLC/IL-2 tumors treated with PDT (crossing, N=10).

our previous report (14). These data suggest that cytokine expression and/or GADD-45 and GADD-153 may affect the antitumor effect of NPe6-PDT.

IL-2 overexpressing cells, LLC/IL-2 cells were resistant to the loss of clonogenicity induced by PDT as compared to the parental LLC cells. In order to elucidate the immunological responses elicited by NPe6-PDT and the mechanisms involved in the effects of this therapy, we examined the effects of cytokine overexpression on the efficacy of PDT by using cytokine gene transfected cells, LLC/IL-2 and LLC/IL-6 cells by clonogenic assay *in vitro*. The survival curves (Fig. 1) indicated that LLC cells overexpressing IL-2, LLC/IL-2 cells, were more resistant to the cytotoxic effects of NPe6-PDT than the parental LLC cells. At the 10% survival level, the expression of IL-2 and IL-6 yielded a dose-modifying factor of 2.0 and 1.2, respectively. These results suggest that IL-2 exerts a marked regulatory effect and inhibits the antitumor effect of NPe6-PDT *in vitro*.

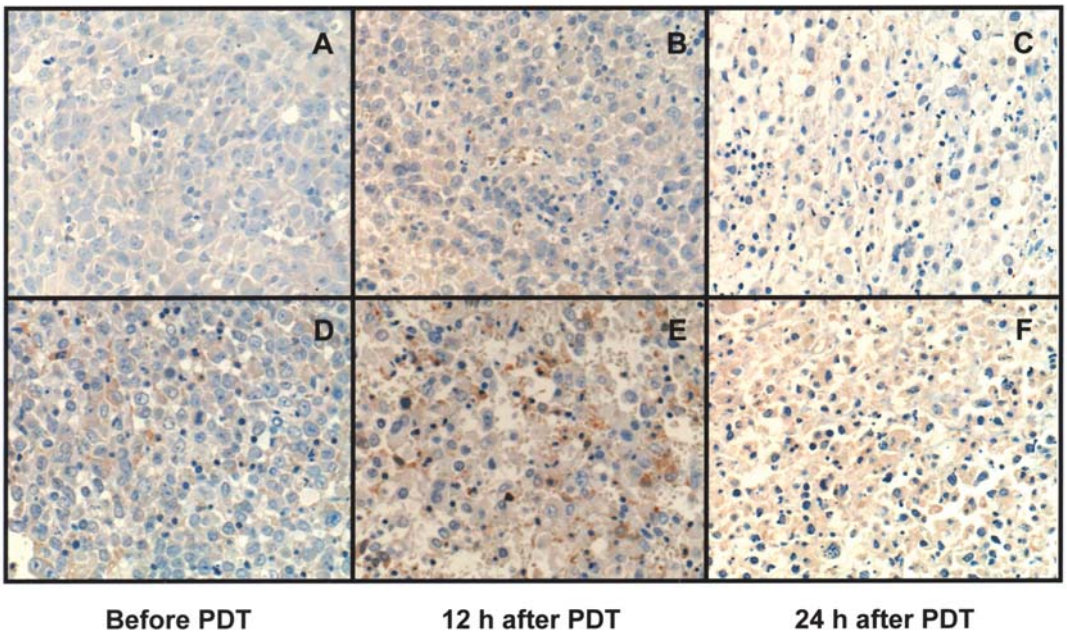

Figure 2. Immunohistochemical staining of LLC tumors (A, B and C) and LLC/IL-2 tumors for IL-2 (C, D and E). LLC or LLC/IL-2 tumors were collected from the mice at various time-points before and after NPe6-PDT (before PDT, and 1h and 24 h after PDT), and immnunological staining was performed using IL-2 rabbit polycolonal antibody sc-7896.

IL-2 expression in LLC/IL-2 tumors. As shown in Table I, induction of IL-2 mRNA expression was observed by 3 h after laser irradiation using the LD_{90} dose *in vitro*. We examined the IL-2 protein expression in the LLC tumors and the LLC/IL-2 tumors by immunohistochemical analysis (Fig. 2). Before PDT, while the LLC/IL-2 tumors showed enhanced expression of IL-2 (Fig. 2D), no such increase in expression was observed in the LLC tumors (Fig. 2A). Twelve hours after PDT, lymphoid cell infiltration was observed in the tumors and enhanced IL-2 expression was found in both LLC and LLC/IL-2 tumors; 24 h after PDT, the IL-2 expression levels were higher in the LLC/IL-2 tumors than in the LLC tumors (Fig. 2B and E). These data indicated that NPe6-PDT induced IL-2 expression in the tumors.

Overexpression of IL-2 was associated with a statistically significant failure of the tumoricidal action of NPe6-PDT as measured by the tumor cure rate. We hypothesized that NPe6-PDT may fail, with recurrence in IL-2 overexpressing, LLC/IL-2 tumors *in vivo*. Fig. 3 shows that while NPe6-PDT (100 J/cm^2) produced a 66.7% cure rate in LLC tumors, the cure rate was only 16.6% in LLC/IL-2 tumors. There was thus a statistically significant difference ($p<0.05$) in the tumoricidal action as measured by the tumor cure rates between LLC tumors and LLC/IL-2 tumors *in vivo*. These data suggest that induction of IL-2 expression caused failure of NPe6-PDT, with tumor recurrence, and that IL-2 may thus play an unfavorable role.

Overexpression of IL-2 induced and promoted GADD-45α and VEGF expression after NPe6-PDT. As shown in Table I, NPe6-PDT induced growth arrest and DNA-damage inducible protein 153 and 45 (GADD-153, GADD-45). Recently, it was reported that GADD-45 protein plays a survival function to protect cells against DNA-damage-inducing agents, including ultra violet (UV) light-induced apoptosis (29). We hypothesized that GADD-45α protein expression may cause resistant to the anti-tumor effects of NPe6-PDT *in vivo*. Therefore, we examined the expression of GADD-45α protein in LLC tumors and LLC/IL-2 tumors by immunohistochemical staining (Fig. 4). Before PDT, GADD-45α expression was higher in LLC/IL-2 tumors than in the LLC tumors (Fig. 4A and D); after 12 h, significant induction was observed in both tumors (Fig. 4B and E). These results suggest that IL-2 expression may be related to the expression of GADD-45α and that they may co-operate in attenuating the anti-tumor effect of NPe6-PDT.

It has been reported that PDT damages the microvasculature and induces a vascular shut-down effect (3,12,30). We examined whether induction of vascular endothelial growth factor (VEGF) expression in the LLC/IL-2 tumors may be involved in the enhanced angiogenesis and tumor recurrence following NPe6-PDT. As shown in Fig. 5, the expression pattern of VEGF was similar to that of GADD-45 in both the LLC and LLC/IL-2 tumors. Before NPe6-PDT, the expression level of VEGF was a little higher in the LLC/IL-2 tumors than in the LLC tumors (Fig. 5A and D). Twelve hours after PDT, significant induction of the genes was noted and the expression level of VEGF was significantly greater in the LLC/IL-2 tumors than in the LLC tumors (Fig. 5B and E). It was induced after NPe6-PDT in both tumors, and in the LLC/IL-2 tumors, in particular, the expression level remained high until 72 h (data not shown). These results suggest that tumor recurrence may be promoted by the enhancement of VEGF expression mediated by IL-2 upregulation.

Discussion

It has been reported that PDT using NPe6 and diode laser induces cell death, inflammatory reactions, immunological responses and damage to the microvasculature (3,11-13,15). In this study, in order to elucidate the immunological responses and the mechanisms underlying the effects of NPe6-PDT, we examined the effects of cytokine gene-transfected cells.

Figs. 1 and 3 show that the IL-2 overexpressing cells, LLC-IL-2 cells were resistant to NPe6-PDT both *in vitro* and *in vivo*. We previously reported that NPe6-PDT induced IL-6, IL-2 mRNA expressions (14) and that there was no statistically significant difference in the anti-tumor effect between LLC/IL-2 cells and the paretal LLC cells as measured by the MTT assay. However, in this study we evaluated the anti-tumor effect by clonogenic assay and in a mouse model. Therefore, the results of this study suggested IL-2 expression may play an unfavorable role in relation to attenuating the anti-tumor effect of NPe6-PDT.

It is unclear how IL-2 expression causes resistance to the anti-tumor effect, and exerting an unfavorable influence, of NPe6-PDT. Recently, it was reported that the vascular-shut-down effect, which induces congestion and thrombus formation in tumor vessels, with degeneration of the tumor vascular endothelial cells, plays an important role in PDT (3,11-13,15,31). We hypothesized that the failure of NPe6-PDT in preventing tumor recurrence, may be attributable to the induction of VEGF expression mediated via upregulation of IL-2 following damage of the microvasculature and hypoxic changes in the tumors. As shown in Fig 3, VEGF expression was high in LLC/IL-2 tumors, significantly so as compared to that in LLC tumors, after NPe6-PDT. Mor *et al* reported that VEGF was secreted upon stimulation by IL-2 and hypoxia (32). Jiang *et al* demonstrated immunohistochemically that VEGF expression increased within the PDT-treated lesions by one week after Photofrin-PDT and remained elevated for a few weeks (31). In our study, high levels of VEGF expression were maintained for a week in the LLC/IL-2 tumors (data not shown). From our data, we conclude that tumor recurrence after NPe6-PDT in the *in vivo* model may be regulated by the enhancement of VEGF expression via IL-2 upregulation (Fig. 6).

As shown in Fig. 2, *in vitro* experiments, clonogenic assay revealed that LLC/IL-2 cells were more resistant to NPe6-PDT as compared to the parental LLC cells. From the DNA microarray analysis, we hypothesized that GADD-45α expression, and not VEGF expression, may be responsilble for this resistance. As shown in Fig. 4, the GADD-45α expression level was higher in LLC/IL-2 tumors than in LLC tumors. Hoffmeyer *et al* reported that GADD-45, a family member of the growth arrest and DNA damage-inducible gene family, was strongly induced by IL-2 (28). Based on these data, we suggest that GADD-45α induced by NPe6-PDT may promote cell survival via IL-2 upregulation (Fig. 6).

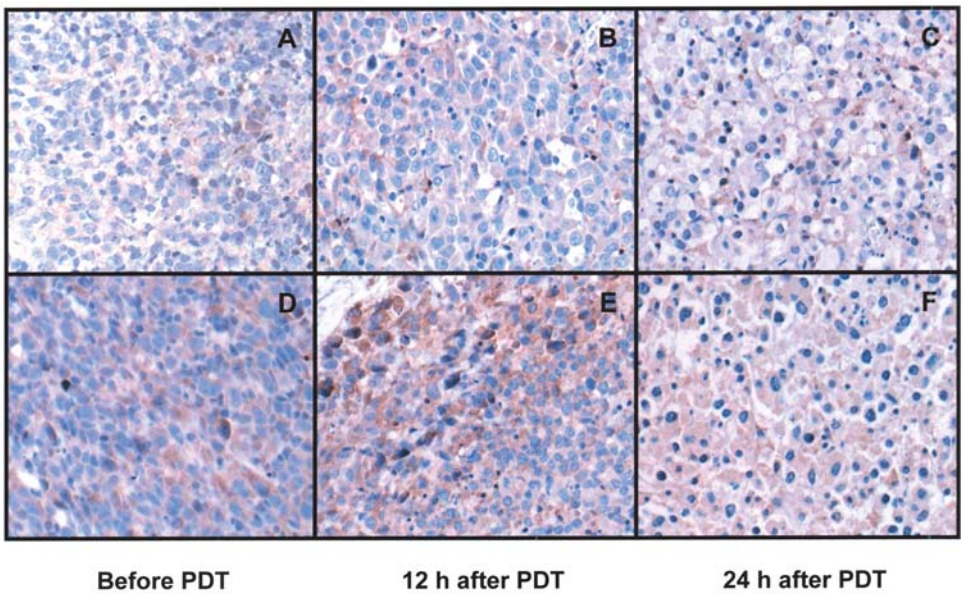

Figure 4. Immunohistochemical staining of LLC tumors (A, B and C) and LLC/IL-2 tumors (C, D and E) for GADD-45α. LLC or LLC/IL-2 tumors were collected from mice at various time-points (before PDT, and 12 and 24 h after PDT), and immunological staining was performed using GADD-45α rabbit polyclonal antibody sc-792.

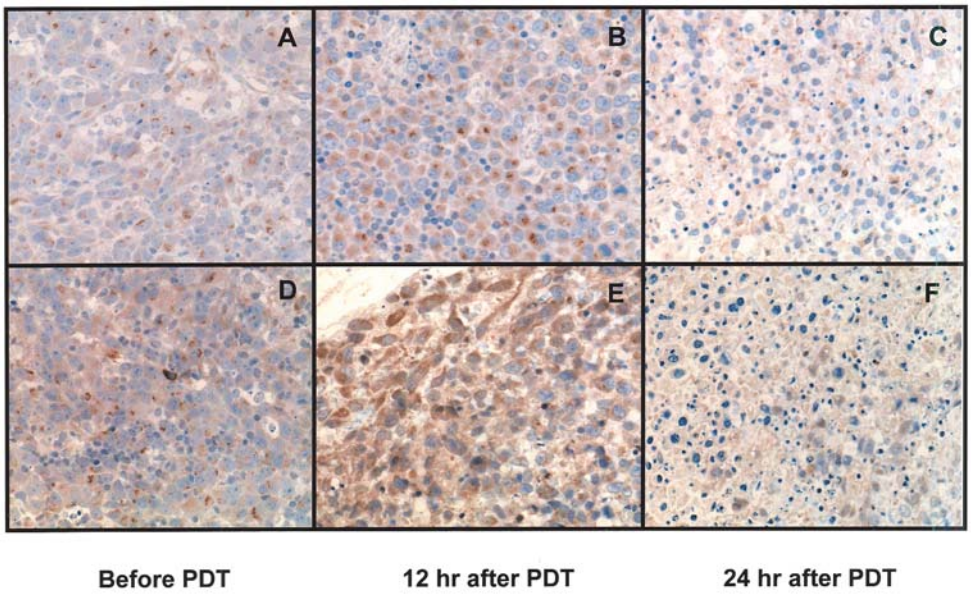

Figure 5. Immunohistochemical staining of LLC tumors (A, B and C) and LLC/IL-2 tumors (C, D and E) for VEGF. LLC or LLC/IL-2 tumors were collected from mice at various time-points (before PDT, and 12 and 24 h after PDT), and immunological staining was performed using VEGF rabbit polyclonal antibody (ab2992; abcam).

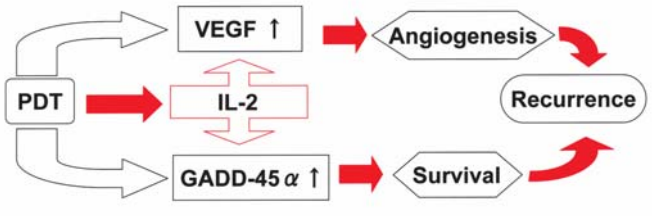

Figure 6. Schema of the interaction of IL-2, VEGF and GADD-45α. Cell survival is promoted by the induction of GADD-45α· by NPe6-PDT cell via IL-2 upregulation in the *in vitro* model. The tumor recurrence after NPe6-PDT is regulated by the enhancement of VEGF expression via IL-2 upregulation in the *in vivo* model.

In conclusion, high levels of GADD-45α and VEGF expression caused tumor recurrence and cell survival via upregulation of IL-2 (Fig. 6). It may be worthwhile investigating whether a combination of NPe6-PDT with IL-2 inhibitor administration may improve the therapeutic effectiveness of NPe6-PDT.

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