Selenium enhances the efficacy of Radachlorin mediated-photodynamic therapy in TC-1 tumor development

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Abstract. Selenium, an essential trace element possessing anti-carcinogenic properties, can induce apoptosis in cancer cells. Our goal was to investigate the enhanced antitumor effects of photodynamic therapy (PDT) plus selenium in TC-1 tumor cells and implanted mice. Cell viability was evaluated at various time intervals after PDT treatment and/or selenium by methyl thiazolyl tetrazolium (MTT) assay. When only PDT treatment was administered to TC-1 tumor cells, TC-1 cell growth recovered over time. On the other hand, co-treatment of PDT and selenium extended the inhibition time of tumor cell growth. Co-treatment of PDT and selenium showed serious morphological changes in TC-1 cells and induced a more apoptotic population by FACS analysis. By signal transduction pathway SuperArray analysis, genes closely involved in the NFκB, p53 and phopholipase C pathways, such as VCAM1, MDM2 and FOS, were significantly downregulated at least 10-fold in TC-1 cells following PDT and selenium cotreatment. In an *in vivo* study, tumor-bearing mice were intravenously injected with Radachlorin 3 h before irradiation with 300 J/cm^2 of light. Selenium was administered daily for 20 days. Combination therapy against the mouse tumors generated by TC-1 cells was more effective than PDT or selenium alone. These data suggest that selenium plus PDT can induce a significant tumor suppression response compared with PDT alone. Additionally, it can be an effective anticancer therapy strategy.

Introduction

Photodynamic therapy (PDT) is an oxygen-mediated, minimally invasive therapeutic modality. It involves the administration of a tumor-localizing photosensitizer that is subsequently activated with light of a specific wavelength, thus causing highly selec-tive photodynamic destruction of tumor cells. The evident advantage of PDT over other conventional cancer treatments such as chemotherapy and radiotherapy is its minimal invasiveness, selective targeting, and reduced toxicity, which allows for repeated treatment (1). Currently, PDT is being successfully used for the treatment of early lung cancers (2,3) and in dermatology for the treatment of non-melanoma skin cancers and precancerous diseases (4). PDT has also been successfully employed to treat early carcinomas of the oral cavity and larynx to preserve normal tissue and improve cure rates (5). Though therapeutic responses are encouraging, recurrences have been noticed due to incomplete PDT, which consequently triggers tumor angiogenesis. Non-homogeneous light distribution, incomplete photosensitizer dosage, and tissue/tumor dynamics are some of the factors that impose constraints on the efficacy of PDT. Moreover, as PDT-induced oxidative stress can cause hypoxic conditions in the surviving tumor cells, it can elicit the expression of angiogenic growth factors and cytokines as an adaptive response (6,7). Hypoxia is also known to reduce tumor sensitivity to radiation therapy and chemotherapy that is associated with decreased local tumor control (8,9). Thus, further study of controlling unwanted growth-stimulatory pathways after PDT is desirable to minimize the risk of harmful adverse effects.

Selenium is an important antioxidant nutrient that supports the production of enzymes that protect cells against oxidant stress (10). Since oxidants can damage DNA, leading to potentially carcinogenic mutations, good selenium status has clearly anti-mutagenic potential. Epidemiological studies have pointed to the decrease of risks for certain cancers in people who have relatively high selenium intakes or who live in regions of the world where soil selenium levels are relatively high. The anti-carcinogenic effects of selenium against leukemia and cancers of the colon, rectum, pancreas, breasts, ovaries, prostate, bladder, lung, and skin have been reported (11). Anti-carcinogenic mechanisms in various cancers are related to reactive oxygen species (ROS) produced by redox cycling, modification of protein-thiols, and methionine mimicry. Principal selenium metabolites, such as hydrogen selenide, methylselenol, and selenomethionine execute anti-carcinogenic effects (12). Selenium compounds also can cause acute toxicity due to DNA strand breaks by generating superoxide at high concentrations of mainly inorganic forms. But many studies using different

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forms and doses of selenium have been reported; selenium doses involved in chemoprevention and tumor growth inhibition remain to be elucidated (13).

To induce effective anti-tumor response with low adverse effects, we attempted to co-treat with PDT and selenium. Combination therapy using low concentrations of photosensitizer and selenium may effectively reduce angiogenesis and recurrence of cancer cells by PDT and selenium toxicity at a high concentration. Additionally, apoptosis of cancer cells and continuous inhibition of cancer cell development may be induced more effectively.

Materials and methods

Ethics statement. All procedure of animal research were provided in accordance with the Laboratory Animals Welfare Act, the Guide for the Care and Use of Laboratory Animals and the Guidelines and Policies for Rodent experiment provided by the IACUC (Institutional Animal Care and Use Committee) in School of Medicine, The Catholic University of Korea (permit no: CUMC-2008-0062-02).

Cell culture and tumor model. A murine cell line, TC-1 (ATCC CRL-2785) was cotransformed by HPV-16 E6/E7 oncoproteins and c-Has-Ras and was cultured in RPMI-1640 medium (Gibco BRL, Rocksville, MD) supplemented with 5% fetal bovine serum (FBS; Gibco BRL). Streptomycin/penicillin (Gibco BRL), L-glutamine (Gibco BRL), 2.2 mg/ml sodium bicarbonate (Sigma, St. Louis, MO), and 0.4 mg/ml G418 disulfate (Duchefa, The Netherlands) were added to the culture medium, and the cells were maintained at 37°C in a 5% CO_2 humid environment.

Specific pathogen-free female C57BL/6 mice 6-7 weeks of age were purchased from Orient Bio (Seongnam, Korea) and maintained in the specific pathogen-free animal facility. Five to six mice were housed per cage under standard conditions. Mice were fed standard laboratory chow and water. The depilated lower dorsa of the mice were injected subcutaneously with $5x10^5$ TC-1 cells to generate the tumor model. Tumor growth was documented regularly by Vanier calipers in three orthogonal dimensions. Tumors were used for experimentation 10-12 days after inoculation when reaching surface diameters of 10-12 mm and thicknesses of 5-6 mm.

Photodynamic theraphy (PDT) and selenium treatment. The photosensitizer, Radachorin, was purchased from RADA-PHARMA group (RADA-PHARMA, Moscow, Russia) and was diluted in PBS buffer to make a 1 mg/ml stock solution. The light source was a diode laser with a 662±2 nm wavelength (ALOD-01, Alcom Medica Ltd., St.-Petersburg, Russia). TC-1 cells were treated with Radachlorin (0.1, 0.15, or 0.2 µg/ml) for 12 h. Cells were washed with PBS buffer and then irradiated with 6.25 J/cm² of light. Selenious acid (SELA, Sigma) was dissolved in PBS buffer and filter-sterilized.

MTT assay. Cell proliferation was determined using the methyl thiazolyl tetrazolium (MTT) assay. TC-1 cells were subjected to PDT, selenium, or PDT plus selenium and further incubated at 37°C and 5% CO_2 in a humidified incubator. After incubation, MTT solution was added to each well. After 4 h, DMSO was added, and then absorbance was measured at 670 nm in an automated spectrophotometer microtiter plate reader (Spectra Max 340, Molecular Devices, CA, USA).

Flow cytometry analysis. For cell cycle analysis, propidium iodide (PI) staining was performed. Cells were collected, washed twice with PBS, and then re-suspended in PI buffer (0.1% sodium citrate, Triton X-100, and 5 µg/ml propidium iodide). RNase A (250 µg/ml) was added to each cell sample, and the cells were stained for 10 min in the dark. Flow cytometry was performed on a FACScan automated system (Becton-Dickinson, Sunnyvale, CA, USA), and data were analyzed using the CellQuest software package and ModFit LT 2.0 program.

To determine apoptosis of TC-1 cells following treatments of PDT and/or selenium, TC-1 cells were collected at 48 h after all treatments and washed twice with PBS. The cells were stained with PI and annexin V-FITC. Annexin V-FITC staining was performed with an apoptosis detection kit (Invitrogen, Camarillo, CA, USA) as described by the manufacturer's instructions. The cells were analyzed using a FACScan flow cytometer (Becton-Dickinson) at 488 nm laser light excitation. The cell populations were observed in a dot plot graphic. The population of apoptotic cells was represented as annexin V-positive and PI-negative cells, the population of necrotic cells was represented as annexin V-negative and PI-positive cells, and the population of late apoptotic/necrotic cells were represented as annexin V-positive and PI-positive cells.

Real-time quantitative PCR (QPCR)/microarray analysis. Total RNA was isolated using TRIzol (Invitrogen Life Technologies, Carlsbad, CA) from TC-1 cells previously treated with PDT, selenium, or PDT plus selenium for 48 h. Reverse transcription was performed using the RT2-First Strand Kit (SuperArray Bioscience Corp., Frederick, MD) according to the manufacturer's protocol. Gene profiling was performed using the RT2-profiler PCR array Mouse Signal Transduction Pathway Finder PCR Array (89 genes including 5 housekeeping genes). The reactions were carried out in a Stratagene Mx 3000P real-time PCR system (Stratagene, La Jolla, CA). The values were obtained for the threshold cycle (C_t) for each gene and normalized using the average of the 5 housekeeping genes on the same array. The resulting values were reported as fold change; only genes showing 3-fold or greater change were considered.

Statistical analyses. Genes that showed differences in their expression levels ≥3.0 fold, were selected for analyses (gene ontology analysis, hierarchical cluster analysis, functional cluster analysis, biological pathway analysis). Hierarchical clustering (Gene Cluster v3.0) and display programs (TreeView) were also used for analysis (http://rana.stanford.edu/software). We performed unsupervised hierarchical clustering based on the most variably expressed genes using the Euclidean distance as the similarity metric and the average linkage method as the between-cluster distance metric. A t-test was also performed to find genes that had changed between PDT alone and selenium alone, and PDT plus selenium treatment. Supervised clustering of experimental samples was performed by reducing the number of genes by statistical analysis. To classify the gene expression profiles, functional analyses and KEGG (Kyoto Encyclopedia for Genes and Genomes) pathway analyses (http://www.genome.jp/kegg/pathway.html) were carried out as previously described
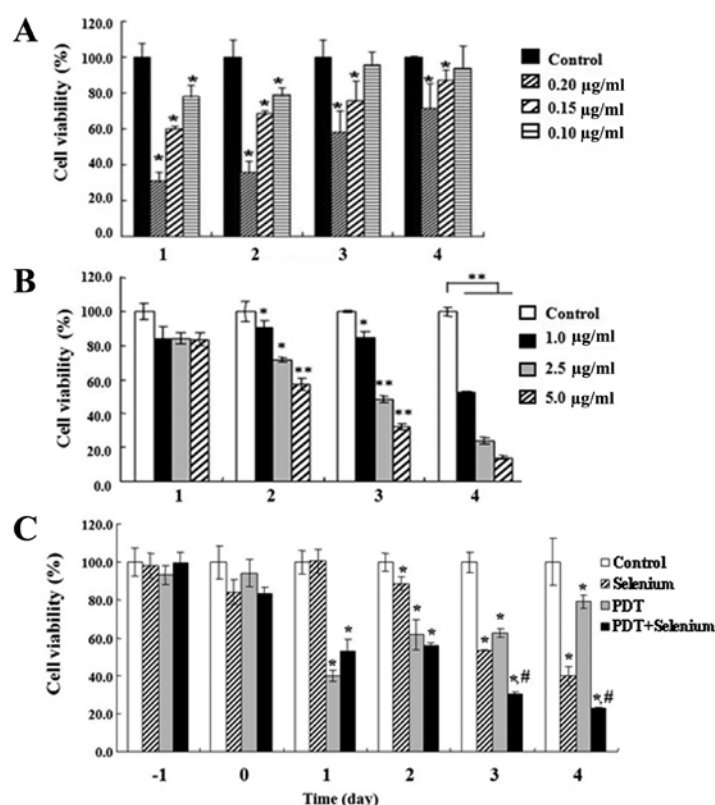
Figure 1. Cell viability of TC-1 cells by Radachlorin PDT and selenium. TC-1 cells were treated with different doses of Radachlorin for PDT (6.25 J/cm^2) (A) and various doses of selenium (B) for 4 days. Cell viability was determined based on the MTT assay as indicated in Materials and methods (columns: bars, SD; *P<0.05 and **P<0.001 compared with the control, by Student's t-test). (C) Cell growth inhibitory effects of Radachlorin PDT plus selenium on TC-1 tumor cell lines. Radachlorin PDT (0.15 μg/ml, 6.25 J/cm^2) and selenium (1 μg/ml) were administered together to TC-1 cells for 4 days. Cell viability was determined by MTT assay (columns: bars, SD; *P<0.05 compared with control; #P<0.05 compared with the PDT group by Student's t-test).

(14,15). To perform a KEGG analysis, differentially expressed genes of each treatment group were used for the calculation of their attribution to pre-defined KEGG signaling pathways and analyzed by pair-wise comparisons. Different number of genes were seen in a given pathway. The Ingenuity Pathway Analysis software (IPA, Ingenuity Systems, Mountain View, CA) was utilized to identify networks of interacting genes and other gene ontology functional groups. The significantly up-regulated and down-regulated functional activities were analyzed according to the biological processes, cellular components and molecular function ontology. Semantically consistent pathway relationships were modeled based on a continual, formal extraction from the public domain literature (www.ingenuity.com/products/pathways_ knowledge.html).

Measurement of tumor size in TC-1 cells implanted in C57BL/6 mice. Mice were divided into 4 groups of at least 5 animals per group. Tumor-bearing mice were given intravenous and intraperitoneal injections of Radachlorin and selenium, respectively. The first group was treated with selenium (dose: 2 μg/kg body weight), the second group was treated with Radachlorin (dose: 10 mg/kg), and the third group was treated with both Radachlorin and selenium. The final two groups were untreated controls. When tumors were ~8-10 mm in mean tumor diameter, Radachlorin (10 mg/kg) was injected into the tail veins of mice 3 h before irradiation. The tumor was irradiated at a fluence of 300 J/cm^2. Following light treatment, selenum was given daily by intraperitoneal injection (2 μg/kg bw).

One control group was only irradiated (no administration of photosensitizer), and the other did not receive any photosensitizer or irradiation. The mice were maintained, and the tumor volumes were measured at two or three day intervals for 23 days. Tumor growth was measured twice a week using calipers and recorded as volume diameter [longest surface length (a), width (b), and height (c), a x b x c].

Statistical analysis. Statistical analysis included ANOVA and the Student's t-test. The values for the different groups were compared. P-values of less than 0.05 or 0.01 were considered significant.

Results

Growth inhibitory effect in tumor cells by PDT, selenium, or PDT plus selenium. We performed cell viability assays (MTT assays) to examine effects of PDT in growth inhibition of TC-1 cells. TC-1 tumor cells were treated with various concentrations of Radachlorin as a photosensitizer and maintained for 12 h. The cells were washed with PBS and then irradiated by 6.25 J/cm^2 of light with a 662±2 nm wavelength emitted from a diode laser. Cell viability was determined by MTT assay. TC-1 cell growth was significantly inhibited in a Radachlorin dose-dependent manner. However, the cell growth that was inhibited by PDT recovered over time after 3-4 days (Fig. 1A). In particular, at 4 days after PDT therapy, growth of TC-1 cells treated with 1 μg/ml Radachlorin PDT recovered up to ~90%. We examined
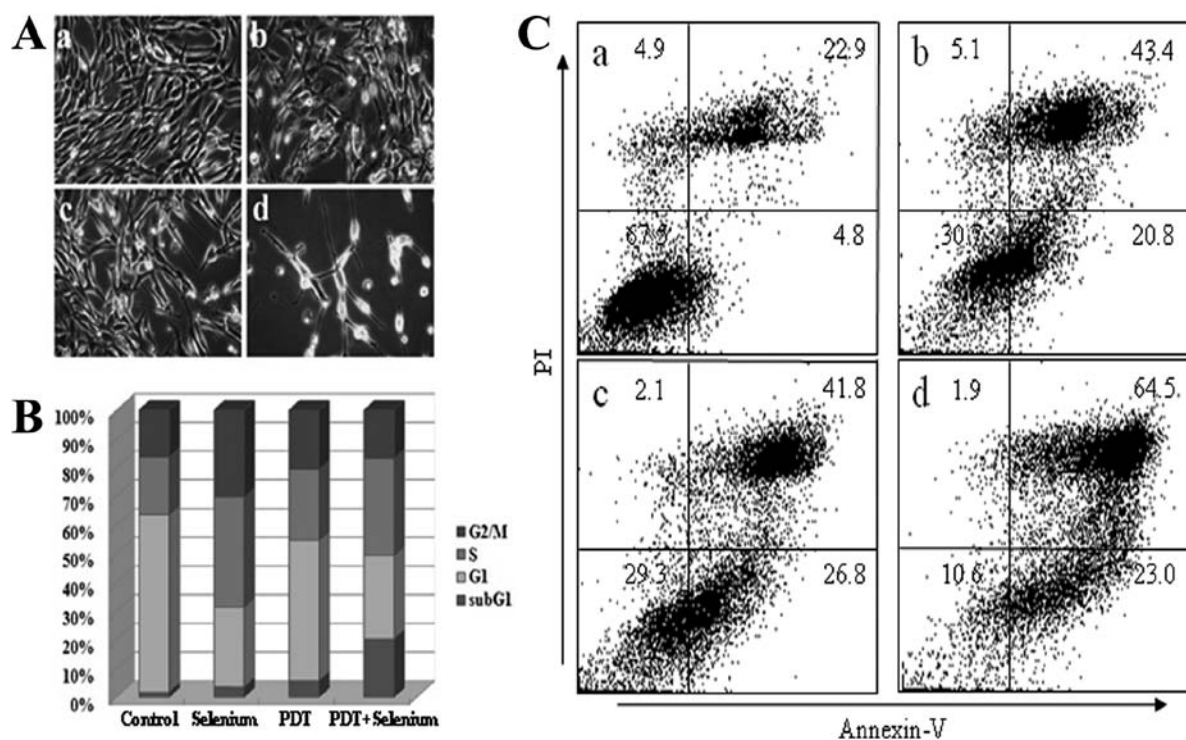
Figure 2. Combination effects of Radachlorin PDT and selenium in apoptosis of TC-1 tumor cells. (A) Morphological change of TC-1 cell lines. TC-1 cells were treated with 0.15 μg/ml Radachlorin PDT and/or 1 μg/ml selenium for 2 days as described in Materials and methods. Cells were observed under a microscope and photographed at x300 (a, non-treated; b, 0.15 μg/ml Radachlorin PDT; c, 1 μg/ml selenium; d, 0.15 μg/ml Radachlorin PDT plus 1 μg/ml selenium). (B) Cell cycle analysis of TC-1 cells following the Radachlorin PDT and/or selenium treatments by FACS. TC-1 cells were cultured with Radachlorin PDT (0.15 μg/ml, 6.25 J/cm^2) and/or selenium for 4 days. Cell pellets were then washed once in ice-cold HEPES buffer and propidium iodide (PI) staining dye was added and incubated in the dark for 15 min. (C) Detection of TC-1 apoptosis using FACS analysis. Cells were exposed to Radachlorin PDT (0.15 μg/ml, 6.25 J/cm^2), selenium (1 μg/ml) and Radachlorin PDT plus selenium for 2 days. The cells were stained with PI and annexin V-FITC and analyzed with a flow cytometer. Percentage (%) of each cell population was determined.

the effect of selenium in TC-1 cell growth inhibition. Selenium is known to possess anticancer properties at low concentration, but it can be genotoxic and possibly carcinogenic at high concentrations. TC-1 cell growth was determined by MTT assay for different concentrations of selenium (Fig. 1B). TC-1 cell growth was significantly suppressed in a selenium dose-dependent manner, and cell growth recovery was not observed, unlike that seen with PDT administration.

To effectively inhibit TC-1 growth using the benefits of PDT and selenium treatment, we attempted combination therapy consisting of PDT treatment with a low concentrations of both Radachlorin and selenium. TC-1 cells incubated with 0.15 μg/ml Radachlorin for 24 h, and selenium (1 μg/ml) were added to the cultured medium immediately before light treatment. Co-treatment of PDT plus selenium significantly reduced TC-1 cell growth compared to PDT or selenium alone (Fig. 1C). Despite the low concentrations of Radachlorin and selenium, TC-1 cell growth was continuously inhibited according to time, and growth recovery of TC-1 cells after PDT was not observed at 3 and 4 days after treatment. These results indicate that co-treatment of PDT and selenium is able to effectively suppress growth of TC-1 tumor cells.

Apoptotic morphology of TC-1 cells by co-treatment with PDT and selenium. We characterized TC-1 cell growth inhibition by co-treatment of PDT and selenium. TC-1 cells were treated with 0.15 μg/ml Radachlorin PDT and/or 1 μg/ml selenium for 2 days.

The cell morphology was observed by microscopy (Fig. 2A). TC-1 cell growth was more inhibited with co-treatment of PDT and selenium than PDT or selenium alone. Co-treatment of PDT and selenium more greatly induced the distorted morphology of TC-1 cells and generated many apoptotic bodies. We performed cell cycle analysis to identify the cell cycle distribution (Fig. 2B). The proportion of cells in the sub-G1 phase increased in TC-1 cells following co-treatment with PDT and selenium compared with those treated with PDT or selenium alone. To ascertain whether TC-1 tumor cells were apoptotically induced by combination treatment, we performed FACS analysis after TC-1 cells were treated with 0.15 μg/ml Radachlorin PDT and/or 1 μg/ml of selenium for 2 days (Fig. 2C). The proportion of cells in apoptosis was significantly higher in TC-1 cells following the combination treatment of PDT plus selenium compared to either single therapy. These data show that combination treatment induces a higher level of apoptosis in TC-1 tumor cells compared with treatment of PDT or selenium alone.

Change in gene expression of TC-1 cells by combination therapy. Apoptosis, the process of programmed cell death in eukaryotic cells, causes expression changes in various genes, as well as changes in cell morphology. Therefore, we performed real-time reverse transcription PCR assays to identify genes with significant changes in expression in TC-1 cells treated with PDT plus selenium. Co-treatment of PDT plus selenium compared with treatment of PDT or selenium alone induced

Table I. Relative expression of target genes in TC-1 cells.

Gene symbol	Fold change		
	Selenium	PDT	PDT + selenium
ATF2	-1.1	-1.8	-9.5
BCL2	-1.2	-2.0	-5.7
NAIP1	6.4	-1.2	24.4
BIRC2	-3.4	-2.7	-84.0
BIRC3	-2.4	-3.6	-19.9
BMP2	-1.3	1.4	-3.4
BRCA1	-1.8	-3.5	-10.7
CCL2	-3.6	-1.9	-3748.3
CCND1	-1.0	1.4	-3.7
CD5	7.8	1.1	34.5
CDH1	4.2	-1.6	20.6
CSF2	3.2	4.9	14.8
CXCL1	4.8	1.4	1.5
CXCL9	8.3	-1.2	16.5
CYP19A1	4.9	-1.2	46.8
EGR1	-2.1	-2.0	-3.9
FAS	-1.3	-2.6	-5.5
FASN	-1.5	-1.1	-3.8
FGF4	5.7	-1.2	32.6
FN1	-2.7	-4.4	-9.9
FOS	-1.7	-1.9	-1391.1
FOXA2	5.2	-1.2	15.1
GREB1	5.7	-1.2	26.3
HHIP	2.1	-1.3	6.2
HK2	-1.4	-1.8	-498.7
HSPB1	23.7	1.3	6.3
ICAM1	-3.3	-2.6	-3.7
IL1A	10.6	1.1	16.2
IL2	6.0	1.1	2.2
IL2RA	4.6	-1.2	13.3
IRF1	-3.8	-3.8	-5.9
LEP	6.1	-1.2	-1.1
LTA	5.0	-1.0	-4.4
MDM2	1.0	-1.3	-8792.2
MMP10	1.5	2.7	-17.2
MMP7	7.2	-1.2	-1.8
NOS2	7.5	1.3	25.2
NRIP1	-1.3	-1.3	-3.7
PPARG	4.5	-1.2	10.8
PTGS2	1.2	-1.5	-1597.9
RBP1	-1.8	-2.6	-3.5
SELE	5.2	5.3	-1.7
SELP	8.4	1.1	-3.0
VCAM1	-1.4	-2.1	-10.9
VEGFA	-1.2	-2.4	-1642.9
WISP1	-1.3	-2.6	-28.1
WNT1	8.5	2.0	-2.3
WNT2	3.9	-1.2	-1.8

many changes in gene expression in TC-1 cells (Table I). Fold changes were relatively high in TC-1 tumor cells co-treated with PDT plus selenium compared with those treated with PDT or selenium alone. To identify signal transduction pathways related to significantly changed genes by co-treatment, they were classified as shown in Table II. Using the signal transduction pathway SuperArray, FOS, HK2, CCL2, MDM2, PTGS2, VEFGA, VCAM1, BIRC2, and BIRC3 were very significantly downregulated at least 10-fold in TC-1 cells following co-treatment. These genes were included in gene groups closely related to the NFκB, p53, and phospholipase C pathways. Furthermore, MDM2, BIRC2, and BIRC3 are anti-apoptosis related genes, and FOS, HK2, VCAM1, CCL2, PTGS2, WISP1, MMP10, and WISP1 are tumor survival or promoting genes. Upregulated genes by co-treatment had relatively low fold changes compared with the fold changes of downregulated genes and were related to cell survival and proliferation mechanisms. These results indicate that the combination of PDT plus selenium can induce a significant tumor suppression response. To detect the differences in the functional profiles, KEGG pathway analyses were carried out. The enhanced cell growth inhibition and antitumor effects were significantly related with gene expression levels of focal adhesion, MAPK pathway, cytokine-cytokine interaction, and cell adhesion pathway (Fig. 3A-E). Significantly down-regulated molecular functions for the combination treatment group was focal adhesion pathway. Each representative gene in the pathway was tested by quantitative PCR (Fig. 3G). These included genes coding for PTEN, AKT, p53, p21, VEGF, PTGS2, and MMP2. Jak-STAT pathway was also studied in the gene sets, but not strictly correlated with the enhanced cell growth inhibition (Fig. 3F).

Suppression of tumor development in TC-1 cells implanted in C57L/6 mice. We measured tumor sizes of TC-1 tumor cell-implanted C57BL/6 mice to determine *in vivo* effects of PDT and selenium combination therapy. TC-1 cell xenografted C57BL/6 mice were grouped in four different groups: control, PDT, selenium, and PDT plus selenium groups (Fig. 4). PDT effectively inhibited in tumor development for a short period (approximately 15 days), but inhibition of tumor growth by PDT seemed to be recovered after ~20 days. After 20 days, growth rates of tumors following PDT treatment compared with growth rates following selenium treatment were relatively increased. In contrast, combination treatment with PDT plus selenium clearly reduced tumor diameters, and this tumor suppression was maintained for 23 days. This result suggests that combination therapy with PDT plus selenium in the tumor-bearing mouse model is effective for *in vivo* tumor suppression as well as for *in vitro* tumor suppression.

Discussion

Photodynamic therapy (PDT) is a relatively recent clinical treatment applied against patients' lesions, including various cancers, and it has already been recognized as a very promising therapeutic method (16). PDT has been applied to improve early stage disease, including lung cancer, brain cancers, head and neck cancers, and non-oncological disorders, such as age-related macular degeneration (17). However, PDT can cause hypoxic conditions in the surviving tumor cells due to

Table II. Effects of co-treatment of PDT and selenium in signal transduction pathway.
A, Down-regulated genes ≤10-fold

Related signal pathway	Gene symbol	Selenium	PDT	PDT + selenium
Calcium and PKC	FOS	-1.7	-1.9	-1391.1
CREB	FOS	-1.7	-1.9	-1391.1
Estrogen	BRCA1	-1.8	-3.5	-10.7
Insulin	HK2	-1.4	-1.8	-498.7
Jak/Src	MMP10	1.5	2.7	-17.2
LDL	VCAM1	-1.4	-2.1	-10.9
	CCL2	-3.6	-1.9	-3748.3
Mitogenic	FOS	-1.7	-1.9	-1391.1
NFκB	VCAM1	-1.4	-2.1	-10.9
	BRIC3	-2.4	-3.6	-19.9
	BRIC2	-3.4	-2.7	-84.0
P53	MDM2	1.1	-1.3	-8792.2
PLC	VCAM1	-1.4	-2.1	-10.9
	FOS	-1.7	-1.9	-1391.1
	PTG2	-1.2	-1.5	-1597.9
Stress	FOS	-1.7	-1.9	-1391.1
Wnt	WISP1	-1.4	-2.6	-28.1
	VEGFA	-1.3	-2.4	-1642.9

B, Up-regulated genes ≤10-fold

Related signal pathway	Gene symbol	Selenium	PDT	PDT + selenium
Selenium Calcium and PKC	CSF2	3.2	4.9	14.8
	IL2RA	4.6	-1.18	13.34
CREB	CYP19A1	4.9	-1.18	46.79
Estrogen	GREB1	5.7	-1.18	26.38
Hedgehog	FOXA2	5.2	-1.18	15.12
Jak-Stat	NOS2	7.5	1.3	25.25
	CXCL9	8.3	-1.18	16.54
LDL	CSF2	3.2	4.9	14.8
NFκB	NOS2	7.5	1.3	25.25
	IL1A	10.6	1.09	16.2
PLC	NOS2	7.5	1.3	25.25
Wnt	FGF4	5.7	-1.18	32.63
	CDH1	4.2	-1.55	20.65
	PPARG	4.5	-1.18	10.84

oxidative stress induction, and it can elicit tumor recurrence and angiogenesis (8,9). Selenium is a dietary essential trace element with biological roles and possesses anticancer properties (11). However, selenium at low concentrations has anti-carcinogenic properties in multiple cancers, including those of the colon, prostate, breast, ovary, and lung, whereas at high concentrations, selenium can generate reactive oxygen species (ROS), which can induce DNA damage (13). Therefore, we tried combination therapy consisting of PDT and selenium to develop a therapeutic method with low adverse effects. We used the TC-1 cervical cell line with overexpression of human papilloma virus (HPV), the E6/E7 oncogene, and the Ha-*ras* oncogene. To prepare the cervical cancer mouse model, the TC-1 cervical cell line was transplanted into C57BL/6 mice. Combination therapy with PDT and selenium was applied both in the TC-1 cell line *in vitro* and tumor-bearing C57BL/6 mice *in vivo*.

TC-1 cell viability was significantly inhibited both in PDT or selenium treatment, but cell growth inhibited by PDT alone recovered over time. We tried to co-treat with Radachlorin PDT plus selenium at low concentrations to prevent adverse effects,
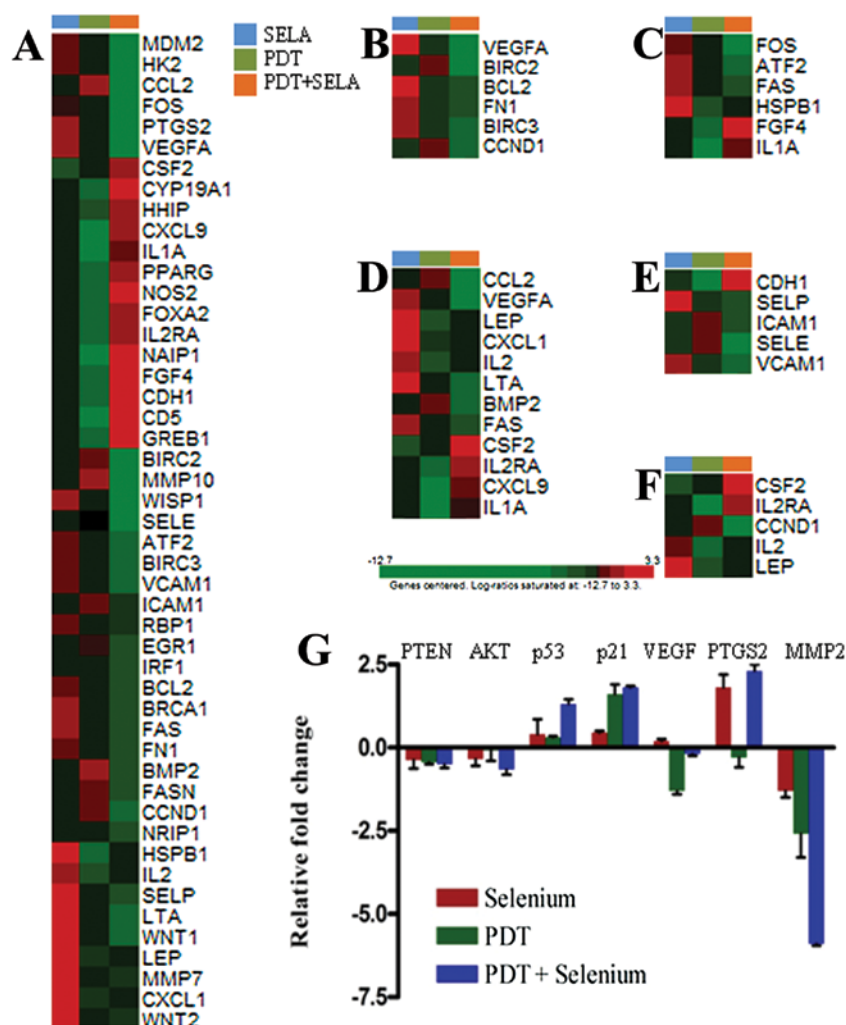
Figure 3. Hierarchical cluster analysis. (A) All the data were median centered and clustered using a hierarchical clustering. A cluster image representing 48 of the genes is shown in matrix format, where rows represent individual genes and columns represent each assay. Each cell in the matrix represents the expression level of a gene in an individual assay. Red and green cells reflect high and low expression levels, respectively. (B) Focal adhesion pathway; (C) MAPK pathway; (D) Cytokine-cytokine receptor interaction pathway; (E) Cell adhesion pathway; (F) Jak-STAT pathway. (G) *In vitro* gene expression changes of TC-1 cells treated with 0.15 µg/ml Radachlorin PDT and/or 1 µg/ml selenium for 1 day. The expression of genes was examined by Q-PCR. The results are presented as transcript levels relative to the level in untreated control cells.
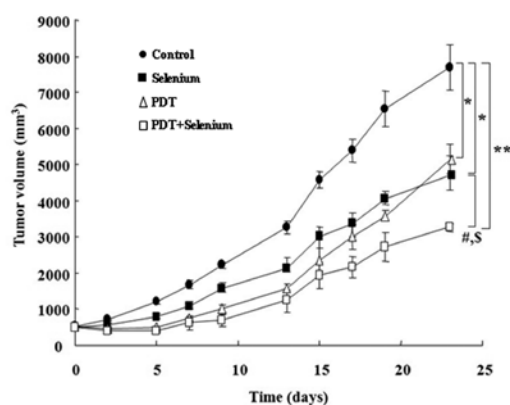

Figure 4. Tumor growth-inhibitory effects of Radachlorin PDT and/or selenium in TC-1 cell-implanted C57BL/6 mice. Each group of mice (n=5) were challenged s.c. with 2x10⁵ TC-1 cells. Tumor-bearing mice were administered PDT and/or peritoneal injections of 2 µg/kg b.w. of selenium for 20 days. For performing PDT, 3 h after intravenously injected with Radachlorin (10 mg/kg bw), tumor sites were exposed to a laser (300 J/cm²). Tumor size was monitored as described in Materials and methods, and then its mean volume was plotted over time. Significant inhibition of tumor growth was measured by ANOVA. *P<0.05 and **P<0.001 compared with the control; #P<0.05, compared with Radachlorin PDT and selenium, P<0.05 compared with selenium.

such as tumor cell growth recovery and cell toxicity. Despite the low concentrations of Radachlorin PDT and selenium, TC-1 cell growth was effectively inhibited by co-treatment, and cell growth recovery did not occur during the time of the experiment. To determine cell arrest and cell apoptosis by combination therapy, we performed cell cycle and apoptosis analyses using a FACS analyzer. Co-treatment of Radachlorin PDT and selenium compared to Radachlorin PDT or selenium alone induced greater apoptotic TC-1 cell morphology and increased the proportion of TC-1 cells in the sub-G1 phase of the cell cycle. In FACS analysis to confirm TC-1 apoptosis by co-treatment, TC-1 cells following co-treatment were observed to have a larger proportion of annexin V-positive and PI-negative cells in a FACS analysis dot plot graph.

We supposed that combination therapy with Radachlorin PDT and selenium should more effectively inhibit growth of TC-1 cancer through higher TC-1 apoptosis induction. Therefore, we tried to identify genes with significant expression changes as a result of combination treatment. Overall, down-regulated genes by co-treatment with PDT and selenium had

relatively higher fold changes than those of upregulated genes. Co-treatment of PDT and selenium downregulated expression of many genes related to the NFκB, p53, and phospholipase C pathways. These genes included groups of anti-apoptotic and anti-tumor genes. Upregulated genes by co-treatment of PDT plus selenium were related with cell survival and proliferation mechanisms. Upregulation of these genes may be a repair mechanism for various types of damage induced by therapies. Finally, we examined effects of combination therapy in tumor tissue using the TC-1 cell-implanted mouse model. Combination treatment with PDT plus selenium clearly reduced tumor diameters, and this tumor suppression was maintained for a long time.

PDT induces apoptosis by production of singlet oxygen (18), and selenium also induces apoptosis at a low concentration. High concentrations of selenium have pro-oxidant toxicity due to DNA strand breaks, and PDT can cause tumor recurrence and angiogenesis by hypoxic conditions. Therefore, we inspected the results for gene expression change in TC-1 cells following co-treatment with low concentrations of Radachlorin PDT and selenium. Genes with relatively high fold changes in significantly downregulated genes were FOS, HK2, CCL2, MDM2, PTGS2, and VEGFA. FOS, an FBJ murine osteosarcoma viral oncogene homolog, is a transcription factor with oncogenic activity and is frequently overexpressed in various tumor cells (19). HK2, hexokinase II, is known as an enzyme catalyzing the initial metabolic step of glycolysis and is predominantly expressed in many cancer cells. Downregulation of the HK2 gene increases the sensitivity of cancer to anti-cancer agents (20). CCL2, a chemokine (C-C motif) ligand 2, is known as a chemoattractant for monocytes and is expressed in at least nine of ten cervical cancer cell lines (21). MDM2, transformed mouse 3T3 cell double minute 2, induces degradation of tumor suppressor p53 protein and inhibits apoptosis of cancer cells by p53 (22). PTGS2, prostaglandin-endoperoxide synthase 2, also known as cyclooxygenase 2 (COX-2), is known to be regulated by the human papillomavirus 16 oncogenes E6 and E7 through EGFR, and high PTGS2 expression is related to advanced diseases, distant metastases, and decreased survival in cervical cancer patients (23). VEGFA, vascular endothelial growth factor A, is known to stimulate the growth of endothelial cells and is a powerful inducer of angiogenesis. VEGF prevents apoptosis of endothelial cells by the lack of serum and induces expression of anti-apoptotic proteins BCL-2 and A1 (24,25). The significant expression reduction of these genes that are closely related to cancer development is consistent with the results for proliferation inhibition and apoptosis of TC-1 tumor cells by combination therapy with PDT and selenium.

BRCA1, MMP10, VCAM1, BIRC2, BIRC3, and WISP1 were also significantly down-regulated in TC-1 cells with co-treatment of PDT and selenium. Deficiency of BRCA1, breast cancer associated gene 1, causes abnormalities in the S-phase checkpoint, the G2/M checkpoint, and centrosome duplication and induces inhibition of cell proliferation and apoptosis (26). Matrix metalloproteinases (MMPs) are involved in several steps of cancer development, and MMP10 is overexpressed in head and neck SCCs (27). Expression of VCAM1, vascular cell adhesion molecule 1, in tumor cells decreases accumulation of T cells in the tumor site by promoting T-cell migration away from tumors. VCAM1-expressing tumor cells are able to avoid immune attack

(28). BIR2 and BIRC3, baculoviral IAP repeat-containing 2 and 3, are known to accelerate tumorigenesis as anti-apoptotic genes (29,30). WISP-1, WNT1 inducible signaling pathway protein 1, is strongly expressed in breast tumors developing in WNT-1 transgenic mice, and overexpression in normal rat kidney fibroblasts induces their transformaiton (31,32). As these genes were also related to cell cycle and transformation of tumors, downregulation of these genes would effectively inhibit development of TC-1 cancer cells.

In conclusion, we have shown that co-treatment with PDT and selenium induces growth inhibition, apoptotic morphology changes, and gene expression changes in TC-1 tumor cells. Treatment with low concentrations of PDT and selenium in combination sufficiently inhibited tumor cell proliferation without growth recovery of cancer cells following PDT alone. These effects may be due to significant down-regulation of anti-apoptotic genes and tumor-promoting genes. These results indicated that combination therapy of selenium and PDT could effectively induce a tumor suppression response compared to PDT or selenium alone. Therefore, we suggested that combination therapy using PDT and selenium can be an approach to induce effective anti-cancer effects.

Acknowledgements

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