Cellular mechanisms of growth inhibition of human endometrial cancer cell line by an antagonist of growth hormone-releasing hormone

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Abstract. The expression of growth hormone-releasing hormone (GHRH) and its receptors has been demonstrated in peripheral tissues as well as CNS. Recently, the functional splice variant SV1 of GHRH receptor was identified in various human cancers and cancer cell lines. Although antineoplastic activity of GHRH antagonists has been clearly demonstrated, the mechanism of action is incompletely understood. The objective of this study was the investigation of direct anti-proliferative effect of GHRH antagonist MZ-5-156 on HEC-1A human endometrial cancer cell line and the elucidation of underlying mechanisms. RT-PCR revealed the expression of mRNA for GHRH and SV1 of GHRH receptor in HEC-1A cells. MZ-5-156, at concentrations between 10^{-7} and 10^{-5} M, had a dose-dependent antiproliferative effect on HEC-1A cells, as determined by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, (MTS) assay. Hoechst 33342 staining and flow cytometric analysis indicated that MZ-5-156, at 10^{-6} M, induced apoptosis in HEC-1A cells after 48 h of treatment. Western blot analysis of apoptosis-related proteins demonstrated that treatment with MZ-5-156 (10^{-6} M) for 48 h significantly increased the protein levels of Fas, phospho-p53 (Ser46), p53AIP1 (p53-regulated Apoptosis-Inducing Protein 1), and caspase-8, -9, and -3, and decreased the protein level of Bcl-2. These results demonstrate that MZ-5-156 can directly inhibit the proliferation of human endometrial cancer cells, which express mRNA for GHRH and SV1 of GHRH receptor, presumably through the induction of p53-dependent apoptosis coupled with the up-regulation of Fas, phospho-p53 (Ser46), p53AIP1, and caspase-8, -9, and -3, and the down-regulation of Bcl-2.

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Introduction

Endometrial cancer is one of the most common malignancies of the female genital tract. In the US, approximately 40,000 new cases of endometrial cancer are expected to be diagnosed in 2007, with an estimated 7,000 deaths (1). Surgery, radiation, chemotherapy, and endocrine therapy with progestin are of limited effectiveness in the treatment of advanced and disseminated endometrial cancer, and new therapeutic approaches are needed (2).

Growth hormone-releasing hormone (GHRH) is secreted by the hypothalamus and upon binding to GHRH receptor (GHRH-R) in the pituitary, stimulates the synthesis and the release of GH. GH, in turn, induces the production of hepatic insulin-like growth factor I (IGF-I) (3,4). IGF-1 is a known mitogen for various cell types and has been linked with malignant transformation, tumor progression, and metastasis of diverse cancers (5). Since 1994, numerous GHRH antagonists including MZ-5-156 have been synthesized in the laboratory of one of us (Andrew V. Schally) for therapeutic use in the management of various malignancies (3,4). These GHRH antagonists were found to inhibit the growth of human ovarian, endometrial, breast, prostatic, colorectal, pancreatic, renal, and lung cancer, osteosarcoma, and non-Hodgkin's lymphoma xenografted into nude mice (3,4,6,7). *In vitro* and *in vivo* studies revealed that GHRH antagonists can inhibit tumor growth through indirect and direct pathways. The indirect mechanism is based on the suppression of the pituitary GH/hepatic IGF-I axis. Thus, GHRH antagonists, by blocking the pituitary GHRH-R, can inhibit the synthesis and release of GH, with the resulting reduction in hepatic IGF-I production. However, much evidence indicates that the principal anti-

proliferative effects of GHRH antagonists are probably exerted directly through the blocking of the stimulatory loop formed by tumoral GHRH and its receptors on tumors, and by the disruption of the autocrine/paracrine production of IGF-I and/or IGF-II on tumors (3,4,8,9). The isolation and sequencing of cDNAs corresponding to the tumoral GHRH-R mRNAs revealed that they are truncated splice variants (SVs) of the pituitary GHRH-R (10). The presence of four SVs of GHRH-R has been demonstrated in various human cancers and cancer cell lines (6,7,9,10-17). Of the four isoforms, SV1 of GHRH-R has the greatest structural similarity to the pituitary GHRH-R and is probably the main SV that mediates the effects of GHRH and its antagonists in tumors (18-20).

The cellular mechanisms involved in the antiproliferative effects of GHRH antagonists on tumor cells have not yet been completely elucidated. Recent studies have shown that, in addition to cAMP, other intracellular second messengers, such as PKC, MAPK, and c-fos and c-jun oncogenes, may participate in the signal transduction pathways of GHRH antagonists mediated by tumoral GHRH-R (16,21-23). It has also been reported that GHRH antagonists induce apoptosis in LNCaP human prostate cancer cell line through the elevation of intracellular free Ca^{2+} levels (24). Although GHRH antagonists have been shown to induce apoptosis in human prostate (LNCaP), colon (HT-29), and breast (MDA-MB-231) cancer cell lines and MXT mouse mammary cancer cells, little is known about the exact apoptotic pathway (24-27).

In the previous *in vitro* and *in vivo* experiments, GHRH antagonist MZ-5-156 (28) inhibited the growth of human ovarian (OV-1063), breast (MDA-MB-468), prostate (PC-3 and DU-145), and pancreatic (SW-1990 and CAPAN-2) cancer cell lines and non-Hodgkin's lymphoma (RL and HT) cell lines (6,17,21,29,30). In the present study, we investigated the expression of SV1 of GHRH-R in HEC-1A human endometrial cancer cell line and the direct effects of GHRH antagonist MZ-5-156 on the growth of HEC-1A cells, including the induction of apoptosis. In order to elucidate the cellular mechanisms by which MZ-5-156 inhibits cancer cell growth, we also examined the expression of apoptosis-related proteins in HEC-1A cells.

Materials and methods

Chemicals. GHRH antagonist MZ-5-156 was synthesized by solid phase methods (28). The chemical structure of MZ-5-156 is [PhAc-Tyr^1, D-Arg^2, $Phe(4\text{-}Cl)^6$, Abu^{15}, Nle^{27}]hGHRH-(1-28)Agm, where PhAc is phenylacetyl, Phe(4-Cl) is 4-chlorophenylalanine, Abu is α-aminobutyric acid, Nle is norleucine, and Agm is agmatine (17). DMEM-F12 medium and fetal bovine serum (FBS) were purchased from Invitrogen Corp. (Carlsbad, CA, USA). All other chemicals, unless otherwise mentioned, were obtained from Sigma (St. Louis, MO, USA).

Cell line and cell culture. HEC-1A human endometrial cancer cell line, which originated from a moderately differentiated papillary adenocarcinoma of the endometrium in a 71-year-old Japanese woman (31), was obtained from American Type Culture Collection (Manassas, VA, USA). The cell line was grown in DMEM-F12 medium supplemented with 10% FBS, 15 mM HEPES buffer, 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B in a humidified atmosphere of 5% CO_2 and 95% air at 37˚C.

RT-PCR. Total RNA was extracted from cultured HEC-1A cells by the acid guanidinium-phenol-chloroform (AGPC) method using Isogen (Nippongene, Toyama, Japan). First-stand cDNA was synthesized in a reaction volume of 20 μl containing 1 μg total RNA by using ReverTra Dash (Toyobo, Tokyo, Japan) according to the manufacturer's instructions. After the reverse transcription reaction, cDNA was amplified to determine GHRH and GHRH receptor SV1 expression using the following primer pair: GHRH, 5'-ATGCAGATGC CATCTTCACCAA-3' (sense) and 5'-TGCTGTCTACCTGA CGACCAA-3' (antisense) (GenBank NM021081); GHRH splice variant receptor (SV1), 5'-TGGGGAGAGGGAAGGA GTTGT-3' (sense) and 5'-GCGAGAACCAGCCACCA GAA-3' (antisense) (GenBank AF282259). PCR was carried out with the Takara Taq (Takara Shuzo Co., Ltd., Otsu, Japan) in a DNA thermal cycler (GeneAmp PCR System 9700; Perkin-Elmer Applied Biosystems, Foster City, CA, USA). After an initial denaturation at 95˚C for 5 min, the samples were submitted to 35 reaction cycles under the following conditions: denaturation for 10 sec at 98˚C, annealing for 2 sec at 60˚C, and extension for 30 sec at 74˚C. PCR amplified products were electrophoresed on a 2% agarose gel containing 0.5 μg/liter ethidium bromide and photographed under UV light. The PCR products were characterized by using a DNA sequencer (ABI PRISM 310 Genetic Analyzer; Perkin-Elmer Applied Biosystems).

Cell viability assay. Cell viability was examined by using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) assay kit (CellTiter 96 Aqueous One Solution Cell Proliferation Assay; Promega Corp., Madison, WI, USA) according to the manufacturer's instructions. Briefly, HEC-1A cells were seeded into 96-multiwell plates (Becton Dickinson and Co., Franklin Lakes, NJ, USA) at a density of $3x10^3$ cells/well in 100 μl of the culture medium. After 24 h, the medium was replaced with fresh FBS-free medium. After an additional 24 h, the medium was replaced with fresh medium containing 2% FBS and various concentrations of GHRH antagonist MZ-5-156, and cell culture was continued for a further 24 or 48, or 72 h. MZ-5-156 was dissolved in dimethyl sulfoxide and diluted with the medium to yield desired concentrations. The final concentration of dimethyl sulfoxide never exceeded 0.05%. Finally, the medium was replaced with 100 μl of fresh medium containing 20 μl of MTS solution and incubated for an additional 3 h. Mitochondrial dehydrogenase enzymes of viable cells converted MTS tetrazolium into a colored formazan product. The optical density of samples was read at 492 nm in the DigiScan Microplate Reader (ASYS Hitech GmbH, Eugendorf, Austria).

Hoechst 33342 staining. Hoechst staining was performed to confirm the apoptotic profile as a result of morphological change in the nucleus in which Hoechst 33342 binds specifically to A-T base region in DNA and emits fluorescence. HEC-1A cells were seeded into 16-well chamber slides (Nalge Nunc, Naperville, IL, USA) at a density of 3000 cells/well in

200 μl of the culture medium. After 24 h, the medium was replaced with fresh FBS-free medium. After an additional 24 h, the medium was replaced with fresh medium containing 2% FBS and 10^{-6} M MZ-5-156. After 48 h incubation, HEC-1A cells were rinsed in PBS (pH 7.4) and fixed with 4% paraformaldehyde in PBS at room temperature for 30 min. Then, cells were rinsed in PBS twice and stained with Hoechst 33342 (10 μg/ml in PBS) for 3 min. The specimens were mounted with Vectashield medium (Vector Laboratories Inc., Burlingame, CA, USA) and photographs were taken at magnification x200 under a fluorescent microscope (Olympus, Tokyo, Japan). The proportion of cells with nuclear fragmentation was calculated by counting the number of stained cells per >200 cells. Three different individuals made these observations 3 times each.

Flow cytomery. HEC-1A cells were seeded into a 10-cm culture dish (Iwaki, Tokyo, Japan) at a density of $1x10^6$ cells/dish in 10 ml of the culture medium. After 24 h, the medium was replaced with fresh FBS-free medium. After an additional 24 h, the medium was replaced with fresh medium containing 2% FBS and 10^{-6} M MZ-5-156, and cell culture was continued for a further 24, 48, or 72 h. Then, the cells were harvested with trypsin (0.05%)/EDTA (0.02%), washed twice with ice-cold PBS (pH 7.4), and fixed with 70% ethanol at -20˚C overnight. After washing twice with ice-cold PBS, the cells were incubated in 0.25 mg/ml ribonuclease solution (Qiagen GmbH, Hilden, Germany) for 30 min at 37˚C and stained with 50 μl/ml propidium iodide for 30 min on ice, followed by filtration through a 40-μm nylon mesh (Becton Dickinson and Co.) to remove cell clumps. A total of 50,000 stained cells per treatment were analyzed in the EPICS XL Flow Cytometry (Beckman Coulter, Inc., Fullerton, CA, USA). Sub-G1 phase represents low-molecular-weight DNA derived from apoptotic cells.

Western blotting. HEC-1A cells were seeded into 10-cm dish at a density of $1x10^6$ cells/dish in 10 ml of the culture medium. After 24 h, the medium was replaced with fresh FBS-free medium. After an additional 24 h, the medium was replaced with fresh medium containing 2% FBS and 10^{-6} M MZ-5-156. After a further 48 h, the cells were harvested with trypsin (0.05%)/EDTA (0.02%) and scraped into the lysis buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.02% sodium azide, 0.1% sodium dodecyl sulfate, 1% Nonidet P-40, and 0.5% sodium deoxycholate for 30 min on ice. Insoluble material was removed by centrifugation at 12,000 x g, for 10 min at 4˚C. The supernatants were recovered, and the protein concentrations were measured using Bio-Rad protein assay reagent (Bio-Rad Lab., Hercules, CA, USA). Equivalent amounts of lysate protein (30 μg) were subjected to 12% SDS-PAGE and electrophoretically transferred onto polyvinylidene difluoride membranes (Millipore Corp., Billerica, MA, USA) by using the Bio-Rad Semi-Dry Electrophoretic Transfer Cell. After blocking non-specific binding sites by incubation for 1 h with Tris-buffered saline (25 mM Tris and 150 mM NaCl, pH 7.6) containing 5% non-fat milk and 0.2% Tween-20, the membranes were blotted with the primary antibodies overnight at 4˚C. Rabbit polyclonal antibodies to phospho-p53 (Ser46) and cleaved caspase-3 and -9 were obtained from Cell Signaling Technology, Inc. (Beverly, MA, USA). Rabbit polyclonal antibody to Fas (C-20); mouse monoclonal antibodies to Bcl-2 and caspase-8 (D-8); and goat polyclonal antibodies to p53AIP1 (p53-regulated Apoptosis-Inducing Protein 1) and actin were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Reactive proteins were detected with horseradish peroxidase-conjugated secondary antibodies (Cell Signaling Technology, Inc.) for 60 min at room temperature and developed with ECL Plus Western blotting detection reagents (Amersham Biosciences, Little Chalfont, UK). The images were scanned and analyzed by the fluorescence scanning system STORM (Molecular Dynamics, Inc., Sunnyvale, CA, USA). The values were normalized to actin levels and then expressed as a percentage of the control value.

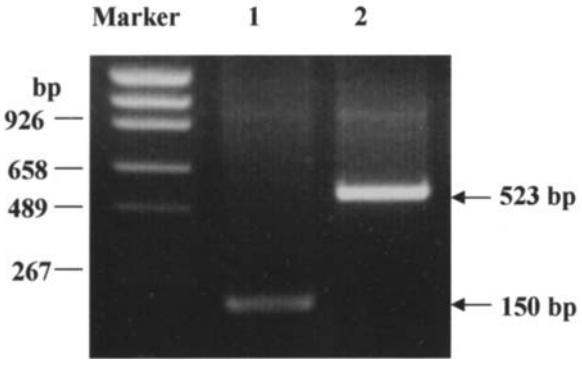

Figure 1. RT-PCR analysis of mRNA expression for GHRH (lane 1) and SV1 of GHRH-R (lane 2) in HEC-1A human endometrial cancer cell line. Sizes of PCR products, predicted on the basis of cDNA sequence, were 150 bp for GHRH and 523 bp for SV1 of GHRH-R. The result is representative of three independent experiments.

Statistical analysis. The data represent the mean ± SEM of at least three independent experiments. The statistical analysis was carried out by Mann-Whitney U test for paired comparison and One-way ANOVA with *post hoc* test for multiple comparisons by using StatView software (SAS Institute Inc., Cary, NC, USA). Repeated-measures ANOVA was applied for analysis of time course study of the effect of MZ-5-156 on HEC-1A cell viability. $P<0.05$ was considered statistically significant.

Results

Expression of mRNA for GHRH and SV1 of GHRH-R in HEC-1A cells. RT-PCR was performed to detect mRNA expression for GHRH and SV1 of GHRH-R in HEC-1A human endometrial cancer cell line. As shown in Fig. 1, an amplified product with the predicted size of 150 bp for GHRH and a product with the predicted size of 523 bp for SV1 of GHRH-R were observed in the cell line. Each PCR product was sequenced and confirmed to be identical to the sequence of GHRH and SV1 of GHRH-R, as previously described (32).

Effect of GHRH antagonist MZ-5-156 on the viability of HEC-1A cells. The effect of MZ-5-156 on HEC-1A cell viability was examined by MTS assay. MZ-5-156, at concentrations between 10^{-7} and 10^{-5} M, produced a dose-dependent

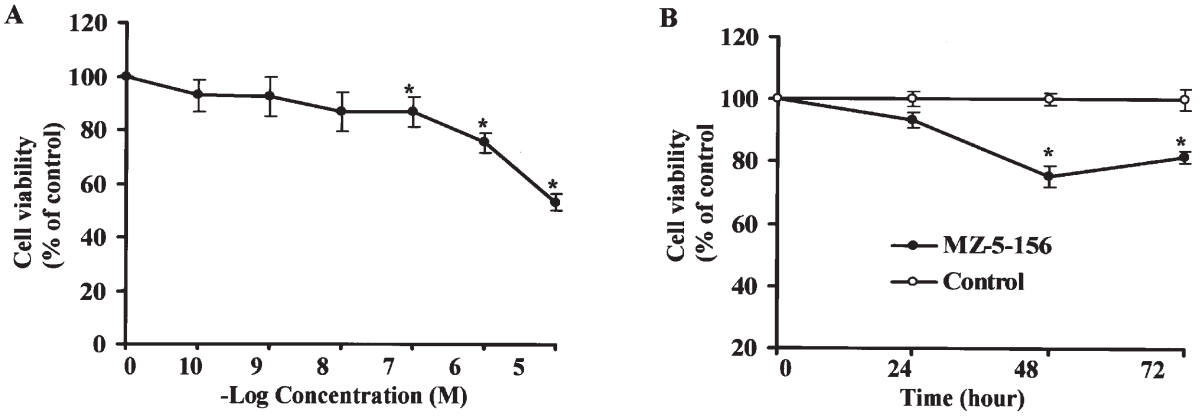

Figure 2. Effect of GHRH antagonist MZ-5-156 on the viability of cultured HEC-1A cells estimated by MTS assay. A, Treatment with MZ-5-156 at concentrations between 10^{-10} and 10^{-5} M for 48 h. Results are shown as the mean percentage of the untreated control at each time ± SEM of 8 wells of three independent experiments. *P<0.05 vs. control. B, Treatment with 10^{-6} M MZ-5-156 for 24, 48, and 72 h. Results are shown as the mean percentage of the untreated control at 0 h ± SEM of 8 wells of five independent experiments. *P<0.05 vs. 0 h.

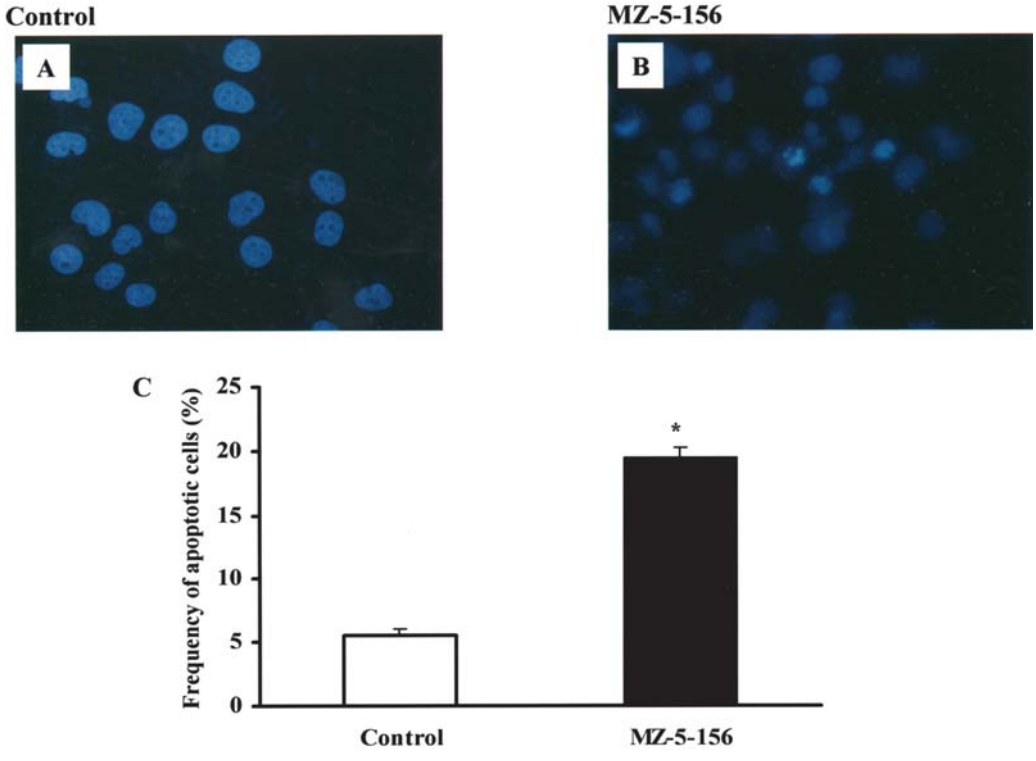

Figure 3. Hoechst 33342 staining of HEC-1A cells cultured with or without MZ-5-156 (magnification x200). A, Control. B, Treatment with MZ-5-156 (10^{-6} M) increased the rate frequency of cells with nuclear fragmentation at 48 h. C, The frequency of cells with nuclear fragmentation by Hoechst 33342 staining was calculated by counting the number of stained cells per more than 200 cells. Results are shown as the mean ± SEM of three independent experiments. *P<0.05 vs. control.

inhibition of HEC-1A cell viability after 48 h of treatment, with the maximal effect (reduction to 52.1±3.2% of the control) being observed at 10^{-5} M (Fig. 2A). The cell viability was significantly reduced to 75.3±3.6% of (0 h) baseline after 48 h incubation with MZ-5-156 (10^{-6} M), and the antiproliferative effect was sustained up to 72 h (Fig. 2B). Control incubation at 24, 48, and 72 h did not result in significant loss of viability.

Induction of apoptosis by GHRH antagonist MZ-5-156 in HEC-1A cells. The effect of MZ-5-156 on the incidence of apoptotic cells was determined by Hoechst 33342 nuclear staining and flow cytometry in cultured HEC-1A cells. The apoptotic cells exhibiting shrunken nuclei, chromatin condensation, and nuclear fragmentation were recognized by Hoechst 33342 nuclear staining (Fig. 3B). The exact frequency of cells with nuclear fragmentation by Hoechst 33342 staining is shown in Fig. 3C. The percentage of dead cells was significantly increased after 48 h incubation with 10^{-6} M MZ-5-156 (19.4±0.8%; P<0.05 vs. control) compared with that of the control (5.6±0.5%). Fig. 4A is a representative

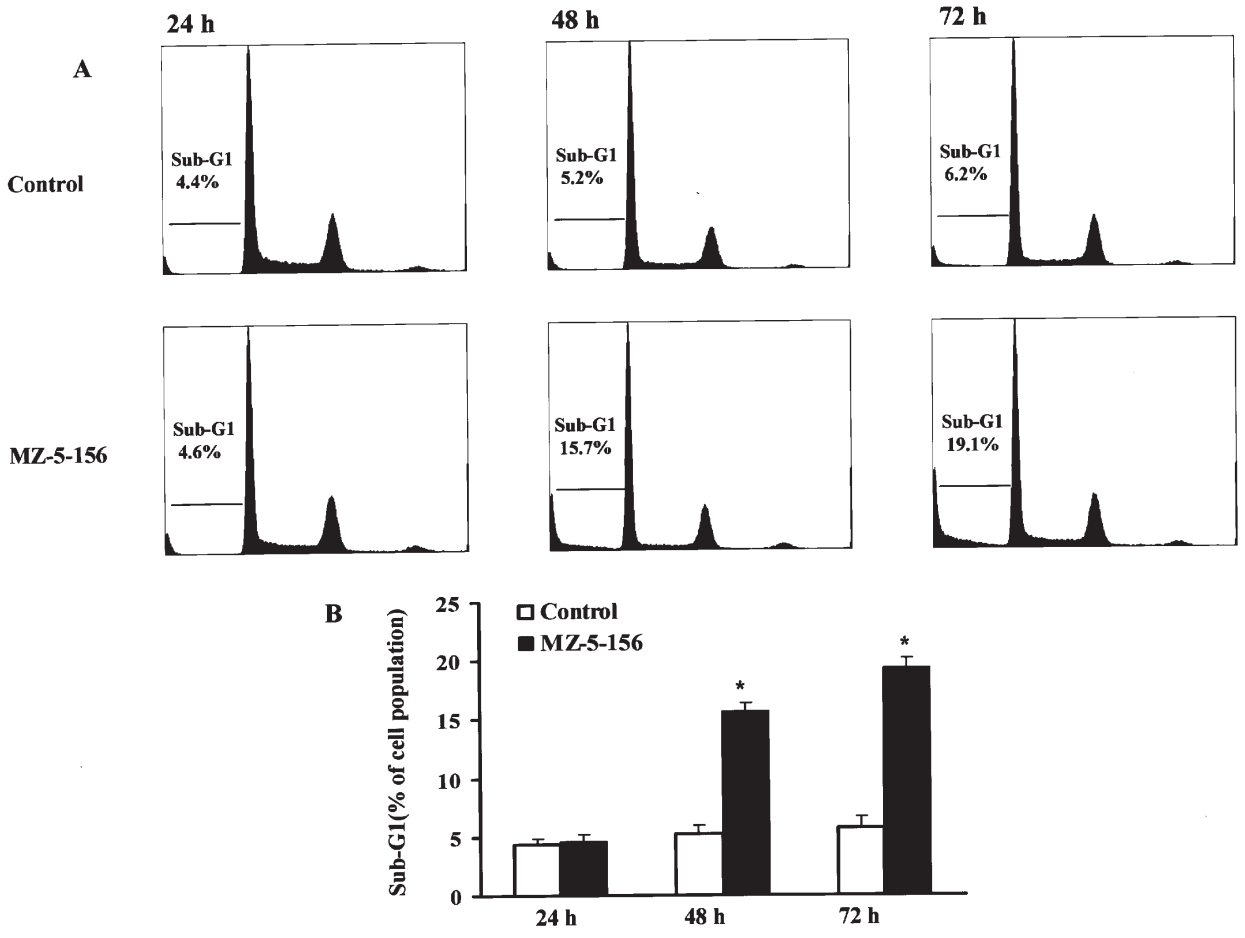

Figure 4. DNA histograms of GHRH antagonist MZ-5-156-treated HEC-1A cells. Cells were incubated with or without MZ-5-156 (10^{-6} M) for 24, 48, and 72 h, and then analyzed by flow cytometry. Sub-G1 indicates an apoptotic cell fraction. A, The result of flow cytometric analysis is representative of six independent experiments. B, Effects of MZ-5-156 on the proportion of Sub-G1 phase. Results are shown as the mean ± SEM of six independent experiments. *P<0.05 vs. control.

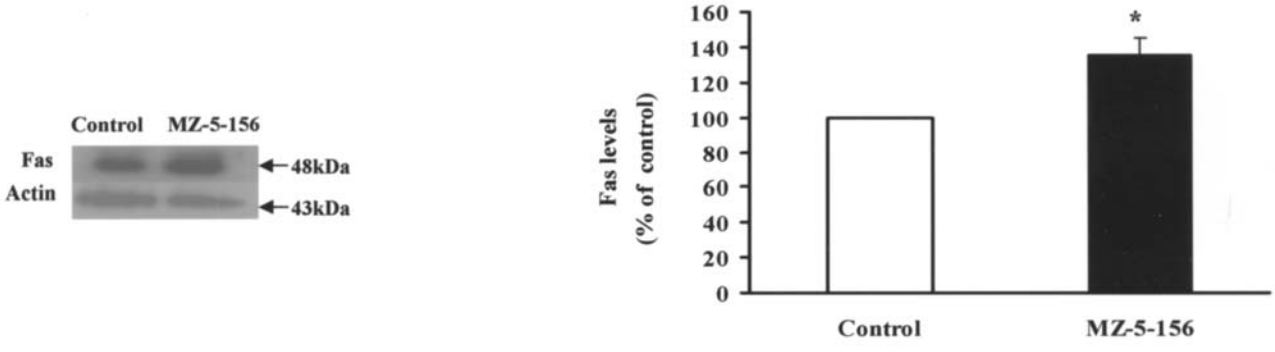

Figure 5. Effect of GHRH antagonist MZ-5-156 on the expression of protein levels of Fas in HEC-1A cells. Cells were incubated with or without MZ-5-156 (10^{-6} M) for 48 h. Protein extracts were subjected to SDS-PAGE followed by Western blot analysis of protein levels of Fas. Results in the left panel are representative of four independent experiments. Results in the right panel show quantitative analysis of Fas levels and are presented as the mean percentage of the untreated control ± SEM of four independent experiments. *P<0.05 vs. control.

result of three independent experiments showing the proportion of sub-G1 phase, an apoptotic cell fraction. As shown in Fig. 4B, flow cytometric analysis revealed that the proportion of sub-G1 phase was significantly increased by the addition of 10^{-6} M MZ-5-156 (15.7±0.7%; P<0.05), compared with that for the control (5.2±0.7%) at 48 h of treatment. At 72 h, the proportion of sub-G1 phase was further increased to 19.1±0.7% (P<0.05), compared with the control value of 6.2±0.7%.

Effect of GHRH antagonist MZ-5-156 on the expression levels of apoptosis-related proteins in HEC-A1 cells. We examined the protein levels of apoptosis-related proteins, including Fas, phospho-p53 (Ser46), p53AIP1, Bcl-2, and caspase-3, -8, and -9 in HEC-1A cells exposed to 10^{-6} M MZ-5-156 for 48 h, by using Western blot analysis. Fig. 5 shows that the expression level of Fas was significantly increased to 135.3±9.5% of the control (P<0.05 vs. control). As depicted in Fig. 6, the

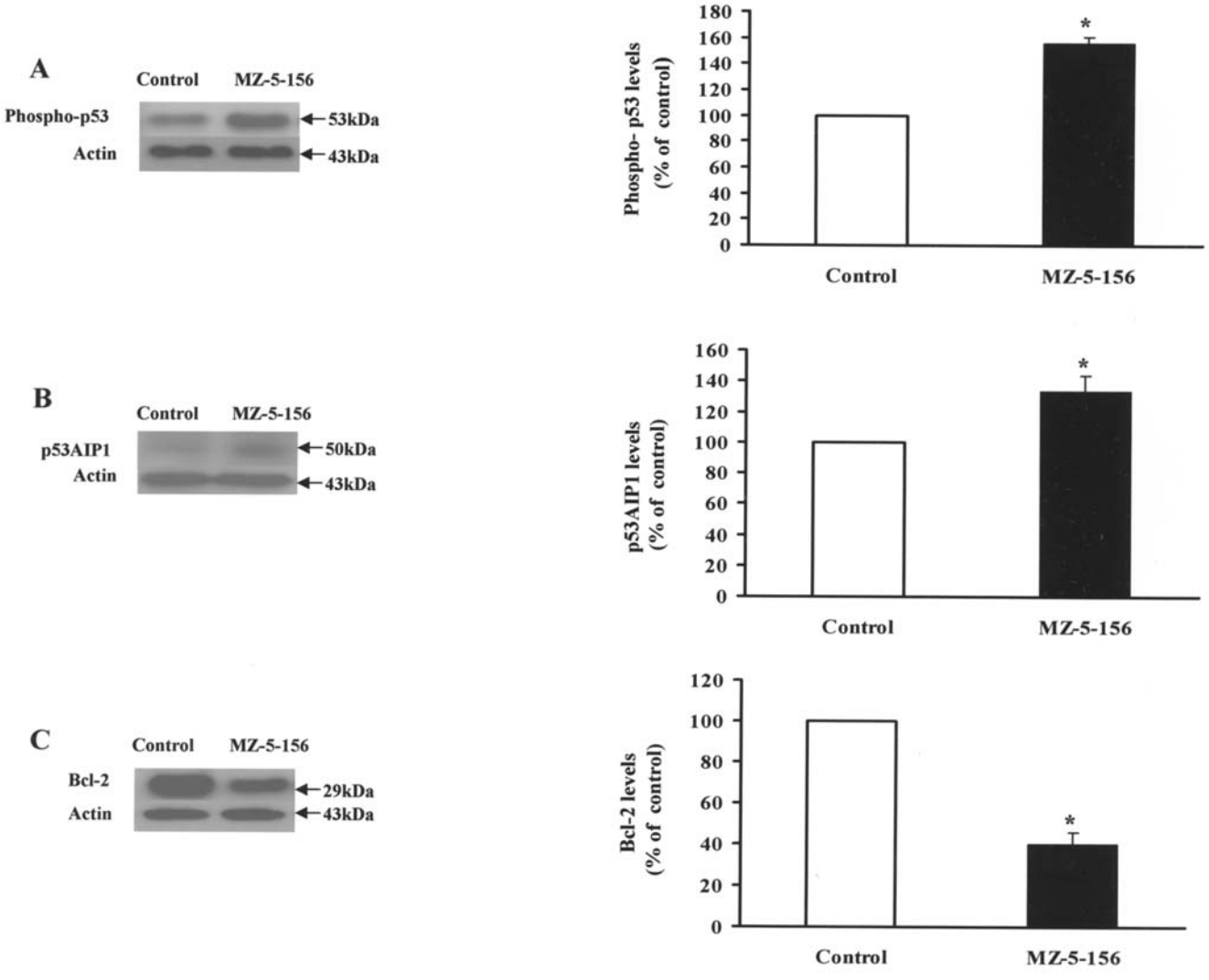

Figure 6. Effect of GHRH antagonist MZ-5-156 on the expression of protein levels of phospho-p53 (Ser46), p53AIP1, and Bcl-2 in HEC-1A cells. Cells were incubated with or without MZ-5-156 (10^{-6} M) for 48 h. Protein extracts were subjected to SDS-PAGE followed by Western blot analysis of protein levels of (A) phospho-p53 (Ser46), (B) p53AIP1, and (C) Bcl-2. Results in the left panels are representative of four independent experiments. Results in the right panels show quantitative analysis of each protein level and are presented as the mean percentage of the untreated control ± SEM of four independent experiments. *P<0.05 vs. control.

expression levels of phospho-p53 (Ser46) and P53AIP1 were significantly increased to 155.4±5.2 and 133.2±10.6%, respectively, whereas Bcl-2 levels were decreased to 40.0±6.1%, compared with the control value (P<0.05). Fig. 7 (left), illustrates representative results, exhibiting the 17-kDa cleavage fragments of activated caspase-3, the 20-kDa cleavage fragments of activated caspase-8, and the 17-kDa cleavage fragments of activated caspase-9, respectively. As shown in Fig. 7 (right), the expression levels of cleaved caspase-3, -8, and -9 were significantly up-regulated to 143.8±13.9, 164.7±10.5, and 182.0±23.5% of the control, respectively.

Discussion

Evidence summarized (3,4) indicates that GHRH acts as an autocrine/paracrine regulator of cancer cell proliferation and that SV1 of GHRH-R is the main functional receptor responsible for mediating the effects of GHRH and its antagonists in tumors. In the present study, using RT-PCR, we demonstrated that mRNA for GHRH and SV1 of GHRH-R is expressed in HEC-1A human endometrial cancer cell line, suggesting that an autocrine/paracrine GHRH loop may be present in the cell line and participate in the regulation of cell growth. All of the SVs of GHRH-R have a retained intronic sequence at the 5'-end but lack the first three exons (10,11). The lack of the first three exons in SV1 results in a tumoral receptor protein, in which most of the large NH2-terminal extracellular domain, characteristic of the pituitary receptor, is truncated. Thus, the mRNA for SV1 encodes a functional receptor protein that binds GHRH and its analogs but is different from the pituitary GHRH-R (10,11). The participation of SV1 in cell proliferation signaling was proven by several studies (18-20). NIH 3T3 mouse fibroblast cells transfected with SV1 displayed augmented sensitivity and mitogenic responses to GHRH analogs, compared to the untransfected cells (18). Besides its ligand-dependent activation, a ligand-independent constitutive activation of SV1 has been demonstrated, by using an anti-sense RNA-based approach on HEC-1A cells, which express SV1 (19).

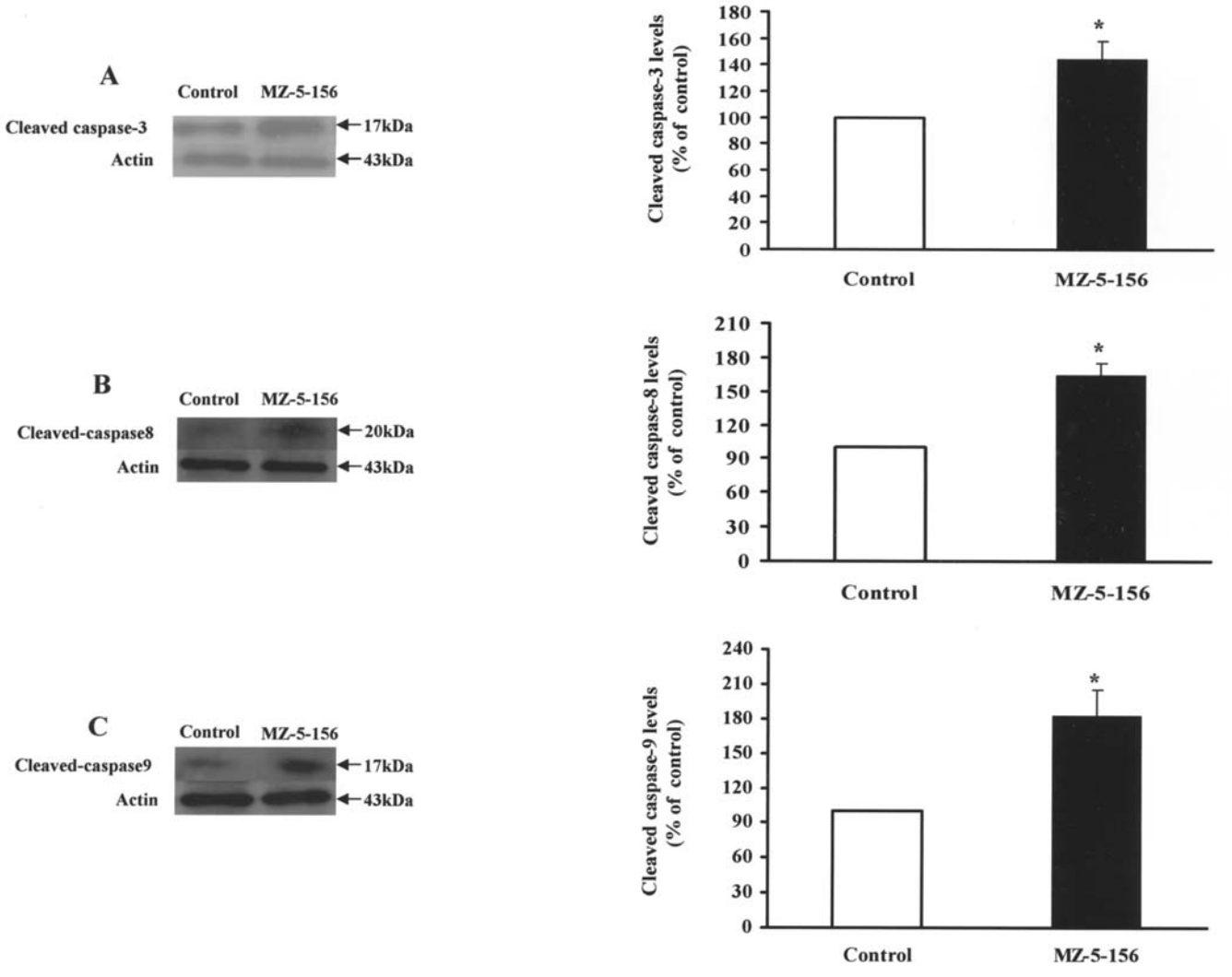

Figure 7. Western blot analysis of protein levels of cleaved caspase-3, -8, and -9 in HEC-1A cells treated with or without MZ-5-156 (10^{-6} M) for 48 h. Cell protein lysates were analyzed by immunoblotting with anti-cleaved caspase-3, anti-caspase-8, and anti-cleaved caspase-9 antibody, which recognizes active cleavage fragments of (A) 17 kDa, (B) 20 kDa, and (C) 17 kDa, respectively. Results in the left panels are representative films exhibiting bands for active forms of caspases with corresponding actin bands. Results in the right panels show quantitative analysis of caspase-3, -8, and -9 cleavage into active forms and are presented as the mean percentage of the untreated control ± SEM of four independent experiments. *P<0.05 vs. control.

The ablation of SV1 reduced the rate of cell proliferation in the absence of exogenous GHRH and decreased their sensitivity to GHRH (19). In MCF-7 human breast cancer cells transfected with the full-length GHRH-R or its SV1, the ligand-independent activity of SV1 was greater than that of GHRH-R and the expression of SV1 appeared to confer oncogenic activity (20). By using real-time PCR, mRNA for the pituitary type of GHRH-R was detected in various human cancers for the first time (32), which confirms and extends the concept that GHRH and its receptors play an important role in the pathophysiology of human cancers (4).

Current studies have shown that GHRH antagonist MZ-5-156 directly inhibits the growth of human ovarian, breast, prostate, and pancreatic cancers and non-Hodgkin's lymphomas (6,17,21,29,30). The present *in vitro* study demonstrated that MZ-5-156 exerts a dose-dependent antiproliferative action in HEC-1A cells, as determined by MTS assay, and induces apoptosis in these cells in a p53-dependent manner, as documented by Hoechst 33342 staining, flow cytometric analysis, and Western blotting. Our study indicates that apoptosis may be one way of explaining the mechanisms of the antiproliferative effect of MZ-5-156 on HEC-1A cells. Although several studies showed that the antiproliferative activity induced by GHRH antagonists might be mediated by stimulation of apoptotic cell death (24-27), the exact apoptotic pathway remained to be elucidated.

Upon exposure to genotoxic stress, the tumor suppressor p53 controls multiple downstream targets that regulate variable cellular outcomes such as cell-cycle arrest, induction of apoptosis, and maintenance of genetic stability by modulation of DNA repair, replication, and recombination machinery (33,34). So far, two separate apoptotic pathways have been described for p53-dependent apoptosis (34). The extrinsic, death-receptor pathway triggers the activation of caspase cascade, and the intrinsic, mitochondrial pathway shifts the balance in the Bcl-2 family towards the pro-apoptotic

members, leading to caspase-mediated apoptosis (34). The extrinsic pathway involves engagement of the death receptors that belong to the tumor necrosis factor (TNF) receptor superfamily and, through the formation of the death-inducing signaling complex (DISC), leads to a cascade of activation of caspases, including caspase-8 and caspase-3, which in turn induce apoptosis. In the extrinsic pathway, activated caspase-8 also induces mitochondrial apoptotic signaling through Bid which is a p53 target (34). p53 can activate the extrinsic apoptotic pathway through the induction of genes encoding transmembranous proteins (35,36). Among p53-downstream genes, Fas (APO-1/CD95), a member of TNF receptor superfamily, is a key component of the extrinsic death pathway (37,38). The present study showed that the protein levels of Fas, and caspase-8 and -3 were increased by treatment with MZ-5-156 in HEC-1A cells, indicating that the extrinsic pathway could be enhanced by GHRH antagonists.

The intrinsic pathway is triggered in response to DNA damage and is associated with mitochondrial depolarization and release of cytochrome *c* from the mitochondria. Cytosolic cytochrome *c* induces formation of the apoptosome by stimulating oligomerization of the adapter protein, apoptotic protease-activating factor 1 (Apaf-1) and, consequently, recruitment of the initiator caspase-9 (39). As a result, activated caspase-9 can then directly cleave and activate caspase-3. The intrinsic apoptotic pathway is dominated by the Bcl-2 family of proteins which controls the release of cytochrome *c* from the mitochondria (34). Bcl-2, a mitochondrial protein, inhibits apoptotic process and promotes cell survival. It is known that Bcl-2 directly or indirectly, prevents the release of cytochrome *c* from the mitochondria (40,41). Over-expression of Bcl-2 can block p53-dependent dissipation of the mitochondrial membrane potential ($\Delta\Psi m$) (40,41). Recently, a new p53-dependent pro-apoptotic gene, p53AIP1, was identified (42). When p53 is phosphorylated at Ser46 in response to severe DNA damage, the expression of *p53AIP1*, a component of the mitochondrial membrane, is induced and followed by depolarization of mitochondrial membrane and sequentially by the release of cytochrome *c* from the mitochondria (43). Ectopic expression of p53AIP1 in the mitochondria induced dissipation of the mitochondrial $\Delta\Psi m$ and release of cytochrome *c* from the mitochondria (43). Interaction between p53AIP1 and Bcl-2 might regulate the mitochondrial $\Delta\Psi m$ by balancing positive and negative effects (43). In the present study, the protein levels of phospho-p53 (Ser46), p53AIP1, and caspase-8, -9, and -3 were increased, and Bcl-2 levels were decreased by treatment with MZ-5-156 in HEC-1A cells, indicating that the intrinsic pathway could also be enhanced by GHRH antagonists.

In conclusion, our study demonstrates that GHRH antagonist MZ-5-156 inhibits directly the growth of HEC-1A human endometrial cancer cells, which express mRNA for GHRH and SV1 of GHRH-R, presumably through the induction of p53-dependent apoptosis coupled with the up-regulation of Fas, phospho-p53 (Ser46), p53AIP1, and caspase-8, -9, and -3, and the down-regulation of Bcl-2. The p53-dependent apoptosis induced by MZ-5-156 can be mediated through both death-receptor pathway and mitochondrial pathway. These results suggest that GHRH antagonists, such as MZ-5-156, are potentially useful in the treatment of endometrial cancer, but further studies followed by clinical trials are necessary to validate this approach.

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