Anti-carcinogenic Activity of Taraxacum Plant. I

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An extract of the roots of Taraxacum japonicum (Compositae) exhibited strong anti-tumor-promoting activities on the two-stage carcinogenesis of mouse skin tumor induced by dimethylbenz[a]anthracene (DMBA) as an initiator and 12-O-tetradecanoyl-phorbol-13-acetate (TPA) as a promoter, as well as on that induced by DMBA and fumonisin B1. Further, the extract exhibited anti-tumor-initiating activity on the two-stage carcinogenesis of mouse skin tumor induced by (±)-(E)-methyl-2-[(E)-hydroxyimino]-5-nitro-6-methoxy-3-hexenamide (NOR-1) as an initiator and TPA as a promoter. These results suggested that an extract of the roots of the Taraxacum plant could be a valuable chemopreventive agent against chemical carcinogenesis.

Key words anti-carcinogenic activity; Taraxacum japonicum; anti-tumor-promoter; anti-tumor-initiator; (±)-(E)-methyl-2-[(E)-hydroxyimino]-5-nitro-6-methoxy-3-hexenamide (NOR-1); fumonisin B1

In the search for potent anti-carcinogenic agents from natural resources, we have researched the anti-tumor-promoting activities of many natural products (flavonoids,1 diterpenoids,2 triterpenoids,3,4 euglobals,3 natural pigments,9 crude drugs7 and kampo prescription8) by employing a two-stage carcinogenesis test on mouse skin using 7,12-dimethylbenz[a]anthracene (DMBA) and 12-O-tetradecanoylphorbol-13-acetate (TPA). Furthermore, it has been reported that some natural products which exhibited anti-tumor-promoting activities on mouse skin carcinogenesis have also exhibited inhibitory effects on mouse pulmonary and hepatic tumors.3–8

On the other hand, many species and subspecies of Taraxacum plants are widely distributed in Japan, and the roots of these plants (T. platycarpum, T. japonicum, etc. Japanese name: Hokoei-kon) have been used as bitter stomachic, diuretic, lactation, anti-mastopathy and anti-inflammatory medicines as folk medicine in China and Japan.9,10 While the leaves of dandelion (T. officinale) have been regarded as a vegetable in Europe, many triterpenoids have been isolated from these leaves and their chemical structures have been reported.11

In the course of our continuing biological study on anti-carcinogenic agents, we investigated in vitro primary screening using the synergistic assay indicated by the inhibitory effect on the induction of Epstein-Barr virus early antigen (EBV-EA), and looked at the anti-tumor-promoting and -initiating activities of Taraxacum japonicum on mouse skin carcinogenesis (induced not only by DMBA/TPA, but also DMBA/fumonisin B1 and (±)-(E)-methyl-2-[(E)-hydroxyimino]-5-nitro-6-methoxy-3-hexenamide (NOR-1)/TPA) 

Fumonisin B1, one of the mycotoxins produced by Fusarium moniliforme, is a common mold associated with corn, has been determined to be a new and non-TPA type tumor-promoter.12,13 This mycotoxin exhibited strong promoting activity in an in vivo carcinogenesis test initiated by DMBA, although, in vitro, it did not show any EBV-EA activation, induction of ornithine decarboxylase activity, protein kinase C activity or enhancement of phospholipid synthesis, unlike TPA. The inhibitory effect of the H2O extract of the Taraxacum plant on two-stage carcinogenesis promoted by fumonisin B1 was also investigated. Further, NOR-1 is known as a stable NO donor and as an initiator of two-stage carcinogenesis of mouse skin papillomas promoted by TPA.13 We also investigated the inhibitory effect of the Taraxacum plant on the two-stage carcinogenesis initiated by an NO donor, NOR-1.

In this paper, the results of an in vitro primary screening test and in vivo two-stage carcinogenesis test on mouse skin tumors are reported.

MATERIALS AND METHODS

Plant Materials The crude drug of the root (Hokoei-kon) was purchased from Kinokuniya, Tokyo, Japan, and identified as Taraxacum japonicum by anatomic analysis. A voucher specimen was deposited in the Herbarium of Showa College of Pharmaceutical Sciences, Machida, Tokyo, Japan.

Preparation of Extracts The dried root of Hokoei (600 g) was extracted with MeOH (3 l) three times for 5 h each, then the MeOH solution was evaporated to dryness to afford 109 g of the MeOH extract. Another Hokoei root (60 g) was extracted with water (0.38 l) for 1 h. The water was lyophilized to give 33.6 g of H2O extract.

Cells EBV genome-carrying lymphoblastoid cells (Raji cells derived from Burkitt's lymphoma) were cultured in RPMI-1640 medium (Nissui) under previously described conditions.4 Spontaneous activation of EBV-EA in our subline Raji cells was less than 0.1%.

Animals Specific pathogen-free female ICR (6 weeks old; for DMBA/TPA induced carcinogenesis test) and Senarc (6 weeks old; for DMBA/fumonisin B1 and NOR-1/TPA induced carcinogenesis test) mice were obtained from Japan SLC, Inc. (Hamamatsu, Japan). These animals were housed 5 per polycarbonate cage in a temperature-controlled room at 24±2°C and given food and water ad libitum.

Chemicals The cell culture reagents, n-butyr acid and other reagents were purchased from Nacalai Tesque, Inc. (Kyoto, Japan). TPA and DMBA were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Fumonisin B1 was a gift from Prof. R. F. Vesonder of the National Center for Agricultural Utilization Research, Agricultural Research Ser-

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vice, USDA (Illinois, U.S.A.), and NOR-1 was purchased from Dojin Chemical (Kumamoto, Japan). EBV-EA positive serum, from a patient with a nasopharyngeal carcinoma (NPC), used for an immunofluorescence test, was a gift from Prof. H. Hattori, the Department of Otorhinolaryngology, Kobe University.

**In Vitro EBV-EA Activation Experiment** The Raji cells were incubated for 48 h at 37°C in a medium containing butyric acid (4 mmol), TPA (32 pmol) and various amounts of the test compounds. Smears were made from the cell suspension, and the EBV-EA inducing cells were stained by means of an indirect immunofluorescence technique. Details of the in vitro assay on EBV-EA activation have been reported previously. In each assay, at least 500 cells were counted and the number of stained cells (positive cells) among them was recorded. Triplicate assays were performed for each data point.

**In Vivo Two-Stage Carcinogenesis Test on Mouse Skin Papillomas Promoted by TPA** The animals were divided into three experimental groups of 15 mice each. The back of each mouse was shaved with surgical clippers, and the mice were topically treated with DMBA (100 μg, 390 nmol) in acetone (0.1 ml) as an initiation treatment. One week after this initiation, papilloma formation was promoted twice a week by the application of TPA (1 μg, 1.7 nmol) in acetone (0.1 ml) to the skin. Group I received this TPA treatment alone, and groups II and III received a topical application of the MeOH and H₂O extracts of the roots of the *Taraxacum* plant (50 μg) in acetone (0.1 ml) 1 h before each TPA treatment, respectively. The incidence and numbers of papillomas were monitored weekly for 20 weeks, as described previously.

**In Vivo Two-Stage Carcinogenesis Test on Mouse Skin Papillomas Promoted by Fumonisin B₁** The animals were divided into two experimental groups of 15 mice each. Initiation with DMBA was carried out by the same method described above. One week after initiation, papilloma formation was promoted twice a week by the application of fumonisin B₁ (36 μg) in acetone (0.1 ml) to the skin. Group I received this fumonisin B₁ treatment alone, and group II received a topical application of the *H₂O* extracts of the roots of the *Taraxacum* plant (360 μg) in acetone (0.1 ml) 1 h before each promotion treatment. The incidence and numbers of papillomas were monitored weekly for 20 weeks.

**In Vivo Two-Stage Carcinogenesis Test on Mouse Skin Papillomas Initiated by NOR-1** The animals were divided into two experimental groups, 15 mice each. The back of each mouse was shaved with surgical clippers, and the mice were treated topically with NOR-1 (90 μg) in acetone (0.1 ml) as an initiation treatment. Group I received a topical application of the *H₂O* extracts from the roots of the *Taraxacum* plant (900 μg) in acetone (0.1 ml) 10 min after initiation treatment. On both groups I and II, one week after initiation, papilloma formation was promoted twice a week by the application of TPA (1 μg) in acetone (0.1 ml) to the skin. The incidence and numbers of papillomas were monitored weekly for 20 weeks.

**RESULTS AND DISCUSSION**

As shown in Table 1, the MeOH extract of the roots of *T. japonicum* exhibited inhibitory effects (100% and 45% inhibition of activation at the concentration of 100 μg and 10 μg/ml, respectively) on the EBV-EA activation induced by TPA without cytotoxicity on Raji cells, even at the highest concentration (more than 60% viability at 100 μg/ml). Also, the *H₂O* extract of this crude drug showed the inhibitory effects similar (more than 80% and 35% inhibition) to the MeOH extract.

The inhibitory effects of MeOH and *H₂O* extract of the roots of *T. japonicum* on two-stage carcinogenesis of mouse skin papillomas induced by combinations of two different types of initiators, DMBA and NOR-1, and two different types of promoters, TPA and fumonisin B₁, were investigated. The incidence (%) of papilloma bearing mice and the average number of papillomas per mouse are presented in Figs. A and B, respectively.

As shown in Figs. 1A and B, the incidence of papillomas in the positive control group, treated with DMBA as an initiator and TPA as a promoter, was highly significant in 100% of the mice after 9 weeks of promotion. Further, more than 4, 8 and 9 papillomas were formed per mouse after 10, 15 and 20 weeks of promotion, respectively. When MeOH and *H₂O* extracts were applied before each TPA treatment, they delayed the formation of papillomas as follows. In both groups, 40 and 73.3% of mice bore papillomas at 10 and 15 weeks of promotion, respectively. Also, less than 2.1, 4.2 and 5.8 papillomas were formed per mouse after 10, 15 and 20 weeks of promotion, respectively. From these results, the extract of the *Taraxacum* plant might be valuable as an anti-tumor-promoter in the chemical carcinogenesis induced by TPA. As the roots of *T. japonicum* would be usually taken in the form of a water decoction, other two-stage carcinogenesis tests were carried out using the *H₂O* extract.

The inhibitory effect of the *H₂O* extract from *T. japonicum* on two-stage carcinogenesis promoted by fumonisin B₁, which has been known as a non-TPA type promoter, was examined, as shown in Figs. 2A and B. In the positive control group, 46.6 and 80% of the mice bore papillomas even at 8 and 9 weeks of promotion, respectively, and all of the mice bore papillomas after 11 weeks of promotion. Further, more than 5 and 8 papillomas were formed per mouse after 15 and 20 weeks of promotion, respectively. When the *H₂O* extract was applied before each fumonisin B₁ treatment, the papilloma formation was significantly delayed (only 20, 40 and 60% of mice bore papillomas after 10, 12 and 16 weeks of promotion, respectively, and 80% of mice bore papillomas even after 20 weeks of promotion), and the number of papillomas per mouse was reduced (only 3.6 and 5.2 papillomas

<table>
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<tr>
<th>Sample</th>
<th>Concentration (μg/ml)</th>
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<tr>
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<td>100</td>
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<tr>
<td>MeOH extract</td>
<td>0.0±0.4* (70%)</td>
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<tr>
<td>H₂O extract</td>
<td>18.7±1.0 (60)</td>
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a) TPA was 20 ng (32 pmol)/ml. b) Values represent relative percentages to the positive control value (100%) (n=3, and ±S.D.). c) Values in parentheses are viability percentages of Raji cells.
were formed per mouse after 15 and 20 weeks of promotion, respectively) compared with the positive control. Therefore, this H₂O extract also apparently inhibits the tumor-promotion induced by the non-TPA type promoter, fumonisin B₁.

One of the stable NO donors, NOR-1, is well known as an initiator of two-stage carcinogenesis of mouse skin papillomas promoted by TPA. The inhibitory effect of the roots of *T. japonicum* on the initiation of NOR-1 was examined and the results are shown in Figs. 3A and B. In the positive control group, 46.6 and 80% of mice bore papillomas after 8 and 10 weeks, and all of the mice bore papillomas after 11 weeks of promotion. Also, 4.6, 6.3 and 7.6 papillomas were formed per mouse after 10, 15 and 20 weeks of promotion. When the H₂O extract was only applied once, at 10 min after initiation treatment by NOR-1, 33.3 and 66.6% of mice bore papillomas even after 11 and 16 weeks of promotion, respectively. Further, this extract strongly reduced the number of papillomas per mouse (only 1.2, 2.7 and 3.9 papillomas were formed per mouse after 10, 15 and 20 weeks of promotion) compared with the positive control group. Therefore, these results strongly suggested that the initiation stage was also inhibited by the extract of *T. japonicum*.

From these results of the two-stage carcinogenesis test, it was concluded that the root of *T. japonicum* inhibited both the initiation and promotion stages on two-stage carcinogenesis and may therefore be valuable as a chemopreventive agent in the treatment of chemical carcinogenesis. It was deduced that the plural constituents of this extract acted on different stages such as initiation or promotion. In studying the combined effects of these constituents, the chemopreventive effects of this extract would be revealed. Detailed investigation of the inhibitory mechanism of *Taraxacum* plants on chemical carcinogenesis is now in progress.

Isolation of the active constituents of these extracts and
their anti-carcinogenic activity on mammary cancer are also being investigated.

REFERENCES AND NOTES

15) A high viability of Raji cells is necessary for in vitro assay using an indirect immunofluorescence technique by antigen-antibody reaction and is beneficial for the following in vivo assay.