GABA\(_\text{A}\) receptor-binding protein promotes sensitivity to apoptosis induced by chemotherapeutic agents

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Abstract. In the present study, the expression of human \(\gamma\)-aminobutyrate type A (GABA\(_\text{A}\)) receptor-binding protein (GABARBP) is downregulated in ovarian cancer cell lines and tissues. We also found that the specific function of GABAPBP was that of a novel pro-apoptotic protein. Both GABARBP and cisplatin suppressed cancer cell proliferation in a concentration-dependent manner. The combined treatment of GABARBP and cisplatin was more effective in inhibiting cell growth, as well as cell migration, than with either drug treatment alone. At the same time, the treatment combination was correlated with the downregulation of cyclin D1 and CDK4, arrested cell cycle progression in the G\(_0\)-G\(_1\) phase and enhancing p53 expression, while also reducing Bcl-2 and Bcl-xL expression. The p53 and p21 promoter luciferase activities were induced by GABARBP, whereas there was no effect on the p53\(^{\text{+/−}}\) and p21\(^{\text{+/−}}\) system. In addition, p53 activity was validated with UV irradiation and siGABARBP. Taken together, our results indicate that GABARBP can regulate the pro-apoptotic activity of cisplatin via the upregulation of p53 expression.

Introduction

Anticancer drugs are mainly used to treat malignancies, such as cancer cell growths. In most cases, chemotherapy generally can be used in addition to other cancer treatments, such as surgery and radiation therapy. Cancer occurs when a damaged cell produces a mutant of the oncogenes and when tumor suppressor genes and the mutant produce more mutants, which is then commonly defined as the uncontrolled growth of cells.

Cisplatin (cis-diaminedichloro platinum) is a well-known platinum-based chemotherapeutic agent, which acts as an activator of apoptosis and an inducer of DNA strand breaking through damage (1,2). It is extensively used to treat various types of cancers, including metastatic ovarian, cervical, sarcoma, head and neck, advanced bladder, thyroid, lymph nodes, skin, colorectal, gastric, pancreatic, metastatic testicular and small-cell lung tumors, either alone or in combination with other anticancer drugs (3-9). Unfortunately, the use of this compound is generally limited by its side effects. Therefore, the goal of this therapy is to improve the quality of life and possibly, to extend the quantity of life while minimizing the side effects (10). On the other hand, multidrug resistance (MDR) is one of the major obstacles responsible for the failure in the treatments using chemotherapy for cancer (11-13). Therefore, treatment strategies to overcome chemoresistance are needed in the treatment of ovarian cancers.

Human \(\gamma\)-aminobutyrate type A (GABA\(_\text{A}\)) receptor-binding protein (GABARBP) plays an important role in the regulation of GABA\(_\text{A}\)-receptor activity. It is a key player in the intracellular trafficking of these receptors (14-16). GABARBP binds to various intracellular proteins, which are all associated with vesicle transport processes, autophagy and apoptosis, which include the cytoskeleton, tubulin (17,18), gephyrin (19,20), the clathrin heavy chain (21), p130/phospholipase C-related inactive protein (PRIP) (22), transferrin receptor (23), Unc-51-like kinase (24), Ras-related protein 24 (25), DEAD (Asp-Glu-Ala-Asp/His) box polypeptide 47 (DDX47) (26), glutamate receptor-interacting protein 1 (GRIP1) (27), calre...
ticulin (28), angiotensin II type 1 (AT1) receptor (29) and transient receptor potential vanilloid (TRPV1) (30). Recently, Schwarten et al (31) identified the proapoptotic protein Nix/Bnip3 to be a potential GABARBP ligand. Also, Alam et al (32) reported that C-terminal processing of GABARBP is not required for the trafficking of the angiotensin II type 1A receptor. GABARBP is downregulated in different breast cancer cell lines, including tumor tissues and is suggested as playing a potential role in GABARBP, acting through a vesicle transport mechanism as a tumor suppressor in breast tumors (33). In contrast, GABARPs are enhanced in adenomas and thyroid tumors and are also frequently expressed in colorectal cancer (34,35). However, important roles of the GABARBP physiological functions and regulation remain unclear.

In the present study, we examined whether GABARBP is generally upregulated or downregulated in ovarian cancer cells and patient tissues. Also, this study was designed to assess the role in regards to the inhibition of the PI3k/Akt signaling regulators, as well as the susceptibility to the effects of GABARBP alone or combined with cisplatin, in ovarian cancer cells. These similar apoptotic signaling pathways for cisplatin could also contribute to a higher effectiveness in chemotherapy when these drugs are used in combination rather than alone. Our major focus was the functional mechanisms of GABARBP on the different agent-mediated cell deaths in human ovarian cancer cells. Furthermore, we show that the PI3k/mTOR pathways are affected by GABARBP and that the expression levels of the Bcl-2 family proteins are regulated by GABARBP in ovarian OVCAR-3 cancer cells. In these observations, we elucidated that the possible intracellular mechanisms of GABARBP have an additive effect on the roles of anticancer drug-induced apoptosis.

Materials and methods

Cell line, culture, reagents and antibodies. Human normal and carcinoma cell lines (HOSE-E6E7, MCF10A, BEAS-2B, SKOV-3, OV-CAR-3 and MCF-7) were purchased from the American Type Culture Collection (ATCC, Manassas, VA) and were grown in M199/MCDB-105 (HOSE-E6E7), DMEM/F12 (MCF10A and BEAS-2B) or DMEM (SKOV-3, OV-CAR-3 and MCF-7) (Life Technologies, Gaithersburg, MD) supplemented with 10% heat-inactivated FBS and penicillin/streptomycin (100 U/ml) in a humidified incubator containing 5% CO₂ at 37°C.

Anticancer drugs and chemicals were obtained from Sigma (St. Louis, MO). The primary antibodies used in this study were anti-GABARBP (Santa Cruz Biotechnology, Santa Cruz, CA), anti-Bax, anti-p53, anti-Bcl-2 (Cell Signaling, Beverly, MA), anti-cyclin D1, anti-p16, anti-p27, anti-p21, anti-CDK1, anti-PDK1, anti-phospho-specific PDK1, anti-Akt, anti-phospho-specific Akt, anti-mTOR, anti-phospho-specific mTOR, anti-p70S6K, anti-phospho-p70S6K, anti-GSK-3β, anti-phospho-specific GSK-3β (Santa Cruz), anti-β-actin (Sigma) and anti-α-tubulin (Santa Cruz). Cell viability was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay system. The MTT assay suggests the fastest and most precise analysis results for cell growth. In brief, cells were seeded at a density of 3.5x10³ cells/well, in 96-well plates. After 24 h, fresh cell culture medium containing 10% FBS, were added and incubated with 20 µl of MTT solution (Sigma, 5 mg/ml) for an additional 4 h at 37°C. After centrifugation, culture medium was removed and dimethyl sulfoxide (DMSO) was added to each well. The amounts of MTT-formazan generated were determined as the absorbance using a microplate reader at 540-570 nm.

Flow cytometry analysis. Cells were detached using trypsin and rinsed twice with PBS. The pellets were re-suspended with ice-cold PBS and fixed by adding ice-cold 70% ethanol. Fixed cells were stored for 1 h at 4°C and then rinsed once with ice-cold PBS. Cells were treated with fluorescein isothiocyanate (FITC)-labeled Annexin V, propidium iodide (PI) (Boehringer-Mannheim, Mannheim, Germany) and RNase A (1 mg/ml) in PBS and incubated for 1 h at 37°C in the dark. All cytometry data were analyzed in the FACSCalibur flow cytometer (Becton-Dickinson, Franklin Lakes, NJ).

Western blot analysis. Cells were harvested, rinsed in PBS, centrifuged and then lysed in a buffer containing protease inhibitor (50 mM Tris, pH 7.2, 150 mM NaCl, 1% Triton X-100, 1 µg/ml leupeptin, 1 µg/ml pepstatin, 2 µg/ml aprotonin, 200 µg/ml phenylmethylsulfonyl fluoride). The supernatant protein concentration was calculated using the Bradford method. Cell lysates were then collected and subsequently resolved with SDS-PAGE gel, then transferred to Immobilon P membranes (Millipore Corp., Billerica, MA) by electroblotting. After blocking, the membranes were probed with primary antibodies. The membranes were then washed thrice in a wash buffer and incubated with horse-radish peroxidase-conjugated secondary antibodies. Blots were visualized using an ECL detection kit (Amersham, Arlington Heights, IL).

Luciferase-reporter gene assay. The p53- and p21-responsive reporter analysis was carried out using the Dual-luciferase assay kit (Promega, Medison, WI) according to the manufacturer's protocol. Briefly, OVCAR-3 cells were transfected with the vector DNA containing p53- and p21-luciferase, in which the luciferase was expressed under each promoter control. The reporter plasmid, p53-Luc, was a kind gift of Dr K. Park (Samsung Medical Center, Korea) and the p21 promoter reporter construct of Dr J. Park (Yonsei University, Korea). OVCAR-3 cells at 80-85% confluence were transiently transfected with each indicated reporter construct. As an internal control to correct for variations in transfection efficiency, 20 ng of pRL-TK (Promega), was co-transfected. After lysis with Reporter lysis buffer (Promega), the cell extracts were assayed for luciferase activity. The luciferase activity was expressed as the ratio of p53-Luc to pRL-TK activity. The luciferase assay was performed in triplicate with three independent experiments.

Reverse transcription-polymerase chain reaction (RT-PCR) for cDNA encoding full-length of human GABARBP. The total RNA from various cancer cell lines and tissues were obtained after total RNA isolation using TRIzol (Invitrogen) and treated with RNase-free DNase (Invitrogen). After purification, the cDNA was synthesized using the M-MLV reverse transcriptase (Invitrogen) and random hexamers (Invitrogen) as primers. The cDNA encoding full-length of human GABARBP was amplified using the following primers: 5'-ATG GCT CTT CCA AGC ATG -3' (forward) and 5'-TGG TTT TCA AGG CGT TTA CG -3' (reverse). The PCR products were separated on a 1.5% agarose gel and visualized under UV light.
using the TRIzol (Invitrogen, Carlsbad, CA) extraction method and full length cDNA, encoding human GABARBP, was synthesized from 1 µg of the total RNA using M-MLV reverse transcriptase (Promega) with the use of random hexamers (Invitrogen).

PCR reactions were carried out using a MyCycler™ Thermal Cycler (Bio-Rad Laboratories Inc., Hercules, CA) as follows: 25 cycles for 1 min at 94°C, 1 min at 56°C and 1 min at 72°C, followed by a final extension step of 10 min at 72°C. The PCR products were sequenced by the dideoxy-mediated chain termination method using a 310-automatic sequencer (ABI 373; Perkin-Elmer, Wellesley, MA).

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control.

Statistical analysis. All data values are presented as the means ± SD or means ± SEM for at least three replicates for each group. Statistical comparisons were carried out using Student’s t-test. The statistical significance was defined as P<0.05 and depicted with an asterisk (*) on each graph.

Results

GABARBP inhibits cell growth in a dose-dependent manner and additively promotes cell death by combination with cisplatin. To explore the biological function of GABARBP during cisplatin-induced cell death, we first checked by using a treatment of cisplatin and the ectopic expression of GABABP in ovarian cancer cells. As shown in Fig. 1A, the ectopic expression with GABARBP significantly reduced OVCAR-3 cell viability in a concentration-dependent manner. Overexpression with 1 µg GABARBP decreased the cell viability by 38%. Similar results were obtained when MCF-7 breast cancer cells were transfected with 1 µg GABARBP (data not shown). The cell viability of OVCAR-3 cells treated with various concentrations (0, 5, 10, 15, 20, 25 and 50 µM) of cisplatin was suppressed in a dose-dependent manner, and to inhibit the cells by 50% more than 20 µM was required. We also investigated the effect of GABARBP on FACS analysis, under the effects of cisplatin. As shown in Fig. 1B, the transfection of GABARBP into
cisplatin-induced cells, GABARBP-transfected cells had an additive effect when compared with the control, GABARAP, cisplatin or siGABARBP alone. All of these results indicate that GABARBP can additively enhance apoptotic sensitivity to the cisplatin-induced signal.

Movement of apoptosis-related machinery and cell cycle distribution. To further clarify the biological mechanism of GABARBP or cisplatin-stimulated cell cycle arrest, the protein levels of the cell death distribution were assessed in OVCAR-3 cells and then validated via cell migration and FACS analysis. The migration of carcinoma cells is a critical process in tumorigenesis. In order to measure the effects of GABARBP, cells were transfected/treated with control (vector only), GABARBP, cisplatin or cisplatin plus GABARBP, respectively. As shown in Fig. 2A, transfection/treatment with GABARBP or cisplatin, compared with the control, had suppressive effects, indicating that GABARBP plus cisplatin additively inhibits cell migration. Consistently, the cell cycle progression was examined using flow cytometry. The etopic expression of GABARBP was disrupted at a faster cell cycle when treated with cisplatin, under the IC_{50} value cells were greatly increased in the G_{0}-G_{1} phase. Co-treatment with cisplatin and GABARBP dramatically increased the G_{0}-G_{1} phase in OVCAR-3 cells, indicating that cell cycle arrest in the G_{0}-G_{1} phase contributes to the sensitization of GABARBP in cisplatin-induced cells (Fig. 2B).

The expression levels of CDK, cyclin D1, p16 and p27 proteins are major factors in cell cycle progression. This is the case, since cell proliferation is directly related to cell cycle progression. Therefore, we next examined the expression of the cell cycle-regulatory member. After treatment with cisplatin plus GABARBP, the expression levels of cyclin D1 and CDK4 were additively reduced when compared with the levels of each, the GABARBP-transfected or cisplatin-induced cells, whereas the CDK inhibitors, p16 and p27, were enhanced (Fig. 2C). Our findings suggest that GABARBP and cisplatin suppresses cell growth through the activation of the G_{1} phase arrest in cancer cells.

GABARBP controls the expression levels of apoptosis-related regulatory proteins and activation of p53 by UV-irradiation. Since p53-associated signaling pathways are well-known as critical modulators of cell death and survival (36-38), they are also known as major regulators of the Bcl-2 family genes (39-44). We investigated as to whether GABARBP altered the expression of the apoptosis-associated proteins. GABARBP dramatically decreased the expression levels of Bcl-xL and Bcl-2 proteins, while the siGABARBP transfectants were rescued. In contrast, the levels of Bax and p53 were increased in the GABARBP-transfected cells. Interestingly, the level of p21 expression was significantly increased by the ectopic expression of GABARBP, which is a well-known target gene of p53 (Fig. 3A). The effects of GABARBP on p53 and p21 transcription were assessed individually using a p53 and p21 promoter construct, which was introduced to the luciferase gene. Both activities significantly enhanced the ectopic expression of GABARBP (Fig. 3B). In HCT116 colon carcinoma cells, the absence of functional p53 and p21, abolished its anti-proliferative activity (Fig. 3C). These results indicate that GABARBP suppresses cell growth via p53.

To further investigate whether GABARBP is essential for p53 activation, we introduced small interfering (si) RNA and antisense (As) methods to silence the expression of GABARBP. As-GABARBP and si-GABARBP reduced the expression of GABARBP, as calculated using RT-PCR (Fig. 3D, upper panel), in HCT116 p53-deficient colon cancer cells. Since As-GABARBP was shown to be more effective than si-GABARBP in blocking the expression of GABARBP, we used As-GABARBP to suppress GABARBP and examined...
the significance of GABARBP in p53 induction. As shown in Fig. 3D, treatment with UV-irradiation promoted the p53 expression levels, whereas the suppression of GABARBP, by As-GABARBP, blocked the induction of p53 (Fig. 3D, bottom panel), supporting the requirement of GABARBP for the increase in p53. These results indicate that GABARBP is a key regulator for the DNA damage-dependent induction of p53.

Effect of GABARBP on inhibiting Akt/mTOR phosphorylation by cisplatin. Akt as a key downstream modulator of PI3K, activates cell growth through a variety of biological mechanisms, such as phosphorylation and the inactivation of pro-apoptotic genes, Bcl-2 and the caspase family (45-47). We next performed immunoblotting of the downstream targets of PI3K after treatment with either GABARBP or cisplatin. With treatment of GABARBP or cisplatin alone, as well as GABARBP plus cisplatin, it was shown that they induced downregulation of phospho-phosphoinositide-dependent protein kinase 1 (PDK-1), phospho-Akt, phospho-mammalian target of rapamycin (mTOR), phospho-ribosomal p70 S6 kinase (70S6K) and phospho-glycogen synthase kinase 3β (GSK-3β), without changing their entire protein levels (Fig. 4). Taken together, the data clearly suggest that GABARBP and cisplatin induce
changes in apoptosis, cell cycle progression and the PI3K signaling pathway regulatory proteins in ovarian carcinoma cells.

Expression of GABARBP in human carcinoma cells and patient tissues. To explore whether a functional connection of GABARBP exists with human cancers, we first observed the mRNA expression of GABARBP with different human carcinoma cells and non-malignant normal cell types by RT-PCR. Among the calculated cell types, the expression level of GABARBP mRNA was enhanced ~8-fold higher in HOSE-E6E7 (6.25-fold), MCF10A (6.95-fold) and three BEAS-2B (9.46-fold) normal cell types than in the two ovarian and one breast carcinoma cell types (Fig. 5A). When we also compared the expression levels of GABARBP, by western blotting, in the three different carcinoma cell types; the band intensities were measured using ImageLab software program. Our results were consistent with those observed by the RT-PCR experiments (Fig. 5B). Therefore, these finding suggest that the GABARBP expression levels may be downregulated in human carcinoma cell types. The results appear to be associated to the functionality of p53 (Fig. 3A). We next assessed the expression levels of GABARBP with tissues obtained from ovarian tumor patients. Among the six different patient samples of the ovarian tumor, low GABARBP expression levels were observed in six of the six patient cases, in contrast to the corresponding normal tissue samples (Fig. 5C). All of these results clearly suggest that low GABARBP expression may be frequently reduced with various human cancer types that include ovarian tumors.

Discussion

Ovarian cancer is one of the most aggressive tumor types, the overall prognosis for advanced cancer patients is poor. One of the reasons for the low survival rate is due to the resistance to many clinical therapies, such as conventional chemotherapy and radiotherapy (48). Conventional chemotherapy is generally used when the tumor has spread or may spread, to all areas of the body. Several previous studies have reported that different types of epigenetic and genetic alterations are included in gynecologic carcinoma (49,50). Inhibition of apoptosis is commonly accepted as one of the major contributing factors to chemoresistance (51). Apoptosis plays an important role for the maintenance of tissue homeostasis and the development and inhibition of tumorigenesis. Generally, regulation of this form of apoptosis comprises of the participation of the p53 and Bcl-2 family proteins. Bcl-2 family members are known as major regulators of apoptosis, including pro- and anti-apoptotic proteins, such as Bcl-2, Bcl-xL, Mcl-1 and Bax (39,52). GABARBP is a modulator of the cellular trafficking of GABA\textsubscript{A} receptor, including the \( \gamma_2 \) subunit (53). Klebig et al (33) reported that GABARBP functions as a putative tumor suppressor gene class II in breast cancer.

In this study, using the cancer model system, we found the new biological mechanisms of GABARBP as a novel potent regulatory factor that can target through a combination of traditional anticancer drugs in the mTOR signaling pathway. GABARBP mRNA and protein expression are dramatically downregulated in ovarian cancers when compared with normal patient tissues (Fig. 5). Ectopic expression of GABARBP greatly inhibits cell proliferation by enhancing cisplatin-induced apoptosis, increased Bax expression and reduced Bcl-xL expression, as well as Bcl-2 in OVCAR-3 carcinoma cells. Tumor suppressor p53 was upregulated in GABARBP-expressing cells (Fig. 3), while NF-\( \kappa \)B expression was downregulated (data not shown), which may give a survival benefit for cancer cells.

The activation of the Bcl-2 protein can be regulated by post-translational modifications, such as phosphorylation that contains Akt and mTOR (54,55). Akt activates mTOR and is regulated by mTOR via a negative and positive feedback system (56), as a critical mediator of cell proliferation, which enhances cell survival through various mechanisms. mTOR,
a serine/threonine kinase, plays a key role in controlling cell cycle progression, protein synthesis and tumor growth. Interestingly, this downstream regulator for PI3K/mTOR, is constitutively activated in various types of human tumors, including ovarian cancer (57-59). Recently, Suvasini and Somasundaram have suggested the pivotal role of the PI3K/Akt signaling pathway in p53-mediated transcription (60). In spite of these reports, biological/physiological mechanisms for therapeutic outcomes are very poor. We have integrated the biological role among these molecules in ovarian tumors. GABARBP inactivated cisplatin-induced dephosphorylation of the PI3K/Akt signaling pathway, including mTOR, p70S6K, as well as GSK-3β expression. The promoter activity of p53 and p21 was enhanced as well as the protein expression levels, while Bcl-2 protein expression was inactivated by GABARBP. Importantly, our results show that GABARBP can additively regulate the expression of the apoptosis regulator gene by upregulation of p53 and the downregulation of NF-κB activity to cisplatin-induced apoptosis in the OVCAR-3 ovarian carcinoma cells. In addition, we found that the GABARBP ectopic expression, significantly reduced the protein levels of the mTOR signaling pathway-associated genes. Taken together, these results clearly indicate that GABARBP and a chemotherapeutic agent have an additive effect on apoptosis and they also provide a biological function of GABARBP during tumor cell apoptosis. Optimization of the use of anticancer agents may offer great chances for further elevating the management of ovarian malignancies because it could help to enhance the quality of life in tumor patients (61).

In conclusion, we further validated a new critical molecular mechanism for GABARBP, a novel powerful pro-apoptotic factor, which can control phosphorylation of Akt/mTOR via the targeting of the p53 signaling pathways. These findings strongly indicate that GABARBP inhibition supports tumorigenesis by suppressing apoptosis in an ovarian cancer system. Our study is the first to show that GABARBP exerts its anticancer potency by increasing the p53-p21 protein expression of the G1 phase of cell cycle progression and cell death in human ovarian tumorigenesis.

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References

Identification of pRb2/p130 decreases characterisation of the trafficking protein Nix directly binds 44.

Heckman CA, Mehew JW and Boxer LM: NF-κB activates NF-κB transcription factor increases Bax expression in 43.


