Thalidomide and irradiation combination therapy increases substance P levels *in vitro*

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Abstract. Thalidomide is an anti-angiogenic agent that is used in the treatment of cancer. However, in many cases, particularly in patients with breast cancer, thalidomide treatment alone is insufficient and must be combined with other drugs or therapies. In the clinical setting, thalidomide is most commonly used in combination with radiation therapy. However, the exact mechanisms of its effect are unkown. Radiotherapy alters the expression of substance P, which is considered a crucial pro-angiogenic peptide. To determine whether thalidomide and radiotherapy in combination overcome the limitations of each as monotherapy, we examined the effects of the combination on the growth of breast cancer cells as well as on the expression of substance P *in vitro*. Mouse breast cancer cells (4T1) and cells produced from metastatic lesions (4THMpc) were treated with radiotherapy (RT) (45 Gy) alone, thalidomide (Thal) (40 μg/ml) alone or combination therapy (40 μg/ml Thal + 45 Gy RT), and compared with control cells. MTS, Live/Dead and trypan blue exclusion assays were used to evaluate the cytotoxic effects of the treatments. The levels of substance P in the conditioned media and in the cell lysates were determined by a substance P ELISA kit, and changes in the protein content were analyzed by Western blotting. Thalidomide alone resulted in a significant inhibition in the growth of the 4T1 (34.1%) and 4THMpc (52.6%) cell lines. RT alone inhibited the growth of the 4T1 (19.2%) and 4THMpc (23.31%) cell lines. The combination therapy enhanced the growth inhibition noted in the 4T1 (47.9%) and 4THMpc (62.03%) cell lines. The expression of substance P in the conditioned media and in the cell lysates increased within 72 h of RT. This increase was significantly enhanced with the combination therapy. These data indicate that thalidomide inhibits breast cancer cell growth and potentiates the anti-tumor effects of radiation at appropriate doses.

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Introduction

Breast cancer is one of the most common neoplasms in women and is a leading cause of cancer-related mortality, resulting in approximately 500,000 deaths worldwide annually (1). Surgery, radiotherapy and chemotherapy are widely used treatment methods for breast cancer. Despite significant improvements in cancer diagnosis and therapy, breast cancer remains a challenging disease to treat, and approximately one quarter of breast cancer patients succumb to the disease. Thus, further investigations into the mechanisms of this disease are required to aid in the development of novel treatments (2).

The formation of new blood vessels by the extension or elaboration of existing vasculature is called angiogenesis. This mechanism plays a central role in both local tumor growth and distant metastasis in breast cancer (3,4). Angiogenesis is regulated by angiogenic and anti-angiogenic factors, and the expression levels of angiogenic factors reflect the aggressiveness of tumor cells. Since the discovery of angiogenic inhibitors, the inhibition of tumor angiogenesis has become a promising strategy for the treatment of cancer, and thousands of patients have received anti-angiogenic therapy to date. Unfortunately, despite their theoretical effects, anti-angiogenic treatments have not proven beneficial in terms of long-term survival. Thus, there is clear need for a new comprehensive treatment strategy combining anti-angiogenic agents with conventional treatments, such as chemotherapy or radiotherapy, in the treatment of cancer (5-7).

Thalidomide (a derivative of glutamic acid that exists as an equal mixture of its enantiomers) was introduced in Europe for the treatment of morning sickness in pregnant women. However, due to its teratogenicity, it was withdrawn from the market in the late 1960s (8). Many years later, D'Amato *et al* revealed that thalidomide inhibits limb development by suppressing angiogenesis via the inhibition of basic fibroblast growth factor (bFGF) and/or vascular endothelial growth factor (VEGF) (8-10). Today, thalidomide is one of the most well-known teratogens in medical history and is clinically recognized as an efficient therapeutic agent for the treatment of various types of cancer; however, the anti-angiogenic mechanism of thalidomide remains unknown.

Certain studies conducted in pre-clinical tumor models have documented the advantages of combining cytotoxic chemotherapeutic agents with radiation therapy. Over the

last few years, significant survival benefits for breast cancer patients have been achieved with the use of postoperative systemic therapies and radiotherapy (11,12). Currently, the majority of early breast cancer patients are routinely managed with breast-conserving surgery followed by radiation therapy and adjuvant systemic therapies, including chemotherapy and hormone therapy. Despite the extensive use of radiotherapy and systemic treatments, the optimal strategy for their use in combination remains unclear, and their mechanisms are unkown (13,14).

Recently, it was confirmed that neuroimmune mechanisms also play a role in the defense against cancer, as well as in its progression. The involvement of the nervous system in the modulation of cancer development and its progression is indicated by clinical and experimental data from various studies. Several retrospective studies of patients who have undergone vagotomy suggest that the loss of various sensory nerve mediators, such as substance P (SP), leads to an increased risk of cancer development (15).

The angiogenesis-related peptide SP is a member of the tachykinin family encoded by the preprotachykinin A (PPT-A) 1 gene (16). SP is generally accepted to be the major neuropeptide involved in neurogenic inflammation, and is the most important neuropeptide in cancer. The PPT-A gene is expressed in many other cell types, such as monocytes, human fibroblasts, keratinocytes, lymphocytes, platelets and tumor cells. SP also induces angiogenesis and local inflammatory responses, which may increase cancer progression and metastases (17). SP seems to have a bidirectional effect on inflammation, tumor growth and carcinogenesis. These bidirectional effects on inflammation and carcinogenesis may be due to the counter-balancing effects of SP fragments and the intact peptide, since the intact peptide is tumorigenic and induces inflammation, whereas fragments produced by peptidases are anti-tumorigenic and anti-angiogenic (18).

To the best of our knowledge, the effect of thalidomide on SP has yet to be investigated. The close interaction between immune system involvement in the development and progression of cancer and the importance of combined therapy requires further research. Thus, whether thalidomide, either alone or in combination with radiotherapy, is capable of altering SP levels in breast cancer cells warrants investigation. Therefore, the present study aimed first to determine the cytotoxic effects of thalidomide, radiotherapy and their combination on the 4T1 cell line and its metastatic derivative line, 4THMpc, and second, to determine the changes in SP expression induced by the treatments.

Materials and methods

Thalidomide. Water-soluble thalidomide (Thal) was purchased from A.G. Scientific (cat. no. T 1020). Thal (100 μg) was dissolved in sterile distilled water and aliquoted into standard Eppendorf tubes at quantities of 500 μl for daily assay. These aliquots were stored at -70°C until use.

Cell lines and in vitro cell culture conditions. 4T1 breast cancer cells and 4THM (4T1 Heart Metastases Post Capsaicin) cells, a cell line obtained from orthotopically transplanted 4T1 breast cancer cells, were used in this study. The cell lines

were a kind gift from Dr Nuray Erin (Akdeniz University, Faculty of Medicine, Antalya, Turkey). Cells were grown as monolayer adherent cultures in plastic cell culture petri dishes (BD, Bedford, MA, USA) in DMEM-F12 (Biochrom, Germany) supplemented with 5% fetal bovine serum (FBS), 2 mM L-glutamine, 1 mM sodium pyruvate and 0.02 mM non-essential amino acids. The cell lines were maintained at 37°C in a humidified atmosphere of 5% CO_2. All cell lines used in this study were tested and shown to be free of mycoplasma contamination.

Thalidomide treatment. To examine the effects of Thal, radiation therapy (RT) and the combination therapy (Thal + RT) on cell growth *in vitro*, initial experiments were performed to determine the optimal cell treatment conditions.

To determine the optimal cell number, 4T1 and 4THMp cells were plated at a density of 1,000 to 20,000 cells/wells. Thirty-six hours after plating, the cells were treated with Thal at concentrations of 2.5, 5, 10, 20 and 40 μg/ml. Four hours after Thal treatment, the cells were irradiated with a single dose of ionizing radiation. After irradiation, the cells were incubated for 24, 48 or 72 h, following which cell growth was determined. The optimal cell density was found to be 5,000 cells/well.

Radiation therapy. To determine the appropriate dose of RT, initial experiments were performed using various doses of ionizing radiation. Cells were seeded at 5,000 cells/well in a 96-well plate. After 36 h, the medium was replaced with fresh medium containing 1% serum, and RT was applied at doses of 5 to 45 Gy. Each cell plate (2 cm thick) was irradiated in a Co-60 teletherapy unit at a distance of 100 cm. In order to achieve a homogeneous dose (+2.5%) at the cell plate, the plate was embedded in water equivalent bolus material and a 0.5-cm-thick bolus material was additionally placed on the cover. Cell viability was measured 24, 48 or 72 h after RT. The optimal dose of irradiation was found to be 45 Gy at 1.5 cm (in the middle of the plate), carried out at a dose rate of ~145 cGy/min.

Determination of cell viability. Cell growth was determined after 72 h of incubation in three sets of experiments: cells treated with Thal alone at a concentration of 40 μg/ml, cells treated with RT at a dose of 45 Gy, and cells treated with a combination therapy of 40 μg/ml Thal + 45 Gy ionizing radiation, applied 4 h after Thal treatment. As a negative control for all assays, 4T1 and 4THMp cells were treated with conditioned medium containing 1% serum.

The proliferation of the 4T1 and 4THMp cells was first determined using the tetrazolium compound 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium, inner salt (MTS) according to the manufacturer's instructions (Cell Titer 96 Aqueous One Solution Cell Proliferation Assay; Promega Corp., Madison, WI, USA). Formasan formation was quantified at an optical density (OD) of 490 nm and compared between groups to determine cell growth and viability. To calculate the percentage of growth inhibition, the following formula was used: growth inhibition (%) = [(mean OD value of the control group - mean OD value of the treatment group)/mean OD value of the control group] x 100%.

To verify the results of the MTS assay, the Live/Dead viability/cytotoxicity kit for mammalian cells (Invitrogen, Eugene, OR, USA) was used. According to the manufacturer's protocol, the 4T1 and 4THMp cells were dually stained with two fluorescently labeled probes that enable the simultaneous determination of live and dead cells in a sample: Calcein AM, which stains live cells since it fluoresces only when cleaved by intracellular esterases, and EthD-1, which identifies dead/dying cells since it exclusively enters cells with disrupted membranes. Fluorescence intensity was measured on a Victor™ X2 Multilabel Plate Reader (PerkinElmer Inc., Waltham, MA, USA) at excitation wavelengths of 560 and 535 nm and emission wavelengths of 645 and 610 nm, respective to each reagent dye. Experiments were repeated five times in four replicative wells. Data from four highly reproducible independent experiments were pooled and used for statistical comparisons using the Student's t-test.

The number of dead cells as well as the total cell number in the 4T1 and 4THMp cell lines were further examined using the trypan blue (0.4% trypan blue in HBSS) exclusion assay. Images were captured under a phase contrast microscope. The percentage of decrease in cell survival was calculated according to the results of four independent highly reproducible experiments. The following formulas were used: for RT, 100 - [no. of live cells in treated (45 Gy RT) RT group/no. of live cells in the untreated control group] x 100; for Thal, 100 - [no. of live cells in the treated (40 μg/ml Thal) group/no.of live cells in untreated-control group] x 100; for the combination therapy, 100 - (no of live cells in the treated (40 μg/ml Thal and 45 Gy RT) RT group/no. of live cells in the untreated RT group] x 100.

Determination of SP levels. Cells were seeded in 6-well plates at a density of 200,000 cells/well then, 36 h after plating, the medium was replaced with 40 μg/ml Thal in medium containing 1% serum. After 4 h, one group of the plates was irradiated in serum-free medium. Conditioned medium was collected 24 h after irradiation, and SP was extracted using the Oasis Extraction Column (Waters Corp., Milford, MA, USA). SP extractions from the cell lysates were examined as previously described without the use of column extraction (19). Briefly, $2x10^6$ purified 4T1 and 4THMpc cell pellets were lysed by incubation in 2% (vol/vol) glacial acetic acid at 95°C for 45 min. Supernatants were dried in a speed vacuum and re-suspended in the sample buffer provided in the SP EIA kit. The SP concentration in both the conditioned medium and cell lysates was measured in duplicate using a sensitive (20 pg/ml detection limit) competitive EIA kit according to the manufacturer's instructions (Substance P Enzyme Immunoassay kit; cat. no. 583751; Cayman, Ann Arbor, MI, USA). Absorbances were read at 420 nm with a microplate reader (Model 450; Bio-Rad, Richmond, CA, USA).

Immunoprecipitation and Western blotting. To ascertain whether alterations in the SP levels of 4T1 and 4THMpc cells were due to changes in SP content, immunoprecipitation and Western blotting were performed as previously described (20). Protein concentrations were determined using the BCA Protein Assay (Pierce, Rockford, IL, USA). The conditioned media were concentrated with an Oasis Extraction Column (Waters Corp.) for immunoprecipitation. Briefly, equivalent protein from each sample was incubated at 4°C overnight with anti-SP (1:10,000) Control samples were incubated with non-immune, species-specific IgG. Immune complexes were selected by incubation at 4°C for 6 h with protein A-Sepharose CL 4B (Sigma). Subsequently, protein A-Sepharose was centrifuged at 4°C for 30 min at 10,000 x g. Pellets were washed with PBS, resuspended in sample buffer, and loaded onto a sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel, and then transferred to a polyvinylidene difluoride membrane (Hybond-P; Amersham Pharmacia Biotech, Piscataway, NJ, USA) using a semi-dry transfer apparatus. Membranes were blocked for 2 h in 5% skimmed milk in PBS containing 0.05% Tween-20, and were then incubated overnight at 4°C with an SP goat polyclonal antibody (1:200, sc-14104; Santa Cruz). The blots were then incubated with an HRP-conjugated secondary antibody (1:5,000 sc-2020; Santa Cruz) and detected using ECL Western blotting detection reagent (ECL Plus kit; Amersham Biosciences). The Ultra Low Range molecular weight marker (M3546; Sigma Chemical Co., St. Louis, MO, USA) was used to determine the molecular weights of the visualized bands. Positive control lanes contained SP (Sigma; cat. no., 6883).

Statistical analysis. All data are presented as the mean ± standard deviation (SD). Analysis was performed using professional statistics software (Graph Pad Software, San Diego, CA, USA). ANOVA with Dunnett's Multiple Comparison post-test and t-tests were used for intergroup comparisons. Statistical analyses of SP levels were performed using either ANOVA followed by the Tukey-Kramer multiple comparison test or the Student's paired t-test for the percentage of alteration values. Graphs were drawn using Sigma Plot version 10.0 (SPSS Inc., USA) and CorelDRAW version X4 (Corel, Co., Minneapolis, MN, USA) software. p<0.05 was considered statistically significant.

Results

In vitro cytotoxic effects of Thal, RT and their combination on 4T1 and 4THMpc cell lines. To determine the effects of Thal, RT and the combination therapy (Thal + RT) on cell growth *in vitro*, initial experiments were conducted to determine the optimal cell density and irradiation dose (data not shown). The optimal cell density was found to be 5,000 cells/well. At this density, none of the tested irradiation doses up to 45 Gy were found to inhibit cell proliferation or to induce cell death. However, the 45-Gy dose of irradiation was determined to have a cytotoxic effect on the cells.

The cytotoxic effects of RT alone, (Fig. 1A) Thal alone (Fig. 1B) and of the combination therapy (Fig. 1C) were evaluated and compared (Fig. 1D) using the MTS assay. The results indicated that after 72 h of treatment, RT at 45 Gy caused a 19.2 and 23.31 % inhibition of growth of the 4T1 and 4THMpc cells, respectively, suggesting that the 4THMpc cells were more resistant to RT than the 4T1 cells. The number of untreated control cells indicated that the 4THMpc cells proliferated much faster than the 4T1 cells. These results were confirmed by the Live/Dead cytotoxicity assay.

To further verify these results, a trypan blue exclusion assay was performed on the 4T1 and 4THMpc cell lines. In
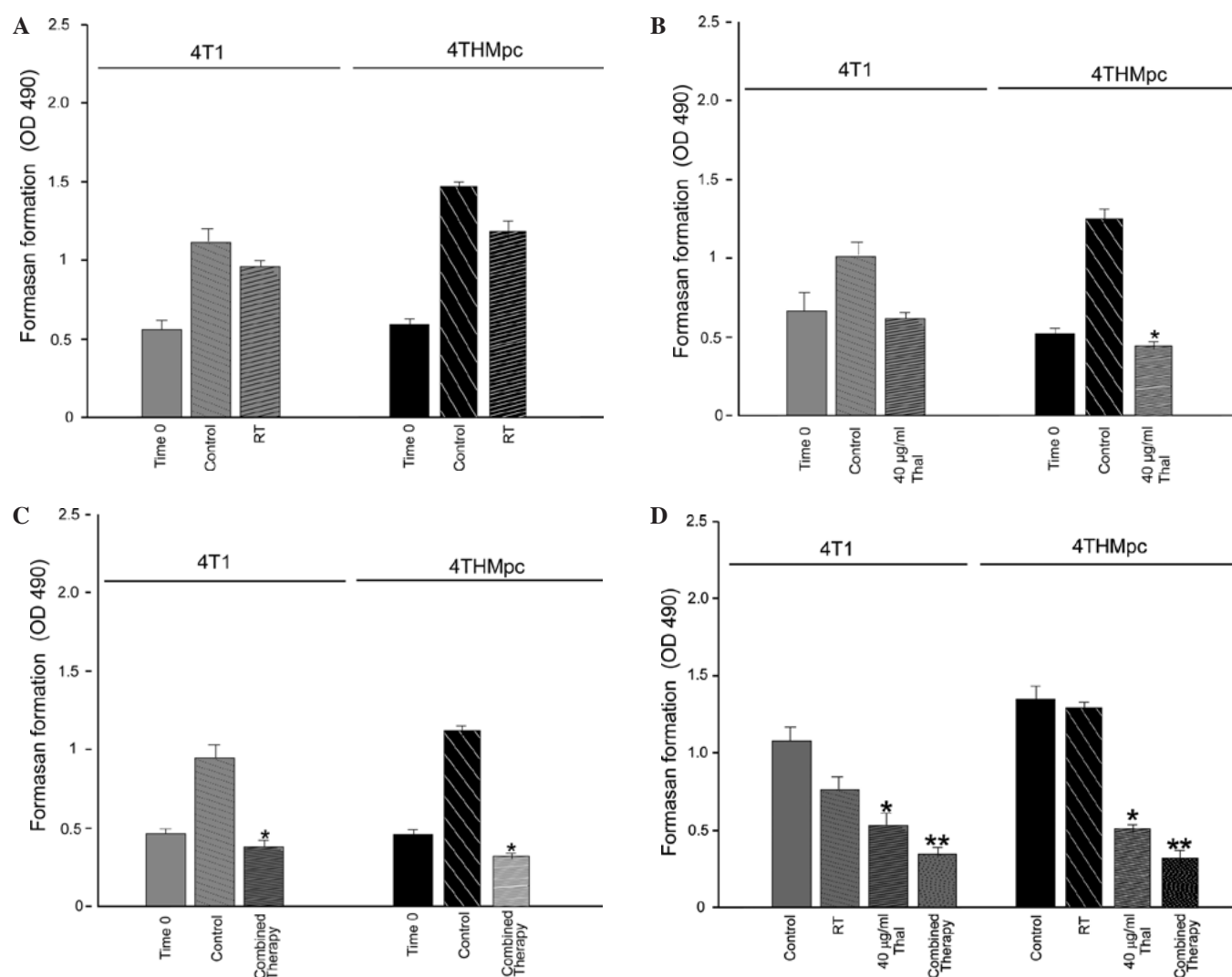
Figure 1. Effect of radiotherapy (RT), thalidomide (Thal) and combination therapy (Thal + RT) on cell growth *in vitro* in the 4T1 and 4THMpc cell lines, determined 72 h after treatment using the MTS assay. (A) Cells treated with 45 Gy of ionizing radiation alone. (B) Cells treated with 40 µg/ml Thal alone. (C) Cells first treated with 40 µg/ml Thal, then after 4 h with 45 Gy ionizing radiation. Time 0 indicates the cell numbers immediately before treatment. (D) Comparison of the effects of RT and Thal on cell viability, either alone or in combination. *p<0.05, significantly different as compared to the control group; Student's t-test.

images captured under a phase contrast microscope 72 h after treatment (Fig. 2), cell death was evident, with an abundance of seemingly condensed apoptotic cells and cell fragments in the Thal- and RT-treated groups.

The results regarding the percentage of decrease in cell survival calculated for each experiment using the Live/Dead cell viability assay and trypan blue exclusion test are summarized in Tables I and II.

SP levels in the media and cell lysates. SP levels were examined in the media and cell lysates from 4T1 and 4THMpc cells 72 h after treatment with Thal alone, RT alone or the combination therapy, at multiple stages. First, time-dependent amounts of basal SP levels were determined in the control cells and also in 2% acetic acid-administered cells, since SP was extracted by an acetic acid extraction method. Second, each sample was divided in two equal amounts; one sample was both acid and column extracted, whereas the other was only acid extracted. Third, a two-step extraction was used in order to measure SP levels. Finally, an experiment was performed in

which the extraction step with either Oasis cartridges or acetic acid was omitted. The supernatants obtained by following any of the steps above were evaporated in a vacuum for SP ELISA. It was not possible to detect SP levels in any repeated experiments using Oasis cartridges or the two-step acetic acid extraction method in order to extract SP from the cell lysates. It is likely that both of these extraction procedures are responsible for the loss of measurable SP in the cell lysates, but not in the conditioned media. Thus, the cartridges and the two-step acetic acid extraction steps were used only in order to extract SP in the conditioned media.

In the 4T1 cell line, no significant differences were noted between the basal SP levels in the cell lysate (150.28±10.2 pg/ml) or the conditioned media (151.12±11.8 pg/ml). When the cells were treated with 40 µg/ml Thal, no significant differences were noted in the cell lysates (150.23±12.9 pg/ml) or in the conditioned media (149.02±13.1 pg/ml) (p<0.05 compared to the control). By contrast, when 45 Gy RT was applied alone, the amount of SP in the 4T1 cells was increased in the 4T1 cell lysates (170.12±10.1 pg/ml) and in the media (60.33±11.8 pg/
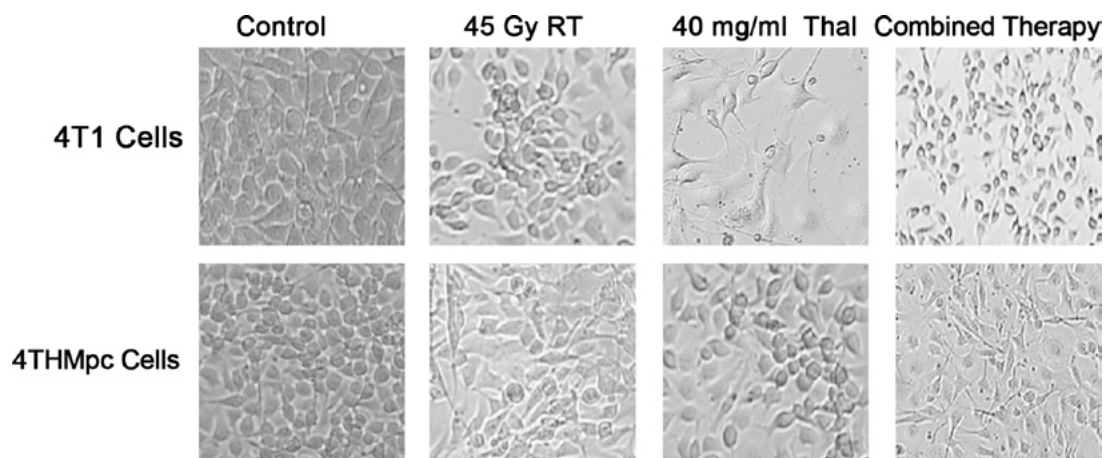
Figure 2. Effects of thalidomide (Thal), radiotherapy (RT) and combination therapy on cell growth. Cells were seeded at 50,000 cells/well in 12 well-plates, and the number of live cells was determined by the trypan blue exclusion assay. Images were captured under a phase contrast microscope.

Table I. Percentage of decrease in cell survival according to the Live-Dead cell viability assay.

	RT		Thal		Combination therapy	
	LC (%)	DC (%)	LC (%)	DC (%)	LC (%)	DC (%)
4T1	71.50±10.02	25.34±11.12	43.25±7.71	51.63±10.34	37.25±11.06	52.75±9.56
4THMpc	63.01±11.22	45.47±10.73	45.72±7.91	50.07±9.35	34.92±10.66	61.27±11.02

LC, percentage of live cells; DC, percentage of dead cells. Experiments were repeated five times in four replicative wells. Cumulative results from four highly reproducible independent experiments are presented as the mean ± SD.

Table II. Percentage of decrease in cell survival according to the trypan blue exclusion assay.

	RT	Thal	Combination therapy
4T1	19.20±3.61	34.1±8.73	47.9±8.95
4THMpc	23.31±7.49	52.6±10.31	62.03±11.57

Experiments were repeated five times in four replicative wells. Cumulative results of four independent highly reproducible experiments are presented as the mean ± SD.

ml) (p<0.01 compared to the control). Notably, the combined therapy (40 µg/ml Thal + 45 Gy RT) significantly increased SP concentrations in both the conditioned media (190.17±9.9 pg/ml) and in the cell lysates (195.28±10.48 pg/ml) (p<0.001 compared to the control). The effects of the treatments on SP levels in the 4T1 cells are summarized in Fig. 3A.

When the results obtained in the 4T1 cells were compared with those obtained in the 4THMpc cells, the 4THMpc cells were found to exhibit a significant increase in SP levels in both the conditioned media and the cell lysates. The only distinct finding noted in the experiments using the 4THMpc cells was that the level of SP in the media decreased in only the Thal-treated cells. According to the results, Thal alone had no effect on the level of SP in the media and cell lysates,

as compared to the control. However, the SP level in the media and cell lysates was significantly increased by the combination therapy. The effects of the treatments on SP levels in the 4THMpc cells are summarized in Fig. 3B. The results clearly indicate that the combination treatment enhanced the effects of Thal on SP levels in both the media and cell lysates.

To verify the results of SP ELISA, changes in SP content were determined by standard Western blotting. As shown in Fig. 4, the thickness or thinness of the bands depending on the amount of SP correlated with the SP ELISA findings.

Discussion

After noting that thalidomide had a teratogenic effect in pregnant women, it was discovered that the drug destroys blood vessels in the fetus. Based on this property, thalidomide was included in the category of anti-angiogenic drugs, and its potential anti-angiogenic and anti-tumoral properties have been demonstrated in animal models. Today, thalidomide is successfully used in the treatment of multiple myeloma, prostate and kidney cancer, and research on the potential use of thalidomide in other types of cancer is currently being carried out (21,22).

Breast cancer is one of the most important global health concerns, with over 1,500,000 new cases diagnosed and over 400,000 deaths occurring annually. Thalidomide alone is not effective in the treatment of metastasic breast cancer, and must be combined with another cytotoxic drug or an alternative therapy (23). While thalidomide is found to be cytotoxic
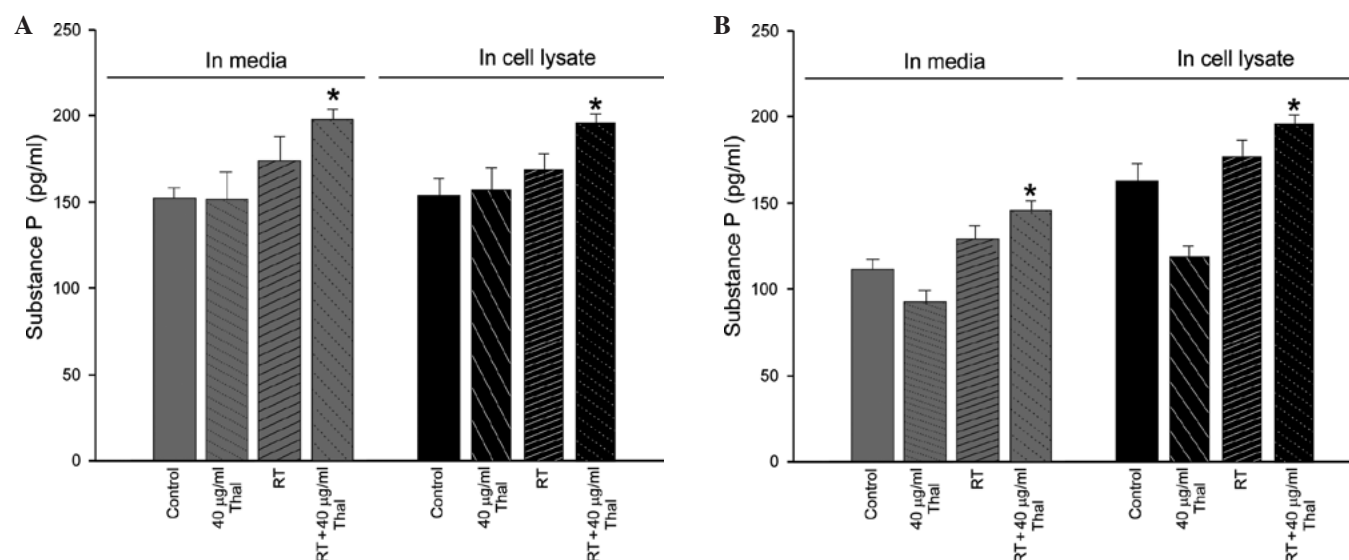
Figure 3. Effects of thalidomide (Thal), radiotherapy (RT) and combination therapy on SP levels *in vitro*, determined 72 h after treatment in conditioned media and cell lysates from (A) 4T1 and (B) 4THMpc cells. *p<0.05, significant difference in SP level as compared to the control group; Student's t-test.
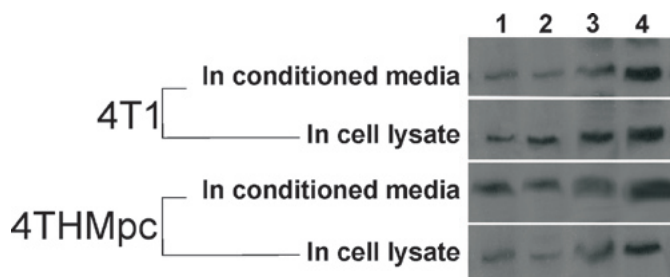

Figure 4. Amount of SP in the conditioned media and in the cell lysates *in vitro*. SP was immunoprecipitated and then characterized by Western blotting. Molecular weights of the visualized bands were ~3 kDa as compared to the marker.

in many cancer cell lines, the growth inhibition effect of thalidomide depends not only on the doses of the drug, but also on the cell type (24).

We first ascertained the cytotoxic dose of thalidomide in 4T1 and 4THMpc mouse breast cancer cell lines. The cytotoxic effects of different doses of thalidomide were evaluated and, according to our test results, thalidomide alone at 40 μg/ml was found to have cytotoxic effects on 4T1 and 4THMpc mouse breast cancer cell lines *in vitro*. In the same experiment, it was found that 4THMpc, a more aggressive form of the cancer, exhibited a more rapid growth than 4T1 cells compared to the control groups. According to their growth rate, thalidomide alone at 40 μg/ml was more effective in 4THMpc cells. At the end of the 72-h incubation period, thalidomide alone, at its cytotoxic dose caused a 34.1±8.73 and 52.6±10.31% inhibition in cell growth in the 4T1 and 4THMpc cells, respectively.

Combining a cytotoxic agent with radiotherapy is the focus of continuing interest to oncologists, and radiotherapy is the most popular therapy, particularly in the treatment of solid tumors. Over the last few years, positive results in breast cancer outcome have been demonstrated with the use of postoperative systemic therapies and radiotherapy. Although these two modalities have been extensively used, the exact mechanisms of their effect remains unknown.

Thus, in the second part of our study, we determined the effects of combination therapy (Thal + RT) on the growth of 4T1 and 4THMpc cells, with the aim of ascertaining the effective irradiation dose on these cell lines. The cells were treated with various doses of irradiation (5, 10 and 20 Gy) alone, and 45 Gy radiation was found to be effective. Mouse breast cancer cells were resistant to both low and conceivable doses of RT. The growth of the 4T1 and 4THMpc cells was not significantly inhibited by low RT doses of 5, 10 and 20 Gy. Thus, we used a relatively high dose of radiation (45 Gy). This high dose 45-Gy radiotherapy alone caused a 19.2±3.61 and 23.31±7.49% inhibition of the growth of 4T1 and 4THMpc cells, respectively. There are no studies in the literature on the effects of irradiation on these cell lines, therefore this was an initial study showing that irradiation has an inhibitory effect on mouse breast cancer cell lines.

To determine the time factor in combination therapy, another set of experiments was designed. Cells were divided into two groups. One group was first treated with Thal followed by RT administration and the other was first treated with RT followed by Thal administration at different time points. The significant results from the different independent trials indicated that 4T1 and 4THMpc cells should be irradiated after Thal treatment, not before nor immediately after. According to our results, 4 h were sufficient for 4T1 and 4THMpc cells to potentiate the efficacy of each treatment.

After determining the effective cytotoxic doses of both Thal and RT alone, and also the suitable treatment times, another set of experiments was designed to evaluate the effects of combined therapy on 4T1 and 4THMpc cells. According to our results, Thal (40 μg/ml) and RT (45 Gy) combination therapy most effectively inhibited the growth of these cells.

It is known that irradiation treatment increases the expression of angiogenic factors (24,25). Chan *et al* revealed that, when combined with anti-angiogenic molecules, the potential

effects of radiation increased, as anti-angiogenic therapy removes pro-angiogenic molecules (26). Thus, to analyze the mechanism of the increased anti-proliferative effects of Thal and RT, we evaluated the changes in the level of the pro-angiogenic peptide, SP. SP is a mitogenic peptide found to induce angiogenesis and tumor cell proliferation (27). Aalto *et al* reported that RT induces SP expression in human breast cancer cell lines (28). Our results confirm this finding: 45 Gy RT alone caused a 13.3 and 6.6% increase in the amount of SP in the media and cell lysate of 4T1 cells, respectively. The specific increase in the levels of SP in response to RT may indicate its importance in the growth of breast cancer cells, and may also explain their metastatic potential after RT. The amount of SP was significantly increased when RT was combined with Thal. Combination therapy caused a 30 and 26.6% increase in the amount of SP in the media and cell lysate of the 4T1 cells, respectively. Similar results were obtained in the 4THMpc cell line. According to our results, 45 Gy RT alone caused a 16.6 and 15.8% increase in the amount of SP in the media and cell lysate of the 4THMpc cells, respectively. Combination therapy caused a 27.27 and 18.75% increase in the amount of SP in the media and cell lysate of the 4THMpc cells, respectively. In addition, Thal alone had no effect on the level of SP in the media, and caused only a 3.3% increase in SP in the cell lysates of 4T1 cells. In the 4THMpc cells, Thal caused an 18.18 and 21.87% decrease in SP in the media and the cell lysates, respectively. The present study demonstrates that high dose irradiation (45 Gy) has systemic side effects, such as an alteration in the amount of neuropeptide (SP) content in breast cancer cells. We suggest that the combination of Thal and RT increases the level of SP, which may potentiate the tumor growth of metastatic breast cancer cells.

In conclusion, this study indicates that thalidomide exhibits anti-proliferative effects against breast cancer cells *in vitro* and potentiates the anti-tumoral effects of radiotherapy. The level of SP observed after radiation therapy alone was increased by the combination of Thal and RT, and this may limit the use of this combination therapy in metastatic breast cancer patients. These data may be helpful in the design of future clinical trials to potentiate the use of anti-angiogenic treatments in combination with other treatment modalities.

Acknowledgements

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