siRNA-mediated inhibition of survivin gene enhances the anti-cancer effect of etoposide in U-937 acute myeloid leukemia cells

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Abstract: Acute myeloid leukemia (AML) is one of the most frequent types of leukemia which mostly affects adult people. Resistance to therapeutic drugs is considered as a major clinical concern resulting in a weaker response to chemotherapy, disease relapse and decreased survival rate. Survivin, a member of Inhibitor of Apoptosis Proteins (IAPs), is associated with drug resistance and inhibition of apoptotic mechanisms in numerous hematological malignancies. In the present study, we examined the combined effect of etoposide and siRNA-mediated silencing of survivin on U-937 acute myeloid leukemia cells. The AML cells were transfected with survivin specific siRNA and gene knockdown was confirmed by quantitative real time PCR and western blotting. Subsequently, U-937 cells were assessed for response to etoposide treatment and apoptosis rate was measured with flow cytometry. The cytotoxic effects in siRNA-etoposide group were measured and compared to etoposide single therapy group. Survivin siRNA effectively knocked down the mRNA and protein levels of survivin, which led to lower cell proliferation and enhanced apoptosis. Furthermore, combined treatment of etoposide and survivin siRNA synergistically increased the cell toxic effects of etoposide and its ability to induce apoptosis.

Key words: Survivin, siRNA, Etoposide, U-937.

Introduction

Acute myeloid leukemia (AML) is a genetically heterogeneous, severe and tremendously progressive hematopoietic malignancy triggered by obstructing differentiation and maturation of granulocytes or monocytes (1). Standard treatment of AML consists primarily of intensive chemotherapy and is done in two phases including induction therapy and post-remission or consolidation therapy (2). Currently, induction therapy includes the first 3 days of cytarabine (3). Consolidation therapy usually consists of high doses of cytarabine given in high doses in combination with other chemotherapeutic agents like etoposide (4). Most of the patients with the experience of initial treatment phase for decreasing the number of malignant cells to an undetectable level (induction therapy) even after significant response to chemotherapy and complete remission, relapse within a few years and the chance for their long-term survival is very low (5). One of the most predominant reasons to interfere the response to chemotherapy is drug resistance, which can be intrinsic in AML patients.

Apoptosis is the process of programmed cell death and is a biological process crucial for the normal development and maintenance of tissue homeostasis (6, 7). Regulation of apoptosis is conducted via proteins with stimulating or suppressing effects. Inhibitor of apoptosis proteins (IAPs) are known as key regulators of apoptosis, cell division and proliferation (8). Members of IAP family in mammals are best known for the suppression of apoptosis. Some of IAPs directly bind to apoptotic proteins and suppress their activity (e.g. cIAP1 and c-IAP2 that bind to TRAF-1 and TRAF-2 or survivin which inhibits caspases activation and consequently apoptosis). Overexpression of the human survivin gene (baculoviral IAP repeat-containing protein 5 or BIRC5) is associated with tumor progression and drug resistance in numerous malignancies like lymphoma and leukemia. Anti-apoptotic characteristic of this protein is a significant growth advantage for tumor cells and is likely to play a central role in resistance to chemotherapy (9, 10). Previous studies have identified a correlation between survivin protein expression and clinical period of disease in a few cancers. This makes survivin expression as a prominent prognostic parameter in related diseases.

In the current study, we analyze the role of survivin knockdown in sensitizing U937 leukemic cells to etoposide treatment. In this regard, we assess the effects of etoposide alone and in combination with survivin siRNA in promoting apoptosis and U937 cell viability.

Materials and Methods

Cell culture

The human myeloid cell line U-937 (ATCC® CRL1593.2™), was purchased from ATCC, USA and

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maintained in RPMI-1640 supplemented with 10% heat-inactivated fetal bovine serum (Sigma-Aldrich, St. Louis, MO, USA), 100 IU/ml penicillin, 100 µg/ml streptomycin and incubated at 37 °C in a 95% humidified atmosphere containing 5% CO₂. In whole experiments, cells were used in the logarithmic growth phase and in triplicates.

siRNA preparation and transfection

The survivin mRNA targeting sequences of siGENOME (5'-CAAAGGAAAACAAAGAUA-3') and a negative control siRNA (Scrambled siGENOME siRNA had no known homology with any human genes) were synthesized by Dharmacon Research, Inc. (Lafayette, CO) using 2°-ACE protection chemistry.

5×10³ cells/well were seeded in six-well plates. Serum and antibiotics free RPMI-1640 medium was used just before transfection. Lipofectamine™2000 reagent (Invitrogen, Carlsbad, CA, USA) was utilized for siRNA transfection according to the manufacturer’s instructions. The final concentration of siRNAs was 80 nM in all experiments. In this regard, siRNAs in the intended concentration and lipofectamine (2.5 µl) were separately diluted in Opti-MEM® I Reduced-Serum Medium (Minimal Essential Medium, Invitrogen) and incubated for 10 min at room temperature (RT). The diluted solutions were then combined and incubated for 20 min at RT. Subsequently, the mixtures were added to the wells containing cells in serum/antibiotics medium. Furthermore, the cells treated with only lipofectamine were considered as siRNA blank control. The cells were then incubated for 6 h at 37 °C in a humidified CO₂ incubator as previously described. After 6 hours, culture medium with 20% of FBS was added to the wells and the cells were incubated under aforementioned conditions. 24h after transfection, Reverse Transcription, quantitative real-time PCR (qRT-PCR) and western blot analysis were exploited to assess the effect of survivin siRNA on gene silencing.

Reverse transcription-PCR

Total RNA was extracted from cells with AccuZol™ reagent according to the manufacturer’s protocol (Bioneer, Daedeok-gu, Daejeon, Korea) and quantified by UV absorbance spectroscopy. Then, 5 µg of total RNA was reverse transcribed using oligo-dT primer and Thermo Scientific Revert Aid First Strand cDNA Synthesis Kit. The cDNA samples were analyzed by performing a gradient RT PCR using the specific primers (5'-GGACGACCGCATCTCTACAT-3', 5'-CAACCGGAAAAGGAAACAGC-3'). β-actin (5'-TCCCTGGAGAA-GAGCTACG-3'; 5'- GTAGTTTCGTGGATGCCACA -3') was used as an internal control. Amplification cycles were: 95°C for 3 min followed by 35 cycles consist of 30 sec at 95°C, 40 sec at a gradient temperature of 54 to 62°C and 30 sec at 72°C. Final extension eventually was performed by heating at 72°C for 5 min. Aliquots of PCR product were electrophoresed on 1.5% agarose gels, and PCR fragments were visualized by ethidium bromide staining.

Quantitative RT-PCR (qPCR)

Quantification of survivin mRNA was performed using Takara SYBR Premix Ex Taq II (TliRNase H Plus, RR820L) in Corbett rotor gene 6000 system. PCR was carried out in 20 µl containing 10 µl of SYBR green reagent, 0.2 µM of each primer, 0.5 µl of template cDNA and 9 µl of nuclease-free water. Beta actin cDNA served for normalization of the reaction. The initial incubation for activation of DNA polymerase (95 °C for 3 min) was followed by 45 cycles at 95 °C for 10 sec, 59 °C for 30 sec and 72 °C for 20 sec. Relative expression of survivin mRNA (RE) was calculated using delta-delta Ct method (11).

MTT assay

In this study, cytotoxicity of the treatments was assessed using MTT assay kit which purchased from the Roche Company. Briefly, the cells were treated with the agents as described above and incubated for 24 h in a humidified CO₂ incubator. Following on, 100 µl of MTT reagent (0.5 mg/ml in PBS) was added to each well and then the plates were returned in the incubator for 4 hrs. The water insoluble formazan crystals were formed during incubation period that solubilized by adding 100 µl of the solubilization buffer (10% SDS in 0.01 M HCl) to each well. After overnight incubation in above mentioned conditions, the absorbances of the solubilized formazan dyes were measured using an ELISA plate reader.

SDS-PAGE and western blotting

Changes in survivin protein expression after siRNA mediated gene knock down was investigated by western blot analysis where Beta actin protein was employed for normalization. Primary and secondary antibodies were purchased from Abcam (Cambridge, MA, UK). 5×10⁴ U-937 cells were seeded in 6 well culture plates. NC, blank control and siRNA (80nM) groups were considered as mentioned before. 24 hours after transfection, the cells were collected by centrifugation at 800 rpm for 7 min and washed using phosphate-buffered saline (PBS, pH= 7.2). Cell pellets were then resuspended in 200 µl cell lysis buffer (1% Triton X-100, 1% SDS, 150 mM NaCl, 50 mM Tris-HCl (pH= 7.4) and 1 mM EDTA (pH= 8). To prevent protease activity, the lysis buffer was supplemented with inhibitor cocktail (Roche Diagnostics GmbH). After 30 min of incubation on ice and alternatively pipetting, the suspensions were centrifuged at 14,000 rpm for 10 min at 4 °C and the supernatants were collected. Total protein concentration was determined using eppendorf Bio Photometer. 200 µg of each protein sample was used for SDS PAGE electrophoresis in tris-glycine running buffer. A 15% gel topped with a 4% polyacrylamide stacking gel was utilized for this purpose and the voltage was adjusted to 120 V. The proteins were then transferred onto a methanol activated Millipore poly vinyl idine difluoride (PVDF) membrane using transfer buffer (0.01% SDS, 25 mMTris-base, 192 mM Glycine and 10% methanol). Nonspecific reactivity was blocked by exposing the PVDF membrane to 4% of skim milk in PBS/Tween-20 (0.05%, v/v). Then the primary antibody was added onto the membrane and incubated for 12 hours at 4 °C (1: 5000 concentrations for Beta actin and 1: 1000 for surviving antibody). Subsequent to washing thrice, the membrane was exposed to the Horse Radish Peroxidase (HRP) conjugated secondary antibody (1: 3000) for two hours at room tem-
perature. Protein detection was performed using the enhanced chemi-luminescence solution (ECL, AmershamPhamacia Biotech Inc, USA). Band densities were measured exploiting ImageJ software (Version 1.49h, national Institutes of Health, Bethesda, Maryland, USA).

Annexin V/PI apoptosis assay

U-937 cells were counted and collected at a density of $1 \times 10^6$ cells/well of 6-well plates and treated with specific siRNAs, NC siRNA, the IC$_{50}$ dose of etoposide and a combination of them, as previously described in the MTT section. Twenty-four hours after transfection, cells were harvested and washed twice with cold PBS. The cell pellets were resuspended in 500 µl of binding buffer containing 1 µl propidium iodide (PI) and 5 µl fluorescein isothiocyanate (FITC)-labeled annexin V solution for 10 min in room temperature and then analyzed using a fluorescence-activated cell sorting (FACS) flow cytometer (BD LSR, Becton-Dickinson, San Jose, CA, USA) and De Novo software. Both Annexin V and PI negative cells were considered as normal, Annexin V positive and PI negative cells were considered as early apoptotic, cells that were both Annexin V and PI positive were considered as late apoptotic as well as early necrotic, and cells that were Annexin V negative and PI positive were considered as mechanically injured during the experiment procedure or late necrotic.

Statistical analysis

To determine the degree of significance, student’s t test was used. Fold reversal was the IC$_{50}$ for cytotoxic drug in NC treated cells divided by the IC$_{50}$ for drug in siRNA-treated cells.

Results

Evaluation of Survivin gene knockdown in U-937 cell line

24 hours after transfection, changes in survivin gene expression in both mRNA and protein levels was assessed using qRT-PCR and western blotting, respectively. Results of the untreated group were considered as 100% and survivin expression in test groups were calculated relative to blank control group. In both experiments, there was not any notable difference between NC siRNA group and untreated cells. However, as shown in Figures 1 and 2 a significant decline in survivin gene expression was evident (the expression level was lowered to 33.1% in mRNA level and 20.8% in protein level).

Chemosensitivity of survivin siRNA to etoposide

The effect of survivin down-regulation on chemosensitivity of the leukemic cells was also investigated. As shown in Figure 3, monotherapy with etoposide induced cytotoxicity in a dose-dependent way. The results of MTT assay showed that survivin siRNA significantly decreased the cell survival rate to 84.01%, compared with the blank control ($P<0.05$). Moreover, etoposide in combination with survivin siRNA further decreased the cell survival rate relative to etoposide or survivin siRNA alone ($P<0.05$).

Surprisingly, the presence of survivin siRNA led to a clear reduction in the IC50 value of etoposide from 2.29 µM to 0.99 µM (Figure 4 and Table 1). The CDI values were also less than 1 in all concentrations, which indicated the synergistic effect between the two agents (Table 2). Notably, transfection with NC siRNA had a minimal effect on the chemosensitivity of the leukemic
cells relative to the etoposide treated cells ($P>0.05$; Figure 3), which further confirms the specific impacts of survivin siRNA.

**Effects of survivin siRNA on U-937 cell growth**

The results of survivin siRNA on cell proliferation are shown in Figure 5. Negative controls and the cells that were transfected with 80 nM of survivin siRNA were monitored 24, 48, 72, 96 and 120 hours after transfection by trypan blue exclusion assay. The NC siRNA group was not found to be different to untreated cells. In contrast, survivin siRNA was led to anti proliferative impact on U937 cells in a time dependent manner. In the first three days, the cell viability was lowered to 86, 74, and 66% respectively. However, since fourth day survival rate did not significantly differ ($≈64\%$).

**Apoptosis analysis by flow cytometry**

In the flow cytometry analysis, as shown in Table 3 and Figure 7, the percentage of annexin V positive cells was 27.55 and 8.52 for etoposide ($IC_{50}$) and survivin siRNA treated, respectively. Interestingly, when cells were subjected to survivin knockdown and concurrent etoposide exposure, population of Annexin V positive cells were increased significantly to 54.84 relative to the siRNA or etoposide treatment (Figure 6). However, no significant alterations in the extent of apoptosis were detected for NC siRNA or NC siRNA plus etoposide groups compared with the blank control group or etoposide alone ($P>0.05$; Figure 6).

**Discussion**

Resistance to therapy is a common clinical obstacle in acute myeloid leukemia (AML). IAP protein family has shown to be involved in the prevention of apoptosis which is a key regulatory mechanism in the elimination of malignant cells and tumors. Survivin as a member of this family is normally expressed in fetal period but its over expression in completely differentiated cells is frequently reported in malignant and cancerous cells (12, 13). According to the literature, the overexpression of survivin gene is directly associated with poor prognosis, tumor development and resistance to chemotherapy drugs in many cancers such as AML (14-16). Previous studies have demonstrated that, survivin gene suppression leads to increased apoptosis and simultaneously sensitizes the cancerous cells to chemotherapeutic agents. In this regards, we utilized RNA interference for specific down-regulation of survivin in U-937 cells to overcome resistance to chemotherapeutic agent, etoposide. Studies confirmed presence of the mutation in survivin promotor was correlated in these cell lines with increased survivin expression at the both mRNA and protein levels, for example, Polymorphisms are located at CDE/CHR repressor elements is the most common
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Mutation with high frequency among cancer cell lines compared to normal cell line controls (17). The results of qRT-PCR and western blot analysis showed that transfection with survivin siRNA could significantly reduce the mRNA levels of survivin and its translated protein, suggesting that survivin siRNA could effectively cleave survivin mRNA and blocked its translation to protein. The results of survivin siRNA on cell growth and proliferation indicated that survivin silencing significantly inhibited the cell growth in U-937 cells. These results suggest that survivin has an important role on cell growth and proliferation in AML cells. Furthermore, the results of MTT assay revealed that pretreatment with survivin siRNA could synergistically reduce the IC50 of leukemic cells to etoposide, demonstrating that survivin downregulation could sensitize U-937 AML cells to etoposide. In order to better understand the effect of survivin on drug resistance in leukemic cells we evaluated the effects of survivin siRNA on the apoptotic effect of etoposide. Our results indicated that treatment with etoposide alone resulted in a significant apoptosis in U-937 cells. Furthermore, down-regulation of survivin led to a remarkable apoptosis and sensitized the U-937 cells to etoposide-mediated apoptosis. However, NC siRNA and lipofectamine treatment had no effects on survivin gene and protein expression, cell growth, IC50 and apoptotic effect of etoposide. The above mentioned results indicated that presence of survivin is vital for survival and cell growth of leukemic cells, therefore inhibiting survivin by siRNA can potentiate these cells to apoptosis and sensitize them to chemotherapeutic agents.

Figure 6. The Change of the Proportion of Annexin V Positive Cells after Treatment with Anticancer Agents. The proportion of annexin V positive cells was measured by flow cytometer using annexin V-FITC and PI staining of U-937 cells after 24 h of treatment with blank control, 50nM of NC siRNA, etoposide (IC50), 50nM of survivin siRNA, 50nM of NC siRNA + etoposide and 50nM of survivin siRNA + etoposide. Dot plots are representative of three experiments.

Figure 7. Percentages of cells gated into quadrants by flow cytometry. Cells (1-12 according to table 4.4) were harvested, transfected, stained with Annexin V-FITC and PI, and analyzed by flow cytometry. Quantification of flow cytometric cell counts in 4 different quadrants is illustrated. UL: upper left, dead cells. UR: upper right, late-stage apoptotic cells. LL: lower left, viable cells. LR: lower right, early stage apoptotic cells. Each experiment was repeated three times, and the values shown are means +/- standard error.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>LL</th>
<th>UL</th>
<th>LR</th>
<th>UR</th>
<th>LR + UR</th>
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<tbody>
<tr>
<td>1  Blank Control</td>
<td>97.17</td>
<td>1.62</td>
<td>0.4</td>
<td>0.81</td>
<td>1.21</td>
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<tr>
<td>2  NC siRNA (50 nM)</td>
<td>96.39</td>
<td>2.8</td>
<td>0.35</td>
<td>0.46</td>
<td>0.81</td>
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<tr>
<td>3  NC siRNA (100 nM)</td>
<td>94.02</td>
<td>1.79</td>
<td>1.4</td>
<td>2.79</td>
<td>4.19</td>
</tr>
<tr>
<td>4  Survivin siRNA</td>
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<td>0</td>
<td>8.11</td>
<td>0.41</td>
<td>8.52a</td>
</tr>
<tr>
<td>5  Etoposide</td>
<td>64.82</td>
<td>7.63</td>
<td>21.41</td>
<td>6.14</td>
<td>27.55a</td>
</tr>
<tr>
<td>6  NC siRNA (50 nM) + Etoposide</td>
<td>60.73</td>
<td>2.06</td>
<td>11.46</td>
<td>25.75</td>
<td>37.21a</td>
</tr>
<tr>
<td>7  NC siRNA (100 nM) + Etoposide</td>
<td>63.67</td>
<td>2.14</td>
<td>27.87</td>
<td>6.32</td>
<td>34.19a</td>
</tr>
<tr>
<td>8  Survivin siRNA + Etoposide</td>
<td>45.07</td>
<td>0.09</td>
<td>50.03</td>
<td>4.81</td>
<td>54.84a</td>
</tr>
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</table>

Table. 3 Flow cytometric Results of U-937 Cells Treated with Anticancer.
Apoptosis or programmed cell death consists of two main pathways, extrinsic (the death receptor-mediated) and intrinsic (mitochondrial) pathways. Both pathways activate caspase 3 which leads to downstream caspase proteins activation and eventually apoptose occurs. It has proven that, the treatment of HL-60 (human leukemia cell line) with etoposide activates both signaling pathways of apoptosis via multiple caspase activities (16, 18-20). The anti-cancer agent etoposide, disturbs DNA topoisomerase II by interfering DNA re-ligation. This result in numerous errors in DNA synthesis at the premitotic stage of cell division. Because of high rate of cell division in cancerous cells, they rely on topoisomerase II enzyme more than normal cells. Consequently, a larger number of errors in DNA synthesis of malignant cells caused by etoposide which results in apoptosis. However, by inhibition of caspase activities and other possible mechanisms, survivin protein blocks the intrinsic and extrinsic pathways and leads to chemo or radiotherapy resistance. On the other hand, survivin is associated with microtubules and the mitotic spindle (16, 20-23). Hence, it could influence the response to microtubule-interacting agents like paclitaxel in cancer cells. These features make Survivin as an attractive therapeutic target in hematological malignancies.

The results of our study showed that siRNA-mediated downregulation of survivin led to the increased apoptotic effect of etoposide, suggesting the possibility of that survivin sensitizes AML cells in a caspase 3-dependent mechanism.

To conclude, our study showed that survivin has a vital role in cell growth, proliferation and drug resistance. Down-regulation of survivin mRNA by siRNA enhanced apoptosis in U-937 leukemic cells and when used in combination with etoposide it synergistically sensitized leukemic cells to apoptotic effect of etoposide. Therefore, we suggest that siRNA-mediated silencing of survivin in combination with chemotherapeutics could be a promising strategy for the treatment of AML in the near future.

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References