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Molecules and Cells

Enhanced Cytotoxicity of 5-FU by bFGF through Up-Regulation of Uridine Phosphorylase 1

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Anti cancer agent 5-FU (Fluoro Uracil) is a prodrug that can be metabolized and then activated to interfere with RNA and DNA homeostasis. However, the majority of administered 5-FU is known to be catabolized *in vivo* in the liver where Dihydropyrimidine dehydrogenase (DPD) is abundantly expressed to degrade 5-FU. The biological factors that correlate with the response to 5-FU-based chemotherapy have been proposed to include uridine phosphorylase (UPP), thymidine phosphorylase (TPP), p53 and microsatellite instability. Among these, the expression of UPP is known to be controlled by cytokines such as TNF-α, IL1 and IFN-γ. Our preliminary study using a DNA microarray technique showed that basic fibroblast growth factor (bFGF) markedly induced the expression of UPP1 at the transcription level. In the present study, we investigated whether bFGF could modulate the expression of UPP1 in osteo-lineage cells and examined the sensitivity of these cells to 5-FU mediated apoptosis.

INTRODUCTION

Pyrimidine nucleotides are one of the two building blocks of nucleic acids and their biosynthesis is known to occur through a de novo pathway. On the other hand, a large portion of the pyrimidines are salvaged from the diet or from the breakdown of nucleic acids and nucleotides (Baynes and Dominiczak, 2005). The relative activity of de novo synthesis and the salvage pathway in the homeostasis of the nucleotide pools varies in different cells and tissues (Moyer et al., 1981).

Pyrimidine phosphorylases, which include Uridine Phosphorylase (UPP, UPase) and Thymidine Phosphorylase (TPase), catalyze either the phosphorolysis or the formation of (fluorinated) pyrimidine nucleosides. In the presence of available substrates, UPP plays an appreciable role in the activation of 5-FU and its prodrug 5′-deoxy-5-fluorouridine (5-DFUR)/capecitabine via anabolism of 5-FU through the pyrimidine salvage pathway or the phosphorolysis of 5-DFUR into 5-FU (Cao and Pizzorno, 2004).

The classical antineoplastic agent, 5-FU, is often used with newer chemotherapies such as IRINOTECAN and OXALIPLATIN to improve the response rates of 5-FU for advanced colorectal cancer from 10-15% to 40-50% (Douillard et al., 2000; Giacchetti et al., 2000; Johnston and Kaye, 2001). Through the enzymatic activity of uridine phosphorylase, orotate phosphoribosyltransferase and thymidine kinase, 5-FU is converted intracellularly to several active metabolites including fluoro(deoxy)uridine monophosphates (F(d)UMP), all of which interfere with RNA and DNA synthesis (Diasio and Harris, 1989; Kim et al., 2008). In contrast, dihydropyrimidine dehydrogenase mediated conversion of 5-FU to dihydrofluorouracil (DHFU) is the rate-limiting step of 5-FU catabolism in normal and tumor cells.

The expression of UPP in different tumor cell lines, such as Colon 26 and HCT-116 has been shown to be induced by TNF-α, IL-1α and IFN-α and γ, and vitamin D3 (Geng et al., 1991; Schwartz et al., 1998; Wan et al., 2006). A similar response to cytokines has been observed for thymidine phosphorylase (TPase) (el Kouni et al., 1990). Induction of UPP expression has also been reported in c-H-ras transformed NIH 3T3 cells. However, the underlying regulatory mechanism of UPP expression remains poorly characterized.

bFGF is knonw to be involved in various steps of biological processes, such as limb and nervous system development, wound healing, and tumor growth. In addition to binding to FGF receptors, it also binds to extracellular heparins to elicit broad mitogenic and angiogenic activity in a variety of cell types. It has been shown that upon the activation of tyrosine kinase activity through the interaction between bFGF and FGF receptors, a cascade of downstream signaling proteins such as ERK, PI3K and Rac1 participate in the epithelial-mesenchymal transition, rearrangement of cytoskeleton, cell proliferation and cell survival (Longley et al., 2003).

Our study revealed that the expression of UPP1 was induced by bFGF at the transcriptional level. Given that UPP1 is an important enzyme that can metabolize the anticancer agent (5-FU) into an active drug, we sought to investigate whether bFGF

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Table 1. Specific induction of UPP1 expression by bFGF

	Mock	bFGF	BMP2	bFGF + BMP2	TNF-a
Uridine phosporylase 1 (NM_009477)	-1.14	82.58	1.10	26.78	1.96
Uridine-cytidine kinase 2 (NM_030724)	-1.13	1.16	1.1	-1.09	1.06
Uridine monophosphate synthase (XP_001169057)	-1.02	1.21	1.01	1.33	-1.04
Thymidylate synthase (NM_021288)	-1.16	1.03	1.09	1.14	1.30

The expression of genes involved in pyrimidine metabolism is analyzed by DNA microarray technique and represented as relative numbers over those of mock treated controls. The numbers indicate fluorescent intensity.

can up-regulate the expression of UPP1 in C2C12 pre-myoblast cells and thus sensitize the cells to 5-FU mediated cell apoptosis.

MATERIALS AND METHODS

Cell culture and reagents

Mouse chondroprogenitor ATDC5 cells and pluripotential mesenchymal precursor cells-C2C12 were maintained in DMEM (Invitrogen, USA) containing 5% and 20% FBS, respectively, (Hyclone, USA) plus 50 units/ml Penicillin and 50 μg/ml Streptomycin (P/S) (Invitrogen, USA). Approximately 5×10^4 cells/cm^2 were plated in the same medium described above for ATDC5 cells or in low serum (2% FBS) medium for C2C12 cells before cytokine stimulation. Total RNAs were prepared using an RNAeasy kit according to the manufacturer's instructions (QIAGEN, USA) and 5 ug total RNA was sent to Macrogen (Korea) for mouse Illumina DNA microarray analysis. The details of this experiment are described in a previous publication (Lee et al., 2008).

Osteosarcoma cell lines of Hos, MG-63 were purchased from Korea Cell Line Bank, Seoul, Korea and maintained in DMEM, MEM media containing 10% FBS and P/S.

bFGF and BMP2 were purchased from R & D Systems (USA) and prepared in solution (10 ug/ml as 1000× stock) and maintained at 4°C until use. Anti-Flag antibody, 5-Fluorouracil (5-FU) and 5-Fluoro-5′ deoxyuridine (5′-dFUrd) were purchased from Sigma Aldrich Co. (USA). PDTC and LY294002 were purchased from Calbiochem (USA) and reconstituted in DMSO as 1000× stock concentrations of 10 mg/ml and 40 mg/ml respectively. Gene specific primers for RT-PCR analysis of mouse UPP1 (forward primer; 5′-GGT TCA GGA GTT GGT GCA GT-3′ & reverse primer; 5′-GCA AAC ACC GAA GAT TCC AT-3′) and UPP2 (forward primer; 5′-GCACACTGCTG-TGATGTT-3′ & reverse primer; CTGCTACAGTTGAACAGA-3′) were purchased from Bioneer (Korea).

RNA isolation and synthesis of cDNA for RT-PCR analysis

Total RNAs were purified using Qiagen's RNAeasy kit as described above. For the preparation of cDNAs, a total of 5 μg of whole RNAs were incubated with oligo dT primer and Super-Script™ First-strand synthesis system (Invitrogen, USA) to generate single strand cDNAs and the reaction was finished by incubating with RNase H for 20 min at 37°C. Using the cDNAs and gene specific primers, 35 cycles of PCR (95°C/30 s → 55-60°C/1 min → 72°C/1 min) were carried out and 5 μl out of the total 25 μl reaction was analyzed by agarose gel electrophoresis.

FACS analysis of 5-FU mediated apoptosis

Flow cytometry was performed to investigate the cell cycle and death profiles of C2C12 cells that were treated with either 5-FU/5-dFUrd and/or bFGF. Both floating and adherent cells were pelleted by centrifugation at 3,200 rpm and 4°C. The pellet was then washed twice with PBS before fixing the cells in 75% Ethanol and staining in Propidium Iodide (Sigma Chemical Co., USA). Stained cells were immediately analyzed by a flow cytometer (Becton Dickson, USA). The percentage of cells in each phase of the cell cycle was analyzed and those in sub-G1 are represented as the percentage.

RESULTS

bFGF increases the expression of uridine phosphorylase 1 gene

When the ATDC5 chondroprogenitor cells were incubated with bFGF and/or BMP2 cytokines, we observed that uridine phosphorylase 1 was noticeably induced by bFGF treatment, while the expression of the other 3 genes (Uridine-cytidine kinase 2, orotate phosphoribosyl transferase, Thymidylate Synthase) were unaltered (Table 1). RT-PCR analysis of UPP1 expression in C2C12 cells demonstrated that a bFGF concentration of 5-10 ng/ml induced UPP1 expression in C2C12 cells (Fig. 1A). In addition, bFGF elicited a similar response in ATDC5 cells. The expression of the mouse UPP2 isoform, which has 64% identity and 78% similarity with UPP1, appeared to be altered by bFGF treatment but its mRNA level was quite low compared to that of UPP1 (Fig. 1A). In addition, osteosarcoma cell lines, such as Hos and MG-63, were tested and found to be responsive to bFGF stimulation in a dose dependent manner. The additional band, which was slightly above the expected size, was shown to be a DNA fragment that contained an additional intron DNA sequence in the middle (Fig. 1B).

bFGF sensitizes the apoptosis of C2C12 premyoblasts induced by 5-FU and 5-dFUrd

5-FU and 5-DFUR anticancer agents are reported to induce cell death either by interfering with RNA and DNA homeostasis or through the induction of the p53 tumor suppressor protein. Over the time window of 12, 24, 36 and 48 h following bFGF and/or 5-FU / 5-DFUR treatment, C2C12 cell death was determined by counting the number of cells in the Sub-G1 phase with FACS. As presented in the upper panel of Figs. 2A and 2B, DMSO and/or bFGF did not cause any appreciable cell death over the entire test period. However, 1 microM of 5-FU dramatically induced cell death approximately 36 to 48 h following treatment, resulting in a total cell death of about 70%. Interestingly, 10 microM of 5-FU was found to exhibit a high cytotoxicity that was independent of bFGF treatment. As presented in the lower panel of Fig. 2A, the combination of 10 ng/ml of bFGF and 1 uM 5-FU was effective at inducing apoptosis in the majority of C2C12 cells after 48 h.

When C2C12 cells were treated with 5-DFUR for 12-48 h with or without bFGF, the overall apoptosis profile was similar to that observed for 5-FU treated cells (the lower panel, Fig. 2A). However, the cytotoxicity profile according to the dosage of the

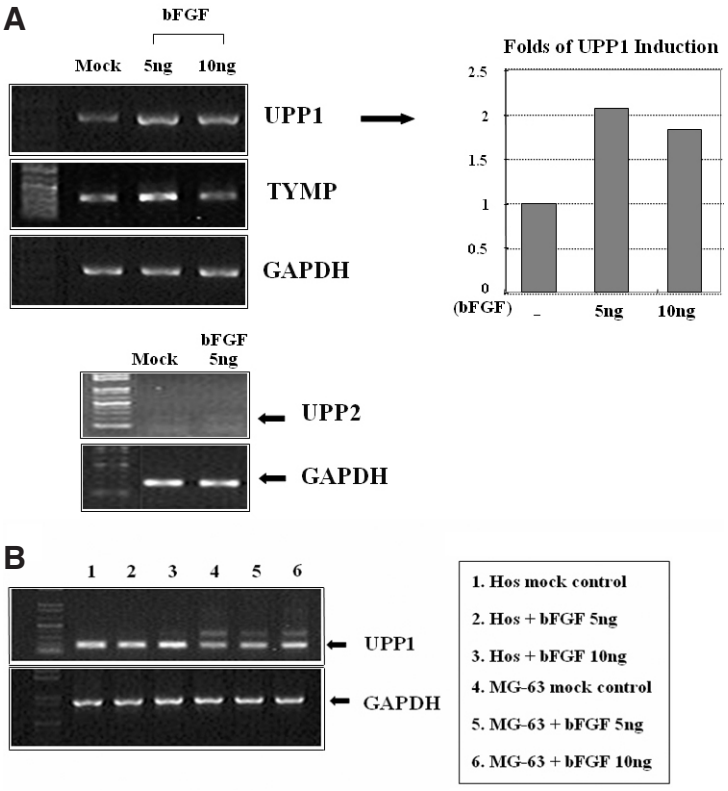

Fig. 1. Expression of UPP1 gene in C2C12 and Osteosarcoma cells is increased in response to bFGF. Expression of mouse UPP1, UPP2, Tymp and GAPDH genes was checked by RT-PCR using a pair of the gene specific primers and cDNAs prepared from total RNAs of C2C12 cells. Expression of GAPDH gene was checked as an internal control.

drug was somewhat different between the groups of cells treated with 5-FU and 5-DFUR. The C2C12 cells tolerated much higher concentrations of 5-DFUR (100 microM). The 24 h after treatment with 10 microM 5-FU, approximately 50% of the cells were in the process of apoptosis, while it took about 42 h to reach this point in the cells treated with 100 microM 5-DFUR.

siRNA mediated down-regulation UPP1 impairs bFGF and 5-FU mediated cell death

In order to test whether the downregulation of UPP1 eliminates the effect of bFGF on 5-FU mediated cell death, the efficiency of several commercially available siRNAs against mouse UPP1 were tested. As shown in panels J and K of Fig. 3, one UPP1 siRNA was found to inhibit UPP1 expression by 40% in the C2C12 cells. C2C12 cells transfected with the UPP1 or mock (= scrambled) siRNA were stimulated with bFGF and then 5-FU before FACS analysis of PI stained cells. As shown in panels A through I of Fig. 3, the level of cell death in cells treated with the UPP1 specific siRNA was approximately 10% lower than the cells treated with the scrambled siRNA control. Interestingly, the UPP1 specific siRNA inhibited 5-FU mediated cell death in the absence of bFGF by a margin of 10% compared to that of the scrambled siRNA, which may reflect the level of reduced expression of endogenous UPP1. It is worth noting that in this experiment the lipofectamine transfection reagent by itself appeared to sensitize the cells for apoptosis to a certain degree.

NFκB is responsible for the UPP1 induction by bFGF

The family of FGF cytokines is well known for their pleiotrophic effect on cellular signaling. In another study conducted by our research group, NFκB and PI3K signaling pathways were found to be important for the induction of the antiapoptotic Bcl2-A1 gene in C2C12 cells (manuscript in preparation). Along this line, we investigated whether these two effectors of bFGF signaling were also important in UPP1 expression. As shown in Fig. 4, the induction of UPP1 transcription by bFGF was suppressed in the presence of 5-10 uM of a chemical inhibitor of NFκB (PDTC). In contrast, an inhibitor of PI3K (LY294002) failed to inhibit UPP1 expression in the presence of bFGF, demonstrating that UPP1 induction by bFGF is dependent on NFkB activity rather than PI3K activity. However, it remains to be investigated whether other signaling pathway(s) downstream of FGF receptors are also required for UPP1 expression.

DISCUSSION

Although 5-FU has been used for more than four decades in the treatment of early to advanced stages of colorectal cancer, its overall response rate in advanced colorectal cancer is quite limited [14; 16]. Thus, strategies to modulate the anticancer activity of 5-FU by decreasing the degradation or increasing the activation of 5-FU have been developed (Longley et al., 2003). Interferons (IFNs), Leukovorin (LV), Methotrexate or inhibitors of dihydropyrimidine dehydrogenase have been reported to generate significantly greater response rates compared with single agent 5-FU, but this did not result in improved overall survival (Greco et al., 1996; Longley et al., 2003; Seymour et al., 1996; Voboril et al., 2004).

In our study, UPP1 was initially identified from DNA microarray analysis as a candidate gene whose expression might be controlled by bFGF. In the present study, we provide evidence to support this early finding. RT-PCR analysis demonstrated that bFGF increases the expression level of UPP1 in osteolineage cell lines such as ATDC5 and C2C12. In addition to UPP1, UPP2 isozyme and thymidine phosphorylase (Tymp) also appeared to be up-regulated at the transcription level by bFGF.

When challenged with 1 uM of 5-FU, bFGF increased the population of cells at the sub-G1 phase (apoptotic cells) com-

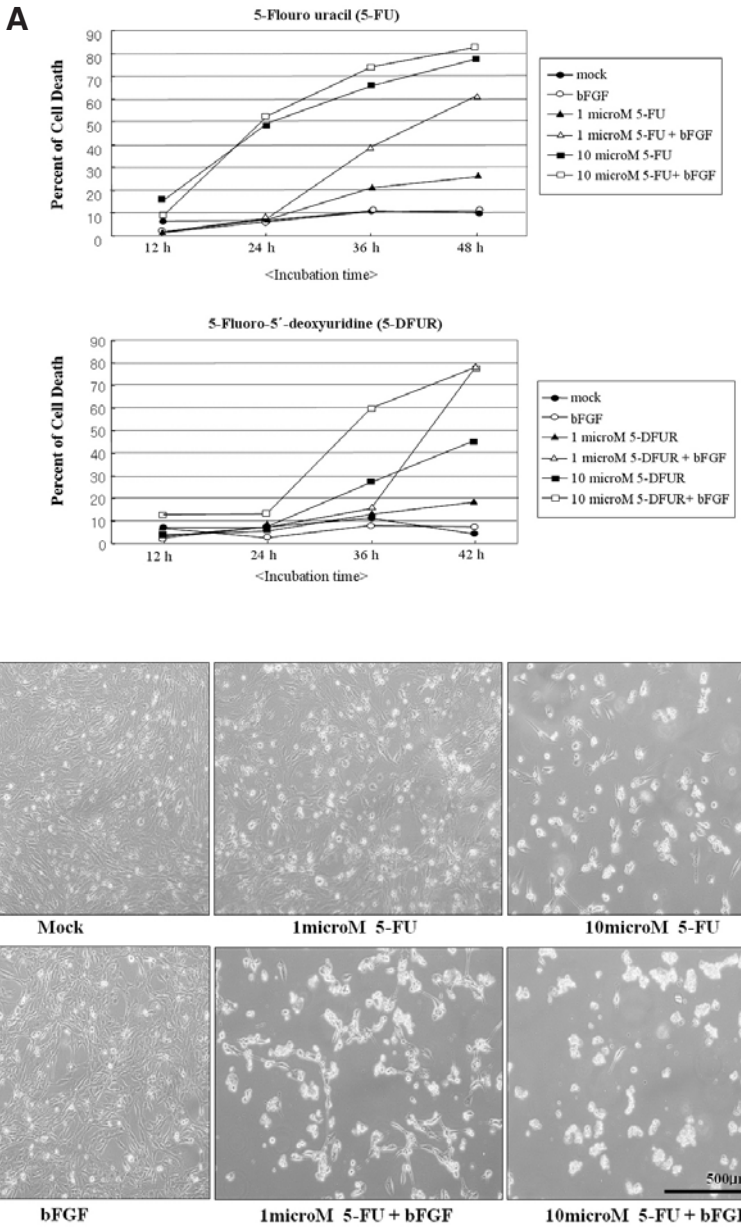

Fig. 2. After treatment of C2C12 cells with bFGF and/or 5-FU/5-DFUR for the indicated time and concentrations, the percent of cell death was checked by FACS method and represented in the Y-axis. (A) bFGF sensitizes C2C12 apoptosis induced by 5-FU/5-DFUR. (B) Light microscopic analysis of C2C12 apoptosis at 48 h after the treatment of 5-FU and/or bFGF.

pared to that of 5-FU or bFGF treated cells. bFGF and 5-FU mediated cell death was more evident between 36 and 48 h after treatment. The high concentration of 5-FU (10 uM) appeared toxic to C2C12 cells over the entire time period regardless of bFGF treatment. In contrast, 5-DFUR was less toxic since C2C12 cells tolerated up to 100 uM of 5-DFUR at which bFGF increased the apoptotic rate of 5-DFUR to as early as 24 h after treatment. Osteosarcoma cell lines such as Hos and MG-63, which have a potential to differentiate into mature osteogenic-lineage cells, were tested with 5-FU and/or bFGF. The 72 to 96 h after 5-FU treatment, both cell lines exhibited a dramatic cytotoxicity in the presence of bFGF (data not shown). This phenomenon may be due to the fact that cell growth rates are inherently different for different cell lines as potentially is the sensitivity to bFGF stimulation.

Along this line, we tried to overexpress UPP1 cDNA in C2C12 cells and then expose them to 1 microM of 5-FU. In these experiments, we found that UPP1 overexpression alone did not confer an increased sensitivity to 5-FU compared to that of the mock control (data not shown). Although the molecular basis of this finding remains unclear, as indicated in the schematic diagram, there are several enzymes, including DPD, UPP, Tymp, and OPRT, that can metabolize 5-FU and also be up-regulated to a varying degree by bFGF. The sum of some or all of these enzymatic activities might be responsible for the bFGF mediated synergism with 5-FU or 5-dFUrd for inducing cell death. Translational upregulation of Tymp expression by 5-FU was reported and this might have resulted in an increased metabolism of 5-FU, irrespective of the UPP1 expression level.

Nevertheless, the siRNA against UPP1 decreased UPP1 expression and under this condition, cells were more vulnerable to 5-FU cytotoxicity, indicating that UPP1 may be a downstream target of bFGF signaling that enhances the cytotoxicity of 5-FU. UPP1 specific chemical inhibitors, such as PTAU, have been reported (Al Safarjalani et al., 2005) and the use of these specific inhibitors may be helpful in further defining the

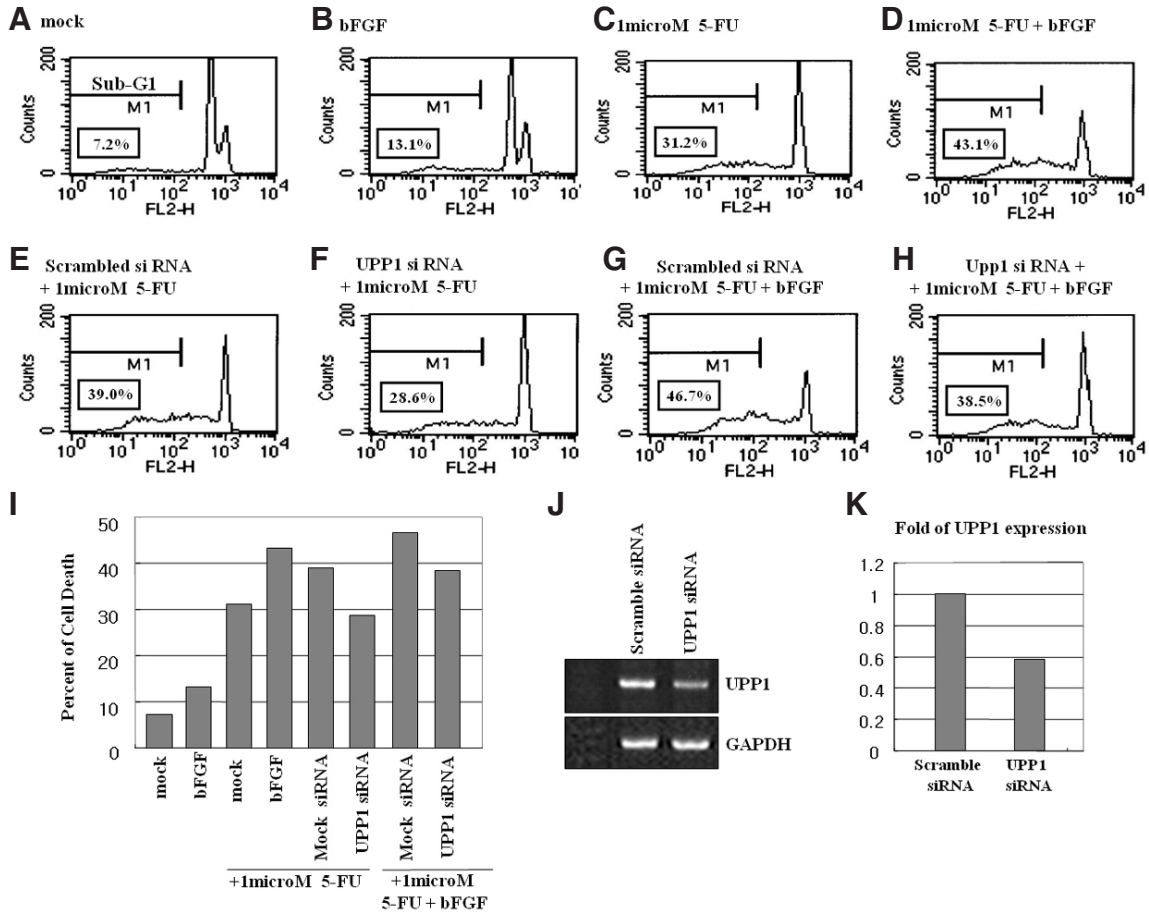

Fig. 3. siRNA mediated down-regulation of UPP1 impairs the cell death induced by bFGF and 5-FU.

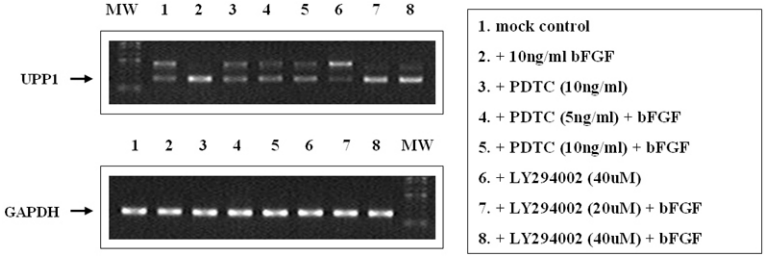

Fig. 4. Inhibition of NFkB impaired the UPP1 induction by bFGF in ATDC5 cells. The ATDC5 chondroprogenitor cells were pretreated for an hour with each chemical inhibitor before bFGF treatment for 8 h. Total RNAs were prepared from each sample for synthesis and RT-PCR. The DNA band indicated with the arrow represents the UPP1 DNA fragment of the expected size (about 200 bp).

precise role of UPP1 as one of the targets of bFGF activity, which increases cellular sensitivity to 5-FU toxicity.

The chemical inhibitor experiments indicated that the induction of UPP1 by bFGF was dependent on NFκB activity, but not PI3K activity. Our previous study using ATDC5 chondroprogenitor cells demonstrated that NFκB and PI3K were critical in the induction of antiapoptotic Bcl2-A1 expression (manuscript in preparation). Thus, the induction of UPP1 and Bcl2-A1 by bFGF appeared to be mediated through different signaling pathways.

The results presented in this study suggest that bFGF may be used in combination with 5-FU to increase the cytotoxicity against cancer cells. However, considering the fact that FGFs are potent mitogens, morphogens, and inducers of angiogenesis, this hypothesis needs to be carefully confirmed in animal models.

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