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Curcumin dramatically enhances retinoic acid-induced superoxide generating activity via accumulation of p47-phox and p67-phox proteins in U937 cells

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ABSTRACT

The membrane bound cytochrome b558 composed of large gp91-phox and small p22-phox subunits, and cytosolic proteins p40-, p47- and p67-phox are important components of superoxide (O_2^-)-generating system in phagocytes and B lymphocytes. A lack of this system in phagocytes is known to cause serious life-threatening infections. Here, we describe that curcumin, a polyphenol responsible for the yellow color of curry spice turmeric, dramatically activates the O_2^--generating system during retinoic acid (RA)-induced differentiation of human monoblastic leukemia U937 cells to macrophage-like cells. When U937 cells were cultured in the presence of RA and curcumin, the O_2^--generating activity increased more than 4-fold compared with that in the absence of the latter. Semiquantitative RT-PCR showed that co-treatment with RA and curcumin slightly enhanced gene expressions of the five components compared with those of the RA-treatment only. On the other hand, immunoblot analysis revealed that co-treatment with RA and curcumin caused remarkable accumulation of protein levels of p47-phox (to 7-fold) and p67-phox (to 4-fold) compared with those of the RA-treatment alone. These results suggested that curcumin dramatically enhances RA-induced O_2^--generating activity via accumulation of cytosolic p47-phox and p67-phox proteins in U937 cells. Therefore, it should have the potential as an effective modifier in therapy of leukemia and/or as an immunopotentiator.

1. Introduction

Phagocytes (neutrophils, eosinophils, monocytes, macrophages and so on) rapidly consume molecular oxygen to generate superoxide anion (O_2^-) upon stimulation [1]; this process is known as "respiratory burst". O_2^- is used for the precursor of microbicidal oxidants (e.g. hydrogen peroxide). At the initial reaction, a membrane-associated protein complex (O_2^--generating system) generates O_2^-, which is released inside phagosomes or outside the cells. Five specific proteins (large gp91-phox and small p22-phox subunits of cytochrome b558, cytosolic p40-phox, p47-phox and p67-phox) and small G-protein Rac are essential for the O_2^--generating system [2]. The system is dormant in resting phagocytes, but is activated during phagocytosis to carry an electron from NADPH to molecular oxygen resulting in O_2^- generation. The importance of this system is emphasized by a genetic disorder known as chronic granulomatous disease, where phagocytes cannot generate O_2^- upon stimulation. In particular, the four genes encoding p22-phox, gp91-phox, p47-phox and p67-phox are very important and indispensable for the O_2^--generating activity as this disorder results from defects in any of the four genes [3]. Since the O_2^--generating system does not act in undifferentiated phagocytes including leukemia, this system is regarded as one of the differentiation indexes in phagocytes [4].

Human leukemia cell lines have been frequently used as models to study both differentiation of leukocytes and therapy of leukemia [5]. Human monoblastic leukemia U937 cells, one of the human leukemic cell lines, lack the O_2^--generating activity observed during leukocyte differentiation [6]. The U937 cells are differentiated to macrophage-like cells in response to various agents: phorbol esters, dimethylsulfoxide, interferon-γ, tumor necrosis factor, vitamin D3 (VD_3) and retinoic acid (RA) [5–7]. Above all, a differentiation therapy using RA has been studied to improve care rates in leukemia including acute promyelocytic leukemia [8]. Now, leukemia therapy using RA has been improved stepwise through elucidation of more effective combination regimen of RA with cytotoxic chemotherapy [9]. Therefore, more effective and safer

Abbreviations: CL, chemiluminescence; FITC, fluorescein isothiocyanate; O_2^-, superoxide anion; PMA, phorbol 12-myristate 13-acetate; RA, retinoic acid; VD_3, vitamin D_3.

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chemical compounds are expected as modifiers in leukemia therapy.

Curcumin [1,7-bis-(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione] is a phenolic compound responsible for the yellow color of turmeric, a curry spice. Since curcumin targets numerous molecules, including transcription factors, growth factors, cytokines and enzymes, it has several prophylactic and therapeutic effects such as anti-inflammatory, -oxidant, -carcinogenic, -thrombotic and -infectious, and cardiovascular protective effects [10–16]. In addition, it has been reported that curcumin is a weak stimulator of differentiation and shows synergistic effects on VD_3- or RA-induced differentiation of HL60 cells [17,18]. However, little is known regarding effect of curcumin on O_2^--generating activity during differentiation of leukocytes. In this study, we show that curcumin dramatically enhances RA-induced O_2^--generating activity via accumulations in the amounts of p47-phox and p67-phox proteins in U937 cells.

2. Materials and methods

2.1. Materials

Phorbol 12-myristate 13-acetate (PMA) (Calbiochem, Darmstadt, Germany), all-trans RA, luminol (Sigma, St. Louis, MO, USA), curcumin (Alexis Biochemicals, San Diego, CA, USA), PMSF (Wako, Osaka, Japan) and Diogenes (National Diagnostics, Atlanta, GA, USA) were obtained. Monoclonal anti-human CD11b antibody (Ancell, Bayport, MN, USA), 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), fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse immunoglobulin (Molecular Probes, Eugene, OR, 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).

2.2. Cell cultures and monocytic differentiation of U937 cells

Human monoblastic leukemia U937 cells were grown in RPMI-1640 culture medium as described [4,6]. To induce monocytic differentiation, U937 cells (1.5×10^6) in 10 ml of culture medium were treated with 1 μM RA in the absence or presence of 5 μM curcumin at 37 °C for 48 h. Surface antigen CD11b was analyzed by flow cytometry as described [4].

2.3. Assay of O_2^- generation

O_2^- was quantified by measuring chemiluminescence (CL) using Diogenes-luminol CL probes. Cells in PBS containing 1 mM $MgCl_2$, 0.5 mM $CaCl_2$, 5 mM glucose and 0.03% bovine serum albumin were stimulated with 200 ng/ml PMA at 37 °C, and the O_2^- generation was measured by TD-20/20 luminometer (Promega, Madison, WI, USA).

2.4. Semiquantitative RT-PCR

Total RNAs were isolated from undifferentiated and differentiated U937 cells. Semiquantitative RT-PCR was performed as described [19,20] using sense and antisense primers listed in Supplementary Table 1, which were synthesized according to the sequence data deposited in GenBank for appropriate genes. 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 Image Gauge software Profile mode (densitometrical analysis mode) using a luminescent image analyzer LAS-1000plus (FUJIFILM, Tokyo, Japan).

2.5. Immunoblotting

Cells (1×10^7) were collected by centrifugation and sonicated 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 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 microliters of loading buffer [0.5 M Tris–HCl (pH 6.8) containing 5% SDS, 20% glycerol] were added to the solubilized membrane fraction. Immunoblotting was performed as described [6,21,22]. Data analyses were carried out using a luminescent image analyzer LAS-1000plus. Human β-actin was used as a control.

3. Results

3.1. Curcumin dramatically enhances RA-induced O_2^--generating activity in U937 cells

We examined effects of curcumin on RA-induced O_2^--generating activity in U937 cells (Fig. 1A). Undifferentiated U937 cells generated a negligible level of O_2^- when stimulated with PMA. As expected, O_2^--generating activity of U937 cells was induced in the presence of RA, but was not induced in the presence of curcumin. On the other hand, very interestingly, RA-induced O_2^--generating activity was dramatically enhanced (to ~400%) by the addition of curcumin. To further examine the effects of curcumin on RA-induced differentiation of U937 cells, we analyzed the expression of CD11b, a marker protein of macrophage on the cell surface, by flow cytometry (Fig. 1B). Expression of CD11b was slightly enhanced by the addition of curcumin. These results suggested that RA and curcumin effectively collaborated to induce O_2^--generating activity rather than differentiation in U937 cells.

3.2. Effects of curcumin on gene expressions of essential components for O_2^--generating system during RA-induced monocytic differentiation

To know the effects of curcumin on gene expressions of five essential components (p22-phox, gp91-phox, p40-phox, p47-phox and p67-phox) for the O_2^--generation, we carried out semiquantitative RT-PCR on total RNAs prepared from undifferentiated, curcumin-treated, RA-treated, and RA and curcumin-co-treated U937 cells. Fig. 2A shows typical semiquantitative RT-PCR profiles for the five components of the O_2^--generating system. Data were indicated as percentages of control values obtained from undifferentiated U937 cells (Fig. 2B). Curcumin alone had little effects on gene expressions of the five components. In RA-treated U937 cells, as expected, gene expressions of gp91-phox and p47-phox remarkably increased (to ~730% and ~1000%, respectively), while those of p22-phox, p40-phox and p67-phox slightly increased (to ~160%, ~125% and ~170%, respectively). After incubation with RA in the presence of curcumin, all genes tested were further increased: p22-phox (to ~220%), gp91-phox (to ~760%), p40-phox

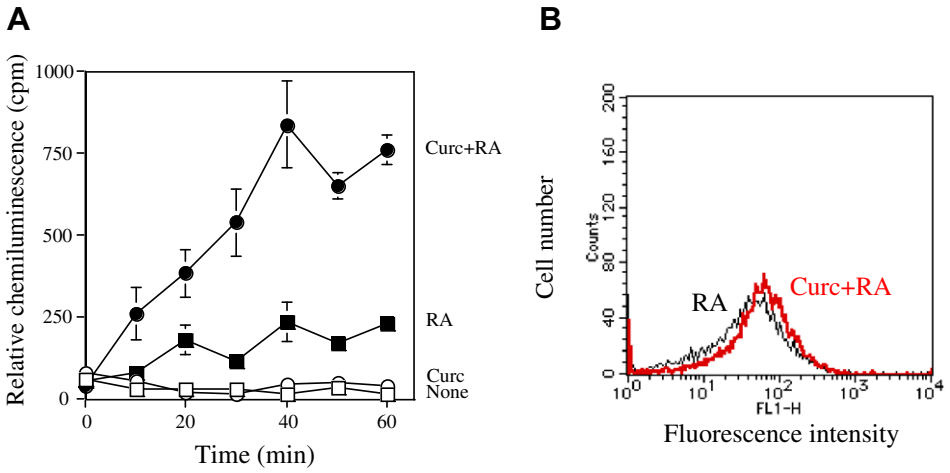

Fig. 1. Influences of co-treatment with RA and curcumin on induction of O_2^--generating activity and expression of cell surface CD11b in U937 cells. (A) Induction of O_2^--generating activity in RA-treated and RA plus curcumin-treated U937 cells. O_2^- generation was determined after culture of the cells for 48 h in the presence of agents (RA and/or curcumin) as in Section 2. Untreated (None, open squares), curcumin-treated (Curc, open circles), RA-treated (RA, closed squares), and RA plus curcumin-treated (Curc+RA, closed circles) U937 cells (2×10^6 cells/ml) were stimulated with 200 ng/ml PMA at 37 °C. PMA-induced CLs were measured every 10 min until 60 min after stimulation. Data represent the averages of three separate experiments and error bars indicate standard deviation. (B) Expression of cell surface CD11b in RA-treated and RA plus curcumin-treated U937 cells. RA-treated and RA plus curcumin-treated U937 cells were incubated with anti-CD11b monoclonal antibody followed by FITC-conjugated anti-mouse antibody, and analyzed by FACSCalibur.

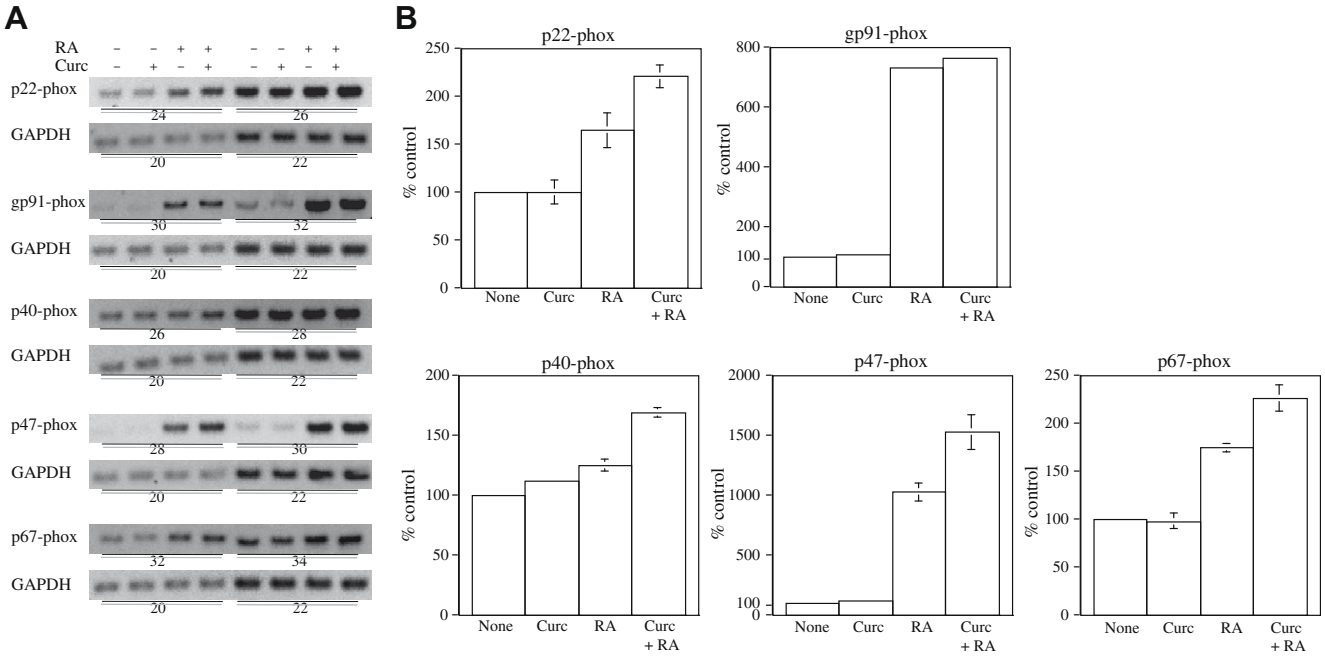

Fig. 2. Influences of co-treatment with RA and curcumin on gene expressions of O_2^--generating system-related factors. Total RNAs were extracted from untreated, curcumin-treated, RA-treated, and RA plus curcumin-treated U937 cells, and mRNA levels of p22-phox, gp91-phox, p40-phox, p47-phox and p67-phox were determined by semiquantitative RT-PCR using appropriate primers. Human GAPDH gene was used as internal controls. The gel images obtained were analyzed by Image Gauge software Profile mode using a luminescent image analyzer LAS-1000plus. (A) Typical semiquantitative RT-PCR profiles for five components of O_2^--generating system. Numbers below the panels indicate PCR cycle numbers. (B) Quantitative mRNA levels of p22-phox, gp91-phox, p40-phox, p47-phox and p67-phox in untreated (None), curcumin-treated (Curc), RA-treated (RA), and RA plus curcumin-treated (Curc+RA) U937 cells. Data calibrated with the internal controls are indicated as percentages of control values obtained from untreated U937 cells, and represent the averages of three separate experiments. Error bars indicate standard deviation.

(to ~170%), p47-phox (to ~1500%) and p67-phox (to ~230%), respectively.

3.3. Co-treatment with RA and curcumin dramatically causes accumulation of cytosolic p47-phox and p67-phox proteins in U937 cells

To examine the effects of curcumin on amounts of five proteins essential for the O_2^--generation (p22-phox, gp91-phox, p40-phox, p47-phox and p67-phox), immunoblot analysis was performed for these proteins using antibody specific for each protein. Fig. 3 shows typical immunoblotting profiles for undifferentiated, curcumin-treated, RA-treated, and RA- plus curcumin-treated U937 cells. Quantitative data obtained from RA plus curcumin-treated U937 cells were indicated as percentages of control values obtained from RA-treated U937 cells. Unexpectedly, curcumin exhibited insignificant influences on protein levels of p22-phox, gp91-phox and p40-phox in the presence of RA. On the other hand, very

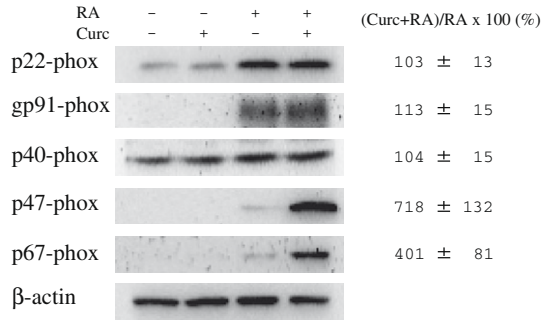

Fig. 3. Influences of co-treatment with RA and curcumin on protein levels of the O_2^--generating system-related factors. Cytosolic (for p40-phox, p47-phox and p67-phox) and membrane (for p22-phox and gp91-phox) fractions were prepared from untreated (None, –, –), curcumin-treated (Curc, +), RA-treated (RA, +), and RA plus curcumin-treated (Curc+RA, +, +) U937 cells, and protein levels were determined by immunoblotting using appropriate antibodies. Human β-actin was used as a control. Typical immunoblotting profiles are shown. Quantitative data obtained from RA plus curcumin-treated (Curc+RA) U937 cells are indicated as percentages of control values obtained from RA-treated (RA) U937 cells, and represent the averages of three separate experiments with errors indicated by standard deviation to the right of each panel.

interestingly, co-treatment with RA and curcumin caused remarkable increases in protein levels of p47-phox (to ~700%) and p67-phox (to ~400%), respectively. These results suggested that curcumin may contribute to the accumulation of these two cytosolic proteins rather than transcriptional activation during differentiation induced by RA.

4. Discussion

In this study, we demonstrated that curcumin dramatically enhances O_2^--generating activity via accumulation of p47-phox and p67-phox proteins during RA-induced monocytic differentiation of U937 cells. In the presence of curcumin, surprisingly, RA-induced O_2^--generating activity was significantly enhanced (to ~400%) during differentiation (Fig. 1A). To clarify molecular mechanisms for remarkable enhancement of O_2^--generating activity by curcumin, we performed semiquantitative RT-PCR and immunoblot analysis of five specific proteins essential for the O_2^--generating system. Semiquantitative RT-PCR showed that co-treatment with RA and curcumin slightly enhanced expressions of these five components compared with those obtained by treatment with RA only (Fig. 2). On the other hand, surprisingly, immunoblot analysis revealed that co-treatment with RA and curcumin caused remarkable accumulations in protein levels of p47-phox (to ~700%) and p67-phox (to ~400%) compared with those obtained by treatment with RA only (Fig. 3). These results suggested not only that curcumin causes drastic accumulation of p47-phox and p67-phox proteins rather than their transcriptional activation during RA-induced differentiation, but also that the remarkable increase of these two proteins may result in enhancing their translocation to membrane and drastically up-regulating O_2^--generating activity. However, further studies are needed to explore how curcumin accumulates amounts of p47-phox and p67-phox proteins.

As mentioned above, numerous studies have suggested that curcumin acts as a modifier for various biofunctions, through its prophylactic or therapeutic effects by targeting a number of molecules [10–16]. Regarding phagocytes, for example, curcumin raises phagocytic activity of macrophages [23], suppresses nitric oxide production in macrophages [24] and regulates apoptotic cell death in U937 cells [25,26]. In addition, curcumin shows synergistic effects on VD_3- or RA-induced differentiation of HL60 cells [17,18]. Therefore, curcumin has been expected for therapy of leukemia by its anti-cancer activity and differentiation-stimulating activity. Our data, together with these previous results, propose that curcumin has the ability as an effective modifier in therapy of leukemia and a potential immunopotentiator via strongly enhancing the O_2^--generating activity in leukocytes. The curcumin-enhanced O_2^--generating activity may rescue leukemia patients from infectious diseases.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2010.03.136.

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