Res Exp Med (1998) 198: 93–99

Research in
Experimental Medicine

Inhibitory effect of Hesperidin on tumour initiation and promotion in mouse skin

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Received: 30 March 1998 / Accepted: 24 April 1998

Abstract. A flavonoid, Hesperidin was evaluated for its ability to inhibit tumour initiation by a polycyclic aromatic hydrocarbon and tumour promotion by a phorbol ester in the skin of CD-1 mice. Subcutaneous application of Hesperidin did not inhibit 7,12-dimethylbenz(a)anthracene-induced tumour initiation but did inhibit 12-O-tetradecanoyl-13-phorbol acetate-induced tumour promotion. Results provide evidence for a potential chemopreventive activity of Hesperidin.

Key words: Hesperidin – Skin tumorigenesis – Mice

Abbreviations: *DMBA* 7,12-dimethylbenz[a]anthracene – *TPA* 12-O-tetradecanoyl-13-phorbol acetate – *FOR* free oxygen radicals

Introduction

Flavonoids are universally distributed among vascular plants. They are ingested in a normal diet in average quantities of 1 g daily [1]. Accumulating evidence lends support to the theory that certain flavonoids may be inhibitors of carcinogenesis [2–4]. Thus, flavonoids were found to inhibit metabolism of carcinogens in vitro in isolated liver microsomes [5]. Furthermore, a number of hydroxylated flavonoids were found to inhibit the mutagenic activity of bay-region diolepoxides of benzo(a)pyrene [6]. Finally, certain flavonoids inhibited chemically induced tumours in experimental animal models both applied topically or in diet [2–4, 7].

In our previous work, we have shown that the extract of grapefruit, one of the citrus fruits, has some inhibitory effects on cancer cells in vivo [8]. The

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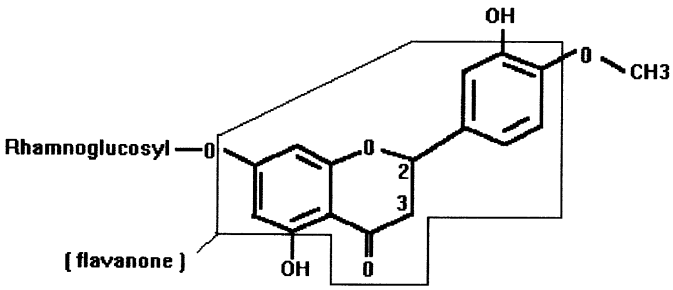

Fig. 1. Hesperidin

flavonoid Hesperidin (7-rhamnoglycosyl mw:610.6 Dalton) (Fig. 1) is found in the edible portion of the majority of citrus fruits, particularly in grapefruit (2–3%) [1].

We then demonstrated one striking feature of Hesperidin; that it plays an important role as a reversible electron transferring substance during ascorbic acid synthesis in nature [9].

It has been also reported that Hesperidin is an effective antioxidative and scavenger of free oxygen radicals (FOR) whose role is well known in carcinogenesis [10–15].

This study aimed to evaluate whether Hesperidin alone has any effect to inhibit experimental skin tumorigenesis. Data indicating that Hesperidin inhibits 7,12-dimethylbenz[a]anthracene (DMBA) and 12-O-tetradecanoyl-13-phorbol acetate (TPA) induced tumorigenesis is summarised in this paper.

Materials and methods

Chemicals

DMBA and TPA were obtained from Sigma Chemical (St. Louis, Mo.) and Hesperidin Phosphoric Acid Sodium Salt from Merck.

Animals and treatments

Female CD-1 mice were obtained from Charles Rivers Breeding Laboratories (Germany). Mice weighed between 24 and 26 g at the beginning of the experiment. They were brought to our animal facility at least 1 week before use. Mice were fed a standard chow diet and drinking water ad libitum. All mice were housed in polypropylene cages in a temperature-controlled room. They were kept on a 12-h light, 12-h dark cycle.

The dorsal region of each mouse was shaved with an electric clipper 2 days prior to experimental use and only those mice in the resting phase of the hair growth cycle were used.

DMBA and TPA were applied topically to the shaved area in 0.2 ml of acetone; control mice were treated with equal volumes of the appropriate vehicle alone. Hesperidin was dissolved in distilled water to prepare 1% (v/w) solutions, which was then injected into animals directly. Hesperidin solution was prepared as stocks, protected from the light and kept at 4°C during the experiment.

Mice were injected 125 µl of 1% hesperidin solution everyday subcutaneously at the dorsal skin. The injections either started 1 week prior to DMBA initiation and ended on in-

itiation day or started 1 day after initiation and continued until the end of the experiment. Hesperidin was injected into the animals 1 h before DMBA or TPA application.

The two-stage, DMBA-TPA-induced mouse skin tumorigenesis protocol was used as previously described [3, 16]. Briefly, skin tumours were initiated by application of 200 nmol of DMBA to the skin. Two weeks following initiation, 10 nmol of TPA was applied to the skin twice a week for the entire duration of the experiment. Mice were carefully checked for the presence of tumours once a week. Callipers measured the tumours and the number of tumours greater than 0.2 μm in size were counted and recorded weekly. The percentage of the mice with tumours in each group was calculated and recorded weekly as tumour incidence. All animals were also weighed each week to monitor their general health during the experiment.

Animals were divided into 4 groups of 20 mice. TPA was applied to the first and second groups twice a week for promotion, after initiation with DMBA in a single dose. Hesperidin was not injected in the first group. Hesperidin was injected daily only for 7 days in the second group before initiation, and DMBA was given on the 7th day after 1 h of Hesperidin injection. TPA was given twice a week for tumour promotion to the third group after tumour initiation with DMBA. Hesperidin was given daily to that group after 1 h of TPA application only during the promotion period. No chemical was given to the 4th group except daily Hesperidin injection for the entire period of the experiment.

At the end of the experiment all animals were killed. Tumours were dissected for histopathological examination. Macroscopic and microscopic evaluations were carried out after autopsy studies in order to observe any side effects.

Statistical evaluation was performed using the Kruskall-Wallis and χ^2 test.

Results

Application of 125 μl of 1% Hesperidin 1 h prior to each bi-weekly application of TPA to mice resulted in substantial tumour inhibition. Tumour incidence was reduced by 50% and the number of papillomas per mouse by 48% after 20 weeks promotion (Fig. 2). Hesperidin significantly inhibited the promotion of visible skin tumours greater than 2 μm in diameter throughout the period from the 12th to the 20th week, with weekly TPA applications. The number of tumours per mouse at week 20 decreased significantly ($P<0.01$, χ^2: 13.1, Kruskall-Wallis test; Fig. 3). Furthermore, those animals receiving Hesperidin during the promotion stage of the tumorigenesis study had a significantly lower percentage of tumours than the control group during the period from the 12th to the 20th week (χ^2 test, $P<0.01$–0.001; Fig. 2) of the experiment.

By contrast, 7 days treatment with the same dose of Hesperidin prior to the single initiating dose of DMBA yielded no significant effect on tumour incidence and tumour multiplicity.

There was no tumour development in group 4. The results of group 4 are excluded from the figures and statistical evaluations.

The application of Hesperidin to the experimental groups did not cause any significant difference in the period between DMBA application and appearance of visible skin tumours.

There was no abnormal finding in autopsy studies and no significant weight loss of mice, during the experiment, indicating that Hesperidin did not have any toxic side effects on the animals.

All tumours were diagnosed as papillomas in histopathological studies.

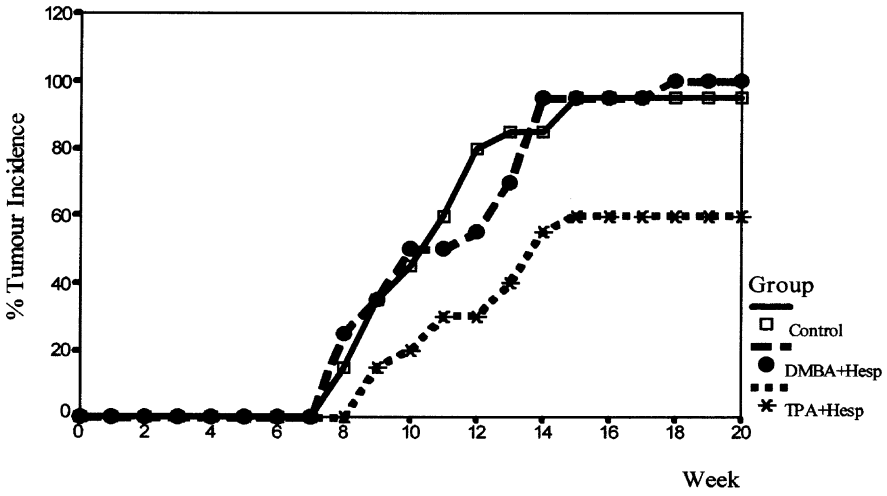

Fig. 2. Tumour incidences of the groups. (*DMBA* 7,12-dimethylbenz[a]anthracene, *Hesp* Hesperidin, *TPA* 12-O-tetradecanoyl-13-phorbol acetate) * $P<0.01$, ** $P<0.001$. Tumour incidence is significantly different for TPA+Hesp group at 12th–20th weeks (χ^2 test)

Discussion

Incomplete reduction of the molecular oxygen by a single electron causes the production of FOR [17].

Recently it has been demonstrated that FOR play a role in cytotoxicity and carcinogenesis as promoter and initiator. They are known to cause mutations, sister chromatid exchanges, and chromosomal deletions [17–19].

It has been found that tumour cells have only a decreased amount of mitochondrial super oxide dismutase, which is an endogen FOR scavenger enzyme [20–21].

Tumour cell biochemistry can be effected positively by the inhibition of promotion, if radical scavenger agents are administered exogenously [21–23].

Some flavonoids have been reported to possess biological properties, e.g. antitumour, antibacterial, antiviral, antimutagenic, and antioxidative properties. Some of these properties derive from the free radical scavenging activities of flavonoids. There are many reports relating to the reactivities of flavonoids with active oxygen species. Furthermore, we could find few reports concerning the relation between antioxidative ability on lipid peroxidation and OH^- scavenging effect of flavonoid in spite of general recognition that lipid peroxidation is initiated by OH^-. A flavonoid Hesperidin has also been reported to have preventive effects on skin inflammation by the way of both scavenging FOR and initiating pre-inflammatory mediators such as prostaglandin, which induce neutrophil chemotaxis [10–15, 24].

We concluded that a flavonoid Hesperidin inhibited the tumour promotion induced by TPA, but did not inhibit the initiation induced by DMBA in CD-1 mouse skin carcinogenesis. The chemopreventive activity may probably be related to the FOR scavenging properties of Hesperidin.

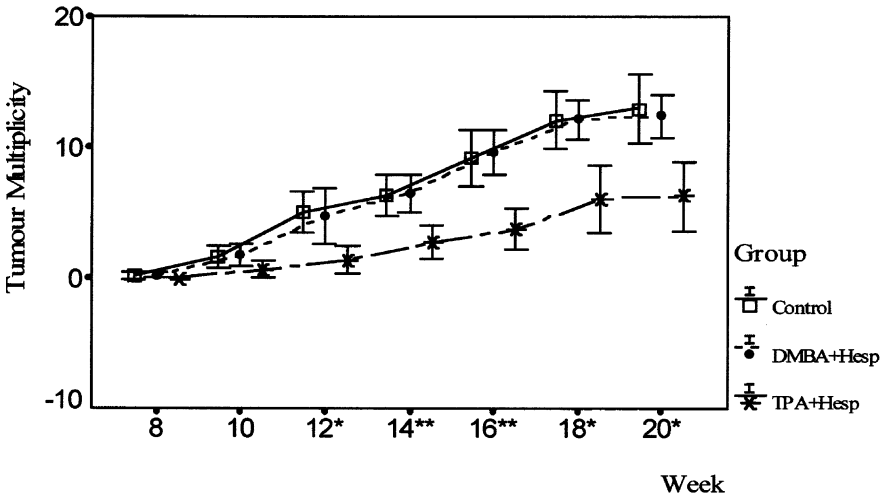

Fig. 3. Mean tumour number of the groups. * $P<0.01$, ** $P<0.0001$. The number of tumours per mouse is significantly different for TPA+Hesp group at 12th, 14th, 16th, 18th and 20th weeks (Kruskal-Wallis test)

Another possible explanation for the effect of Hesperidin may involve the electron transfer reactions in aerobic respiratory systems.

Any degeneracy in the electron transfer chain that supplies the aerobic system in the inner membrane of mitochondria of a cell causes the failure of electron transfer to oxygen. In this way, the cell may be induced to supply mostly anaerobic energy, which may cause mutation in the DNA of the mitochondria and in the nucleus. It is also known that the electron chain is either broken or damaged in most of the cancer cells and the cells turn to a glycolysis pathway from aerobic respiration when transformed into malignant phenotype [25].

It has been shown that Hesperidin plays a role in the synthesis of ascorbic acid in nature due to its capacity as a reversible electron transferring substance [9]. It has been found that the specific potential energy of Hesperidin is E°: 0.715-volt [26]. We hypothesised that the defect occurring in the electron chain flow in the mitochondria of the cancer cell may be compensated by Hesperidin.

In conclusion, the properties of scavenging FOR produced by the tumour induction agents and the restorative effect in the damaged electron transfer chain due to photocatalysis, may be the possible mechanisms resulting in the chemopreventive effect of Hesperidin in carcinogenesis. It appears that further research is required to determine how Hesperidin interferes with radicals and thereby prevents tumour development or tumour promotion.

Acknowledgment. We wish to thank Dr. Canan Alatlı from the Institute of Oncology of Istanbul University for performing histopathological studies.

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