Oncology Research, Vol. 16, pp. 507–516
Printed in the USA. All rights reserved.
Copyright © 2007 Cognizant Comm. Corp.

0965-0407/07 $90.00 + .00
E-ISSN 1555-3906
www.cognizantcommunication.com

Actin-Sequestering Protein, Thymosin-Beta-4 (TB4), Inhibits Caspase-3 Activation in Paclitaxel-Induced Tumor Cell Death

Eun-Yi Moon,*† Ji-Hee Song,† and Kyu-Hwan Yang‡

*Department of Bioscience and Biotechnology, Sejong University, Seoul 143-747, Korea
†Department of Functional Genomics, Korea Research Institute of Bioscience and Biotechnology (KRIBB), Taejeon 305-806, Korea
‡Gachon Bionano Research Institute, Kyungwon University, Kyunggi 461-701, Republic of Korea

(Submitted June 25, 2007; revision received August 1, 2007; accepted August 14, 2007)

Thymosin-beta-4 (TB4) as an actin-sequestering peptide has been detected outside of cells in blood plasma or in wound fluid. TB4 induces tumor metastasis and paclitaxel resistance, which is the most significant obstacle to successful therapy in tumors. Here we investigated the inhibitory effect of TB4 peptides on tumor cell death by paclitaxel. The effect of TB4 peptides was assayed by the measurement of caspase-3 activity, G_2/M arrest, and Bcl-2 phosphorylation. Cell survival rate was increased and caspase-3 activity was decreased by the treatment with TB4 peptides. In contrast, small interfering RNA (siRNA) of TB4 inhibited cell viability and augmented caspase-3 activity. Significant changes in Bcl-2 phosphorylation were detected by TB4 peptide treatment or by the overexpression of TB4 gene in Hela cells. The reduced population in G_2/M phase by TB4 peptide treatment was correlated with the decreased expression of cyclin B1. The data were confirmed in gastric tumor cell lines, SNU 638 (low TB4 level) and SNU 668 (high TB4 level), which were established from clinically isolated gastric tumors. In conclusion, soluble TB4 peptides produced in cancer cells could be an obstacle to treat tumors with paclitaxel. Therefore, TB4 could be a novel target to control paclitaxel resistance.

Key words: Thymosin-beta-4 (TB4); Paclitaxel; Hela cell; siRNA; Caspase-3; Bcl-2

INTRODUCTION

Thysmosin-beta-4 (TB4) is a small, naturally occurring 43-amino acid protein. TB4 is the most abundant member of the beta-thymosins, a family of highly conserved polar 5-kDa peptides (1,2). TB4 presents in high concentrations in almost every cell (1). TB4 is detected outside of cells in blood plasma or in wound fluid. Several biological effects are attributed to TB4, oxidized TB4, or to the fragment, acSDKP, possibly generated from TB4 (1). TB4 is the major G actin-sequestering molecule in mammalian cells (3–6) and can be cross-linked to some proteins including fibrin and collagen (7). This provides a mechanism to increase the local concentration of TB4, where TB4 may contribute to wound healing (8,9), angiogenesis (10–12), bone marrow stem cell proliferation (1), and inflammatory responses (1,13,14). TB4 is associated with increased invasion and the distant metastasis of human carcinoma by increasing motility of the cells (15,16). TB4 overexpression also provides tumor cells advantages such as immune evasion and resistance to anticancer drug-induced apoptosis and facilitates their survival during metastasis and chemotherapy (17).

Paclitaxel, an antitumor drug that is demonstrating encouraging activity in human malignancies, is likely to play a major role in cancer chemotherapy. The microtubule cytoskeleton is reorganized in the presence of paclitaxel and extensive parallel arrays or stable bundles of microtubules are formed in cells growing in tissue culture (18). Paclitaxel blocks cells in the G_2/M phase of the cell cycle, and such cells are unable to form a normal mitotic apparatus (19–21). Paclitaxel activated Raf-1, phosphorylated anti-apoptotic Bcl-2 protein, which lead to apoptotic cell death (22–24). Many reports show that caspase-3 is involved in paclitaxel-induced apoptosis in various cell types (25–30).

Caspases are crucial mediators of programmed cell death (apoptosis). Among them, caspase-3 is a frequently activated death protease in a remarkable tissue-, cell type-, or death stimulus-specific manner, catalyzing the specific cleavage of many key cellular proteins. Caspase-3 is also essential for some of the characteristic changes in cell morphology and certain biochemical

Address correspondence to Eun-Yi Moon, Department of Bioscience and Biotechnology, Sejong University, Seoul 143-747, Korea. Fax: +82-2-466-8768; E-mail: eunyimoon@sejong.ac.kr or eunyimoon@yahoo.com

events associated with the execution and completion of apoptosis. Pathways to caspase-3 activation are either dependent on or independent of mitochondrial cytochrome c release and caspase-9 function (31–33). Recent study showed that decreased TB4 was involved in the induction of apoptosis by antitumor drugs (34). Overexpressed TB4 in Hela cells induces paclitaxel resistance by elevating the extracellular-regulated kinase (ERK) activity (35). TB4 may function as an antiapoptotic agent by inhibiting the release of cytochrome c from mitochondria and by suppressing the activation of caspases (14). Caspase-3 activation was reduced in TB4-overexpressed colon carcinoma cells, SW480 (17). However, little is known about whether TB4-induced paclitaxel resistance was resulted from the inhibition of caspase-3 activity.

Here, we investigated how TB4 peptides play a role in the increased cell survival after paclitaxel treatment. We examined paclitaxel-induced apoptotic activation in the presence or absence of TB4 peptides, especially by comparing caspase-3 activity. Data showed that TB4 peptide treatment inhibited paclitaxel-induced cell death through the decrease of caspase-3 activity.

MATERIALS AND METHODS

Reagents

Thymosin-beta-4 (TB4) peptide was kindly provided from Dr. Hynda Kleinman, Gorge Washington University, USA. Monoclonal anti-Bcl-2 antibodies (6C8) were from Pharmingen; monoclonal anti-tubulin antibodies were from Sigma; polyclonal anti-cyclin B1, cyclin A, and cytochrome C antibodies and HRP-conjugated goat anti-hamster IgG were from Santa Cruz; polyclonal anti-caspase-3 antibodies were from StressGen; *N*-acetyl (Ac)-DEVD-*p*-nitroanilide (NA) was from Calbiochem. Except where indicated, all other materials were obtained from Sigma Chemical Company (St. Louis, MO).

Cell Culture

Cervical cancer cell line, Hela, and gastric tumor cell lines, SNU 638 and SNU 668 (36), were obtained from the Korea Research Institute of Bioscience and Biotechnology (KRIBB) cell bank (Taejeon, Korea). Stable TB4-overexpressed Hela cells were prepared by methods of a previously described protocol (35). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (GIBCO, Carlsbad, CA), 2 mM L-glutamine, 100 U/ml penicillin, and 100 U/ml streptomycin.

To knock down TB4 expression, confluent cells were transfected with TB4-siRNA duplex using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). siRNA sequence was 5′-ccgatatggctgagatcga-3′, which was provided from Ahram Biosystem (Seoul, Korea). After cells were incubated for 16 h, paclitaxel was added to the cultures. Cells were incubated for an additional 20 h and cell density was measured by MTT assay described below. Cells were also used for the analysis of TB4 expression and caspase-3 activation by RT-PCR or Western blot analysis, respectively.

MTT Assay

We quantified paclitaxel-treated cell survival using colorimetric assay described for measuring intracellular succinate dehydrogenase content with MTT [3(4, 5-dimethyl-thiazol-2-yl)-2, 5-diphenyl tetrazolium bromide] (37). Confluent cells were cultured with various concentrations of paclitaxel for 48 h. Cells were then incubated with 50 μg/ml of MTT at 37°C for 2 h. Formazan formed was dissolved in dimethyl sulfoxide (DMSO). Optical density (OD) was read at 540 nm.

Caspase-3-Like Activity Assay

The colorimetric assay was carried out by means of a previously described protocol (23). Briefly, caspase-3-like activity was measured in the mixture of assay buffer [100 mM HEPES (pH 7.5), 5 mM EDTA, 0.1% (3-cholamidopropyl-dimethylammonio)-1-propane-sulfonic acid (CHAPS), 5 mM DTT, and 20% glycerol], cell lysates, and 100 mM NA. After incubation for 1 h, absorbance was read at 405 nm. According to the manufacturer's instruction, the caspase-3 substrate can also be cleaved by caspases-6, -7, -8, and -10.

RT-PCR

RNA was isolated from each experimental group using TRIZOL (Invitrogen). cDNA was synthesized from 1 μg of total RNA, using oligo-dT_{18} primers and superscript reverse transcriptase (Promega, Madison, WI) in a final volume of 21 μl. For standard PCR, 1 μl of the first-strand cDNA product was then used as a template for PCR amplification with Taq DNA polymerase (Bioneer, Taejeon, Korea). PCR amplification proceeded as follows: 30–33 thermocycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s, using oligonucleotides specific for hTB4 (forward: atg tct gac aaa ccc gat atg gc, reverse: tta cga ttc gcc tgc ttg ctt c), and hGAPDH (forward: gaa ggt gaa ggt cgg agt c, reverse: gaa gat ggt gat ggg att tc).

Western Blot Analysis

Cells were lysed in ice-cold lysis buffer, containing 0.5% Nonidet P-40 (v/v) in 20 mM Tris-HCl (pH 8.3), 150 mM NaCl, protease inhibitors [2 μg/ml aprotinin, pepstatin, and chymostatin; 1 μg/ml leupeptin and pepstatin; 1 mM phenylmethyl sulfonyl fluoride (PMSF)],

and 1 mM Na_4VO_3. Lysates were incubated for 30 min on ice prior to centrifugation at 14,000 rpm for 5 min at 4°C. Proteins in the supernatant were denatured by boiling for 5 min in sodium dodecyl sulfate (SDS) sample buffer. Proteins were separated by 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and then transferred to nitrocellulose membranes. Following this transfer, equal loading of protein was verified by Ponceau staining. The membranes were blocked with 5% skim milk in Tris-buffered saline with Tween 20 (TBST) [10 mM Tris-HCl (pH 7.6), 150 mM NaCl, 0.5% Tween 20], then incubated with the indicated antibodies. Bound antibodies were visualized with HRP-conjugated secondary antibodies with the use of enhanced chemiluminescence (ECL) (Amersham, Piscataway, NJ).

Flow Cytometric Analyses

Cell cycle was analyzed by flow cytometry that was performed with CELLQuest™ software in FACScalibur™ (Becton Dickinson, San Jose, CA). For the analysis of cell, cells were fixed in 40% ethanol on ice for 30 min and then incubated with propidium iodide (50 μg/ml) and RNase (25 μg/ml) at 37°C for 30 min. Cell cycle of 10,000 cells was analyzed using flow cytometry.

Statistical Analyses

Experimental differences were tested for statistical significance using ANOVA and Student's *t*-test. A value of $p < 0.05$ was considered to be significant.

RESULTS

TB4 Peptides Inhibit Paclitaxel-Induced Hela Cell Death

To investigate the role of TB4 peptides in paclitaxel-induced tumor cell death, we treated Hela cells with paclitaxel in the presence or absence of TB4 peptides. As shown in Figure 1, paclitaxel-induced cell death was inhibited by coincubation with TB4 peptides. Cell survival rate was about 20% increased by TB4 peptide treatment at 20 h after the treatment with 2 μM paclitaxel. It suggests that cellular responsiveness to paclitaxel treatment could be regulated by the existence of soluble TB4 peptides in Hela cell culture medium.

We investigated whether paclitaxel treatment showed apoptotic changes in Hela cells. Paclitaxel (2 μM) induced chromosomal condensation compared to control cells (data not shown). Caspase-3, which is the converting enzyme of various apoptotic signaling pathways (33), was dose-dependently or time-dependently activated by paclitaxel treatment (data not shown). Its activation was indicated by the increased 19 kDa caspase-3 cleavage product or the decreased procaspase-3 amount.

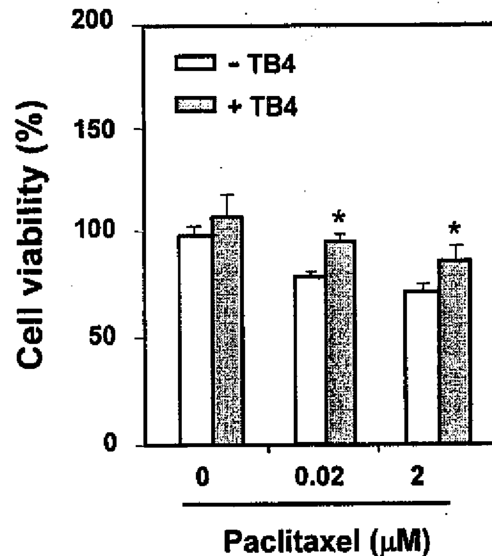


Figure 1. Thymosin-beta-4 (TB4) peptide reduced cell viaility. Hela cells were plated at a density of 2×10^4 cells/ml. Cells were treated with paclitaxel (0.02 or 2 μM) in the presence or absence of TB4 peptide (1 μg/ml). Cells were incubated for 24 h and the cell viability was measured by MTT assay described in Materials and Methods. Bar graph depicts TB4-untreated (open bars) and TB4-treated cells (filled bars). Data represent mean ± SED. *$p < 0.05$, significantly different from TB4-untreated control.

In the following studies, we focused on the changes of caspase-3 activity to define the effect of TB4 peptides on apoptosis pathways.

TB4 Peptides Inhibit Caspase-3 Activation

To investigate the effect of TB4 peptides on paclitaxel-induced caspase-3 activation, Hela cells were treated with paclitaxel in the presence or absence of TB4 peptides. As shown in Figure 2, paclitaxel-induced caspase-3 activation was reduced by coincubation with TB4 peptides. Paclitaxel-induced 19 kDa caspase-3 cleavage product was reduced by coincubation with TB4 peptides (Fig. 2A). According to the manufacturer, the caspase-3 substrate can be cleaved by caspase-6, -7, -8, and -10. Caspase-3 activity in the presence of TB4 peptides was lower than that in the absence of TB4 (Fig. 2B), which was consistent with the reduced production of 19 kDa active caspase-3 in the presence of TB4 peptides, time dependently (Fig. 2C). Many tumor cells are in apoptosis at 18-h incubation. Among them, some might be already dead cells. This could explain why caspase-3 activities are a little reduced at 18 h. Data suggest that cellular responsiveness to paclitaxel could be regulated by extracellular soluble TB4 peptide level.

To confirm the effect of TB4 on paclitaxel-induced caspase-3 activation, small interfering RNA (siRNA)

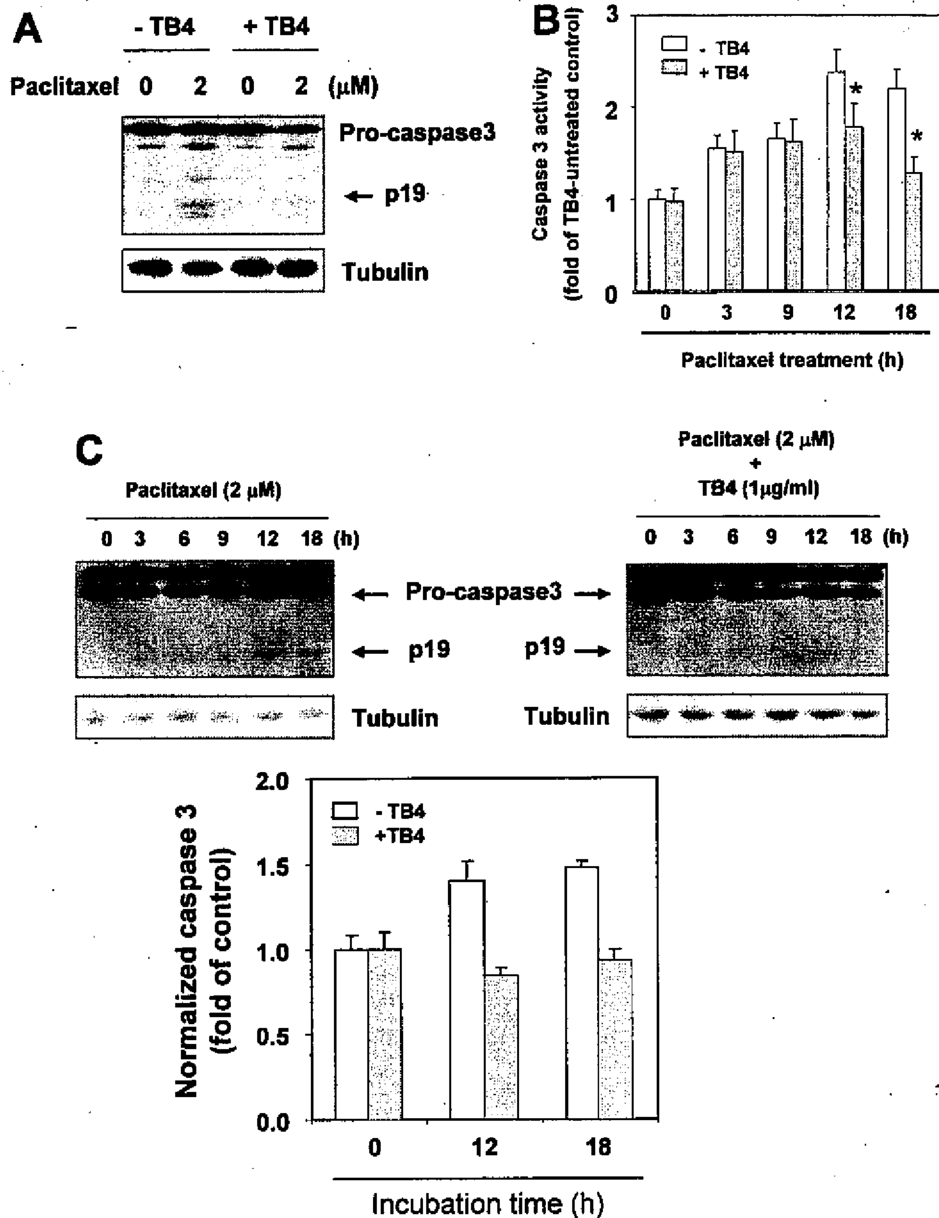


Figure 2. Thymosin-beta-4 (TB4) peptide reduced apoptotic cell death. (A) Hela cells were treated with paclitaxel (2 μM) in the presence or absence of TB4 for 18 h. Cell lysates were prepared and caspase-3 activation was detected by Western blot analysis described in Materials and Methods. Full-length procaspase-3 and the caspase-3 cleavage product p19 are indicated by arrows. (B, C) Cells were treated with paclitaxel (2 μM) in the presence or absence of TB4 (1 μg/ml) for an appropriate time. Cells were assessed for caspase-3-like activity with the substrate Ac-DEVD-pNA by means of a colorimetric assay. Data in the bar graphs represent mean ± SED. *$p < 0.05$, significantly different from Hela cell growth control (B). Caspase-3 activation was detected by Western blot analysis described in Materials and Methods. Full-length procaspase-3 and the caspase-3 cleavage product p19 are indicated by arrows (C, top). Density of p19 bands by the treatment with paclitaxel for 12 or 18 h was quantified with NIH image analysis software (version 1.62) and compared with control density. Fold increase of active caspase-3, p19, is represented with bar graph (C, bottom).

was used to silence TB4 expression in Hela cells. TB4-siRNA inhibits TB4 transcript in Hela cells (Fig. 3A). TB4-siRNA enhanced caspase-3 activation in paclitaxel-treated Hela cells, which was measured by colorimetric caspase-3 activity assay and Western blot (Fig. 3B, C). Cell survival rate was also reduced by blocking the TB4 expression with siRNA, compared to nonsilencing control siRNA-treated group (Fig. 3D). It suggests that cellular responsiveness to paclitaxel could be regulated by TB4 transcript level in Hela cells.

TB4 Peptides Inhibit G_2/M Arrest and Bcl-2 Phosphorylation by Paclitaxel Treatment

Many reports described that paclitaxel arrested cell cycle at G_2/M phase (19–21). Prior reports also showed that Bcl-2 protein was phosphorylated on G_2/M-arrested cells and phosphorylated Bcl-2 protein showed as upper-shifted band on SDS-PAGE (23,24,38). Our data also showed that cell cycle was arrested at G_2/M phase and antiapoptotic Bcl-2 protein was phosphorylated by the incubation with paclitaxel, which resulted in cytochrome C release through the increase of mitochondrial membrane permeability (data not shown). Paclitaxel treatment augmented cyclin B1 expression to control G_2/M phase but no changes were detected in cyclin A expression (data not shown).

To investigate whether the decrease of paclitaxel-induced apoptotic cell death by TB4 peptides resulted from the altered percentage of G_2/M arrest, cell cycle in Hela cells was analyzed after the treatment with paclitaxel in the presence or absence of TB4 peptides. Figure 4A showed that cell cycle arrest on G_2/M was reduced by the incubation with TB4 peptides. Reduced percentages of G_2/M arrest by TB4 peptides were consistent with the decrease of Bcl-2 phosphorylation and cyclin B1 expression (Fig. 4B). Similar data were obtained us-

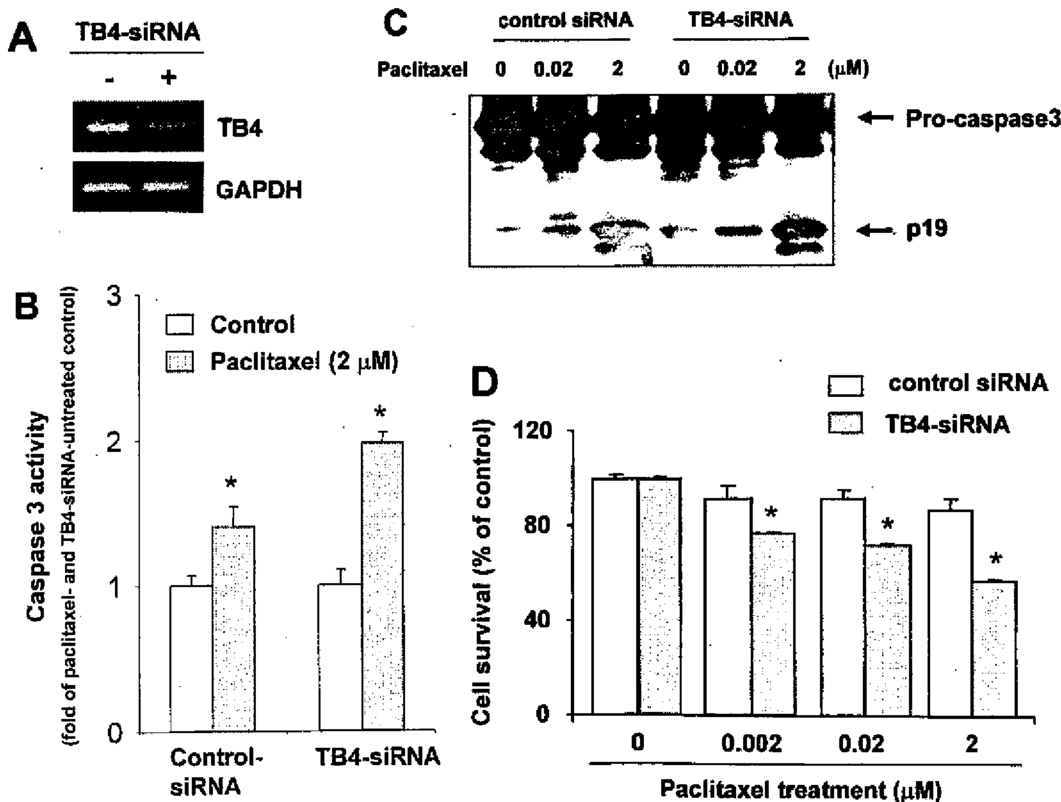
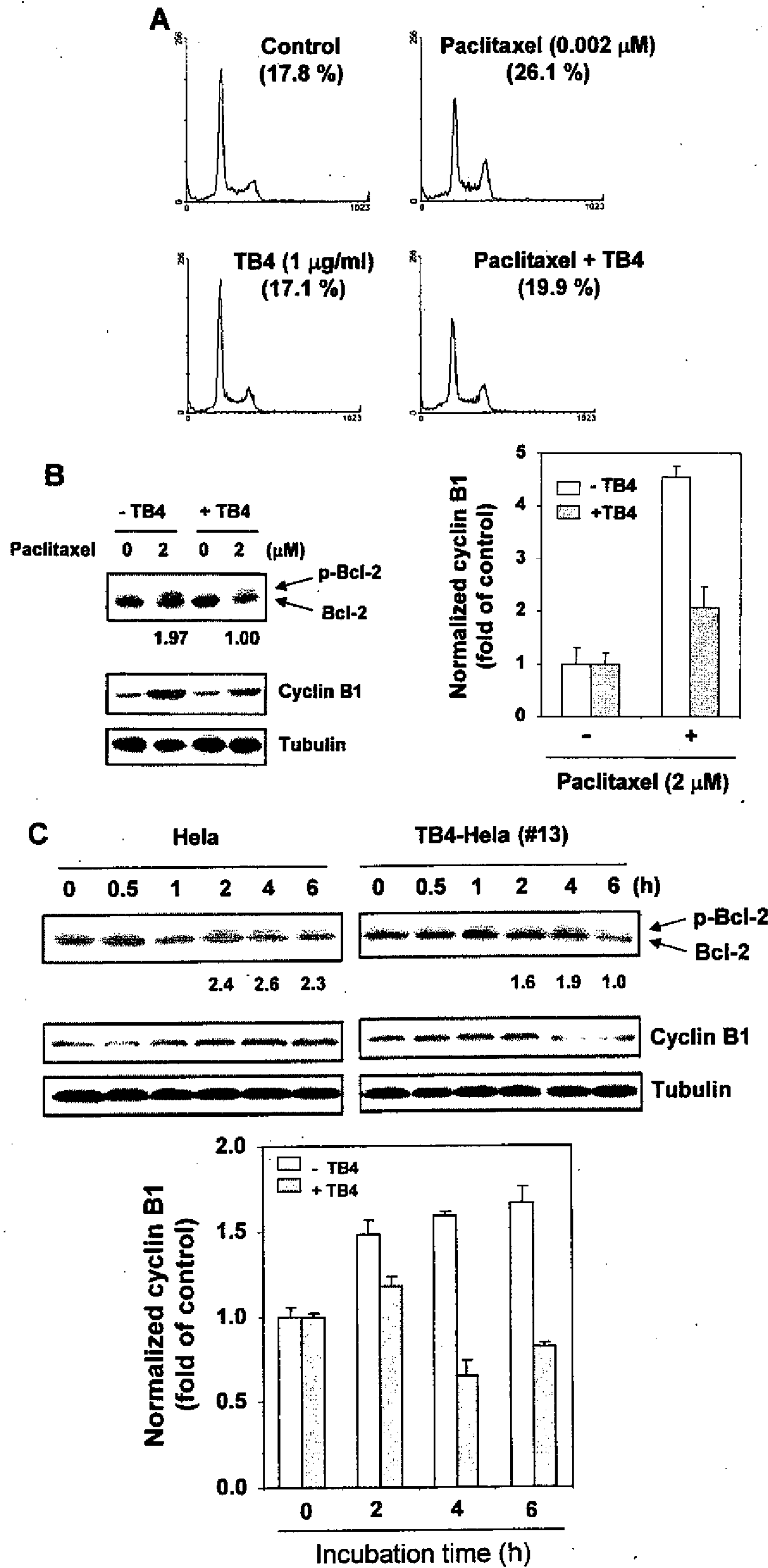


Figure 3. Apoptosis in paclitaxel-treated cells increased by silencing the TB4 gene expression with TB4-siRNA. (A) TB4 expression was attenuated by TB4-siRNA transfection. TB4 mRNA level was measured by RT-PCR. (B) Paclitaxel (2 μM)-treated cells were assessed for caspase-3-like activity with the substrate Ac-DEVD-pNA by means of a colorimetric assay. Data in bar graph represent mean ± SED. $*p < 0.05$, significantly different from Hela cell growth control. (C) Caspase-3 activation was detected by Western blot analysis described in Materials and Methods. Full-length procaspase-3 and the caspase-3 cleavage product p19 are indicated by arrows. (D) TB4-siRNA was transfected into Hela cells using Lipofectamine 2000. Then, 18 h later, cells were incubated with paclitaxel (0.0002, 0.02, and 2 μM) for an additional 24 h. Cell density was measured by MTT assay described in Materials and Methods. Data in bar graph represent mean ± SED. $*p < 0.05$, significantly different from nonsilencing control siRNA-transfected group.


A
Control
(17.8 %)
Paclitaxel (0.002 μM)
(26.1 %)
TB4 (1 μg/ml)
(17.1 %)
Paclitaxel + TB4
(19.9 %)
B
- TB4
+ TB4
Paclitaxel 0 2 0 2 (μM)
p-Bcl-2
Bcl-2
1.97 1.00
Cyclin B1
Tubulin
Normalized cyclin B1
(fold of control)
- TB4
+TB4
Paclitaxel (2 μM)
C
Hela
TB4-Hela (#13)
0 0.5 1 2 4 6
0 0.5 1 2 4 6 (h)
p-Bcl-2
Bcl-2
2.4 2.6 2.3
1.6 1.9 1.0
Cyclin B1
Tubulin
Normalized cyclin B1
(fold of control)
- TB4
+ TB4
Incubation time (h)

ing TB4-overexpressed Hela cells (Fig. 4C). Although the percentage of G_2/M arrest is dependent on time and concentration of paclitaxel, G_2/M arrest and Bcl-2 phosphorylation are the same event independent of paclitaxel-treated time and concentration (data not shown). It suggests that the inhibition of caspase-3 activation by TB4 peptides was mediated by the reduced cell cycle arrest on G_2/M and the decreased Bcl-2 phosphorylation, which might affect mitochondrial membrane permeability.

Caspase-3 Activation Was Associated With TB4 Expression Level

To reaffirm the effect of TB4 on caspase-3 activation, we used SNU 638 and SNU 668 cell lines that were established from clinically isolated gastric tumors. TB4 expression in SNU 638 was lower than that in SNU 668 (Fig. 5A). Figure 5B shows that the growth rate in SNU 638 was higher than that in SNU 668. Even though we reported that cell growth rate was increased by TB4 overexpression in Hela cells (35), these data suggest that tumor cell growth rate was independent of TB4 expression level. In addition, cell death rate in SNU 638 was higher than that in SNU 668 (Fig. 5C). Caspase-3 activation was detected by the production of caspase-3 cleavage product p19 and it was higher in SNU 638 than in SNU 668 (Fig. 5D). These are consistent with the data that cell survival rate was correlated with the TB4 expression level (35).

DISCUSSION

The development of paclitaxel resistance in tumors is one of the most significant obstacles to successful therapy. A recent report described that cDNA subtraction revealed increased expression of six genes, including clathrin heavy chain, alpha3-tubulin, a neuroblastoma-specific thymosin-beta, the ribosomal protein L7a, HLA-B associated transcript 3, and collagen IIIalpha1 in the paclitaxel-resistant larynx carcinoma cell line HLaC79 cell line (39). Overexpressed TB4 in Hela cells induced paclitaxel resistance by elevating the basal ERK activity (35). TB4 was involved in the induction of apoptosis by antitumor drugs (34). TB4 functions on apoptotic caspase activation when tumor cells were treated with microtubule-related antitumor drug such as paclitaxel (34). We investigated whether TB4 peptide treatment affects paclitaxel-induced apoptosis. Data show that TB4 peptides inhibited the rate of G_2/M arrest and Bcl-2 phosphorylation by paclitaxel, resulting in the reduced caspase-3 activation. It might be associated with the induction of paclitaxel-resistance by TB4. These results implicated that TB4 peptides could be a cellular response regulator to stresses such as antitumor drug treatment.

Unfortunately, no receptors to TB4 peptide have been found. However, it is still possible for TB4 to affect apoptosis through binding cell membrane. Another way is as follows. TB4 peptide could be digested to smaller optimum size of oligopeptide and then it could go through the cell membrane into the intracellular compartment to modulate apoptotic signaling molecules.

Given that many molecules were changed by TB4 (27–30,40,41), it is possible for caspase-3 to be inhibited through TB4-associated signaling pathways. According to our previous data that overexpressed TB4 in Hela cells induces paclitaxel resistance by elevating the basal ERK activity, ERK is the first candidate to inhibit caspase-3. This is comparable to previous reports that microtubule damage by some anticancer drugs can activate ERK survival pathway (42–44). In androgen-independent prostate cancer cells, C-81 LNCaP, inhibition of ERK by PD98059, but not U0126, plus docetaxel resulted in enhanced growth suppression by an additional 20% compared to the sum of each agent alone ($p < 0.02$) (44).

In addition, Raf-1 is the second candidate to inhibit caspase-3, because downregulation of Raf-1 kinase in MCF-7 cells, which can be induced through sustained

FACING PAGE
Figure 4. Alteration of paclitaxel-induced G_2/M arrest and various molecular changes. (A) Cells were incubated with paclitaxel (0.002 μM) in the presence or absence of TB4 (1 μg/ml) for an appropriate time. Cells were stained with propidium iodide and analyzed by flow cytometry. Numbers in parentheses are the percentage of G_2/M-arrested cell population. These data are representative of results obtained with three independent experiments. (B) Cells were incubated with paclitaxel (2 μM) in the presence or absence of TB4 for 18 h. Cell lysates were prepared with paclitaxel-treated Hela cells. Various molecular changes were detected by Western blot analysis described in Materials and Methods. Density of phosphorylated Bcl-2 and cyclin B1 bands was quantified with NIH image analysis software (version 1.62). Fold phosphorylation of Bcl-2 is numerically indicated under each paclitaxel-treated band. Fold expression of cyclin B1 is represented with bar graph (right). (C) TB4-overexpressed Hela (TB4-Hela) and control Hela cells were treated with paclitaxel (2 μM) for 0.5, 1, 2, 4, and 6 h. Cell lysates were analyzed with SDS-PAGE. Molecular changes were detected by Western blot analysis. Density of phosphorylated Bcl-2 and cyclin B1 bands in each time point was quantified with NIH image analysis software (version 1.62) and compared with control density. Fold phosphorylation of Bcl-2 is numerically indicated under each paclitaxel-treated band for 2, 4, and 6 h. Fold expression of cyclin B1 is represented with bar graph (bottom).

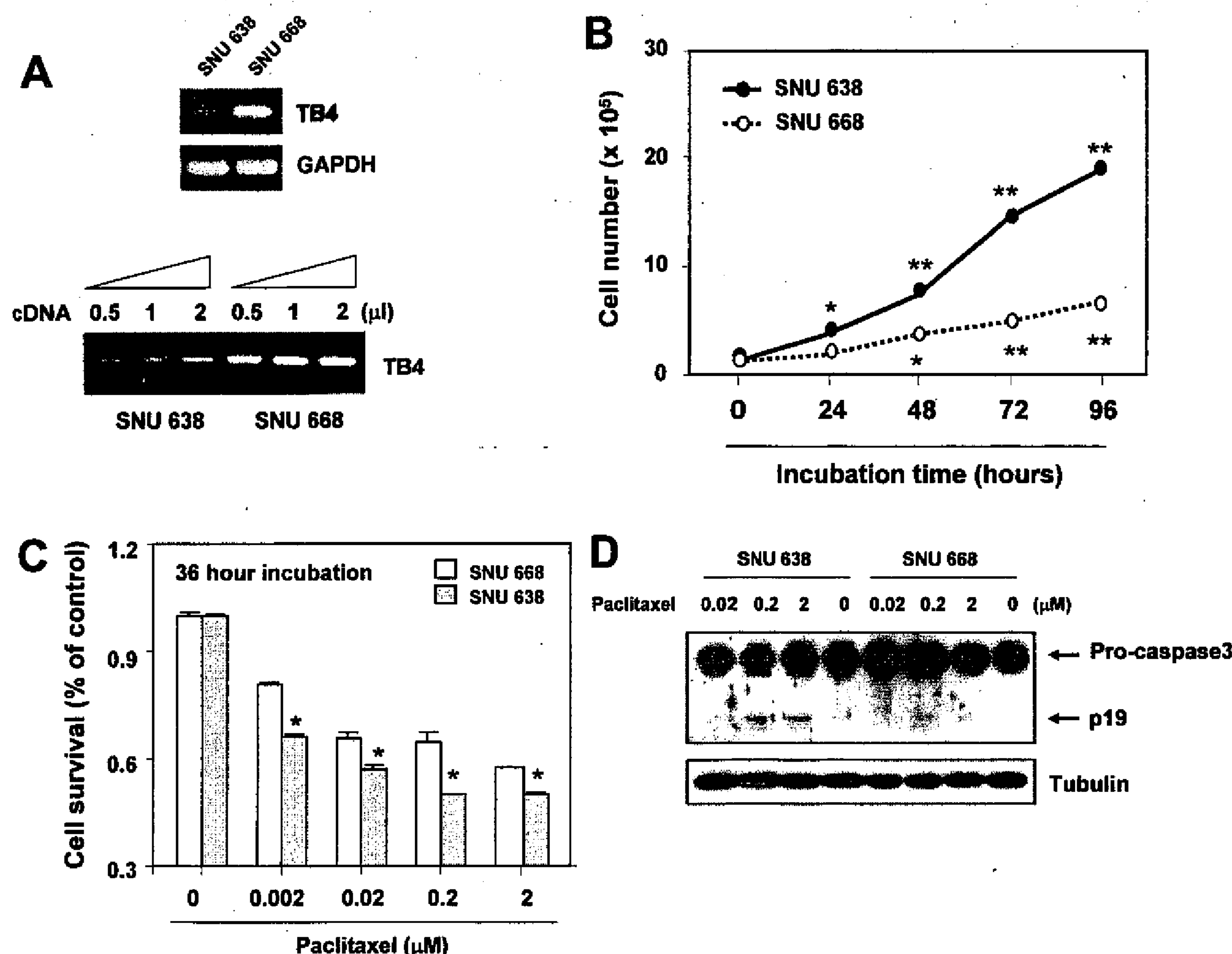


Figure 5. Comparison of apoptotic cell death between SNU 638 and SNU 668 cancer cells that were established from clinically isolated stomach cancer and showed a different TB4 expression level. (A) RNA was purified from SNU 638 and SNU 668 cells. TB4 mRNA level was measured by RT-PCR. Various amounts of cDNA (0.5, 1, and 2 µl) were used to compare TB4 expression in SNU 638 and SNU 668 cells (bottom). (B) Growth curve is depicted for SNU 668 (open circles) and SNU 638 cells (filled circles). Cells were plated at a density of 2×10^4 cells/ml. Cell number was counted at 24, 48, 72, and 96 h after plating cells. Data represent mean ± SED. $*p < 0.05$; $**p < 0.01$, significantly different from cell growth control (0-h incubation). (C) Cells were incubated with paclitaxel (0.002, 0.02, 0.2, or 2 µM) for 48 h. The cell viability was measured by MTT assay described in Materials and Methods. Bar graph depicts TB4-untreated (open bars) and TB4-treated cells (filled bars). Data represent mean ± SED. $*p < 0.05$, significantly different from TB4-untreated control. (D) Cell lysates were prepared from paclitaxel (0.02, 0.2, and 2 µM)-treated SNU 638 and SNU 668 cells. Caspase-3 activation was detected by Western blot analysis. Full-length procaspase-3 and the caspase-3 cleavage product p19 are indicated by arrows.

ERK activation, may contribute to the development of acquired resistance (45). Paclitaxel-induced Raf-1 activation accompanied Bcl-2 phosphorylation (38). Our data also showed that the amount of Bcl-2 phosphorylated by paclitaxel treatment was reduced by TB4 treatment. This indicates that Raf-1 activation could be decreased by TB4, although CDC2 (46), protein kinase A (24), and JNK (47) are the other kinases responsible for phosphorylation of Bcl-2.

In addition to the above kinases, other molecules are possible candidates to reduce caspase-3 activation. Decreased apoptosis by TB4 was also reported in mouse cells, which was mediated by the reduced phosphorylation of pp125FAK and twice as much paxillin associated with pp125FAK (48). TB4 formed a functional complex with PINCH and integrin-linked kinase (ILK), resulting in the activation of the survival kinase Akt (also known as protein kinase B) in the heart (10). This should be further clarified in the future.

In conclusion, even though we could not clarify all those explanations, the data demonstrate that TB4 inhibits paclitaxel-mediated apotosis and induces paclitaxel

presistance. It is required to define a detail mechanism of action on the inhibition of apoptosis and the induction of paclitaxel resistance by TB4 peptides. Our data for the first time suggest that TB4 involved in tumor growth and metastasis by cytoskeleton reorganization (11,12) plays a crucial role to decide cell death or survival, and TB4 peptide-mediated caspase-3 inhibition leads to paclitaxel resistance in TB4 peptide-treated Hela cells.

ACKNOWLEDGMENTS: We thank Dr. Hynda Kleinman, George Washington University, for providing TB4 peptides. This work was supported by grants from National Cancer Control Program, Ministry of Health and Welfare, Korea.

REFERENCES

1. Huff, T.; Muller, C. S.; Otto, A. M.; Netzker, R.; Hannappel, E. beta-Thymosins, small acidic peptides with multiple functions. Int. J. Biochem. Cell Biol. 33:205–220; 2001.
2. Low, T. L.; Hu, S. K.; Goldstein, A. L. Complete amino acid sequence of bovine thymosin beta 4: A thymic hormone that induces terminal deoxynucleotidyl transferase activity in thymocyte populations. Proc. Natl. Acad. Sci. USA 78:1162–1166; 1981.
3. Domanski, M.; Hertzog, M.; Coutant, J.; Gutsche-Perelroizen, I.; Bontems, F.; Carlier, M. F.; Guittet, E.; van Heijenoort, C. Coupling of folding and binding of thymosin beta4 upon interaction with monomeric actin monitored by nuclear magnetic resonance. J. Biol. Chem. 279: 23637–23645; 2004.
4. Hertzog, M.; van Heijenoort, C.; Didry, D.; Gaudier, M.; Coutant, J.; Gigant, B.; Didelot, G.; Preat, T.; Knossow, M.; Guittet, E.; Carlier, M. F. The beta-thymosin/WH2 domain; structural basis for the switch from inhibition to promotion of actin assembly. Cell 117:611–623; 2004.
5. Hertzog, M.; Yarmola, E. G.; Didry, D.; Bubb, M. R.; Carlier, M. F. Control of actin dynamics by proteins made of beta-thymosin repeats: The actobindin family. J. Biol. Chem. 277:14786–14792; 2002.
6. Safer, D.; Elzinga, M.; Nachmias, V. T. Thymosin beta 4 and Fx, an actin-sequestering peptide, are indistinguishable. J. Biol. Chem. 266:4029–4032; 1991.
7. Huff, T.; Otto, A. M.; Muller, C. S.; Meier, M.; Hannappel, E. Thymosin beta4 is released from human blood platelets and attached by factor XIIIa (transglutaminase) to fibrin and collagen. FASEB J. 16:691–696; 2002.
8. Malinda, K. M.; Goldstein, A. L.; Kleinman, H. K. Thymosin beta 4 stimulates directional migration of human umbilical vein endothelial cells. FASEB J. 11:474–481; 1997.
9. Malinda, K. M.; Sidhu, G. S.; Mani, H.; Banaudha, K.; Maheshwari, R. K.; Goldstein, A. L.; Kleinman, H. K. Thymosin beta4 accelerates wound healing. J. Invest. Dermatol. 113:364–368; 1999.
10. Bock-Marquette, I.; Saxena, A.; White, M. D.; Dimaio, J. M.; Srivastava, D. Thymosin beta4 activates integrin-linked kinase and promotes cardiac cell migration, survival and cardiac repair. Nature 432:466–472; 2004.
11. Cha, H. J.; Jeong, M. J.; Kleinman, H. K. Role of thymosin beta4 in tumor metastasis and angiogenesis. J. Natl. Cancer Inst. 95:1674–1680; 2003.
12. Goldstein, A. L. Thymosin beta4: A new molecular target for antitumor strategies. J. Natl. Cancer Inst. 95:1646–1647; 2003.
13. Philp, D.; Huff, T.; Gho, Y. S.; Hannappel, E.; Kleinman, H. K. The actin binding site on thymosin beta4 promotes angiogenesis. FASEB J. 17:2103–2105; 2003.
14. Sosne, G.; Siddiqi, A.; Kurpakus-Wheater, M. Thymosin-beta4 inhibits corneal epithelial cell apoptosis after ethanol exposure in vitro. Invest. Ophthalmol. Vis. Sci. 45: 1095–1100; 2004.
15. Wang, W. S.; Chen, P. M.; Hsiao, H. L.; Wang, H. S.; Liang, W. Y.; Su, Y. Overexpression of the thymosin beta-4 gene is associated with increased invasion of SW480 colon carcinoma cells and the distant metastasis of human colorectal carcinoma. Oncogene 23:6666–6671; 2004.
16. Huang, Y.; Sheikh, M. S.; Fornace, Jr., A. J.; Holbrook, N. J. Serine protease inhibitor TPCK prevents Taxol-induced cell death and blocks c-Raf-1 and Bcl-2 phosphorylation in human breast carcinoma cells. Oncogene 18:3431–3439; 1999.
17. Hsiao, H. L.; Wang, W. S.; Chen, P. M.; Su, Y. Overexpression of thymosin beta-4 renders SW480 colon carcinoma cells more resistant to apoptosis triggered by FasL and two topoisomerase II inhibitors via downregulating Fas and upregulating Survivin expression, respectively. Carcinogenesis 27:936–944; 2006.
18. Jordan, M. A.; Wilson, L. Microtubules as a target for anticancer drugs. Nat. Rev. Cancer 4:253–265; 2004.
19. Horwitz, S. B. Taxol (paclitaxel): Mechanisms of action. Ann. Oncol. 5(Suppl. 6):S3–6; 1994.
20. Martin, V. Overview of paclitaxel (TAXOL). Semin. Oncol. Nurs. 9:2–5; 1993.
21. Rowinsky, E. K.; Onetto, N.; Canetta, R. M.; Arbuck, S. G. Taxol: The first of the taxanes, an important new class of antitumor agents. Semin. Oncol. 19:646–662; 1992.
22. Blagosklonny, M. V. Antiangiogenic therapy and tumor progression. Cancer Cell 5:13–17; 2004.
23. Moon, E. Y.; Lerner, A. PDE4 inhibitors activate a mitochondrial apoptotic pathway in chronic lymphocytic leukemia cells that is regulated by protein phosphatase 2A. Blood 101:4122–4130; 2003.
24. Srivastava, R. K.; Srivastava, A. R.; Korsmeyer, S. J.; Nesterova, M.; Cho-Chung, Y. S.; Longo, D. L. Involvement of microtubules in the regulation of Bcl2 phosphorylation and apoptosis through cyclic AMP-dependent protein kinase. Mol. Cell. Biol. 18:3509–3517; 1998.
25. Chen, T.; Pengetnze, Y.; Taylor, C. C. Src inhibition enhances paclitaxel cytotoxicity in ovarian cancer cells by caspase-9-independent activation of caspase-3. Mol. Cancer Ther. 4:217–224; 2005.
26. Fawcett, H.; Mader, J. S.; Robichaud, M.; Giacomantonio, C.; Hoskin, D. W. Contribution of reactive oxygen species and caspase-3 to apoptosis and attenuated ICAM-1 expression by paclitaxel-treated MDA-MB-435 breast carcinoma cells. Int. J. Oncol. 27:1717–1726; 2005.
27. Osaki, S.; Nakanishi, Y.; Takayama, K.; Pei, X. H.; Ueno, H.; Hara, N. Transfer of IkappaBalpha gene increase the sensitivity of paclitaxel mediated with caspase 3 activation in human lung cancer cell. J. Exp. Clin. Cancer Res. 22: 69–75; 2003.
28. Oyaizu, H.; Adachi, Y.; Taketani, S.; Tokunaga, R.; Fukuhara, S.; Ikehara, S. A crucial role of caspase 3 and cas-

pase 8 in paclitaxel-induced apoptosis. Mol. Cell. Biol. Res. Commun. 2:36–41; 1999.
29. Pan, J.; Xu, G.; Yeung, S. C. Cytochrome c release is upstream to activation of caspase-9, caspase-8, and caspase-3 in the enhanced apoptosis of anaplastic thyroid cancer cells induced by manumycin and paclitaxel. J. Clin. Endocrinol. Metab. 86:4731–4740; 2001.
30. Perkins, C. L.; Fang, G.; Kim, C. N.; Bhalla, K. N. The role of Apaf-1, caspase-9, and bid proteins in etoposide- or paclitaxel-induced mitochondrial events during apoptosis. Cancer Res. 60:1645–1653; 2000.
31. Kluck, R. M.; Martin, S. J.; Hoffman, B. M.; Zhou, J. S.; Green, D. R.; Newmeyer, D. D. Cytochrome c activation of CPP32-like proteolysis plays a critical role in a Xenopus cell-free apoptosis system. EMBO J. 16:4639–4649; 1997.
32. Chauhan, D.; Pandey, P.; Ogata, A.; Teoh, G.; Krett, N.; Halgren, R.; Rosen, S.; Kufe, D.; Kharbanda, S.; Anderson, K. Cytochrome c-dependent and -independent induction of apoptosis in multiple myeloma cells. J. Biol. Chem. 272:29995–29997; 1997.
33. Porter, A. G.; Janicke, R. U. Emerging roles of caspase-3 in apoptosis. Cell Death Differ. 6:99–104; 1999.
34. Iguchi, K.; Usami, Y.; Hirano, K.; Hamatake, M.; Shibata, M.; Ishida, R. Decreased thymosin beta4 in apoptosis induced by a variety of antitumor drugs. Biochem. Pharmacol. 57:1105–1111; 1999.
35. Oh, S. Y.; Song, J. H.; Gil, J. E.; Kim, J. H.; Yeom, Y. I.; Moon, E. Y. ERK activation by thymosin-beta-4 (TB4) overexpression induces paclitaxel-resistance. Exp. Cell Res. 312:1651–1657; 2006.
36. Park, J. G.; Yang, H. K.; Kim, W. H.; Chung, J. K.; Kang, M. S.; Lee, J. H.; Oh, J. H.; Park, H. S.; Yeo, K. S.; Kang, S. H.; Song, S. Y.; Kang, Y. K.; Bang, Y. J.; Kim, Y. H.; Kim, J. P. Establishment and characterization of human gastric carcinoma cell lines. Int. J. Cancer 70:443–449; 1997.
37. Denizot, F.; Lang, R. Rapid colorimetric assay for cell growth and survival. Modifications to the tetrazolium dye procedure giving improved sensitivity and reliability. J. Immunol. Methods 89:271–277; 1986.
38. Blagosklonny, M. V.; Giannakakou, P.; el-Deiry, W. S.; Kingston, D. G.; Higgs, P. I.; Neckers, L.; Fojo, T. Raf-1/bcl-2 phosphorylation: A step from microtubule damage to cell death. Cancer Res. 57:130–135; 1997.
39. Schmidt, M.; Schler, G.; Gruensfelder, P.; Hoppe, F. Differential gene expression in a paclitaxel-resistant clone of a head and neck cancer cell line. Eur. Arch. Otorhinolaryngol. 263:127–134; 2006.
40. Lu, K. H.; Lue, K. H.; Liao, H. H.; Lin, K. L.; Chung, J. G. Induction of caspase-3-dependent apoptosis in human leukemia HL-60 cells by paclitaxel. Clin. Chim. Acta 357:65–73; 2005.
41. Lu, K. H.; Lue, K. H.; Chou, M. C.; Chung, J. G. Paclitaxel induces apoptosis via caspase-3 activation in human osteogenic sarcoma cells (U-2 OS). J. Orthop. Res. 23: 988–994; 2005.
42. Seidman, R.; Gitelman, I.; Sagi, O.; Horwitz, S. B.; Wolfson, M. The role of ERK 1/2 and p38 MAP-kinase pathways in taxol-induced apoptosis in human ovarian carcinoma cells. Exp. Cell Res. 268:84–92; 2001.
43. Suyama, H.; Igishi, T.; Sano, H.; Matsumoto, S.; Shigeoka, Y.; Nakanishi, H.; Endo, M.; Burioka, N.; Hitsuda, Y.; Shimizu, E. ERK activation and subsequent RB phosphorylation are important determinants of the sensitivity to paclitaxel in lung adenocarcinoma cells. Int. J. Oncol. 24: 1499–1504; 2004.
44. Zelivianski, S.; Spellman, M.; Kellerman, M.; Kakitelashvilli, V.; Zhou, X. W.; Lugo, E.; Lee, M. S.; Taylor, R.; Davis, T. L.; Hauke, R.; Lin, M. F. ERK inhibitor PD98059 enhances docetaxel-induced apoptosis of androgen-independent human prostate cancer cells. Int. J. Cancer 107:478–485; 2003.
45. Lee, M.; Koh, W. S.; Han, S. S. Down-regulation of Raf-1 kinase is associated with paclitaxel resistance in human breast cancer MCF-7/Adr cells. Cancer Lett. 193:57–64; 2003.
46. Furukawa, Y.; Iwase, S.; Kikuchi, J.; Terui, Y.; Nakamura, M.; Yamada, H.; Kano, Y.; Matsuda, M. Phosphorylation of Bcl-2 protein by CDC2 kinase during G2/M phases and its role in cell cycle regulation. J. Biol. Chem. 275:21661–21667; 2000.
47. Yamamoto, K.; Ichijo, H.; Korsmeyer, S. J. BCL-2 is phosphorylated and inactivated by an ASK1/Jun N-terminal protein kinase pathway normally activated at G(2)/M. Mol. Cell. Biol. 19:8469–8478; 1999.
48. Niu, M.; Nachmias, V. T. Increased resistance to apoptosis in cells overexpressing thymosin beta four: A role for focal adhesion kinase pp125FAK. Cell Adhes. Commun. 7:311–320; 2000.