Department of Biochemistry, College of Medicine, Chung-Ang University, Seoul, Korea

Enhanced effects of citrate on UVB-induced apoptosis of B16 melanoma cells

YUN-MI JEONG, JU EUN LEE, SU YEON KIM, HYE-YOUNG YUN, KWANG JIN BAEK, NYOUN SOO KWON, DONG-SEOK KIM

Received April 29, 2009, accepted June 6, 2009

Dong-Seok Kim, Ph.D., Department of Biochemistry, College of Medicine, Chung-Ang University, 221 Heukseok-dong Dongjak-gu, Seoul 156-756, Korea
ds_kim@cau.ac.kr

Pharmazie 64: 829–833 (2009) *doi: 10.1691/ph.2009.9622*

Ultraviolet (UV) radiation is a major risk factor for the development of melanoma. Recent studies have reported that the intake of citrate-containing juices may reduce the risk of cancer. Thus, we investigated the effects of citrate on UVB-irradiated B16 murine melanoma cells. B16 cells had more evident apoptotic features with the combination of citrate/UVB than by citrate or UVB alone; cell death of HaCaT human keratinocytes was not observed with citrate/UVB. Western blot analysis demonstrated that citrate/UVB led to phosphorylation of the stress signaling proteins, such as c-Jun N-terminal kinase (JNK) and p38 mitogen-activated protein kinase (MAPK). Furthermore, citrate/UVB caused activation of caspase-9/-3 as well as cleavage of poly(ADP-ribose) polymerase (PARP). Correspondingly, cell cycle analysis showed that citrate/UVB clearly increased the sub-G_0/G_1 phase, which indicated apoptotic cell death of B16 cells. Therefore, our study has demonstrated that sub-lethal doses of citrate enhanced the apoptotic cell death of melanoma cells under UVB irradiation. From these results, we suggest that citrate might reduce the risk of developing melanoma induced by UVB.

1. Introduction

Citrate, a water-soluble organic acid, is an intermediary metabolite of the tricarboxylic acid (TCA) cycle and regulates the metabolism of almost all living organisms (Lawrence et al. 2004). Citrate is present at high levels in fruit and vegetable juices, such as grapefruit, citrus, and tomato juices (Haleblian et al. 2008; Jensen et al. 2002; Yilmaz et al. 2008). Interestingly, it has been reported that intake of these juices may reduce the risk of cancer (Giovannucci 1999; Manners 2007). However, the effects of citrate on cancer cells have not been well-studied. Therefore, we investigated the potential effects of citrate on cancer cells.

Malignant melanoma, a type of skin cancer, is known to develop from melanocytes and frequently metastasizes to other organs, such as lymph nodes, lung, liver, brain, and bone (MacKie 1998). Malignant melanoma is characterized as a very aggressive cancer resistant to existing therapies (surgery, biological therapy, chemotherapy, or radiotherapy). If melanoma could be detected at an early stage, it could be removed and/or treated easily with current therapy. However, when melanoma is diagnosed at an advanced stage, it is very hard to treat with current therapies because its spreads to organs nearby (Chin et al. 2006). Of note, the incidence of melanoma has doubled every decade for the last 30 years, indicating a more rapid increase than for any other type of cancer in the world (MacKie 1998).

Several researchers have explored whether anticancer drugs, including TNF-related apoptosis-inducing ligand (TRAIL), tumor suppressor gene, and indole-3-carbinol (I3C), can augment radiation-induced toxicity (Kim et al. 2001, 2006). These studies have reported that the combination of chemopreventive components with light promotes cell death. For example, I3C and curcumin enhances UVB-induced apoptosis via activation of the caspase pathways in G361 human melanoma and HaCaT human keratinocytes (Kim et al. 2006; Park and Lee 2007). It is known that excessive UVB exposure increases the risk of malignant melanoma in human skin (MacKie 1998). However, high doses of ionizing or ultraviolet (UV) radiation have adverse effects that lead to the destruction of normal cells and tissue. In particular, UVB is a major risk factor for the development of melanoma. UVB induced DNA damage is generally believed to play a critical role in the pathogenesis of melanoma because DNA damage is known to lead to mutations and carcinogenesis. In the present study, the effects of citrate on B16 melanoma cell death were tested in the presence of low dose UVB, which does not induce apoptosis, but may function as a carcinogen. Moreover, we evaluated the effects of citrate/UVB on stress-mediated signaling pathways as well as apoptotic pathways. Our results suggest interesting insights into the melanoma preventive effects of citrate; these need further in-depth investigations for melanoma preventive strategies.

Abbreviations: I3C, indole-3-carbinol; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; PARP, poly(ADP-ribose) polymerase; TRAIL, TNF-related apoptosis-inducing ligand; UVB, ultraviolet B.

2. Investigations and results

2.1. Effects of citrate or UVB on B16 cell viability

First of all, to determine the effect of citrate, B16 melanoma cells were treated with citrate at 0.1–20 mM for 24 h. The cell viability

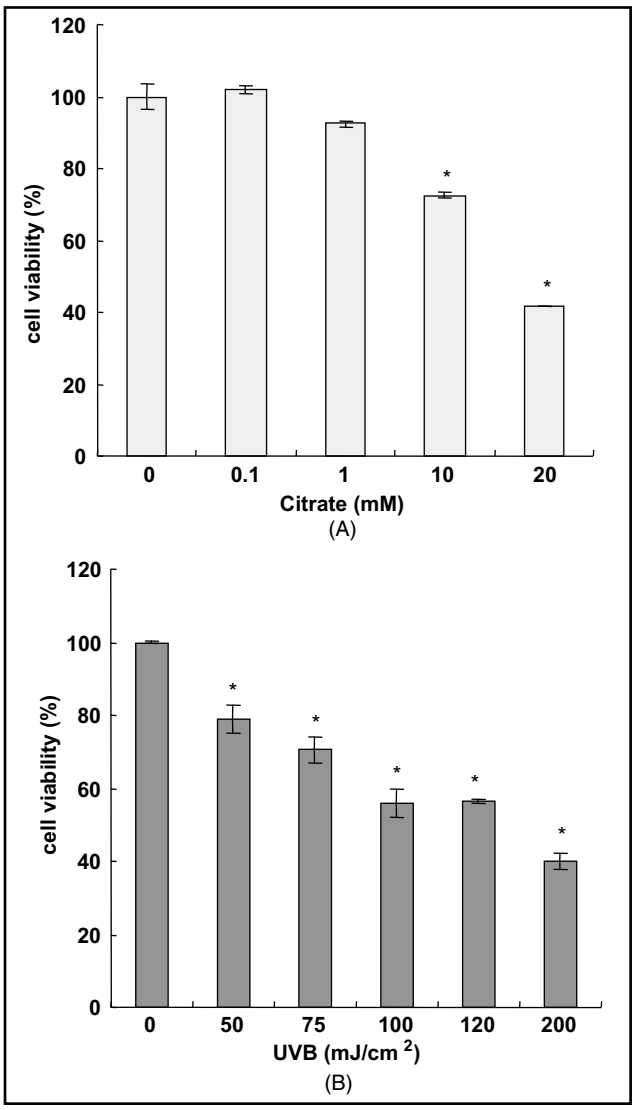

Fig. 1: The cell viability in citrate- (A) and UVB-treated (B) B16 cells. After serum starvation, cells were treated with varying concentrations of citrate (0, 0.1, 1, 10, and 20 mM) or UVB (0, 50, 75, 100, 120, and 200 mJ/cm^2). After 24 h, cell viability was measured by crystal violet staining assay. The data represent the means ± S.D. of triplicate assays, expressed as percentages of the control. Each experiment was repeated at least twice independently, and the representative results are shown. * $P < 0.01$ compared to the untreated control

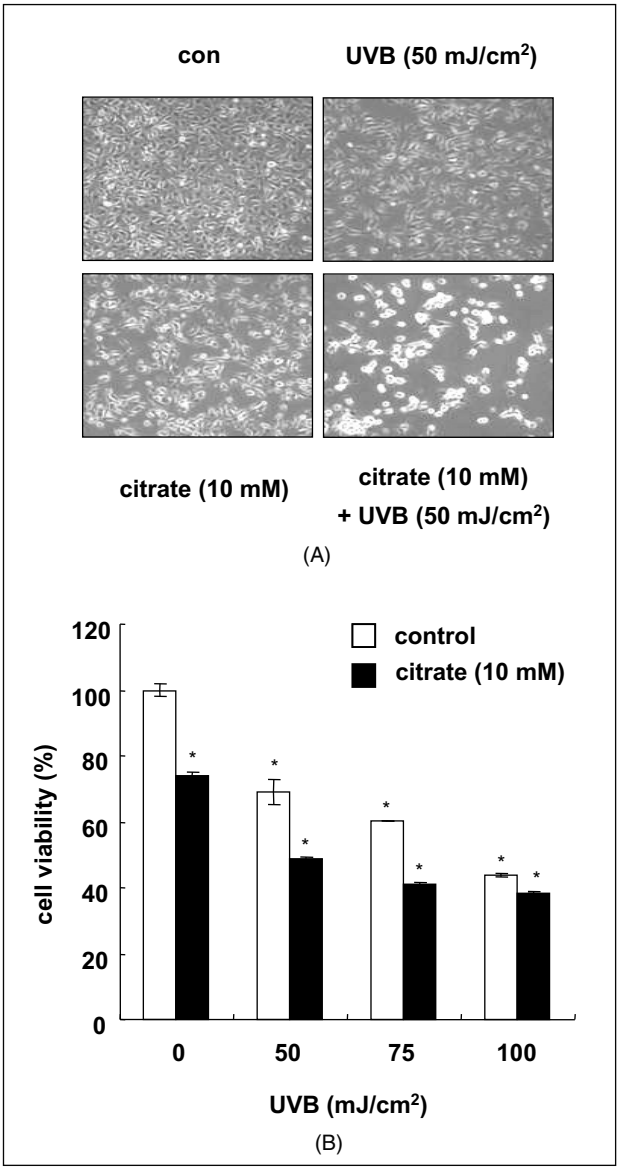

Fig. 2: Effects of citrate on UVB-irradiated B16 cells. (A) Morphology of B16 cells after citrate and/or UVB; con, untreated. (B) After UVB exposure at 0, 50, 75, and 100 mJ/cm^2, citrate (10 mM) was added. After 24 h, cell viability was measured by crystal violet staining assay; the absence (□) and the presence (■) of citrate**.** The data represent the means ± S.D. of triplicate assays expressed as percentages of the control. Each experiment was repeated at least twice independently, and representative results are shown. * $P < 0.01$ compared to the untreated control

was determined by crystal violet staining. As shown in Fig. 1A, we observed that citrate treatment significantly decreased B16 cell viability in a concentration-dependent manner. The mortality of B16 cells treated with citrate at 10 mM was approximately 27%. Citrate at 20 mM was highly toxic to melanoma cells. We then assessed the effect of UVB on B16 cells. When B16 cells were exposed to the indicated dose of UVB, as described in the Materials and Methods section, UVB induced B16 cell death in a dose-dependent fashion (Fig. 1B).

2.2. Effects of citrate/UVB on B16 and HaCaT cell viability

To evaluate whether citrate and UVB work synergistically, we investigated the effect of citrate on UVB-irradiated B16 cells. In the presence of citrate at 10 mM, the mortality of UVB-irradiated B16 cells decreased 2-fold more than that of cells treated with citrate or UVB alone (Figs. 2A and 2B). These data suggest that citrate acts synergistically with UVB-induced damage in B16 cells. To further investigate the role of citrate on the viability of the major normal cells in skin, HaCaT keratinocytes were treated with citrate/UVB. In contrast to B16 cells, citrate/UVB exhibited no cytotoxicity compared with the control (Fig. 3A and 3B).

2.3. Effects of citrate/UVB in stress and apoptotic cell signaling pathways

Various studies have shown that c-Jun N-terminal kinase (JNK) and/or p38 mitogen-activated protein kinase (MAPK) activation is required for apoptosis (Lippens et al. 2009). To demonstrate whether citrate/UVB activates the JNK and/or p38 MAPK pathway in B16 cells, JNK and p38 MAPK phosphorylation was detected by Western blotting. As shown in Figure 4A, UVB irradiation did not stimulate phosphorylation of JNK and p38 MAPK. However, citrate alone slightly induced JNK and p38 MAPK phosphorylation. In contrast, citrate strongly augmented the phosphorylation of p38 MAPK and JNK after UVB-irradiation (Fig. 4A).

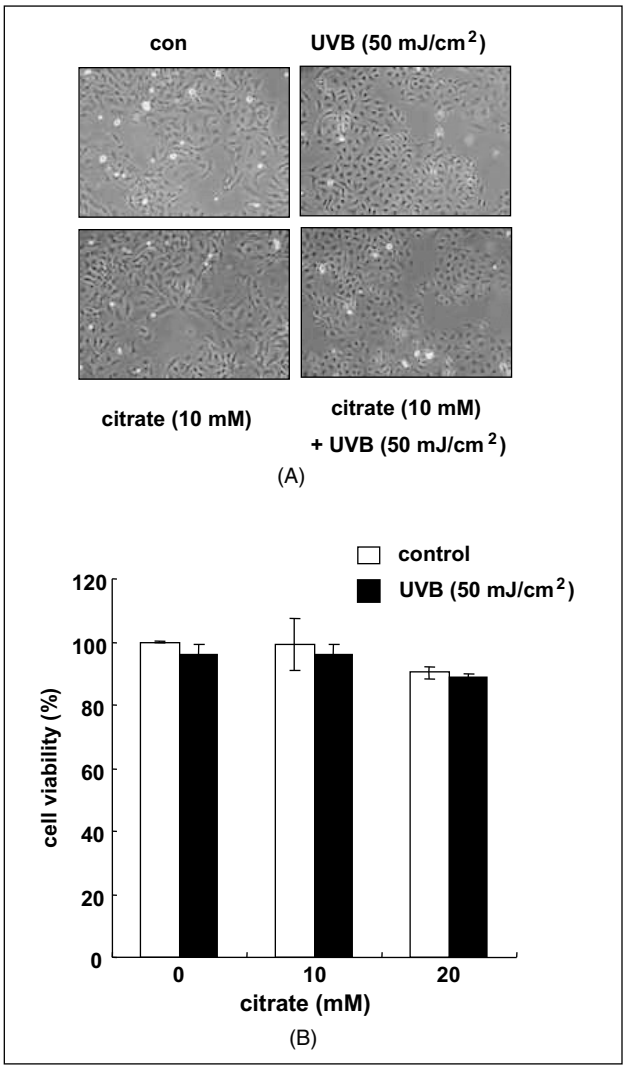

Fig. 3: Effects of citrate on UVB-irradiated HaCaT cells. (A) Morphology of HaCaT cells after citrate and/or UVB; con, untreated. (B) After UVB exposure at 50 mJ/cm^2, cells were treated with citrate (0, 10, and 20 mM). After 24 h, cell viability was measured by a crystal violet staining assay; the absence (□) and in the presence (■) of UVB. Data represent the means ± S.D. of triplicate assays expressed as percentages of the control. Each experiment was repeated at least twice independently, and the representative results are shown.
* $P < 0.01$ compared to the untreated control

A variety of death-stress stimuli lead to activation of caspases (caspase-8, -9, and -3) and the cleavage of poly(ADP-ribose) polymerase (PARP), which result in apoptosis of cells. Thus, we investigated whether citrate/UVB induces the activation of caspase-8, -9, and -3 and the cleavage of PARP in B16 cells. Caspases are known to become active when they are cleaved into fragments. We used anti-caspase-8 and anti-caspase-9 antibodies directed against the precursor forms, whereas we used anti-caspase-3 directed against the cleaved form. After citrate or UVB treatment alone, activation of caspase-8, -9, and -3, and PARP were not detected by Western blotting. However, the combination of citrate/UVB promoted the activation of caspase-3 and -9, and the cleavage of PARP, while caspase-8 was not altered (Fig. 4B).

2.4. Effects of citrate/UVB on cell cycle distribution

To further identify the apoptotic feature of citrate/UVB, we evaluated the cell cycle by fluorescence activated cell sorter analysis. There were little effects of citrate or UVB alone on the cell cycle. In contrast, citrate/UVB was most effective in increasing

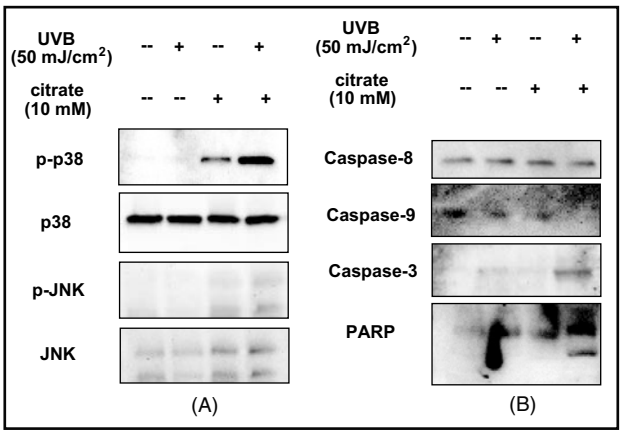

Fig. 4: Effects of citrate/UVB on the JNK/p38 and apoptotic pathways. (A) After serum starvation, B16 cells were treated with citrate (10 mM) and/or UVB (50 mJ/cm^2). Cells were collected at the time points indicated after citrate/UVB treatment. Cell lysates were then subjected to Western blotting with antibodies against phospho-specific JNK or p38 MAPK. Equal protein loadings were confirmed by reaction with phosphorylation-independent JNK or p38 MAPK antibodies, respectively. (B) After serum starvation, B16 cells were treated with citrate (10 mM) and/or UVB (50 mJ/cm^2). In 6 h after citrate/UVB treatment, cells were collected. Cell lysates were then subjected to Western blotting with antibodies against caspase-8, caspase-9, caspase-3, or PARP

sub-G_0/G_1 (Fig. 5), indicating an increase in apoptosis of B16 cells.

3. Discussion

UVB is a well-known risk factor for melanoma. To prevent melanoma, the American Academy of Dermatology recommends sun protection practices; specifically, generously apply sunscreen to all exposed skin every day, avoid deliberate tanning, and get vitamin D safely through a healthy diet that includes vitamin supplements (Dajani et al. 2005). These guidelines have

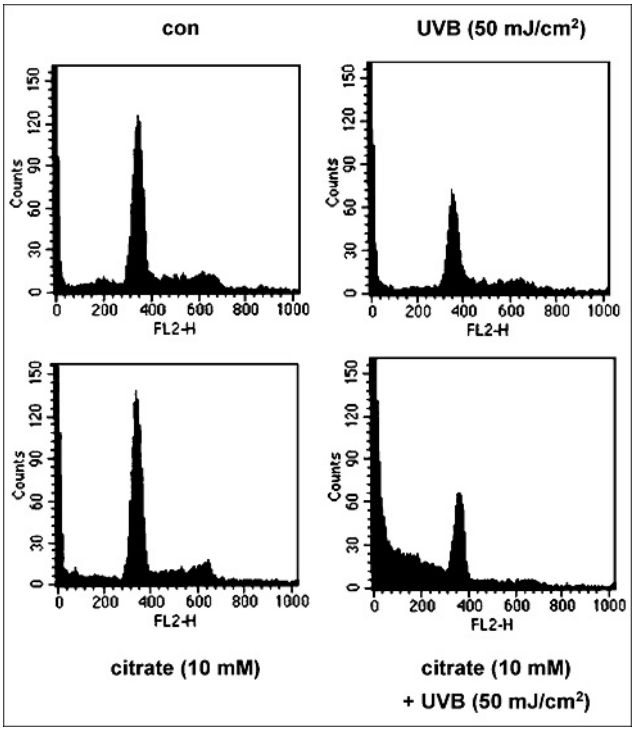

Fig. 5: Effects of citrate/UVB on cell cycle. B16 cells were treated with citrate (10 mM) and/or UVB (50 mJ/cm^2). After 24 h, cell cycle analysis of B16 cells was performed by FACS, as described in the Materials and Methods section; con, untreated

been developed to improve melanoma prevention, but clinical practice is not sufficient in fight against melanoma. Recent studies have demonstrated the anti-cancer effects of dietary compounds (genistein, 3,3′-diindolylmethane, indole-3-carbinol [I3C], curcumin, (–)-epigallocatechin-3-gallate [EGCG], and resveratrol; Sarkar and Li 2006). Moreover, these compounds have been reported to show an enhanced effect on chemotherapy or radiotherapy, as well as cancer prevention, by modifying the activity of cell proliferation and survival pathways (Sarkar and Li 2006). For example, I3C, one component of cruciferous vegetables, also exhibits a chemopreventive effect on liver, colon, and breast cancers (Sarkar and Li 2004). We have also reported that I3C enhances UVB-induced apoptosis via changes in the Bax/Bcl-2 level in G361 human melanoma cells (Kim et al. 2006). This investigation suggested that I3C may prevent the development of UVB-induced melanoma (Kim et al. 2006). In this concept, we demonstrated that citrate also promotes the apoptotic cell death of UVB-irradiated B16 cells.

Citrate supplementation improves urinary citrate levels more than 2-fold without changing total urinary volume, and may be useful as adjunctive treatment for patients with hypocitraturic calcium nephrolithiasis (Seltzer et al. 1996). Furthermore, high concentrations of citrate inhibit phosphofructokinase, the pyruvate dehydrogenase complex, and succinate dehydrogenase of the TCA cycle (Halabe Bucay 2007). Recently, it has been suggested that administering citrate to cancer patients might slow metabolism via the inhibition of glycolysis, and thus might improve cancer treatment (Halabe Bucay 2007). Another study has reported that citrate augments the protective effect of orally administered bismuth sub-nitrate against the nephrotoxicity of cis-diamminedichloroplatinum (Kondo et al. 2004). Our results also showed that citrate plays a role in the augmentation effect on UVB-irradiated B16 cells. Therefore, we suggest that citrate may protect against the risk of developing UVB-induced melanoma.

The JNK and p38 pathways can be activated by many stress stimuli, such as UVB (Lippens et al. 2009). In the present study, we found that citrate/UVB strongly induced the phosphorylation of JNK and p38 MAPK (Fig. 4A). In accordance with our results, previous studies reported that p38 MAPK and JNK activation is involved in the apoptotic effects of UVB (Lippens et al. 2009). Moreover, activation of the stress-/apoptotic-signaling pathways in cancer cells is an important strategy for the treatment of cancer (Sarkar and Li 2006). The typical apoptotic features are DNA fragmentation, the activation of caspases, and the cleavage of PARP (Lippens et al. 2009; Sarkar and Li 2006). Our data showed that citrate/UVB strongly activated caspase-9 and -3, and cleaved PARP (Fig. 4B). Furthermore, citrate/UVB also increased the sub-G_0/G_1 cells (Fig. 5). These results explain why citrate can increase apoptosis of UVB-irradiated B16 cells.

In summary, the present study demonstrated that citrate markedly enhances UVB-induced apoptosis through the activation of the p38 and JNK pathways. This finding therefore might provide a new and exciting strategy for the prevention of developing melanoma induced by UVB.

4. Experimental

4.1. Materials

Citrate was obtained from Sigma (St. Louis, MO, USA). Antibodies that recognize phospho-specific JNK, total JNK, phospho-specific p38, and total p38 were obtained from Cell Signaling (Danvers, MA, USA). Antibodies against caspase-3, caspase-9, caspase-8, and actin were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA), and anti-PARP antibody was obtained from BD Pharmingen (San Diego, CA, USA).

4.2. Cell cultures

B16 murine melanoma cells were obtained from the Korean Cell Line Bank (Seoul, Korea), and HaCaT human keratinocytes (Boukamp et al. 1988) were purchased from Deutsches Krebsforschungszentrum (Heidelberg, Germany). The cells were grown in DMEM supplemented with 10% FBS, 50 μg/ml streptomycin, and 50 μg/ml penicillin at 37 °C in 5% CO_2.

4.3. Cell viability

Cells were observed under a phase contrast microscope (Olympus Optical Co., Tokyo, Japan) and photographed with a DCM300 digital camera for a microscope (Scopetek, Inc., Hangzhou, China), which was supported by ScopePhoto software (Scopetek, Inc.). Cell viability was assessed using crystal violet staining assays (Kim et al. 2006). After treating with the test substances for 24 h, the culture medium was removed. Cells were stained with 0.1% crystal violet in 10% ethanol for 5 min at room temperature and then rinsed 4 times. The crystal violet retained by the adherent cells was extracted with 95% ethanol, and absorbance was determined in lysates at 590 nm using an ELISA reader (VERSAMax; Molecular Devices, Sunnyvale, CA, USA).

4.4. Treatment conditions

The source of UVB was from Sankyo Denki Company Ltd (Hiratsuka, Kanagawa, Japan). To evaluate the cytotoxicity of citrate or UVB on cells, B16 cells (1×10^6 cells per well) were prepared on 6-well plates. After serum starvation, various concentrations of citrate (0 – 20 mM) or UVB (0 – 200 mJ/cm^2) were used in treatment for 24 h. For the combination of citrate/UVB, the cells were irradiated with UVB (50 mJ/cm^2), then citrate (0 – 20 mM) was added. After 24 h, cell viability was measured by the crystal violet staining assay. Control cells were maintained in the same conditions without the addition of citrate or UVB exposure.

4.5. Cell cycle analysis

Cells were trypsinized, adjusted to 5×10^5 – 1×10^6 cells/tube, washed with ice cold phosphate-buffered saline (PBS), and re-suspended in 2 ml of ethanol. After incubation at 4 °C for 1 h, the ethanol was removed, and 100 μl of ribonuclease solution (10 mg/ml) was added to each test tube. The tubes were then re-incubated at room temperature for 30 min, and 500 μl of analysis solution (37 mM EDTA and 0.1% Triton X-100 in PBS) and 100 μl of propidium iodide solution (400 μg/ml) were then added. Samples were stored in the dark at 4 °C and analyzed by flow cytometry (FACScan; Becton Dickinson, San Jose, CA, USA). The proportion of cells in the sub-G_0/G_1 phases with low DNA content were regarded as apoptotic cells.

4.6. Western blotting

Cell lysates were prepared in M-PER mammalian protein reagent (Pierce, Rockford, IL, USA) containing a complete protease inhibitor mixture (Roche, Mannheim, Germany). Samples were separated on 12% SDS-polyacrylamide gels and were transferred to polyvinylidene fluoride (PVDF) membranes, which were blocked with 5% dried milk in PBS containing 0.4% Tween 20. The blots were incubated with the appropriate primary antibodies at a dilution of 1:1000. Membrane-bound primary antibodies were detected by using secondary antibodies conjugated with horseradish peroxidase and chemiluminescent substrate (Pierce). The images of the blotted membranes were obtained using a LAS-1000 lumino-image analyzer (Fuji Film, Tokyo, Japan).

4.7. Statistics

Differences among treatments were assessed by analysis of variance (ANOVA), followed by Dunnett's test. P values of <0.01 were taken to be significant.

Acknowledgements: This research was supported by the Chung-Ang University Research Grants in 2009.

References

Boukamp P, Petrussevska RT, Breitkreutz D, Hornung J, Markham A, Fusenig NE (1988) Normal keratinization in a spontaneously immortalized aneuploid human keratinocyte cell line. J Cell Biol 106: 761–771.

Chin L, Garraway LA, Fisher DE (2006) Malignant melanoma: genetics and therapeutics in the genomic era. Genes Dev 20: 2149–2182.

Dajani Z, Swetter SM, Demierre MF, Geller AC (2005) Sun protection factor content and warning statements for sunless tanning products: an examination of retail outlets and the Internet. J Am Acad Dermatol 53: 919–920.

Giovannucci E (1999) Tomatoes, tomato-based products, lycopene, and cancer: review of the epidemiologic literature. J Natl Cancer Inst 91: 317–331.

Halabe Bucay A (2007) The biological significance of cancer: mitochondria as a cause of cancer and the inhibition of glycolysis with citrate as a cancer treatment. Med Hypotheses 69: 826–828.

Haleblian GE, Leitao VA, Pierre SA, Robinson MR, Albala DM, Ribeiro AA, Preminger GL (2008) Assessment of citrate concentrations in citrus fruit-based juices and beverages: implications for management of hypocitraturic nephrolithiasis. J Endourol 22: 1359–1366.

Jensen HD, Krogfelt KA, Cornett C, Hansen SH, Christensen SB (2002) Hydrophilic carboxylic acids and iridoid glycosides in the juice of American and European cranberries (Vaccinium macrocarpon and V. oxycoccos), lingonberries (V. vitis-idaea), and blueberries (V. myrtillus). J Agric Food Chem 50: 6871–6874.

Kim DS, Jeong YM, Moon SI, Kim SY, Kwon SB, Park ES, Yoŭn SU, Park KC (2006) Indole-3-carbinol enhances ultraviolet B-induced apoptosis by sensitizing human melanoma cells. Cell Mol Life Sci 63: 2661–2668.

Kim MR, Lee JY, Park MT, Chun YJ, Jang YJ, Kang CM, Kim HS, Cho CK, Lee YS, Jeong HY, Lee SJ (2001) Ionizing radiation can overcome resistance to TRAIL in TRAIL-resistant cancer cells. FEBS Lett 505: 179–184.

Kondo Y, Himeno S, Satoh M, Naganuma A, Nishimura T, Imura N (2004) Citrate enhances the protective effect of orally administered bismuth subnitrate against the nephrotoxicity of cis-diamminedichloroplatinum. Cancer Chemother Pharmacol 53: 33–38.

Lawrence CL, Botting CH, Antrobus R, Coote PJ (2004) Evidence of a new role for the high-osmolarity glycerol mitogen-activated protein kinase pathway in yeast: regulating adaptation to citric acid stress. Mol Cell Biol 24: 3307–3323.

Lippens S, Hoste E, Vandenabeele P, Agostinis P, Declercq W (2009) Cell death in the skin. Apoptosis 14: 549–569.

MacKie RM (1998) Incidence, risk factors and prevention of melanoma. Eur J Cancer 34 (Suppl 3): S3–6.

Manners GD (2007) Citrus limonoids: analysis, bioactivity, and biomedical prospects. J Agric Food Chem 55: 8285–8294.

Park K, Lee JH (2007) Photosensitizer effect of curcumin on UVB-irradiated HaCaT cells through activation of caspase pathways. Oncol Rep 17: 537–540.

Sarkar FH, Li Y (2004) Cell signaling pathways altered by natural chemopreventive agents. Mutat Res 555: 53–64.

Sarkar FH, Li Y (2006) Using chemopreventive agents to enhance the efficacy of cancer therapy. Cancer Res 66: 3347–3350.

Seltzer MA, Low RK, McDonald M, Shami GS, Stoller ML (1996) Dietary manipulation with lemonade to treat hypocitraturic calcium nephrolithiasis. J Urol 156: 907–909.

Yilmaz E, Batislam E, Basar M, Tuglu D, Erguder I (2008) Citrate levels in fresh tomato juice: a possible dietary alternative to traditional citrate supplementation in stone-forming patients. Urology 71: 379–383; discussion 383–374.