

Endostatin enhances radioresponse in breast cancer cells via alteration of substance P levels

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Abstract. Radiotherapy is widely used in the treatment of cancer. On the other hand, endostatin is considered to be a potent inhibitor of angiogenesis. Therefore, to test whether ES combined with RT overcomes the limitations of each monotherapy the present study investigated the effects of endostatin (ES), radiotherapy (RT) or combination therapy on the growth of mouse breast cancer cells as well as on the expression of substance P *in vitro*. The breast cancer cell lines 4T1 and 4THMpc were treated with recombinant murine ES (0.5, 1, 2, 4 and 8 µg/ml) alone, RT (45 Gy) alone or as a combination therapy. Cell proliferation was evaluated using an MTS assay and the results were verified by the Live/Dead assay. Immunoprecipitation and Western blotting analysis were performed to determine whether the substance P levels of the two cell lines occurred due to substance P content. Results showed that ES alone resulted in a low but significant inhibition in the growth of 4T1 and 4THMpc cell lines (27.63 and 21.75%, respectively). RT alone inhibited the growth of 4T1 (30.81%) and 4THMpc (39.64%) cells. A combination of ES with RT enhanced growth inhibition in the cells (83% in 4T1 and 80% in 4THMpc). The amount of substance P, both in the conditioned media and the cell lysates, increased within 72 h after RT. This increase was inhibited when ES and RT were applied in combination. These data indicate that ES inhibits the *in vitro* growth of breast cancer cells and potentiates the anti-tumor effects of RT at appropriate doses via alteration of the amount of substance P and thus an increase of radioresponse.

Introduction

Breast cancer is the most frequently diagnosed type of cancer in female individuals (1). Despite widely used treatment methods, such as surgery, radiotherapy (RT) and chemotherapy, breast cancer remains a challenging disease to treat, since a poor

survival rate is observed in patients with locally advanced or metastatic breast cancer (2).

Angiogenesis involves the formation of new blood vessels from existing ones, and is a mechanism that is involved in tumor growth and metastasis (3). Consequently, inhibition of the angiogenic process is crucial in arresting tumor growth. Endostatin (ES), a 20-kDa C-terminal proteolytic fragment of collagen XVIII, is known as a potent inhibitor of angiogenesis *in vitro* (4).

Findings of various studies have shown that the addition of anti-angiogenic agents to RT increases clinical efficacy (5). Reports have suggested that ES may effectively enhance the effects of RT for tumor cells *in vitro* (3,6). The exact mechanisms of this type of synergistical effect have yet to be clarified. Therefore, attention is currently focused on gaining a better understanding of specific molecular pathways of any combination in order to modify the survival of tumor cells and to enhance the efficacy of the individual treatment involved.

Since the emergence of the relationship between neuro-peptides and cancer, novel approaches were suggested in the treatment of cancer (7). Substance P (SP) has been identified as a member of the tachykinin family, a class of small peptides that is widely distributed in the central and peripheral nervous system. SP was found to be involved in the generation or progression of various physiological and pathological conditions (8). Besides acting as a neurotransmitter, SP is thought to be involved in a number of processes related to oncogenesis (9). However, the role of SP during metastatic growth remains to be elucidated.

To the best of our knowledge, the effect of ES on the SP pathway has yet to be examined. ES is thought to affect various cellular signaling pathways. Thus, we posed the question of whether ES as a monotherapy or in combination with RT is capable of changing SP levels in breast cancer cells. However, since a limited number of studies examining the effects of ES and RT currently exist, the aim of the present study was to determine the antiproliferative effects of ES, RT and their combination on breast cancer cells, and to determine ES, RT and combination therapy-induced changes in SP expression.

Materials and methods

Recombinant murine ES. Recombinant murine ES (rmES) in citrate phosphate buffer (17 mM citric acid, 66 mM sodium

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phosphate dibasic and 59 mM sodium chloride; pH 6.2) was purchased from Sigma (Sigma Chemical Co., St. Louis, MO, USA).

Cell lines and in vitro culture conditions. 4T1 breast cancer cells and a cell line developed from orthotopically transplanted 4T1 breast cancer cells, cardiac metastases of capsaicin-treated mice that denominated as 4THM (4T1 Heart Metastases Post Capsaicin), were used in the experiments. 4THM cells have been shown to exhibit more metastatic potential than 4T1 cells (10). The 4T1 and 4THMpc cell lines were a generous gift from Dr Nuray Erin (Akdeniz University, Medicine Faculty, Antalya, Turkey). Cells were maintained 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% CO₂. Both the 4T1 and 4THMpc cell lines used in this study were tested and found to be free of mycoplasma contamination.

Cell viability. To assess the effects of rmES, RT and the combination (rmES + RT) on cell growth *in vitro*, initial studies were performed to determine the optimal treatment conditions. Cells were plated at a density ranging from 1,000 to 30,000/wells (data not shown). Subsequently, the most applicable cell number per well was found to be 10,000 cells. Two sets of experiments were designed to evaluate the effects of each treatment alone and as combination therapy. After 36 h of plating, the cells were treated with rmES only at 0.5, 1, 2, 4 and 8 µg/ml in serum-free medium and medium supplemented with 1% FBS, with eight repeats of each treatment, or RT (45 Gy) only. The cells were then treated with rmES + RT, with RT being applied 4 h after rmES. Vehicle (citrate phosphate buffer) and plain medium were used as negative controls.

Radiotherapy. Experiments were performed to determine the optimal dose of RT. Briefly, each cell plate (2-cm thick) was irradiated in the Co-60 teletherapy unit at a distance of 100 cm. 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 placed on the cover of the plate. The optimal dose of irradiation was found to be 45 Gy at 1.5 cm (in the centre of the plate) and the dose rate at RT was ~145 cGy/min.

Cell viability. Cell proliferation was determined using the tetrazolium compound 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy-phenyl)-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). Cell viability was measured at 24, 48 and 72 h after treatments. Formation of formazan was determined at an optical density (OD) 490 nm and was compared between the groups. To calculate the percentage of growth inhibition the following formula was used: growth inhibition (%) = [(mean OD value of control group - mean OD value of treatment group)/mean OD value of control group] x 100%

The Live/Dead Viability/Cytotoxicity Kit for Mammalian Cells (Invitrogen, Eugene, OR, USA), was used to verify

results. Cells were dually stained with the probes Calcein AM and EthD-1, according to the manufacturer's protocol. The probes enabled the simultaneous determination of viable and dead cells, respectively. Fluorescent intensity was measured on an LS55 Luminescence Plate Reader (PerkinElmer Inc., Waltham, MA, USA) at 560- and 535-nm excitation, and 645- and 610-nm emission wavelengths, respective to each reagent dye.

After cell proliferation was determined using the MTS and Live-Dead assays, the viable and dead cell numbers (50,000 cells/well in 12-well plates) were assessed using a trypan blue (0.4% trypan blue in HBSS) exclusion test. The percentage of viable cells from each well after 72 h incubation was obtained using the equation: % viable cells = (VC/TC) x 100, where VC = viable cell number counted and TC = total cell number counted (stained + unstained cells). The amount of total cell cells number (TC) per ml was calculated using the formulas: total cells number/ml = average of total cell number in four big squares x dilute multiple x 10⁴. Additionally, to determine the number of viable cells, the formulas used were: for RT, 100 - [number of viable cells in RT-treated (45 Gy) group/number of viable cells in untreated control group] x 100; for rmES, 100 - [number of viable cells in rmES-treated (4 µg/ml) group/number of viable cells in untreated-control group] x 100; and for the combination therapy, 100 - [number of viable cells in rmES + RT treated (4 µg/ml and 45 Gy) group/number of viable cells in untreated RT-receiving group] x 100.

Determination of substance P concentrations. Cells were dually seeded in 25-cm cell culture petri dishes at a density of 2,000,000 cells/well and treated with vehicle or rmES in serum-free medium 36 h after plating. After 4 h, one group of the dishes was irradiated in serum-free medium. Conditioned medium was collected 72 h after RT, and SP was extracted using the Oasis Extraction Column (Waters Corp., MA, USA). SP extractions from the cell lysates were examined as previously described without column extraction (11). SP concentrations in both the conditioned medium and cell lysates were measured in duplicate using a sensitive (3.9 pg/ml detection limit) competitive ELISA kit according to the manufacturer's instructions (Catalog No. 583751; Cayman, Ann Arbor, MI, USA). Absorbances were read at 420 nm using a microplate reader (Thermolabsystem, Chantilly, VA, USA).

Immunoprecipitation and Western blotting. To determine whether changes in the SP levels of 4T1 and 4THMpc cells were due to changes in the SP content, immunoprecipitation and Western blotting were performed as previously described (12). Protein concentrations were determined using the BCA Protein Assay kit (Pierce, Rockford, IL, USA). 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 phosphate-buffered saline, resuspended in sample buffer, loaded on to a sodium dodecyl sulphate-polyacrylamide gel electrophoresis gel, and transferred onto polyvinylidene difluoride membranes (Hybond-P; Amersham

Table I. Cumulative results of the Live-Dead cell viability assay^a.

	RT		rmES		rmES + RT	
	LC (%)	DC (%)	LC (%)	DC (%)	LC (%)	DC (%)
4T1						
SF	49.21±11.01	69.33±14.76	36.14±9.605	51.63±18.40	42.30±13.18	79.05±8.90
S	89.64±6.21	52.85±7.43	56.42±5.34	86.42±9.31	24.57±6.93	98.54±5.13
4THMpc						
SF	54.10±10.43	62.12±9.21	39.15±4.55	61.74±7.91	44.30±17.24	78.10±9.65
S	74.92±6.42	48.91±1.67	51.70±3.07	72.13±2.73	28.17±1.56	95.66±2.29

^aExperiments were repeated five times with four replicative wells and the percentage of viable and dead cells was calculated as described in Live/Dead Viability/Cytotoxicity Kit for Mammalian Cells (Invitrogen) Technical Datasheet with the results of highly reproducible four independent experiments. LC, DC, SF and S refer to live-cell, dead-cell, serum-free media and media with 1% fetal calf serum, respectively.

Pharmacia Biotech, Piscataway, NJ, USA) using semidry transfer apparatus. Membranes were blocked for 2 h in 5% skimmed milk in phosphate-buffered saline containing 0.05% Tween-20, and incubated overnight at 4°C with the SP goat poly-clonal antibody (1:200, sc-14104; Santa Cruz). The blots were then incubated with HRP-conjugated secondary antibody (1:5,000 sc-2020; Santa Cruz) and detected using the ECL Western blotting detection reagent (ECL Plus kit; Amersham Biosciences, Amersham, NJ, USA). An ultra low range molecular weight marker (M3546, Sigma Chemical Co.) was used to determine the molecular weights of the visualized bands.

Statistical analysis. Data were presented as the mean ± SEM. Analysis was performed using a professional statistics software program (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 for the SP levels were performed using either ANOVA followed by a Tukey-Kramer multiple comparison test or the Student's paired t-test on the percentage of alteration values. The graphs were drawn using Sigma Plot version 10.0 (SPSS Inc., Chicago, IL, USA) and CorelDRAW version X4 (Corel, Co., MN, USA) softwares. P<0.05 was considered to be statistically significant.

Results

rmES combined with radiotherapy significantly inhibits in vitro proliferation of 4T1 and 4THMpc cells. Experiments were performed to determine the acute effects of rmES alone, RT alone as well as the combination therapy rmES + RT on 4T1 and 4THMpc cells. However, no difference was found between the proliferation of rmES-treated and untreated cells up to a threshold concentration of 4 µg/ml for 72 h. Additionally, the results of the MTS assay showed that none of the tested RT doses up to 45 Gy effectively inhibited cell proliferation or induced cell death (data not shown).

RT (45 Gy) alone caused 27.63 and 21.75% inhibition in the growth of 4T1 and 4THMpc cells, respectively. This result is noteworthy since a higher rate of proliferation of 4THMpc cells was observed. In particular, the number of 4T1 cells

was increased by 3.8-fold, whereas that of 4THMpc cells was increased by 7.2-fold after 72 h of treatment (Fig. 1A). Time 0 bars in Fig 1 show the formation of formazan, which serves as an indicator of viable cell numbers, prior to treatment. rmES (4 µg/ml) alone caused 30.81 and 39.64% inhibition in the growth of 4T1 and 4THMpc, respectively (Fig. 1B). The results indicate that 4THMpc cells are more sensitive to rmES than 4T1 cells. Particularly, after 72 h of treatment, the number of 4T1 cells was increased by 4.1-fold, whereas that of 4THMpc cells was increased by 9.2-fold. The higher rates of proliferation of the 4THMpc cells noted in this study may provide an explanation for the sensitivity of 4THMpc cells to rmES.

As shown in Fig. 1C, rmES increased the cytotoxic effects of RT. While rmES alone and RT alone caused 30.81 and 27.63% inhibition, respectively, in the growth of 4T1, the combination of rmES + RT yielded an 83% inhibition. On the other hand, 39.64 and 21.75% inhibition, respectively, in the growth of 4THM were caused by rmES and RT treatments alone whereas the combination resulted in an 80% inhibition (Fig. 1D).

The growth rates of 4T1 and 4THMpc cells were also determined 1 week after rmES + RT treatment to determine whether RT-induced cell death is a late event. Notably, although an increase in the viability of the cells treated only with rmES or RT was noted, a time-dependent decrease occurred in the cell viability of rmES + RT-treated groups of the two cell lines (Fig. 1E). The marked decrease in the number of viable cells a week after treatment suggests that the impact of combination is ongoing.

Furthermore, the dead and total cell numbers were determined 72 h after treatment using the trypan blue exclusion test. As shown in Fig. 2, rmES alone increased the number of dead cells and changed the total cell number, indicating that rmES alone not only induces cell death, but also inhibits cell proliferation. Images captured under a contrast phase microscope at a magnification of x200 showed cell death, with the abundance of apparently condensed apoptotic cells and cell fragments in the rmES-treated group (Fig. 3). Similar results were obtained using the Live/Dead Assay. The percentage of decrease in cell survival was calculated to determine the

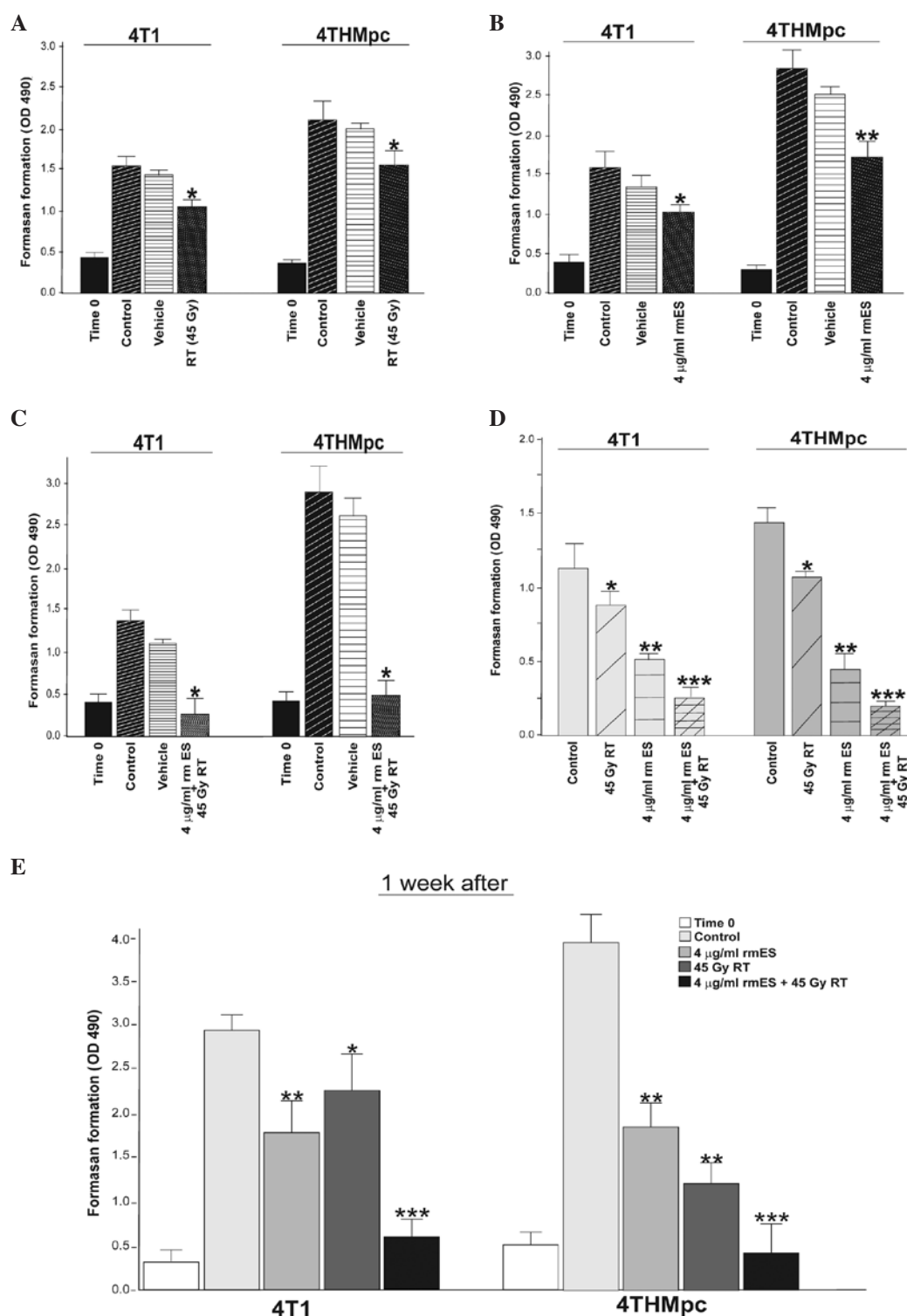


Figure 1. Effects of radiotherapy (RT), endostatin (ES) and combination therapy on cell growth *in vitro* in the 4T1 and 4THMpc cell lines as determined using MTS solution 72 h after treatment. (A) Cells were treated with 45 Gy of RT. (B) 4T1 and 4THMpc cells were treated with 4 µg/ml rmES. (C) 4T1 and 4THMpc cells were initially treated with 4 µg/ml rmES and 4 h after rmES treatment, 45 Gy RT was applied. Time 0 bars shown cell numbers prior to RT. (D) A comparison of the effects of RT and ES on cell viability, either alone or in combination is shown. (E) Time-dependent changes in the viability of 4T1 and 4THMpc cells following rmES (4 µg/ml) + RT (45 Gy) treatment. *P<0.05, significantly different as compared to control group, Student's t-test.

average of four independent experiments (Table I). The effects of rmES changed depending on the ratio of FBS in complete medium (Table II).

To determine the effects of rmES on metastatic cells, the experiments were repeated using 4THMpc cells. Similar results were obtained to those of rmES alone in 4T1 cells since

rmES alone was found to be efficacious in decreasing cell survival and appeared to induce further cell death in 4THMpc as compared to 4T1 cells. Images captured under a contrast phase microscope showed the occurrence of condensed cells and fragmentary cell remnants (Fig. 4). The decrease in viable cell numbers following combination therapy clearly demon-

Table II. Cumulative results of the trypan blue exclusion test.^a

	RT	rmES	rmES + RT
4T1			
SF	7.42±2.82	35.46±14.66	44.21±6.78
S	10.35±3.71	43.57±4.29	66.42±8.81
4THMpc			
SF	19.52±9.57	46.52±11.33	62.75±13.24
S	25.07±6.42	38.29±3.07	71.82±2.73

^aExperiments were repeated five times with four replicative wells and the percentage of decrease in cell survival was determined. SF and S refer to serum-free media and media with 1% fetal calf serum, respectively.

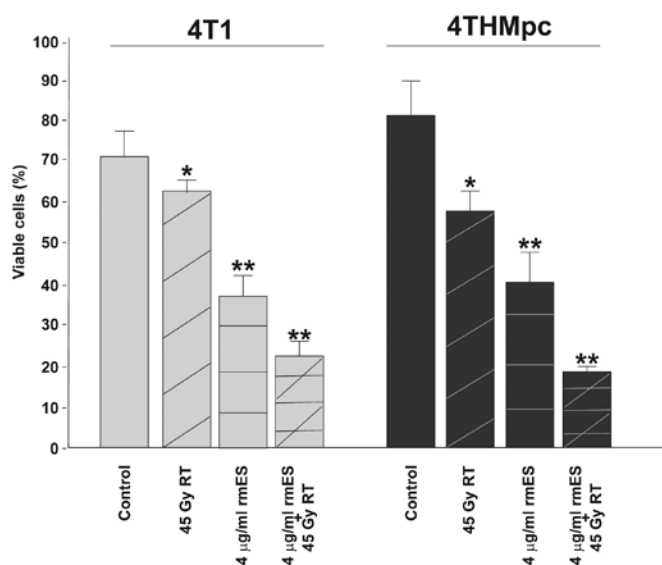


Figure 2. Changes in the percentages of viable cell numbers following RT, rmES or RT + rmES treatments are shown. Cells were seeded in 12 well-plates (50,000 cells/well) and the number of viable cells were determined using the trypan blue exclusion test. *P<0.05 and **P<0.01, significantly different as compared to the control group, Student's t-test.

strates that rmES also enhances the cytotoxic effects of RT in metastatic 4THMpc cells.

Effects of combination therapy of recombinant murine endostatin with radiotherapy alters substance P levels in the media and cell lysates. To determine the effects of ES alone RT alone and the combination therapy on SP levels within 4T1 and 4THMpc cells, the conditioned media were examined after 72 h of treatment at multiple stages. First, time-dependent amounts in basal SP levels were determined in the control cells as well as in 2% acetic acid-administered cells. Second, each sample was divided into two equal amounts. One of the samples was extracted by both acid and column, whereas the other sample was extracted by acid only. Third, a two step-extraction was used to measure the SP levels. In the final stage an experiment was performed in which the extraction step was omitted either with Oasis cartridges or with acetic acid. No SP

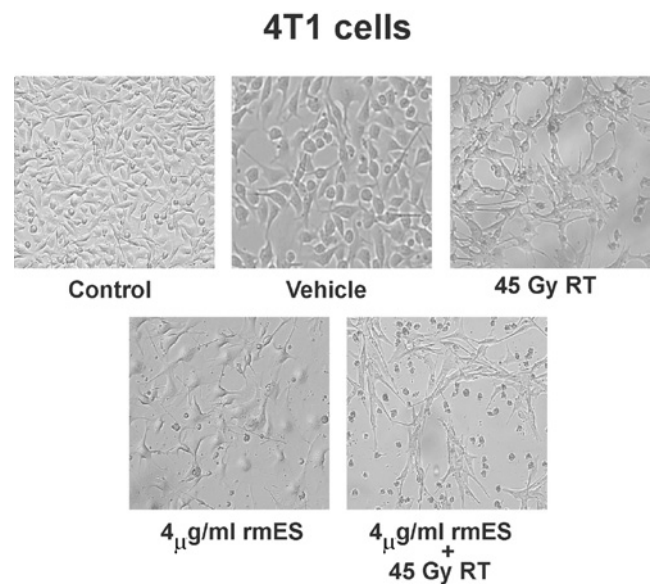


Figure 3. Appearance of the 4T1 cells seeded in 12 well-plates (50,000 cells/well) under a contrast phase microscope (magnification, x200).

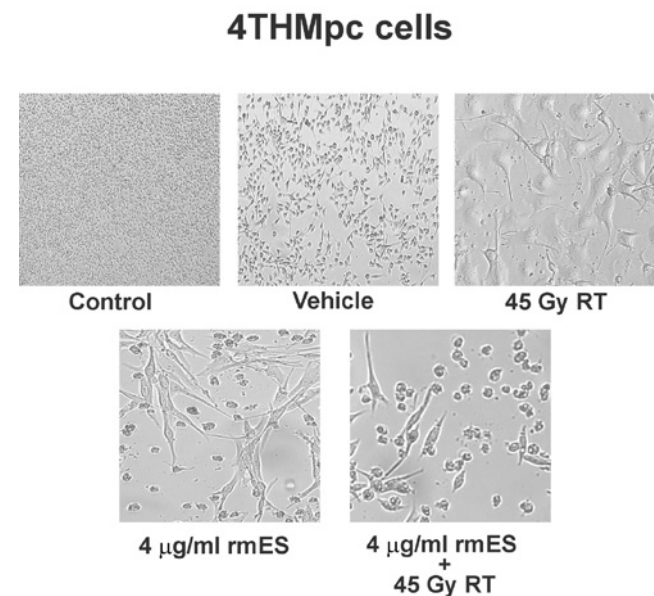


Figure 4. Appearance of 4THMpc cells seeded in 12 well-plates (50,000 cells/well) under a contrast phase microscope (magnification, x200).

level was detected in any of the repeated experiments when Oasis cartridges or two-step acetic acid extraction methods were used to extract SP from cell lysates. Therefore, the two extraction procedures are responsible for the loss of measurable SP in cell lysates, but not in the conditioned media. Thus, the cartridges and two-step acetic acid extraction steps were only used to extract SP in the conditioned media.

To determine the amount of basal SP levels either in the cells or in the conditioned media, 4T1 cells potentially required longer incubation times of at least 24 h for extraction. No significant difference was found between the basal SP levels in the 4T1 cell line. The SP amount this cell line for the cell lysates and conditioned media were found to be 147.35±11.6

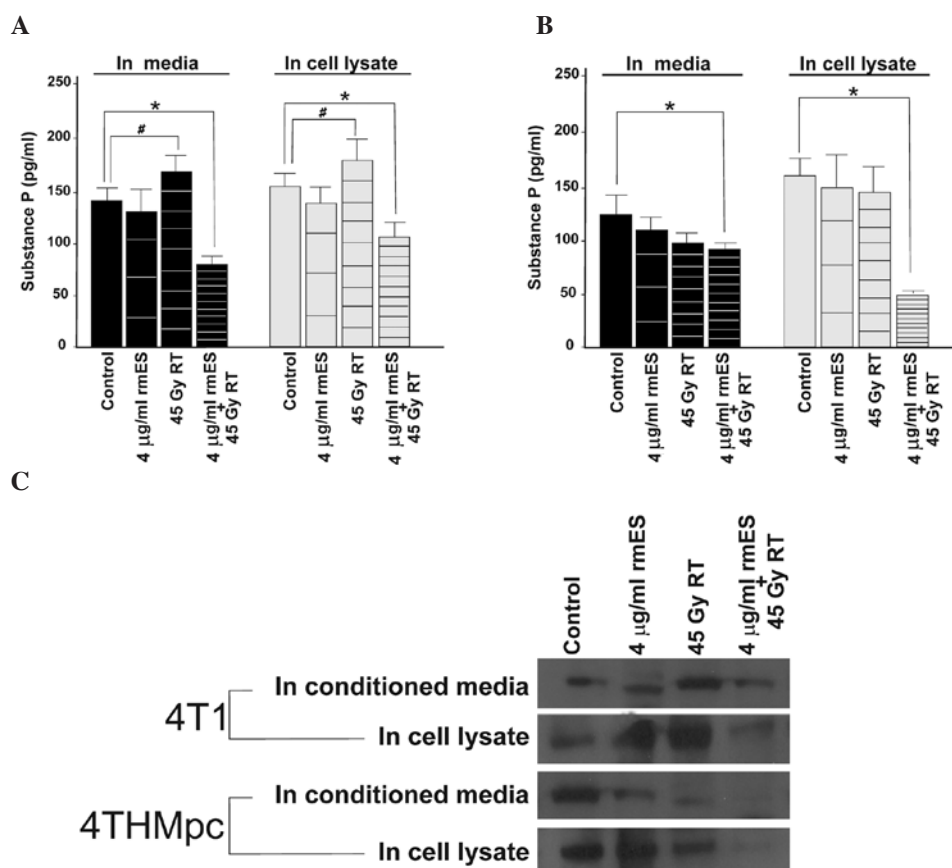


Figure 5. Effects of recombinant murine endostatin (rmES), radiotherapy (RT) and their combination on substance P (SP) levels *in vitro*. (A and B) Changes in the concentration of SP both in the conditioned media and cell lysates as determined 72 h after treatment of 4T1 and 4THMpc cells, respectively, with rmES alone, RT alone or their combination. * and # indicate a statistically significant decrease and increase in the concentration of SP, respectively. *, #P<0.05 indicates a significant difference as compared to the control group, Student's t-test. (C) Amount of SP in the conditioned media and cell lysates *in vitro*. SP was immunoprecipitated and Western blotting was applied. The bands were visualized and their molecular weights were found to be ~3 kDa as compared to the marker.

and 152.04 ± 13.01 pg/ml, respectively (Fig. 5A). The amount of SP released from 4T1 cells was significantly decreased when the cells were treated with only 4 μ g/ml rmES (132.11 ± 15.6 pg/ml in the conditioned media and 147.02 ± 13.7 pg/ml in the cell lysates, $p < 0.05$). By contrast, when RT was applied alone (Fig. 5A), the amount of SP was increased (174.25 ± 13.7 pg/ml in the media and 181.28 ± 14.2 pg/ml in the 4T1 cell lysates, $p < 0.01$). The combination therapy significantly reduced SP concentration in both the conditioned media and cell lysates (77.30 ± 10.3 pg/ml in the media and 112.14 ± 11.76 pg/ml in 4T1 cell lysates, $p < 0.001$).

4THMpc cells exhibited a marked result profile (Fig. 5B). There was a statistically notable difference in the amount of SP between the conditioned media and cell lysates of the control groups (120.11 ± 12.74 and 163.41 ± 11.91 pg/ml, respectively; $p < 0.001$). rmES treatment of 4THMpc cells alone did not yield a statistically significant difference in the SP levels either in the conditioned media (112.46 ± 10.97 pg/ml) or in the cell lysates (150.22 ± 14.10 pg/ml) when compared to the relative controls. However, a considerable increase was also determined between the amounts of SP (pg/ml) in the conditioned media and cell lysates of rmES-treated 4THMpc cells. Our data firmly indicate that when RT was administered alone, there was a decreased release of SP into the conditioned media of 4THMpc cells (101.05 ± 10.81 pg/ml, $p < 0.05$)

whereas, the amount of SP in the cell lysates (148.35 ± 13.06 pg/ml) was found to be unchanged. The combination therapy resulted in a marked decrease of the SP levels [97.67 ± 9.11 pg/ml in the conditioned media ($p < 0.01$) and 51.86 ± 7.25 pg/ml in the cell lysates ($p < 0.001$)]. In this context, the study results indicate that the combination therapy exhibited a marked decrease in the SP levels of the media and cell lysates of the 4THMpc cells as compared to the 4T1 cells. Changes in SP content were determined by Western blotting. As shown in Fig. 5C, the thickness or thinness of the bands depending on the amount of SP is correlated with the results of SP ELISA.

Discussion

Our results demonstrate for the first time that ES has direct antiproliferative effects on 4T1 and 4THMpc breast cancer cell lines *in vitro*. However, the antiproliferative effect of ES on breast cancer cells appears to be restricted by the concentration, since there is a narrow range for a therapeutic dose of ES for breast cancer cells. These findings are consistent with the hypothesis that an optimal dose range of ES is required for effective tumor growth inhibition (13-17). Results of studies have shown that the *in vitro* antiproliferative effects of ES are dependent on the tumor cell lines in which these effects are examined. Although findings of certain studies

have shown that ES does not inhibit tumor cell proliferation, other studies have documented the opposite effect (18-26). For example, in their study, Dkhisssi *et al* reported that 5 μ g/ml rmES exerts *in vitro* antiproliferative and apoptotic effects on HT29 and C51 colon cancer cell lines at the end of 72 h of treatment (24). Additionally, results by Hajitou *et al* showed that ES inhibits *in vitro* EF43.fgf-4 mouse breast cancer cell proliferation via the inhibition of VEGF expression (25). Consequently, the antiproliferative effect of rmES are considered to vary depending on factors, such as tumor cell type, the number of tested cells, incubation period and concentration ranges. The present study demonstrated that the amount of serum in the conditioned media, while rmES is applied to tumor cells, significantly alters the antiproliferative effects.

Therefore, rmES treatment induces cell death rather than inhibiting cell proliferation, suggesting that ES treatment is more suitable as adjuvant therapy or in combination with RT. Results of this study confirm those of previous studies and demonstrate that rmES potentiates the cytotoxic effects of RT (5,6,27-30). Of note, however, is that the results do not indicate the benefits of combination therapy. Conflicting results were obtained from the combination of rmES and RT prior to determining the appropriate doses and time period of dose administration. Hanna *et al* have demonstrated that low doses of ES (1,000 ng/ml) and RT (19 Gy) enhance the cytotoxic effects on endothelial cells, but not on tumor cells (30). The lack of interactive tumor cell killing may be due to the low ES concentrations tested. Since 4T1 and 4THMpc cells appear to be resistant to both low and moderate doses of rmES and RT, we did not observe any antiproliferative effects or any interaction up to the appropriate doses. Even with relatively high doses (45 Gy), RT alone caused 27.63 and 21.75% growth inhibition in 4T1 and 4THMpc cells, respectively. However, the combination therapy enhances the growth inhibition rate of 4T1 and 4THMpc cells to 83 and 80%, respectively. In addition, the synergistical effects obtained from the combination changed depending on the administration time of the RT after rmES treatment. Results from various independent trials emphasize the fact that 4T1 and 4THMpc cell lines had to be irradiated either before or concomitant to administration of rmES treatment. In the present study, 4 h were sufficient for 4T1 and 4THMpc cells to potentiate the efficacy of each treatment. On the basis of our results, it is concluded that the success of the combination therapy may change depending on which treatment has been applied primarily and when follow-up treatment is implemented.

RT is known to increase the expression of angiogenic factors (6,31,32). In their study, Chan *et al* indicated that when combined with antiangiogenic molecules, the potential effects of RT increase due to the fact that anti-angiogenic therapy eliminates the proangiogenic molecules (3). Findings by Itasaka *et al* indicated that ES treatment decreases the tumor cell expression of IL-8 and VEGF, and therefore potentiates the antitumoral effects of RT (6). Aalto *et al* reported that RT induces SP expression in human breast cancer cell lines (33). Our results confirm the finding by Aalto *et al* and demonstrate that 45 Gy RT alone caused 26.87 and 29.23% increases in the amount of SP in the media and cell lysates of 4T1 cells, respectively. The present study demonstrates that high-dose RT (45 Gy) has systemic side effects, such as altering the

SP content in breast cancer cells. In addition, the increased amount of SP may potentiate tumor cell growth after RT treatment. The specific increase obtained in the levels of SP in response to RT may indicate a significant role played by SP in the growth of breast cancer cells as well as explain the metastatic potential of these cells. However, when RT is combined with rmES, a reduction in cell viability is achieved in a manner proportional to the decreased amount of SP.

In conclusion, the present study indicates that ES exhibits antiproliferative effects against breast cancer cells *in vitro* and potentiates the antitumoral effects of RT. The increased amount of SP after RT therapy alone is decreased by the combination of rmES and RT, and this multimodality therapy may therefore overcome the limitations of the individual therapy, resulting in superior antiproliferative activity. This appears to be a novel functional mechanism of ES possibly mediated by altering the concentration of SP in tumor cells in combination with RT.

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