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Water-soluble antioxidants improve the antioxidant and anticancer activity of low concentrations of curcumin in human leukemia cells

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Curcumin (Cur) is a promising antioxidant and anticancer drug, but several recent studies indicate that Cur exerts its anticancer activity through promoting reactive oxygen species (ROS) generation. In the present study, concentration-dependent regulation of Cur on cell proliferation, viability and ROS generation, and effect of water-soluble antioxidants ascorbic acid (ASA), N-acetyl-cysteine (NAC) and reduced glutathione (GSH) on the antioxidant and anticancer activity of Cur were investigated in human myeloid leukemia cells (HL-60 cells). We found that although Cur concentration- and time-dependently decreased the proliferation and viability of cells, its effect on ROS generation (as indicated by the level of malondialdehyde, MDA) varied with its concentrations. I.e., low concentrations of Cur diminished the ROS generation, while high Cur promoted it. Combined with the opposite effect of 50 μM H_2O_2 on low or high Cur-induced MDA alteration, cell proliferation arrest and cell death, these results proved that low Cur exerted its anticancer activity through diminishing ROS generation in HL-60 cells, while high Cur through promoting ROS generation. Further studies showed that all water-soluble antioxidants ASA, NAC and GSH significantly enhanced both the antioxidant and the anticancer activity of low Cur. Considering that the extra accumulation of ROS is harmful to normal cells, the data presented here indicate that instead of using high doses, combining low doses of Cur with water-soluble antioxidants is a better strategy for us to improve the anticancer activity of Cur.

1. Introduction

Curcumin (diferuloylmethane, Cur), a polyphenol derived from the plant Curcuma longa, has been recognized as a promising anticancer drug due to its efficient induction of proliferation arrest and cell death (including apoptosis and necrosis) in a variety of tumor cells (Aggarwal et al. 2003; Anto et al. 2002; Roy et al. 2002; Kim et al. 2001; Hadi et al. 2000; Bhaumik et al. 1999). Like most polyphenols, although Cur is a naturally occurring antioxidant, it exhibits prooxidant properties under certain conditions (Sakano et al. 2002; Galati et al. 2002; Kim et al. 2001; Bhaumik et al. 1999; Nogaki et al. 1998; Wang et al. 1996). Interestingly, both antioxidant and prooxidant activities are considered to be involved in the anticancer properties of Cur (Aggarwal et al. 2003; Khar et al. 2001; Hadi et al. 2000; Bhaumik et al. 1999). Our previous study found that polyphenols scavenge ROS at low concentrations, but promote ROS generation at higher concentrations (Wang et al. 1996), suggesting that Cur might exert anticancer activity via diminishing ROS at low concentrations or via enhancing ROS at high levels. Coincidently, higher concentrations of Cur (such as at 50 μM) were found to promote ROS generation in different studies (Sakano et al. 2002; Galati et al. 2002; Kim et al. 2001; Bhaumik et al. 1999), while low Cur (such as at 10 μM) usually diminishes ROS generation (Chan et al. 2003; Joe et al. 1994). Considering that the generation of ROS might damage normal cells, trying to improve the anticancer activity of low doses of Cur instead of using high doses, is obviously useful.

Since the combination of different antioxidants can obviously improve their net antioxidant and biological activities (Chen et al. 2004b; Wedgwood et al. 2003; Kang et al. 2000; Chow, 1988), and the combination of Cur with water-soluble antioxidants, such as ascorbic acid (ASA), has been found to synergistically scavenge free radicals *in vitro* (Jovanovic et al. 2001), we suppose that a combination of low doses of Cur with water-soluble antioxidants may be an efficient strategy to improve its antioxidant and anticancer activity. To address this, the effect of water-soluble antioxidants ASA, N-acetyl-cysteine (NAC) and reduced glutathione (GSH) on Cur-caused ROS accumulation, cell proliferation and viability alteration was investigated in human leukemia HL-60 cells.

2. Investigations, results and discussion

2.1. Anticancer effect of Cur in human leukemia cells

Treating cells with Cur resulted in a concentration- and time-dependent arrest in the proliferation of HL-60 cells (Fig. 1A and 1B). After treatment of cells with Cur at no

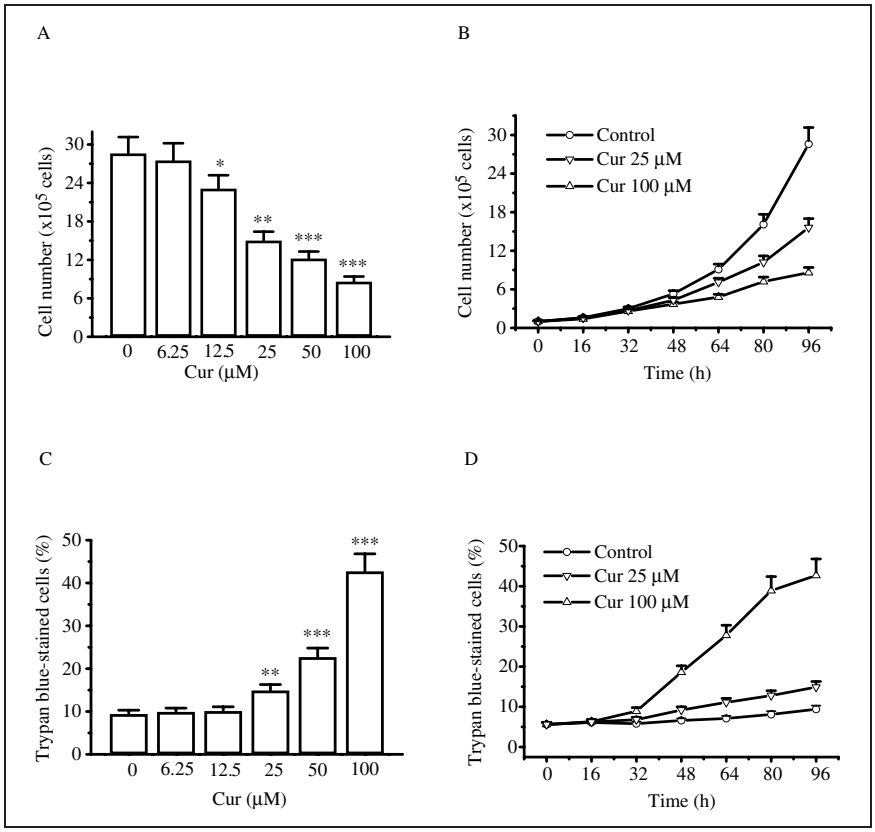

Fig. 1:
Effect of Cur on the proliferation and viability of HL-60 cells. Cells were incubated with different concentrations of Cur for 96 h in A and C, with 25 or 100 μM of Cur for the indicated times in B and D. Means ± SD of three parallel experiments was indicated, n = 3×3 cultures per conditions, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.01$ vs. the control group in A and C

less than 25 μM, the trypan blue exclusion assay showed up to 45% of cells were dyed by trypan blue, indicating the loss of cell membrane integrity and cell death (Fig. 1C), this increase of cell death also exhibited a time-dependent manner (Fig. 1D).

2.2. Effect of Cur on the accumulation of ROS in vivo

To evaluate the effect of Cur on ROS accumulation *in vivo*, the level of lipid peroxidation (LPO, as indicated by MDA content), resulting from the reaction between ROS and unsaturated fatty acids (Gutteridge et al. 1990), was studied in HL-60 cells. As indicated in Fig 2A, treating cells with low doses of Cur (not more than 25 μM) significantly decreased the amount of MDA, while high doses (50 and 100 μM) increased it (Fig. 2A). This decrease or increase of LPO exhibited a time-dependent manner (Fig. 2B). These results suggested that low or high doses of Cur might exert its anticancer activity through oppositely regulating the ROS generation.

2.3. Effect of H_2O_2 on Cur-induced lipid peroxidation and cytotoxicity

In order to investigate whether the alteration of ROS is involved in the cytotoxicity of Cur, we tested whether the anticancer activity of low or high Cur could be diminished or enhanced by the addition of H_2O_2, which was believed to consume the antioxidant capacity of antioxidants (such as low Cur in this study), but enhance the oxidative damage caused by a pro-oxidant (such as Cur in high doses). We found that H_2O_2 at 50 μM did not significantly affect ROS accumulation (Fig. 3A), cell proliferation and viability in HL-60 cells (Fig. 3B, 3C), while higher concentrations (such as 100 μM) showed an obvious effect on both ROS accumulation and cell proliferation (data not shown); thus H_2O_2 at 50 μM was selected in this study. As we expected, H_2O_2 at 50 μM significantly counteracted or enhanced the effects of low or high Cur on ROS accumulation (Fig. 3A), cell proliferation (Fig. 3B), and survival (Fig. 3C) in HL-60 cells.

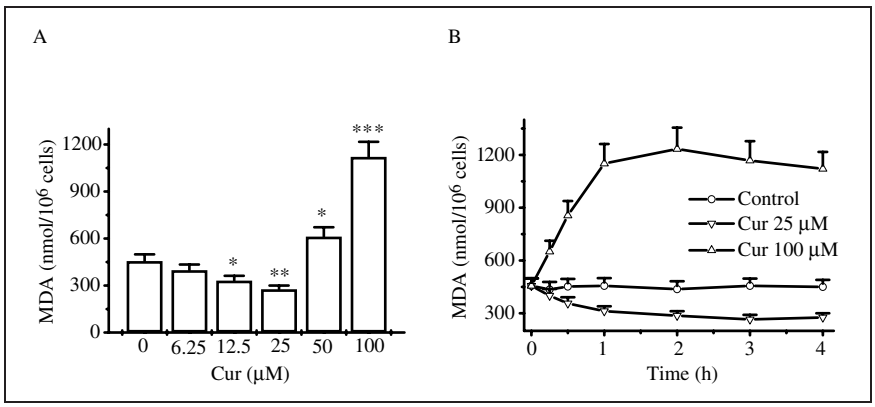

Fig. 2:
Effect of Cur on lipid peroxidation in HL-60 cells. Cells were incubated with different concentrations of Cur for 4 h in A, or with 25 or 100 μM of Cur for the indicated times in B. Means ± SD of three parallel experiments was indicated, n = 3×3 cultures per conditions, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. the control group in A

Previous studies indicate that ROS are not only involed in carcinogenesis, but also in the prevention and cure of cancer (Wedgwood and Black, 2003; Deshpande and Irani, 2002; Kang et al. 2000; Kang et al. 1999). On one hand, ROS act at different stages of carcinogenesis (Deshpande and Irani, 2002). On the other hand, either diminishing (Chen et al. 2004a; Wedgwood and Black, 2003; Kang et al. 2000; Kang et al. 1999) or enhancing (Kang et al. 2001; Khar et al. 2001; Bhaumik et al. 1999) ROS generation is involved in the induction of proliferation arrest, re-differentiation, apoptosis or necrosis of tumor cells, thus both antioxidant and prooxidant therapy are potential anticancer strategies. In fact, as we have known, to keep a balance between ROS generation and scavenging *in vivo* is found important for the proliferation and viability of cells in all aerobic animals (Martindale and Holbrook, 2002; McCord, 1998; Burdon, 1995).

Different studies showed that both antioxidant and prooxidant activity are involved in the anticancer activity of Cur (Aggarwal et al. 2003; Khar et al. 2001; Hadi et al. 2000; Bhaumik et al. 1999). Interestingly, in this study, Cur exerts its anticancer activity in the same system through either diminishing ROS at low concentrations or increasing ROS at high concentrations. Although the anticancer activity of high doses is obviously higher than that of low doses, considering the possible damage to normal cells caused by extra ROS accumulation *in vivo*, to improve the anticancer activity of low Cur, especially through improving the antioxidant activity of Cur is obviously helpful.

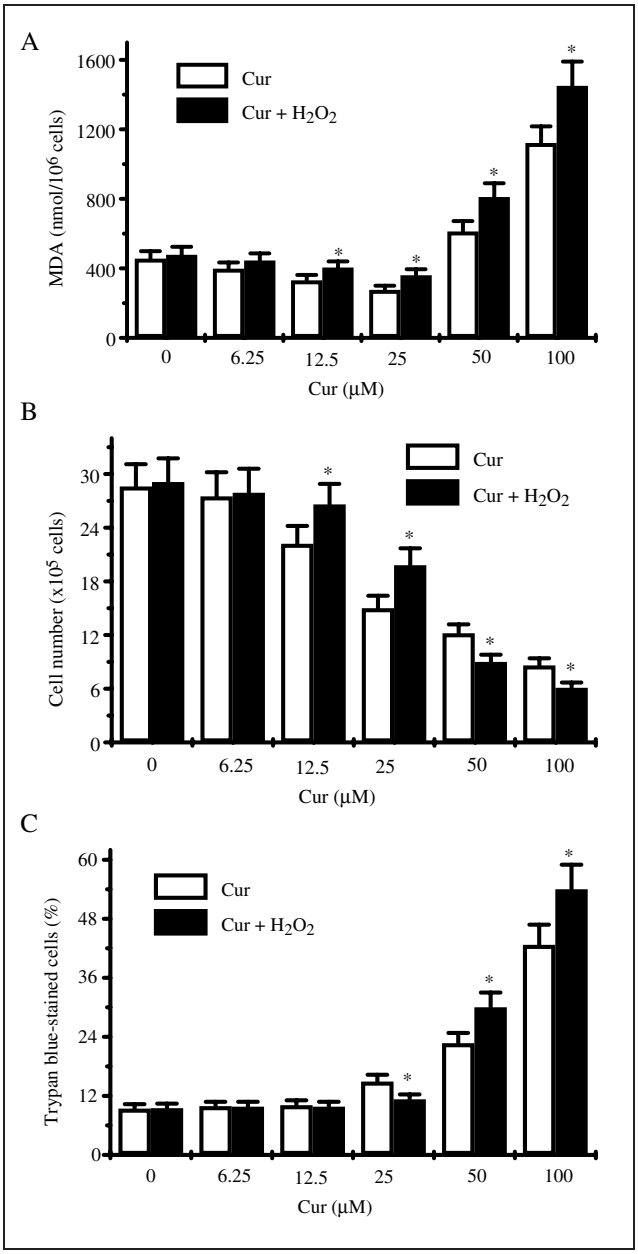

Fig. 3: Effect of H_2O_2 on the antioxidant and anticancer activity of Cur. Cells were treated with Cur in the absence or presence of 50 μM of H_2O_2 for 4 h. H_2O_2 diminished or enhanced the ROS diminution (A), cell proliferation arrest (B), and the increase of trypan blue-stained cells (C) triggered by low or high Cur, respectively. Means of three parallel experiments are displayed (means ± SD), n = 3×3 cultures per conditions, * indicates $p < 0.05$ as compared with the corresponding Cur control

2.4. Enhancement of ASA, NAC and GSH on the antioxidant and anticancer activity of Cur in low doses

Combination of different antioxidants, especially the combination of lipid- and water-soluble antioxidants has been found to significantly improve their net antioxidative and other biological activities (Chen et al. 2004b; Wedgwood et al. 2003; Jovanovic et al. 2001; Kang et al. 2000; Chow, 1988). For example, the combination of the water-soluble ASA and the lipo-soluble DL-α-tocopherol has been proven to synergistically scavenge ROS and induce tumor cell proliferation arrest and differentiation (Kang et al. 2000). Since Cur is lipo-soluble, we tested the antioxidant and anticancer activity of its combination with water-soluble antioxidants. All water-soluble antioxidants used here, ASA at 1 mM, NAC at 500 μM, or GSH at 250 μM, diminished ROS accumulation in HL-60 cells (Fig. 4A), but did not significantly affect cell proliferation and viability (Fig. 4B, 4C). Since higher concentrations of these antioxidants clearly affected the cell proliferation and viability, while lower concentrations showed no scavenging effect on ROS in cells (data not shown), the listed concentrations of water-soluble antioxidants were used in the following studies. Our data showed that all these water-soluble antioxidants not only significantly enhanced the scavenging effect of low Cur on ROS (Fig. 4A), but also significantly enhanced its anticancer activity, as indicated by their enhancement on low Cur-induced cell proliferation arrest (Fig. 4B) and trypan-blue penetration (Fig. 4C). These results indicated that certain amounts of ROS are critically needed for the proliferation and viability of HL-60 cells, while water-soluble antioxidants improve the anticancer activity of Cur through synergistically scavenging ROS with Cur.

In summary, although additional experiments are needed to clarify the detailed mechanism on the anticancer activity of low or high doses of Cur, our present study proved that 1), ROS are differently involved in the anticancer activity of Cur, simply, low doses of Cur exert an anticancer effect through scavenging ROS, while high doses act mainly through promoting ROS generation; 2), combining with water-soluble antioxidants is a useful strategy to improve the antioxidant and anticancer activity of low doses of Cur. In addition, since polyphenols with similar structures possess similar antioxidant and pro-oxidant properties (Wang et al. 1996), our present data may also suggest a good strategy to improve the antioxidant and anticancer activity of other polyphenols with structures similar to Cur.

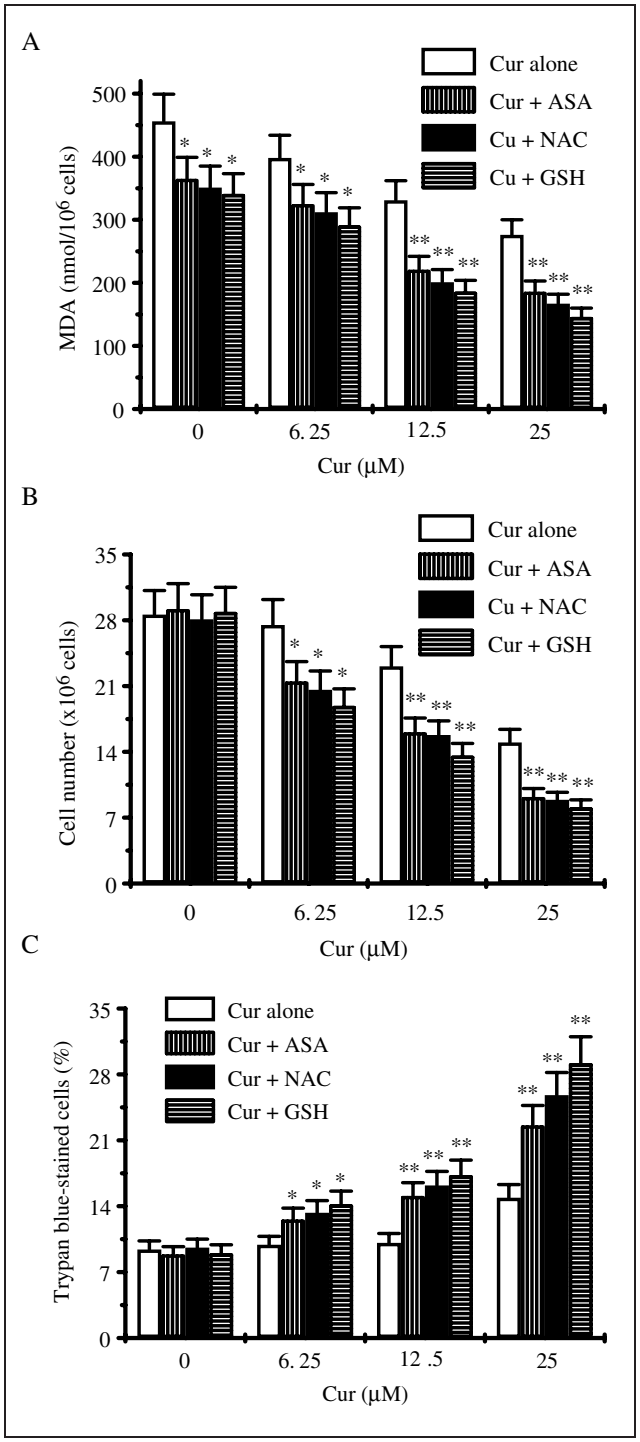

Fig. 4: Effect of ASA, NAC, and GSH on the antioxidant and anticancer activity of low Cur. Cells were treated with different concentrations of Cur alone, or the combination of Cur with ASA at 1 mM, NAC at 500 μM, or GSH at 250 μM, for 4 h in the MDA assay or for 96 h in the other assays. Water-soluble antioxidants significantly enhanced the ROS generation (A), cell proliferation arrest (B) and cell death (C) caused by low concentrations of Cur (no more than 25 μM). Means of three parallel experiments are displayed (means ± SD), $n = 3 \times 3$ cultures per conditions, *, ** indicates $p < 0.05$ or $p < 0.01$ as compared with the corresponding Cur control

3. Experimental

3.1. Reagents

Curcumin (Cur), ascorbic acid (ASA), N-acetyl-cysteine (NAC), reduced glutathione (GSH), trypsin, trypan blue, 1,1,3,3-tetramethoxypropane and thiobarbituric acid were purchased from Sigma (Sigma, St. Louis, MO), RPMI-1640 was purchased from Gibco (Santa Clara, CA). All other reagents are of analytical grade.

3.2. Cell culture and treatment

Human leukemia cells (HL-60) were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (equivalent to 100 units/ml and 100 μg/ml, respectively) at 37 °C in a humidified atmosphere containing 5% CO_2. After culturing the cells (1×10^5 cells/ml) for 24 h, the culture medium was aspirated and replaced with new medium containing Cur, ASA, NAC, GSH, and/or H_2O_2 where indicated, after that, the different cultures were replaced with fresh medium containing the corresponding reagents every 24 h.

3.3. Determination of cell proliferation and viability

Cells at 1×10^5/ml were cultured for 24 h, then treated with Cur and/or other agents at the indicated concentrations, three dishes for each of differently treated cells were collected every 16 h in the first 4 days, the total and dead cells were counted using the trypan blue stain exclusion method under a phase-contrast microscope.

3.4. The malondialdehyde (MDA) assay

After different treatment, HL-60 cells were collected and suspended in the lysis buffer (Kang et al. 2003). And the suspension was collected after pipetting the cell mixture up and down for 20 times. MDA amount in the obtained suspension was immediately evaluated using a colorimetric assay as described previously (Ohkawa et al. 1979). All the analyses were performed in triplicate.

3.5. Statistical analysis

Statistical analysis was performed by analysis of variance (ANOVA post-hoc Bonferroni), and $p < 0.05$, 0.01, or 0.001 were denoted as *, **, or ***, respectively.

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