Fluoxetine-induced Apoptosis in Hepatocellular Carcinoma Cells

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Abstract. Background: In addition to being used to treat mental disorders, a serious complication of cancer, antidepressants have been reported to improve cancer patient immunity, inhibit cell growth and have an antitumor effect on various cancer cell lines. We investigated the apoptotic effect of fluoxetine against the Hep3B human hepatocellular carcinoma cell line. Materials and Methods: After treatments of Hep3B cells with fluoxetine, we measured cell viability, reactive oxygen species (ROS), mitochondrial membrane potential (MMP) and activation of mitogen-activated protein kinases (MAPK). Results: Fluoxetine reduced the viability of cancer cells, induced loss of MMP and formation of ROS, reduced expression of extracellular signal-regulated kinase 1/2 and increased expression of c-JUN N-terminal kinase and p38 MAPK. N-Acetylcysteine, an oxidant-scavenger, and 1,2-bis (o-aminophenoxy) ethane-N,N',N'-tetraacetic acid (BAPTA-AM), an intracellular Ca2+ chelator, prevented fluoxetine-induced modulation of MAPK. Conclusion: Fluoxetine appears to exhibit an apoptotic effect against Hep3B cells through the loss of MMP, formation of ROS and modulation of MAPK activities.

Hepatocellular carcinoma (HCC) is a primary type of hepatic tumor, an aggressive malignancy with high prevalence, and the sixth most common cancer worldwide (1). HCC represents 5% of all cancers with the annual number of cases exceeding 500,000 worldwide (2). HCC cells have extreme chemoresistance and low selectivity to chemotherapy drugs. In addition, chemotherapy drugs kill normal cells as well as tumor cells, leading to significant adverse effects (3). To overcome the weaknesses of traditional anticancer chemotherapy, alternative or complementary medicine such as combined treatments with therapeutics for other diseases or new agents prepared from natural products is drawing attention as a potential new approach to anticancer therapy.

Antidepressants are clinically prescribed to patients for management of depression and psychiatric disorders (4), and many also exhibit substantial benefit in various types of chronic pain (5). Interestingly, recent studies have documented the anticancer effects of antidepressants in a variety of solid tumor types and cancer cell lines (6). Selective serotonin re-uptake inhibitors (SSRI), including fluoxetine, can inhibit growth of various cancer cell lines from lung, colon, neuroblastoma and breast (6, 7). Fluoxetine can improve cancer patient immunity and life quality, and extend their life expectancy (8, 9). Furthermore, fluoxetine was reported to be a highly effective chemosensitizer (10) that is synergistic with anticancer drugs in overcoming multidrug resistance (11). Fluoxetine has also been reported to have an apoptotic effect against ovarian cancer cell lines by inducing mitochondrial membrane permeability (MMP) changes (12) and induce preventive and complex effects against colon cancer development in rats (13). In contrast, several studies have linked fluoxetine with cell proliferation and an increased risk of developing cancer (12), while having no effect on cell survival and growth of cancer cells and tumor growth in vivo (14).

In the present study, we demonstrated that fluoxetine induces apoptosis in Hep3B cells, an HCC cell line. To elucidate the mechanism of fluoxetine-induced apoptosis in Hep3B cells, we investigated cell viability, reactive oxygen species (ROS), MMP, and activation of mitogen-activated protein kinases (MAPK).
Materials and Methods

Cell culture and reagents. Hep3B cells were obtained from the Korea Cell Line Bank (KCLB, Seoul, Korea) and grown in Dulbecco’s modified Eagle’s medium nutrient mixture F-12 HAM (DMEM F-12 HAM) supplemented with 10% fetal bovine serum (Sigma-Aldrich, St. Louis, MO, USA), 5 mM L-glutamine, 50 U/ml penicillin and 50 μg/ml streptomycin in a humidified 5% CO2-95% air environment at 37˚C. Fluoxetine was purchased from Enzo Life Sciences (Plymouth Meeting, PA, USA), and 2,7'-dichlorodihydrofluorescein diacetate (DCFH-DA) was purchased from Molecular Probes (Eugene, OR, USA). 4′,6-Diamidino-2-phenylindole (DAPI) and 5,5′,6,6′-tetra-chloro-1,1′,3,3′-tetraethyl benzimidazolyl-carbocyanine iodide (JC-1) were purchased from Enzo Life Sciences.

Cell viability assay. Hep3B cells were grown on 96-well plates (5000 cells/well) and cultured for 24 h in DMEM F-12 HAM medium containing 10% fetal bovine serum. After treatment with fluoxetine (10-100 μM) for 24 h, the culture medium was discarded and cell viability was assessed with the aid of a Cell Counting Kit-8 (CCK-8; Enzo Life Sciences). Briefly, 100 μl CCK-8 reagent were added to each well at a 1:10 ratio to cell culture medium. After a 2-h incubation in a humidified atmosphere with 5% CO2 at 37˚C, the absorbance was read at 450 nm using a spectrophotometer (Spectra Max M5; Molecular Devices, Sunnyvale, CA, USA).

Nuclear staining with DAPI. Hep3B cells were seeded on coverslips and cultured for 48 h in DMEM F-12 HAM medium containing 10% fetal bovine serum. After the treatment with fluoxetine (10-100 μM) for 24 h, the cells were washed three times with ice-cold PBS and fixed with 70% ethanol for 1 h. The cells were washed with PBS and counterstained with DAPI mounting medium for 15 min at room temperature. Apoptotic nuclei were then visualized under a fluorescence microscope (IX-81; Olympus Corp., Tokyo, Japan).

MMP assessment by JC-1 staining. Disruption of the MMP is an early event in reactive nitrogen species-induced apoptosis. Mitochondria depolarization is specifically indicated by JC-1, which is a cationic dye that exhibits potential-dependent accumulation in mitochondria, indicated by a fluorescence emission shift from green to red as the mitochondrial membrane becomes more polarized (15). After a 48-h incubation of the Hep3B cells in a Petri dish (1×10 4/well), the Hep3B cells were treated with fluoxetine (10-100 μM) for 24 h. After incubation, Hep3B cells were washed three times with ice-cold PBS and scraped with a cell scraper. The harvested cells were lysed for 30 min at 4˚C in RIPA buffer containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na2EDTA, 1 mM EGTA, 1% NP-40, 1% sodium deoxycholate, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na4VO4 and 1 μg/ml leupeptin. The sample was then sonicated for five pulses at a 40% using a Sonics & Materials Ultrasonic Processor (USA), and then transferred to a 0.5 ml microfuge tube. The sample was heated to 100˚C for 7 min and placed briefly on ice. Then, 30 μL of the supernatant were loaded onto a sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel. After electrophoresis, the protein was electrotransferred to a Hybond-ECL polyvinylidene fluoride membrane (SurModics Inc. Eden Prairie, MN, USA). The membrane was blocked with Tris-buffered saline (20 mM Tris and 140 mM NaCl, pH 7.6) containing 0.1% Tween 20 (TBST) and 4% milk at room temperature for 1 h. The membrane was then incubated overnight with a monoclonal rabbit anti-rat antibody (Cell Signaling Tech., Danvers, MA, USA) against total or phosphorylated extracellular signal-regulated kinase 1/2 (ERK1/2), c-JUN N-terminal kinase (JNK) or p38 MAPK as the primary antibody at a 1:1000 dilution in TBS with 5% milk at 4˚C. The blot was washed three times for 10 min in TBST at room temperature. The membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (Cell Signaling Tech.) for 60 min. The blot was washed three times for 15 min in TBST (three times for 5 min each), and the bands were detected using enhanced chemiluminescence. Representative western blots were scanned by a Bio-Rad ChemiDoc XRS and the images quantified in Quantity One 4.5.0 software (Bio-Rad, Hercules, CA, USA).

Western blot analysis of MAPKs. After a 48-h incubation of the Hep3B cells in a Petri dish (1×10 4/well), the Hep3B cells were treated with fluoxetine (100 μM) with/without N-αcetylcysteine (NAC) (10 mM) and (acetoxyethyl)methyl-I-Z bis (o-aminophenoxo) ethane N,N,N′,N′-tetra-acetic acid (BAPTA-AM) for 24 h. After incubation, Hep3B cells were washed three times with ice-cold PBS and scraped with a cell scraper. The harvested cells were lysed for 30 min at 4˚C in RIPA buffer containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na2EDTA, 1 mM EGTA, 1% NP-40, 1% sodium deoxycholate, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na4VO4 and 1 μg/ml leupeptin. The sample was then sonicated for five pulses at a 40% using a Sonics & Materials Ultrasonic Processor (USA), and then transferred to a 0.5 ml microfuge tube. The sample was heated to 100˚C for 7 min and placed briefly on ice. Then, 30 μL of the supernatant were loaded onto a sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel. After electrophoresis, the protein was electrotransferred to a Hybrid-ECL polyvinylidene fluoride membrane (SurModics Inc. Eden Prairie, MN, USA). The membrane was blocked with Tris-buffered saline (20 mM Tris and 140 mM NaCl, pH 7.6) containing 0.1% Tween 20 (TBST) and 4% milk at room temperature for 1 h. The membrane was then incubated overnight with a monoclonal rabbit anti-rat antibody (Cell Signaling Tech., Danvers, MA, USA) against total or phosphorylated extracellular signal-regulated kinase 1/2 (ERK1/2), c-JUN N-terminal kinase (JNK) or p38 MAPK as the primary antibody at a 1:1000 dilution in TBS with 5% milk at 4˚C. The blot was washed three times for 10 min in TBST at room temperature. The membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (Cell Signaling Tech.) for 60 min. The blot was washed three times for 15 min in TBST (three times for 5 min each), and the bands were detected using enhanced chemiluminescence. Representative western blots were scanned by a Bio-Rad ChemiDoc XRS and the images quantified in Quantity One 4.5.0 software (Bio-Rad, Hercules, CA, USA).

Statistical analysis. Results are expressed as mean±standard error of the mean (SEM). The data were analyzed by Student’s t-test or analysis of variance (ANOVA) with the Bonferroni post-hoc test, where appropriate, using Prism 5.03 (GraphPad Software Inc., San Diego, CA, USA). A p-value<0.05 was considered significant.
Results

Effect of fluoxetine on the cell viability of Hep3B cells. Fluoxetine led to a significant reduction in the number of cells, as shown in Figure 1A. To characterize the cell death induced by fluoxetine, we examined the nuclear morphology of dying cells with a fluorescent DNA-binding dye, DAPI. After a 24 h treatment with fluoxetine, cells clearly exhibited condensed and fragmented nuclei, indicative of apoptotic cell death (indicated by white arrows).

In transmitted light images, the concentration-dependent death of the Hep3B cells in the presence of fluoxetine was examined. As shown in Figure 1B, treatment with fluoxetine induced a decrease in cell viability in a dose-dependent manner. For fluoxetine at 10, 30 and 100 μM, the viability values were 98.3±15.2, 80.4±4.3 and 30.4±3.4%, respectively.

Effect of fluoxetine on MMP of Hep3B cells. MMP is an important parameter of mitochondrial function that is used as an indicator of cell health. In order to determine the cause of reduced mitochondrial activity in the presence of fluoxetine, we analyzed cells using the MMP-sensing dye, JC-1. Figure 2A shows the uptake of JC-1 by viable Hep3B cells, as measured by the increase in monomer (green) and aggregate (orange-red) fluorescence. Fluoxetine-induced mitochondrial depolarization is specifically indicated by a shift in JC-1 fluorescence from red to green in Hep3B cells. To corroborate these results, we also detected the fluorescence of the cationic dye JC-1 by spectrophotometry. As shown in Figure 2B, fluoxetine induced a dramatic decrease in the ratio of JC-1 (red/green ratio). Treatment with fluoxetine at 30 μM yielded a ratio that was 81.5±7.0% of the control, and fluoxetine at 100 μM yielded a ratio that was 56.6±8.4% of the control (p<0.005).

Effect of fluoxetine on ROS of Hep3B cells. Because intracellular ROS is considered to be a death signal in apoptosis (16), we demonstrated that ROS participates in fluoxetine-induced apoptosis. DCFH-DA was used to measure ROS levels in fluoxetine-treated Hep3B cells. As shown in Figure 5, the ROS level notably increased in Hep3B cells at 30 μM (125.6±4.6%) and 100 μM (262.3±14.7%) (p<0.005).

Effect of fluoxetine on the MAPKs expression of Hep3B cells. To identify the involvement of ERK1/2, JNK or p38 MAPK, the levels of phosphorylated as well as of total forms were determined in Hep3B cells treated with fluoxetine. Phosphorylation of JNK and p38 was clearly increased in fluoxetine-treated Hep3B cells, whereas the phosphorylation level of ERK1/2 was inhibited. Using densitometry, the percentage variations in the 30 μM fluoxetine-treated group compared to the control group (100%) were determined to be 109.6±3.0% for p-p38 MAPK, 108.4±1.2% for p-JNK and 84.7±9.9% for p-ERK. Likewise, the 100 μM fluoxetine-treated group resulted in values of 59.7±7.1% for p-ERK1/2, 199.4±10.5% for p-p38 APK, and 143.6±1.7% for p-JNK.

To demonstrate whether ROS and intracellular Ca2+ participate in fluoxetine-induced apoptosis, NAC or BAPTA-AM was added to the Hep3B cells growing in the presence of fluoxetine. NAC treatment markedly attenuated fluoxetine-induced modulation of MAPK. Using densitometry, 10 mM NAC treatment inhibited the fluoxetine-induced increase in phosphorylation of p38 and JNK (as a percentage of fluoxetine-induced phosphorylation of 100%: 76.4±28.9% and 76.5±18.8%, respectively). Also, NAC treatment inhibited the fluoxetine-induced decrease in p-ERK. BAPTA-AM at 5 μM significantly attenuated the fluoxetine-induced increase in p-p38 and p-JNK MAPK (as a percentage of fluoxetine: 79.4±26.1%, 76.2±19.3%, respectively). BAPTA-AM also inhibited the fluoxetine-induced decrease in p-ERK (Figure 5).

Discussion

In the present study, we demonstrated that fluoxetine had a potential effect on apoptosis in the specific HCC cell line. We showed that fluoxetine induced cell death, loss of MMP, formation of ROS, decrease in anti-apoptotic ERK1/2 protein and increase in proapoptotic JNK and p38 MAPK in Hep3B cells.

Although fluoxetine, used as an anti-depressant for cancer patients, has been reported to have an apoptotic effect against ovarian cancer cell lines (12) and colon cancer (13), it has also shown to increase risk of promoting cancer (12), but also been had no effect on cell survival and growth in cancer cells (14). A recent study showed that fluoxetine, when used alone, had little effect on cytotoxicity but had a remarkable effect on breast cancer cells when combined with adriamycin or paclitaxel (11). In the present study, fluoxetine (10-100 μM) induced a dose-dependent antiproliferative effect in Hep3B cells. After fluoxetine treatment at standard therapeutic doses (40 mg/day, 30 days) as an antidepressant, plasma levels reached around 1 μM (9), which is much lower than the concentrations necessary to induce apoptosis in Hep3B cells, as observed in our study. However, the fluoxetine tissue levels were up to 20-fold higher than in plasma, as reported for brain tissue, because of its lipophilic properties (17). Indeed, fluoxetine has been used for the treatment of mental disorders for extended periods of time at doses higher than 100 mg a day, without significant side-effects (18). Fluoxetine has a very broad safety range; serious side-effects in humans occur when administered at doses higher than 75-times the therapeutic doses for depression (19). Therefore, relatively high doses of fluoxetine, at concentrations shown to induce apoptosis in Hep3B cells in our study, could be used alone or as an adjunct treatment for cancer, with acceptable side-effects.
ROS are constantly generated and eliminated in biological systems and play important roles in a variety of normal biochemical functions and abnormal pathological processes (20). Mitochondrial damage causes ROS to be released from mitochondria, since mitochondria are the major source of ROS. On the other hand, overproduction of ROS causes an imbalance of pro- and antioxidative processes, thus creating a phenomenon known as oxidative stress that results in mitochondria damage, loss of MMP and the execution of apoptosis (21). In the present study, a high level of intracellular ROS production was observed in fluoxetine-treated Hep3B cells. In addition, our results showed that fluoxetine caused a decrease in MMP in Hep3B cells. Reduction of MMP is among the very first intracellular events preceding the execution phase of apoptosis via the mitochondria-mediated death pathway (22).

The evidence suggests that ROS are important signaling molecules regulating MAPK activity (23). Several studies have demonstrated that most anticancer agents and ionizing radiation that can induce ROS generation can also concurrently activate MAPK pathways in multiple cell types (24, 25). In addition, recent research has shown that phosphorylation of MAPK plays an important role in the apoptotic cascade of various cancer cell lines (26). We showed that fluoxetine causes an increase in phosphorylation of JNK and p38 MAPK but a decrease in phosphorylation of ERK1/2 in Hep3B cells. It is universally accepted that activation of ERK1/2 enhances cell proliferation (27), but activation of JNK and/or p38 MAPK induces cell death and apoptosis (28, 29). Furthermore, activation of p38 kinase has also been implicated in the mechanism of apoptosis triggered by chemotherapeutic agents (30). It was reported that fluoxetine inhibited the growth of lung (A549) and colon (HT29) cancer cells as a result of inhibition of ERK1/2 activation (6).

In several cell types, mitochondria also serve as a very efficient Ca\(^{2+}\) buffer, taking up substantial amounts of cytosolic Ca\(^{2+}\) at the expense of MMP. As a consequence of Ca\(^{2+}\) uptake, mitochondria can suffer Ca\(^{2+}\) overload, triggering the opening of the permeability transition pore (PTP), which is associated with apoptosis via the mitochondrial pathway, and necrosis due to mitochondrial damage (31). Opening of PTP has been shown to be promoted by thiol oxidation and inhibited by antioxidants, lending support for a role of ROS in pore opening (32). Furthermore, it has been demonstrated that mitochondrial Ca\(^{2+}\) uptake can lead to free radical production (33). In order to determine that the production of intracellular ROS or the accumulation of intracellular Ca\(^{2+}\) is essential for fluoxetine-induced apoptosis, we further investigated whether NAC, an oxidant-scavenger, and BAPTA-AM, an intracellular Ca\(^{2+}\) chelator, can protect against fluoxetine-induced modulation of MAPK. Our results showed that NAC and BAPTA-AM both inhibit the fluoxetine-induced decrease in antiapoptotic ERK1/2 protein and increase in proapoptotic JNK and p38 MAPK in Hep3B cells.

In view of the above arguments and the new data presented herein, we propose that fluoxetine-induced apoptosis results from the production of intracellular ROS, the accumulation of intracellular Ca\(^{2+}\), the loss of MMP, a decrease in antiapoptotic ERK1/2 protein and an increase in proapoptotic JNK and p38 MAPK proffers in Hep3B cells. Therefore, fluoxetine may be a potential antiproliferative agent against HCC, although further research must be carried out to fully investigate this possibility.

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References


Figure 4. The effects of fluoxetine on mitogen-activated protein kinases (MAPK) expression in Hep3B cells. The amount of MAPK was measured by western blot analysis. This image shows typical changes in MAPK levels after exposure to fluoxetine (A). The blots were quantified by scanning densitometry. The data are reported as a mean±SEM (n=4) for each group (B). ***p<0.005: vs. control, Bonferroni’s post-hoc test.

Figure 5. The effects of fluoxetine with/without N-acetylcysteine (NAC) and (acetoxymethyl)-1.2 bis (o-aminophenoxy) ethane N,N,N',N'-tetra-acetic acid (BAPTA-AM) on mitogen-activated protein kinases (MAPK) expression in Hep3B cells. The amount of MAPK was measured by western blot analysis. This image shows typical changes in MAPK levels after exposure to fluoxetine (A). The blots were quantified by scanning densitometry. The data are reported as a mean±SEM (n=4) for each group (B). ***p<0.005: vs. control; ###p<0.005: vs. control, Bonferroni’s post-hoc test.


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