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Research article

DOWNREGULATION OF KDR EXPRESSION INDUCES APOPTOSIS IN BREAST CANCER CELLS

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Abstract: Angiogenesis plays a crucial role in the growth, invasion and metastasis of breast cancer. Vascular endothelial growth factors (VEGFs) and their receptors (VEGFRs) are the key regulators of tumor angiogenesis. VEGFR-2, known as the kinase insert domain receptor (KDR), is a key receptor involved in malignant angiogenesis. We previously showed that knocking down KDR with short interference RNA (KDR-siRNA) markedly decreased KDR expression and suppressed tumor growth in a xenograft model. However, the mechanisms underlying the anti-cancer effects of KDR-siRNA are not clearly understood. This study aimed to elucidate the molecular mechanisms that induce apoptosis in human breast cancer MCF-7 cells after transfection with KDR-siRNA. We studied the effects of KDR-siRNA on proliferation, apoptosis, anti-apoptotic and pro-apoptotic proteins, mitochondrial membrane permeability, cytochrome c release and caspase-3 activity. The results indicated that KDR-siRNA treatment significantly inhibited the proliferation and induced the apoptosis of MCF-7 cells, reduced the levels of the anti-apoptotic proteins, Bcl-2 and Bcl-xl, and increased the level of the pro-apoptotic protein Bax, resulting in a decreased Bcl-2/Bax ratio. KDR-siRNA also enhanced the mitochondrial membrane permeability, induced cytochrome c release from the

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Abbreviations used: Apaf-1 – apoptotic protease-activating factor-1; BSA – bovine serum albumin; DMEM – Dulbecco's modified Eagle's medium; KDR – kinase domain receptor; MTT – 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazo-liumbromide; SDS – sodium dodecyl sulphate; siRNA – short interfering RNA; VEGF – vascular endothelial growth factor; VEGFR – vascular endothelial growth factor receptor

mitochondria, upregulated apoptotic protease-activating factor-1 (Apaf-1), cleaved caspase-3, and increased caspase-3 activity in MCF-7 cells. Furthermore, KDR-siRNA-induced apoptosis in MCF-7 cells was blocked by the caspase inhibitor Z-VAD-FMK, suggesting a role of caspase activation in the induction of apoptosis. These results indicate that the Bcl-2 family proteins and caspase-related mitochondrial pathways are primarily involved in KDR-siRNA-induced apoptosis in MCF-7 cells and that KDR might be a potential therapeutic target for human breast cancer treatments.

Keywords: Apoptosis, Breast cancer, MCF-7 cells, Cytochrome c, Caspase-3, Mitochondrial pathway, KDR, VEGF, Short interfering RNA, siRNA

INTRODUCTION

Breast cancer is one of the most common cancers in women, accounting for 23% of the total cancer cases. It is the primary reason for cancer-related death in women accounting for 14% of all cancer deaths [1]. Breast cancer has replaced cervical cancer as the leading cause of cancer death among women in the metropolitan centers of China [2, 3].

Although the exact cause of breast cancer is not fully understood, increasing evidence indicates that angiogenesis plays a crucial role in breast cancer growth, invasion and metastasis. Breast cancer is known to be an angiogenesis-dependent disease [4]. Vascular endothelial growth factors (VEGFs) and their receptors (VEGFRs) are emerging as the key regulators of tumor angiogenesis. VEGFs can stimulate angiogenesis and increase the permeability of capillaries after binding to VEGFRs [5–7].

VEGFR-2, also known as kinase insert domain receptor (KDR), is a key receptor involved in malignant angiogenesis. Its activation promotes a number of other cellular changes that lead to tumor development and metastases [8, 9]. KDR is highly expressed in breast cancer [10], suggesting that breast cancer is potentially treatable by reducing KDR expression. For instance, recent studies showed that the monoclonal antibody ramucirumab, which specifically targets KDR, appears to be effective at treating breast cancer. Ramucirumab induces tumor responses and stabilizes the tumor size for extended periods in patients pretreated in phase 1 and 2 clinical trials [11].

Short interfering RNA (siRNA) is a natural phenomenon of the inhibition of gene expression. It provides us with an effective and simple method to specifically downregulate target genes [12]. We previously showed that KDR-siRNA suppressed tumor growth in a xenograft model [13], suggesting that KDR-siRNA might be an effective approach for the treatment of breast cancer.

Apoptosis, also known as programmed cell death, is involved in the normal development of multicellular organisms and homeostasis. The deregulation of apoptosis is considered one of the characteristics of malignant tumors. Inducing tumor cell apoptosis has become an effective strategy for cancer treatment [14, 15]. Most previous studies about KDR have centered on its role in cell proliferation.

However, the precise function of KDR in apoptosis is largely unknown. In this study, we investigated the effect of knocking down KDR on apoptosis in breast cancer MCF-7 cells using KDR-siRNA, and the possible molecular mechanisms underlying KDR-siRNA-mediated effects on apoptosis. These may help us find an effective treatment strategy for breast cancer and understand the pathogenesis of breast cancer.

MATERIALS AND METHODS

Materials

MCF-7 cells were obtained from the China Peking Union Cell Culture Center. Dulbecco's modified Eagle's medium (DMEM) was from Gibco. Lipofectamine 2000 and TRIzol Reagent were from Invitrogen. Antibodies for Bcl-2, Bcl-xl, Bax, apoptotic protease-activating factor-1 (Apaf-1), and ß-actin were from Santa Cruz Biotechnology, and antibodies for cleaved caspase-3 were obtained from Cell Signaling Technology. The caspase inhibitor, Z-VAD-FMK, was purchased from R&D. Caspase-3 Colorimetric Assay Kit and Hoechst Staining Kit were the products of the Beyotime Company. All the other chemicals and regents were obtained from local commercial sources.

Cell culture

MCF-7 cells were grown in DMEM supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin and 100 µg/ml streptomycin in a cell culture incubator at 37ºC with 5% CO_2.

siRNA sequences

The Promega software system was used to select siRNA against KDR mRNA (GI: 11321596). After a BLAST search, the KDR-siRNA and scrambled siRNA (siRNAscr used as a negative control sequence, because it has no known homology to human genes) were designed as follows:
5'-GCCACCAUGUUCUCUAAUATT-3' (sense) and 5'-UAUUAGAGAACA UGGUGGCAT-3' (anti-sense) for KDR siRNA; and 5'-UUCUCCGAACGU GUCACGUTT-3' (sense) and 5'-ACGUGACACGUUCGGAGAATT-3' (anti-sense) for scrambled siRNA.
In order to enhance the stability of siRNA, the sense strands were methylated, while the anti-sense strands were not modified. All of the siRNA sequences were chemically synthesized by Shanghai Genepharma Co. Ltd.

Transient transfection of siRNA oligonucleotides

One day before transfection, cells were seeded at a density of 30,000 cells in 0.5 ml of growth medium without antibiotics in 24-well plates so that cells would grow to 50–60% confluence at the time of transfection. MCF-7 cells were transfected with or without KDR-siRNA or siRNAscr via Lipofectamine 2000 according to the manufacturer's instructions. Three groups were set up:

(a) The 100 nmol/l KDR-siRNA group;
(b) The 100 nmol/l siRNAscr group (serving as a negative control); and
(c) The untransfected control (UT, serving as a blank control).

Flow cytometry

For the determination of the effects of siRNA on KDR expression, flow cytometry was performed. In brief, cells were collected 48 h after transfection, pelleted, and resuspended in PBS with 1% bovine serum albumin (BSA). After permeabilization with 1% saponin for 15 min, the cells were incubated with KDR rabbit polyclonal antibodies (sc504, 1:200, Santa Cruz) diluted in PBS with 1% BSA or the control antibodies, rabbit IgG (sc2012, 1:200, Santa Cruz), for 2 h. Next, cells were washed with PBS and the cells were incubated with FITC-labeled secondary antibodies for 1 h. Finally, the fluorescence in each sample (at least 1×10^6 cells) was measured using flow cytometry (Becton Dickinson).

Total RNA extraction and semi-quantitative PCR

Total RNA was extracted from cultured MCF-7 cells using Trizol reagent (Invitrogen, USA) according to the manufacturer's instructions, and RT-PCR was performed using a reverse transcription system and Taq DNA Polymerase (Promega). The cDNA obtained for KDR was amplified using PCR, as was the cDNA for GAPDH, a housekeeping gene that served as a control. The sequences used for primers were as follows:
5'-CTGGCATGGTCTTCTGTGAAGCA-3' (sense) and 5'-AATACCAGTG GATGTGATGCGG-3' (antisense) for KDR (795 bp); and 5'-CGTGGAAGG ACTCATGACCA-3' (sense) and 5'-TCCAGGGGTCTTACTCCTTG-3' (antisense) for GAPDH (509 bp).
For RT-PCR, the program consisted of an initial denaturation at 95ºC for 2 min, followed by amplification for 35 cycles (95ºC for 1 min to denature, 64ºC for 1 min to anneal, and 72ºC for 2 min to extend) with a final extension step at 72ºC for 10 min. Products were then separated by means of electrophoresis on a 1% agarose gel and the bands were visualized using UV light.

Cell proliferation assay

Cell proliferation was determined using the 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide (MTT) assay. Briefly, MCF-7 cells (6,000 cells per well) were plated in 96-well culture plates and treated with siRNA-KDR at different concentrations ranging from 50 to 200 nmol/l in fresh medium for 48 h. Next, the medium was aspirated and MTT (50 µl 5 mg/ml in PBS) was added to each well. Incubation was continued for additional 4 h at 37ºC. Thereafter, 150 µl DMSO was added to each well to dissolve the purple formazan precipitates. The absorbance was measured at 492 nm with a microplate reader. The cell proliferation after siRNA treatment was expressed as the percentage of UT control cells.

Detection of apoptotic cells by Hoechst 33258 staining

Cells were plated on cover slips placed in 24-well plates, and treated with or without 100 nmol/l KDR-siRNA or siRNAscr for 48 h. After gentle washing with cold PBS, the cells were fixed using 4% polyformaldehyde for 1 h, washed again in PBS, and stained with 0.5 ml Hoechst 33258 (10 ug/ml) for 10 min at 37ºC in the dark. Morphological changes in the cells were examined under a fluorescence microscope. The percentage of apoptotic cells was determined with at least 200 cells per treatment group. Apoptotic cells were defined on the basis of morphological alterations, including cell shrinkage, chromatin condensation and fragmentation, nuclear membrane blebbing, and the form of the apoptotic body.

Measurement of mitochondrial membrane potential

This assay was performed according to the instructions in the Cytochrome c Releasing Apoptosis Assay Kit (Biovision). Briefly, after treatment, MCF-7 cells were pelleted by centrifugation. Cell pellets were resuspended with 1 ml cytosol extraction buffer mix containing DTT and protease inhibitor, and incubated for 10 min on ice. Cells were then homogenized with a Dounce grinder. After homogenization, unbroken cells and large debris were removed by centrifugation at 800 × g for 10 min at 4ºC. The supernatants containing mitochodria were further centrifuged. The resulting supernatants were saved as cytosolic extracts at –70ºC. The pellets were resuspended with 100 µl extraction buffer mix containing DTT and protease inhibitor, and saved as mitochondrial fractions. Then we loaded 30 µg cytosolic and mitochondrial fractions isolated from MCF-7 cells on 12% standard sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). Western blot was done with cytochrome c antibody (1:2000 dilution; Biovision). The same blot was reprobed for α-tubulin. The mitochondrial damage was also evaluated using JC-1 dye. After treatment with or without 100 nmol/l KDR-siRNA or 100 nmol/l siRNAscr for 48 h, as described above, all of the MCF-7 cells were incubated with the dual emission dye JC-1 for 30 min at 37ºC. The numbers of cells exhibiting green and red fluorescence were quantified using a flow cytometer (Becton Dickinson), and the data were analyzed with the ModFit software.

Western blot analysis

To analyze the protein levels of Bcl-2, Bcl-xl, Bax, caspase-3 and Apaf-1, MCF-7 cells were plated in 6-well plates at a density of 1.2×10^5 cells/well and treated with 100 nmol/l KDR-siRNA or 100 nmol/l siRNAscr, as described above. 48 h after transfection, whole cell lysates were prepared from cultured cells with lysis buffer containing protease inhibitors. The western blot was carried out following the standard protocol. Briefly, 50 µg protein samples were resolved by 12% standard SDS-PAGE and transferred to nitrocellulose membranes. After overnight blocking with 4% non-fat milk at 4ºC, the membranes were incubated with primary antibody for 1 h at room temperature. The membranes were

incubated with rabbit polycolonal anti-Bcl-2, anti-Bax, anti- Bcl-xl, anti-Apaf-1 antibody (1:300 Santa Cruz), and rabbit anti-human cleaved caspase-3 monoclone antibody (1:1000 Cell Signaling Technology), for 1 h at room temperature. Then the membranes were washed with PBST three times and incubated with goat anti-rabbit IgG conjugated with horseradish peroxidase for 1 h at room temperature. The expression levels of these proteins were normalized with that of β-actin. The band intensities of these proteins were quantified with a Gel EDAS analysis system (Cold Spring USA Corporation).

Caspase-3 activity assay

The caspase-3 activity was measured using a caspase-3 colorimetric assay kit. Cells were pelleted and resuspended in cell lysis buffer. After incubation on ice for 10 min, the lysed cells were centrifuged. The supernatant was incubated with the caspase substrate, AC-DEVD-pNA. The absorbance of the reaction product p-nitroaniline (pNA) was quantified using a spectrophotometer at 405 nm. By comparing the absorbance of pNA for the KDR-siRNA group to that for the UT group, the relative change in caspase-3 activity could be determined.

Quantification of apoptotic cells

MCF-7 cells were treated with the general caspase inhibitor, Z-VAD-FMK (20 μmol/l), for 2 h, followed by transfection with siRNAs for 48 h. The transfected cells were harvested to analyze the apoptotic cells using Annexin V-Fluos staining kit (Roche Applied Science) and a flow cytometer (Becton Dickinson).

Statistical analysis

All of the data are expressed as means ± SD. Student's t-test was used to assess the statistical significance. A value of $P < 0.05$ was considered statistically significant. Treatments of cells were performed in triplicate wells and the experiments were repeated three times.

RESULTS

KDR-siRNA inhibits KDR expression in MCF-7 human breast cancer cells

Cells were transfected with siRNA and harvested after 48 h, and KDR protein was examined by flow cytometry. Flow cytometry data presented lower protein expressions of KDR in MCF-7 cells treated with siRNA compared to MCF-7 cells treated with siRNAscr (Fig. 1A). There was no difference in KDR protein expression between siRNAscr-treated cells and the untreated (blank control) cells. To confirm flow cytometry results and better characterize the knockdown of KDR at the mRNA level, semi-quantitative RT-PCR was performed. The KDR-siRNA in MCF-7 cells induced a marked decrease in KDR mRNA level compared with the blank control group (Fig. 1B). GRPDH mRNA expression showed no difference between the groups. siRNAscr showed no function for the RNAi method. This revealed successful transfection of KDR-siRNA into MCF-7 cells and specific reduction of KDR expression at the protein and mRNA levels.

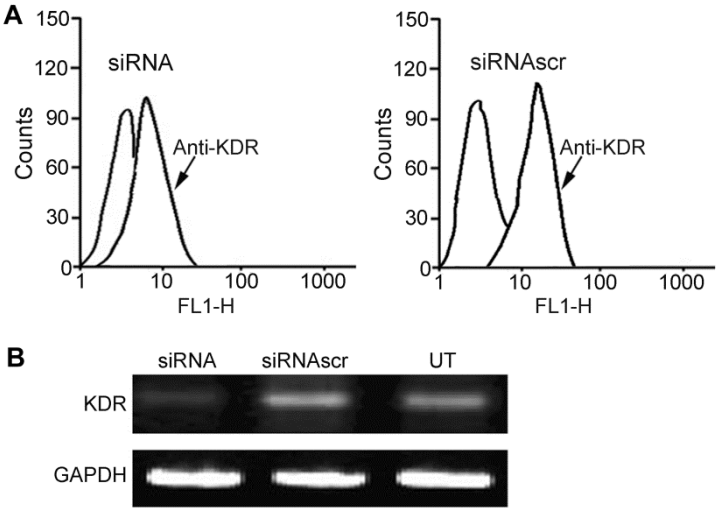

Fig. 1. The effects of KDR-siRNA on KDR expression in MCF-7 breast cancer cells. A – KDR-siRNA treatment decreased the protein expression levels of KDR in MCF-7 cells analyzed by flow cytometry. B – KDR-siRNA treatment decreased the KDR mRNA levels in MCF-7 cells analyzed by RT-PCR. The results are representative of three independent experiments.

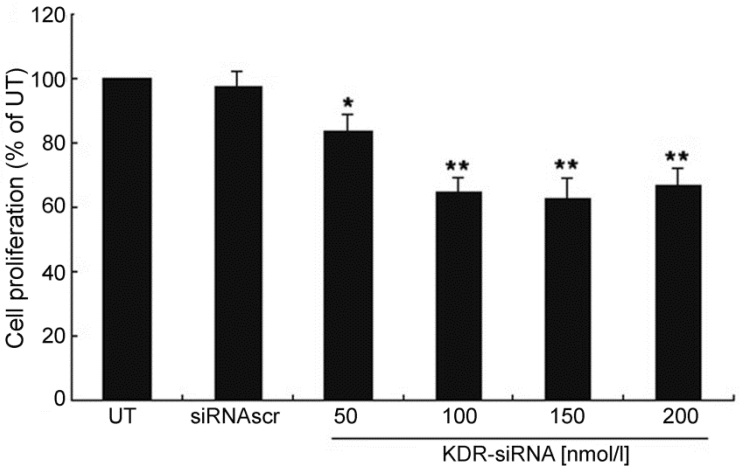

Fig. 2. The effects of KDR-siRNA on the proliferation of MCF-7 cells. Cells were treated with the indicated concentrations of KDR-siRNA, and the inhibitory effect on cellular proliferation was determined using the MTT assay as described in the Materials and Methods section. The results were expressed as percentages of cell numbers relative to that for untreated cells (UT). The data are the means ± SD (n = 5). *$P < 0.05$ (vs. UT group); **$P < 0.01$ (vs. UT group).

KDR-siRNA decreased the cell proliferation of MCF-7 cells

A preliminary screening was conducted to measure the effect of KDR-siRNA at concentrations of 50, 100, 150 and 200 nmol/l on cellular proliferation. As shown in Fig. 2, the proliferation of MCF-7 cells treated with 100 nmol/l KDR-siRNA was significantly reduced (by 36%) compared with the proliferation for the UT group ($P < 0.01$). Higher concentrations of KDR-siRNA (> 100 nmol/l) did not result in further reduction of cell proliferation. However, the control siRNAscr had no effects on MCF-7 cell proliferation compared to the UT control group ($P > 0.05$). Thus, 100 nmol/l KDR-siRNA was selected to examine its effects on apoptosis, anti-apoptotic and pro-apoptotic proteins, mitochondrial membrane permeability, the release of cytochrome c, Apaf-1 expression, and caspase-3 expression.

KDR-siRNA-induced cell apoptosis

To determine whether KDR-siRNA transfection could induce apoptosis in MCF-7 cells, Hoechst 33258 staining was used to detect the apoptotic cells. As shown in Fig. 3, the percentage of apoptotic cells in the KDR-siRNA treatment group was 29.4%, whereas it was 4.0% in the UT group and 4.5% in the siRNAscr-treated group.

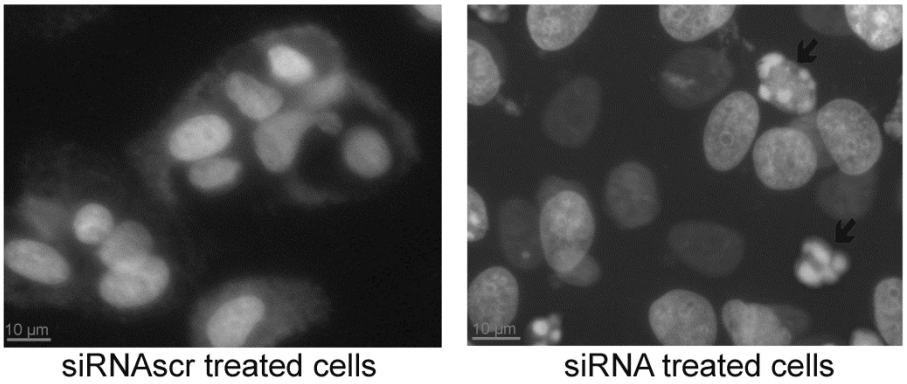

Fig. 3. The effects of KDR-siRNA on the morphological changes of MCF-7 cells. The cells were stained with Hoechst 33258 and visualized under a fluorescence microscope. The apoptotic bodies and nucleic fragments (indicated with arrows) were stained deep blue, and the normal cells were stained light blue. The nuclei of the cells appeared normal, round and large with regular contours in the siRNAscr groups. Cells with smaller nuclei and condensed chromatin were rare. By contrast, the nuclei of the cells exposed to KDR-siRNA appeared hyper-condensed.

KDR-siRNA downregulated Bcl-2 and Bcl-xl expression, but upregulated Bax expression

Bcl-2 and Bcl-xl are anti-apoptotic proteins. Bax is a pro-apoptotic protein. Treatment of MCF-7 cells with KDR-siRNA reduced the expression levels of Bcl-2 and Bcl-xl by > 40% compared with those of the UT group. At the same

time, the level of proapoptotic Bax protein increased 2.1-fold (Fig. 4), resulting in a significantly decreased Bcl-2/Bax ratio ($P < 0.01$). Because the Bcl-2/Bax ratio is a determining factor for apoptosis, the decreased Bcl-2/Bax ratio indicates that KDR-siRNA treatment induces apoptosis in MCF-7 cells.

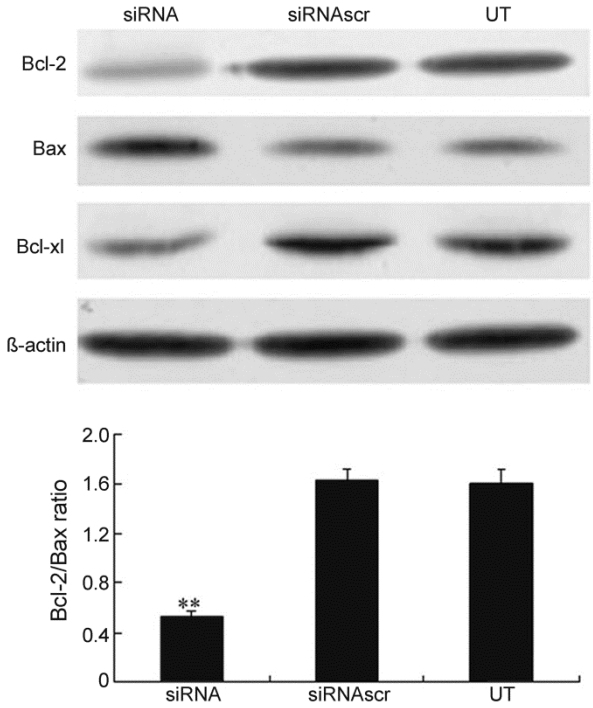

Fig. 4. The effects of KDR-siRNA on the levels of the Bcl-2 family proteins in MCF-7 cells. A – KDR-siRNA treatment decreased the protein expression levels of Bcl-2 and Bcl-xl, and increased the protein expression level of Bax in MCF-7 cells as analyzed via western blot. B – Quantification of the results from panel A. Shown here are the ratios of Bcl-2/Bax as determined by the relative intensities of protein bands. Each bar represents the mean ± SD of three experiments. $^{**}P < 0.01$ (vs. UT group).

KDR-siRNA increased the release of cytochrome c from the mitochondria and the mitochondrial membrane permeability

KDR-siRNA treatment significantly increased the level of cytochrome c in the cytoplasm (Fig. 5A), and there was a significant corresponding decline in the level of mitochondrial cytochrome c. However, siRNAscr had no effect on the release of cytochrome c. Mitochondrial membrane permeability was assessed via JC-1 staining. KDR-siRNA treatment caused a decrease in the number of cells with intact mitochondria (red color), and an increase in the number of cells with depolarized mitochondrial membranes (green color; Fig. 5B).

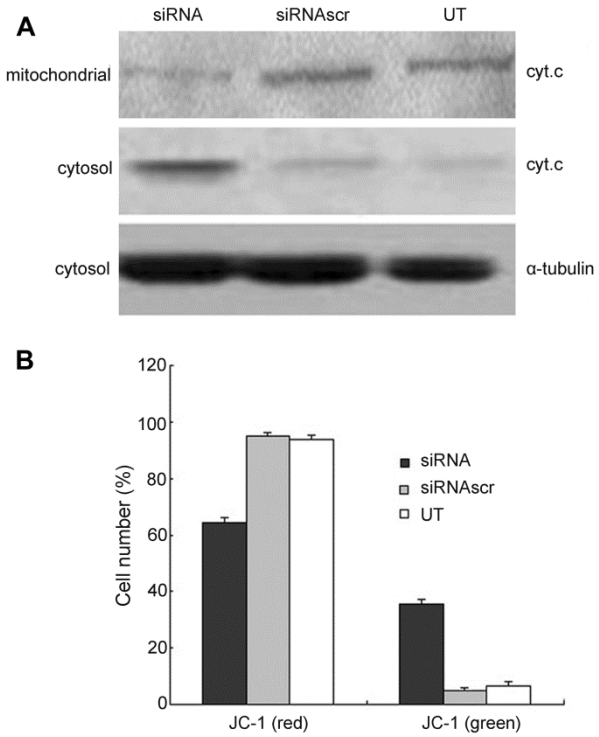

Fig. 5. The effects of KDR-siRNA on cytochrome c release from the mitochondria and mitochondrial membrane potential. A – Cells were treated with KDR-siRNA for 48 h and subjected to subcellular fractionation. The cytochrome c levels were analyzed via western blot, and normalized to α-tubulin. B – Cells were loaded with JC-1 and analyzed using flow cytometry. The cells with normal polarized mitochondrial membranes were stained red and those with depolarized mitochondrial membranes were stained green. The data are expressed as percentages of the total cell number. Each bar represents the mean ± SD (n = 3).

KDR-siRNA induced Apaf-1 expression and caspase-3 cleavage in MCF-7 cells

Cytosolic cytochrome c can interact with Apaf-1 and activate caspases. Activation of caspase-3 (by cleaving it to 17- and 19-kDa fragments) leads to apoptosis. For these reasons, we investigated whether the induction of apoptosis by KDR-siRNA treatment is associated with the induction of Apaf-1 and the activation of caspases.

We found that KDR-siRNA increased the levels of Apaf-1 and cleaved caspase-3 (Fig. 6A). We also measured caspase-3 activity using a chromogenic caspase-3 assay kit. Fig. 6B showed that KDR-siRNA treatment increased the caspase-3 activity as compared with that of the siRNAscr and UT groups. These results indicate that KDR-siRNA efficiently induces apoptosis in MCF-7 cells by inducing the expression of Apaf-1 and activation of caspase-3 cleavage.

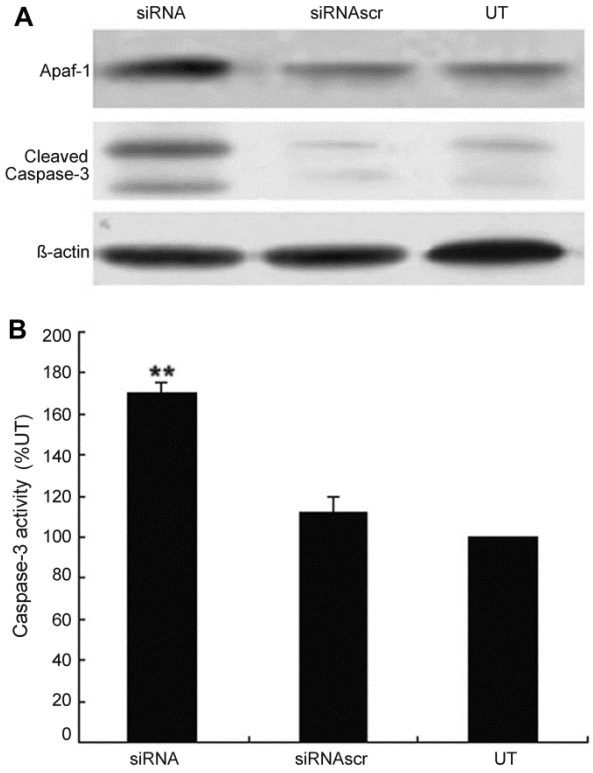

Fig. 6. The effects of KDR-siRNA on the Apaf-1 and caspase-3 levels in MCF-7 Cells. A – KDR-siRNA increased the expression levels of apoptotic protease-activating factor-1 (Apaf-1) and cleaved caspase-3 in MCF-7 cells, as analyzed via western blot. The caspase-3 antibody recognizes the cleaved products of caspase-3. B – KDR-siRNA treatment increased the caspase-3 activity, which was measured with a caspase-3 colorimetric assay kit. The results were expressed as percentages of caspase-3 activity relative to the UT control. The bars represent means ± SD (n = 5). $^{**}P < 0.01$ (vs. UT control).

Inhibition of caspases prevented KDR-siRNA-induced apoptosis in MCF-7 cells

Because KDR-siRNA activates caspase-3, which might be involved in KDR-siRNA-induced apoptosis, it is of interest to examine whether the suppression of caspases could block KDR-siRNA-induced apoptosis in MCF-7 cells. KDR-siRNA induced significant levels of apoptosis in MCF-7 cells (Fig. 7), reaching 27.6% after 48 h transfection, while the apoptosis rates of untransfected control cells (UT) and siRNAscr-transfected MCF-7 cells were 5.9% and 6.5%. Pretreatment of MCF-7 cells with a caspase inhibitor (Z-VAD-FMK) decreased KDR-siRNA-induced apoptosis to 13.8%. This observation indicates that KDR-siRNA-induced apoptosis requires the activation of caspases, including caspase-3.

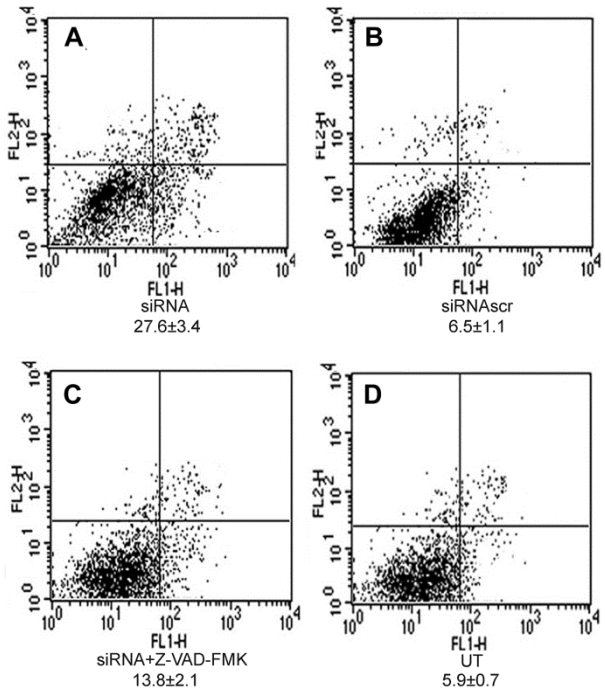

Fig. 7. The effects of a caspase inhibitor on KDR-siRNA-induced apoptosis in MCF-7 cells. Pretreatment of MCF-7 cells with Z-VAD-FMK significantly inhibited KDR-siRNA-induced apoptosis ($P < 0.05$).

DISCUSSION

In accordance with our previous results showing that KDR-siRNA markedly decreased KDR gene expression and suppressed tumor growth in the xenograft model [13], using the siRNA-targeting KDR gene, we were able to silence the expression of KDR at the protein and mRNA levels. However, the molecular mechanisms underlying the anti-cancer effects of KDR-siRNA are not clearly understood.

The loss of balance between the apoptosis and anti-apoptosis signal pathways plays a key role in the pathogenesis of a variety of cancers, including breast cancer. The process of apoptosis has distinct morphological characteristics and generally involves energy-dependent biochemical mechanisms that eliminate damaged, aged or otherwise abnormal cells [16]. The lack of response to apoptotic stimuli results in tumor growth and progression as well as resistance to most oncologic therapies [17]. It has been reported that the inhibition of apoptosis induces mitotic progression in cancer cells [18]. The suppression of apoptosis is considered one of the possible mechanisms of tumor progression, and many anti-cancer treatments act through their induction of apoptosis.

Therefore, it is of interest to understand whether the induction of cell apoptosis is involved in the anti-cancer effect of KDR-siRNA.

In this study, we observed that KDR-siRNA at concentrations of 100 nmol/l or higher significantly inhibited the cell proliferation of MCF-7 cells (by 36%) and that KDR-siRNA treatment significantly induced apoptosis in these cells. The apoptotic pathways are tightly controlled by a variety of regulators, including the Bcl-2 family proteins, caspases, and the inhibitor of apoptosis protein (IAP) family [19–21]. In this study, we investigated the molecular mechanism underlying the effect of KDR-siRNA on breast cancer cells. Our results demonstrated that knocking down KDR expression with siRNA reduced breast cancer cell survival, a process in which Bcl-2 family members might be involved. The Bcl-2 family is composed of both anti-apoptotic and pro-apoptotic members that elicit opposing effects on the mitochondria. Here, we showed that KDR-siRNA treatment reduced the Bcl-2 and Bcl-xl levels, but increased the Bax level in MCF-7 cells.

It is known that the ratio of anti-apoptotic protein level to pro-apoptotic protein level, such as the Bcl-2 to Bax ratio, is critical for the induction of apoptosis, and that the ratio of Bcl-2 to Bax in a cell determines its susceptibility to apoptosis [22]. The permeability of the mitochondrial membrane is precisely regulated by the Bcl-2 family proteins [23, 24]. Anti- and pro-apoptotic Bcl-2 family proteins reside either within the cytoplasm or on the outer membrane of the mitochondria. We found that the alteration in the Bcl-2 to Bax ratio caused an increase in mitochondrial membrane permeability and the release of cytochrome c from the mitochondria into the cytosol. Cytosolic cytochrome c can interact with Apaf-1, which leads to the activation of caspase-3 [25].

In this study, we found that KDR-siRNA treatment decreased the Bcl-2 to Bax ratio in MCF-7 cells, increased mitochondrial membrane permeability, induced the release of cytochrome c, increased the expression of Apaf-1, and increased the protein level and activity of cleaved caspase-3. Because caspase-3 is the ultimate executioner caspase essential for the nucleic changes associated with apoptosis [26], it is reasonable to infer that the elevated level of activated caspase-3 is responsible for the increased apoptosis in MCF-7 cells. The role of caspase activation in KDR-siRNA-induced apoptosis was further confirmed by the prevention of KDR-siRNA-induced apoptosis in MCF-7 cells through pre-treatment with the caspase inhibitor Z-VAD-FMK.

The results of this study indicate that KDR-siRNA-induced apoptosis in MCF-7 cells primarily involves the Bcl-2 family proteins, cytochrome c, Apaf-1, and caspase-3. However, the precise molecular mechanisms underlying the effects of KDR-siRNA treatment on apoptosis in MCF-7 cells need to be characterized in more detail.

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