Synergistic effect of survivin-specific small interfering RNA and topotecan in renal cancer cells: Topotecan enhances liposome-mediated transfection by increasing cellular uptake

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Abstract. Survivin, an inhibitor of apoptosis (IAP) protein detected in many tumors but not in most normal differentiated tissues, has been widely recognized as an attractive target for cancer therapy. We previously showed that survivin expression is associated with cell proliferation. Although liposome-mediated transfection of survivin-specific siRNA decreases survivin expression and cell proliferation, these effects are limited in part by the low efficiency of the transfection. In the present study we therefore investigated the possibility of better suppressing survivin expression and cell growth by using treatments combining survivin-specific siRNA and the topoisomerase I inhibitor topotecan. Survivin-specific siRNA and topotecan given simultaneously inhibited survivin expression and cell proliferation synergistically, but topotecan alone or topotecan and siRNA given metachronously did not alter survivin expression. We hypothesized that topotecan increases the efficiency of siRNA transfection by increasing cellular uptake, and we confirmed this hypothesis by using fluorescein-labeled siRNA. Combination therapy using survivin-specific siRNA and topotecan should thus show a synergistic effect due to increased cellular uptake of siRNA and offer an attractive approach for treatment of advanced renal cancer.

Introduction

Renal cancer accounts for 2-3% of adult cancers and for 2% of all cancer-related deaths (1). Approximately half of the patients with renal cancer have metastases at the time of diagnosis or will develop them during the clinical course (2). The outcome of the treatment of advanced renal cancer is disappointing. Conventional cytotoxic chemotherapy and radiotherapy are largely ineffective (3), response rates to interferon (IFN)-α and IFN-ß treatments range from 10 to 20% (4), and long-lasting responses to interleukin (IL)-2-based therapy are obtained in only 4-5% of the treated patients (5). Although there have been numerous attempts to increase these low response rates by using combination therapies, such as vinblastine and IFNs, the reported effects have not been dramatic (6,7). Further improvement awaits the development of a more effective systemic therapy.

Survivin is one of the inhibitor of apoptosis (IAP) proteins detected in many tumors but is absent in most normal differentiated tissues (8). Unlike other IAPs, survivin contains a single BIR and lacks a carboxyl-terminal RING finger (8). Survivin is expressed in the G2-M phase in a cell cycle-dependent manner, and binds directly to mitotic spindle microtubules (9). It has also been shown to inhibit the processing of procaspases-3 and -7 and to specifically bind both active caspases (10). Survivin thus inhibits apoptotic stimuli and its expression is associated with carcinogenesis, tumor progression, and decreased survival (11). It has therefore been widely recognized as an attractive target for cancer therapy, and the suppression of survivin expression by antisense oligonucleotides or small interfering RNA (siRNA) is a promising approach to selective cancer therapy (12-14).

We have previously shown that survivin expression is associated with the proliferation of renal cancer cells, but their proliferation was not completely inhibited by survivin-specific siRNA (15). The transfection efficiency of liposome-based gene delivery is generally low and complete suppression of survivin expression is not always achieved. One might thus expect to achieve more thorough inhibition of survivin expression and cell proliferation by using combination therapies exploiting siRNA and chemotherapeutic agents.

In the present study we investigated the response of renal cancer cells to treatment with a combination with survivin-specific siRNA and the topoisomerase I inhibitor topotecan. We found a synergistic effect of survivin-specific siRNA and topotecan on the suppression of survivin expression and cell growth. We also found that topotecan enhanced liposome-mediated transfection of siRNA by increasing cellular uptake. The synergistic effect of this combination treatment is thought to be due in part to topotecan increasing the cellular uptake of siRNA. Combining survivin-specific siRNA and topotecan is thus a promising approach to the treatment of advanced renal cancer.

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Materials and methods

Cell culture. A KU19-20 cell line, derived from human renal cancer, was established at Keio University. Cells were grown in RPMI-1640 medium containing 10% fetal bovine serum and 0.3% penicillin-streptomycin (Invitrogen, Carlsbad, CA, USA).

Treatment with survivin-specific siRNA and topotecan. Cells were plated in 6-well (for Western blot analysis) or 96-well (for cell proliferation assay) culture plates one day before treatment so they would reach about 70% confluence. The survivin-specific siRNA or non-sense siRNA (Santa Cruz Biotechnology, Santa Cruz, CA, USA) (each 100 nM) was mixed with Lipofectamin 2000 (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instruction and administered to the cells in the RPMI-1640 medium containing topotecan (Calbiochem, San Diego, CA, USA) at concentrations ranging from 0 to 1 μg/ml. Cells were maintained in this medium for 24 or 48 h and then assayed for survivin suppression and cell proliferation.

For metachronous treatment, cells were first treated with survivin-specific siRNA for 24 h and then cultured in the medium with or without topotecan for another 24 h before being subjected to Western blot analysis.

Cell proliferation assay. Cells were plated in 96-well culture plates 24 h prior to treatment and then incubated under the indicated conditions for 48 h after which cell proliferation was determined using the MTT assay.

Western blot analysis. Samples were mixed with loading buffer, resolved by 12.5% SDS-PAGE, and transblotted onto nitrocellulose membranes. After blocking with 5% dry skim milk, the membranes were incubated for an hour or overnight with anti-survivin rabbit polyclonal antibody (Alpha Diagnostic International, San Antonio, TX, USA) before being incubated with a biotinylated secondary goat anti-rabbit antibody (Bio-Rad Laboratories, Hercules, CA, USA) at room temperature. The membranes were then incubated for 1 h in streptavidin-biotinylated alkaline phosphatase complex (Bio-Rad Laboratories) at room temperature. Immunoreactivity was detected using an alkaline phosphatase substrate kit (Bio-Rad Laboratories).

Cell cycle analysis. At indicated time intervals after treatment with survivin-specific siRNA and/or topotecan as described above, KU19-20 cells were washed with PBS and harvested by trypsinization. After they were resuspended in citrate buffer and stained with propidium iodide according to the method by Vindelov *et al* (16), they were analyzed by flow cytometry using the Cell Quest program for calculation of the different cell cycle phases.

Measurement of cellular uptake of siRNA. KU19-20 cells were transfected with fluorescein-labeled non-sense siRNA (Santa Cruz Biotechnology) as described above. They were cultured in the RPMI-1640 medium with or without topotecan (0-1 μg/ml) for 48 h, washed with PBS, and harvested by trypsinization. Cellular uptake of fluorescein-labeled siRNA was measured by flow cytometry using the Cell Quest program.

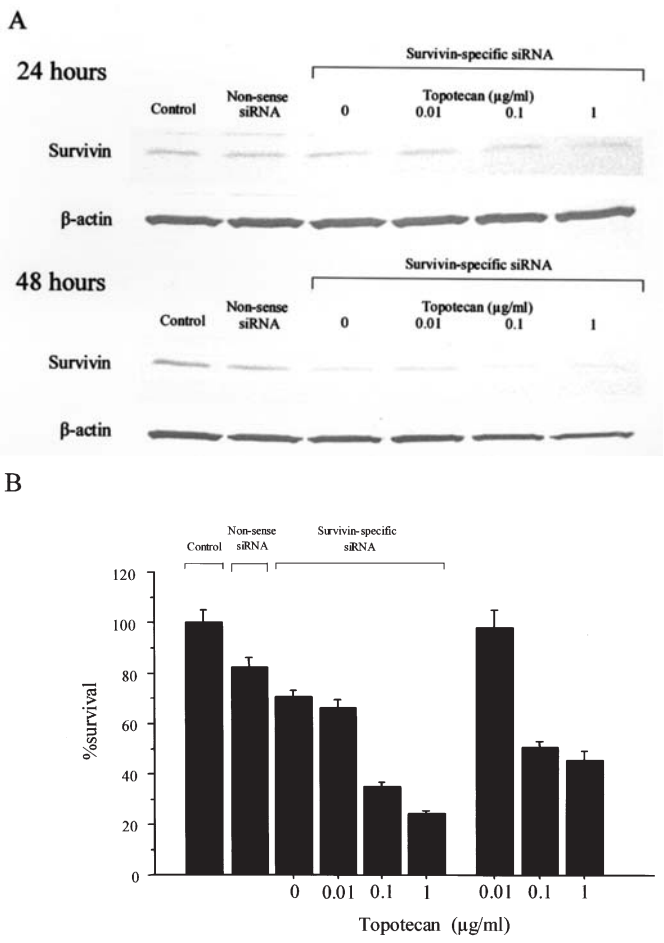

Figure 1. Inhibition of survivin expression and cell proliferation by simultaneous treatment with survivin-specific siRNA and topotecan. (A) Survivin Western blot analysis after 24- and 48-h treatment. Survivin expression was further suppressed by the simultaneous treatment with siRNA and topotecan in a dose-dependent manner. (B) Cell viability using MTT assay after 48-h treatment. Cell proliferation was synergistically inhibited by the combination of siRNA and topotecan.

Results

Successful uptake of siRNA under the conditions used in this study was confirmed in a previous study by using fluorescein-labeled siRNA and an epifluorescence microscope (15). In the present study we found that a more thorough inhibition of survivin expression and cell proliferation could be obtained by treating the cells with both survivin-specific siRNA and topotecan. Simultaneous treatment with siRNA and topotecan for 24 or 48 h inhibited survivin expression more than did treatment with siRNA alone (Fig. 1A) and the 48-h combination treatment inhibited cell proliferation synergistically (Fig. 1B).

The results of the simultaneous treatment with survivin-specific siRNA and topotecan indicated that topotecan increased the efficiency of siRNA transfection and/or topotecan itself decreased the expression of survivin. To explore the mechanism of the topotecan-induced enhancement of the decline in survivin expression, we investigated the effects of metachronous treatment with survivin-specific siRNA and topotecan. If the enhancement were due to increased cellular uptake, it would not occur when siRNA and topotecan were administered metachronously.

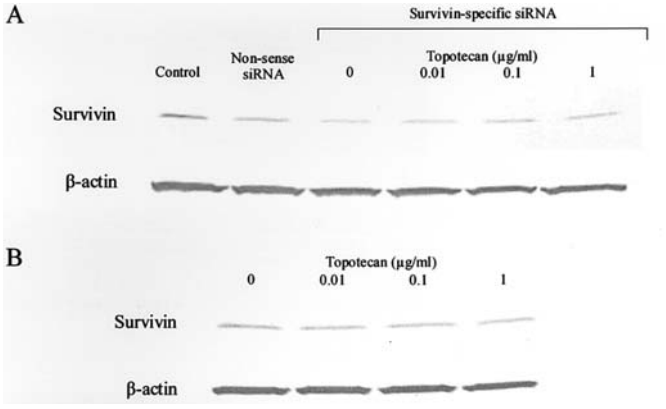

Figure 2. Effect of metachronous treatment with survivin-specific siRNA and topotecan or topotecan alone on survivin expression. (A) Survivin Western blot analysis after metachronous treatment with siRNA (24 h) and topotecan (24 h). Different from the simultaneous treatment, no further suppression of survivin was seen by the combination of siRNA and topotecan. (B) Survivin Western blot analysis 48 h after treatment with topotecan alone. Topotecan itself had no effect on survivin espression.

When the cells were treated first with survivin-specific siRNA for 24 h and then with topotecan for 24 h (metachronous treatment), no further suppression of survivin was observed (Fig. 2A). Administration of topotecan alone for 48 h also did not alter survivin expression (Fig. 2B). From these results we inferred that topotecan itself had no effect on survivin expression but enhanced the transfection of siRNA.

Cell cycle analysis comparing the treatment with topotecan alone and the simultaneous administration of survivin-specific siRNA and topotecan for 48 h showed that the combination treatment increased the sub-G1 fraction, which included apoptotic cells induced by the suppression of survivin, without markedly changing other cell cycle phases. This result supports the hypothesis that topotecan increases the survivin suppression due to siRNA. The effect of the 48-h treatment with topotecan on cell cycle varied with concentration. The treatment with 0.1 μg/ml topotecan led to accumulation of the S-phase fraction, whereas the treatments with 0.01 and 1 μg/ml topotecan increased the G1 fraction (Fig. 3).

To find out if topotecan increased transfection efficiency by increasing the cellular uptake of siRNA, we evaluated the changes in the cellular uptake of siRNA by using fluorescein-labeled non-sense siRNA. Because the cellular uptake of siRNA was increased by the simultaneous administration of topotecan (Fig. 4), we therefore inferred that one mechanism

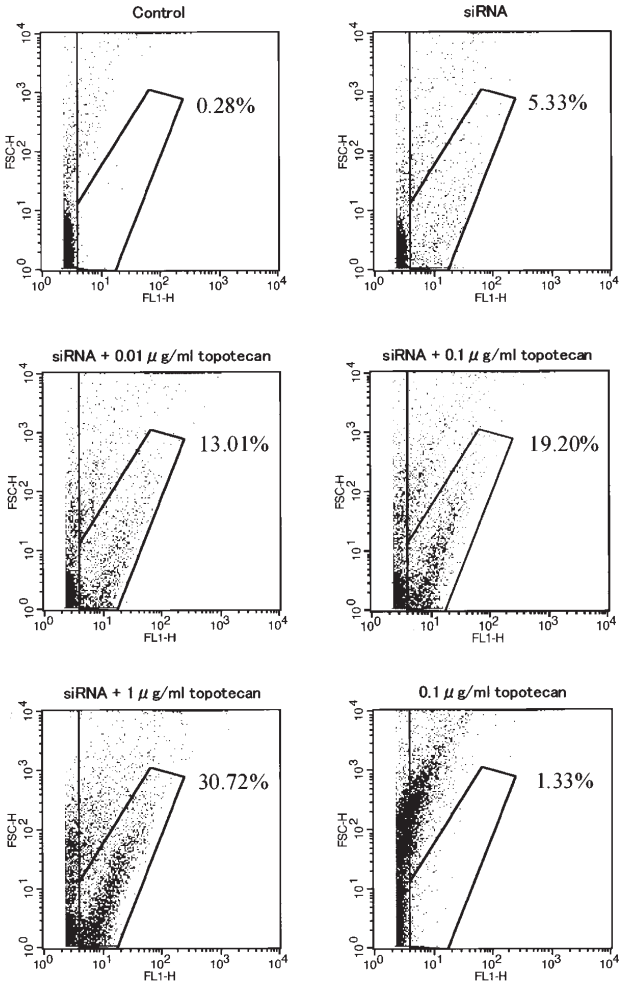

Figure 4. Analysis of cellular uptake of siRNA by flow cytometry 48 h after administration of fluorescein-labeled siRNA and topotecan. Percentage of the cells which incorporated liposome-fluorescein-labeled siRNA complex is measured. By the simultaneous administration of siRNA and topotecan, cellular uptake of siRNA was increased in a topotecan dose-dependent manner.

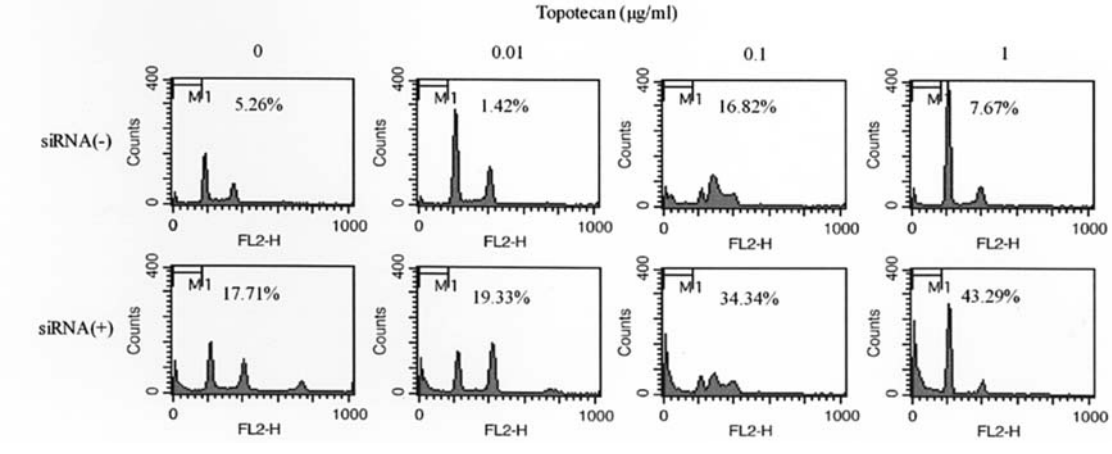

Figure 3. Cell cycle analysis 48 h after treatment with survivin-specific siRNA and/or topotecan. Percentage of the cells in the sub-G1 phase is shown. The simultaneous treatment with siRNA and topotecan increased cells in the sub-G1 fraction in a dose-dependent manner.

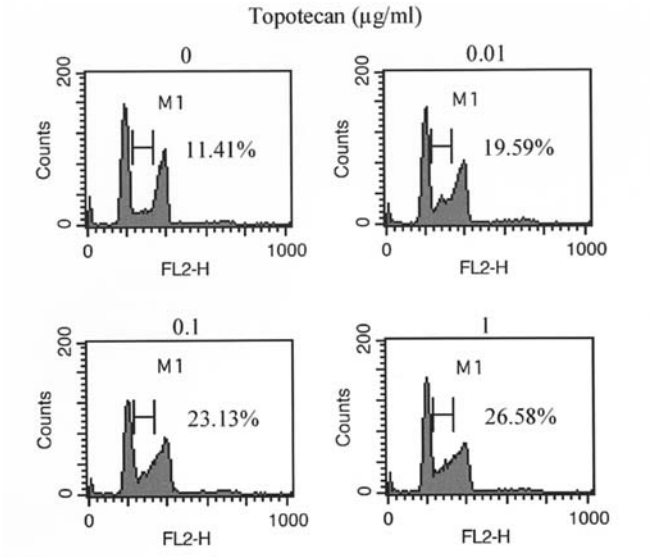

Figure 5. Cell cycle analysis 6 h after treatment with topotecan. Percentage of the cells in the S-phase is measured. The S-phase fraction was increased by topotecan in a dose-dependent manner.

of the synergistic effect of survivin-specific siRNA and topotecan is topotecan-increased uptake of siRNA.

Thinking that topotecan-induced changes in the cell cycle in the early phase of transfection would shed light on the mechanism of the increased cellular uptake of siRNA, we evaluated the change in the cell cycle after 6-h treatment with topotecan. Cell cycle analysis revealed that the 6-h treatment with topotecan increased the S-phase fraction in a dose-dependent manner (Fig. 5). The accumulation in the S-phase may be one of the mechanisms of increased cellular uptake of siRNA.

Discussion

Survivin, which is highly expressed in cancer cells and undetectable in terminally differentiated tissues, may play an important role in carcinogenesis (8). Its expression in renal cancer cells is associated with cell proliferation (15), and survivin may be a promising target for gene therapy in advanced renal cancer.

RNA interference (RNAi) is an evolutionarily ancient mechanism of gene regulation in eukaryotes and has become a powerful tool for probing gene function. The general mechanism of RNAi involves the cleavage of double-stranded RNA (dsRNA) to short siRNAs. This processing is catalyzed by Dicer, a highly conserved dsRNA-specific endonuclease that is a member of the RNase III family. The siRNAs are then incorporated into the RNA-induced silencing complex (RISC) and guide the destruction or repression of complementary mRNAs (17). Recent studies have found that knockdown of survivin expression by siRNA decreases cell survival in human sarcoma (14) and renal cancer (15). Furthermore, liposome-mediated systemic delivery of siRNA has been shown to inhibit exogenous and endogenous gene expression *in vivo* (18). Suppression of survivin expression by siRNA would thus seem to be a promising means of selective cancer therapy.

In the present study survivin expression was inhibited by the liposome-mediated transfection of survivin-specific siRNA, but cell proliferation was not completely suppressed. Similar results have been obtained in other studies using antisense oligonucleotides or siRNA (13-15,19). Although survivin mRNA has been reported to be one of the four most highly expressed transcripts in tumor cells (20) and would thus code for one of the major regulatory proteins in cancer cells, it is natural that other molecules and different signaling pathways also play important roles in the proliferation of cancer cells. Also, because transfection efficiency usually differs between cell types and cell conditions, survivin expression would not always be suppressed completely. We postulated that cancer cell damage could be enhanced by combining the use of survivin-specific siRNA with the use of chemotherapeutic agents.

Topotecan is a topoisomerase I inhibitor whose effectiveness in patients with advanced renal cancer has so far been assessed only in one small phase II trial (21). Ramp *et al* have however shown not only that clinically relevant doses of topotecan are effective in human renal cancer cell lines but also that in these lines topotecan is more effective than 5-fluorouracil (22). The present study showed that topotecan is also effective in another line of human renal cancer cells and that it inhibits cell proliferation in a dose-dependent manner. Cell cycle effects of topotecan seem to vary with topotecan concentration, with the duration of treatment, and probably also with the type of cells treated. One-hour treatment with topotecan increased the number of lung cancer cells in the S-phase (23). In lung fibroblast cells, treatment with 0.05 μM topotecan mainly caused an accumulation of the cells in the S-phase 24 h after treatment, and subsequently 60% of the cells were arrested in the G2/M phase (24). In the KU19-20 cells the 6-h treatment with topotecan increased the S-phase fraction, whereas the 48-h treatment increased the G1- and S-phase fractions by amounts dependent on the topotecan concentration.

The combination of survivin-specific siRNA and topotecan reduced cell proliferation and survivin expression synergistically, and the cause of this synergistic effect was examined by investigating the influence of topotecan on survivin expression. Treatment with topotecan alone, however, did not affect survivin expression. The effect of topotecan on survivin expression has been little investigated and the only report of which we are aware is that topotecan decreases the expression of survivin in prostate cancer cells (25), which is inconsistent with our results.

Treatment with topotecan alone did not influence survivin expression, and metachronous administration of survivin-specific siRNA and topotecan did not suppress survivin expression more than did treatment with survivin-specific siRNA alone. Simultaneous administration of survivin-specific siRNA and topotecan, in contrast, suppressed survivin expression synergistically. We therefore hypothesized that topotecan could increase the cellular uptake of siRNA. The results of flow cytometry indicated the cellular uptake of siRNA was increased by the simultaneous administration of topotecan. The cell cycle analysis also revealed that the sub-G1 fraction, which corresponded to apoptotic cells induced by the suppression of survivin, was increased by the simultaneous administration of topotecan. Thus, transfection

efficiency appeared to be increased by topotecan. Because non-viral vectors have been shown to have a relatively low transfection efficacy (26), numerous studies have explored ways to increase transfection efficiency. According to these studies, transfection efficiency is increased chiefly by improving intracellular transport and/or cellular uptake of vectors. Topoisomerase II inhibitors were shown to enhance the integration of exogenous vectors including liposome by promoting the integration process into human chromosomes (27). Steroids enhance liposome-mediated gene transfer by improving the intracellular transport to the nucleus (28). Insulin has the potential to increase the efficiency of lipofection correlated with the percentage of the S-phase fraction (29). In a mouse model, irradiation was shown to increase the uptake of liposome-DNA complexes in several organs (30). The increased S-phase fraction seen after 6-h treatment with topotecan in the present study may explain the improvement of transfection efficiency. To our knowledge, this is the first report that topotecan can increase transfection efficiency by increasing the cellular uptake of liposome-siRNA complexes.

Survivin also plays an important role in the resistance of carcinomas to chemotherapy and/or radiotherapy (31-34), and the suppression of survivin expression sensitizes cancers to chemotherapy and radiotherapy (34,35). Pennati *et al* found that ribozyme-mediated inhibition of survivin expression increases the sensitivity of human melanoma cells to topotecan (36). Another mechanism of the synergistic effect of survivin-specific siRNA and topotecan seen in the present study might be sensitization of cancer cells to topotecan by decreased expression of survivin.

In conclusion, the synergistic effect of this combination treatment is due to topotecan increasing the cellular uptake of siRNA. The combination of topotecan and survivin-specific siRNA thus offers an attractive approach to therapy of advanced renal cancer, and topotecan is a useful agent worthy of also being evaluated in the treatment of other types of cancer.

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