Exp Oncol 2006
28, 3, 209–215

Experimental ONCOLOGY

EXPRESSION OF CLUSTERIN, XIAP AND SURVIVIN, AND THEIR CHANGES BY CAMPTOTHECIN (CPT) TREATMENT IN CPT-RESISTANT PC-3 AND CPT-SENSITIVE LNCaP CELLS

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Aim: **Clusterin and IAPs (inhibitor of apoptosis proteins), such as survivin and XIAP, are known to be related to chemo-resistance in several cancer cells. In the current study, we investigated their expression levels in human prostate cancer cell lines, LNCaP and PC-3 which are sensitive and resistant to camptothecin (CPT), topoisomerase I inhibitor, respectively.** ***Methods:*** **LNCaP and PC-3 cells were cultured in the presence of CPT, cell death was evaluated using Hoechst 33342 and propidium iodide (PI) double staining. The expression of clusterin, XIAP and survivin on mRNA and protein levels was investigated by semi-quantitative RT-PCR and Western blotting, respectively.** ***Results:*** **Our data showed that 24 h treatment of LNCaP cells with 0.5 and 3.0 μM CPT resulted in higher number of apoptotic cells, than that in PC-3 cells. Western blot analysis revealed that the clusterin level in PC-3 cells was 5-fold higher than that in LNCaP cells. In contrast, XIAP expression level in PC-3 cells was lower than that in LNCaP cells, and survivin levels were similar in these two cell lines. Treatment with 0.5 and 3.0 μM CPT resulted in the reduced survivin and XIAP expression in both cell lines, while clusterin expression remained unchanged in LNCaP cells, but was increased in PC-3 cells.** ***Conclusion:*** **The results suggest that clusterin may take a greater part in CPT-resistance than survivin and XIAP in PC-3 cells.**
Key Words: **LNCaP, PC-3, clusterin, XIAP, survivin, camptothecin.**

Prostate cancer is the most commonly diagnosed malignancy in male. Although early prostate cancer is eminently treatable due to hormone dependency, androgen withdrawal results in incurability of its advanced stage because of the inevitable emergence of androgen-independent cells within few years [1]. Thus hormone-refractory prostate cancer has long been recognized as a chemo-resistance disease, and there is a great need novel therapeutic strategies that target the molecular basis of hormone refractory chemo-resistance of prostate cancer. Recently, it was reported that clusterin and inhibitors of apoptosis proteins (IAPs) are related to chemo-resistance in some cancer cells including prostate cancer [2–6]. Furthermore, the targeted downregulation of XIAP or survivin genes have been shown to directly sensitize cancer cells to apoptosis induced by various conventional chemotherapeutic drugs [7, 8]. Clusterin (also known as ApoJ, TRPM-2, and SGP-2) is a secretory glycoprotein known as an anti-apoptotic protein [9–14]. Survivin and XIAP belong to a family of IAPs that have been shown to prevent chemotherapy-induced apoptosis by inhibiting caspase [15, 16].

Camptothecin (CPT) specifically targets topoisomerase I (Top1), which is required for maintenance of double helical structure of DNA [17]. Studies by Wang et al. and our data [18, 19] demonstrated that CPT induces growth inhibition and apoptosis in a time- and dose-dependent manner in androgen-sensitive prostate cancer cells (LNCaP), but not in androgen–resistant PC-3 cells. However, the precise mechanism underling the different sensitivity to CPT between these cell types remains to be explored. As an approach to gain some insight into the CPT-resistance, we have examined the expression levels of clusterin, survivin and XIAP and the changes in during CPT treatment in LNCaP and PC-3 cells.

Received: July 28, 2006.
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Abbreviations used: ApoJ – apolipoprotein J; CPT – camptothecin; IAPs – inhibitors of apoptosis proteins; PI – propidium iodide; TopI – topoisomerase I; TRPM-2 – testosterone-repressed prostate message 2.

MATERIALS AND METHODS

Cell culture. LNCaP and PC-3 human prostate cancer cells were cultured in RPMI 1640 (Nikken Bio Medical Laboratory, Kyoto, Japan) supplemented with 10% fetal bovine serum (Sigma, USA) under a humidified atmosphere of 5% CO2 and 95% air at 37 °C. (S)-(+)-camptothecin (CPT) (Sigma, USA) dissolved in DMSO was added to the cell cultures with final DMSO concentration less than 0.03% v/v, which had no significant effect on the growth and differentiation of LNCaP and PC-3 cells. 0.03% DMSO treatment was used as control.

Cell death assay. To identify apoptotic or necrotic cell death, double staining with Hoechst 33342 and propidium iodide (PI) was performed. Cells were stained with 5 μg/ml Hoechst 33342 (Calbiochem, San Diego, CA, USA) and 2 μg/ml PI (Invitrogen, Carlsbad, CA, USA) for 20 min. After washing with phosphate-buffered saline (PBS), cells were observed under a fluorescence microscope, Olympus BX-51 (Olympus, Tokyo, Japan). To evaluate the type of cell death, over 500 cells were analyzed.

Western blotting. Antibodies used were rabbit polyclonal anti-survivin (Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit polyclonal anti-clusterin γ chain (Upstate, Charlottesville, VA, USA), rabbit polyclonal anti-XIAP (Cell Signaling Technology, Beverly MA, USA) and mouse monoclonal anti-β-tubulin (Sigma). Cells washed

with PBS were resuspended in RIPA buffer (10 mM Tris-HCl pH7.4, 1% NP-40, 0.1% sodium deoxycholate, 0.1 SDS, 150 mM NaCl, 1 mM EDTA) containing the protease inhibitor cocktail (Sigma, USA), and stored on ice for 30 min. After centrifugation at 13,000 rpm for 30 min at 4 ˚C, the resultant supernatant was used as protein sample. Each protein content was measured with a DC protein assay kit (BIORAD, USA). 30 μg of each protein sample was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to PVDF membrane. The membrane was blocked with 5% non-fat dry milk in 0.1 M Tris buffered saline containing 0.1% Tween-20 for 30 min, and incubated overnight with primary antibodies at 4 ˚C. The immunocomplex was detected with horseradish peroxidase conjugated anti-mouse or anti-rabbit IgG (Amersham Biosciences, USA) using an ECL system (Amersham Biosciences).

Semi-quantitative RT-PCR. Total RNA was isolated from LNCaP cells and PC-3 cells using a Quick Prep Total RNA Extraction Kit (Amersham Biosciences) according to manufacturer's instruction. 2 μg of total RNA of each sample were reverse transcribed by using Super Script II RNase H-reverse transcriptase and oligo(dT) primer (Invitrogen). Primers and reaction conditions for each gene are summarized in Table 1. The housekeeping transcript, elongation factor-1α (EF-1α), was used as a control for standardization [20]. Each PCR product was separated by electrophoresis on 2 % (w/v) agarose gel, and then visualized by ethidium bromide staining. To measure the density of each band, we use computer-imaging analysis (BIO-1D Vilber Lourmat, France).

RESULTS

CPT sensitivity in LNCaP and PC-3 cells. We examined CPT sensitivity in LNCaP cells and PC-3 cells using Hoechst 33,342 and PI double staining (Fig. 1). At 0.5 μM of CPT, nuclear fragmentation and PI positive staining were observed in LNCaP cells, but not in PC-3 cells. When LNCaP cells were treated with 0.5 μM and 3.0 μM of CPT for 24 h, PI positive cells (necrotic or late apoptotic) and PI negative cells with nuclear fragmentation (apoptotic) were observed. At 0.5 μM CPT, less than 10% of cell death was mainly due to necrosis/late apoptosis, whereas at 3.0 μM cell death was enhanced up to over 20% of total cells (~70% necrotic/late apoptotic and ~30% apoptotic) (Fig. 1, B). In contrast, in PC-3 cells the effect of CPT treatment on cell death was much less pronounced.

Expression of clusterin, XIAP and survivin in prostate cancer cells. To investigate the mechanism underlying the different susceptibility of LNCaP cells and PC-3 cells to CPT, we examined expression levels of the

Table. PCR Primers and Conditions

Gene	5' primer	3' primer	Annealing Temperature (°C)	Cycle
Clusterin	AATACAACGAGCTGCTAAAGTCCT	AATTTAGGGTTCTTCCTGGAGACT	62	25
Survivin	AGAGGAACATAAAAAGCATTCGTC	ACAGGAGCACAGTTGAAACATCTA	62	27
XIAP	GCAGATCTAGTGAATGCTCAGAAA	TACTTGGTAGCAAATGCTAATGGA	62	29
EF-1α	CTCAGGTGATTATCCTGAACCATC	AACAGTTCTGAGACCGTTCTTCCA	60	22

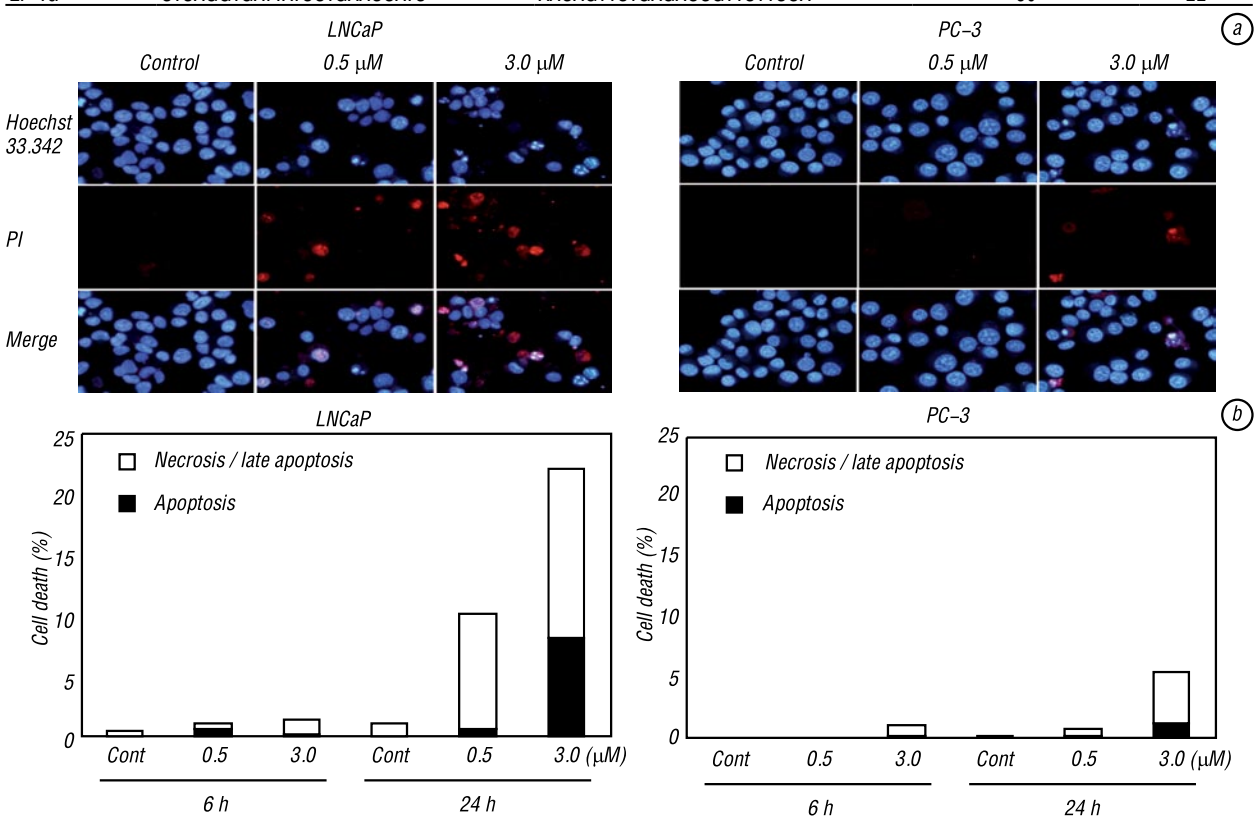

Fig. 1. CPT-induced cell death in prostate cancer cell lines. (*a*) Morphological examination of CPT-induced cell death in LNCaP and PC-3 cells. Cell death was examined by the Hoechst 33342 and PI double staining. After 24 h incubation with 0.5 μM and 3.0 μM CPT, cells were stained and examined by fluorescence microscopy. (*b*) Time course of cell death in LNCaP and PC-3 cells. Both cells were exposed to 0.5 μM and 3.0 μM CPT for different time intervals (6, 24 h). After Hoechst 33,342 and PI double staining, over 500 cells were analyzed in each experiment

proteins that exert the anti-apoptotic action by Western blotting. α-clusterin with 60 kDa has been known as anti-apoptotic protein in normal state [21]. Its expression in LNCaP cells was marginal while it is much higher in PC-3 cells (Fig. 2, *a*). The XIAP expression level was slightly higher in LNCaP cells compared to PC-3 cells (Fig. 2, *b*). As for survivin, there was no significant difference in both cell lines.

Changes in expression levels of clusterin, XIAP and surviving by CPT treatment. We examined CPT-induced changes of clusterin and its mRNA by Western blotting and semi-quantitative RT-PCR. Interestingly,

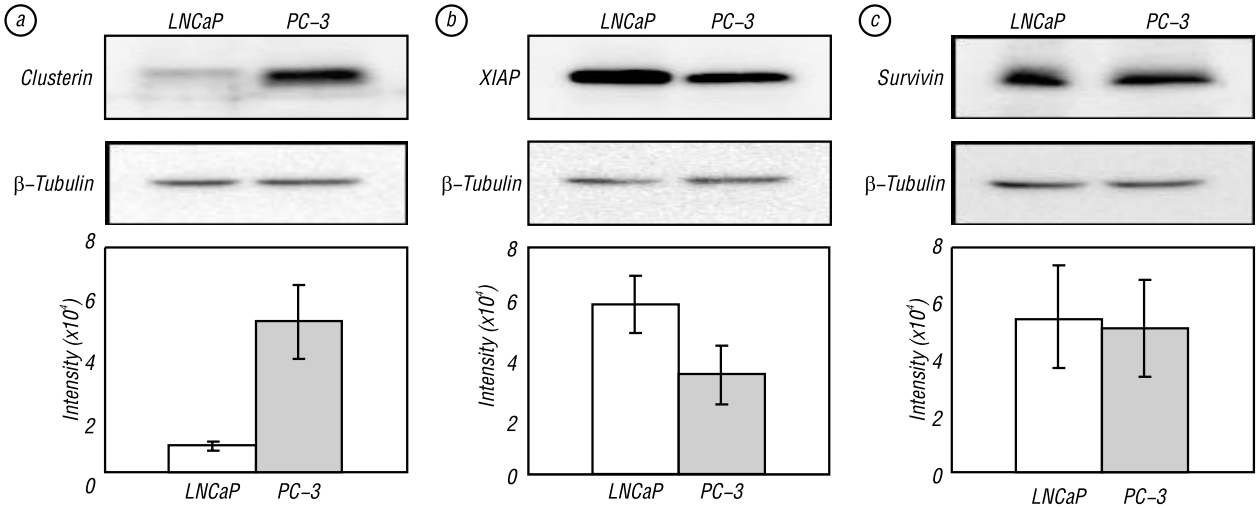

Fig. 2. Clusterin and IAPs expression levels in LNCaP and PC-3 cells analyzed by Western blotting with polyclonal antibodies to human clusterin (*a*), XIAP (*b*) and survivin (*c*). Values represent means ± SD (bars) from three independent experiments. The blots are representative of three separate experiments

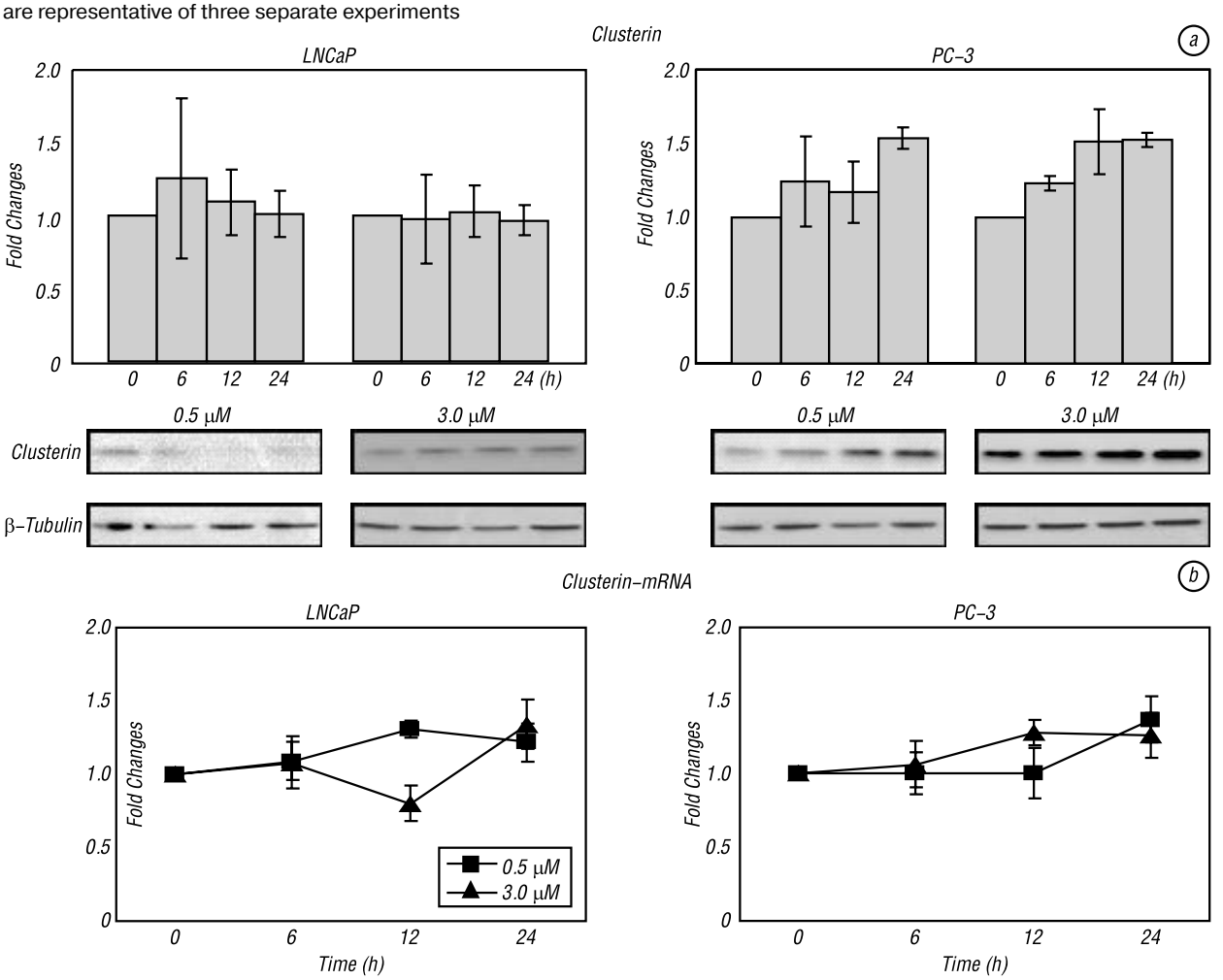

Fig. 3. Expression of clusterin in LNCaP and PC-3 cells upon CPT treatment. (*a*) Time course of the protein expression of clusterin analysed by Western blotting with the specific antibody for human clusterin. Fold changes were determined from relative intensity compared to the control. Values represent means ± SD (bars) from three independent experiments. The blots represent one of three separate experiments with similar results. (*b*) Time course of expression of clusterin mRNA. Fold changes were determined from relative intensity to the control. Values represent means ± SD (bars) from three independent experiments

clusterin protein expression appeared to increase by CPT treatment at 0.5 μM and 3.0 μM in PC-3 cells in a time-dependent manner (Fig. 3, *a*). The temporal profile of its mRNA expression was reflected in the protein expression pattern (Fig. 3, *b*). However, clusterin expression in LNCaP cells did not change upon CPT treatment.

CPT-induced changes were also examined for protein and mRNA of XIAP. After treatment with 0.5 μM and 3.0 μM CPT, the content of XIAP protein were shown to be decreased in both cell lines in a time-dependent manner. The general patterns of the XIAP protein changes in LNCaP and PC-3 cells were compatible with those of mRNA changes (Fig. 4).

Further, we have examined survivin expression levels in LNCaP cells and PC-3 cells exposed to 0.5 μM and 3.0 μM CPT. CPT treatment resulted in reduced protein expression level in both cell lines. It's interesting to note that in LNCaP cells there was a marked decrease in protein expression level of survivin upon 24 h-incubation with 0.5 μM CPT, while the treatment with 3.0 μM CPT caused only a small decrease (Fig. 5). The profile of expression levels was similar for proteins and respective mRNAs. In PC-3 cells, there was a trend to decreased survivin expression during treatment with either 0.5 μM or 3.0 μM CPT.

As it is shown here, the expression levels were discordant between proteins and mRNAs, suggesting the transcriptional and/or post-transcriptional regulation of protein expression.

DISCUSSION

Prostate cancer is the most common in men and the major leading cause of cancer-related death in males. Prostate cancer is characterized by the androgen-dependent growth at the early stage and can be effectively treated by hormone ablation [1]. However, prostate cancer cells often become androgen-independent and refractory to chemotherapy. Despite abundant investigations, the molecular mechanism underlying the resistance to chemotherapy-induced apoptosis remains poorly understood. For *in vitro* studies, androgen-sensitive LNCaP and androgen-resistant PC-3 cell lines have been most commonly used as a model system to search for biochemical or molecular differences between these two cell lines [1, 22]. Wang et al. [18] have demonstrated that LNCaP cells produced ceramide, a key apoptotic mediator, by CPT treatment while PC-3 cells were defective in the ceramide generation, and concluded that the

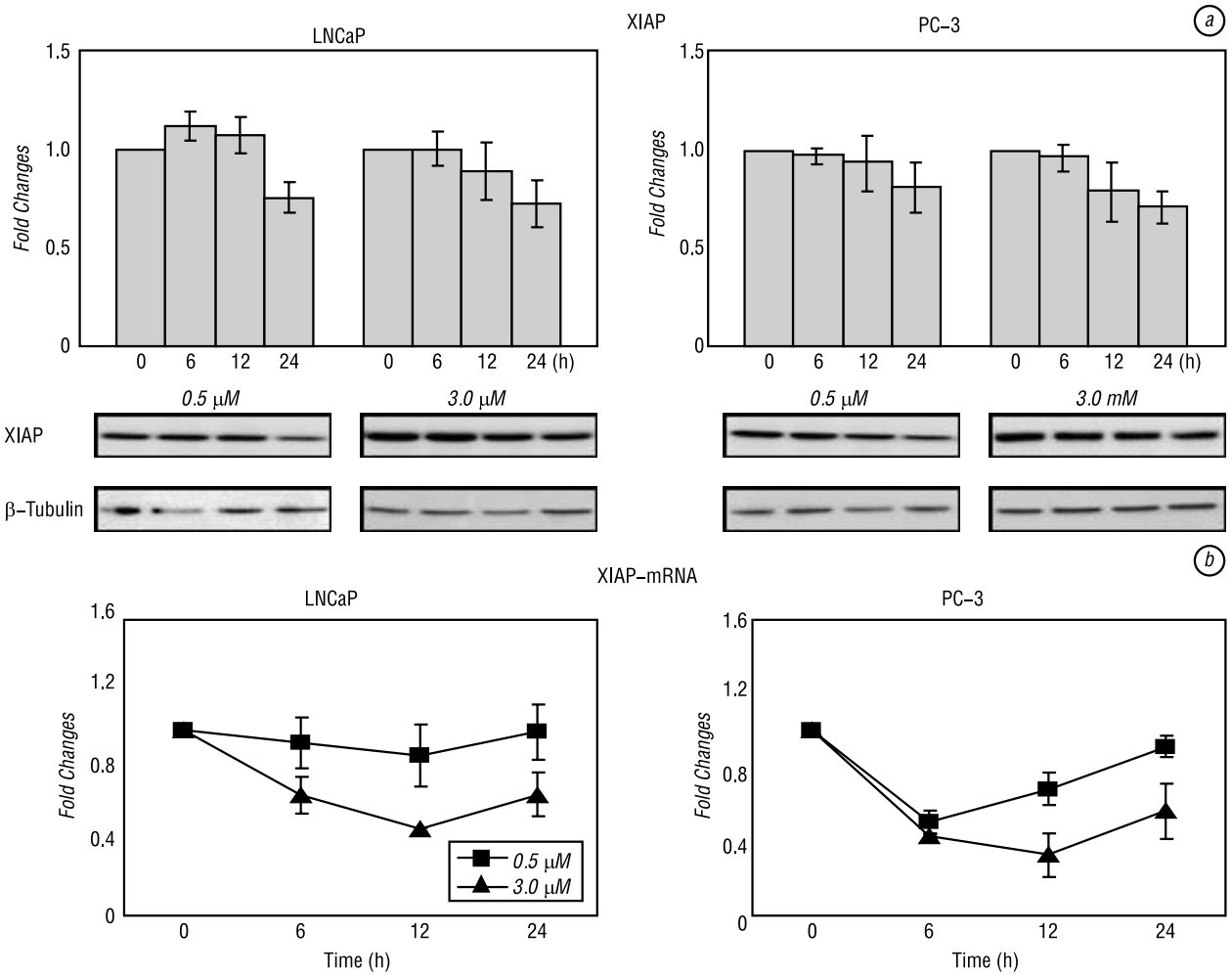

Fig. 4. Expression of XIAP in LNCaP and PC-3 cells upon CPT-treatment. (*a*) Time course of the protein expression of XIAP analyzed by Western blotting with the specific antibody for human XIAP. Fold changes were determined from relative intensity to the control. Values represent means ± SD (bars) from three independent experiments. The blots represent one of three separate experiments with similar results. (*b*) Time course of expression of XIAP mRNA analized by RT-PCR. Fold changes were determined from relative intensity to the control. Values represent means ± SD (bars) from three independent experiments

loss of ceramide formation was responsible for the anti-apoptotic response of PC-3 cells. Our study using ceramide synthetase inhibitor suggested that ceramide generated via its de novo synthesis may not play a primary role in CPT-induced apoptotic cell death in LNCaP cells [19]. Recently, studies by Pchejetski et al. and our report [23, 24] have shown that expression and activity of oncogenic sphingosine kinase 1 were higher in PC-3 cells than in LNCaP cells and that the enzyme expression level was enhanced by CPT-treatment in PC-3 cells. On the other hand, we have previously performed the comparative proteomic analysis to examine differential protein expression in PC-3 and LNCaP cells [25]. Several proteins preferentially expressed in PC-3 cells were identified; they include annexin A1, glutathione-S-transferase (GST) pi and glucose-regulated protein (GRP) 78/Bip which are thought to be involved in the anti-apoptotic response. However, because of the low level expression, we were unable to detect other anti-apoptotic proteins including clusterin and inhibitor of apoptosis proteins (IAPs) known as endogenous caspase inhibitors. Therefore, in the current study, we have examined and compared the expression profiles of clusterin, XIAP and survivin, and their changes by CPT-treatment by Western blotting and RT-PCR analysis.

Clusterin, also known as apolipoprotein J or testosterone-repressed prostate message 2 (TRPM-2), is a cytosolic glycoprotein that has multiple functions including regulation of apoptosis [21]. There is accumulating evidence suggesting that clusterin is an anti-apoptotic protein associated with chemoresistance. In fact, recent study has shown that clusterin-overexpressing cells are highly resistant to CPT- and etoposide-mediated apoptosis [26]. We have observed a striking difference in clusterin protein expression between LNCaP and PC-3 cells, as shown in Fig. 2, *a*; abundant in PC-3 cells and marginal in LNCaP cells. This predominant expression of clusterin may at least in part account for resistance to CPT in PC-3 cells but not in LNCaP cells. Moreover, it is of great interest to note that the expression of clusterin protein was enhanced in a time-dependent fashion within 24 h following CPT-treatment in PC-3 cells. This enhanced clusterin expression was considered to be regulated at the transcriptional regulation.

The IAP proteins XIAP and survivin expression were shown to be at the nearly same level, which is distinct

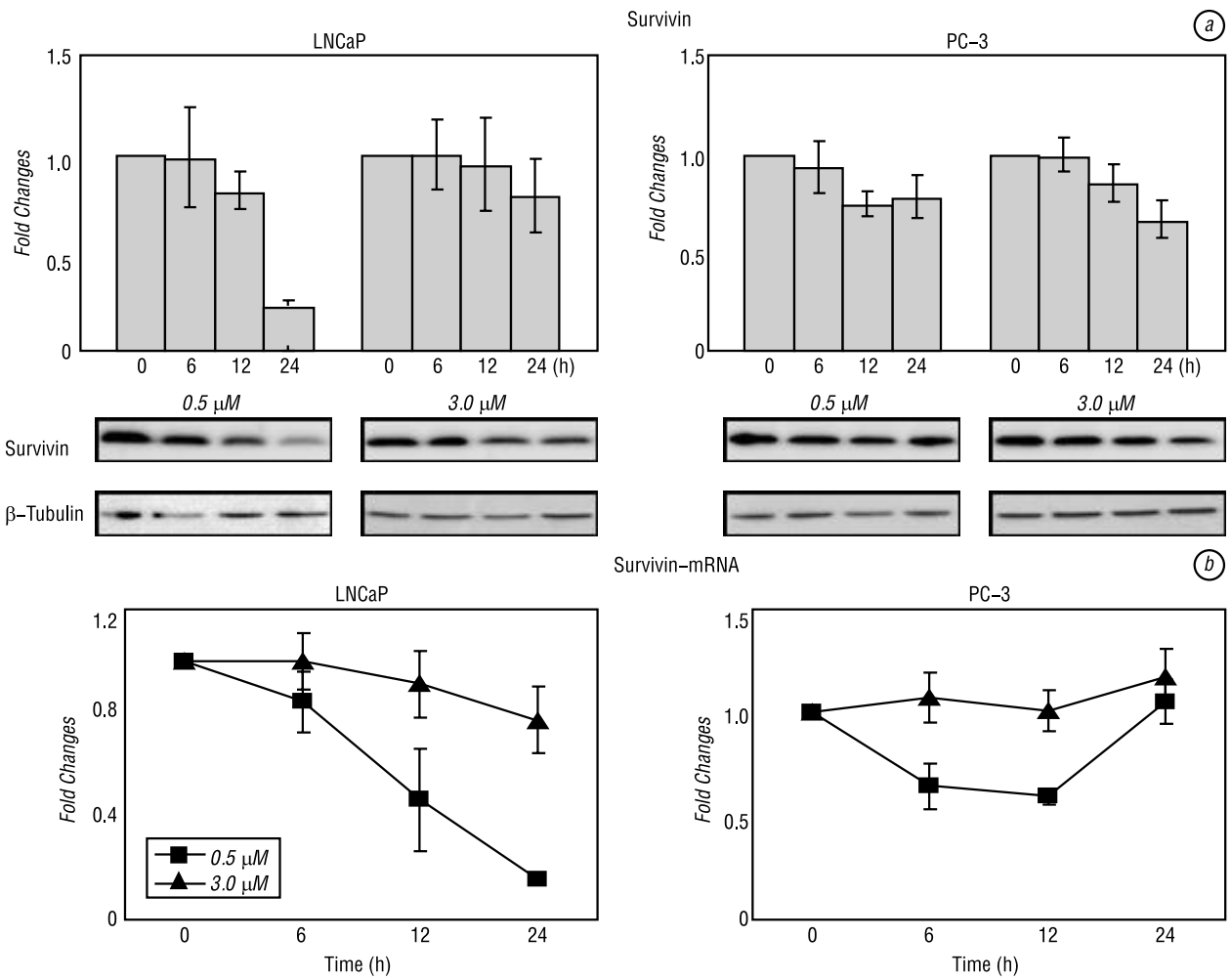

Fig. 5. Expression of survivin in LNCaP and PC-3 cells upon CPT-treatment. (*a*) Time course of the protein expression of surviving analyzed by Western blotting with the specific antibody for human survivin. Fold changes were determined from relative intensity to the control. Values represent means ± SD (bars) from three independent experiments. The blots represent one of three separate experiments with similar results. (*b*) Time course of survivin mRNA expression analyzed by RT-PCR. Fold changes were determined from relative intensity to the control. Values represent means ± SD (bars) from three independent experiments

from clusterin. Furthermore, in sharp contrast to clusterin, CPT-induced up-regulation of XIAP and survivin was not observed in PC-3 cells as well as LNCaP cells. Instead, the content of both anti-apoptotic proteins was reduced in a time-dependent fashion in both cell lines. The down-regulated expression of these proteins is favoring for induction of the apoptotic process. Unexpectedly, the survivin level was markedly reduced in LNCaP cells exposed to 0.5 μM CPT compared to 3.0 μM CPT-treated cells, and this profound decrease in the protein level was well correlated with mRNA expression (Fig. 5). At present, we have no adequate explanation for this finding and further experiments are needed to address this phenomenon.

Taken together, the comparative investigations of the expression profiles for three anti-apoptotic proteins provide at least in part some evidence suggesting that clusterin may take a greater part in resistance to CPT in PC-3 cells, compared to XIAP and survivin.

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ЭКСПРЕССИЯ КЛАСТЕРИНА, XIAP И СУРВИВИНА В КЛЕТКАХ РАКА ПРЕДСТАТЕЛЬНОЙ ЖЕЛЕЗЫ, УСТОЙЧИВЫХ И РЕЗИСТЕНТНЫХ К КАМПТОТЕЦИНУ

Цель: **известно, что кластерин и белки-ингибиторы апоптоза семейства IAP, такие как сурвивин и XIAP, связаны с химиорезистентностью опухолевых клеток. Задача исследования — определить уровни экспрессии указанных белков в линиях опухолевых клеток предстательной железы, чувствительных (LNCaP) и устойчивых (PC-3) к действию камптотецина (CPT) — ингибитора топоизомеразы I.** ***Методы:*** **клетки LNCaP и PC-3 инкубировали с CPT, после чего анализировали количество погибших клеток с использованием двойного окрашивания реактивом Херста 33 342 и пропидиум-иодидом. Экспрессию кластерина, XIAP и сурвивина на уровне белка и мРНК оценивали методами Вестерн-блоттинга и полуколичественного РТ-ПЦР соответственно.** ***Результаты:*** **установлено, что вследствие 24-часовой инкубации клеток с 0,5 и 3,0 μM CPT определяют более значительный процент гибели клеток линии LNCaP, чем таковых PC-3. Данные Вестерн блот анализа выявили, что в клетках линии PC-3 количество кластерина в 5 раз больше, а XIAP — меньше, чем в клетках линии LNCaP, в то время как экспрессия сурвивина одинакова в обеих клеточных линиях. Инкубация клеток с 0,5 и 3,0 μM CPT приводила к снижению уровня сурвивина и XIAP в обеих линиях. Экспрессия кластерина на уровне мРНК и белка не изменялась при действии CPT на клетки линии LNCaP, однако повышалась в клетках линии PC-3.** ***Выводы:*** **полученные данные свидетельствуют о том, что в развитии резистентности к камптотецину клеток линии PC-3 кластерин может играть более важную роль, чем сурвивин и XIAP.**

Ключевые слова: **LNCaP, PC-3, кластерин, XIAP, сурвивин, камптотецин.**