

Modulation of Uridine Phosphorylase Gene Expression by Tumor Necrosis Factor- α Enhances the Antiproliferative Activity of the Capecitabine Intermediate 5'-Deoxy-5-fluorouridine in Breast Cancer Cells

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ABSTRACT

Uridine phosphorylase (UPase) has been shown to play an important role in the antineoplastic activity of 5-fluorouracil (5-FU) and in the anabolism of its oral prodrug, capecitabine, through the conversion of 5'-deoxy-5-fluorouridine (5'-DFUR) into 5-FU. In this study, we investigated the effect of tumor necrosis factor- α (TNF- α) on UPase gene expression and 5'-DFUR antiproliferative activity and elucidated the involved signal transduction pathway. Our data indicate that TNF- α significantly induced UPase mRNA expression and its enzymatic activity in EMT6 murine breast cancer cells, leading to an enhanced cytotoxicity of 5'-DFUR. This is further confirmed by an increased incorporation of 5'-DFUR-originated 5-FU nucleotides into nucleic acids. To clarify the mechanism of TNF- α -induced UPase expression, we first observed the effect of

TNF- α on the UPase promoter activity with a series of 5'-deleted promoter-luciferase constructs. Transient transfection analysis showed that the TNF- α -inductive pattern in EMT6 cells was consistent with the presence of a nuclear factor- κ B (NF- κ B) binding element (–1332/–1312 bp) in the UPase promoter region. Furthermore, electrophoretic mobility shift assays, supershift, and cotransfection assays revealed that the activation of p65 was responsible for UPase induction by TNF- α . Finally, the induction of UPase by TNF- α could be suppressed by PS-341, a NF- κ B inhibitor. In summary, TNF- α efficiently induces UPase gene expression through a NF- κ B subunit p65-dependent pathway enhancing cell sensitivity to 5'-DFUR. The elucidation of this regulation mechanism may aid in the clinical use of 5-FU-based chemotherapy.

Uridine phosphorylase (UPase), which can reversibly convert uridine to uracil, plays a crucial role in the activation of fluoropyrimidines such as 5-fluorouracil (5-FU), its prodrug 5'-deoxy-5-fluorouridine (5'-DFUR), and in "the uridine rescue" of normal tissues (Pizzorno et al., 2002; Cao and Pizzorno, 2004). Our previous studies have shown that the abrogation of UPase activity in murine embryonic stem cells significantly reduced the cell sensitivity to 5-FU, 5'-DFUR, and phosphonacetyl-L-aspartic acid compared with wild-type embryonic stem cells (Cao et al., 2002). In UPase $-/-$ mice, the loss of UPase activity resulted in greater increase in

uridine levels in plasma and tissues, reduction of 5-fluorouracil host toxicity, and alteration of the anesthetic effect of pentobarbital (Cao et al., 2005). Compared with adjacent normal tissues, UPase activity has been shown to be elevated in various human solid tumors, including breast carcinomas (Liu et al., 1998; Kanzaki et al., 2002), colorectal carcinomas (Katsumata et al., 2003), oral squamous carcinomas (Maehara et al., 1990; Miyashita et al., 2002), melanoma tissue (Leyva et al., 1983), and lung adenocarcinomas (Maehara et al., 1990), providing a rationale for targeting this enzyme for tumor-specific modulation of fluoropyrimidines activity. Very little is currently known about the regulatory mechanisms of UPase gene expression. Some investigators have indicated that cytokines such as tumor necrosis factor- α (TNF- α), interleukin-1 α (IL-1 α), and interferon- α and - γ (IFN- α and - γ) can induce UPase gene expression with consequent improvement of 5-FU and 5'-DFUR antiproliferative activity

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ABBREVIATIONS: 5'-DFUR, 5'-deoxy-5-fluorouridine; EMSA, electrophoretic mobility shift assay; 5-FU, 5-fluorouracil; IFN, interferon; IL, interleukin; NF- κ B, nuclear factor- κ B; TNF- α , tumor necrosis factor- α ; TPase, thymidine phosphorylase; UPase, uridine phosphorylase; PS-341, bortezomib; bp, base pair(s); BAU, benzylacyclouridine.

(Eda et al., 1993), but the molecular regulatory mechanism has not been described extensively.

Uridine has been used as a biochemical modulator to reduce 5-FU host toxicity and escalate 5-FU dosage to improve the therapeutic index of this pyrimidine antimetabolite (Peters, 1988; Martin et al., 1989; Seiter et al., 1993). This has been based on the hypothesis that the host toxicity of 5-FU is mainly related to RNA dysfunction caused by 5-FU incorporation, whereas its antitumor activity is ascribed to thymidylate synthase inhibition and resultant DNA damages (Kufe and Major, 1981; Major et al., 1982; Longley et al., 2003). Unfortunately, this potentially useful clinical practice has been limited by the dose-related toxicities of uridine, because the administration of large doses of the pyrimidine nucleoside are required to maintain therapeutic plasma uridine concentration because of its rapid clearance catalyzed by UPase. In this study, we confirmed the induction of UPase gene expression by TNF- α , elucidated the regulatory mechanisms, and evaluated the effect of UPase modulation on 5'-DFUR antiproliferative activity, an intermediate in the enzymatic activation of capecitabine, an oral prodrug of 5-FU recently approved by the FDA for the treatment of advanced breast and colon cancers (Miwa et al., 1998; Blum, 1999; Hoff et al., 2001; Van Cutsem et al., 2001).

Materials and Methods

Reagents and Antibodies. Bacteria-derived mouse recombinant TNF- α with a specific activity of $>1 \times 10^7$ U/mg, IL-1 α with a specific activity of $>5 \times 10^9$ U/mg, and IFN- γ with a specific activity of $>1 \times 10^7$ U/mg were purchased from Chemicon, Inc. (Temecula, CA). The following polyclonal or monoclonal antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA): anti-p65, anti-p50, and anti-c-Rel. I κ B inhibitor PS-341 is a product of Millennium Pharmaceuticals (Cambridge, MA). 5'-DFUR was purchased from Sigma-Aldrich (St. Louis, MO) and dissolved in water at a stock concentration of 50 mM. 6-[3 H]5'-DFUR (3.2 Ci/mmol) and 6-[3 H]uridine (16.2 Ci/mmol) were obtained from Moravsek Biochemicals Inc. (Brea, CA).

Cells and Culture Conditions. EMT6 cells, a murine breast cancer cell line (kindly provided by Dr. Sarah Rockwell, Yale University, New Haven, CT), were maintained in Waymouth's MB 752/1 medium (Invitrogen, Carlsbad, CA) with 15% fetal bovine serum, 100 U/ml penicillin, and 100 U/ml streptomycin (Sigma) at 37°C in humidified atmosphere of 5% CO₂/95% air.

Northern Blot Hybridization. Total RNA was extracted with TRIzol reagents (Invitrogen, Carlsbad, CA). Total RNA, 15 μ g/lane, was electrophoretically separated on a 1.2% formaldehyde-agarose gel, blotted to Hybond-N⁺ nylon membranes (GE Healthcare, Little Chalfont, Buckinghamshire, UK), and fixed at 80°C for 2 h. The membranes were subsequently hybridized with ³²P-labeled mouse UPase cDNA and β -actin cDNA probes (Ambion, Austin, TX) in an ULTRAhyb ultrasensitive hybridization buffer (Ambion) for 18 h, washed, and exposed to X-ray films at -70°C. Bands in the autoradiogram were quantitated by means of filmless autoradiographic analysis. The UPase levels were normalized to that of β -actin levels in each lane to correct for the amount of total RNA loaded.

UPase Enzymatic Activity Assay. UPase enzymatic activity was measured by the conversion of the natural substrate uridine to uracil using thin-layer chromatographic separation. At the same time, activities of thymidine phosphorylase (TPase), orotate phosphoribosyl transferase, and uridine kinase were analyzed as described previously (Cao et al., 2002).

In Vitro Cytotoxicity Assay. The antiproliferative activity of 5'-DFUR was assessed by cell proliferation kit II [sodium 3'-[1-ph-

nylamincarbonyl]-3, 4-tetrazolium]-bis (4-methoxy-6-nitro) benzene sulfonic acid hydrate]. In brief, 1000 EMT6 cells/well were plated in 96-well tissue culture plates. The following day, the cells were pretreated with 20 ng/ml TNF- α for 24 h (inducing UPase expression) followed by the addition of different concentrations of 5'-DFUR to the medium for an additional 72 h. Each concentration point was replicated in eight wells, and all experiments were conducted minimally in triplicate.

Incorporation of [3 H]5'-DFUR into Nucleic Acids. Cells (1×10^5 /well) were plated in six-well plates. After overnight incubation, the cells were pretreated with 20 ng/ml TNF- α for 24 h, followed by the addition of 30 or 100 μ M 5'-DFUR with 5 μ Ci/well of [3 H]5'-DFUR for an additional 4 h. DNA and total RNA were extracted at the end of the treatment with TRIzol reagents (Invitrogen) according to the manufacturer's instructions. Purified RNA and DNA were dissolved in 400 μ l of distilled water, respectively. The amount of incorporated 5'-DFUR anabolites (pmol/ 10^6 cells) was calculated after determining the specific activity 5'-DFUR relative to each experiment.

Transfection and Luciferase Assays. The transfection was conducted in triplicate in six-well plates. Approximately 10^5 cells/well were seeded for 24 h before transfection. Two micrograms of supercoiled plasmid DNA (5'-deleted mouse UPase promoter constructs, p65/pc-DNA-3, p50/pc-DNA-3, and c-rel/pc-DNA-3) and an internal control (pRL-TK plasmid) were cotransferred into cells using LipofectAMINE reagent (Invitrogen). Vector-alone transfected cells were used as a negative control. The cells were incubated in transfection buffer (serum-free medium) for 3 h and then incubated in Waymouth's medium with 15% fetal bovine serum. After 24 h, the cells were treated with or without 20 ng/ml TNF- α for an additional 24 h. Firefly luciferase and the control *Renilla reniformis* luciferase were detected using a dual luciferase detection kit (Luciferase Assay System; Promega, Madison, WI).

Electrophoretic Mobility Shift Assay. Nuclear extracts were prepared as described previously in detail (Lin et al., 1998). Electrophoretic mobility shift assay (EMSA) was conducted according to a modified protocol provided by the manufacturer (Promega). Double-stranded DNA probes containing the NF- κ B element located at -1339 to -1320 bp of the UPase promoter [5'-CATTGTTGGGTTT-TCCCTTC-3' (potential binding region is in bold)] and the c-Rel element located at -71 to -58 bp (5'-AGGAAGGACCCTGA-3') were synthesized by the Oligonucleotide Synthesis Facility of the Department of Pathology of the Yale University School of Medicine. Consensus NF- κ B oligonucleotides were provided by the manufacturer (Promega). For binding specificity determination, a 100-fold excess of unlabeled or mutant NF- κ B and c-Rel oligonucleotides was added as specific or nonspecific probes in the binding reaction. For the supershift assays, nuclear extracts were incubated with antibodies against p50, c-Rel, or p65 of NF- κ B for 45 min on ice before the labeled probes were added. The reaction products were separated onto a 6% nondenaturing polyacrylamide gel and visualized by autoradiography.

Statistical Analysis. Data shown in the figures and tables are reported as the mean \pm S.D. of at least three experiments. Differences between groups were compared using the Student's *t* test. Differences resulting in *P* values <0.05 were considered to be statistically significant.

Results

Induction by TNF- α of UPase Gene Expression in Murine Breast Tumor Cells. Watanabe et al. (1995) reported that the expression of the UPase gene was elevated in various human cancer cell lines by a combination of the three cytokines, TNF- α , IL-1 α , and IFN- γ , and vitamin D₃. To investigate the molecular mechanism, we initially treated mouse mammary EMT6 cells with individual cytokines, in-

cluding TNF- α , IL-1 α , or IFN- γ alone. UPase mRNA expression and enzymatic activity were determined to estimate the inductive efficiency of each cytokine. As shown in Fig. 1, A and B, TNF- α (20 ng/ml) caused a significant increase of UPase mRNA level, with consequent elevated enzymatic activity, IFN- γ alone induced a significant but variable increase of UPase gene products, and IL-1 α produced only a minimal change in UPase expression. The concurrent increase in both UPase mRNA and enzyme activity indicates that TNF- α stimulates UPase activity at the transcriptional level. Analysis with different concentrations of TNF- α revealed that the UPase stimulation by TNF- α was dose-dependent, with maximal induction at 40 ng/ml (Fig. 1C). However, the death of EMT6 cells exposed to TNF- α was also dose-dependent. We observed that the majority of the cultured cells would die in medium containing 40 ng/ml of TNF- α alone during 72 h of exposure. Therefore, we decided to choose 20 ng/ml as the standard concentration of TNF- α for all our studies because it caused only minimal cell death. The activity levels of the other enzymes involved in the metabolism and activation of 5-FU, including TPase, uridine kinase, and orotate phosphoribosyl transferase, were at or below the limit of detection and were not affected when the cells were treated with individual cytokines (data not shown).

Enhanced Sensitivity to 5'-DFUR of EMT6 Cells Treated with TNF- α . Capecitabine has no antiproliferative activity on its own before its activation in the liver to 5'-DFUR and subsequent metabolism to 5-FU by pyrimidine nucleoside phosphorylases, UPase, and TPase (Ishitsuka et al., 1980; Armstrong et al., 1983). Therefore, we tested di-

rectly the effect of cytokines on the antiproliferative activity of 5'-DFUR in EMT6 cells. After pretreatment with TNF- α (20 ng/ml) for 24 h (induction of UPase expression), the sensitivity of EMT-6 cells to 5'-DFUR significantly increased by 2.5-fold with the IC₅₀ value that decreased from 25 to 10 μ M (Fig. 2). This difference in sensitivity was consistent with an increased incorporation of 5'-DFUR anabolites into RNA and DNA (Table 1) compared with nontreated EMT6 cells using both 30 and 100 μ M concentrations of the fluorinated antimetabolite. However, when the cells were simultaneously exposed to 5'-DFUR and the specific UPase inhibitor BAU (50 μ M), the increased sensitivity and incorporation of 5'-DFUR anabolites were abolished, indicating that the increased antiproliferative activity is directly associated with the induction of UPase by TNF- α .

Identification of Functional NF- κ B Binding Site in UPase Promoter. To understand the molecular mechanism of TNF- α -induced UPase gene expression, we performed a motif analysis of the UPase promoter. The results indicated the presence of two NF- κ B putative binding elements, sequence 5'-GGGTTTTC-3' at -1332 to -1323 bp and 5'-GGGTCCTTC-3' at -70 to -62 bp. To test the functionality of these elements, we first observed the relationship between the kinetics of NF- κ B activation (indicated by EMSA) and UPase induction by TNF- α . As shown in Fig. 3A, TNF- α treatment resulted in a biphasic NF- κ B activation in EMT6 cells with a rapid activation of NF- κ B occurring within 15 min and a maximum peak at 30 min, as reported previously in other cell types (Han and Brasier, 1997; Hoffmann et al., 2002). The second peak of NF- κ B activity took place between 8 and 12 h from the initial exposure to TNF- α . The enzymatic

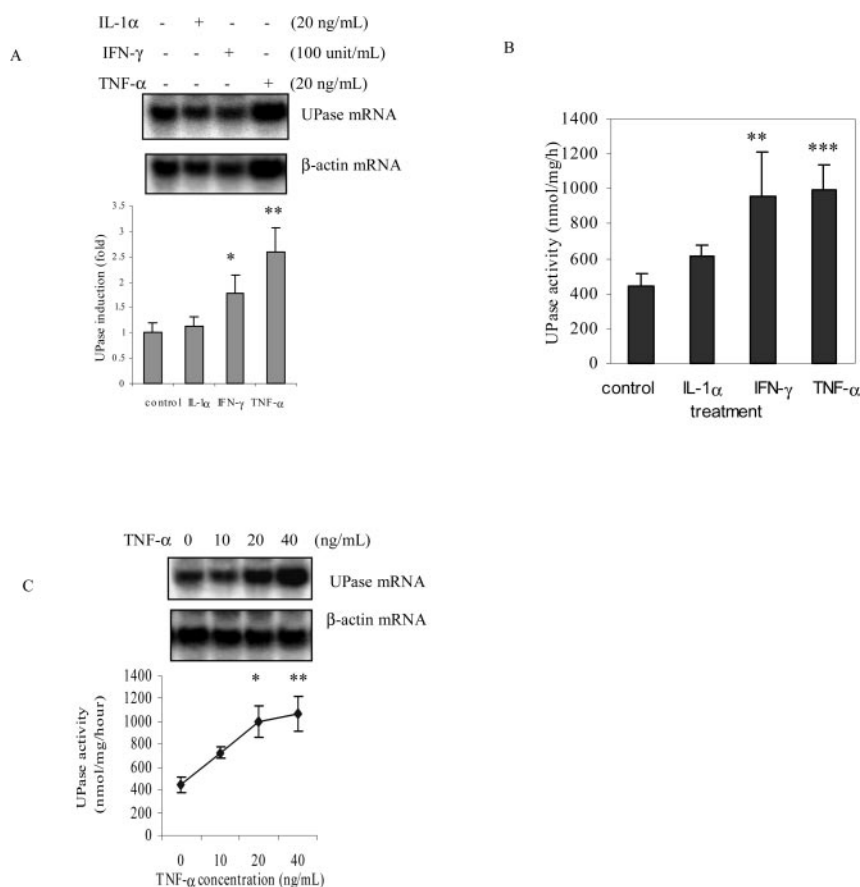


Fig. 1. Effects of IL-1 α , IFN- γ , and TNF- α on UPase gene expression and enzymatic activity in EMT6 cells. A, EMT6 cells were treated with IL-1 α (20 ng/ml), IFN- γ (100 U/ml), and TNF- α (20 ng/ml) for 24 h. Total RNA was extracted and analyzed (15 μ g/lane) for UPase mRNA levels by Northern blot. Signal intensities were quantified by densitometry and normalized to the β -actin mRNA level and expressed as fold over control. B, UPase activity was analyzed in EMT6 cell treated with IL-1 α , IFN- γ , and TNF- α respectively. C, EMT6 cells were treated with different concentrations of TNF- α (0, 10, 20, and 40 ng/ml) for 24 h. UPase mRNA expression and enzymatic activity were analyzed. Results were expressed as mean value \pm S.D. ($n = 4$). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ compared with control. The blots represent single representative experiments, and the graphs are the mean \pm S.D. of at least three experiments.

activity of UPase followed a similar biphasic pattern with a rapid induction within 15 min after exposure to TNF- α that reached a maximum peak of activity at 30 min, indicating the relationship between NF- κ B activation and UPase induction. The elevated UPase activity was sustained for 24 h (Fig. 3B), possibly because of the longer half-life of the UPase protein.

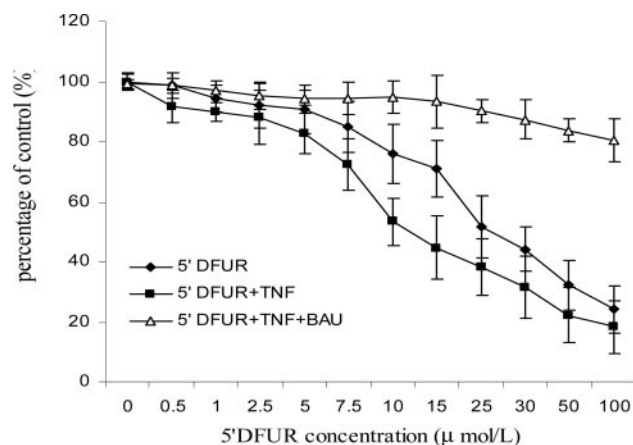


Fig. 2. Effect of TNF- α on the antiproliferative activity of 5'-DFUR in EMT6 cells. EMT6 cells were pretreated with 20 ng/ml TNF- α for 24 h followed by the addition of different concentrations of 5'-DFUR with or without 50 μ M BAU to the culture for another 72 h. The amount of viable cells was determined by cell proliferation kit II (Roche Diagnostics, Indianapolis, IN).

TABLE 1

TNF- α exposure enhances the incorporation of 5'-DFUR into nucleic acids

EMT6 cells were incubated with 20 ng/ml TNF- α for 24 h followed by exposure to 5'-DFUR (30 and 100 μ M) with or without 50 μ M BAU for an additional 4 h. Total RNA and DNA were extracted and analyzed for radioactivity as indicated under *Materials and Methods*. Each value represents the mean of three separate experiments.

Treatment	RNA (<i>n</i> = 3)		DNA (<i>n</i> = 3)	
	<i>pmol / 10⁶ cells</i>			
5'-DFUR, $\mu\text{mol/l}$	30	100	30	100
5'-DFUR	8.56 \pm 1.00	18.49 \pm 0.70	1.06 \pm 0.05	8.63 \pm 0.30
5'-DFUR + TNF	31.61 \pm 4.28, <i>P</i> < 0.001	48.44 \pm 3.29, <i>P</i> < 0.001	1.99 \pm 0.07, <i>P</i> < 0.05	13.5 \pm 0.61, <i>P</i> < 0.05
5'-DFUR + TNF + BAU	0.03 \pm 0.01, <i>P</i> < 0.001	0.08 \pm 0.01, <i>P</i> < 0.001	0.02 \pm 0.00, <i>P</i> < 0.001	0.03 \pm 0.00, <i>P</i> < 0.00

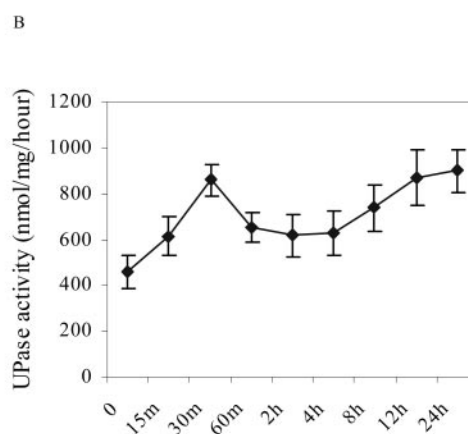
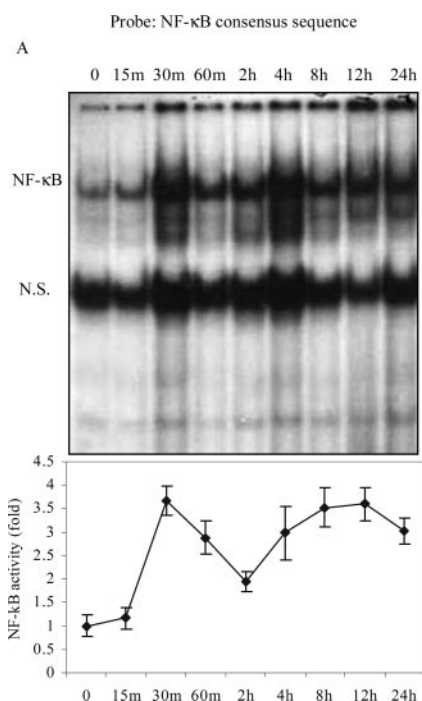


Fig. 3. TNF- α induced biphasic patterns of NF- κ B DNA binding activity and UPase enzymatic activity in EMT6 cells. Cytoplasmic and nuclear proteins were extracted from EMT6 cells treated with 20 ng/ml of TNF- α for 0 min, 15 min, 30 min, 60 min, 2 h, 4 h, 8 h, 12 h, and 24 h. A, nuclear proteins (10 μ g) were used for EMSA using the consensus NF- κ B binding sequence as a probe. B, cells extracts were used for measurement of UPase activity. N.S., nonspecific binding. The blots represent single representative experiments whereas the graphs are the mean \pm S.D. of at least three experiments.

completely abolished the formation of this complex (Fig. 5A), indicating the NF- κ B specificity of this complex.

To further confirm the specificity of the DNA protein complexes and identify the active subunit in the NF- κ B complex, we performed a supershift assay with specific antibodies against p65, p50, and c-Rel. Preincubations of nuclear extracts with these antibodies indicated that the presence of the p65 antibody, not p50 or c-Rel antibodies, retarded the mobility of the specific complex (Fig. 5B). These data suggest that the NF- κ B p65 was the subunit responsible for TNF- α regulation of UPase expression at the promoter level. No specific complexes were formed with oligonucleotide corresponding to the element located at -70 to -62 bp (data not shown).

The Effect of PS-341 and p65 Gene Cotransfection on UPase Promoter Activity. The specificity of NF- κ B p65 regulation on TNF- α -induced UPase expression was further confirmed by investigating the effect of the NF- κ B inhibitor PS-341 (Sunwoo et al., 2001) and p65 gene cotransfection on UPase activity. As expected, the block of NF- κ B function by bortezomib (PS-341) resulted in the inhibition of UPase mRNA expression and UPase enzymatic activity in a concentration-dependent fashion (Fig. 6, A and B). In addition, we also cotransfected the UPase promoter construct p-1619 containing the putative NF- κ B binding site at -1332 to -1323 bp or p-1081 without this NF- κ B binding site with p50, p65, and c-Rel expression vectors into EMT6 cells, respectively. As shown in Fig. 7, the introduction of p65 greatly stimulated the UPase promoter activity, but p50 and c-Rel did not. It is more interesting that PS-341 could also efficiently inhibit the

p-1619 construct activity induced by either p65 transfection or TNF- α addition. Neither cotransfection with p65, p50, and c-Rel nor TNF- α exposure significantly affected the activity of the construct p-1081 lacking the NF- κ B binding site at -1332 to -1323 bp.

Discussion

The murine UPase promoter region contains several putative regulatory elements for cytokine-induced transcriptional factors, oncogenes, and tumor suppressor gene factors (i.e., p53) (Cao et al., 1999). Because of the lack of canonical TATA or CAAT box in the UPase promoter, the presence of these transcriptional regulatory elements may contribute to the initiation of UPase transcription. We have elucidated previously the regulatory mechanism of the tumor suppressor gene p53 on UPase gene expression, in which p53 represses UPase gene expression by specifically binding to a p53 consensus site at -303 and -294 bp in the UPase promoter (Zhang et al., 2001). This has provided an explanation for the critical role of p53 regulation of pyrimidine nucleotide pools. In the current study, we examined the influence of TNF- α on UPase expression in EMT6 cells and the resultant effect on 5'-DFUR antiproliferative activity. Furthermore, we elucidated the regulatory mechanism of TNF- α on UPase expression.

Motif analysis of the UPase promoter region indicated the presence of two NF- κ B binding sites located at -1332 to -1323 bp and -70 to -62 bp, respectively, of the UPase promoter region. To evaluate their functional status and

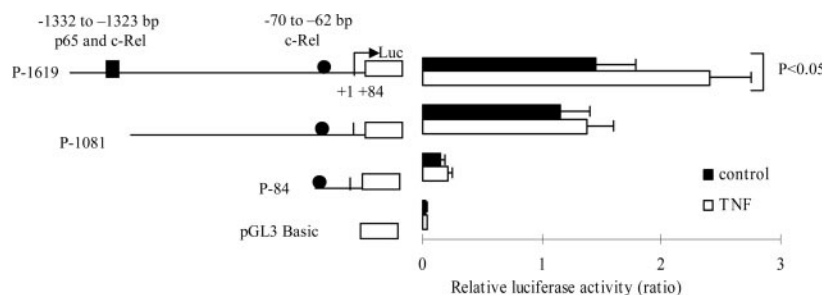


Fig. 4. Putative NF- κ B binding sites in the UPase promoter region and their effect on TNF- α induction of promoter activity. Left, location of NF- κ B elements in the UPase promoter sequence and schematic representation of the 5'-flanking region of the UPase/luciferase gene constructs used in the transient transfection analysis of promoter activity. Right, luciferase activity by TNF- α induction. The UPase luciferase constructs were cotransfected with plasmid pRL-TK as internal control into EMT6 cells. Twenty-four hours after transfection, the cells were stimulated with TNF- α (20 ng/ml) for 24 h before the determination of the luciferase activity. The relative luciferase activity was expressed as -fold over an internal control, and shown as the means \pm S.D. ($n = 3$). *, $P < 0.05$ compared with control.

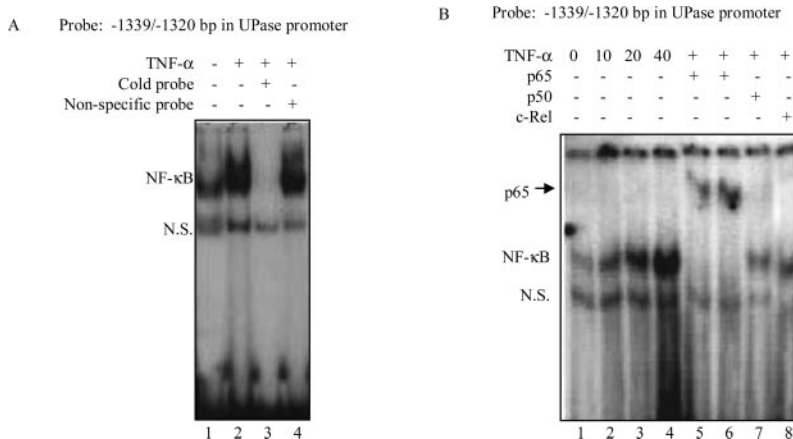


Fig. 5. Effect of TNF- α on the nuclear factor binding to the NF- κ B element in the UPase gene promoter. A, nuclear extracts (10 μ g) from EMT6 cells treated with 20 ng/ml of TNF- α for 24 h were incubated with 32 P-labeled probes including NF- κ B p65 element and analyzed by EMSA to determine NF- κ B activity. A 100-fold excess of specific or nonspecific unlabeled oligonucleotides were added as competitors. B, EMSA was performed with 10 μ g of nuclear proteins from EMT6 cells treated with different concentration of TNF- α (0, 10, 20, and 40 ng/ml) for 24 h. The antibodies of the NF- κ B family members, p65, p50, and c-Rel, were used for the supershift assay. Specific NF- κ B binding complexes, nonspecific binding (N.S.), and free probe are shown.

possible involvement in TNF- α -induced UPase gene expression, we conducted a promoter activity assay with a series of deleted UPase promoter-luciferase constructs. The results indicated that the presence of the NF- κ B binding element at -1332 to -1323 bp conferred the TNF- α inducibility of the UPase promoter. The EMSA assays further confirmed this finding. The sequence at -1332 to -1323 bp displayed a specific binding with nuclear proteins prepared from EMT6 cells, and this binding activity was positively related to the TNF- α treatment. The element at -70 to -62 bp showed no binding activity. These data suggest that TNF- α may induce UPase expression first via the activation of the NF- κ B pathway, which later stimulates UPase expression through DNA sequence-specific binding to the element at -1332 to -1323 bp.

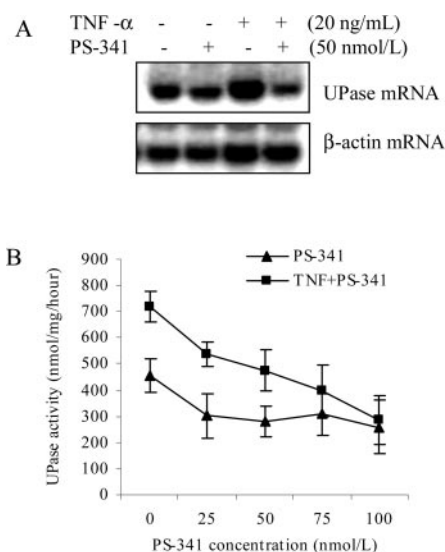


Fig. 6. Inhibition of UPase gene expression by PS-341. A, total RNA (15 μ g) from EMT6 cells treated with TNF- α (20 ng/ml) and PS-341 (50 nM) for 24 h were analyzed for UPase mRNA level by Northern blot. B, EMT6 cells were treated with or without TNF- α (20 ng/ml) and PS-341 (0, 25, 50, 75, and 100 nM) for 24 h. Enzymatic activity of UPase in EMT6 cells was assayed as reported under *Materials and Methods*.

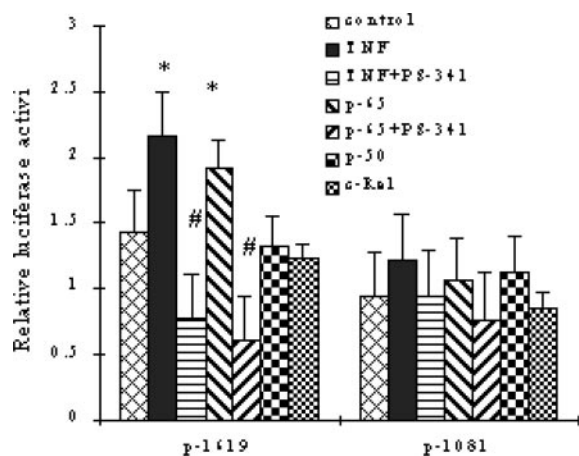


Fig. 7. Effect of the introduction of p65 gene and PS-341 on the UPase promoter activity. p65, p50, and c-Rel plasmid DNA were cotransformed in EMT6 cells with constructs p-1619 containing the NF- κ B element, and p-1081 without the NF- κ B element, respectively. The transformed cells were treated with or without TNF- α (20 ng/ml) or PS-341 (50 nM) for 24 h, respectively. Results are expressed as mean value \pm S.D. ($n = 3$). *, $P < 0.05$ compared with control; #, $P < 0.05$ compared with TNF- α alone or p65 group.

The NF- κ B family includes five distinct subunits: NF- κ B1 (p50/p105), NF- κ B2 (p52/p100), RelA (p65), RelB, and c-Rel. The NF- κ B subunits can undergo either homo- or heterodimerization in the cytoplasm before activation, but the p50/p65 heterodimer is the predominant one. Upon stimulation, either of the subunits moves into the nuclei and activates the effectors' gene expression (Leong and Karsan, 2000). To identify the subunit(s) involved in UPase gene expression, we performed supershift assays with specific antibodies against p65, p50, and c-Rel. The results indicated that only the presence of the anti-p65 antibody caused the retardation of shift bands, indicating that it is the NF- κ B subunit p65 that contributes to the TNF- α -induced UPase expression. This is further confirmed by p65 gene cotransfection with the UPase promoter-luciferase constructs containing the -1332 to -1323 bp element (p-1619), which led to a significant elevation of luciferase activity. However, p65 expression had no effect on the UPase promoter activity in the construct without the -1332 to -1323 bp element (p-1081). In addition, we also observed the effect of an NF- κ B inhibitor, PS-341, on endogenous UPase expression and UPase promoter-luciferase construct activity in response to TNF- α stimulation. As expected, PS-341 efficiently inhibited TNF- α -induced endogenous UPase gene expression and UPase promoter activity. Taken together, our data suggest that TNF- α induces UPase gene expression via the activation of NF- κ B subunit p65 and subsequent binding to the element located at -1332 to -1323 bp in the murine UPase promoter.

Fluoropyrimidines are a group of antimetabolite agents widely used in the treatment of breast, gastrointestinal, and head/neck tumors; however, their clinical success has been limited by serious host toxicity because of their poor tumor selectivity. Over the years, a major effort has been dedicated to the tumor-specific modulation of their action. Capecitabine is an oral agent designed to generate 5-FU preferentially in tumor tissues (Schilsky, 2000). The tumor selectivity of capecitabine has been confirmed in both tumor xenografts (Ishikawa et al., 1998) and human colon carcinoma tissues (Schüller et al., 2000). Schüller et al. found that the concentration of 5-FU in primary colorectal tumors was, on average, 3.2 times higher than in adjacent healthy tissue and 21.4-fold higher than that in plasma. 5'-DFUR, an intermediate metabolite, is the critical step that provides tumor specificity to capecitabine through its conversion to 5-FU catalyzed by pyrimidine phosphorylases UPase and TPase. It has been well documented that both UPase and TPase are both induced in tumor tissues (Leyva et al., 1983; Maehara et al., 1990; Saeki et al., 1997; Liu et al., 1998; Kanzaki et al., 2002; Miyashita et al., 2002; Yang et al., 2002; Katsumata et al., 2003; Shimabukuro et al., 2005). Whereas the regulatory mechanism of UPase expression was still unclear, the mechanism of TPase regulation has been reported previously. Several studies have indicated that TPase expression is stimulated by TNF- α (Goto et al., 2001; Zhu et al., 2002). In our study using the EMT6 model, we found that the basal activity of TPase in these cells is barely detectable, and the TNF- α treatment has no effect on its activity level, indicating that TPase expression is not TNF- α -inducible in this cell line. Therefore, the increased 5'-DFUR toxicity in EMT6 cells by TNF- α has to be entirely ascribed to the induction of UPase.

In summary, this study not only provides direct evidence for the role of UPase in the activation of fluoropyrimidines in

general and more specifically of 5'-DFUR but also elucidates the mechanism leading to the elevated expression of the pyrimidine enzyme after TNF- α stimulation. This study also provides the first direct evidence of TNF- α involvement in the regulation of fluoropyrimidine-activating enzymes and warrants a systematic evaluation of the expression of TNF- α and pyrimidine phosphorylases in tumors to properly design a therapeutic approach that exploits the tumor-selective activation of these fluorinated antimetabolites.

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