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Suppression of active oxygen-induced cytotoxicity by flavonoids

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Abstract—The flavonoids quercetin, kaempferol, catechin and taxifolin suppressed cytotoxicity of active oxygen species (O_2^- and H_2O_2) on Chinese hamster V79 cells. Cytotoxicity of active oxygen species was assessed with a colony formation assay. The flavonoids prevented the decrease in the number of colonies caused by H_2O_2 or O_2^- at concentrations which were not themselves cytotoxic. There was a very substantial difference in the dose-dependency of the protective effects brought about by quercetin and kaempferol in contrast to catechin and taxifolin. The structure–activity relationship revealed that either the *o*-dihydroxy structure in the B ring or certain structures in the A and C rings of the flavonoids are necessary for the protective activities.

Flavonoids are polyphenolic secondary metabolites found in a wide variety of plant sources such as vegetables, herbs, nuts and teas. Various chemical and biochemical effects of flavonoids have been reported. For example, flavonoids scavenge free radicals, e.g. O_2^-, ·OH, N_3·, *t*-BuO· and LOO· [1]. Polyphenols, including flavonoids, inhibit lipoxygenase, cyclooxygenase and lipid peroxidation [2]. Diverse effects of the flavonoids on immune and inflammatory cell functions have been reviewed extensively [3]. Furthermore, flavonoids have antihemolytic activities [4], inhibit the oxidation of low density lipoproteins [5] and prevent the cytotoxicity of oxidized low density lipoproteins on lymphoid cell lines [6]. But there is no report that flavonoids directly suppress active oxygen-induced mammalian cytotoxicity. Recently, we found that polyphenols, such as nordihydroguaiaretic acid, caffeic acid methyl ester, gallic acid esters and gossypol, prevent mammalian cytotoxicity caused by H_2O_2 [7–9]. Here we show that flavonoids also have suppressive effects against cytotoxicity caused by active oxygen species (H_2O_2 and O_2^-).

Materials and Methods

Reagents. H_2O_2 was obtained from Mitsubishi Gas Chemical Co. Ltd (Tokyo, Japan). Quercetin and hypoxanthine were purchased from Nacalai Chemicals Ltd (Kyoto, Japan). Kaempferol, taxifolin, xanthine oxidase from bovine milk (EC 1.2.3.2), catalase from bovine erythrocyte (EC 1.11.1.6) and superoxide dismutase (SOD*) from bovine liver (EC 1.15.1.1) were obtained from the Sigma Chemical Co. (St Louis, MO, U.S.A.). Catechin was purchased from Tokyo Kasei Kogyo Co. Ltd (Tokyo, Japan). All other chemicals were reagent grade and were used without further purification.

Colony formation assay. Cytotoxic effects of active oxygen species and the flavonoids and protective effects of the flavonoids against active oxygen-induced cytotoxicity were assessed by a colony-formation assay, one of the most reliable methods of assessing cytotoxic effects [10]. In our experiments we avoided direct reactions of flavonoids with active oxygen species in the medium in order to evaluate the real effects of the flavonoids in the cell or the cell membrane. Chinese hamster lung fibroblast V79 cells were seeded in 60-mm petri dishes (200 cells/dish) and incubated in 5 mL of minimum essential medium (MEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) in a humidified atmosphere of 5% CO_2 in air at 37°. After changing the medium to 5 mL of MEM free of FBS, a 5 mM flavonoid ethanol solution (up to 40 μL) was added to the medium, and the cells were incubated for 4 hr. After being washed with HEPES-buffered saline (HBS, pH 7.3), the cells were treated with active oxygen species (60 μM of H_2O_2 or a combination of 50 μM of hypoxanthine plus 0.025 U of xanthine oxidase) in 5 mL of HBS for 30 min. After culture in MEM supplemented with 10% FBS for 5 days, the number of colonies was counted as described previously [7–9]. The survival (% of control) was calculated by dividing the number of colonies of the cells treated with flavonoids and/or active oxygen species by the number of colonies of the untreated control cells. The results are expressed as the means and standard deviations (SD) of four separately treated cultures. The data were analysed by Student's *t*-test, comparing the groups with active oxygen species and the groups treated with flavonoids prior to challenge with active oxygen species.

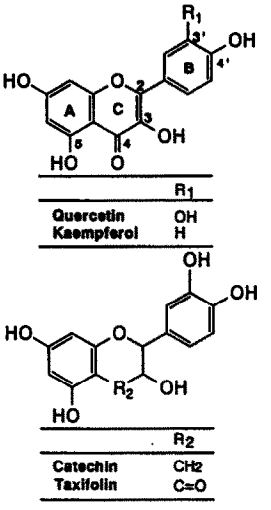

Fig. 1. Structures of flavonoids.

Results and Discussion

We first determined the dose dependence of the cytotoxic effects of the flavonoids themselves, whose structures are depicted in Fig. 1. The results in Fig. 2 indicate that quercetin and kaempferol were toxic at concentrations above 100 μM. On the other hand, catechin and taxifolin were not toxic at concentrations up to 200 and 1000 μM, respectively. We next investigated the protective effects of flavonoids against active oxygen-induced cytotoxicity at the

* Abbreviations: MEM, minimum essential medium; SOD, superoxide dismutase; FBS, fetal bovine serum; HBS, HEPES-buffered saline.

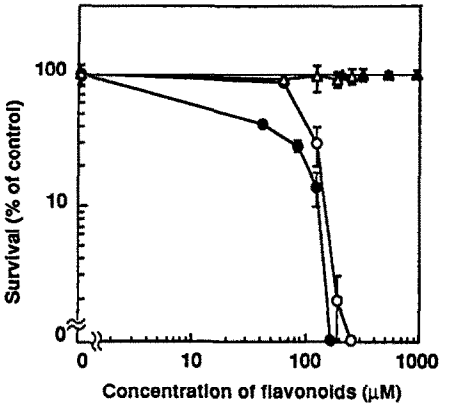

Fig. 2. Dose–effect curves of survival of the cells treated with the flavonoids. V79 cells were treated with each flavonoid in MEM (−FBS) at 37° for 4 hr. After culture in MEM (+FBS) for 5 days, the number of colonies was counted. (●) Quercetin, (○) kaempferol, (▲) catechin, (△) taxifolin.

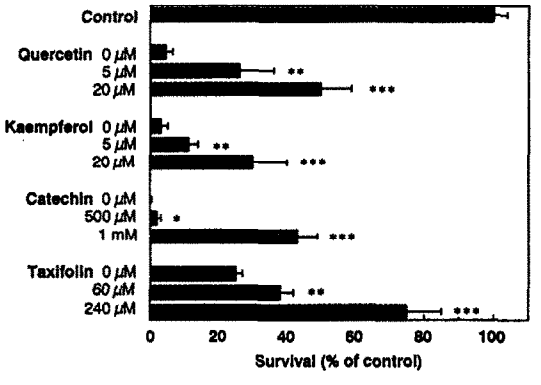

Fig. 3. Dose effects of the flavonoids on the cytotoxicity of H_2O_2. The cells were treated with each flavonoid for 4 hr. After being washed with HBS, the cells were treated with H_2O_2 (60 μM) in 5 mL of HBS for 30 min as described in Materials and Methods. After culture in MEM (+FBS) for 5 days, the number of colonies was counted. The data were analysed by Student's *t*-test, comparing the groups treated with and without each flavonoid prior to challenge with H_2O_2. Note the difference of the concentrations of each flavonoid. *P < 0.05, ** P < 0.01, ***P < 0.001.

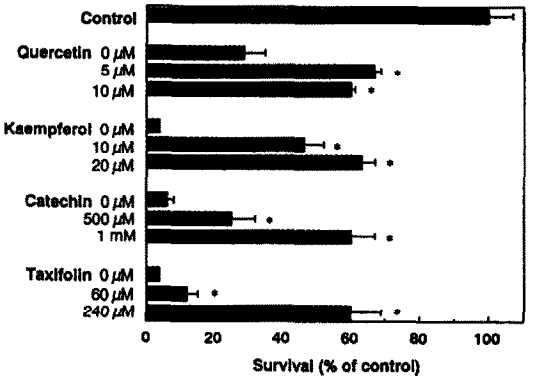

Fig. 4. Dose effects of the flavonoids on the cytotoxicity of O_2^-. The cells were treated with each flavonoid for 4 hr. After being washed with HBS, the cells were treated with 50 μM of hypoxanthine and 2.5×10^{-2} U of xanthine oxidase in 5 mL of HBS for 30 min. After culture in MEM (+FBS) for 5 days, the number of colonies was counted. The data were analysed by Student's *t*-test, comparing the groups treated with and without each flavonoid prior to challenge with the O_2^- generation system. Note the difference of the concentrations of each flavonoid. *P < 0.001.

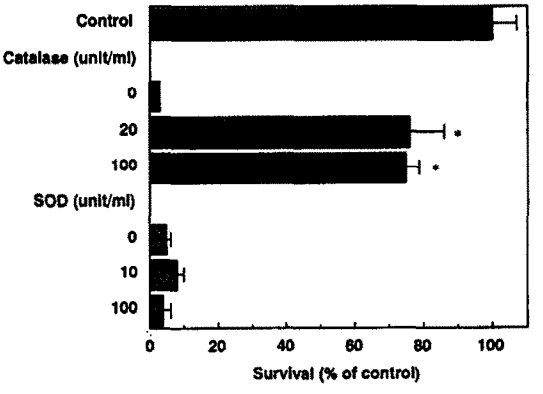

Fig. 5. Effects of catalase and SOD on the cytotoxicity of O_2^-. The cells were incubated in MEM (−FBS) for 4 hr. After the medium was changed to 5 mL of HBS, the cells were treated with 50 μM of hypoxanthine and 2.5×10^{-2} U of xanthine oxidase in the presence or absence of catalase or SOD. After culture in MEM (+FBS) for 5 days, the number of colonies was counted. The data were analysed by Student's *t*-test, comparing the groups challenged with the O_2^- generation system in the absence of the enzymes and those challenged with the O_2^- generation system in the presence of each enzyme.

concentrations so that each flavonoid was itself not toxic. As shown in Fig. 3, all flavonoids used in our experiments had protective effects against the cytotoxicity of H_2O_2. Quercetin and kaempferol showed protective effects at concentrations above 5 μM. On the other hand, much higher concentrations of catechin and taxifolin were necessary to prevent the cytotoxicity of H_2O_2. Under these conditions, 40 μL of ethanol used as a vehicle for flavonoids had neither enhancing nor inhibitory effects on the cytotoxicity of active oxygen species (data not shown). Similar dose dependence of the protective effects of these flavonoids was observed when the cells were challenged with O_2^- (Fig. 4). There was a very substantial difference in the dose dependence of the protective effects brought about by quercetin and kaempferol in contrast to catechin and taxifolin. This difference might be ascribed to their structures depicted in Fig. 1.

Since O_2^- is unstable in the medium and H_2O_2 is produced after rapid disproportionation of O_2^-, we investigated the effects of catalase and SOD on the cytotoxicity caused by

O_2^-. As shown in Fig. 5, catalase inhibited the cytotoxicity and SOD did not. These results similar to those previously reported [11, 12] indicate that H_2O_2 is more critical for the damage of the cells in the hypoxanthine–xanthine oxidase system, which produced O_2^- in the medium. Since it is difficult to maintain reproducibility of the dose dependence of cytotoxic effects of H_2O_2 and O_2^-, the results of each flavonoid in the two systems as shown in Figs 3 and 4 cannot be compared exactly. However, the similar tendency of the dose dependence in the two systems suggests that the mechanism of the protective effects of the flavonoids against the cytotoxicity of H_2O_2 and O_2^- added in the medium might be the same. Bors *et al.* [1] reviewed a capability of flavonoids to scavenge superoxide. They found that flavonoids scavenge O_2^- but only with very low rate constants. Therefore, there is little possibility that the flavonoids incorporated in the cell membranes react with O_2^- formed in the medium. Our results only show that the flavonoids are effective for preventing the cytotoxicity of O_2^- produced outside of the cells.

We have already suggested that the *o*-dihydroxy structure of polyphenols was essential for protection against H_2O_2-induced cytotoxicity [8], because antioxidants bearing only one phenolic OH, such as ferulic acid methyl ester and α-tocopherol, had no protective effects. The result that kaempferol also suppresses active oxygen-induced cytotoxicity seems an exception to the above role. Bors *et al.* [1] indicated that flavonoids bear three structural groups for radical scavenging potential and antioxidative potential: (a) the *o*-dihydroxy structure in the B ring; (b) the 2,3-double bond in conjugation with the 4-oxo function in the C ring; (c) the 3- and 5-hydroxyl groups with the 4-oxo function in the A and C rings. Since kaempferol has the (b) and (c) groups (Fig. 1), its preventive effects against the cytotoxicity of active oxygen species can be ascribed to these structural properties. On the other hand, the *o*-dihydroxy moiety in the B ring seems essential for the protective effects of catechin, which lack the (b) and (c) groups. Recently, Krishna *et al.* [13] have reported that 1,2-dihydroxybenzene-3,5-disulfonate (Tiron), a metal chelator bearing the *o*-dihydroxy structure, protects V79 cells against active oxygen-induced cytotoxicity. They suggested that Tiron protects the cells by either scavenging O_2^- or binding transition metal ions or both. Since flavonoids can also chelate transition metal ions by the (a) and (c) groups, there is a possibility that iron chelation accounts for the protection.

In conclusion, the flavonoids used in our experiments can be divided into two groups: (i) quercetin and kaempferol; (ii) catechin and taxifolin. Dose dependence and the main effective moiety in their structures for protection against the cytotoxicity of active oxygen species seem to be the same in each group. The effects of flavonoids on various parameters of cells treated with active oxygen species should be investigated further.

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