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Combination with water-soluble antioxidants increases the anticancer activity of quercetin in human leukemia cells

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While accumulation of reactive oxygen species (ROS) is believed to be harmful to organisms, recent studies show that quercetin (QU), a promising antioxidant and anticancer drug, exerts its anticancer role through either diminishing or promoting ROS generation under different conditions. In the present study, it was investigated whether the water-soluble antioxidants ascorbic acid (ASA), N-acetyl-cysteine (NAC) and reduced glutathione (GSH) can enhance both the antioxidant and anticancer activity of quercetin in human myeloid leukemia cells (HL-60 cells). Proliferation, viability and ROS accumulation (indicated by the level of malondialdehyde, MDA) was significantly decreased by QU in HL-60 cells. 50 μM H_2O_2 markedly attenuated the antioxidant and anticancer activity of QU, proving that diminution of ROS accumulation considerably contributes to the QU-induced decrease of HL-60 cells proliferation and viability. When the effects of water-soluble antioxidants were tested, ASA at 1 mM, NAC at 500 μM, or GSH at 250 μM significantly enhanced QU-mediated proliferation arrest, cell death and ROS diminution. These results indicate that certain amounts of ROS are critical for the proliferation and viability of HL-60 cells, while water-soluble antioxidants enhance the anticancer activity of QU through scavenging ROS. Combing QU with water-soluble antioxidants could be a useful strategy to improve the anticancer activity of QU by increasing its antioxidant activity.

1. Introduction

Quercetin, a common, dietary flavonoid found in onions, apples, and tea (Morand et al. 1998; Hollman et al. 1997), has been found to show anticancer activity in different cancer cells (Chen et al. 2004; Cipak et al. 2003; Kaneuchi et al. 2003; Chan et al. 2003; Lee et al. 2002; Feng et al. 2001). For example, it significantly induces the proliferation arrest, redifferentiation, apoptosis or necrosis in leukemia cells (Chen et al. 2004; Cipak et al. 2003; Liesveld et al. 2003; Kang et al. 1997; Uddin et al. 1995; Kuo et al. 2002; Kim et al. 2000; Kawaii et al. 1999). Like most polyphenols, QU is a naturally occurring antioxidant (Morand et al. 1998; Hollman et al. 1997; Duthie et al. 2000), but exhibits prooxidant properties under certain conditions (Dickancaite et al. 1998; Lapidot et al. 2002 a, 2002 b). Interestingly, among various mechanisms suggested for the anticancer activity of QU (Chen et al. 2004; Kaneuchi et al. 2003; Lee et al. 2002; Feng et al. 2001; Kang et al. 1997; Uddin et al. 1995; Dickancaite et al. 1998; Lapidot et al. 2002 a, 2002 b; Casagrande et al. 2001; Lee et al. 2002), both the antioxidant and pro-oxidant activities are included. Since extra ROS accumulation is harmful to normal cells (Datta et al. 2000), to treat cancer relying on the antioxidant potency of QU is obviously better than using its pro-oxidant activity.

The redox characteristics and the anticancer activity of QU have been found to be correlated with its concentrations (Kaneuchi et al. 2003; Dickancaite et al. 1998; Lapidot et al. 2002 a, 2002 b; Skibola et al. 2000), simply, in low concentrations, QU mainly acts as an antioxidant but sometimes possesses limited anticancer activity (Kaneuchi et al. 2003; Dickancaite et al. 1998; Lapidot et al. 2002 b), on the contrary, in high concentrations, QU shows strong anticancer activity but sometimes initiates the generation of ROS (Dickancaite et al. 1998; Lapidot et al. 2002 a, 2002 b; Skibola et al. 2000). Here, we tried to find a way to enhance the antioxidant and anticancer activity of QU.

Combination of different antioxidants has been proven to be able to significantly improve their net antioxidative and biological activities (Kang et al. 2000; Wedgwood et al. 2003; Chow 1988), thus to combine with other antioxidants may be efficient to improve the antioxidant and anticancer activity of QU. Since QU is liposoluble (Morand et al. 1998; Hollman et al. 1997), and the combination of curcumin (another famous liposoluble polyphenol) with water-soluble antioxidants is found to synergistically scavenge free radicals *in vitro* (Jovanovic et al. 2001), we suppose that combining QU with water-soluble antioxidants may enhance its antioxidant and anticancer activity. To address this, the effect of the water-soluble antioxidants ascorbic acid (ASA), *N*-acetyl-cysteine (NAC) and reduced glutathione (GSH) on

the antioxidant and anticancer activity of QU was investigated in human leukemia HL-60 cells.

2. Investigations, results and discussion

2.1. Anticancer effect of QU in human leukemia cells

Treating cells with QU markedly decreased cell proliferation in a concentration – (Fig. 1A) and time-dependent manner (Fig. 1B). At the same time, the trypan blue exclusion assay showed that QU treatment significantly increased the trypan blue-stained cells, indicating a loss of cell membrane integrity and cell death (Fig. 1C). This increase of cell death also exhibited a time-dependent manner (Fig. 1D). When cells were treated with QU at 50 μM for 16 or 48 h, cell proliferation decreased by about 35 or 55%, while the trypan blue-stained cells increased by about 3 or 15%, respectively.

2.2. Involvement of ROS diminution in the anticancer activity of QU

Among various mechanisms described for the anticancer activity of QU (Cipak et al. 2003; Kaneuchi et al. 2003; Chan et al. 2003; Lee et al. 2002; Feng et al. 2001), QU is found to exert its anticancer activity by either diminishing or promoting ROS generation (Chen et al. 2004; Feng et al. 2001; Dickancaite et al. 1998; Lapidot et al. 2002 a, 2002 b). Thus we detected if the antioxidant or pro-oxidant activity is involved in the anticancer activity of QU in this study. For this purpose, according to a previous report (Gutteridge et al. 1990), which stated that lipid peroxidation (LPO, as indicated by MDA content) resulted from the direct interaction between *in vivo* ROS and unsaturated fatty acids, this was used as one representative of *in vivo* ROS accumulation. Fig. 2A shows that treating cells with gradually increased QU led to a concentration-dependent decrease in the concentration of MDA. QU treatment caused a rapid, followed by a sustained decrease in the amount of MDA (Fig. 2B), suggesting the involvement of the antioxidant activity in QU's anticancer activity in HL-60 cells. In order to confirm this, we tested whether the anticancer activity of QU could be attenuated by the addition of H_2O_2, which was believed to consume the antioxidant capacity of QU. We found that H_2O_2 at 50 μM did not significantly affect the ROS accumulation (Fig. 3A), cell proliferation and viabil-

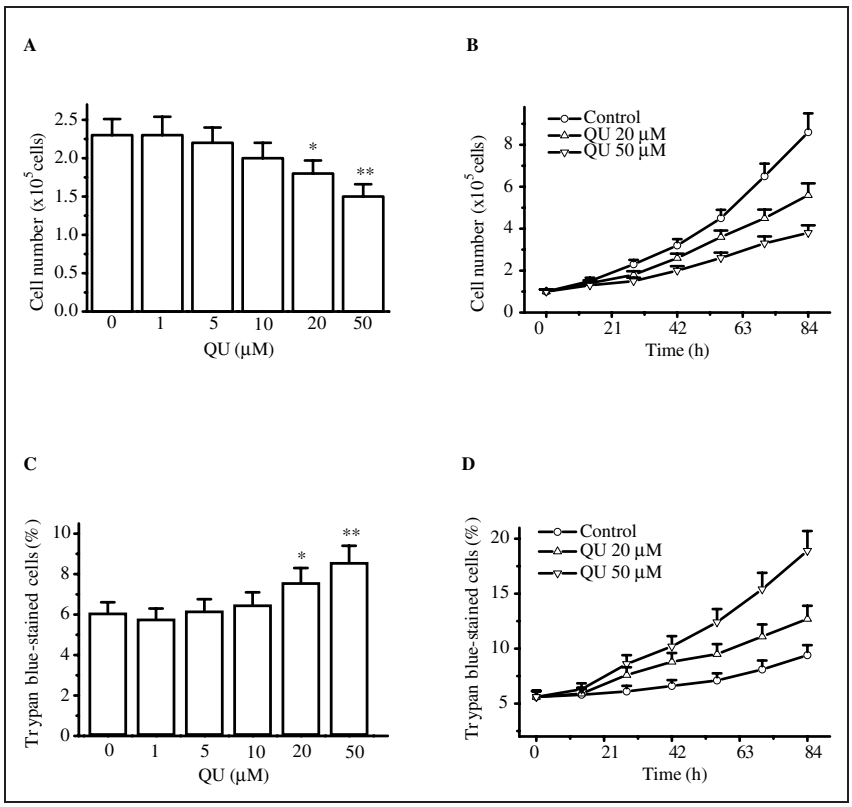

Fig. 1:
Effect of QU on the proliferation and viability of HL-60 cells. Cells were incubated with different concentrations of QU for 16 h in A, C; or with 20 or 50 μM of QU for the indicated times in B, D. Then cell number (A, B) and trypan blue-stained cells (C, D) were measured. Means ± SD of three 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 and C

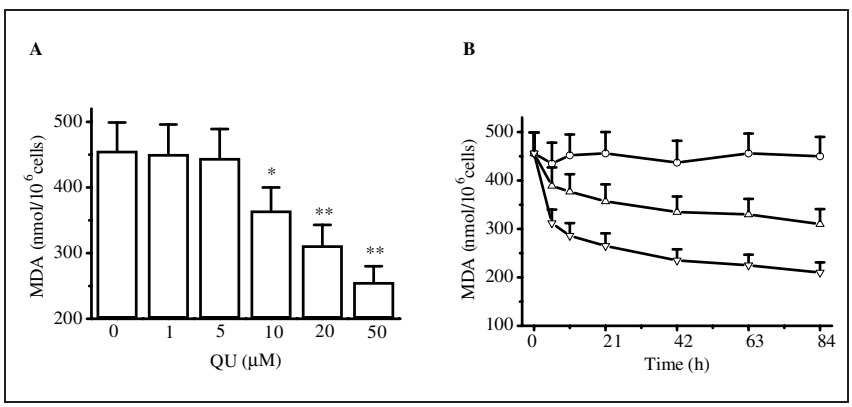

Fig. 2:
Effect of QU on lipid peroxidation in HL-60 cells. Cells were incubated with different concentrations of QU for 16 h in A, or with 20 or 50 μM of QU for the indicated times in B. Means ± SD of three parallel experiments was indicated, n = 3 × 3 cultures per conditions, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. the control group in A. Open circle, up-triangle and down-triangle represents the group treated with 0, 20 or 50 μM of QU, respectively in B

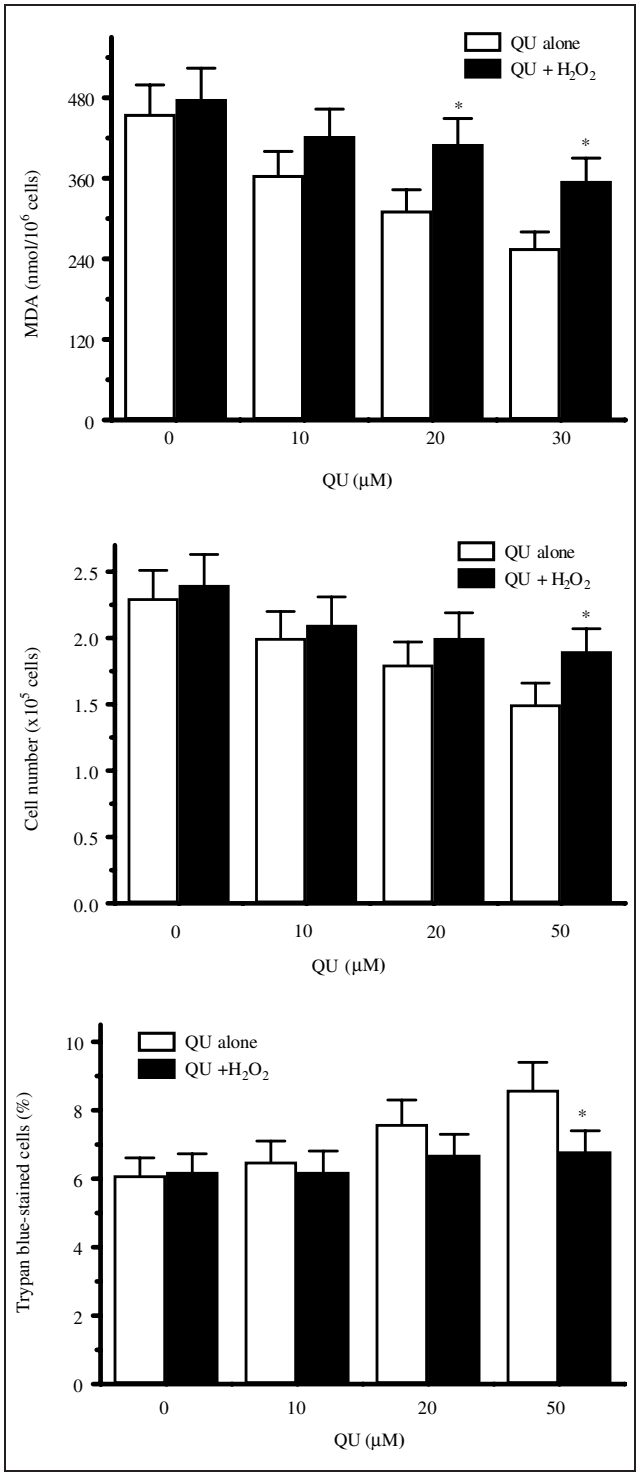

Fig. 3: Effect of H_2O_2 on the antioxidant and anticancer activity of QU. Cells were treated with QU in the absence or presence of 50 μM of H_2O_2 for 16 h. H_2O_2 attenuated the ROS diminution (A), cell proliferation arrest (B), and the increase of trypan blue-stained cells (C) triggered by QU. Means of three parallel experiments are displayed (means ± SD), n = 3×3 cultures per conditions, * indicates $p < 0.05$ as compared with the corresponding QU control

ity in HL-60 cells (Fig. 3B, 3C), while higher concentrations (such as 100 μM) showed obvious effects on both ROS accumulation and cell proliferation (data not shown). Thus H_2O_2 at 50 μM was selected in this study. As expected, H_2O_2 at 50 μM significantly counteracted the effects of QU on the ROS attenuation (Fig. 3A), cell proliferation arrest (Fig. 3B), and the increase of trypan blue-stained cells (Fig. 3C). These results further confirmed the important role of the antioxidant capacity in the anticancer effect of QU.

Consistent with our results, 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 (Burdon 1995; McCord 1998; Martindale and Holbrook 2002). Considering the automatic generation of ROS and the low superoxide dismutase activity in tumor cells (Oberley 1982; Burden 1994), improving the antioxidant activity of QU may be also able to improve its anticancer activity. Thus we tried to find a way to improve both the antioxidant and the anticancer activity of QU.

2.3. Enhancement of water-soluble antioxidants on the antioxidant and anticancer activity of QU

Previous studies found that a combination of different antioxidants, especially the combination of lipid- and water-soluble antioxidants significantly improves their antioxidative and biological activities (Kang et al. 2000; Wedgwood et al. 2003; Chow 1988; Jovanovic et al. 2001). For example, the combination of the water-soluble ASA and the lipo-soluble DL-α-tocopherol (Kang et al. 2000), or the combined superoxide dismutase/catalase mimetic (Wedgwood et al. 2003) has been proven to synergistically scavenge ROS and induce tumor cell proliferation arrest, differentiation or apoptosis. Since QU is lipo-soluble (Morand et al. 1998; Hollman et al. 1997), we tested the antioxidant and anticancer activity of the combination of QU with water-soluble antioxidants. All water-soluble antioxidants used here, ASA at 1 mM, NAC at 500 μM, or GSH at 250 μM, diminished the ROS accumulation in HL-60 cells (Fig. 4A), but did not significantly affect the proliferation and viability of cells (Fig. 4B, 4C). Since higher concentrations clearly affected cell proliferation and viability, while lower amounts showed no scavenging effect on the ROS in HL-60 cells (data not shown), the above listed concentrations of water-soluble antioxidants were used. Our data showed that all these compounds not only significantly enhanced the scavenging effect of QU on ROS (Fig. 4A), but also obviously enhanced its anticancer activity, as indicated by their enhancement on QU-induced cell proliferation arrest (Fig. 4B) and trypan-blue penetration (Fig. 4C). For example, the antioxidant and anticancer activity of the combination of 10 μM QU with water-soluble antioxidant is even higher than that of 50 μM QU alone. Considering the possible initiation of ROS generation by high concentrations of QU under certain conditions (Dickancaite et al. 1998; Lapidot et al. 2002 a, 2002 b; Skibola et al. 2000), these results indicate that instead of using high amounts of QU, a combination of low doses of QU with water-soluble antioxidants may better improve its anticancer activity.

In summary, our present study proved that 1), certain amounts of ROS are critical for the proliferation and viability of HL-60 cells, the antioxidant capacity of QU plays an important role, in its anticancer activity, and water-soluble antioxidants can improve both the antioxidant and anticancer activity of QU; 2), given the fact that polyphenols with similar structures possess similar redox properties (Wang et al. 1996), our data suggest a useful strategy to improve not only the antioxidant and anticancer activities of QU, but also those of polyphenols with structures similar to QU.

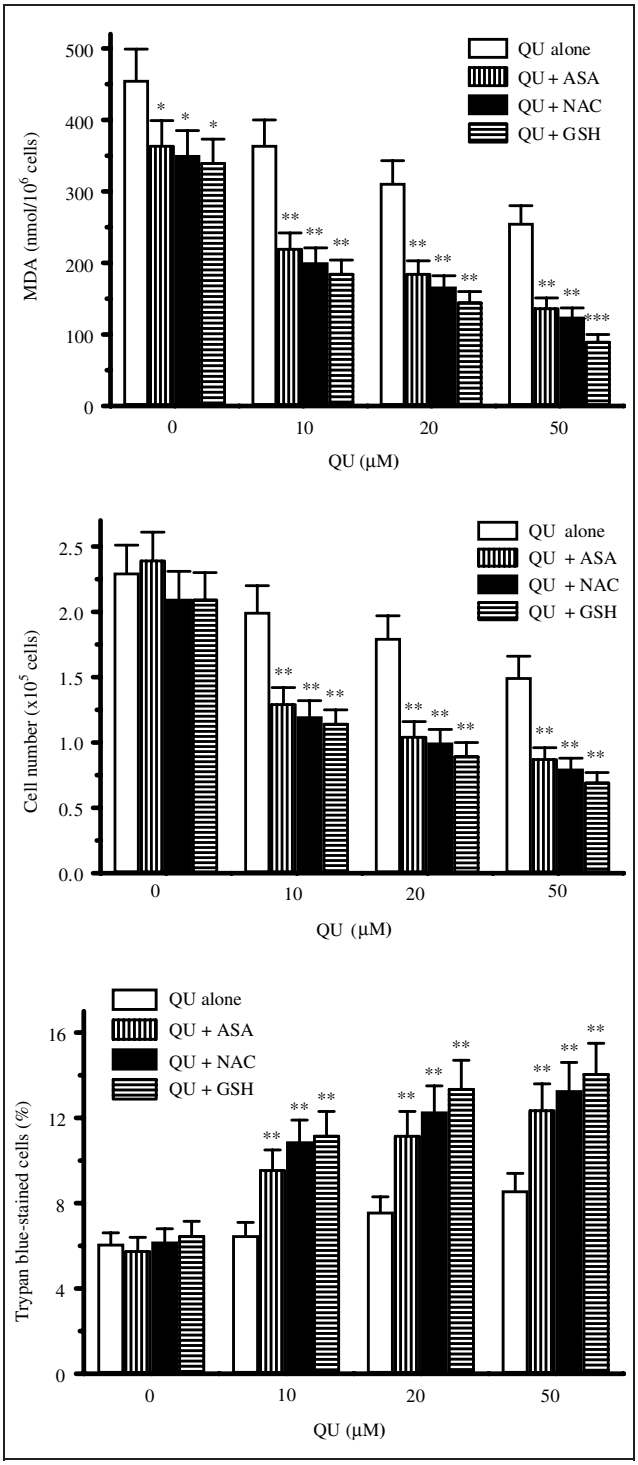

Fig. 4: Effect of water-soluble antioxidants ASA, NAC, and GSH on the antioxidant and anticancer activity of QU. Cells were treated with different concentrations of QU alone, or the combination of QU with ASA at 1 mM, NAC at 500 μM, or GSH at 250 μM, for 16 h. Water-soluble antioxidants significantly enhanced the effect of QU on the ROS diminution (A), cell proliferation (B), and the increase of trypan blue-stained cells (C). Means of three parallel experiments are displayed (means ± SD), n = 3×3 cultures per conditions, *, ** indicates $p < 0.05$ or $p < 0.01$ as compared with the corresponding QU control

3. Experimental

3.1. Reagents

Quercetin, *N*-acetyl-cysteine, reduced glutathione, 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 (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 mg/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 quercetin (QU), ascorbic acid (ASA), reduced glutathione (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 QU 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. Malondialdehyde (MDA) assay

After different treatment, HL-60 cells were collected and suspended in the lysis buffer (Kang et al. 2003). 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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