Anti-apoptotic effect of clusterin on cisplatin-induced cell death of retinoblastoma cells

HYUN BEOM SONG1,2, HYOUNG-OH JUN1, JIN HYOUNG KIM1, YOUNG SUK YU1,3, KYU-WON KIM4, BON HONG MIN5 and JEONG HUN KIM1,3

1Fight against Angiogenesis-Related Blindness Laboratory, Clinical Research Institute, Seoul National University Hospital; 2Department of Biomedical Sciences, Seoul National University Graduate School; 3Department of Ophthalmology, College of Medicine, Seoul National University; 4Department of Pharmacology, Seoul National University; 5Department of Pharmacology, College of Medicine, Korea University, Seoul, Republic of Korea

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Abstract. Clusterin is a cytoprotective chaperone protein that is known to protect various retinal cells. It was also reported to be overexpressed in several types of malignant tumors, whose chemoresistance correlates with the expression of clusterin. Herein, we investigated the effect of clusterin on cisplatin-induced cell death of retinoblastoma cells. Firstly, evaluation of clusterin expression demonstrated that it was highly expressed in human retinoblastoma tissues and cell lines (SNUOT-Rb1 and Y79) particularly in the area between viable cells around vessels and necrotic zones in the relatively avascular area in human retinoblastoma tissues. Furthermore, the effects of cisplatin on retinoblastoma cells were evaluated. Cisplatin (1 µg/ml) significantly affected cell viability of SNUOT-Rb1 cells by inducing caspase-3-dependent apoptosis. Notably, the cell death due to cisplatin was prevented by 5 µg/ml of clusterin administered 4 h prior to cisplatin treatment by inhibiting cisplatin-induced apoptosis. Furthermore, overexpression of clusterin exerted its anti-apoptotic effect on cisplatin-induced apoptosis, and effectively prevented cisplatin-induced cell death. These data suggest that clusterin, found to be overexpressed in human retinoblastoma, may exert anti-apoptotic effects on cisplatin-induced apoptosis and prevent cell death. Therefore, clusterin can contribute to cisplatin resistance of retinoblastoma.

Introduction

Retinoblastoma is the most common intraocular malignant solid tumor in childhood (1). Traditionally, external beam radiotherapy or enucleation was previously the mainstay of treatment, but emerging evidence of an increased risk for secondary cancers with external beam radiation has shifted our management strategies (2). Recently, systemic chemotherapy has become the primary approach to salvage eyes (3). Although chemotherapy regimens vary between institutions, cisplatin (cis-diammine dichloro platinum) is widely used for the treatment of retinoblastoma (4-6). It interacts with DNA to form DNA adducts leading to intranuclear or interstrand cross-links, which impair proper DNA replication and activate apoptotic pathways (7,8). Cisplatin treatment led to initial success in the treatment of solid neoplasms, but many patients exhibit intrinsic resistance (9). Moreover, a significant fraction of initially sensitive cancers eventually develop chemoresistance (10). Similarly, primary chemotherapy frequently fails to achieve success on eyes with advanced retinoblastoma, which finally results in enucleation (11). Thus, eyes with advanced stages are in need of more effective chemotherapy to reduce the tumor volume for local therapy, eventually saving the globes.

Clusterin is a sulfated glycoprotein of 75-80 kDa encoded by a single gene, which undergoes a maturation process finally resulting in the secreted heterodimeric form consisting of α- and β-chains (12). Clusterin has been viewed as a cytoprotective chaperone protein that is upregulated during many types of stress and exerts a putative role in the quality control of protein folding (13). Recently, we demonstrated that clusterin protects against blood-retinal barrier dysfunction in diabetic retinopathy, ischemia-induced cell death of human retinal endothelial cells (HRECs) and oxidative stress-induced apoptosis of retinal cells including retinal pigment epithelial cells, astrocytes and HRECs (14-17). Notably, these protective roles of clusterin were also proven in malignant neoplasms by demonstrating that its expression was correlated with chemoresistance and poor survival in ovarian and cervical cancer (18,19).

Based on our previous studies concerning its protective role in the retina and data regarding its association with chemoresistance, we investigated the role of clusterin in cisplatin-induced apoptosis of retinoblastoma cells. In the present

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study, overexpression of clusterin in human retinoblastoma tissues and cells was confirmed. We also demonstrated that exogenous supplement and overexpression of clusterin attenuated cisplatin-induced apoptosis of retinoblastoma cells.

Materials and methods

Human retinoblastoma tissues. Three patients with unilateral group E retinoblastoma, the most extensive stage according to the International Classification of Retinoblastoma (ICRB) (11), and one patient with bilateral retinoblastoma (group B and E by ICRB) were included in this study. The patients had no evidence of metastasis and underwent enucleation of eyes with group E retinoblastoma without prior systemic chemotherapy or local treatments at 8.3±3.3 months of age. All human retinoblastoma tissue samples were obtained with informed consent and approval by the Institutional Review Board for Clinical Research at the Seoul National University Hospital complying with the tenets of the Declaration of Helsinki.

Culture of human retinoblastoma cells. Human retinoblastoma cell line Y79 (American Type Culture Collection, Rockville, MD, USA) and SNUOT-Rb1, which was established by our group and is distinguished from Y79 by adherent growth and rapid proliferation (16), were incubated in RPMI-1640 medium (WelGENE Inc., Daegu, Korea), supplemented with 10% fetal bovine serum (Gibco-BRL, Rockville, MD, USA) and 100 µg/ml streptomycin (Invitrogen Life Technologies, Carlsbad, CA, USA) at 37°C in a moist atmosphere of 95% air and 5% CO₂. We replaced the medium every third day and checked the cultured tumor cells daily under a phase-contrast microscope (Carl Zeiss, Chester, VA, USA). If needed, 5 µg/ml of clusterin (provided by B.H. Min, Korea University, Seoul, Korea) and/or 1 µg/ml of cisplatin (Sigma-Aldrich, St. Louis, MO, USA) were administered.

Immunohistochemistry. The enucleated eyes were fixed in formalin, embedded in paraffin, and then sectioned (4 µm). The slides were de-paraffinized and incubated with proteinase K at 37°C. After blocking endogenous peroxidase activity with hydrogen peroxide and blocking non-specific binding with a blocking kit (Zymed Laboratories Inc., South San Francisco, CA, USA), slides were incubated overnight with rabbit anti-clusterin antibody (1:100; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) at 4°C, followed by a biotinylated goat anti-rabbit antibody (Dako, Glostrup, Denmark), revealed by the avidin-biotin complex (Vectastain kit; Vector Laboratories, Burlingame, CA, USA) at 37°C, and incubated on ice for 1 h and centrifuged at 20,000 x g for 30 min. The precipitate was subjected to heparin-Sepharose column chromatography (GE Healthcare Life Sciences). Fractions containing clusterin were subjected to heparin-Sepharose column chromatography (GE Healthcare Life Sciences). The obtained clusterin was finally purified by a clusterin monoclonal antibody affinity chromatography (cytogen bromide-activated Sepharose 4B; Sigma-Aldrich). The eluted protein was dialyzed and lyophilized before being stored at -80°C.

Transfection of pcDNA expressing clusterin. For transfection, the Lipofectamine Plus™ reagent (Invitrogen Life Technologies) was used following the manufacturer's instructions. Briefly, 8 µl of Plus™ reagent and 5 µg of plasmid DNA (pcDNA-CLU; provided by B.H. Min) were suspended with 487 µl of RPMI-1640 medium without serum and antibiotics [RPMI(-)], and incubated for 15 min at room temperature. Lipofectamine (12 µl) was mixed with 488 µl of RPMI(-). The Lipofectamine suspension was then mixed with the Plus-pcDNA mixture, and incubated for 15 min at room temperature. The mixtures of 1 ml RPMI(-) containing 5 µg of pcDNA were added to 2.5x10⁴ cells and incubated for 3 h at 37°C. Then, the medium was replaced with RPMI-1640 medium. The time when pcDNA was added to cells was defined as time 0. After 24 h of induction, cisplatin treatment was carried out.

Cell viability assay. Cell viability was evaluated by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. SNUOT-Rb1 cells were seeded into each well of 48-well plates at a concentration of 2x10⁴ cells/well. After incubation for 24 h, cells were treated with various concentrations of cisplatin. After incubation for the following 24 h, MTT solution was added to each well at a final concentration of 0.2 mg/ml. After incubation at 37°C for 2 h, the medium was carefully removed, and DMSO was added to solubilize the formazan produced by the viable cells. Optical density values at 540 nm were measured by a microplate spectrophotometer (Molecular Devices, Sunnyvale, CA, USA). Three independent experiments were performed for each experimental condition.

Trypan blue dye exclusion was also used to assess the viability of cells following clusterin treatment. Viable cells were counted on a Luna™ automated cell counter (Logos Biosystems, Gyunggi-Do, Korea). Three independent experiments were performed for each experimental condition.

Western blot analysis. Cell lysates were obtained by resuspending cells in radioimmunoprecipitation assay (RIPA) buffer (Tris 50 mM pH 7.4; NaCl 150 mM; SDS 0.1%; Na deoxycholate 0.5%; Triton X-100 1%; Cell Signaling Technology, Inc., Danvers, MA, USA) with a complete protease inhibitor cocktail (Roche Molecular Biochemicals, Indianapolis, IN, USA), incubated on ice for 1 h and centrifuged at 20,000 x g for 30 min at 4°C. After measurement of the protein concentration using a BCA protein assay kit (Pierce Biotechnology Inc., Rockford, IL, USA), equal amounts of protein from the supernatant were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 7% Tris-Tricine gel (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and transferred to nitrocellulose membranes (Amersham Hybond ECL; GE Healthcare Life Sciences, Buckinghamshire, UK). Fractions containing clusterin were subjected to heparin-Sepharose column chromatography (GE Healthcare Life Sciences). The obtained clusterin was finally purified by a clusterin monoclonal antibody affinity chromatography (cytogen bromide-activated Sepharose 4B; Sigma-Aldrich). The eluted protein was dialyzed and lyophilized before being stored at -80°C.

Purification of clusterin from human serum. Clusterin was purified from fresh normal human plasma as described in our previous studies, in compliance with the Declaration of Helsinki (14,17,20). Human plasma supplemented with 0.5 mM phenylmethylsulfonyl fluoride (PMSF) was precipitated by adding 12-23% polyethylene glycol (PEG, MW 3350; Sigma-Aldrich) overnight at 4°C. The precipitate was dissolved and subjected to dimethylamino ethanol-Sepharose column chromatography (GE Healthcare Life Sciences, Buckinghamshire, UK). Fractions containing clusterin were subjected to heparin-Sepharose column chromatography (GE Healthcare Life Sciences). The obtained clusterin was finally purified by a clusterin monoclonal antibody affinity chromatography (cytogen bromide-activated Sepharose 4B; Sigma-Aldrich). The eluted protein was dialyzed and lyophilized before being stored at -80°C.
After being blocked with diluted 5% dry skim milk for 1 h, the membranes were rinsed and incubated with specific antibodies against clusterin and β-actin in PBS-T (PBS containing 0.1% Tween-20; Bio-Rad Laboratories, Inc.) overnight at 4˚C. The primary antibody was removed by washing the membranes with PBS-T and incubation for 1 h with horseradish peroxidase-conjugated secondary antibodies.

4,6-Diamidino-2-phenolindole staining. We performed 4,6-diamidino-2-phenolindole (DAPI; Sigma-Aldrich) staining for cell viability analysis. SNUOT-Rb1 cells (1x10⁶ cells) were plated in 6-well plates and cultured for 24 h. The cells were treated with cisplatin (1 µg/ml) and/or clusterin (5 µg/ml). After 24 h of incubation, cells were fixed and stained with 10 µg/ml of DAPI (Sigma-Aldrich). After incubation for 5 min in the dark, the cells were washed and observed under a fluorescence microscope (Leica, Wetzlar, Germany).

Statistical analysis. Statistical analyses were performed using SPSS software version 18.0 (SPSS Inc., Chicago, IL, USA). A P-value <0.05 was considered to indicate a statistically significant result. Values are expressed as means ± SD.

Results

Expression of clusterin in human retinoblastoma. To investigate the expression of clusterin in clinical samples, we performed immunohistochemcal staining with an anti-clusterin antibody on tissues from enucleated human eyeballs with retinoblastoma. Microscopic examination showed that all the retinoblastoma tissues consisted of viable cells around blood vessels and zones of necrosis relatively far from the vessels. Clusterin was highly expressed in the retinoblastoma particularly at the peripheral areas of viable cells, adjacent to the necrotic zones, while barely detectable in the area with numerous Flexner-Wintersteiner rosettes marked by central lumen with surrounding cells showing cytoplasmic extensions (Fig. 1A). Counterstaining with methyl green revealed that clusterin was mainly stained in the cytoplasm.

Furthermore, we assessed the expression of clusterin in human retinoblastoma cell lines. As shown in Fig. 1B, clusterin was detected in SNUOT-Rb1 and Y79 cells by western blot analysis. Following evidence of clusterin expression in human retinoblastoma tissues and SNUOT-Rb1 cells, we further evaluated the effect of clusterin on cisplatin-induced cell death in SNUOT-Rb1 cells.

Induction of apoptotic cell death by cisplatin treatment on retinoblastoma cells. To investigate the effect of cisplatin on retinoblastoma cell death, we performed a cell viability assay under a condition of gradually increasing concentrations of cisplatin for 24 h. As shown in Fig. 2A, at least 1 µg/ml of cisplatin was required to significantly affect the cell viability of SNUOT-Rb1 cells (P<0.05). We determined the concentration with enough chemotherapeutic activity as 1 µg/ml for further experiments.

As cisplatin is known to impair proper DNA replication and activate apoptotic pathways (7,8), apoptosis was evaluated in SNOT-Rb1 cells. After 24 h of cisplatin treatment, cleaved caspase-3 was increased (Fig. 2B). Thus, cisplatin induced the apoptotic death of retinoblastoma cells.

Effect of exogenous clusterin on cisplatin-induced apoptotic cell death. To determine the effect of exogenous clusterin on cisplatin-induced apoptotic cell death, trypan blue dye exclusion was carried out following treatment with cisplatin and/or exogenous clusterin. Clusterin was administered 4 h prior to the cisplatin treatment. The viability of SNUOT-Rb1 cells...
SONG et al.: EFFECT OF CLUSTERIN ON CISPLATIN-INDUCED APOPTOSIS OF RBL CELLS

was not affected by 5 µg/ml of exogenous clusterin (Fig. 3A). Cisplatin (1 µg/ml) significantly decreased the viability, but the exogenous supplement of clusterin effectively prevented cisplatin-induced cell death (Fig. 3A). The results were confirmed by DAPI staining of the culture plates. Condensed and fragmented nuclei (arrow) with decreased cell numbers in the cisplatin-treated group reflected cell death by cisplatin, which was completely abrogated by the supplement of exogenous clusterin (Fig. 3B).

To evaluate the mechanism by which clusterin protects retinoblastoma cells from cisplatin-induced cell death, the inhibitory effect of clusterin on caspase-3 activity was assessed by western blot analysis. As demonstrated in Fig. 3C, increased activity of caspase-3 by cisplatin treatment was dramatically attenuated by cotreatment with 5 µg/ml of clusterin. These data suggest that exogenous clusterin inhibits apoptosis induced by cisplatin in retinoblastoma cells.

Effect of clusterin overexpression on cisplatin-induced apoptotic cell death. Given that clusterin is overexpressed in human retinoblastoma tissues and cells as determined in the present study, we evaluated the effect of clusterin overexpression on cisplatin-induced apoptotic cell death. (A) SNUOT-Rb1 cells transfected with pcDNA-CLU were incubated for 24 h and analyzed for the expression of clusterin. β-actin served as the loading control. Figures were selected as representative data from 3 independent experiments. (B) After 24 h of incubation following transfection, SNUOT-Rb1 cells were treated with cisplatin (1 µg/ml). Cell viability was measured 24 h after the treatment by an MTT assay. The data represent the means ± SD of the results from 3 independent experiments (*P<0.05). (C) Transfected cells treated with cisplatin (1 µg/ml) for 24 h were harvested and analyzed by western blot analysis using antibodies against clusterin and cleaved caspase-3. β-actin served as the loading control. Figures were selected as representative data from 3 independent experiments.
overexpression on cisplatin-induced cell death. SNUOT-RbI cells were transfected with pcDNA-CLU using empty vectors as a control, and underwent a cell viability assay. Cells transfected with pcDNA-CLU exhibited increased expression of clusterin (Fig. 4A). The cell viability assay demonstrated that transfected cells exhibited significantly less cell death following cisplatin treatment (Fig. 4B). The effect of clusterin overexpression on apoptosis was also evaluated by western blot analysis of cleaved caspase-3. As shown in Fig. 4C, increased activity of cleaved caspase-3 following cisplatin treatment was attenuated in cells transfected with pcDNA-CLU. Thus, clusterin overexpression inhibits cisplatin-induced apoptotic cell death similar to that of exogenous clusterin.

Discussion

In the present study, we demonstrated the overexpression of clusterin in human retinoblastoma tissues and cells. Cisplatin treatment induced retinoblastoma cell death, which was prevented with the supplement or overexpression of clusterin by inhibiting cisplatin-induced apoptosis. Therefore, expression of clusterin in retinoblastoma exerts an anti-apoptotic effect against cisplatin-induced apoptotic cell death.

Retinoblastoma usually grows so rapidly that oxygen demands often exceed its blood supplies, which results in extensive necrosis in relatively avascular areas (21). Notably, clusterin was highly expressed in the area between viable cells around vessels and necrotic zones. We previously demonstrated the upregulation of clusterin in hypoxic condition in HRECs and astrocytes (17) and the protective role of clusterin against ischemia-induced cell death of HRECs (20). Taken together, clusterin is probably upregulated to protect retinoblastoma cells from ischemia-induced cell death.

Clusterin has been reported to be overexpressed in several types of malignant tumors, whose chemoresistance is related with clusterin expression (18,19,22). The mechanism of the enhancement of chemoresistance by clusterin is known to be associated with its inhibitory effect on apoptosis, which is consistent with our data indicating changes in cleaved caspase-3 expression induced by cisplatin treatment. Clusterin depletion by small interfering RNA was found to result in disruption of the Ku70-Bax complex, activation of Bax and translocation of Bax into mitochondria to induce cytochrome c release and apoptosis (23,24). Moreover, overexpression of clusterin increased phosphorylation of Akt and its target protein Bad, and decreased cytochrome c release and apoptosis (12). On the other hand, downregulation of Bax and enhanced activity of Akt are known mechanisms involved in the inhibition of apoptotic signals in cisplatin-resistant tumor cells (7). Taken together, clusterin overexpression may exert anti-apoptotic effects resulting in the change of cisplatin-sensitive tumor cells into cisplatin-resistant cells.

The association of clusterin and cisplatin resistance was revealed in previous studies. Transfection of the clusterin gene into human renal cell carcinoma cells enhanced their resistance to cisplatin in vitro and in vivo (25), which is consistent with our data on the effect of clusterin overexpression. The administration of clusterin-specific antisense oligonucleotides enhanced cisplatin sensitivity of KoTCC-1 human bladder tumors in vitro and in vivo (26). Based on these preclinical data, clinical studies are underway. Phase II trials of cuxirsine (OGX-011), a second generation antisense oligonucleotide targeting clusterin, in combination with conventional chemotherapy have been carried out in patients with chemotherapy-naive advanced non-small cell lung cancer and metastatic castration-resistant prostate cancer progressing after initial docetaxel therapy (27,28). This type of approach can be applied to retinoblastoma. Although the toxicity of the combination did not differ from what is reported for conventional chemotherapy in the study mentioned above (27), there are safety issues to consider before applying the treatment to retinoblastoma, as clusterin is reported to protect against ischemia-induced cell death of HRECs, oxidative stress-induced apoptosis of retinal pigment epithelial cells and blood-retinal barrier dysfunction in diabetic retinopathy (14-16).

In the present study, following evidence of the expression of clusterin in human retinoblastoma, we demonstrated that exogenously administered or overexpressed clusterin inhibited cisplatin-induced apoptosis in human retinoblastoma cells. Clusterin could play a role in the chemoresistance of retinoblastoma to cisplatin and could be applied as an adjuvant treatment modality to conventional chemotherapy for retinoblastoma.

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