Korean J. Food & Nutr. Vol. 21. No. 4, 416～424 (2008)

THE KOREAN JOURNAL OF
한국식품영양학회지
FOOD AND NUTRITION

The Effect of Blueberry on ROS Accumulation and Cell Death in Human Normal Breast Epithelial(MCF10A) and Breast Cancer(MCF7) Cells

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블루베리가 정상유선세포와 유방암세포의 ROS 축적과 세포사멸에 미치는 영향

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Abstract

In an effort to elucidate the differential actions of blueberry(BB) in both normal and cancer cells, we utilized human breast cell lines to assess the accumulation of radical oxygen species(ROS) and ROS-associated apoptosis in both human normal breast epithelial(MCF10A) and breast cancer(MCF7) cells. BB extract was added to the cultures at a final concentration of 20 ㎍/㎖ for 0(control), 6, 12, and 24 hr intervals. The MCF10A cells evidenced no marked ROS accumulation in the presence of BB, whereas the MCF7 cells evidenced clear ROS accumulation upon BB treatment from 12 hours forward. The number of dying or dead cells did not increase in the BB-treated MCF10A cell groups, whereas that number increased profoundly from 12 hr forward. Furthermore, the expression levels of certain stress-related, and pro- and anti-apoptotic gene products evidenced differential responses to BB treatment between the MCF10A and MCF7 cell groups. These results indicate that the components of BB extract differentiate cancer cells by not preventing ROS accumulation within cells and by inducing ROS-associated cell death in cancer cells. However, no marked ROS accumulation or induction of cell death was noted in the normal breast epithelial cells. The fact that BB extract exerted a differential effect on cancer cells opens further directions of research regarding the specific components that exert the differential BB-mediated effects in the selective prevention of normal cells and therapy for cancer tissues in the physiological body.

Key words: blueberry, breast cancer, ROS, cell death, MCF10A, MCF7 cell.

INTRODUCTION

A variety of berry-bearing plant fruits are known to be rich in phytochemicals, including flavonoids and various phenolic acids[1]. These components have been previously proposed to exert beneficial effects in diverse diseases, including coronary heart disease[2,3], stroke[4], and lung cancer[5]. In particular, it has been demonstrated that a flavonoid family, the anthocyanins, exerts a broad spectrum of beneficial health and cancer preventive effects[6]. One of many studies[7] has shown that blueberry contains the highest total flavonoid and phenolic acid content among the 19 berry species thus far examined. The blueberry(BB) has been long known to enhance the nervous system and mentality, and even to stimulate the survival of transplanted brain tissue[8]. However, studies of its anti-cancer and anti-mutagenic effects have only recently been undertaken, and several previous reports indi-

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cate that BB inhibits the induction of carcinogenesis and exerts anticancer effects in certain studied cancer cell types[9~12]. Although the liver[13] and other various cell types[14,15] have been utilized to demonstrate its effects on cells, breast cancer cells have not been examined thoroughly from the perspective of radical oxygen species(ROS) accumulation and cell death *in vitro*. In particular, it remains unknown as to whether BB also inhibits cell proliferation and eventually kills normal breast cells, as well as breast cancer cells. Thus, it remains to be determined whether BB has differential effects on ROS accumulation and its associated cell death between normal breast and breast cancer cells.

In this manuscript, we assessed the effects of BB extract on ROS accumulation and cell death quantitatively in both normal breast epithelial MCF10 and cancer MCF7 cells under similar conditions in order to demonstrate that both cells respond differently to BB *in vitro*. These differential effects on normal and cancer cells may provide an open road to further research into the manner in which naturally-derived components of fruit plants might be utilized in future as a cancer-preventive and cancer-therapeutic supplement safely, thereby circumventing the selectivity problems associated with currently-used anticancer drugs.

MATERIALS AND METHODS

1. Cell Culture

The MCF10A and MCF7 cells were acquired from the American Tissue Culture Collection(Manassas, VA, USA). The MCF10A cells were cultured in a mixture(1:1) of Dulbecco's modified Eagle's medium(DMEM) and F12 medium containing 5% horse serum, 0.5 ㎍/㎖ hydrocortisone, 10 ㎍/㎖ insulin, 20 ng/㎖ epidermal growth factor, antibiotics including 100 U/㎖ penicillin and 100 ㎍/㎖ streptomycin, 100 ng/㎖ cholera toxin, and 300 ㎍/㎖ L-glutamine(all obtained from Sigma Chemical Co, St Louis, MO, USA) at 37℃ under an atmosphere of 5% CO_2[16]. The human breast cancer MCF7 cells were similarly cultured in DMEM containing 10% fetal calf serum, 100 U/㎖ penicillin and 100 ㎍/㎖ streptomycin, but in the absence of insulin[17]. When the cells were 70% confluent, they were subcultured after trypsinization, blocked with serum-containing medium, and washed with phosphate-buffered saline(PBS, pH 7.4).

2. Preparation of Blueberry(BB) Extract and Determination of BB Concentration Used

Frozen BB(Chefs Pride Pty Ltd, Silverwater, NSW, Australia) was obtained from a local market, crushed by gentle pressure and homogenized in a mixer without any fluid addition[9]. The BB homogenate was then centrifuged at 2,000 × g for 15 min prior to 15 min of centrifugation at 7,000 × g to remove any cellular debris and possible microbes. The supernatant was further processed for 1 h under ultracentrifugation at 45,000 × g at 4℃ to eliminate minute particles and aggregates. The final supernatant was obtained and considered as a BB extract for use in this study. The final concentration(20 ㎍/㎖) of BB extract used was determined in preliminary experiments that considered cell survival, toxicity, treatment duration and pH change in the medium when added to cells at varying concentrations. It was aliquoted in Eppendorf tubes and stored at −80℃ until use.

3. Measurement of ROS Accumulation

ROS accumulation in the cell was detected using 5-(6-)chloromethyl -2', 7'dichlorodihydro-fluorescein diacetate(DCFDA: Molecular Probes, Invitrogen, Carlsbad, CA, USA) in accordance with the provided instructions. The cells were cultured in black-walled and clear-bottomed tissue culture-treated 96-well microplates(Corning Inc, Corning, NY, USA) to avoid well-to-well crossover during fluorescent measurements. At intervals of BB treatment the cells were washed with warm PBS and incubated for 20 min with 800 nM DCFDA. The cells were washed twice more with PBS. The fluorescence of hydrolyzed 2,7-dichlorofluorescein(DCF) bound to intracellular ROS was analyzed using a Zenyth3100 multiwell plate reader(Anthos Labtec Instruments, Wals, Austria) with excitation and emission wavelengths set at 485 ㎚ and 535 ㎚, respectively[18]. The reading values were then transferred to a Microsoft XP Excel worksheet on a PC. The data were expressed as the fold increase in DCF fluorescence over the untreated control.

4. Fluorescence Microscopy for ROS Detection

ROS accumulation in the cell was detected via the method described above. After three extensive washings in cold PBS, the ROS-stained cells were inspected, and their images were digitally recorded with a Nikon Eclipse fluorescence microscope(TE200-U, Nikon Cooperation, Tokyo, Japan) equipped with a FLEX(Diagnostic Instruments, Inc., Sterling Heights, MI, USA) using SPOT software.

5. Flow Cytometry for Measurements of ROS and Cell Death

To evaluate the ROS levels among the treated cell populations,

measurement of accumulated ROS was also similarly conducted by staining the cells with 800 nM DCFDA in PBS. The DCF fluorescence was detected in channel FL-1(530 ㎚ filter and 30 ㎚ bandpass) after laser excitation at a wavelength of 488 ㎚ under a Becton-Dickinson FACS calibur flow cytometer(Becton Dickinson, Mountain View, CA, USA) using Cell Quest software (Becton Dickinson). For the quantitation of cell death, the PI-stained cells were also analyzed similarly using an appropriate filter block for emitted red light.

6. Detection of Cell Death by Dual Staining

In order to measure the cell death, the cells were trypsinized and collected in Eppendorf tubes from 12-well dishes without any loss of floating cells. The cells were stained for 20 min with a mixture of 10 ㎍/㎖ of Hoechst33342 and 1 ㎍/㎖ of propidium iodide(PI) solution in PBS. The stained cells were subsequently washed twice in fresh medium via centrifugation prior to inspection for PI staining for cell death under Nikon fluorescence microscope.

7. Reverse Transcription(RT)-Polymerase Chain Reaction (PCR)

Cells under various treatment regime were lysed with Trizol (Invitrogen) to isolate total RNAs. After the addition of chloroform at a 1/5 volume of Trizol used, the cell lysates were thoroughly mixed by vortexing and centrifuged for 15 min at 16,000 × g at 4℃. The Upper clear phase solution was harvested and mixed with an equal volume of ice-cold isopropanol. After 15 min of centrifugation at 10,000 × g, the precipitated RNA was washed with 75% ethanol and dissolved in double distilled water[19]. The isolated RNAs were then further digested by 1 U of RQ1 DNAse(Promega, Madison, WI, USA) in accordance with the manufacturer's instructions. The first-strand cDNA was synthesized from 1 ㎍ of DNAse treated total RNA using oligo-dT in a volume of 20 ㎕ at 37℃ for 45 min and 95℃ for 5 min. Two ㎕ of the first strand cDNA was used for PCR in a 50 ㎕ reaction mixture. PCR was conducted using the respective primer pairs of Hsp70, PrxI, PrxII, Cox-2, Bcl-2, Bax, Bak, Casp-3 and Casp-9 and GAPDH. Densitometric analysis was conducted using TINA 20 software for semi-quantitation in three replicate experiments.

8. Western Blotting

For Western blotting analysis, 30 ㎍ of the total cellular proteins from the cell lysates were loaded in each lane and separated on 5～10% gel prior to transfer to nitrocellulose membranes. The membranes were then incubated for 1 h with 5% skim milk in TBS containing 0.02% Tween 20(TBST) to avoid non-specific binding. They were then incubated with the respective primary antibodies, and subsequently with secondary antibodies conjugated to alkaline phosphatase. After extensive washing, immunoreaction was initiated via the addition of a color detection solution containing nitro blue tetrazolium, 5-bromo-4-chloro-3-indolyl phosphate, and levamisole in TBST. The positive bands were also semi-quantitatively analyzed from three replicate blottings using the TINA 20 program.

9. Statistical Analysis

All data are presented as the means±standard deviations from at least three replicate experiments, including cytochemical, RNA and protein analyses whenever possible. The differences between the mean values of the experimental groups were evaluated via Duncan's multiple range test at a level of $p<0.05$ for statistical significance.

RESULTS

1. ROS Accumulation in MCF7 and MCF10A Cells

In order to assess the effects of BB on breast cancer MCF7 and normal epithelial MCF10A cells, a predetermined 20 ㎍/㎖ of BB(data not shown) was added to the cells for 0, 6, 12 and 24 hr. Fluorimetric quantitation of total ROS accumulated within the cells was presented indirectly in Fig. 1. The control group containing a vehicle(dimethylsulfoxide, DMSO) alone evidenced a level similar to that in PBS alone(data not shown), thereby suggesting that neither the DMSO used for dissolving DCFDA nor the PBS used for cell suspension induced any ROS production. The levels of ROS increased according to the durations of BB treatment in MCF7 cells, except for BB at 24 hr, when the ROS level decreased slightly. This may be induced by the oxidative alteration of BB added to the culture for 12 hr, although we were unable to determine the extent of oxidative change occurring in BB. By way of contrast, ROS levels decreased gradually according to the durations of BB treatment in MCF10A cells.

In an effort to demonstrate the extent of ROS accumulation in the corresponding cell population of both cell lines used, the DCFDA-stained cells were immediately inspected under a fluore-

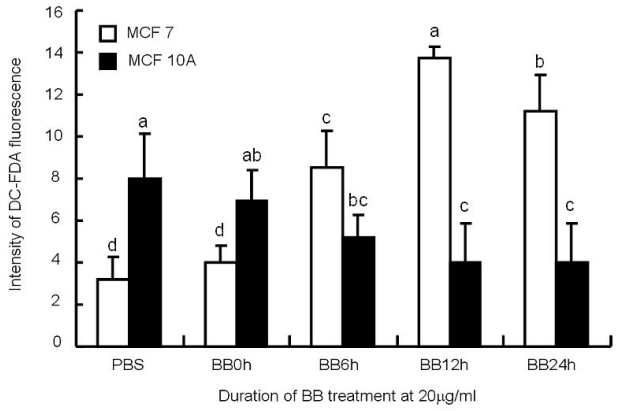

Fig. 1. Quantitative analysis of BB effects on ROS accumulation in breast cancer MCF7 and normal breast epithelial MCF10A cells. Similar number of cells were cultured in a 96-well dish. Twenty g/mℓ BB extract were added to each well for 0(washed away immediately after the BB addition), 6, 12 and 24 hr, respectively. Cells were washed with PBS at each end point, and DC-FDA was used according to the Materials and Methods. PBS groups were served as negative controls for DC-FDA addition. The levels of DCFDA-positive ROS accumulated were measured by a fluorimeter in three replicates in a 96-well microplate. The intensities of fluorescence were presented as fold of background fluorescence. The different letters on top of the each value show significant difference at $p<0.05$ level.

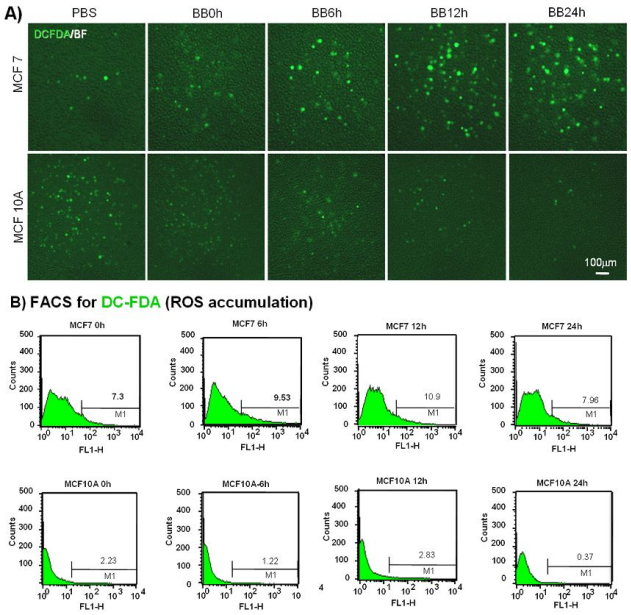

Fig. 2. The effects of blueberry(BB) extract addition on the production radical oxygen species(ROS) in breast cancer MCF7 and normal breast epithelial MCF10A cells. A similar procedure was undertaken as described in the Fig. 1 legend. Instead of measuring the fluorescence, image and FACS analyses were performed. A) A marked difference is found between MCF7 and MCF10A cells in the DCFDA-positive cells. Pairs of ROS-positive cells and corresponding bright fields(BF) were shown as merged images. B) FACS analysis of DC-FDA-stained for ROS accumulation was also shown in parallel.

scence microscope. At the given intervals(0, 6, 12 and 24 hr), the representative extent of ROS accumulation among individual cells was noted in both the MCF7 and MCF10A cells to compare different responses to BB treatment as observed in pairs of ROS-positive cells, and corresponding bright fields(BF) were visualized as merged images(Fig. 2A). Whereas a gradual increase was noticeable in MCF7 cells depending on the duration of BB addition to the culture, a gradual decrease was noted in the MCF10A cells with prolonged BB treatment.

Cell sorting for DCFDA-stained cells by FACS also demonstrated a similar difference between MCF7 and MCF 10A cells at given intervals of BB treatment(Fig. 2B). The area of clustered cells presented is clearly quantitatively different between the MCF7 and MCF10A cells, thereby demonstrating that far fewer cells evidenced ROS accumulation, and that the intensity of DCFDA staining for ROS was also relatively weak in the MCF10A cells.

2. Cell Death in MCF7 and MCF10A Cells

The effects of the addition of BB extract on cell death was also subsequently examined to reveal whether higher ROS accumulation is associated with cell death using dual staining with Hoechst33342 and propidium iodide(PI) together(Fig. 3A). At 6 hr, breast cancer MCF7 cells began to evidence an increasing number of PI-positive cells, achieving a peak value at 12 hr. By way of contrast, normal breast epithelial MCF10A cells evidenced a reduction in the number of PI-positive cells, there by suggesting that BB treatment selectively prevents the death of normal MCF10A cells. However, a small increase in cell death was noted at 24 hr in MCF10A cells.

In order to assess quantitative cell clustering showing PI-positive cell population among BB-treated MCF7 and MCF10A cells, we also conducted a FACS analysis of PI-staining for the analysis of ROS-associated cell death(Fig. 3B). In the control groups of MCF7 and MCF10A cells, the peak of all cells killed by formaldehyde-fixation shifted to the right side, demonstrating that nearly 99% of the cells were PI-positive. The cell clustering

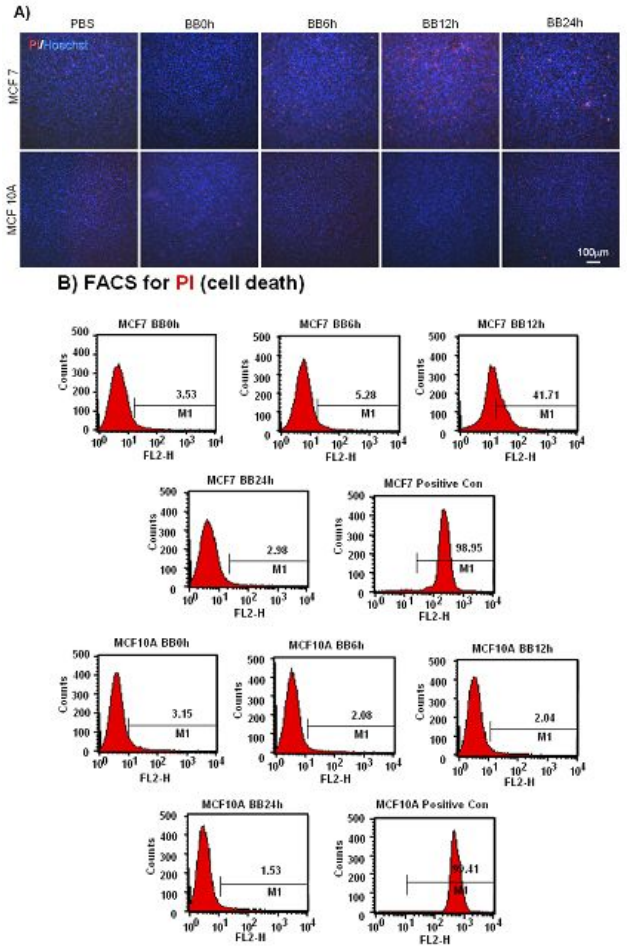

Fig. 3. The effects of blueberry(BB) extract addition on the cell death revealed by propidium iodide(PI)-positive cells. A similar procedure was undertaken as described in the Fig. 1 legend. However, the cells were stained using a vital dye(Hoechst33342) and propidium iodide that stains only dead cell nuclei as described in Materials and Methods. A) Hoechst33342 staining. The pairs of stained cells were presented as merged images. B) FACS analysis of PI- staining for cell death.

peak in the MCF7 cells shifted rapidly to the right side at up to 12 hr of BB treatment, revealing that 41 and 71% of cells were dying or dead. By way of contrast, the shift of peak to the right side in MCF10 A cells was discernable, evidencing a value of only 2.04% at 12 hr and 1.53% dying or dead at 24 hr, respectively.

3. m RNA Gene Expressions in MCF7 and MCF10A Cells

In an effort to determine whether the differential cellular effects

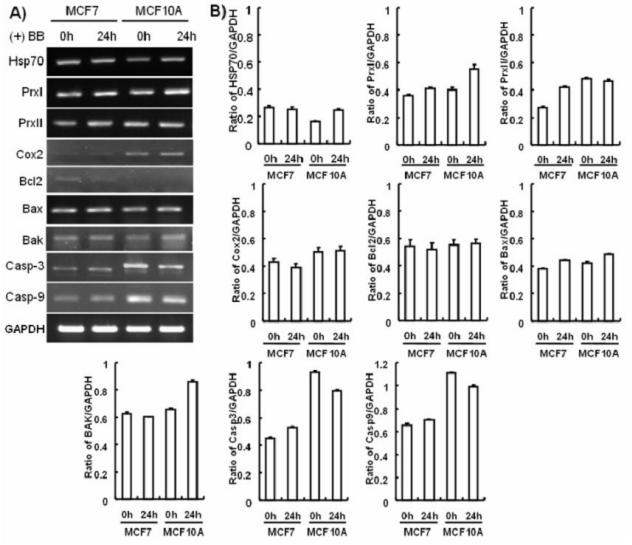

Fig. 4. RT-PCR analysis showing differential responses in MCF7 and MCF10A cells upon BB treatment. Cells were treated with 20 g/mℓ BB extract for either 0 or 24 hr. From total RNA, cDNAs were synthesized according to the method provided in Materials and Method. Using gene-specific primer pairs PCR was carried out to visualize PCR products on agarose gel. A) Representative RT-PCR results. B) Semi-quantitation of the results.

of BB on ROS accumulation and cell death in both cell lines, the mRNAs of several stress-related, and pro- and anti-apoptotic genes were analyzed via RT-PCR(Fig. 4). As noted in the case of Hsp70 expression, the basic level of Hsp70 and PrxI mRNAs was unaltered upon BB treatment in MCF7 cells, whereas the levels of the two gene transcripts increased upon BB treatment in MCF10A cells. Levels of PrxII are quite similar in both cell types, both with and without BB treatment. Whereas the level of Cox-2 gene expression is markedly lower in the MCF7 cells, expressions of Bcl-2 were detectable only in the MCF7 cells, and also evidenced reduced levels in the BB-treated group, but were undetectable in the MCF10A cells, regardless of BB treatment. Among genes in the mitochondrial pathway, the level of Bax gene expression responded to BB treatment only in the MCF10A cells, whereas no response was observed in the MCF7 cells upon BB treatment. The basic levels of Casp-3 and Casp-9 expressions were clearly different between the two cell lines. The expression patterns of Casp-3 and Casp-9 did not change significantly in MCF7 cells upon BB treatment. However, the levels of both gene expressions were clearly reduced in the MCF10A cells.

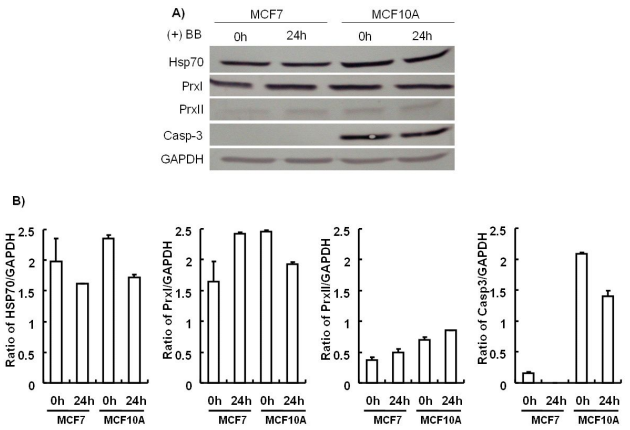

Fig. 5. Protein expression levels of Hsp70, PrxI, PrxII, Casp-3, in MCF7 and MCF10A cells treated with BB extract at 0 h and 24 h. Cells were treated with 20 g/㎖ BB extract for either 0 or 24 hr. Cell lysates were fractionated on 10% SDS-PAGE, and subsequently transferred to nitrocellulose membrane, followed by immunodetection using corresponding specific antibodies. A) Expression patterns in MCF7 and MCF10A cells with or without BB for 24 hr. B) Semiquantitation of the expressing proteins.

4. Expressions of Selected Proteins in MCF7 and MCF10A Cells

The levels of selected protein expression were finally assessed to determine whether both MCF7 and MCF10A cell lysates express similar patterns of the proteins prior to(0 hr) and after 24 hr of BB treatment. The levels of Hsp70 and PrxI proteins were reduced in normal breast epithelial MCF10A cells upon BB treatment in a manner similar to that observed in their mRNA expressions(Fig. 5). However, breast cancer MCF7 cells evidenced unexpected protein expression patterns. The levels of Casp-3 were detectable only in the MCF10A, and not in the MCF7 cells. Upon BB treatment, Casp-3 expression was reduced in the MCF10A cells, just as was the case in the stress-related Hsp70 and PrxI protein expressions. PrxII levels were increased in the MCF7 cells upon 24 hr of BB treatment, whereas no clear difference was detected in MCF10A cells(24 hr group, Fig. 5).

DISCUSSION

In our assessment of differential BB effects on breast cancer MCF7 and normal epithelial MCF10A cells, we noted that the levels of ROS evidenced completely opposite responses in both cell lines with a gradual increase in MCF7 and reduction in the MCF10A cells, respectively. Cell sorting for DCFDA-stained cells by FACS also demonstrated a similar difference between MCF7 and MCF10A cells at intervals of BB treatment, thereby suggesting that far fewer cells evidenced ROS accumulation, and that the intensity of DCFDA staining for ROS was also relatively weak in the MCF10A cells. From this experiment, it was believed that the MCF7 and MCF10A cells respond differently to the components of added BB not only in ROS accumulation, but also in ROS-associated cell death.

Many previous reports reported experimental evidence for the direct or indirect involvement of ROS in apoptosis in normal and cancer cell lines[20~22]. For example, overexpressed ROS scavenging enzyme genes delay apoptosis, whereas their corresponding siRNA knockdown of the genes results in increased cell death[21]. In our subsequent experiments, the effects of BB extract demonstrated that higher ROS accumulation is associated with cell death(Fig. 3A). MCF7 cells began to evidence an increasing number of PI-positive cells, reaching a peak value at 12 hr. Therefore, we concluded that the increased ROS levels induce MCF7 cells to undergo apoptotic cell death when BB-treatment stress is prolonged. By way of contrast, normal breast epithelial MCF10A cells evidenced a reduction in the number of PI-positive cells, thereby suggesting that BB treatment selectively prevents the death of normal MCF10A cells. It was also determined that a higher proportion of MCF7 cells were dying or dead. However, BB treatment appears to prevent ROS accumulation in the cells and apoptosis in MCF10A cells, evidencing a rescuing effect of cells by reducing the levels of ROS in the cells. Similar beneficial effects of polyphenol components derived from fruits have been demonstrated in a variety of normal cell lines, including H9C2 cardiomyocytes[23]. It has been noted that this activity of BB extract was diminished at 24 hr. This may be attributable to the fact that the activity of BB added to the cells would not function up to 24 hr, probably as the result of the oxidative alteration of active components present in BB, although we did not further explore the oxidative alteration of BB components added to the cultures during the treatments.

Several stress-associatedand pro- and anti-apoptotic gene expression[24~28] analyses have also suggested that the basic levels of several genes involved in cell survival and apoptosis differ between the MCF7 and MCF10A cells, and that their responses in gene expression patterns are dissimilar between the two cell lines upon BB treatment. It has also been proposed that the components of BB inhibit the caspase-induced apoptotic pathway

in normal MCF10A cells, but that there may be other unknown cause(s) of cell death in MCF7 cells. Finally, the results of the western blot analysis of Hsp70 and PrxI proteins similarly suggests that the beneficial effects of BB components on the elimination of accumulated ROS from cells and the subsequent prevention of associated apoptosis in those cells can be clearly observed by comparing before and after BB treatment in MCF10A cells.

It has been previously reported[24] that radiation-ionized MCF7 cells evidenced upregulated PrxII expression. In our experiment, PrxII levels were similarly increased in MCF7 cells upon 24 h of BB treatment, whereas no clear change was detectable in MCF10A cells(BB 24 hr group, Fig. 4). This enzyme has been known in many cell types to perform a function in reducing peroxides[24]. The finding that ROS accumulation increased in MCF7, but decreased in MCF10A cells upon BB treatment in our experiment further indicate that BB becomes toxic to MCF7 cells, but does well in MCF10A cells. Thus, two different gene regulations in both MCF7 and MCF10A cells are considered to be unexpected cell physiological responses occurring upon BB treatment. According to a previous report[29], the anthocyanin fraction of sweetpotatoes is cytotoxic to prostate cancer cells via both caspase-dependent and caspase-independent activations. In our study, the MCF7 cells responded unexpectedly, suggesting the deregulation of basic cell physiological responses. By way of contrast, the downregulated expression levels of the three examined proteins suggest that MCF10A cells appear to respond in a general cell physiological manner upon BB treatment. This unexpected deregulation upon BB treatment implies multiple cell death pathways in MCF7 cells, as discussed earlier.

Several lines of beneficial and more active berries, including BB, have been reported in a variety of cancer cells. For example, the presence of antioxidative, anticancer, and antimutagenic effects have been supported by experimental evidence using partially characterized fruit fractions[6,10,11,14]. In particular, recent studies regarding the anticancer effects of BB also showed that active apoptotic induction occurs in several human cancer cell lines, including colon cancer cells[12,13,15]. However, no studies have yet been conducted to observe differential effects in the aspects of ROS accumulation and its associated apoptosis in cancer and normal cells together to discover different responses of cancer cells from those in normal cells.

According to the results of our study, therefore, it was proposed that the actions of components present in BB are effective both in increasing ROS accumulation and subsequently inducing apoptosis in MCF7 cells, but also in both reducing ROS accumulation and subsequently preventing apoptosis in MCF10A cells. These differential effects of BB components, and perhaps other fruit components as well, have yet to be elucidated. These two aspects of the pro- and anti-apoptotic effects of BB components in two different cell types may indicate that certain naturally-derived food supplements used to prevent and treat cancer are probably safe and beneficial to the normal cells present in the body, thus circumventing the non-selectivity problem of current therapeutic anticancer drugs. Nature has probably been trying to teach us the lesson that a mix of components from what we eat is truly the most effective naturally occurring therapeutic strategy.

CONCLUSIONS

In this study, we attempted to determine whether there were differential actions of blueberry(BB) in human normal breast epithelial(MCF10A) and breast cancer(MCF7) cells. To this end, we assessed ROS accumulation and associated apoptotic induction. Both qualitative and quantitative analyses were employed via cytochemistry, FACS, fluorimetry, RT-PCR, and western blotting at a final concentration of 20 ㎍/㎖ BB extract for 0(control), 6, 12, and 24 hr intervals. MCF7 and MCF 10A cells evidenced different responses to the components present in BB added, in terms of ROS accumulation, cell death, and expression levels of several selected stress-related and pro- and anti-apoptotic genes upon BB treatment.

All of the results evidencing different levels of gene expressions indicate that both cells may respond differently to BB treatment as was observed in the ROS and cell death analysis. According to the results of our RT-PCR analysis, it was also demonstrated that both MCF7 and MCF10A cells maintain different basic levels of expression in some of the examined genes. The results also show that cell death induced by the components of the added BB may occur in accordance with known apoptotic pathways, but may also involve other unknown action (s) at present.

ACKNOWLEDGEMENTS

This work was supported by the DukSung Women's University Research Fund 2007.

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(2008년 11월 10일 접수; 2008년 12월 4일 채택)