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Original Article

Sulforaphane displays the growth inhibition, cytotoxicity and enhancement of retinoic acid-induced superoxide-generating activity in human monoblastic U937 cells

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ABSTRACT — Sulforaphane [1-isothiocyanato-4-(methyl-sulfinyl)butane] is an isothiocyanate derivative from cruciferous vegetables, with anti-proliferative actions on various cancer and tumor cells. In this paper, we envisaged the effects of sulforaphane on various functions (growth inhibition, cytotoxicity and enhancement of O_2^--generating activity) of human monoblastic leukemia U937 cells. Sulforaphane showed strong cytotoxicity, resulting in inhibition of proliferation in a dose-dependent manner. In addition, cell differentiation induced by 1 μM *all-trans* retionic acid (RA) remarkably caused the enhanced resistance against cytotoxicity of sulforaphane. Moreover, the RA-induced O_2^--generating activity was also enhanced by sulforaphane in a dose dependent manner. When U937 cells were cultured in the presence of 1 μM RA and 2 μM sulforaphane, the O_2^--generating activity increased more than 2.5-fold compared with that in the absence of the latter. Semiquantitative RT-PCR showed that co-treatment with RA and sulforaphane slightly enhanced transcription of only p47-phox gene among five essential components (p22-phox, gp91-phox, p40-phox, p47-phox and p67-phox) for the O_2^--generating system in phagocytes. On the other hand, immunoblot analysis revealed that co-treatment with RA and sulforaphane caused accumulation of protein levels of p47-phox (upto ~130%) and p67-phox (upto ~240%) compared with those of the RA-treatment alone. These results indicated that sulforaphane may enhance the RA-induced O_2^--generating activity in U937 cells via accumulation of p47-phox and p67-phox proteins. These data suggested that sulforaphane may serve as an effective drug for leukemia treatment.

Key words: Sulforaphane, Cytotoxicity, Proliferation, Differentiation, Superoxide, U937

INTRODUCTION

Phytochemicals, in the form of plant-derived chemical compounds, are responsible for many biological activities. On such phytochemical, namely sulforaphane [1-isothiocyanato-4-(methyl-sulfinyl)butane] is an isothiocyanate compound derived from cruciferous vegetables, i.e. broccoli and its sprouts and is synthesized by hydrolysis of sulforaphane glucosinolate (Posner *et al.*, 1994). In higher plants, sulforaphane protects them from infections

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by various bio-invaders such as aphids, ticks, bacteria or nematodes (Melrose, 2019). In recent times, sulforaphane attracted as an effective carcinogenesis prophylactic drug (Zhang *et al.*, 1992; Tortorella *et al.*, 2015). Moreover, in human being, sulforaphane displays the multiple interesting biological functions: such as anti-cancer effects (Li *et al*., 2011; Amin and Shankar, 2015; Dandawate *et al*., 2016; Leone *et al*., 2017; Jiang *et al*., 2018; Sita *et al*., 2018), antioxidant functions (Negrette-Guzman *et al*., 2013), anti-*Helicobacter pylori* activity (Yanaka, 2017), photo aging prevention (Sikdar *et al.*, 2016), prevention of obesity (Martins *et al*., 2018), and general health benefits (Vanduchova *et al*., 2019). On the other hand, although numerous reports concerning to the chemopreventive and chemotherapeutic properities of solid tumors are available, there is little information on the properities of sulforaphane in leukemia cells (Choi *et al*., 2008; Fimognari *et al*., 2008; Suppipat and Lacorazza, 2014; Fimognari *et al*., 2014; Shih *et al*., 2016; Shang *et al*., 2017; Brown *et al*., 2017; Koolivand *et al*., 2018). Unfortunately, the understanding of the effects of sulforaphane on human leukemia cells with mechanisms is incompletely explored till now.

Phagocytes including macrophages generate superoxide anion (O_2^-) to exclude various invading microbes. While cytochrome b558 composed of p22-phox and gp91-phox consists in the membrane, p40-phox, p47-phox and p67-phox are in cytosol. On various stimuli, these five proteins are assembled on the membrane (formation of NADPH oxidase), resulting in O_2^--generation. Human monoblastic leukemia U937 cells differentiate to macrophage-like cells by *all-trans* retinoic acid (RA), and aquire the O_2^--generating activity (Kikuchi *et al*., 1994; Kikuchi *et al*., 1996; Kikuchi *et al*., 2010; Kikuchi *et al*., 2011; Kikuchi *et al*., 2018a; Kikuchi *et al.*, 2019). Of course, gene expression of each the five essential components for the O_2^--generation activity (p22-phox, gp91-phox, p40-phox, p47-phox and p67-phox) is significantly up-regulated.

In this paper, we investigated the effects of sulforaphane on various cell functions of U937 cells. It is interesting that sulforaphane generates reactive oxygen species, resulting in apoptosis in U937 cells (Choi *et al*., 2008). Here, we revealed that sulforaphane shows growth inhibition and cytotoxicity against U937 cells. In addition, we also showed that RA-induced differentiation of U937 cells brought about the enhanced resistance against sulforaphane. Moreover, our data suggested that sulforaphane moderately enhances the RA-induced O_2^--generating activity via accumulation of cytosolic p47-phox and p67-phox proteins in U937 cells.

MATERIALS AND METHODS

Materials

Sulforaphane (Cayman Chemical, MI, USA), RPMI-1640 culture medium and trypan blue solution (Gibco Laboratories, MD, USA), RA and luminol (Sigma, St Louis, MO, USA), phorbol 12-myristate 13-acetate (PMA) (Calbiochem, Darmstadt, Germany), PMSF (Wako, Osaka, Japan), Diogenes (national Diagnostics, Atlanta, GA, USA), fetal bovine serum (FBS) (JRH Biosciences, KS, USA) and plasmocin (InvivoGene, CA, USA) were obtained. Monoclonal anti-gp91-phox antibody, monoclonal anti-p47-phox antibody, monoclonal anti-p67-phox antibody (BD Biosciences, San Jose, CA, USA), anti-p40-phox antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and horseradish peroxidase-conjugated rabbit anti-mouse or rabbit immunoglobulin (DAKO, Inc., Glostrup, Denmark) were used. Monoclonal anti-human p22-phox antibody (449) was kindly provided by Dr. Roos and Dr. Verhoeven (Sanquin Research, and Landsteiner Laboratory, Academic Medical Centre, University of Amsterdam, The Netherlands).

Cell culture and treatment with sulforaphane

Human monoblastic leukemia U937 cells (RCB0435) were provided by the RIKEN BRC through the National Bio-Resource Project of the MEXT, Japan. Cells were grown in RPMI-1640 culture medium containing 10% FBS and 5 μg/mL plasmocin as described (Kikuchi *et al*., 2018a; Kikuchi *et al*., 2018b; Kikuchi *et al.*, 2019). Cells (1.0×10^6) in 5 mL of culture medium were incubated in the absence or presence (1, 2 or 5 μM) of sulforaphane at 37°C. A hemocytometer was used to count total cells under a microscope. Viable cells were counted by the trypan blue dye exclusion method (Kikuchi *et al*., 2018b).

Cultivation of RA-treated U937 cells with sulforaphane

Cells (1.0×10^6) in 5 mL of culture medium were incubated in the absence or presence of 1 μM RA for 48 hr at 37°C . The RA-untreated or -treated cells (1.0×10^6) were resuspended in 5 mL of fresh culture medium, incubated in the presence of 2 or 5 μM sulforaphane at 37°C. Viable cells were counted by the trypan blue dye exclusion method (Kikuchi *et al*., 2018b).

Assay of O_2^- generation

Cells (1.0×10^6) in 5 mL of culture medium were incubated with 1 μM RA in the absence or presence of sulforaphane (1 or 2 μM) for 48 hr at 37°C. O_2^- was quan-

tified by measuring chemiluminescence (CL) using Diogenes-luminol CL probes as described (Kikuchi *et al*., 2011; Kikuchi *et al*., 2018a; Kikuchi *et al.*, 2019). Cells (1 x 10^6 cells) were washed by PBS, and resuspended in 1 mL PBS containing 1 mM $MgCl_2$, 0.5 mM $CaCl_2$, 5 mM glucose and 0.03% bovine serum albumin. These cells were stimulated with 200 ng/mL PMA at 37°C, and the O_2^- generation was measured by Lumat^3 LB9508 luminometer (Berthold Technologies, Bad Wildbad, Germany).

Semiquantitative RT-PCR

Total RNA was isolated from the cells. Semiquantitative RT-PCR was performed as described using specific sense and antisense primers of five components essential for the O_2^--generation system: p22-phox, gp91-phox, p40-phox, p47-phox and p67-phox (Kikuchi *et al*., 2011; Kikuchi *et al*., 2018a; Kikuchi *et al.*, 2019). Human GAPDH gene was used as internal controls. PCR products were subjected to 1.5% agarose gel electrophoresis. Data obtained by semiquantitative RT-PCR before reaching the plateau were analyzed by Quant-AMZ software (TotalLab., Newcastle upon Tyne, UK) using a luminescent image analyzer STAGE-5100 (AMZ System Science, Osaka, Japan).

Immunoblotting

Cells (5 x 10^6) were collected by centrifugation and disrupted by a sonicator in 100 μL of 50 mM Tris-HCl buffer, pH 7.5, containing 0.25 M sucrose, 2 mM EDTA and 1 mM PMSF. Cell lysates were centrifuged and supernatants obtained (cytosolic fractions) were treated with 10% trichloroacetic acid, collected by centrifugation, dissolved in 100 μL of 0.5 M Tris-HCl (pH 6.8) containing 2.5% SDS, 10% glycerol, and 5% 2-mercaptoethanol, and heated at 100°C for 5 min. Precipitates of cell lysates (membrane fractions) were suspended 50 μL of a solubilizing solution containing 9 M urea, 2% Triton X-100 and 5% 2-mercaptoethanol. Fifty μL of loading buffer [0.5 M Tris-HCl (pH 6.8) containing 5% SDS, 20% glycerol] was added to the solubilized membrane fraction. Immunoblotting was carried out as described Kikuchi *et al*., 2011, Kikuchi *et al*., 2018a, Kikuchi *et al.*, 2019). Data analyses were performed using a luminescent image analyzer STAGE-5100. Human β-actin (for cytosolic fractions) and Na^+/K^+-ATP ase (for membrane fractions) were used as controls (Kikuchi *et al.*, 2019).

Statistical analysis

Data obtained from proliferation assays, cell viability assays, semiquantitative RT-PCR and immunoblotting are presented as averages of three separate and independent experiments. Error bars indicate standard deviation. Statistical differences were calculated with Student's *t* test.

RESULTS AND DISCUSSION

First, to study the growth inhibition and cytotoxicity caused by sulforaphane, we examined the effects of commercially available sulforaphane on proliferation and viability of U937 cells. As shown in Fig. 1A, the proliferation rates of U937 cells were decreased in a dose dependent manner. In particular, the number of the cells was dramatically decreased by treatment with 5 μM sulforaphane. Similarly, the viability of U937 cells was also decreased in a dose dependent manner, and dramatically reduced at 5 μM sulforaphane (to ~85% at 8 hr, to ~50% at 20 hr, to ~25% at 32 hr and to ~10% at 44 hr) (Fig. 1B). These results suggested that sulforaphane shows strong cytotoxicity and resulted in inhibition of proliferation. Our findings confirmed the previous report in which sulphoraphane induces apoptotic cell death in a dose dependent manner (Choi *et al.*, 2008).

In order to study the influences of the RA-induced monocytic differentiation on sensitivity of U937 cells against sulforaphane, we measured viability of RA-untreated or -treated U937 cells cultured in the presence of 2 or 5 μM sulforaphane. As shown in our previous report, the RA-induced differentiation of U937 cells caused the enhanced resistance against 2-hydroxychalcone, 2'-hydroxychalcone and isoliquiritigenin (Kikuchi *et al*., 2018b). Viability of RA-untreated (undifferentiated) or RA-treated (differntiated) U937 cells was not affected during culture in the absence of sulforaphane for upto 48 hr (data not shown). As expected, the RA-treated U937 cells showed distinct resistance against 2 or 5 μM sulforaphane as compared to the RA-untreated U937 cells (Fig. 2). These results suggested that cell differentiation may bring about the enhanced resistance against cytotoxicity of sulforaphane in U937 cells.

We further evaluted the effects of sulforaphane on the RA-induced O_2^--generating activity in U937 cells. Therefore, we measured the O_2^--generating activity of U937 cells cultured with RA in the absence or presence of 1 or 2 μM sulforaphane. When U937 cells were cultured in the presence of RA and sulforaphane, the O_2^--generating activity increased compared with that in the absence of the latter (to ~2.6-fold at 2 μM) (Fig. 3). The RA-induced O_2^--generating activity was enhanced by sulforaphane in a dose dependent manner. In contrast, the O_2^--generating activity was not induced in the presence of sulforaphane

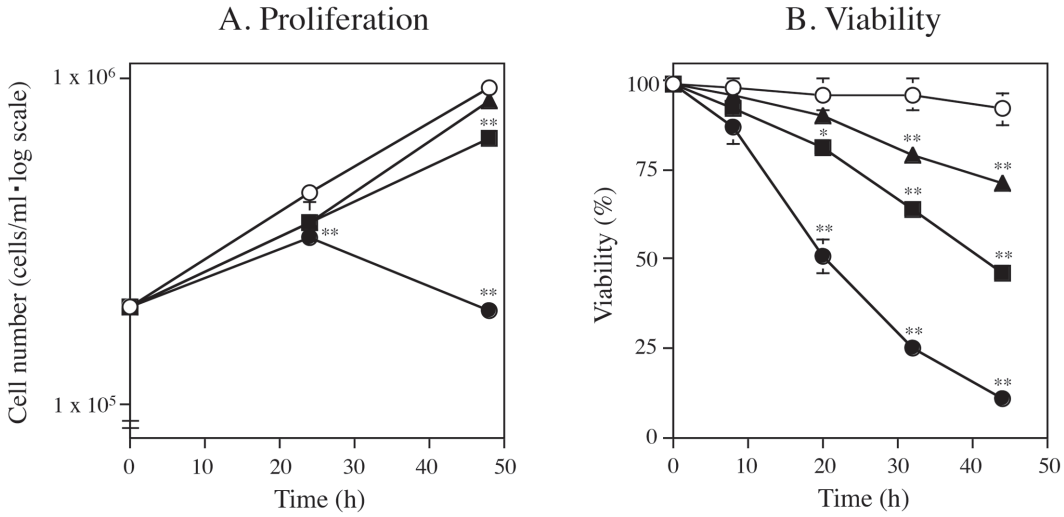

Fig. 1. Influences of sulforaphane on proliferation and viability of U937 cells. (A) Proliferation. Cells (1.0 x 10^6) in 5 mL of culture medium were incubated without (open circles) or with 1 (closed triangles), 2 (closed squares) or 5 μM (closed circles) sulforaphane for upto 48 hr. Cell counts were performed by a hemocytometer under a microscope at the indicated times in the graph. Data represent the averages of three separate experiments. Statistical differences were calculated using Student's *t* test. **, $p < 0.01$ compared with the data for without sulforaphane. (B) Viability. Cells (1.0 x 10^6) in 5 mL of culture medium were incubated without (open circles) or with 1 (closed triangles), 2 (closed squares) or 5 μM (closed circles) sulforaphane for upto 44 hr. Viable cells were counted by the trypan blue dye exclusion method at the indicated times in the graph. Data represent the averages of three separate experiments. Error bars indicate standard deviation. Statistical differences were calculated using Student's *t* test. *, $p < 0.05$; **, $p < 0.01$ compared with the data of without sulforaphane.

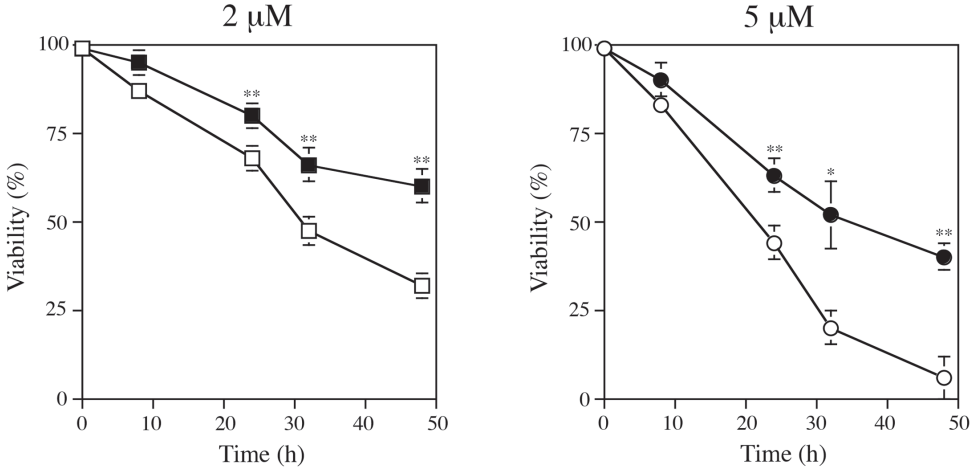

Fig. 2. Influences of RA-induced monocytic differentiation on sensitivity against sulforaphane. Cells (1.0 x 10^6) in 5 mL of culture medium were incubated in the absence or presence of 1 μM RA for 48 hr. Untreated (open symbols) and RA-treated (closed symbols) cells (1.0 x 10^6) were resuspended in 5 mL of fresh culture medium, incubated with 2 (squares) or 5 (circles) μM sulforaphane for upto 48 hr. Viable cells were counted by the trypan blue dye exclusion method at the indicated times in the graph. Data represent the averages of three separate experiments. Error bars indicate standard deviation. Statistical differences were calculated using Student's *t* test. *, $p < 0.05$; **, $p < 0.01$ compared with the data of untreated cells.

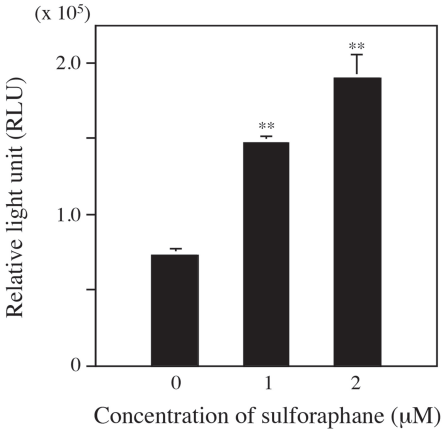

Fig. 3. Influences of sulforaphane on the RA-induced O_2^--generating activity in U937 cells. O_2^--generation was determinated after culture of the cells with 1 μM RA in the absence or presence (1 or 2 μM) of sulforaphane for 48 hr. PMA-induced CL was measured at 10 min after stimulation using Lumat^3 LB9508 luminometer. Quantitative data represent the averages of three separate experiments. Error bars indicate standard deviation. Statistical differences were calculated using Student's *t* test. **, $p < 0.01$ compared with the data of RA-treated (without sulforaphane) U937 cells.

alone (data not shown).

In the next experiments, we have studied the transcriptions of the differnt essential components (p22-phox, gp91-phox, p40-phox, p47-phox and p67-phox) for the O_2^--generation. Hence, we performed semiquantitative RT-PCR with total RNAs prepared from RA-treated and RA and 2 μM sulforaphane-co-treated U937 cells. Quantitative data were indicated as percentages of control values obtained from RA-treated U937 cells (Fig. 4A). Among these five essential components, only transcription of p47-phox of RA and sulforaphane-co-treated U937 cells was slightly increased (upto ~130%) compared to that of RA-treated U937 cells. However, unexpectedly, the transcription levels of other four factors were not altered by addition of sulforaphane.

Finally, to know the effects of sulforaphane on the amounts of proteins of the five essential components, we carried out immunoblotting with proteins prepared from RA-treated and RA plus 2 μM sulforaphane-co-treated U937 cells using antibody specific for each protein. Quantitative data were indicated as percentages of control values obtained from RA-treated U937 cells

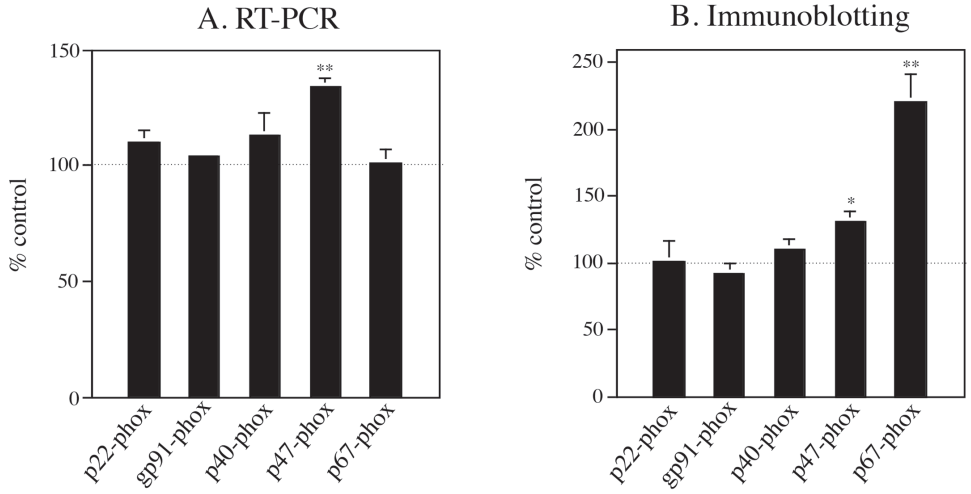

Fig. 4. Influences of sulforaphane on gene expression of the O_2^--generating system-essential components. (A) RT-PCR. The mRNA levels of p22-phox, gp91-phox, p40-phox, p47-phox and p67-phox were determined by semiquantitative RT-PCR using total RNAs extracted from RA-treated and RA plus 2 μM sulforaphane-treated U937 cells. Data obtained by semiquantitative RT-PCR before reaching the plateau were analyzed by Quant-AMZ software using a luminescent image analyzer STAGE-5100. Quantitative data calibrated with the internal controls (human GAPDH gene) are indicated as percentages of control values obtained from the RA-treated (without sulforaphane) U937 cells, and represent the averages of three separate experiments. Error bars indicate standard deviation. **, $p < 0.01$ compared with the data of RA-treated (without sulforaphane) U937 cells. (B) Immunoblotting. The protein levels of p22-phox, gp91-phox, p40-phox, p47-phox and p67-phox were determined by immunoblotting with appropriate antibodies using protein samples from RA-treated and RA plus 2 μM sulforaphane-treated U937 cells. Data were analyzed by Quant-AMZ software using a luminescent image analyzer STAGE-5100. Quantitative data are indicated as percentages of control values obtained from the RA-treated (without sulforaphane) U937 cells, and represent the averages of three separate experiments. Error bars indicate standard deviation. *, $p < 0.05$; **, $p < 0.01$ compared with the data of RA-treated (without sulforaphane) U937 cells.

(Fig. 4B). Interestingly, co-treatment with RA and sulforaphane brought about increases in protein levels of not only p47-phox (upto ~130%) but also p67-phox (upto ~230%) compared with those of the RA-treated alone. In our previous study, co-treatment with RA and curcumin caused remarkable accumulations in protein levels of p47-phox and p67-phox despite it slightly enhanced transcription levels of genes of these two cytosolic factors (Kikuchi *et al*., 2010). Therefore, the effects of sulforaphane may also resemble the action of those of curcumin concerninig to the expression of p47-phox and p67-phox. These results suggested that sulforaphane may enhance the RA-induced O_2^--generating activity in U937 cells via accumulation of p47-phox and p67-phox proteins. However, further experiments should be needed to elucidate how sulphoraphane accumulates the amounts of these protein factors and enhances the O_2^--generating activity in U937 cells.

Our findings in this study revealed that sulforaphane shows growth inhibition, cytotoxicity and enhancement of the RA-induced O_2^--generating activity in human monoblastic leukemia U937 cell. As is well known, RA-treatment has been studied to improve care rates in leukemia (Nasr *et al*., 2009; Nowak *et al*., 2009). Our results suggested that sulforaphane in combination with RA may serve as effective modifiers for leukemia treatment. Therefore, we can expect that studies on both cytotoxicity and differentiation-enhancing activity of sulforaphane will become more important for development of leukemia treatment or immunopotentiator.

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Conflict of interest---- The authors declare that there is no conflict of interest.

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