The growing incidence of prostate cancer and the traditional use of *Rubus coreanus* Miquel (RCM) for prostate health led us to compare RCM extracts and to test their efficacy in inhibiting the growth of prostate cancer cells differing in androgen dependency. Ethanol extracts of unripe RCM (EUR) were more effective in reducing cell viability than water extracts or ripe RCM. EUR-induced growth inhibition, as indicated by significant reductions in numbers of proliferating cells and decreases in the protein levels of proliferating cell nuclear antigen (PCNA), cyclin D1 and CDK4, was greater in the androgen-dependent LNCaP cells than in the androgen-independent DU145 cells. EUR also induced mitochondrial-mediated apoptosis in prostate cancer cells by reducing Bcl-2 and Bcl-\alpha levels, but increased Bax levels. Nevertheless, the LNCaP cells were more sensitive to EUR-induced apoptosis and displayed sub-G1 and late apoptotic cell populations, whereas the DU145 cells did not. Our findings suggest that EUR suppresses the growth of prostate cancer cells by anti-proliferative and/or pro-apoptotic effects, and that these effects are stronger in androgen-dependent cells.

**Key words:** *Rubus coreanus* Miquel; prostate cancer; proliferation; apoptosis

Prostate cancer is the most commonly diagnosed solid tumor and the second leading cause of cancer death in North America.\(^1,2\) It is a multi-factorial disease with a complex and unclear etiology including both genetic and environmental factors. Current therapies, including surgery, chemotherapy, and radiation therapy have limited efficacy in the treatment of advanced prostate cancer.\(^3\) Androgen-ablative therapy is commonly used to control metastatic prostate cancer, but most prostate tumors progress rapidly and become hormone-resistant.\(^4\) Disease-free survival is not significantly improved by radical prostatectomy. Hence, more studies are warranted to improve outcomes for this disease.

Deregulation of the fine balance between cellular proliferation and apoptosis is the hallmark of cancer. The expression and sequential activation of cell cycle dependent cyclins, cyclin dependent kinases (CDKs), and CDK inhibitors (CDKIs) regulate cell proliferation in eukaryotic cells.\(^5\) Cyclins, and the corresponding specific CDKs regulate the progression of the various phases of the cell cycle. The association of cyclin D isoforms with CDK4 and CDK6 leads to activation of CDK4 and CDK6 and progression through the early G1 phase of the cell cycle. This activation of CDKs is inhibited by CDKIs, including p21\(^{WAF1}\) and p27\(^{KiP1}\). Apoptosis or programmed cell death is a self-defense mechanism to remove potentially dangerous or damaged cells, such as tumor cells. Prostate cancer cells have been reported to be sensitive to the apoptosis induced by chemotherapy.\(^6\) Promotion of apoptosis, inhibition of proliferation, or both in prostate cancer cells can lead to improved prognoses for this disease.

Recent epidemiological studies suggest that dietary intake of fruits and vegetables can be beneficial in many cancers, including prostate cancer.\(^9\) Dietary and nutritional factors might be important modulating factors in the development of prostate cancer. Hence, it has been proposed that chemoprevention by dietary modulation can reduce the development of prostate cancer. *Rubus coreanus* Miquel (RCM), one of almost 100 genera in the family *Rosaceae*, is a type of red raspberry from southern Korea and China.\(^10\) The unripe fruit of RCM are used in traditional herbal medicine for the treatment of prostatism, diabetes mellitus, impotence, spermatorrhea, enuresis, and asthma.\(^11-13\) RCM has also been reported to have anti-osteoporosis, anti-stomach disorder, and anti-inflammatory effects.\(^14-17\) It has been found that the functional constituents of RCM, including polyphenols, niga-ichigoside F1, and gallic acid, have antioxidant capacities that scavenge peroxyl radicals, and have anticarcinogenic and antinociceptive effects. In particular, several lines of evidence indicate that RCM is a regulator of human cancer development, including gastric cancer and colon cancer.\(^11,18\)

Even though numerous biological activities of RCM have been reported, there is limited evidence for the effect of RCM on prostate function and its chemopreventive mechanisms in prostate cancer. Based on existing evidence, the principal hypothesis of this study was that RCM controls cell growth and apoptosis and plays an important chemopreventive role in prostate cancer. Our data indicate that RCM exerted antitumor effects by inhibiting cell proliferation and inducing apoptosis.
Materials and Methods

Preparation of RCM extracts. Unripe and ripe fruits of RCM were collected from May to June of 2010 in the Gokseong area of South Korea and freeze-dried. Four different types of extract were prepared from the RCM fruits: (i) aqueous extracts of unripe fruit (WUR), (ii) aqueous extracts of ripe fruit (WR), (iii) 50% ethanol extracts of unripe fruit (EUR), and (iv) 50% ethanol extracts of ripe fruits (ER). In brief, freeze-dried unripe and ripe fruits were pulverized and extracted in a solution of 30 g of berries/L of distilled water or 50% ethanol by heating at 45°C for 1.5 h. The supernatants were collected and frozen overnight at −80°C and dried with a lyophilizer.

RCM treatment. RCM was dissolved in dimethyl sulfoxide (DMSO) at a final concentration of 100 mg/mL, and the concentration of DMSO in the culture medium was 0.4% (v/v). Stock solutions of RCM were aliquoted and kept at −20°C for storage.

Cell culture. Two human prostate cancer cell lines, DU145 and LNCaP, were obtained from the Korean Cell Line Bank (Seoul, Korea). All the cell lines were cultured in RPMI 1640 medium containing 10% fetal bovine serum (v/v), 5% penicillin (100 U/mL), and streptomycin (100 μg/mL; Invitrogen, Carlsbad, CA) at 37°C in 5% CO2 and 95% air.

Cell viability assay. Cells were treated with increasing doses of RCM extracts for 24, 48, or 72 h. Cell survival was determined by adding 500 μg/mL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to each well and incubating this for another 3 h at 37°C. After removal of the medium, the cells were lysed with DMSO. The absorbance was measured at a wavelength of 560 nm using a microplate reader (Molecular Devices, Sunnyvale, CA).

Cell counting. The number of viable cells was determined by staining the cell population with trypan blue. DU-145 (3 × 10^4/mL) or LNCaP cells (9 × 10^4/mL) were plated onto 12-well plates. The following day, various doses of WUR and of EUR were treated for 24 h, 48 h, or 72 h. After incubation of the cells for the designated times, they were suspended and stained with trypan blue. The numbers of unstained (viable) and stained (dead) cells were counted by hemocytometer.

Cell apoptotic analysis. Annexin V-FITC Apoptosis Detection Kit I (BD Pharmingen, San Diego, CA) was used following the manufacturer’s instructions. Briefly, cells were harvested at the completion of treatment, rinsed with PBS, and stained by annexin V-FITC and propidium iodide (PI, 50 μg/mL) double staining. Analysis was performed on a FACS Calibur flow cytometer (BD Company, San Jose, CA) with CellQuest software.

Western blot. After RCM treatment, cells were collected and lysed in radioimmunoprecipitation assay (RIPA) buffer containing protease inhibitors. Immunoblot assay was carried out as described previously with antibodies against PCNA, cyclin D1, Cdk4, Bax, and β-actin (Santa Cruz Biotechnology, Santa Cruz, CA), and an antibody against Bcl-xL, from Cell Signaling (Danvers, MA).

Statistical analysis. Data are presented as means ± standard deviation (SD) from at least three independent experiments. Cell viability and cell counting data were analyzed statistically by two-way analysis of variance (ANOVA), followed by Duncan’s multiple range test. In addition, the difference in cell viability between WUR and EUR treatment was analyzed by Student’s t-test. Cell cycle, apoptosis, and western blot data were evaluated by one-way ANOVA by Duncan’s multiple range test using SPSS Statistics V.17.0. A value of p < 0.05 was considered statistically significant.

Results

RCM decreased prostate cancer cell viability and growth

The ripe RCM extracts failed to show any effect on prostate cancer cell viability, while the unripe RCM extracts showed a significant dose-dependent reduction in cell viability (Fig. 1A). EUR displayed greater effects on cell viability. We observed that EUR significantly reduced cell viability as compared to control as well as to WUR at concentrations of 200 and 400 μg/mL after 72 h of incubation (p < 0.05). This indicates that EUR was more effective in decreasing cell viability than the water extracts. Hence, we used EUR for the remaining experiments to investigate the mechanism of their effects on prostate cancer.

EUR was examined for its effect on the growth of DU145 and LNCaP prostate cancer cells. It significantly decreased the number of proliferating cells as early as 24 h at a concentration of 200 μg/mL as compared to the control group for both cell types (Fig. 1B). However, EUR had no effect on normal prostate epithelial cell (RWPE-1) viability at any dose (data not shown). In addition, the anti-proliferative effect of EUR appeared to be dose-dependent, because the number of cells decreased more dramatically when treated with 400 μg/mL as compared to 200 μg/mL of EUR. On the other hand, we observed that 400 μg/mL of EUR resulted in 10% viability of LNCaP cells at 24 h, while 40% viability was observed in the DU145 cells for the same incubation time, as compared to the respective control cells (Fig. 1B). These data indicate that EUR has anti-proliferative effects on prostate cancer cells regardless of androgen sensitivity.

EUR increased the sub-G1 population in the LNCaP cells but not in the DU145 cells

We attempted to determine whether the decreased growth of prostate cancer cells in response to EUR treatment was due to disturbances in cell-cycle progression. EUR elicited significant changes in the proportions of cells at the sub-G1 and G0/G1 phases only in the LNCaP cells (Fig. 2A). These changes were dose-dependent, since the LNCaP cells treated with 200 μg/mL of EUR displayed a significantly greater proportion at the sub-G1 phase with a subsequent further decrease at the G0/G1 phase in comparison to those treated with 100 μg/mL of EUR (Fig. 2A). On the other hand, the same doses of EUR did not significantly affect the proportion of DU145 cells at any phase of the cell cycle, including sub-G1. Together, these results suggest that EUR affects cell-cycle phase only in LNCaP cells, probably by increasing apoptotic cell populations in a dose-dependent manner.

EUR affected the protein levels of genes in G1-to-S phase progression in LNCaP cells

At a dose as low as 100 μg/mL of EUR, the LNCaP cells showed a significant reduction in the protein levels of the cyclin D1 and CDK4 genes (Fig. 2B). This dose of EUR did elicit a moderate change in cyclin D1 protein levels in the DU145 cells, but without affecting CDK4 protein expression (Fig. 2B). Further reductions in cyclin D1 and CDK4 proteins were observed in LNCaP cells at higher doses of EUR (200 μg/mL), whereas a modest reduction was found in the DU145 cells (Fig. 2B). The DU145 cells were more sensitive to low doses of EUR (100 μg/mL) as to PCNA protein expression, but high doses of EUR diminished the PCNA protein levels in the LNCaP cells far more than in
the DU145 cells (Fig. 2B). Overall these findings provide evidence that EUR decreased the abundance of the proteins necessary for the G1-to-S transition and for cellular proliferation in prostate cancer cells. But the EUR effects were greater on the LNCaP cells than on the DU145 cells, especially at higher doses (200 µg/mL).

EUR triggered early changes in apoptosis in prostate cancer cells

EUR at 200 µg/mL generated a significant amount of early apoptotic cells (Annexin V-FITC+, PI−) in both LNCaP cells and DU145 cells, as compared to the corresponding untreated cells (Fig. 3A). However, the proportion of early apoptotic cells was greater in the LNCaP cells (12%) than in the DU145 cells (3.5%). A
lower dose of EUR (100 µg/mL) resulted in early apoptosis only in the LNCaP cells, to an extent comparable to the effects of a higher dose of EUR (200 µg/mL) (Fig. 3A). Furthermore, the higher dose of EUR (200 µg/mL) increased advanced apoptotic populations (Annexin V-FITC+, PI+) in the LNCaP cells but not in the DU145 cells (Fig. 3A). Even if there was a tendency for EUR treatment to induce advanced apoptosis in the DU145 cells, it was not statistically significant (Fig. 3A). This indicates that 48 h of treatment with EUR at a concentration of 100 µg/mL is sufficient to promote apoptotic changes only in LNCaP cells, and that at least 200 µg/mL of EUR is required for early apoptotic membrane changes in DU145 cells.

**EUR modulated levels of proteins associated with apoptosis**

Molecular events leading to morphological changes in apoptosis were examined after EUR treatment. Both LNCaP cells and DU145 cells decreased in levels of the anti-apoptotic protein Bcl-2 when treated with 200 µg/mL of EUR. In the case of Bcl-xL, another anti-apoptotic protein, EUR at 100 µg/mL was sufficient to elicit a remarkable decrease in protein levels in both prostate cell lines (Fig. 3B). On the other hand, the expression of pro-apoptotic factor Bax increased at 200 µg/mL of EUR in both cell types. In addition, both the LNCaP and the DU145 cells exhibited increases in the cleaved form of procaspase-9, a form of active caspase-9, in a dose-dependent manner in response to treatment with EUR. These results suggest that EUR has an effect on the protein expression of genes related to apoptosis in prostate cancer cells regardless of androgen dependency, thereby the activation of apoptotic pathways.

**Discussion**

The increasing frequency of prostate cancer in Korea and the traditional use of RCM as a preventive supplement against various cancers lead us to search for dietary supplements that might prevent and/or delayed its incidence. We investigated the potential of EUR as an anti-cancer supplement, and we propose that it can aid in the inhibition of prostate cancer cell growth.

Prostate cancer cells are classified into two types depending on androgen sensitivity: androgen-dependent and androgen-independent. Androgens are hormones that are important to prostate cell survival, proliferation, and differentiation. Although a lack of androgens normally triggers prostate cell death, deprivation of androgens can generate a persistent, but small number of surviving cells that can progress in an androgen-
independent state. These types of prostate tumor cells are considered more aggressive. In the current study, we used LNCaP cells as a model of androgen-dependent prostate cancer cells and DU145 cells as a model of androgen-independent prostate cancer cells to test the efficacy of EUR as to prostate cancer cells. EUR showed an androgen-independent growth inhibitory effect on both LNCaP and DU145 cells (Fig. 1B), but the potency of EUR was greater in androgen-dependent prostate cancer cells than in androgen-independent cells (Fig. 1B). This was further demonstrated by the larger decrease in the expression of PCNA, a cell proliferation marker, in the EUR-treated LNCaP cells than in the EUR-treated DU145 cells (Fig. 2B).

Androgens are crucial regulators of the G1-to-S phase transition. When deprived of androgen, prostate cancer cells arrest at the early G1 phase with a concomitant decrease in D cyclins. The EUR-driven decrease in the protein levels of cyclin D1 and CDK4, a complex that controls cell-cycle progression at the G1 phase, was also significantly greater in the androgen-dependent LNCaP cells than in the androgen-independent DU145 cells. This effect was prominent at higher doses of EUR (200 µg/mL) (Fig. 2B). The slight reduction in cyclin D1, CDK4, and PCNA protein levels in the DU145 cells (Fig. 2B) appeared to be insufficient to elicit changes in cell-cycle profiles in those cells (Fig. 2A). On the other hand, a reduction in the proportions of cells at the sub-
A  Annexin V

B  Apoptosis regulating protein expression

Fig. 3.  Continued.
G1, and G0/G1 phases 48 h after treatment with EUR was clearly detected in the LNCaP cells (Fig. 2A). The reduced G0/G1 population can be attributed to the appearance of a sub-G1 apoptotic population (Fig. 2A). In sum, it is likely that G1 phase cell cycle progression was disturbed in the LNCaP cells but marginally maintained in the DU145 cells (Fig. 2A). This suggests that EUR-driven inhibition of growth of LNCaP cells is mediated by G1 phase arrest and might also be mediated through an androgen-dependent pathway.

One of the apoptotic pathways is activated in response to DNA damage or other cellular stresses. It involves the release of cytochrome c from the mitochondria and the formation of apoptosome, which then activates caspase 9, leading to the activation of caspases 3, 6, and 7.28) Caspase activation leads to cleavage of target cellular substrates and promotes apoptotic cell death.29) EUR treatment appears to induce mitochondria-mediated apoptosis in prostate cancer cells, regardless of androgen dependency. In both the LNCaP and the DU145 cells, EUR increased the levels of pro-apoptotic protein (Bax) and decreased the levels of anti-apoptotic proteins (Bcl-2 and Bcl-XL) (Fig. 3B). Another early apoptotic change was the exposure of phosphatidylserine on the outside of the cells, which was also detected in both LNCaP and DU145 cells (Fig. 3A), but the cell line-differential response to EUR was recognized as a late apoptotic feature. Advanced apoptosis was detected only in the LNCaP cells (Fig. 3A). Late stages of apoptotic cells displaying DNA fragmentation were detected in the sub-G1 population.30) Again only LNCaP cells, not DU145 cells, produced significant amounts of sub-G1 apoptotic cells (Fig. 2A). Thus, EUR is likely to be pro-apoptotic in prostate cancer cells but able to elicit apoptosis with much greater effect in LNCaP cells than in DU145 cells.

These differences in apoptotic susceptibility to apoptosis-inducing agents were also reported in previous studies,31) perhaps due to altered apoptotic signaling. For instance, enhanced expression of caspase inhibitors might promote the survival of androgen-independent cancer cells.32,33) Sustained Bcl-2 expression might allow cancer cells to resist anti-cancer therapies.34–36) However, a lack of Bcl-2 expression has been reported for DU145 cells,37) but this does not explain why this type of cell is more insensitive to the effects of EUR than LNCaP cells. Secondly, the expression of inhibitors of apoptosis proteins (IAPs), which are recently recognized anti-apoptotic players, have been implicated in resistance to apoptotic agents and in cancer cell
survival.38,39) Indeed, cellular inhibitor of apoptosis 2 (cIAP-2) expression is greater in apoptotic-resistant DU145 cells than in apoptotic-prone LNCaP cells.31) Therefore DU145 cells might better resist EUR-induced apoptosis than LNCaP cells due to cIAP-2 expression.

The identification of EUR as a pro-apoptotic agent in prostate cancer cells is in accordance with the findings of previous in vitro studies demonstrating that RCM extracts are cytotoxic or pro-apoptotic in human hepatocarcinoma cells, cervical cancer cells,40) gastric carcinoma cells,18) and colon cancer cells.13) In addition, the anti-cancer effects of EUR in prostate cancer cells were exerted by promoting G1 phase arrest and decreasing proliferation, which provided further potency when the cells maintained androgen sensitivity. Since EUR is not a pure compound, it would be interesting to identify biologically active constituents to induce pro-apoptotic and anti-proliferative effects in prostate cancer cells. Nevertheless, our data indicate that EUR can be a useful agent in prostate cancer treatment with EUR at lower doses might help to lower other anti-cancer drugs. Furthermore, dietary supplementation with EUR at lower doses might help to lower the risk of prostate cancer development. These applications should be investigated further by in vivo studies.

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References
