GROβ and its downstream effector EGR1 regulate cisplatin-induced apoptosis in WHCO1 cells

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Abstract. Cisplatin is one of the most widely used chemotherapeutic agents employed for treatment of a wide variety of solid tumors, including human esophageal squamous cell carcinoma (ESCC). However, a major limitation of cisplatin-based chemotherapy of ESCC is the rather low-effective rate. Understanding the molecular events of limited efficacy of cisplatin-based chemotherapy of ESCC could lead to strategies resulting in improved therapeutic benefits. The CXC chemokine family has been reported to be related to inflammatory reaction, injure recovery, cell proliferation, apoptosis and even to be involved in the regulation of chemotherapeutic agent-induced apoptosis. CXCL2 chemokine, also known as GROβ (growth-related gene product β), belongs to the CXC chemokine group. The known functions of GROβ are related to attracting neutrophils to sites of inflammation, modulation of the neurotransmitter release, cell proliferation and apoptosis. However, little is known about the relationship between GROβ and chemotherapeutic agent-induced apoptosis. This study was designed to provide insights into the possible role of GROβ in the regulation of cisplatin-induced apoptosis in ESCCs. We report here that inhibition of expression of GROβ can decrease cisplatin-induced apoptosis in WHCO1 cells. EGR1 is a downstream factor regulated by GROβ. Silencing expression of EGR1 can also decrease cisplatin-induced apoptosis in WHCO1 cells. The activation of caspase 9 was delayed in cells in which GROβ and EGR1 were knocked down after cisplatin treatment. All these results indicate that GROβ and its downstream factor EGR1 are involved in regulating cisplatin-induced apoptosis in WHCO1 cells, and during this process the intrinsic apoptotic pathway is activated. It may be useful to examine the expression levels of GROβ and EGR1 in ESCC patients to select those likely to respond well to cisplatin.

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Introduction

Esophageal cancer (EC) is one of the most common cancers worldwide. In China, South Africa and some other developing countries, most of the EC patients were diagnosed as ESCC histologically (1,2). Surgery, radiotherapy or the combination of both are the most commonly used strategies to manage ESCC. At the same time, cisplatin-based chemotherapy is also a widely used regimen for patients conferred to preoperative chemotherapy or advanced patients of ESCC (3). But, the rather low effectiveness is the major problem in cisplatin-based chemotherapy of ESCC, only 35-55% of patients who receive cisplatin-based treatment had their tumor mass reduced by >50% (3). The development of chemoresistance is a major hurdle. A better understanding of the molecular events underlying the limited efficacy of cisplatin-based chemotherapy of ESCC may be helpful to identify therapeutic targets and improve the treatment efficiency to prolong the survive time of patients, thus needs further investigation (4).

CXC chemokines represent a superfamily of small cytokines with chemoattractant properties. They affect cells by activating surface receptors, which are seven-transmembrane-domain G-protein-coupled receptor (5). Evidence suggest that members of this group are correlated with inflammatory reaction (6), injure recovery (7), cell proliferation (8,9), apoptosis (10) and one even involved the regulation of chemotherapeutic agent-induced apoptosis (11,12). CXCL12 increases resistance of small-cell lung cancer cells to chemotherapy by activation its receptor CXCR4 (13). CXCL4, CXCL7 were able to protect hematopoietic cells from the toxicity of chemotherapeutic agents (14). Combination of CXCL9, CXCL10 plus cisplatin induced apoptosis significantly in colon carcinoma (CT26) and Lewis lung carcinoma (LL/2c) murine models (11,12). It seems that the members of this family play different roles in regulation chemotherapeutic agents-induced apoptosis.

CXCL2 chemokine, also known as GROβ (growth-related gene product β), belongs to the CXC chemokine group. Its receptor is the CXCR2 (15). The known functions of GROβ are related to fibroblast differentiation, angiogenesis, stem cell

mobilization, attracting neutrophils to sites of inflammation and modulation of the neurotransmitter release (16-20). Additional evidence suggests that GROβ is involved in cell proliferation and apoptosis by activation many signal pathways. GROβ could activate the sphingomyelin pathway or JNK1 and induced apoptosis in proliferating cells (21-22). GROβ also induce the activation of MEK/ERK signal pathway and promote cell proliferation (15,20,21,23). However, little is known about the relationship between GROβ and chemotherapeutic agent-induced apoptosis. This study was designed to provide insight into a possible role of GROβ in the regulation of cisplatin-induced apoptosis in ESCC. Its downstream factor EGR1 was also investigated.

Materials and methods

Chemicals. Dulbecco's modified Eagle's medium (DMEM) was purchased from Invitrogen (Carlsbad, CA, USA); fetal bovine serum (FBS) was obtained from HyClone (Logan, UT). Goat anti-GROβ primary antibody (sc-1375) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA); rabbit anti-EGR1 and anti-caspase 9 primary antibody were purchased from boster biological technology (Wuhan, China) and Cell Signaling Technology (Danvers, MA, USA), respectively. Mouse anti-actin antibody (A5316) and 4',6-diamidine-2'-phenylindole dihydrochloride (DAPI) were obtained from Sigma (St. Louis, MO, USA). Human GROβ ELISA development kit was purchased from peprotech (Rocky Hill, NJ, USA). Annexin V-FITC apoptosis detection kit was from Beijing Baosai Biotechnology (Beijing, China). Selective apoptotic DNA ladder detection kit was from Beijing Bioteke Corporation (Beijing, China)

Cell culture and drug treatment. The human ESCC cell WHCO1 and stable transfected cells: WHCO1-pcDNA3.1, WHCO1-pcDNA3.1-GROβi and WHCO1-pcDNA3.1-EGR1i were gifted by Dr Denver Hendricks (15). Cells were grown in DMEM medium supplemented with 10% fetal bovine serum (FBS), and maintained in humidified 5% CO_2 at 37˚C.

For treatment with cisplatin, cells were plated at $1.5x10^5$ per well in 2 ml DMEM in a six-well plate. Twenty-four hours later, the medium was changed and cisplatin was added into the medium to the final concentrations of 40 μM. Cells were incubated at 37˚C for certain time and then used for further experiment.

Western blot. Total cells were lysated with the buffer (1% SDS, 10 Mm Tris-Cl, pH 7.6, 20 g/ml aprotinin, 20 g/ml leupeptin and 1 mM AEBSF). The protein concentrations were determined using Bradford method. Protein (20 μg) was separated on 12% of SDS-PAGE gels and transferred to PVDF membranes. After blocked with 10% non-fat milk, the membranes were incubated with the first antibodies at 4˚C overnight. After washing three times, the membranes were incubated with horseradish peroxidase-conjugated second antibodies at room temperature for 1 h. The signals were developed with the ECL kit and using anti-actin antibody as an internal control.

Immunofluorescence and fluorescence microscope imaging. Cells were fixed with 4% paraformaldehyde, permeabilized with 0.06% of Triton X-100 and blocked in 2% BSA. After incubation with primary antibody at 4˚C overnight, cells were incubated with second antibody for 2 h at room temperature. Nuclei were stained with DAPI.

ELISA analysis for GROβ. For detection of GROβ in protein lysate of WHCO1-pcDNA3.1-GROβi cells and vector control cells, the human GROβ ELISA development kit was used according to the manufacturers' protocol. The amount of bound conjugate was determined by adding 100 μl of substrate solution to each well, then incubated at room temperature for color development. Color development was monitored with a Model 680 microplate reader (Bio-Rad Lab. Inc., Hercules, CA) at 405 nm with wavelength at 650 nm.

Assessment of chromatin condensation. The cells were plated at $1.5x10^5$ per well in 2 ml DMEM in a six-well plate. After 40 μM cisplatin treatment for 24 h, cells were collected and incubated with 2 μg/ml of DAPI (formaldehyde, 10% NP40, PBS) for 5 min. The apoptotic nuclei (intensely stained, fragmented nuclei and condensed chromatin) were observed by using a fluorescent microscope (Nikon E400).

Detection of apoptosis by flow cytometry (FCM). To evaluate the apoptotic rate of different cells, Annexin V-FITC apoptosis detection kit was used according to manufacturer's instruction. Briefly, cells were collected and resuspended in 200 μl binding buffer. Then 10 μl Annexin V-FITC, 5 μl PI were added to the suspension and reacted for 15 min at room temperature. After that, 300 μl of binding buffer was added and detected using a flow cytometer.

Apoptosis as detected by DNA ladder. Apoptotic DNA ladders were detected in WHCO1, WHCO1-pcDNA3.1, WHCO1-pcDNA3.1/GROβ RNAi and WHCO1-pcDNA3.1/EGR1 RNAi cells using selective apoptotic DNA ladder detection kit as per manufacturer's instruction.

Determination of caspase 9 activities. Cells were treated with 40 μM cisplatin for 0 and 12 h. After that, the cleaved fragments of caspase 9 were detected by Western blotting using specific antibodies that detect the intact band as well as the corresponding cleaved fragments. Anti-actin antibody was used as an internal control.

Statistical analysis. All statistical comparisons were performed using SPSS 16.0 software. Student t-test was used to compare differences in the frequency between the two groups or association among different variants. $P<0.05$ was considered statistically significant.

Results

RNA interference silences GROβ expression in WHCO1 cells. To examine possible role of GROβ in the regulation of cisplatin-induced apoptosis in WHCO1 cells, RNA interference method was used to decrease the expression of GROβ. Previous real-time RT-PCR analysis confirmed >95% ($P<0.05$) reduction in GROβ mRNA levels in the GROβ RNAi clone relative to the vector control (15). Here, the effect of RNA

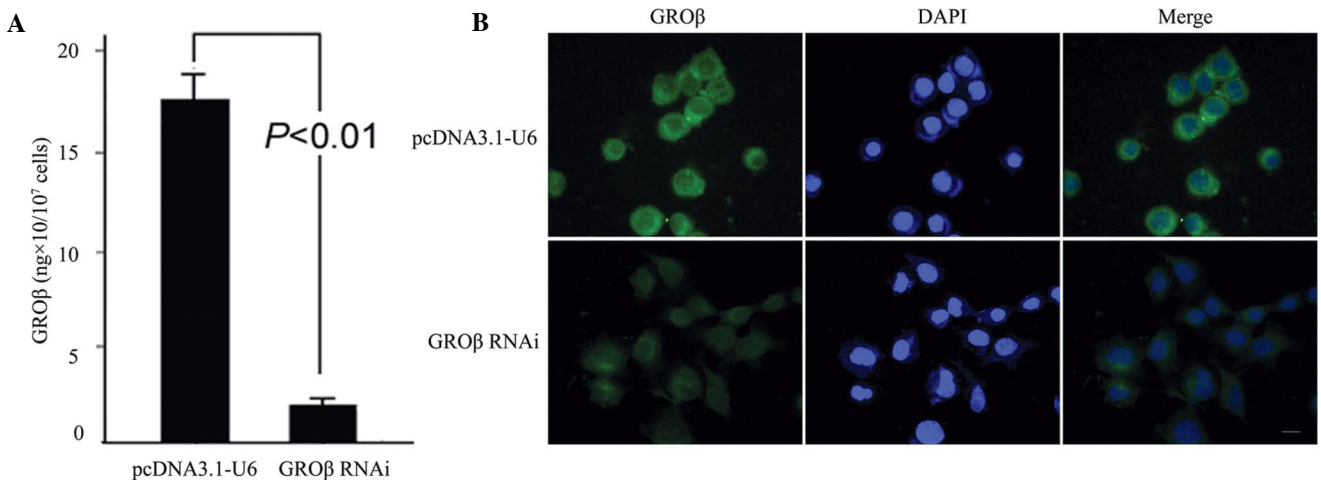

Figure 1. GROβ is down-regulated by RNA interference. GROβ RNAi clone was shown to display a reduction in GROβ protein levels relative to the vector control measured by ELISA method (A) and immunofluorescence method (B).

interference was detected by ELISA and immunofluorescence methods in GROβ protein lever. The result showed that the GROβ RNAi expressing clone displayed lower levels of cytoplasm GROβ than the cells transfected with its vector control, indicating that GROβ expression levels had been successfully suppressed in this clone (Fig. 1).

Inhibition of GROβ expression decreases cisplatin-induced apoptosis in WHCO1 cells. We compared the apoptotic morphology and apoptotic rate between GROβ RNAi clone and its vector control induced by cisplatin. DNA staining method was used to assess chromatin condensation in GROβ RNAi cells and vector control cells after treatment with 40 μM cisplatin for 24 h. The result showed that GROβ RNAi cells displayed less apoptotic nuclei (intensely stained, fragmented nuclei and condensed chromatin) than vector control cells (Fig. 2a, b). Then the apoptosis rate of GROβ RNAi cells and vector control cells induced by cisplatin was detected through flow cytometry method and Annexin V and PI immunofluorescence (Fig. 2A and B). The result indicated that the apoptosis rate of GROβ in RNAi cells (25.73±2.01%) was lower than the control (36.78±2.22%) (P<0.01, Fig. 2C) It seemed that inhibition of GROβ expression could decrease cisplatin-induced apoptosis in WHCO1 cells (the experiments were performed in triplicate).

EGR1 is a downstream factor regulated by GROβ. Previously, it was reported that using anti-GROβ antibody to decrease GROβ in the medium could reduce EGR1 (early growth regulator 1) mRNA levels in treated cells (15). EGR1 is a transcription factor regulating transcription of target genes and also correlates with drug-induced apoptosis in cancer cells. We detected the expression of EGR1 in GROβ RNAi cells and control cells by Western blot (Fig. 3). The result showed that EGR1 expression lever was reduced in GROβ RNAi cells. It seemed that EGR1 was a downstream factor regulated by GROβ.

Silencing expression of EGR1 also decreases cisplatin-induced apoptosis in WHCO1 cells. Then we reduced the expression of EGR1 by RNA interference (Fig. 3) and compared the apoptotic morphology and apoptotic rate induced by cisplatin between EGR RNAi cells and control cells. The representative results in Fig. 4a and b showed that EGR1 RNAi cells displayed less condensed, intensely stained and fragmented nuclei than vector control cells. Meanwhile, the apoptotic rate of EGR1 RNAi cells (18.65±2.01%) was lower than the control (36.78±2.22%) (P<0.01, Fig. 4a and b). Silencing expression of EGR1 also decreased cisplatin-induced apoptosis in WHCO1 cells (the experiments were performed in triplicate).

GROβ and EGR1 knock-down inhibits the DNA ladder formation, respectively. In the process of apoptosis, internucleosomal DNA could be fragmentized as the unit of nucleosome. So the detection of DNA fragmentation can be used as an important indicator of apoptosis. We dectected the apoptotic DNA ladders in WHCO1, WHCO1-pcDNA3.1, WHCO1-pcDNA3.1/GROβ RNAi and WHCO1-pcDNA3.1/EGR1 RNAi cells after treatment with 40 μM cisplatin for 24 h. As shown, laddered DNA was seen at the lines of wild-type and vector control but litter occurred in GROβ or EGR1 knock-down strains (Fig. 5).

Caspase 9 activation is delayed in GROβ and EGR1 knock-down cells after cisplatin treatment. Cisplatin induced intrinsic apoptotic pathway through which caspase 9 was activated. So we checked the activation of caspase 9 in GROβ RNAi, EGR1 RNAi and control cells after treatment with 40 μM cisplatin for 0, 12 h. No cleavages of caspase 9 were found in any type of cells at the very beginning of cisplatin treatment. After 12 h of culture, caspase 9 was found activated in control cells rather than in GROβ or EGR1 knocked down cells (Fig. 6). Caspase 9 activation was delayed in GROβ and EGR1 knock-down cells after cisplatin treatment.

Discussion

Cisplatin is one of the most widely used chemotherapeutic agents employed for treatment of a wide variety of solid

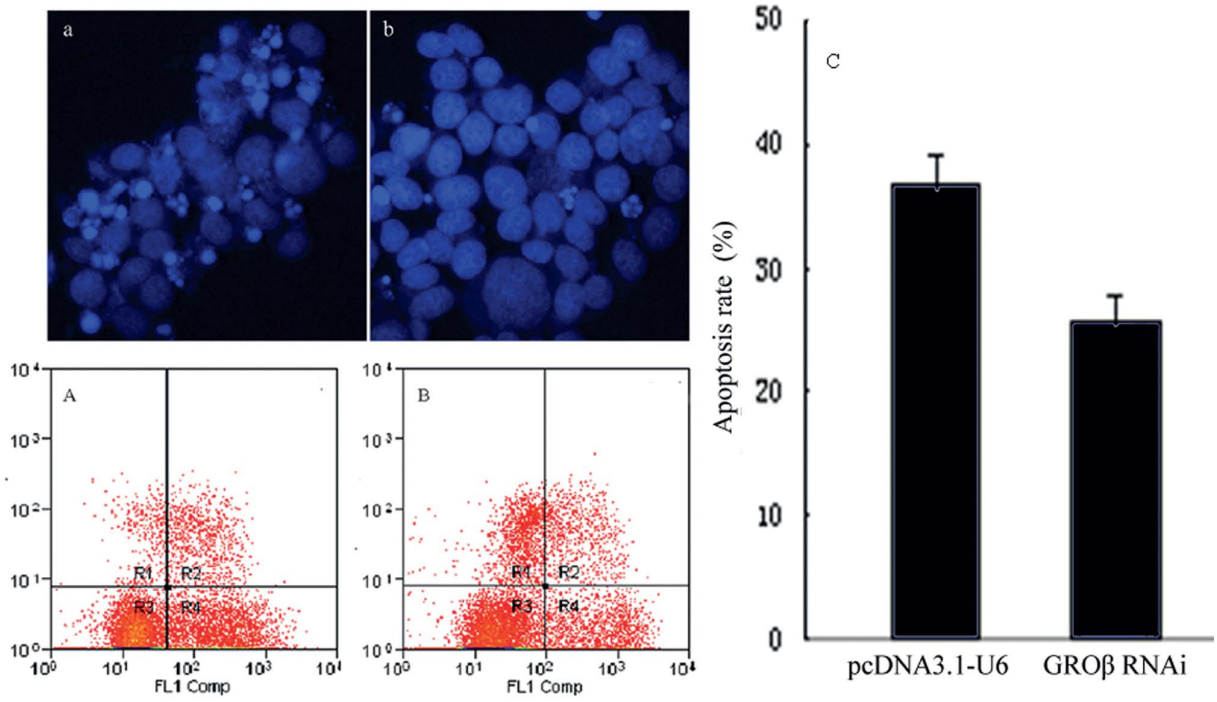

Figure 2. Reduced expression of GROβ decreases the apoptosis induced by cisplatin. (a, b: DAPI staining apoptosis nuclear of WHCO1-pcDNA3.1 and WHCO1-pcDNA3.1/GROβ RNAi cells. A, B: FCM measured the difference of apoptosis in WHCO1-pcDNA3.1 and WHCO1-pcDNA3.1/GROβ RNAi cells. C: the apoptosis rate detected by FCM.)

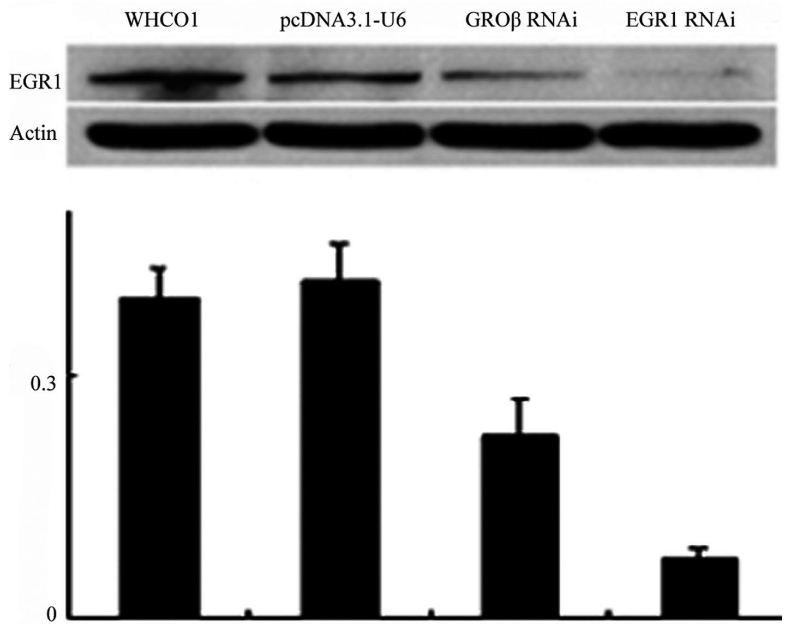

Figure 3. The expression of EGR1 was detected in WHCO1, WHCO1-pcDNA3.1, WHCO1-pcDNA3.1/GROβ RNAi and WHCO1-pcDNA3.1/EGR1 RNAi cells by Western blot. The upper panel showed the representative Western blot results. The lower bar graph is the result of densitometric analysis of three independent experiments, plotting the degree of EGR1 levels in these four types of cells.

tumors, including human ESCC. The main mechanism of action of cisplatin is mediated by its interaction with DNA to form intrastrand crosslink adducts, and then activates DNA damage-mediated cell apoptosis (24). However, a major limitation of cisplatin-based chemotherapy of ESCC is the rather low effectiveness. So understanding the molecular events of limited efficacy of cisplatin-based chemotherapy of ESCC could lead to strategies to improve therapeutic benefits. Previous results showed that members of CXC chemokine family could play different roles in regulation

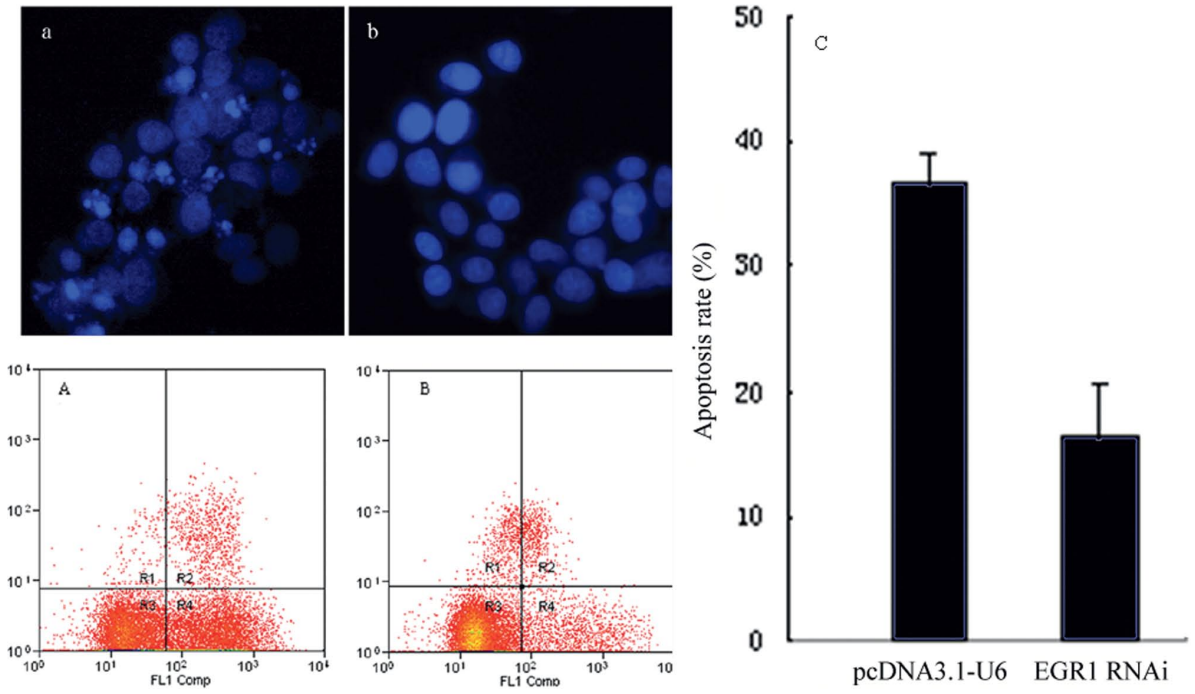

Figure 4. Silencing expression of EGR1 also decreases cisplatin-induced apoptosis in WHCO1 cells. (a, b: DAPI staining apoptosis nuclear of WHCO1-pcDNA3.1and WHCO1-pcDNA3.1/EGR1 RNAi cells. A, B: FCM measured the difference of apoptosis in WHCO1-pcDNA3.1 and WHCO1-pcDNA3.1/EGR1 RNAi cells. C: the apoptosis rate detected by FCM.)

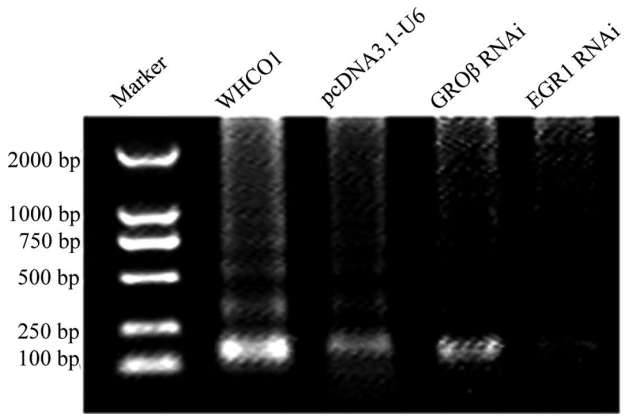

Figure 5. After treatment with 40 μM cisplatin for 24 h, apoptotic DNA was selectively extracted in WHCO1, WHCO1-pcDNA3.1, WHCO1-pcDNA3.1/GROβ RNAi and WHCO1-pcDNA3.1/EGR1 RNAi cells, respectively, then DNA was subjected to 0.8% agar for electrophoresis. As shown, laddered DNA was seen at the lines of wild-type and vector control, but not in GROβ or EGR1 knock-down strains.

cisplatin-induced apoptosis. Combination of CXCL9, CXCL10 plus cisplatin could induce apoptosis significantly in colon carcinoma (CT26) and Lewis lung carcinoma (LL/2c) murine models (25,26), while CXCL12 suppressed the rate of apoptosis induced by cisplatin in adenoid cystic carcinomas (ACC) cells (27). Other reports also showed that treatment of tumor cells with cisplatin could result in a substantial increase in the production of CXCL8, CXCL1 and CXCL 2 (28,29).

Here, we focused on the expression of CXCL2 (GROβ) in the regulating of cisplatin-induced apoptosis. RNA interference method was used to decrease the expression of GROβ and the apoptosis induced by cisplatin was compared between GROβ RNAi clone and the vector control. Our result showed that inhibition of expression of GROβ by RNA interference could decrease cisplatin-induced apoptosis in WHCO1 cells. GROβ was reported to activate many signal pathways, such as JNK (21,22) and MEK/ERK signal pathway (15,20,21,23). Many of these pathways were correlated to cisplatin-induced

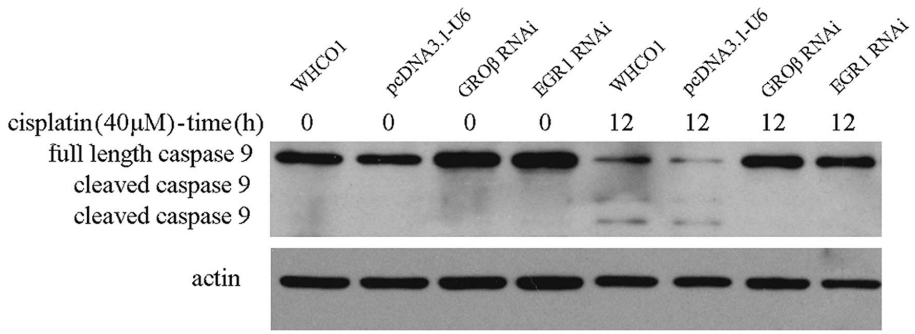

Figure 6. Cells were treated with 40 μM cisplatin for 0, 12 h, the activation of caspase 9 was detected by Western blot. After 12 h treatment with cisplatin, caspase 9 was found activated in control cells rather than in GROß or EGR1 knocked down cells.

apoptosis. JNK activation is involved in apoptotic cell death in sarcoma cell lines following stimulation with cisplatin (30). Inhibition of JNK activity delayed the onset of apoptosis induced by cisplatin (31). Acquisition of cisplatin resistance by OAW42-R ovarian carcinoma cells was associated with the loss of ERK activation in response to cisplatin. A new synthetic compound 2,3-DCPE, which was described to induce ERK activation, increased the cytotoxic effect of cisplatin in OAW42-R resistant cells (32). ERK activation plays an active role in mediating cisplatin-induced apoptosis of HeLa cells and lung A549 cells. Down-regulation of ERK led to an inhibition of cisplatin-induced apoptosis, whereas enhancement of ERK activity-facilitated cell death (33). The activation of these pathways by GROβ may be important for its way in regulating cisplatin-induced apoptosis. This needs further investigation.

Previously it has been demonstrated that using anti-GROβ antibody to decrease GROβ in the medium, EGR1 mRNA level was reduce in treated cells (15). Our result also showed that EGR1 protein expression lever was reduced in GROβ RNAi cells. EGR1 seemed to be a downstream factor regulated by GROβ. EGR1 is a zinc finger transcription factor. It is stimulated by many environmental signals and plays essential roles in cell proliferation and apoptosis (34). Evidence also suggested that EGR1 correlated with chemotherapy- and radiotherapy-induced apoptosis in cancer cells (35,36). EGR-1 knockdown in cultured multiple myeloma cells enhanced their resistance to bortezomib (37). Construction of pcDNA3.1-EGR1-TRAIL (pEGR1-TRAIL) recombinant plasmid and evaluation of its effect on human colon cancer cell line SW480 has been reported. pEGR1-TRAIL can sensitize SW480 cells to radiation, and the radiosensitization is related to cell cycle changes and apoptosis mediated by up-regulation of TRAIL expression (38). In our experiment, silencing expression of EGR1 reduced cisplatin-induced apoptosis in WHCO1 cells. GROβ and its down-stream factor EGR1 might play an important role in regulating cisplatin-induced apoptosis in WHCO1 cells.

Cisplatin induced the intrinsic apoptotic pathway during which caspase 9 was activated (39,40). Our result also showed caspase 9 was activated after 40 μM cisplatin treatment for 12 h in WHCO1 cells and empty vector-transfected WHCO1 cells. But no cleavage of caspase 9 was found in GROβ or EGR1 knocked down cells. Caspase 9 activation in GROβ and EGR1 knock-down cells was delayed after cisplatin treatment. This result further confirmed the role of GROβ and EGR1 in the regulation of cisplatin-induced apoptosis in WHCO1 cells.

In conclusion, we found that inhibition of expression of GROβ decreased cisplatin-induced apoptosis in WHCO1 cells. EGR1 was a downstream factor regulated by GROβ. Silencing expression of EGR1 could also decrease cisplatin-induced apoptosis in WHCO1 cells. Caspase 9 activation was delayed in GROβ and EGR1 knock-down cells after cisplatin treatment. All these results indicate GROβ and its downstream factor EGR1 could be involved in regulation cisplatin-induced apoptosis in WHCO1 cells, during which intrinsic apoptotic pathway was activated. It may be useful to examine the expression level of GROβ and EGR1 in ESCC patients to select those likely to respond well to cisplatin (41).

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