Combination Effect of Bowman-Birk Inhibitor and α-Tocopheryl Succinate on Prostate Cancer Stem-Like Cells

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Summary

The reoccurrence of androgen-dependent prostate cancer after anti-androgen therapy mainly depends on prostate cancer stem-like cells. To reduce the risk, it is important to delete the cancer stem-like cells. Furthermore, to induce differentiation of cancer stem-like cells is critical to abrogate stemness of the cells. Therefore, we tried to investigate a possibility on the establishment of a new effective therapy to eradicate the cancer stem-like cells via the induction of differentiation in this study. Prostate cancer stem-like cells from an androgen-dependent prostate cancer cell line (LNCaP cell) had severe resistance against an anti-androgen therapeutic agent. We selected Bowman-Birk inhibitor (BBI) from soybeans reported as a chemopreventive agent in prostate cancer to differentiate the cancer stem-like cells and α-tocopheryl succinate (TOS) known as a mitocan to induce effectively cytotoxic effect against the cancer stem-like cells. In fact, only TOS treatment had cytotoxic effect against the cancer stem-like cells, but the addition of BBI treatment to the cells treated with TOS reinforced TOS-mediated cytotoxicity in the cancer stem-like cells. This reinforcement coincided with the combination-enhanced apoptosis in the stem-like cells. Also, we confirmed caspase9-caspase3 cascade mainly contributed to the enhancement of the cytotoxicity in the stem-like cells caused by the combination, indicating that the reinforcement of BBI on TOS-mediated apoptosis via mitochondria related to the enhancing cytotoxic effect of the combination on the prostate cancer stem-like cells. Overall, it seems that the combination is an effective new approach to reduce the reoccurrence of prostate cancer targeting prostate cancer stem cells.

Key Words

androgen, prostate cancer, stemness, vitamin E derivative, cytotoxicity, soybeans

In recent Japan, there is an increasing tendency about the incidence as well as the mortality of prostate cancer year by year (1). As an early stage, androgen deprivation therapy is effective to suppress prostate cancer cell growth (2), but the duration of the therapy induces the appearance of malignant phenotypes such as severe chemoresistance in prostate cancer cells called castration-resistance (3). Thus, it is urgently and absolutely required to establish a new effective prevention against prostate cancer.

Recent reports have strongly supported the cancer stem cell hypothesis and have indicated that the stem cells may be the primary causes of tumor initiation, progression, reoccurrence, metastasis and resistance to cancer treatment (4). Prostate cancer stem cells are also suggested to be responsible for castration-resistant phenotype and the subsequent poor prognosis for patients with advanced prostate cancer (5). Thus, to complete prostate cancer prevention, it may be effective to establish eradicative approach against prostate cancer stem cells. It has been reported that cancer cells have chemoresistance based on ultimately undifferentiated status of the cancer stem cells, so any agent-mediated induction of differentiation in the stem cells may lead to abrogation of the resistance (6). After the treatment inducing differentiation of the cancer stem cells, it is assumed that the chemoresistance in the stem cells can be reduced due to less stemness. Then, this treatment procedure is critical to eradicate the cancer stem cells, finally leading to establishment of a new and effective strategy against the reoccurrence of cancer.

It is well known that Bowman-Birk inhibitor (BBI) from soybeans acts as an anticarcinogenic protease inhibitor (7), and BBI-mediated negative growth control against prostate cancer cells depends on the induction of connexin (Cx) 43, which is a tumor-suppressive gene in prostate cancer (8). Cx43 can keep differentiation status in prostate normal cells via the formation of gap junctions necessary for allowing the passage of small growth-regulatory signal molecules between contacting cells (9). Also, we have reported that BBI-
induced expression of Cx43 contributes to differentiation and reduction of some stem-like phenotypes in the prostate cancer stem-like cells (10). On the other hand, α-tocopheryl succinate (TOS) triggers selective apoptosis in cancer cells and cancer stem-like cells, due to affecting mitochondria complex II by interfering with the function of ubiquinone (11). Additionally, TOS treatment shows significant reduction in prostate cancer cell xenograft growth on nude mice, indicating that TOS acts as a chemotherapeutic agent in human prostate cancer (12). Overall, it seems to be possible that the differentiation of prostate cancer stem-like cells by BBI and subsequent treatment of TOS are effective to eradicate prostate cancer stem-like cells. Therefore, the present study was undertaken to clarify this hypothesis.

**Materials and Methods**

**Reagents.** All cultures and chemicals were obtained from Nacalai Tesque, Inc. (Kyoto, Japan), unless otherwise indicated. Fetal bovine serum (FBS), Bowman-Birk inhibitor (BBI) and α-tocopheryl succinate (TOS) were purchased from Sigma-Aldrich (St. Louis, MO). Caspase inhibitor set (caspase3 inhibitor, Z-Asp-Glu-Val-Asp-fluoromethylketone; caspase8 inhibitor, Z-Ile-Glu(OMe)-Thr-Asp(OMe)-fluoromethyl-ketone; caspase9 inhibitor, Z-Leu-Glu(OMe)-His-Asp(OMe)-fluoromethylketone) was from Enzo Life Sciences Inc. (Farmingdale, NY). All antibodies were obtained from Cell Signaling Technology Inc. (Beverly, MA).

**Cell culture and cancer stem-like cell preparation.** LNCap cells, a representative human androgen-dependent prostate cancer cell line (ATCC, Manassas, VA), were routinely grown in RPMI1640 supplemented with 10% FBS, 50 IU/mL penicillin, and 50 μg/mL streptomycin at 37°C in a humidified atmosphere with 5% CO₂. To isolate LNCaP stem-like cells, we utilized the tumor-
sphere forming capacity of the cells in a three-dimensional (3D) culture system using a low attachment culture plate (Thermo Fisher Scientific, Kanagawa, Japan), as mentioned previously (13). Since the tumor-sphere formation capacity of LNCaP stem-like cells until four passages in a two-dimensional culture was retained, the stem-like cells up to four passages were used. For the experiments, exponentially growing cells were used. The stem-like cells were plated on culture plates and cultured for 24 h to permit the cells to adhere. After attachment, the stem-like cells were cultured in DMEM/F12 (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) supplemented with 2% FBS containing each reagent (BBI and TOS) for each indicated concentration period, and subsequently each parameter was determined.

**Cell growth and apoptosis analysis.** Cell growth and apoptosis analysis were determined by a WST-8 assay and an Annexin V-FITC Apoptosis Detection Kit (Nacalai Tesque, Inc.) using Becton Dickinson FACSalibur HG (Japan BD, Fukushima, Japan), respectively.

**Immunoblotting.** After each treatment, cell lysates were prepared in a Laemmli sample buffer (Bio-Rad, Hercules, CA) including a phosphatase inhibitor cocktail and a protease inhibitor cocktail (Nacalai Tesque). After centrifugation, the supernatants (10 μg total protein content) were separated by 15% SDS-PAGE and transferred onto a PVDF membrane (ATTO, Tokyo, Japan). After overnight incubation with Blocking One (Nacalai Tesque), the membrane was incubated with the indicated primary antibody for 1 h at room temperature, followed by a secondary antibody incubation for 1 h at room temperature. Detection of each band was accomplished using Chemi-Lumi One Super (Nacalai Tesque) and a cooled CCD camera-linked Cool Saver System (ATTO). A two-dimensional densitometric evaluation was performed using ATTO Image Analyzer System (ATTO). Molecular sizing was estimated using Protein Dual Color Standards, and protein concentration was determined by DC Protein Assay Kit (both obtained from Bio-Rad).

**Statistical analysis.** Differences among groups were analyzed by one-way ANOVA followed by the Tukey-Kramer test, and differences between two groups were analyzed one-way ANOVA followed by Student’s t-test. All statistic analyses were performed using Ekuseru-Toukei software (Social Survey Research Information Co., Ltd., Tokyo, Japan). Differences with p-values of 0.05 or less were considered significant.

**Results and Discussion**

As mentioned above, to establish a new approach which can eradicate cancer stem cells finally leads to setting up an effective preventive strategy against the reoccurrence of several types of cancers. The cancer stem cells have severe chemoresistance mostly due to their undifferentiated status (14), so as a first step, it is absolutely required to reduce the chemoresistance via the induction of differentiation in cancer stem cells. Subsequently, to cause an effective cytotoxicity on the differentiated cancer stem cells may be important to completely eradicate the stem cells. In this context, the present study was undertaken to confirm this possibility.

First, we evaluated the resistance of LNCaP stem-like cells against an anti-androgen agent, flutamide. As shown in Fig. 1A, treatment of LNCaP cells with flutamide (0–100 nM) showed a dose-dependent inhibition of cell viability, on the contrary, about 50% of LNCaP stem-like cells had the resistance in high doses (60–100 nM), indicating that anti-androgen therapy was ineffective towards the stem-like cells. Being different from the anti-androgen treatment, the combination of BBI and TOS (BBI, 200 μg/mL and TOS, 30 μM) showed a significant decrease of cell viability comparing with TOS treatment (Fig. 1B). Furthermore, the treatment dose of BBI was fixed as 200 μg/mL in this study, because cell growth control by BBI reached a plateau in this dose (data not shown). This result indicated that the combination of BBI and TOS could be a valid approach to eradicate LNCaP stem-like cells. Next, we investigated if the combination effect could depend on the induction of apoptosis. As shown in Fig. 2A, the combination treatment of BBI and TOS enhanced the induction of apoptosis comparing with other groups (apoptotic cell percentage; control, 6.88%; BBI, 13.6%; TOS, 26.5%; BBI+TOS, 36.2%). Also, the cleaved level of caspase3 (a marker of caspase3 activation) in BBI+TOS group showed the highest level among all groups (Fig. 2B), and the difference of the cleaved level in the groups showed a similar tendency with that of percentages of early apoptotic, late apoptotic and dead cells in total cells, as shown in Fig. 2A. This observation indicated that the cleaved level of caspase3 closely related to cell death in LNCaP stem-like cells. Finally, to clarify which apoptotic pathway relates to the apoptosis, we evaluated the apoptotic pathway, using several caspase inhibitors (Fig. 2C). As a result, the inhibitors towards caspase3 and caspase9 but not caspase8 partly restored cell viability in LNCaP stem cells treated with the combination of BBI and TOS. These results suggested that caspase9-caspase3 pathway contributed to the cytotoxic effect of the combination on LNCaP stem-like cells.

Cancer stem cells generally locate G0 phase in cell cycle and are inactive for cell cycle progression, so the stem cells can be insensitive to conventional chemotherapeutic agents targeting cell cycle progression (14). Based on this report, it is reasonable to consider a possibility that anti-cancer agents having cytotoxicity on cancer cells irrespective of cell cycle progression can be effective to eradicate the cancer stem cells. From this possible hypothesis, we selected TOS as a candidate to delete prostate cancer stem cells. The detailed reason why we selected TOS are as follows: 1) there are many reports that TOS acts as a potential anti-cancer agent towards several types of cancers, especially prostate cancers, with less toxicity in non-tumorigenic cells (15, 16); 2) TOS has selective and strong cytotoxicity against cancer cells as a mitocan targeting mitochondria via the inhibition of complex II irrespective of cell cycle progression (17). Additionally, a recent report has demonstrated that TOS kills breast tumor-initiating cells (can-
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Collectively, it seems to be possible that TOS also eradicates LNCaP stem-like cells due to mitochondria-initiated apoptosis. In fact, we confirmed that TOS effectively induced apoptosis in LNCaP stem-like cells, via the activation of mitochondria-dependent apoptotic pathway, that is, caspase9-caspase3 pathway (19).

To reinforce TOS-induced cytotoxicity against LNCaP stem-like cells, as mentioned above, the induction of differentiation in the stem-like cells may be an effective procedure. Since we have reported that BBI could induce differentiation in LNCaP stem-like cells via the restora-
tion of Cx43-dependent functions such as gap junctional intercellular communication (GJIC) (10), we selected this agent as a differentiated agent on the stem-like cells in this study. In fact, we observed that co-treatment of BBI with TOS effectively enhanced TOS-induced cytotoxicity on LNCaP stem-like cells in this study. It is generally established that Cx-mediated tumor-suppressive effect depends on GJIC-dependent and independent functions (20). Cx-mediated GJIC maintains cellular homeostasis between neighboring cells via a direct transfer of several second messengers and small molecules like calcium ions (21), and the GJIC contributes to a tumor-suppressive effect of Cx gene and enhancement of cytotoxicity induced by anti-cancer agents (22). From these reports, it seems that BBI-enhanced TOS-induced cytotoxicity on LNCaP stem-like cells is partly associated with the restoration of Cx43-mediated GJIC. In actually, we have reported that BBI treatment induces the expression of Cx43 and restore the localization in cell membrane in our other study (10). In addition to this effect, GJIC-independent function may closely relate to TOS-induced cytotoxicity on LNCaP stem-like cells via mitochondria-initiated apoptosis. In a recent study, the restoration of Cx43 expression in mesothelioma cells enhances anti-cancer agent-induced apoptosis via the activation of c-Jun N-terminal kinase (JNK)-Bax axis (23). As explained, TOS acts as a mitocan via the activation of Bax to induce mitochondria-initiated apoptotic pathway (17). Thus, BBI-induced Cx43 expression may reinforce the activation of Bax by TOS treatment and finally lead to the mitochondria-initiated apoptosis in LNCaP stem-like cells. Also, with respect to the negative effect of the combination of BBI and TOS on normal cells, we can speculate that the combination has less toxic effect on normal cells comparing with cancer cells based on the following reason. A previous report has shown that TOS shows a selective toxicity on breast tumor cells but not normal breast cells having Cx43-driven GJIC (24, 25). Additionally, we have observed that BBI had no effect on Cx43 expression in non-tumorigenic cells (26). Thus, it seems that the combination of BBI and TOS is a promising treatment to eradicate prostate cancer stem-like cells with less toxicity. However, further study is absolutely required to establish the effectiveness of this combination as a new preventive treatment for the reduction of recurrence of prostate cancer after anti-androgen therapy.

In conclusion, the combination of BBI and TOS can be a promising approach to reduce the risk of recurrence of prostate cancer after anti-androgen therapy by targeting prostate cancer stem cells.

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