Specific positive and negative effects of FLIP on cell survival in human prostate cancer

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We demonstrate here for the first time novel positive and negative effects of the FLICE-like inhibitory protein (FLIP) on human prostate cancer cell survival. A proteasome inhibitor, MG132, mediated cell cycle arrest at G2/M and apoptosis through p38 activation. Interestingly, FLIP was stabilized by MG132 and interacted with Raf-1, resulting in enhancement of p38 signals and cytotoxicity. In contrast, overexpression of FLIP inhibited ubiquitylation and proteasomal degradation of β-catenin, resulting in increase of the target gene cyclin D1, colony formation and invasive activity. Immunohistochemical analysis and in vitro experiments in primary culture showed FLIP to be overexpressed, statistically associated with expression of β-catenin/cyclin D1 in metastatic cells, the FLIP/β-catenin/cyclin D1 signals contributing to colony formation and invasion, which were canceled by FLIP knock down. In contrast, MG132-induced cytotoxicity including apoptosis was strongly inhibited by reduction of FLIP. Taken together, the results indicate that FLIP plays an important role in development of metastatic prostate cancer by inhibiting proteasomal degradation of β-catenin, whereas it is mainly involved in proteasome inhibitor-mediated cell cycle arrest and apoptosis through activating the Raf-1/p38 pathway. Furthermore, proteasome inhibitors may be effective drugs for advanced prostate cancers overexpressing FLIP.

Introduction

It is well recognized that the FLICE-like inhibitor protein (FLIP) promotes cancer cell survival by inhibiting apoptosis involving death-receptors such as Fas and tumor necrosis factors (1,2). On the other hand, transient overexpression of FLIP has been reported to bind to Raf-1 and activate extracellular signal regulating kinase (ERK) and nuclear factor kappa B (NFκB), both of which have pro-survival or anti-apoptotic activity in various types of cancer cells (3,4), and it remains unclear whether the FLIP-mediated ERK/NFκB signals physiologically function in cancer cells. In addition, Tai et al. (5) recently showed ERK and NFκB activation to be unchanged or even decreased in resting FLIP transgenic T-lymphocytes. While FLIP was found to protect against cell death induced by a diverse group of chemotherapeutic drugs (6), Engels et al. (7) reported no inhibitory effect of FLIP overexpression on apoptosis mediated by anticancer drugs. These discrepancies seem to be dependent on the level of FLIP expression and vividly indicate its multiple biological functions under different conditions.

The majority of proteins essential for cell cycling system or cell survival are degraded through the ubiquitin conjugation system and proteasomes in cancer cells (8,9). Recent studies have focused on inhibition of the ubiquitin-proteasome pathway as a new target for anticancer therapy both in vitro and in vivo (10,11): for example, the synthetic proteasome inhibitor, PS341 possesses pharmacological characteristics well suited for clinical testing in patients (12). Several molecules that affect tumor growth, including p53 and the cyclin dependent kinase inhibitors, p21 or p27, (13,14) are also regulated by the ubiquitylation-proteasome pathway (15), and their accumulation due to the action of proteasome inhibitors can promote apoptosis or cell cycle arrest. However, inhibition of ubiquitylation-proteasome degradation is also implicated in increased cell growth: for example, when β-catenin accumulates in the nucleus due to inhibition of its ubiquitylation, up-regulation of target oncogenes can occur with enhancement of malignant transformation (16,17). Moreover, the ubiquitylation-proteasome system tightly regulates the activities of growth factor receptors and this can impact on tumorigenecity (18,19). Thus, opposing roles of ubiquitin-proteasome signals may be played in the progression stage of human cancer. In the present study, we focused on dual oncogenic and anti-oncogenic effects of FLIP in prostate cancer cells associated with the ubiquitylation-proteasome pathway. In addition, we estimated pathological correlations using human samples from radical prostatectomy or resected specimens of metastatic cancer.

Materials and methods

Cell culture, plasmids and chemicals

The human prostate cancer cell line, DU145, was purchased from American Type Culture Collection (Manassas, VA) and cultured in RPMI supplemented with 10% fetal bovine serum. Explants of primary culture of metastatic and localized prostate cancer cells were established from prostatectomy specimens (total six clones) or metastatic lesions (lung, 2 and bone, 1), collected as previously reported (20). In brief, hematoxylin-eosin staining samples of frozen sections from tissue specimens were prepared and pathological examinations were performed. Only areas containing prostate cancer were obtained and the cells were digested with collagenase type I, and passed thorough 100 and 40 μM nylon mesh filters. After washing, the cells were treated with trypsin, Dnase I, then they were cultured under 5% CO2 in 20% serum plus medium. To confirm their nature as prostate cancer cells, we checked positive immunohistochemical staining for prostate specific antigen (DAKO, Kyoto, Japan) and alpha-methylacyl CoA racemase (P504S) (DBS, CA) but not for basal cell markers including p63 (DAKO) and high molecular weight cytokeratin (34βE12) (Enzo Diagnostics, NY) following second to third passages. Primary and metastatic prostate cells were used at the sixth passage. Human full length cDNA for the human FLIP long isoform (1.21) was prepared by reverse transcription PCR and inserted into pME18S (pME18S-FLIP). To establish stable cell lines, the pTK-Hyg vector (Clonetech Laboratories Japan, Ltd., Tokyo, Japan), harboring the hygromycin-resistant gene, was co-transfected with...
pME18S-FLIP (21, 22). MG132 and anti-cyclin D1 antibody were purchased from Calbiochem (San Diego, CA, USA); anti-phospho-p38 kinase (Thr180/Tyr182) and anti-β-catenin antibodies were from Cell Signaling (MA, USA); anti-ubiquitin and anti-FLIPS/L antibodies were from Santa Cruz Biotech. (CA, USA); and anti-actin from Oncogene Research Products (Darmstadt, Germany). The p38 inhibitor, SB202190, was from BIOMOL Res. Labs (PA, USA).

Preparation of cell lysates, immunoprecipitation, and immunoblot analysis
Cells were washed once with PBS and suspended in lysis buffer (40 mM Hepes (pH 7.4) with 10% glycerol, 1% Triton X-100, 0.5% Nonidet P-40, 150 mM NaCl, 50 mM NaF, 20 mM b-glycerolphosphate, 1 mM EDTA, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, and 0.1 mM vanadate) with a protease inhibitor mixture (1 μg/ml aprotinin, leupeptin, and pepstatin). For immunoprecipitation, cell lysates were incubated with 5 μg of anti-Raf-1 antibody for 12 h at 4°C and precipitated with protein A–sepharose (Amersham Pharmacia Biotech, Japan, Ltd., Tokyo, Japan). Cell lysates or immunoprecipitants were resolved in SDS-polyacrylamide gels and transferred to polyvinylidene difluoride membranes (Millipore, Ltd., Bedford, MA, USA). The membranes were blocked in TBST buffer (20 mM Tris-HCl (pH 7.5) containing 150 mM NaCl and 0.1% Tween 20) with 5% skim milk at room temperature for 1 h, and then incubated with the indicated primary antibody for 1 h, washed with TBST, and incubated with horseradish peroxidase-conjugated anti-mouse IgG (Amersham Pharmacia Biotech, Japan, Ltd.). After further washing with TBST, peroxidase activity was detected on X-ray films using an enhanced chemiluminescence detection system.

Transfection with FLIP siRNA and antisense Raf-1 oligonucleotides
Cells were seeded and grown in 80 to 90% confluence, then they were transfected with FLIP siRNA (5'-AAT TCA AGG CTC AGA GAG-3') and the control RNA, or nonsense oligonucleotides (NSOs) (5'-TCA CAT TGG TTA GCC GT-3') and antisense oligonucleotides (ASOs) (5'-TCC CGT C TG TCA GT CAT GCA TT-3') for Raf-1 (23, 24). Each transfection was performed using LipofectAmine (Invitrogen Japan, Tokyo, Japan) according to the manufacturer’s protocol. At 48 h thereafter, cells were stimulated with the indicated reagents.

Cell cycle analysis
Cells were stimulated with MG132 for 12 h. After incubation, cells were harvested, centrifuged, fixed in 80% ethanol, re-suspended in phosphate-buffered saline containing 50 μg/ml propidium iodide, 0.1% Nonidet P-40, and 100 μg/ml RNase A (Sigma), and incubated for 1 h. The cell cycle was analyzed by flow cytometry as previously described (25, 26) and the percentage of cell cycle distribution (G1, S and G2/M phases) calculated as mean ± standard deviations (SD).

Cell viability assay
Cells were cultured in medium containing fetal bovine serum for 96 h. After incubation, MTS [(3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphonyl)-2H-tetrazolium, inner salt] reagent (Promega, Tokyo, Japan) was added and optical absorbance at 490 nm was measured using a microplate reader (2, 26).

Colony forming assays
Colony forming assays were performed as described previously (20). Cells were seeded into 10-cm dishes and were incubated for 14 days. Colonies were fixed in methanol/acetic acid and stained with trypan blue. The number of colonies consisting of more than 50 cells was scored.

Matrigel invasion assays
In vitro invasion assays were performed using Matrigel-coated wells (11 μg per filter; 8 μm, pore size) as detailed previously (27). Briefly, 1 × 10^5 cells

![Fig. 1. p38 dependent cell cycle arrest at G2/M and apoptosis due to proteasome inhibition. (A) The prostate cancer cell line, DU145, was preincubated in serum free medium for 16 h (performed for all following experiments using DU145 cells), then stimulated with dimethyl sulfoxide (DMSO) or MG132 at the indicated concentrations for 36 h. After stimulation, cell viability was analyzed by MTT assay as described in the Materials and methods, and expressed as mean percentages ± standard deviations of absorbance of cells without stimulation (control). (B) For the left panel, DU145 cells were treated with or without a p38 inhibitor, SB202190 (SB) for 30 min, then were stimulated with 2.5 μM of MG132 for the indicated times (left upper panel) or 12 h (left lower panel). For the right panel, cells were stimulated by DMSO or 2.5 μM of MG132 for 36 h in the presence or absence of SB pretreatment. Cell viability was analyzed by MTT assay. (C) DU145 cells were stimulated as described for (B), right panel. After 24 h, the cell cycle was examined by flowcytometric analysis and the percentages of cells in G1, S, G2/M phase were calculated as described in the Materials and methods. (D) DU145 cells were stimulated as described in (B) or (C). After 36 h-stimulation, DNA ladder formation (left panel), PARP cleavage and percentages of apoptotic cells were examined as described in the Materials and methods.]
were placed in the insert. After incubation at 37°C for 24 h, the chambers were scrubbed with a cotton bud to remove non-invaded cells and invading cells were fixed and stained with the Giemsa method. Counts in fields/well were then made under a microscope at a magnification of ×100. Each experiment was repeated three times.

Apoptosis assays
Apoptosis was analyzed with the following: (i) PARP cleavage, (ii) DNA fragmentation and (iii) cells in sub-G1 phase. (i) After treatment with indicated reagents, cells were collected for lysis and cleavage of full length of PARP was determined by western blotting using anti-PARP antibody (Cell signaling), (ii) Cells (5 × 10⁶) were stained with 50 μg/ml Rnase A (Sigma), and incubation for 1 h, apoptotic cells were determined by their hypochromatic, sub-G1, staining profiles and numbers of cells were analyzed by flow cytometry (21).

Reverse transcription-PCR
Total RNA was extracted using Trizol reagent and subjected to reverse transcription and PCR using Ready-to-Go beads (Pharmacia) according to the manufacture protocol. The PCR primers were FLIPL, sense 5'-AAT TCA AGG CTC CAT GCC ATC AC-3', antisense, 5'-GCC AGA AAC TCT GCT GTT-3', and glyceraldehydes-3-phosphate dehydrogenase sense 5'-ACC ACA GTC CAT GCC ATC AC-3', and antisense, 5'-TCC ACC ACC CTG TTG CTG TA-3'. PCR products were analyzed on 1.5% agarose gels and photographed under UV light (22). We examined expression and the distribution of FLIP, b-catenin and cyclin D1 using well characterized and previously documented antibodies (31,32). After deparaffinization, sections were heated for 5 min in 10 nM sodium citrate and 20 μg/ml Rnase A and proteinase K, and DNA was extracted and loaded onto 2% agarose gels and photographed under UV light (22).

Immunohistochemistry and evaluation
In vitro kinase assays
Cells (5 × 10⁵ cells) were lysed and immunoprecipitated with anti-Raf-1 antibodies. The resulting immunoprecipitates were incubated with 5 μg of MEK1 (Santa Cruz Biotech.) in 25 μl kinase reaction buffer (40 mM Hepes (pH 7.5) with 20 mM MgCl₂, 20 mM b-glycerol phosphate, and 0.1 mM vanadate) containing 25 μM ATP and 2.5 μCi of [γ-³²P] ATP for 30 min at 30°C. Reactions were terminated by adding 7 μl of Laemmli’s sample buffer and boiling for 5 min. A portion (20 μl) of each sample was separated on a 12% SDS-polyacrylamide gel and autoradiographed (28).

Tissue samples and histopathology
A total of 90 primary prostate carcinomas, obtained by radical prostatectomy, and 20 metastatic carcinomas were examined in this study. Fixation and tissue processing was as described in a previous report (29). In brief, sections through the entire prostate were fixed in 10% formalin and embedded in paraffin. Serial sections were cut and mounted for immunohistochemical analyses and histopathological evaluation according to the Gleason system for prostate carcinomas (30).

Immunohistochemistry and evaluation
We examined expression and the distribution of FLIP, b-catenin and cyclin D1 using well characterized and previously documented antibodies (31,32). After deparaffinization, sections were heated for 5 min in 10 mM sodium citrate buffer (pH 6.0) in a pressure cooker. They were then incubated with antibodies for 16 h at 4°C and reactions were visualized using a Histofine SAB-PO kit and diaminobenzidine as the chromogen (Nichirei, Tokyo, Japan), with hematoxylin counterstaining. The percentages of cells positive for FLIP, nuclear accumulation of b-catenin or cyclin D1 were determined by examination of at least 1000 cells.

Fig. 2. The role of FLIP stabilization in proteasome-inhibitor-induced cytotoxicity. (A) DU145 cells were stimulated by MG132 at the indicated concentrations for 12 h. After extraction, protein (upper panel) and mRNA (lower panel) levels were examined by western blotting and RT-PCR, respectively. Actin or G3PDH expression was used as a control. (B) and (C) DU145 cells were transfected with control and FLIP₅ siRNA. After 72 h cultivation, cells were stimulated with DMSO or 2.5 μM of MG132 for 12 h (B) or 36 h (C) and expression of FLIP, p38 and phosphorylated p38 was examined by western blotting (B). Cell viability was analyzed by MTT assays (C). (D) DU145 cells were co-transfected with the hygromycin resistance gene and a plasmid expressing FLIP₅. After incubation in the presence of hygromycin B, four stable clones No. 1, 3, 7 and 10, were obtained. The expression of FLIP was demonstrated using anti-FLIP₅ antibody (upper panel). We selected a control clone (HygB) and two stable clones No.1 and 7. They were stimulated by 2.5 μM of MG132 for the indicated times and phosphorylation of p38 was assessed by western blotting (lower panel). (E) The control (HygB) and stable clones No. 1 and 7 were stimulated by DMSO or 2.5 μM of MG132 for 36 h and cell viability were analyzed by MTT assay.
Statistical analysis

The Wilcoxon’s rank sum test was used to analyze the distribution of percentages of positive cells for FLIP, nuclear β-catenin or cyclin D1 in relation to the morphology. The Spearman correlation test was performed to analyze correlations between expression of FLIP and nuclear β-catenin or cyclin D1. Statistical analyses were carried out using the Fisher’s exact test supplemented by the Bonferroni procedures as described previously (20). Results were considered significant if the P value was <0.01.

Results

Proteasome inhibition induces cell cycle arrest through the p38 pathway

The proteasome inhibitor, MG132, reduced cell proliferation of the human prostate cancer line, DU145, in a dose-dependent manner (Figure 1A). 2.5 μM MG132 rapidly activated p38 and increased cytotoxicity, which were completely suppressed by the specific inhibitor for p38, SB202190 (Figure 1B). As shown in Figures 1C and D, MG132 arrested DU145 in the G2/M phase and caused apoptosis, these again being strongly abrogated by the p38 inhibitor. Taken together, the results indicate the proteasome inhibitor to induce G2/M cell cycle arrest and apoptosis through p38 activation.

FLIP plays an important role in proteasome inhibitor-mediated cytotoxicity

As shown in Figure 2A, protein expression of the long isoform of FLIP (FLIP L) was upregulated by MG132 in a dose-dependent manner, but the mRNA level was not changed, suggesting stabilization of FLIP L by proteasome inhibition. When DU145 cells were transfected with the siRNA for FLIP L, upregulation was completely canceled, moreover, both MG132-induced p38 activation and cytotoxicity were significantly inhibited (Figures 2B and C). Next, we constructed clones obtained from DU145 cells stably expressing FLIP L and tried to estimate its role in p38 activation and cytotoxicity. A control clone carrying the hygromycin B resistant gene (HygB) and stable clones (1 and 7) overexpressing FLIP L were selected (Figure 2D upper panel). In the control clone, p38 was rapidly activated, but the activity was decreased within 12 h after stimulation by MG132. In contrast, p38 was similarly activated in both stable clones No.1 and 7, but the activity was very sustained (Figure 2D lower panel). Consistently, cell growth arrest by MG132 was significantly enhanced by FLIP L overexpression (Figure 2E). Similar data were obtained with cells stimulated by lactacystin, well known to be an irreversible 20S/26S inhibitor (data not shown). Under stimulation by MG132 or lactacystin, expression of the short isoform of FLIP was not changed and its overexpression had no effect on cell survival (data not shown). Thus, the data indicated that FLIP L plays a critical role in proteasome inhibitor-induced p38 activation and its dependent cytotoxicity.

FLIP L-mediated activation of Raf-1 is necessary for proteasome inhibitor-induced cytotoxicity

We investigated the influence of FLIP/Raf-1 interaction on the molecular mechanism of MG132-induced cell growth arrest. FLIP L was upregulated by 2.5 μM of MG132 for 12 h, and interacted with Raf-1, resulting in activation. Kinase activity of Raf-1, estimated with reference to the phosphorylation level of the substrate, MEK1, was strongly reduced on FLIP L knockdown by siRNA transfection (Figure 3A). Raf-1 expression was suppressed on transfection with the antisense oligonucleotide, and MG132-induced p38 activation and cell growth arrest were completely suppressed (Figures 3B and C).
**FLIP overexpression induces β-catenin-mediated cancer cell growth and invasion signals in prostate cancer**

Total protein expression of β-catenin was significantly increased by overexpression of FLIP L in DU145 cells, resulting in increased nuclear accumulation and up-regulation of the target gene, cyclin D1 (Figure 4A). When a control clone was treated with the proteasome inhibitor, MG132, ubiquitylation of β-catenin could be observed, but

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Fig. 4. FLIP enhances β-catenin-mediated signaling. (A) Expression of β-catenin or cyclin D1 in cytoplasmic or nuclear fractionated cell extracts from control (HygB) and stable DU145 clones (No. 1 and 7) were analyzed by western blotting. Lamin A/C was used as a control for the nuclear fraction. (B) Control (HygB) and the stable clones (No. 1 and 7) were treated with or without 20 μM of MG132 for 6 h, then, immunoprecipitated with anti-β-catenin antibodies and examined by western blotting using anti-ubiquitin or anti-β-catenin antibodies. (C) and (D) Colony formation and matrigel invasion of the control (HygB) and stable clones (No. 1 and 7), analyzed as described in the Materials and methods. Colony forming activity was expressed as mean ratios ± standard deviations of colony formation of cells without gene transfection (control).

Fig. 5. Immunohistochemical analysis of FLIP/β-catenin/cyclin D1 in human prostate cancers. Localized and metastatic cancers were obtained by radical prostatectomy and surgical resection, respectively. Staining was with anti-FLIP, anti-β-catenin and anti-cyclin D1 as described in the Materials and methods. Original magnification: ×200.
this was strongly reduced and β-catenin was stabilized in stable clones overexpressing FLIPL (Figure 4B). Moreover, colony formation and matrigel invasion in such clones were more elevated than in the control case (Figures 4C and D). Cell growth was increased by FLIP overexpression, but the degree only ranged from 1.5 to 1.7 (data not shown), which was less than that for invasion activity (3.4–3.8). The results indicate that FLIPL elevates cytoplasmic and nuclear accumulation of β-catenin by reducing ubiquitylation-proteasomal degradation and thereby promotes cell survival and invasion of human prostate cancer cells.

**Discussion**

We here presented evidence that FLIPₐ has negative effects on cell survival and growth signals in human prostate cancer cells derived from localized and metastatic prostate cancer specimens. We constructed 5 and 3 primary clones for localized and metastatic prostate cancers, respectively, and we selected two clones for each (clones 2 and 5, localized cancer; clones 1 and 3, metastatic cancer). Consistent with the results of immunohistochemical analysis, FLIP, β-catenin and cyclin D1 were all overexpressed in metastatic prostate cancer cells but were little expressed in primary cases (Figure 7A). In metastatic cancer cells, reduction of FLIP expression by transfection with siRNA resulted in downregulated of both β-catenin and cyclin D1 (Figure 7B). Consistently, colony formation and matrigel invasion were significantly inhibited by reduction of FLIPL (Figure 7C). Thus, FLIPₐ-mediated β-catenin/cyclin D1 signals are necessary for progression of human metastatic prostate cancer cells. In contrast, reduction of FLIPL made metastatic cancer cells highly resistant to MG132-induced growth arrest as well as apoptosis (Figure 7D), suggesting major involvement of FLIPₐ-mediated signals in proteasome inhibitor-induced cytotoxicity. Taken together, FLIPₐ has opposing effects on human metastatic prostate cancer cells associated with the ubiquitylation-proteasome pathway.

**FLIPₐ and the related β-catenin/cyclin D1 are overexpressed in metastatic prostate cancers**

We investigated FLIPₐ/β-catenin/cyclin D1 expression in human prostate cancer samples. As shown in Figures 5 and 6A, positive percentages for FLIPₐ, nuclear accumulation of β-catenin and cyclin D1 in 20 metastatic cancers were statistically higher than those in 90 primary cases (metastatic cancer versus primary cases, FLIPₐ: 38.1 ± 3.0% versus 4.2 ± 0.4%; β-catenin: 34.2 ± 1.4% versus 4.4 ± 0.3%; cyclin D1: 29.5 ± 2.4% versus 4.0 ± 0.5%; P < 0.001). In addition, FLIPₐ and β-catenin/cyclin D1 positivity correlated in metastatic prostate cancers (FLIPₐ/β-catenin correlation: γ = 0.911, FLIPₐ/cyclin D1 correlation: γ = 0.938; P < 0.001) (Figure 6B).

**FLIPₐ-mediated signals function in human metastatic prostate cancer cells**

Finally, we investigated whether FLIPₐ-mediated signals physiologically function in human prostate cancer cells...
However, when stimulated with a proteasome inhibitor, FLIP\textsubscript{L} is stabilized and interacts with Raf-1, then activates downstream p38 activation and cytotoxicity. Lan \textit{et al.} (34) recently demonstrated that involvement of Raf-1 in progression to malignancy requires p38 activation, which contributes to Raf-1-dependent upregulation of NF\textkappaB (35). These data suggest p38 activation is downstream of Raf-1, but the authors emphasized only tumorigenic functions of Raf-1/p38 signals in cancer cells. However, it is widely known that p38 has functions leading to cell cycle arrest or apoptosis induced by various stresses. In line with this, we found that inhibition of Raf-1/p38 by an antisense oligonucleotide of Raf-1 or a p38 inhibitor to cancel proteasome inhibitor-induced cytotoxicity. Therefore, we conclude that Raf-1, as well as FLIP, has opposing effects on cell growth: it contributes to cancer development, but may elevate cytotoxicity depending on the extracellular stress. Casanovas \textit{et al.} (36,37) reported that p38 phosphorylates cyclin D1 at Thr-286 under stress conditions, leading to its degradation. We also found that FLIP\textsubscript{L}-mediated upregulation of cyclin D1 was inhibited by MG132, but this inhibition was canceled by p38 inhibition. Taken together, our results indicate that FLIP\textsubscript{L} is the key molecule to induce cyclin D1, but in the presence of a proteasome inhibitor, it is an important molecule effectively mediating cell cycle arrest through not only activation of p38 but also downregulation of cyclin D1 in prostate cancer cells. Interestingly, caspase 8 was proteolytically activated by MG132, but interaction of FLIP with procaspase 8 was not observed (data not shown). We conclude that death receptor- or death receptor-inducing signaling complex-independent cleavage of caspase 8 is important downstream of FLIP-related apoptotic signaling, which is supported by the recent report by Gilot \textit{et al.} (38) indicating caspase 8 processing as a possible effector of death receptor-independent FLIP activities. A number of conflicting reports as to Raf-1 and FLIP\textsubscript{L} interactions have accumulated. Kataoka \textit{et al.} (4) showed FLIP\textsubscript{L} interacted with Raf-1 on transient transfection without any treatment. However, in our study, FLIP\textsubscript{L} did not interact with Raf-1 until cancer cells were stimulated by MG132 in both stable DU145 clones and human metastatic prostate cancer cells overexpressing FLIP\textsubscript{L}. The discrepancy could be due to differences in the expression level of FLIP\textsubscript{L} in line with earlier reports (5). Raf-1-related signals here had no effects on cell growth arrest due to other genotoxic agents, including etoposide or irradiation (data not shown). The cytotoxic effects of FLIP\textsubscript{L}/Raf-1/p38 signals thus seem to be specific for proteasome inhibitors. We recently demonstrated that phosphorylation and nuclear localization of FLIP interacting protein, FADD, are closely
associated with G2/M cell cycle arrest and cell growth suppression (20,25). In the case of stimulation with MG132, FADD was phosphorylated at 194 serine and G2/M cell cycle arrest was significantly inhibited with mutant FADD, in which 194 serine was replaced by alanine (data not shown), suggesting involvement of FADD phosphorylation in proteasome inhibitor-induced cytotoxicity. However, the roles of phosphorylated FADD in FLIP/p38 signals have yet to be identified and should be further investigated.

In addition to anti-oncogenic effects, we found here that FLIP\textsubscript{L} can exert oncogenic influence and upregulate β-catenin survival signaling. Evidence that ubiquitylation of β-catenin is suppressed by the long isoform of FLIP has been provided by Naito et al. (39), but their data did not indicate physiological or pathological significance in human cancer. In the current study, FLIP\textsubscript{L}-mediated amplification of β-catenin/cyclin D1 signals enhanced cell viability and invasion by prostate cancer cells. Moreover, we suggest for the first time that the FLIP\textsubscript{L}/β-catenin pathway and the target oncogene, cyclin D1, contribute to the development of metastatic prostate cancers given the results of immunohistochemical analysis of human prostate cancer tissues: FLIP\textsubscript{L} expression, nuclear accumulation of β-catenin and cyclin D1 expression were thus strongly increased in metastatic prostate cancer specimens but not in primary cases, and significant correlations between FLIP\textsubscript{L} and β-catenin/cyclin D1 were observed. In vitro experiments using metastatic prostate cancer cells clearly indicated overexpressed FLIP\textsubscript{L} to contribute to upregulation of β-catenin/cyclin D1, resulting in enhanced cell survival and invasion activity, as well as promotion of MG132-induced cytotoxicity. Thus, FLIP\textsubscript{L} has positive and negative effects on prostate cancer progression associated with the ubiquitin-proteasome pathway: in the absence of a proteasome inhibitor, FLIP\textsubscript{L}/β-catenin/cyclin D1 signals promote prostate cancer progression, but with proteasome inhibition, FLIP\textsubscript{L}/Raf-1/p38 signaling is predominant, resulting in cell cycle arrest and apoptosis induction (Figure 8). In addition, we indicated for the first time that FLIP\textsubscript{L}-mediated signals might physiologically function in metastatic cancer cells not only in vitro but also in vivo. FLIP\textsubscript{L} is a key molecule for prostate cancer development and metastasis, therefore, FLIP\textsubscript{L}-targeting chemotherapy using proteasome inhibitors might have utility for advanced cases.

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### Conflict of Interest Statement

None declared.

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