

MINI-REVIEW

Search for Naturally-occurring Antioxidative Chemopreventors on the Basis of the Involvement of Leukocyte-derived Reactive Oxygen Species in Carcinogenesis

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Abstract

Chemoprevention with food phytochemicals is currently regarded as one of the most attractive strategies for cancer control. We have been continuously working on the identification and characterization of chemopreventive phytochemicals extracted from a diverse range of edible plants. Recently, we have utilized a convenient assay, the tumor promoter-induced superoxide generation test, with differentiated HL-60 cells for primary screening, and performed chemical studies of antioxidative food phytochemicals. Here, we report our criteria for evaluation of new types of chemopreventors on the basis of the involvement of leukocyte-derived reactive oxygen in carcinogenesis.

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Key words: chemoprevention, phytochemicals, reactive oxygen species, antioxidant, inflammation, leukocyte

Introduction

A number of epidemiological surveys and animal experiments have demonstrated that ingestion of some "antioxidative" constituents occurring in vegetables and fruits may contribute to reduction of the incidence of cancer in humans (Bertram et al., 1987; Wattenberg, 1985). Antioxidants in biological systems can be classified into at least four groups on the basis of their mechanisms of action; 1) radical scavengers that react directly with reactive oxygen species (ROS), 2) inhibitors of ROS generating enzymes, for instance, active in expression of enzyme proteins and assembly of enzyme components, etc. 3) inhibitors of enzyme reactions including pseudo-substrates and modifiers of the active sites of enzyme, and 4) inducers of antioxidative enzymes. Radical scavengers have been considered as promising candidates as chemopreventors because they have been found to strongly inhibit oxidative reactions both *in vitro* and *in vivo*. Therefore, dietary radical scavengers such as α -tocopherol, ascorbic acid, β -carotene and simple phenolics have attracted a great deal of attention (Huang et al., 1992).

Among these dietary antioxidative phytochemicals, β -carotene, a major carotenoid occurring widely in green-

yellow vegetables and fruit, is one of the most extensively studied agents for chemoprevention on account of its low toxicity and cancer preventive effects demonstrated in various animal models and epidemiological surveys (Malone, 1991; Peto et al., 1981). To date, no beneficial effects of β -carotene in terms of chemoprevention in humans have been reported except in the Linxian Study (Blot et al., 1993). It is evident that β -carotene is a mere phytochemical not exclusively representing the cancer preventive potential of vegetables. Moreover, some radical scavengers exert not only weak anti-tumor promoting activity but also carcinogenic activity in rodents when given at high doses (Ito and Hirose, 1989; Weisburger, 1992). Hence, it is necessary to search for new types of chemopreventive agents that have antioxidative properties rather than acting as radical scavengers: i.e., inhibitors of ROS generation, by scrutinizing a diverse variety of edible plants and their components. ROS generating inhibitors, including both enzyme induction and enzyme activity inhibitors, should suppress oxidative stress and hence tumorigenesis in rodents more effectively than radical scavenging-type antioxidants, because the former can inhibit generation of some types of ROS at earlier stages and may not allow the subsequent

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Development of an *in vitro* assay for ROS generation

ROS have been suggested to play important roles in carcinogenesis (Perchellet et al., 1995). The close relationship between the generation of ROS including superoxide (O_2^-) by phagocytic cells in inflammatory processes and tumor promotion is generally accepted. Among the inflammatory cells, polymorphonuclear leukocytes (PMNs) are particularly adept at generating and releasing ROS, including O_2^- , hydrogen peroxide (H_2O_2), hypochloric acid (HOCl), singlet oxygen (1O_2) and hydroxyl radicals ($\bullet OH$) (Hurst and Barrette, 1989; Ramos et al., 1992; Steinbeck et al., 1992). The generation of O_2^- by PMNs is attributable to the activation of a plasma-membrane enzyme, NADPH oxidase. Utilization of O_2^- -derived H_2O_2 by myeloperoxidase (MPO) results in the formation of HOCl, further reaction of which with H_2O_2

generates 1O_2 . In addition, $\bullet OH$ has been demonstrated to be generated from the interaction of HOCl with O_2^- , and this can randomly react with biological components such as lipids or DNA bases intracellularly.

To search for inhibitors of ROS generation, we conducted the O_2^- generation assay in the differentiated human promyelocytic leukemia cell line HL-60. Since HL-60 cells can be differentiated by retinoic acid or dimethylsulfoxide into granulocyte-like cells expressing both MPO activity and the O_2^- generating NADPH oxidase system (Thompson et al., 1988), we utilized this cell line as a model of PMNs. Differentiated HL-60 cells can generate massive amounts of O_2^- in response to phorbol ester stimulation.

We preliminarily screened 20 species of edible plants from Japan and Thailand for inhibition of 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced O_2^- generation in differentiated HL-60 cells. Thai plants exhibited significantly higher potential in terms of cancer chemoprevention than common Japanese edible plants

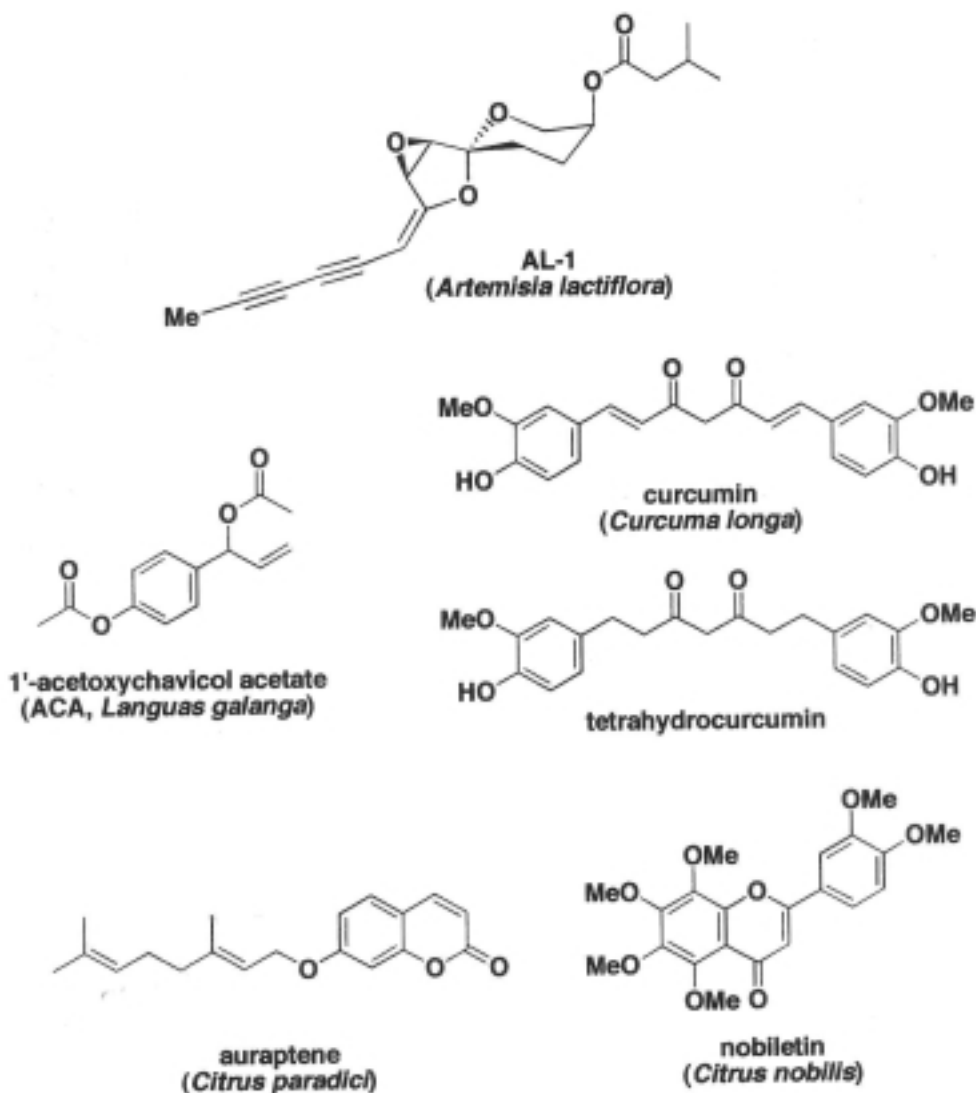


Fig. 1 Chemical structures of inhibitors of O_2^- generation from edible plants

(Murakami et al., 1998). Among these, the methanol extract of Thai mugwort (*Artemisia lactiflora*) showed the highest inhibitory activity. The active constituents, diacetylenic spiroketal enol ethers including AL-1 (Fig. 1), were then isolated and identified (Nakamura et al., 1998a). These compounds showed no O_2^- radical scavenging potential, suggesting that they are inhibitors of ROS generating enzyme activities. The acetylenic compounds are well-known to have various biological activities (for example; nematocidal, antibiotic, insect repellent) (Yano, 1983). Although there have been only a few studies of the medicinal or pharmacological activities of acetylenes, the finding that compounds carrying the acetylene structure possess potent inhibitory activity against ROS generation is important. Some phytochemicals from tropical gingers, including 1'-acetoxychavicol acetate (ACA) and curcuminoid, also potently inhibited O_2^- generation in differentiated HL-60 cells (Murakami et al., 1996; Nakamura et al., 1998b; 1998c). Interestingly, these O_2^- generation inhibitors have been reported to have chemopreventive effects against inflammation-related carcinogenesis such as colon and skin cancer in rodents (Huang et al., 1994; Murakami et al., 1996; Nakamura et al., 1999; Tanaka et al., 1997). In addition, we recently screened 31 citrus fruits (Murakami et al. 2000a), intake of which has been shown to act suppressively towards several types of cancer in epidemiological surveys (Steinmetz and Potter, 1991), for inhibition of O_2^- generation. The inhibitory activities of peel parts were largely found to be higher than those of the corresponding juice sac parts. In particular, the peel portion of Dancy tangerine (*Citrus tangerina*) showed the most marked inhibitory activity. *Citrus* fruits are widely known to contain a variety of chemopreventive agents, e.g. terpenoids including limonene, flavonoids such as hesperidin and glyceroglycolipids. Of citrus constituents, nobiletin, a polymethoxyflavonoid from *Citrus nobilis*, and auraptene, a coumarin derivative widely occurring in citrus juices (e.g. grapefruit), have been found to exhibit strong inhibitory

effects against O_2^- generation and suppress skin carcinogenesis (Murakami et al., 1997; 2000b). Further studies on the protective potentials of auraptene and nobiletin against chemical carcinogenesis and oxidative stress in several rodent models are currently underway.

***In vivo* ROS generation system (double TPA application model).**

ROS production by double or multiple TPA treatments is closely associated with the metabolic activation of proximate carcinogens (Kensler et al., 1987; Ji and Marnett, 1992) and increased levels of oxidized DNA bases (Wei and Frenkel, 1991; 1992; 1993), and thus skin tumor development. Therefore, we have developed a short-term assay for skin oxidative stress named the double TPA application model (Nakamura et al., 1998b; 1998c; 1999). Double application of phorbol esters was required to trigger production of ROS including H_2O_2 , lipid hydroperoxide and peroxy radicals in mouse skin. Inhibitory effects of the *in vitro* O_2^- generation inhibitors, AL-1, ACA, curcuminoids, and a soybean isoflavone genistein, which showed a much weaker inhibitory effect on O_2^- generation (Nakamura et al., 1998b), on double TPA treatment-induced H_2O_2 formation in mouse skin are summarized in Table 1. Each total inhibition of H_2O_2 formation was determined in mice pretreated with the test compound before each TPA treatment. The total inhibitory effects of AL-1, ACA and curcumin were significant (IE > 50%), while the weakly active derivative tetrahydrocurcumin (THC) showed a weaker inhibitory effect as compared with curcumin. In addition, genistein unexpectedly suppressed H_2O_2 formation.

The results of previous studies suggested that each application induces two distinguishable biochemical events, i.e. priming and activation (Ji and Marnett, 1992). The former is characterized by recruitment of inflammatory cells such as PMNs by chemotactic factors to inflammatory regions

Table 1 Inhibitory Effects of Chemopreventive Food Phytochemicals on TPA-induced H_2O_2 Production in Mouse Skin and O_2^- Generation in Cells

| Compound | H_2O_2 inhibition in mouse skin by pretreatment in | | | O_2^- inhibition in HL-60 |
|-----------|--|---------------|------------------|-----------------------------|
| | both phases | priming phase | activation phase | (IC ₅₀ , mM) |
| AL-1 | ++ | - | ++ | 7.6 |
| ACA | ++ | - | ++ | 4.3 |
| Genistein | ++ | + | - | 102 |
| Curcumin | ++ | + | + | 12 |
| THC | + | N.T.* | N.T.* | 29 |

Backs of female ICR mice (7 weeks old) were shaved with surgical clippers two days before each experiment. All test compounds (810 nmol/100 ml in acetone) were topically applied to the shaved area of dorsal skin 30 min before each or either application of TPA solution (8.1 nmol/100 ml in acetone). The second TPA treatment was performed 24 h after the first. Determination of H_2O_2 in the mouse epidermis was performed by the phenol red-horseradish peroxidase method [Nakamura et al., 1998b].

++: Inhibitory effect (IE) ≥ 50%, +: 50% > IE ≥ 20%, -: 20% > IE. *Not tested.

and edema formation. The latter is the process of activation of neutrophils or other oxidant-producing cells including keratinocytes, in which the second TPA application induces oxidative stress. To distinguish whether test compounds inhibit the priming or activation phase in the double TPA application model, experiments were conducted in such a way that the test compounds were coadministered with either the first (priming) or second (activation) application of TPA. Interestingly, the strong *in vitro* O₂⁻ generation inhibitors, but not genistein, exhibited significant suppression of the activation phase, but when applied only with first TPA treatment they did not show sufficient inhibitory effects (Table 1). A positive correlation was observed between inhibition of the activation phase *in vivo* and suppression of O₂⁻ generation *in vitro*. We previously found that a xanthine oxidase inhibitor, allopurinol, did not inhibit double TPA application-induced H₂O₂ formation in mouse skin (Nakamura et al., 1998b), suggesting that the NADPH oxidase system of neutrophils rather than the epithelial xanthine oxidase system is dominant for the O₂⁻ generating potential in double TPA-treated mouse skin. Thus, AL-1 and ACA may mainly and directly act against TPA-induced leukocyte activation to inhibit oxidative stress in mouse skin. Moreover, further biochemical and histological studies clearly demonstrated that ACA can effectively suppress TPA-induced lipid peroxidation, potentially occurring downstream of O₂⁻ generation, and inhibit cell proliferation and hyperplasia (Nakamura et al., 1998b). These results strongly suggested that leukocyte activation resulting in ROS generation coupled to excessive production of chemotactic factors may play an important role in chronic inflammation and hyperplasia in mouse skin. Conversely, O₂⁻ generation inhibitors are agents that effectively suppress chronic inflammation and thus tumor development.

In addition to the leukocyte inactivation agents, inhibition of leukocyte infiltration into inflammatory regions is also effective in oxidative stress control in mouse skin. Some flavonoids and curcuminoids are well-known to act not only as oxygen radical scavengers but also to be anti-inflammatory agents inhibiting arachidonic acid metabolism. In fact, genistein and curcumin significantly suppressed single TPA application-induced edema formation and leukocyte infiltration, while the activation phase-specific inhibitors (AL-1 and ACA) did not (Nakamura et al., 1998b; 1998c). It was thus suggested that genistein and curcumin may inhibit H₂O₂ formation via inhibition of chemotactic action, which regulates leukocyte infiltration.

Conclusions

The O₂⁻ generation test in differentiated HL-60 cells and the double TPA application model in mouse skin have been shown to be useful for the evaluation of the effectiveness of antioxidative agents. It should be noted that O₂⁻ generation inhibitors have been shown to exhibit chemopreventive activities against inflammation-related carcinogenesis in

rodent experiments. Regulation of leukocytes in inflammatory regions has thus been proved to be an effective strategy for oxidative stress control and cancer chemoprevention. Further identification and analyses of novel inhibitors from edible plants are currently in progress in our laboratory.

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