The effect of siRNA-Egr-1 and camptothecin on growth and chemosensitivity of breast cancer cell lines

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Abstract. We have examined the effects of a siRNA targeting the Egr-1, alone or in combination with the breast cancer therapeutic camptothecin (Cpt), in suppressing breast cancer cell survival and anchorage-independent growth in the breast cancer cell lines SK-BR3 and MCF-7. In mammary and lung tumors, as well as most normal tissues, Egr-1 expression is low, suggesting a possible relation between the low levels of Egr-1 and the development of mammary neoplasias. However, analyses of the expression of Egr-1 in breast carcinoma cells, SK-BR3 and MCF-7 demonstrated a relatively high expression of the endogenous Egr-1 in these cells. To investigate the effect of the blocking of the endogenous Egr-1 in breast cancer cells, we used small interfering RNA (siRNA) against Egr-1 alone or in combination with Cpt, and expected that the cell sensitivity to chemotherapeutic drug would increase, when blocked with the Egr-1 gene and treated with Cpt. Thus, we performed *in vitro* experiment to clarify the effect of Egr-1 on tumor cell lines growth. We made control and siRNA-Egr-1 using vector plasmids and then transfected SK-BR3 and MCF-7 cells. After treating the cells with siRNA-Egr-1, the cell lines were assayed with Cpt to confirm the effect of Egr-1 siRNA using the cell expression of mRNA and protein, proliferation assay and anchorage activity with soft agar. Human SK-BR3 and MCF-7 breast carcinoma cell growth and capacity of anchorage transfected with siRNA-Egr-1 or treated with Cpt was slower than that of the control group. This effect was increased when the cells were given simultaneously siRNA and camptothecin. The results strongly suggest that siRNA-Egr-1 alone or in combination with camptothecin could be a potent antineoplastic agent in suppressing the growth of breast tumor despite the known role of Egr-1 as a tumor-suppressor in several other types of human cancers.

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Introduction

It is predicted that cancer will become the first cause of death in industrialised nations within this century (1). In this aspect, combinatorial methods have had an enormous impact in biomedical research, namely, for providing a rapid and efficient route to large number of compounds for screening (2). Despite advances in cancer therapy there are thousands of patients suffering from cancer for which no treatments exist. Hence, there is still a high demand for new anticancer drugs exhibiting improved efficiency and selectivity for their use in combined therapy strategies. The development of molecular and cellular biology tools has made possible the set up of simple *in vitro* assays, susceptible to automation, thus bringing about the possibility of rapid screening of hundreds of compounds (2,3). One of these compounds involved in the development of cancer is the transcription factor early growth response gene-1 (Egr-1) (4). Egr-1 is an example of the immediate-early gene group (e.g., c-fos, c-jun), which exhibits *de novo* transcription within 15-30 min after stimulation of fibroblast and other cell types (4-6). The transcription factor, Egr-1, is rapidly induced by growth factors to transduce the proliferative signal (7). The induction of Egr-1 by external stimuli is generally transient but appears to be sustained in some prostate tumor cell lines and tumors, suggesting that Egr-1 stimulates tumor cell growth and that could have an important function because its expression level increases with the degree of malignancy as measured by the Gleason grade of the tumor (7,8). In mammary and lung tumors, as well as most normal tissues, Egr-1 expression is low (8-11). In addition Egr-1 overexpression is correlated with the loss of its co-repressor NAB2 in primary prostate carcinoma (12,13). This disruption of the balance between Egr-1 and NAB2 expression results in a high Egr-1 transcriptional activity in prostate carcinoma cells (14). On the contrary, in breast, lung and brain tumors, Egr-1 expression is often reduced and when re-expressed, results in growth suppression (4,5,8,14). Egr-1 also plays a role in tumor progression, through the hypoxic signal generated in growing tumors. Egr-1 is highly induced under these conditions and its activities stimulated angiogenesis and improved survival

of tumor cells (4,5,8,14). Camptothecin is a cytotoxic plant alkaloid that exhibits a broad spectrum of anti-neoplastic activity against solid tumors (15-18). The synthesis of camptothecin analogues exhibiting high antitumor activity of the drug, increasing the chemotherapeutic potential of the drug (18-20). The cellular target of the drug is the DNA topoisomerase I (20-22). DNA topoisomerase I is ubiquitous in eukaryotes and plays an important role in a number of cellular processes involving DNA, including transcription, recombination and DNA replication (20-22). There is a variety of camptothecin analogues and in general, there is a strong correlation between the cytotoxic activity of a particular camptothecin analogue in cell culture and animal model systems, and its ability to interfere with the catalytic activity of DNA topoisomerase I (17,18,22).

On the other hand, RNA interference (RNAi) was originally identified as an endogenous mechanism for post-transcriptional gene silencing in plants and nematodes and is now an established technique for experimental 'knockdown' of gene expression to establish function in mammalian cells (23-25). RNAi has typically been achieved in cultured mammalian cells by transfection of chemically synthesized gene-specific 21 nt small interfering RNAs (siRNAs). Introduction of these molecules leads to reduced expression of specific mRNAs, thus permitting the analysis of a knockdown phenotype without the need for time-consuming gene-targeting studies. The effect of siRNA involves post-transcriptional gene silencing via a process in which double-stranded RNA (dsRNA) inhibits gene expression in a sequence-dependent manner through degradation of the corresponding mRNA. Its blocking action on gene expression has been successfully observed in rat and human cells cultured *in vitro*, and the knockdown of genes in cells has been achieved (25-30). In addition, the recent development of retroviral constructs in which the polymerase-III H1-RNA gene promoter synthesizes siRNA-like transcripts, allows stable expression of siRNA, thus rendering this technology applicable as gene transfer (29-32).

Our results argue that targeting the nuclear factor Egr-1 could have potential as a sensitizer of tumors to chemotherapeutic treatment. We showed that combined treatment of SK-BR3 and MCF-7 adenocarcinoma cells with siRNA-Egr-1 and camptothecin results in a greater than additive loss of viability, anchorage, expression and increased apoptosis. Taken together, these data indicate an additive to synergistic effect of siRNA-Egr-1 and Cpt in suppressing the growth of breast carcinoma cell lines SK-BR3 and MCF-7.

Materials and methods

Cell lines and culture. Human breast cancer cell lines SK-BR3 and MCF-7 cells were obtained from American Type Culture Collection. MCF-7 and SK-BR3 cells were cultured in DMEM with 10% fetal bovine serum (FBS), 1% L-glutamine, and 0.1% penicillin/streptomycin. Cells were maintained at 37˚C in a 5% CO_2 incubator. Camptothecin (C9911 Sigma), was dissolved in DMSO (Dimethyl sulphoxide hybri-max), (Sigma, D2650) to achieve a stock solution of 10 mM. In each experiment, the drug was diluted to 10 μM in a transparent growth medium (RPMI, 1% PenStrep, 10% FCS, w/o riboflavin, w/o phenol red, Biological Industries, Kibbutz Beit Haemek, Israel).

siRNA preparation and transfection of short interfering RNA. siRNA oligonucleotides with two thymidine residues (tt) at the 3' end of the sequence were designed for Egr-1 (sense, 5'-CAGCAGCAGCAGCAGCAGCtt-3'-hairping-TTCA AGAGA- antisense, 5'-GCTGCTGCTGCTGCTGttttta-3'). Cells were treated in parallel with a non-silencing-siRNA (sense, 5'-UUCUCCGAACGUGUCACGUtt-3'; antisense, 5'-ACGUGACACGUUCGGAGAAtt-3') as control oligonucleotides were synthesized by Shanghai Genechem Co. These cells were cultured in medium without antibiotics, and 24 h before transfection resulting in a confluence of the cell monolayer by 50-70%. Specific pTER-Egr-1 siRNA or non-silencing siRNA (70 nmol) were mixed with lipofectamine 2000™ (Invitrogen) according to manufacturer's recommendation and added to the cells. After 6 h at 37˚C, the medium was changed, and the cells were cultivated in RPMI-1640 supplemented with 10% heat-inactivated FCS (30).

3-(4,5-methylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay. SK-BR3 and MCF-7 breast carcinoma cell lines ($5x10^5$) were placed onto 96-well plates in RPMI-1640 containing 10% FBS in a final volume of 0.1 ml. The next day, the cells were treated with the pTER siRNA. MTT was added (20 ml/well of 5 g/l solution in PBS) after culture for 24, 48 and 72 h. When incubated at 37˚C for 4 h, the reaction was stopped by addition of 100 ml DMSO. The reaction product was quantified by measuring the absorbance at 490 nm using an ELISA reader (Wallac 1420 Victor 2, Victor Co., Finland) and Software HT-Soft (Perkin-Elmer). All samples were assayed repeatedly in 6-wells (30).

Reverse transcription polymerase chain reaction. SK-BR3 and MCF-7 breast carcinoma cells lines ($5x10^5$) were seeded onto 6-well plates. Total RNA was extracted 48 h after transfection using TRIzol reagent. Reverse transcription was performed using one step RT-PCR kit. The primers of Egr-1 were 5'-AACAGTGGCAACACCTTGTG-3' (forward primer) and 5'-ACTGGTAGCTGGTATTGAGG-3' (reverse primer). The primers of human ß-actin were 5'-TCACCAACTGGGA CGACAT-3' (forward primer) 5'-GAAGTCCAGGGCGA CGTAG-3' (reverse primer). Thermal cycle conditions were as follows: 42˚C for 30 min, 94˚C for 2 min, followed by 28 cycles of 94˚C 15 sec, 55˚C 30 sec, 72˚C 1 min, with a final extension at 72˚C for 10 min. RT-PCR products were visualized by ethidium bromide-stained agarose gels (30,33).

Western immunoblot analysis. SK-BR3 and MCF-7 breast carcinoma cells lines ($5x10^5$) were seeded onto 6-well plates. Forty-eight hours after transfection, cells were collected and washed twice by cold PBS, and each well was treated with 50 ml lysis buffer (2 mmol/l Tris-HCl pH 7.4, 50 mmol/l NaCl, 25 mmol/l EDTA, 50 mmol/l NaF, 1.5 mmol/l Na_3VO_4, 1% Triton X-100, 0.1% SDS, supplemented with protease inhibitors 1 mmol/l phenylmethylsulfonylfluoride, 10 mg/l pepstatin, 10 mg/l aprotinin, and 5 mg/l leupeptin) (all from Sigma). Protein concentrations were determined using the Bradford protein assay. Equal amounts of protein

(40 mg) were separated on a 15% SDS polyacrylamide gel and transferred to a nitrocellulose membrane (Hybond C, Amersham, Freiburg, Germany). Membranes were blocked in 5% non-fat dry milk in TBS for 1 h at room temperature and probed with rabbit anti-Egr-1 antibodies (dilution, 1:500 Santa Cruz Biotechnology, USA) overnight at 4˚C. After 3 times washing with TBS containing 0.1% Tween-20, membranes were incubated with anti-rabbit IgG-horseradish-peroxidase (1:5000, Santa Cruz Biotechnology), and developed by luminol mediated chemiluminescence (Appylgen Technologies Inc., China). To confirm equal protein loading, membranes were reprobed with a 1:1000 dilution of an anti-actin antibody (Santa Cruz Biotechnology). Densitometric analyses were performed using Scion Image software (30,33).

Soft agar colony formation assay. The effects of Egr-1 siRNA and camptothecin on colony formation ability of the breast carcinoma cell lines SK-BR3 and MCF-7 were assessed by soft agar colony formation assay. The assay was done in 6-well plates; in each well, 2 ml of 0.5% agar (in culture medium) was layered in the bottom followed by 1 ml of 0.38% agar as the top layer. Approximately 2000 cells were then plated over the top layer. The cells were treated with Egr-1 siRNA and after 48 h treated with camptothecin 2.5 μM and maintained at 37˚C in a humidified 5% CO_2 atmosphere. At 14 days post-treatment, plates were assessed for size and number of colonies. Colonies (in a representative field) are indicated (30).

Results

The ability to turn off individual genes at will in growing cells porvides a powerful tool for elucidating the role of a particular gene, and for therapeutic intervention when that gene is overexpressed or mutated. Since the discovery of siRNAs as the key mediator of RNA-induced gene silencing, RNAi has rapidly evolved to become a tool for gene regulation in mammals (26) and has been applied since then to inhibit the expression of a wide variety of target genes. One of them, the Egr-1 gene expressing a transcription factor with important functions in the regulation of growth and differentiation, is involved in the transformed phenotype of several cancer types (34,39-45).

Egr-1 activity in breast carcinoma cell lines SK-BR3 and MCF-7 treated with various stimuli. Egr-1 is among the early response nuclear factors that play an important role in the regulation of several genes. To determine the activity of Egr-1 in BK-BR3 (Fig. 1A) and MCF-7 (Fig. 1B) breast carcinoma cell lines, we treated the cells with various stimuli. Without FCS, FCS 10%, arsenite, TNF-α PMA and Cpt. FCS 10%, arsenite TNF-α and PMA strongly induce the expression of Egr-1 protein in the breast carcinoma cell lines. In contrast, the medium without FCS and Cpt induces a marginal expression of Egr-1 protein in both cell types.

Effect of Egr-1 siRNA and Cpt on cell proliferation of SK-BR3 and MCF-7 breast cell lines. Treatment of proliferating SK-BR3 and MCF-7 breast carcinoma cell lines with combinations of camptothecin and siRNA directed against the Egr-1 nuclear factor causes additive anti-proliferation (Fig. 2).

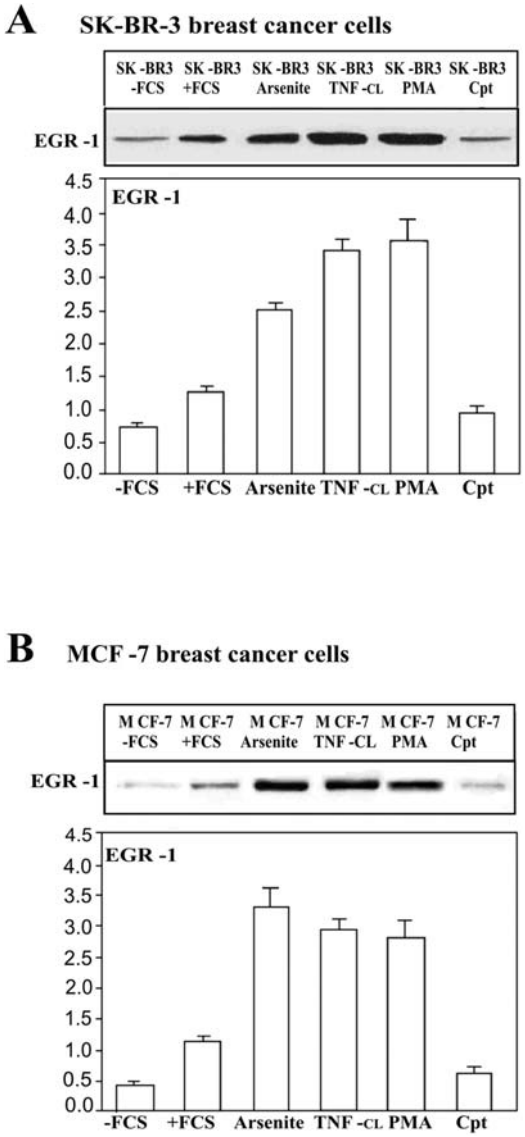

Figure 1. Expression of Egr-1 in breast carcinoma cell lines SK-BR3 and MCF-7 treated with various stimuli. To determine the expression of Egr-1 in breast carcinoma cell lines, the SK-BR3 and MCF-7 cells were treated without and with FCS, Arsenite, TNF-α PMA and camptothecin (Cpt). Egr-1 was examined 1 h after treatment by Western blot assay. Arsenite, TNFα, and PMA strongly induced Egr-1 activation, compared to the cells treated with Cpt and without FCS which were only marginal. One of two similar experiments is shown.

One day after plating, the cells were transfected with lipofectamine plus siRNA-Egr-1 or scrambled siRNA for 5 h, after which the medium was replaced with fresh medium containing 1 μM or 5 μM Cpt. After 3 days, viable cell number were measured and plotted against drug concentrations. The results showed a strong and additive effect of siRNA in conbination with Cpt compared to each compound alone. Our results appear consistent with other studies and show that the inhibition of Egr-1 could be a method to decrease the therapeutically used drug concentrations, minimizing the toxic side effect.

Inhibition of Egr-1 mRNA expression in the SK-BR3 and MCF-7 human carcinoma cell lines by siRNA and camptothecin. To examine the specific effect of siRNA-Egr-1,

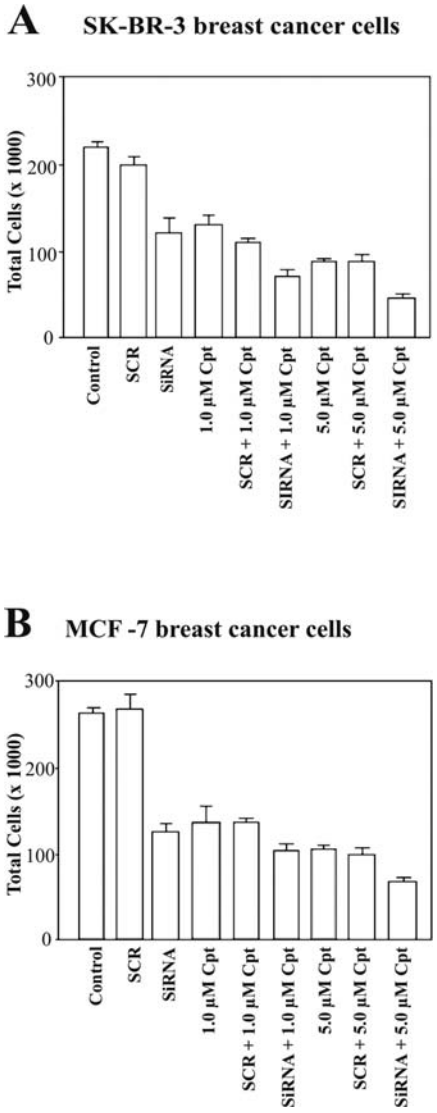

Figure 2. Effect of siRNA against Egr-1, camptothecin and the combination of both on the expression of Egr-1 protein. Western blot assay of Egr-1 protein expression in SK-BR3 (A) and MCF-7 (B) cell lines. In A, lane 1 untreated; lane 2 treated with an Egr-1 expression plasmid; lane 3 treated with siRNA-Egr-1; lane 4 treated with Cpt, and lane 5 treated with the combination of siRNA-Egr-1 and Cpt. Data are expressed as mean ± SD of three experiments, Egr-1 siRNA group vs. expressing Egr-1 group, P<0.01; Egr-1 siRNA group vs. untreated group, P<0.01.

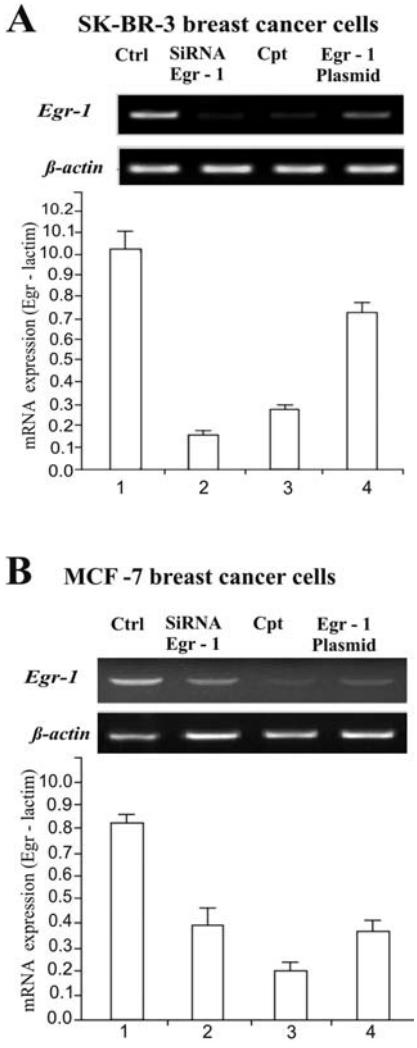

Figure 3. Expression of Egr-1 mRNA in breast carcinoma cell lines SK-BR3 and MCF-7. Egr-1 mRNA expression in RT-PCR assay in SK-BR3 (A) and MCF-7 (B) cell lines. In A, lane 1 untreated; lane 2 treated with siRNA-Egr-1; lane 3 treated with Cpt, and lane 4 treated with an Egr-1 expression plasmid. Data are expressed as mean ± SD of two experiments, Egr-1 siRNA group vs. expressing Egr-1 group, P<0.01; Egr-1 siRNA group vs untreated group, P<0.01.

camptothecin and the combination of both on mRNA expression in SK-BR3 and MCF-7 breast carcinoma cell lines, the Egr-1 mRNA level was determined quantitatively with RT-PCR analysis (Fig. 3).

In Fig. 3A, SK-BR3 cells and in Fig. 3B, MCF-7 breast carcinoma cell lines. Egr-1 mRNA were strongly expressed in both breast carcinoma cells lanes as reflected by RT-PCR in the control (lane 1) and in the expression Egr-1 plasmid (lane 4). However, SK-BR3 treated with specific siRNA-Egr-1 (lane 2) and Cpt (lane 3) strongly inhibited the expression of mRNA. The inhibition rate of mRNA was 82% (lane 2) and 70% for lane 3. In contrast, the inhibition rate of mRNA in MCF-7 cells treated with siRNA was only of 51.8 for lane 2 and 73.5% for lane 3. ß-actin mRNA signals were uniform in all analysed samples.

Effect of siRNA against Egr-1, camptothecin and the combination of both on the expression of Egr-1 protein. Western blot assay of Egr-1 protein expression in SK-BR3 (Fig. 4A) and MCF-7 (Fig. 4B) cell lines. In Fig. 4 lane 1 untreated; lane 2 treated with an Egr-1 expression plasmid; lane 3 treated with siRNA-Egr-1; lane 4 treated with Cpt; and lane 5 treated with the combination of siRNA-Egr-1 and Cpt. Egr-1 protein expression in SK-BR3 (A) and MCF-7 (B) cells transiently transfected with the empty vector and with the Egr-1 expression vector exhibited a strong Egr-1 band by Western blotting while, the cells treated with siRNA-Egr-1 or Cpt alone, strongly decrease the expression of Egr-1 protein. However, the combination of siRNA-Egr-1 and Cpt further decrease the expression of Egr-1 protein. ß-actin protein signals were uniform in all analysed samples.

Effect of Egr-1 siRNA on cell tumorigenicity. To address whether Egr-1 expression maintains the SK-BR3 and MCF-7 tumorigenic phenotype after treatment with siRNA and Cpt

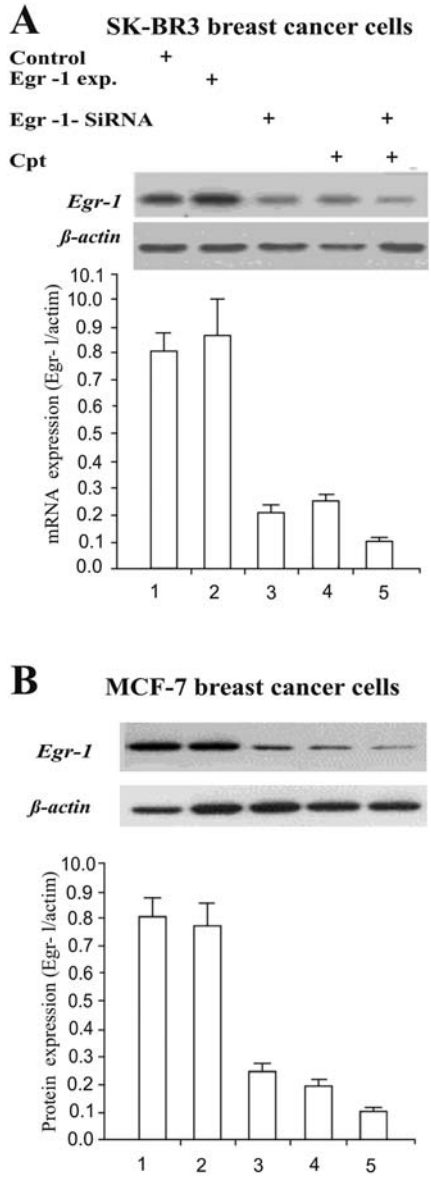

Figure 4. Cell viability: treatment of proliferating SK-BR3 and MCF-7 breast carcinoma cell lines with combinations of camptothecin and siRNA directed against the Egr-1 nuclear factor causes additive antiproliferation. One day after plating, the cells were transfected with lipofectamine plus siRNA-Egr-1 or scrambled siRNA for 5 h, after which the medium was replaced with fresh medium containing 1 μM or 5 μM Cpt. After 3 days, viable cell number were measured and plotted against drug concentrations One of two similar experiments is shown.

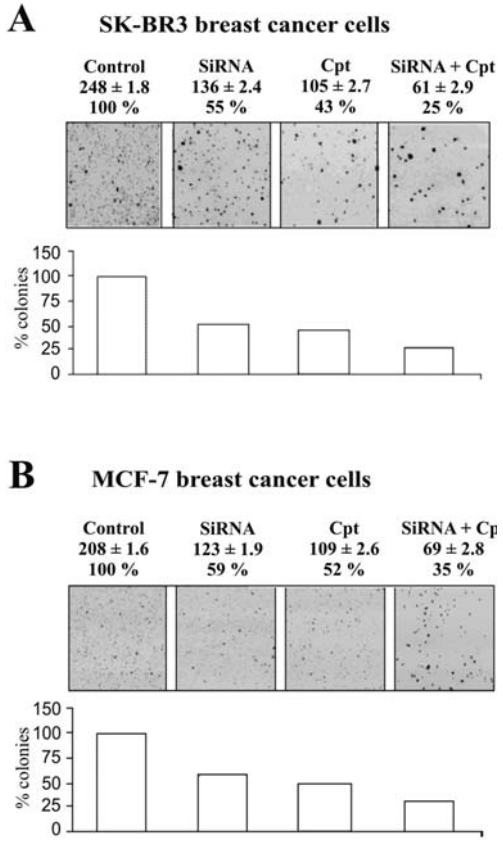

Figure 5. Colony-formation assays in soft agar. SK-BR3 and MCF-7 breast carcinoma cell lines (5000) were seeded onto 6-well plates containing 2 ml of 0.5% agar in the bottom and 1 ml of 0.38% as the top layer. After 14 days of treatment with siRNA-Egr-1 alone or in combination with Cpt, the cells were stained using crystal violet. Analysis of the appearance of the foci was performed after 2 weeks. One of three similar experiments is shown.

alone and the combination of both, we performed a soft agar assay (Fig. 5). SK-BR3 and MCF-7 transfected with the empty vector (lane 1) with the Egr-1 expression plasmid (lane 2), with siRNA-Egr-1 (lane 3) with Cpt (lane 4) and with the combination of siRNA-Egr-1 and Cpt expressing cells were plated in soft agar. As expected control expressing cells (SK-BR3 and MCF-7) grew in soft agar. By contrast, Egr-1 siRNA and camptothecin reduced the ability of these cells to form colonies in soft agar. However, the combination of siRNA-Egr-1 with Cpt was more effective in decreasing the ability of these cells to form colonies in soft agar. Together, these data demonstrate that Egr-1 siRNA can induce SK-BR-3 and MCF-7 growth arrest and loss of tumorigenic potential.

Discussion

In this study, the effects of combining siRNA-Egr-1 with camptothecin, an anticancer drug, were examined. Among the cell types used in our experiments, we observed that the simultaneous exposure to siRNA and Cpt was synergistic in breast carcinoma cells lines SK-BR3 but only additive in MCF-7. It seems that the synergistic effect observed was not a general phenomenon but rather cell line-specific. Camptothecins have antitumor activity based on their binding to and inhibition of topoisomerase I, a nuclear enzyme which reduces torsional stress during DNA replication and which has an important role in DNA replication (21,35,36). Therefore, we examined the combined effects of siRNA targeting Egr-1 alone or in combination with camptothecin in breast SK-BR3 and MCF-7 carcinoma cell lines. It has been reported that Egr-1 can suppress human tumor cell growth and growing evidence suggests that Egr-1 functions as a tumor suppressor gene (37). The role of Egr-1 has been demonstrated in several kind of tumor cells, including human fibrosarcoma, glioblastoma and breast cancer cell lines (37,38), glioblastoma (39), neuroblastoma (10), hepatoma (40), esophageal cancer (41) and leiomyoma (42). In addition, Egr-1 can regulate the coordinated expression of a set of genes, including *PTEN* (43), *TGF-β1*, *Bcl-2*, *fibronectin* (*FN*) and *plasminogen activator inhibitor-1* (*PAI-1*)

(44), all of which are important to normal growth control (44). There is evidence suggesting that many zinc finger proteins function as tumor suppressors or as negative regulators of cell proliferation attracting considerable attention as a possible target for breast cancer treatment. Degradation of mRNA mediated by siRNA is a powerful means of specifically knocking down the expression of a target gene (31,32). RNAi is an evolutionarily conserved phenomenon in which gene expression is suppressed by the introduction of homologous double-stranded RNAs (dsRNAs). Synthetic siRNA can trigger an RNA interference response in mammalian cells and induce strong inhibition of specific gene expression (45). siRNA can transiently mediate specific degradation of homologous messenger RNA (mRNA), silencing the expression of the targeted gene (46,47). Since Egr-1 is important for the survival of various human tumors, Egr-1 siRNA could become an effective therapeutic agent for tumors with overexpression of Egr-1.

In the present study, we have demonstrated that a siRNA against the mRNA of Egr-1 alone or in combination with Cpt blocked the production of the *Egr-1* protein through blocking the transduction process of the mRNA-Egr-1. The siRNA-Egr-1 and Cpt independently blocked *Egr-1* transduction, while the non-silencing Egr-1 did not. siRNA against Egr-1 mRNA prevents the expression of Egr-1 through promoting the degradation of the Egr-1 mRNA while camptothecins have antitumor activity based on their binding to and inhibition of topoisomerase I, a nuclear enzyme which reduces torsional stress during DNA replication and which has an important role in DNA replication. These events could lead to consequent activation of *Egr-1* target genes, such as PTEN, TGF-ß1, p53, and fibronectin, which serve to maintain normal growth regulation (37-44).

We found that the protein level of the endogenous Egr-1 was decreased in breast carcinoma cells by the expression of siRNA against the Egr-1 transcription product, which resulted in the suppression of the mRNA-Egr-1 proteins and consequently decreased the activity of Egr-1 in the cells. Our results demonstrate that the siRNA, and camptothecin in combination could efficiently suppress the Egr-1 expression in SK-BR3 and MCF-7 carcinoma cells compared with the untreated cells. We observed that SK-BR3 and MCF-7 carcinoma cells transfected with siRNA-Egr-1 alone or in combination with Cpt grew slowly as compared with the control groups. Both, siRNA-Egr-1 and camptothecin showed anti-proliferation function. RT-PCR and Western blot findings demonstrated that Egr-1 mRNA and protein expression were strongly reduced in presence of siRNA, Cpt or the combination suggesting that the Egr-1 gene was blocked in SK-BR3 and MCF-7 breast carcinoma cells transfected with siRNA-Egr-1 or treated with Cpt at the level of transcription and protein expression.

Based on combination indices, synergistic decreases in viability and anchorage capacity ocurred for siRNA-Egr-1 alone or in combination with Cpt for SK-BR3 and MCF-7 cell lines. The above-mentioned findings confirm not only that chemically synthesized siRNAs can specifically block Egr-1 gene expression but also in combination with Cpt, decreacing cell anchorage and inhibiting the growth of breast carcinoma cell lines. However, the additive and synergic nature of the results obtained for addition of two components that inhibit the activity of SK-BR3 and MCF-7 by independent mechanisms are mutually exclusive despite the fact that they could work together.

In conclusion, our data suggest that Egr-1 gene can be regarded as a very good target gene in genetic therapy for breast carcinomas and the use of siRNA-Egr-1 alone or in conbination with known drugs deserves further investigations as an approach to cancer therapy.

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