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Vitamin C Enhances the Effect of Etoposide to Inhibit Human Prostate Cancer Growth *in vitro*

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Vitamin C+etoposide 복합투여에 의한 전립선 암세포 성장 억제의 상승 효과

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ABSTRACT

Etoposide (Eto) is chemotherapeutic compounds that is currently used in the treatment of metastatic prostate cancer but new therapeutic agents are needed for the treatment of androgen-independent prostate cancer. The objective of the present study was to determine whether vitamin C (VC), the antioxidant, plays a role in regulating the growth of prostate cancer cell lines and whether VC has synergistic effect to tumor cell killing by chemotherapeutic drugs. Androgen-dependent LNCaP and androgen-independent DU-145 prostate cancer cell lines were used in this study. Both cells presented increase of dose- and time-dependent cytotoxicity in Eto-treated cultures. The combined treatment with Eto and VC significantly increased the percentage of apoptotic cells compared to Eto-treated cells ($p<0.05$). The present findings demonstrated that VC inhibited the growth of prostate cancer cell lines by Eto-mediated cytotoxicity and induced apoptosis. These results suggest that the chemotherapeutic effect of Eto on prostate cancer can be enhanced by VC.

Keywords : Prostate cancer, Apoptosis, Etoposide, Vitamin C

INTRODUCTION

Prostate cancer is a leading cause of cancer-related death in men (Jemal et al., 2007), that can be successfully treated with radical surgery, radiation therapy or androgen blockade, if the malignant tissue is confined within the prostate. However, for the androgen-independent metastatic disease, no sussessful treatment is available yet (Diaz & Patterson, 2004). According to the kinetics of tumor growth, an increase on a neoplastic cell population is the result of the imbalance between the two processes controlling tissue homeostasis, that is, cell proliferation and cell death. Apoptosis therefore comprises a critical intrinsic cellular defense mechanism against tumorigenic growth which, when suppressed, may contribute to malignant development (Kerr et al., 1994). Multiple genetic and epigenetic factors have been implicated in the oncogenesis and progression of prostate cancer, but the molecular mechanism underlying the disease remain largely unknown (Kyprianou, 1994; Tu et al., 1996; Lu et al., 1999). A wide variety of cytotoxic

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agents with different intracellular targets can induce the uniform phenotype of apoptosis (Kerr et al., 1972). This implies that the cytotoxic activity of anticancer drugs is not solely dependent on specific drug-target interactions but also on the activity of an apoptotic machinery (Borner et al., 1995; Kawamura et al., 1996; Frost et al., 1999). Several chemotherapeutic drugs like cisplatin (CDDP), etoposide (Eto), doxorubicin (DXR) induce programmed cell death or apoptosis (Eastman, 1990; Skladanowski & Konopa, 1993; Okamoto-Kubo et al., 1994). Eto, topoisomerase II inhibitor has been reported to cause the death of prostate cancer cells at least, in part, by increasing ceramide levels and activating caspase cascades (Sumitomo et al., 2002). The combined gefitinib, tamoxifen and etoposide also caused a higher rate of apoptotic death of prostate cancer cells as compared to single agent (Mimeault et al., 2006). The antioxidant, VC has been suggested to play a protective role in development of cervical intraepithelial neoplasia (Kwasniewsk et al., 1996). However, its role in the treatment of prostate cancer has not been studied.

The present investigation was undertaken to determine whether combination treatment using VC with Eto might inhibit more effectively the growth of prostate cancer cells and induce a higher rate of apoptotic death in not only androgen-dependent LNCaP but also androgen-independent DU-145 cells compared to Eto alone.

MATERIALS AND METHODS

1. Cell culture

Two human prostate cancer cell lines, LNCaP and DU-145 cell lines were used in all experiments. Cells were grown in RPMI 1640 (ICN Biomedical, Aurora, OH) supplemented with 10% fetal bovine serum (GIBCO, BRL), 4% penicillin-streptomycin (GIBCO, BRL) under standard condition in a water-saturated atmosphere of 5% CO_2 in air. The cultures were treated with 0, 1, 10, 100 μg/mL of Eto alone or in 10 μg/mL of Eto with 10 μg/mL of VC. Cells were examined by light inverted microscopy and TEM to assess morphological changes of apoptosis.

2. Growth kinetics and cell viability

Cells (2×10^5) were plate on culture flasks. Medium was changed after 24 hr and supplement with Eto alone or in combination with VC at varying concentrations for 24, 48, 72 hr. After treatments, the cells were washed with PBS, trypsinized, collected, and resuspended in fresh medium, and viable cells were counted using 0.4% trypan blue staining. Non-stained cells were regarded as viable, and blue cells were considered non-viable.

3. Percentage of apoptotic cells

Defined as (number of nonviable cells/total cell number) × 100

4. Percentage of cytotoxicity

$$\text{Defined as } 1 - \frac{\text{No. of experimental viable cells}}{\text{No. of control viable cells}} \times 100$$

5. Transmission electron microscopy (TEM)

The cells were prepared as above, fixed in 2% formaldehyde. Then, after a washing in 0.1 M PBS, they were fixed with 2.5% glutaraldehyde (0.1 M PBS, pH 7.4), treated with 1% osmium tetroxide (0.1 M PBS pH 7.4), fixed, dehydrated in ethanol, and finally embedded in epoxy resin. Thin section (60 nm thickness) were sliced, and then were double-stained with uranyl acetate and lead citrate. The sections were then examined under a JEM-2000Fx electron microscope (JEOL, Tokyo, Japan).

6. Flow cytometry

LNCaP and DU-145 cells were grown at a density of about 2×10^5 cells on 60 mm dishes as described above. The cells treated with 10 μg/mL of Eto or in combination with 10 μg/ mL of VC for 24 hr, were centrifugated at 1,000 rpm for 5 min, and washed in PBS. Pallet was resuspended in 425 μL PBS and 25 μL propidium iodide. 50 μL NP40 in 1% PBS were added, prior to cytometry analysis (Becton-Dickinson FACSCalibur).

7. Statistical analysis

Statistical analysis was performed using the Student's t-test to determine significant differences between treatment groups, with p values < 0.05 indicating statistically significant differences.

RESULTS AND DISCUSSION

Apoptosis in the prostate or in prostate cancer cells (especially androgen-dependent cells) can be induced by androgen ablation, antiandrogens, irradiation, and chemotherapeutic agents (Sklar et al., 1993; Kwon & Lee, 1999). Other steroid

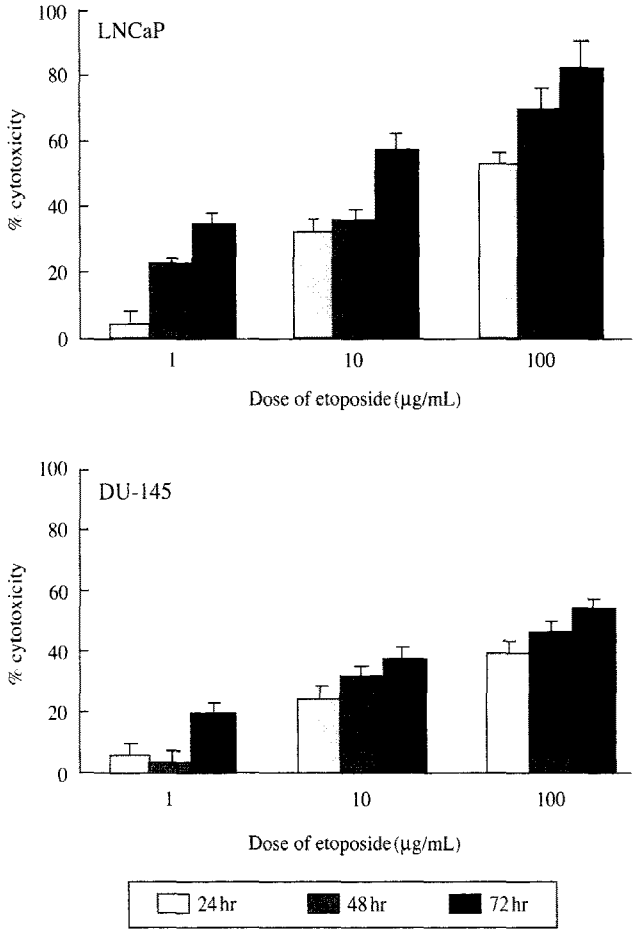

Fig. 1. The percentage of cytotoxicicity was measured after 24 hr, 48 hr, and 72 hr incubation with different dose of Eto by the trypan blue dye exclusion test. Columns, means of three different experiments performed each in duplicate; bars, SD.

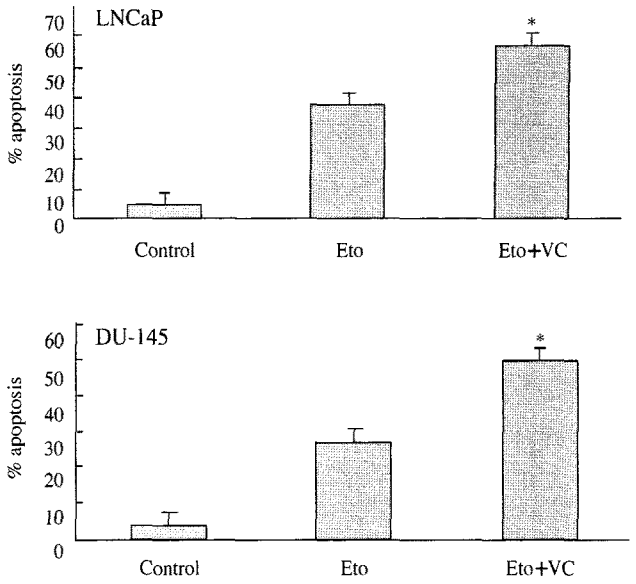

Fig. 2. Synergistic interaction of Eto and VC in LNCaP and DU-145 cell lines. The percentage of apoptotic cells in LNCaP and DU-145 cells treated with 10 μg/mL of Eto alone or in combination with VC for 24 hr. The presence of VC causes a significant increase in the percentage of apoptotic cells when compared to Eto treated cells. Data shown as means and standard deviation (SD), are given after examination by three different experiments. Asterisks mean significant differences between Eto and combination treatment ($p < 0.05$).

hormones such as glucocorticoids, can also repress apoptosis in the rat ventral prostate (Rennie et al., 1989). Eto is chemotherapeutic compound currently used in clinical trial for metastatic prostate cancer (Smith et al., 2003; McKeage & Keam, 2005).

In the present study, Eto treatment of LNCaP cells resulted in a dose-and time-dependent cytotoxicity, accompanied by induction of apoptosis (Fig. 1). As shown in Fig. 1, Eto at 100 μg/mL effected significantly different levels of cytotoxicity. In a second series of experiments, this study examined whether VC plays a role in regulating the growth of androgen-dependent LNCaP and androgen-independent DU-145 prostate cancer cell lines and whether VC has synergistic effect to tumor cell killing by chemotherapeutic drugs.

VC in cervical carcinoma HeLa cells increased the susceptibility/apoptosis induced by CDDP and Eto, priming with low doses of VC can have a significant additive effect particularly with low dose of in vivo achievable chemotherapeutic drugs, as shown by increased apoptosis (Ruddy et al., 2001). But the combination of high doses of VC and CDDP could decrease the effect of chemotherapy. The reason for this is not known although it is known that VC at low doses acts as antioxidant and at high doses as a pro-oxidant (Sestili et al., 1996). However, this finding is in congruence with a report, which states that hydrogen peroxide lowers the degree of cell killing by cisplatin (Shacter et al., 2000).

The combined treatment with low dose (10 μg/mL) of Eto and low dose (10 μg/mL) of VC for 24 hr increased the percentage of apoptotic cells from 37.1±3.6% (LNCaP), 26.1± 4.7% (DU-145) in Eto-treated cultures to 56.4±2.8% (LNCaP), 48.7±3.1% (DU-145) respectively in Eto plus VC treated cells (Fig. 2). These results demonstrate that VC significantly inhibited the growth of both LNCaP and DU-145 prostate cancer cell lines by Eto-mediated cytotoxicity ($p < 0.05$).

Apoptosis is morphologically characterized by several features, such as cell shrinkage, separation from neighboring cells, nuclear condensation, nuclear membrane breakdown, cytosol membrane blebbing, and cytolysis (Kyprianou & Isaacs, 1988; English et al., 1989). Cells shrink due to a loss of cytoplasmic volume in the initial phase of the apoptotic progress. In the following phase, the plasma membrane ruffle

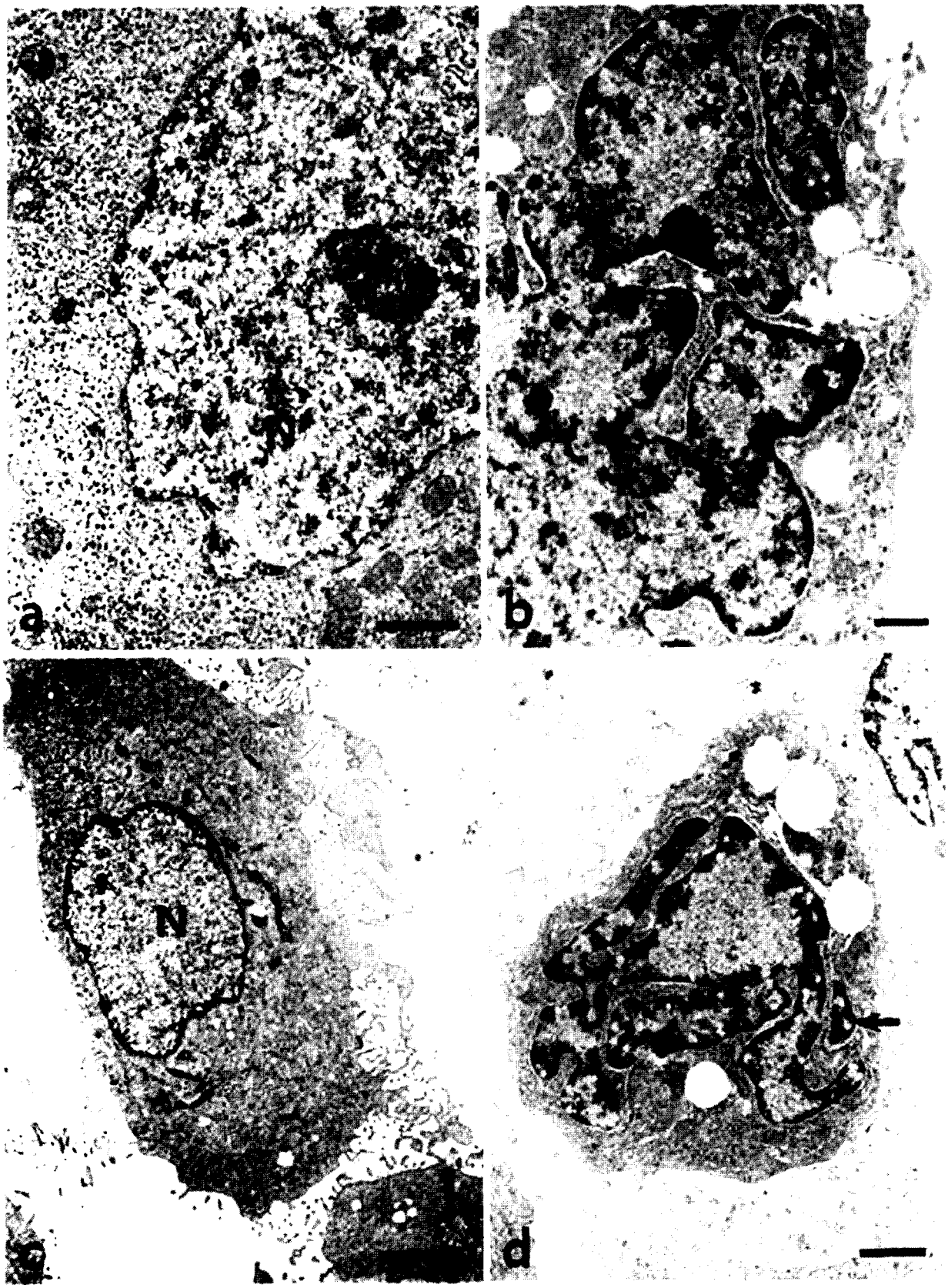

Fig. 3. Effects of Eto plus VC on the ultrastructural morphologies of LNCaP (a, b) and DU-145 (c, d) cells. Photographs were taken with an transmission electron microscope. Scale bars are 1 μm (a), 500 nm (b), 2 μm (c) and 1 μm (d). Untreated cells show unremarkable feature (a, c), whereas after 10 μg/mL of Eto with 10 μg/mL of VC exposure for 24 hr, cells exhibit condensation of nuclear chromatin and apoptotic bodies (b, d). AB and arrow; Apoptotic body, N: Nucleus.

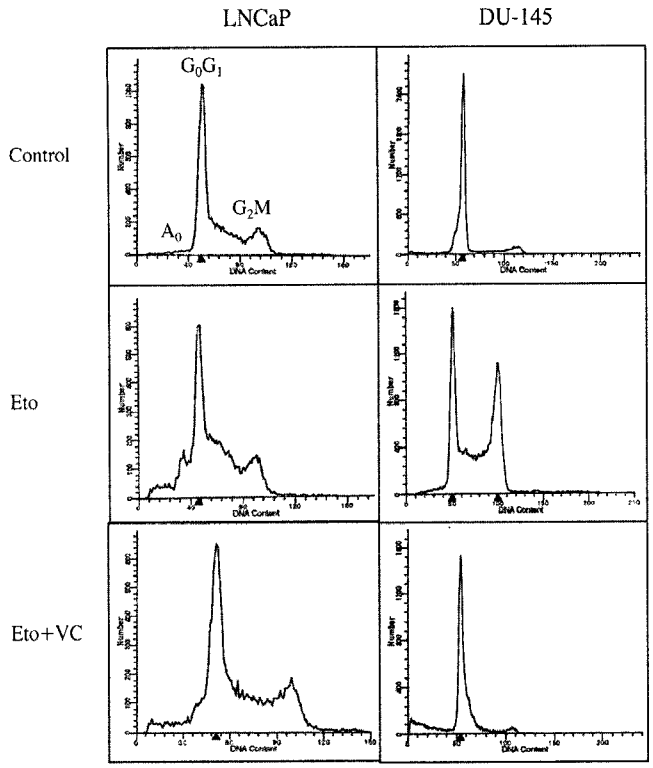

Fig. 4. Cell cycle analysis of LNCaP and DU-145 cells exposed to 10 μg/mL of Eto alone or in combination with 10 μg/mL of VC.

and blebb. In the third phase, progressive degeneration of residual nuclear and cytoplasmic structures is observed (Salido et al., 2001). In the present study, transmission eletron microscopy confirmed the apoptosis of the cultured prostatic cells. Control cells were round with well-preserved nuclear membrane and cytoplasm (Fig. 3a, c). By contrast, the cells after treatment with 10 μg/mL of Eto and 10 μg/mL of VC induced extensive nuclear condensation and fragmentation, as the appearance of apoptotic bodies in prostate cancer cells (Fig. 3b, d).

To establish the percentage of apoptotic cell death, the flow cytometric analyses also were made and the apoptotic cell number in the sub-G1 phase, apoptotic region (A_0) was quantified. As shown in Fig. 4, the treatment of the Eto alone or in combination with VC for 24 hr stimulated LNCaP and DU-145 cells caused an increase in apoptotic cell population compared to untreated control.

Altogether, combination treatment using low dose of Eto plus low dose of VC resulted in a significant synergistic cytotoxic activity in both cell lines. Synergy was much higher in low dose than that in high dose required to achieve similar cytotoxicity by Eto used alone.

The data from recent trials have revealed that the combined use of gefitinib, tamoxifen, or etoposide with antiandrogen and chemotherapeutic treatments resulted in the potential clinical benefit in patients with metastatic hormone refractory prostate cancer (Berruti et al., 2005; Lissoni et al., 2005; Penne et al., 2005; Mimeault & Batra, 2006). Thus, it appears that the combination of low doses of gefitinib, tamoxifen and etoposide with the antiandrogen and/or chemotherapeutic treatments could represent a more effective and safer regimen than use of each drug alone, to eliminate the metastatic and androgen-independent prostate cancer cells, which show some properties with intermediate cancer progenitor cells, and thereby preventing disease relapse (Mimeault et al., 2006).

Additional investigations are certainly needed to understand the precise mechanism of the synergy observed between VC and Eto.

In conclusion, combination treatment using low dose of Eto plus low dose of VC revealed a enhancement of cell growth inhibition and apoptosis in prostate cancer cells. These studies indicated that VC may play a significant role in androgen-dependent and androgen-independent prostate cancer. Therefore, these results suggest that the chemotherapeutic effect of Eto on prostate cancer can be enhanced by VC.

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<국문초록>

전립선암은 남성 사망의 주된 원인이 되는 치명적인 질병으로 남성호르몬 의존형과 비의존형이 있다. Etoposide (Eto)는 현재 전립선암을 치료하는 데 사용하고 있으나 남성호르몬 비의존형에는 치료 성공률이 낮아서, 보다 효과적인 치료제 개발이 절실히 요구되어왔다. 본 연구는 항산화제인 vitamin C (VC)가 전립선 암세포에 어떠한 역할을 하는지 알아보고자 남성호르몬 의존형-전립선 암세포인 LNCaP와 비의존형 암세포인 DU-145에 비교적 낮은 농도의 Eto와 VC를 복합처리한 결과, Eto만을 투여한 것과 비교하여 암세포의 성장이 현저하게 억제되었고, apoptosis의 발생률 역시 유의적으로 증가하였다 ($p<0.05$). 이러한 결과는 VC가 전립선암 치료제로 사용하고 있는 Eto의 효과를 증가시킬 수 있음을 강력히 시사하는 것이다.