Effects of propolis on 7,12-dimethylbenz (a)-anthracene-induced skin tumors and on life-span of tumor-bearing mice

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Abstract

We examined the effects of "propolis", which is a resinous material gathered by honey bees from buds and bark, on benign skin tumor papillomas induced by an application of 7,12-dimethylbenz (a) anthracene (DMBA) in mice. Propolis was given to mice as an ointment by skin-application or an oral drug by gastric-tube feeding. Activities of thymidylate synthetase and thymidine kinase, enzymes in *de novo* and salvage pathways for pyrimidine nucletide synthesis, respectively, were suppressed in papillomas in mice given oral administration of propolis, followed by prolongation of the life-span of the mice, compared with the control.

Key words Propolis, mice, papilloma, life-span, DNA-synthesizing enzymes.

Abbreviations DMBA, 7,12-dimethylbenz(a)anthracene ; TK, thymidine kinase ; TS, thymidylate synthetase.

Introduction

Propolis is a resinous material gathered by honey bees from the buds and bark of certain trees and plants, and used inside their hives.[1-3] The name of "propolis" comes from the Greek "pro", in front of, and "polis" meaning town or city, and bees use it to fill and seal cracks and crevices of their hives against invasion of other insects and the weather. When an intruder is too large to move, *e.g.*, a large moth or a mouse, it is first killed and encased into propolis, resulting in mummification without rotting. In prehistoric times, surgery was made possible by the use of propolis as an antiseptic. It was obtained from wild bee nests and used with honey as a dressing for wounds and leg ulcers, and throat lozenges, soaps and cosmetics. As recently as the Boer War, roughly a century ago, an ointment of propolis and vaseline was used to treat battle wounds. Essentially propolis is a natural resin with a pleasant aromatic smell, and usually composed of about 55% balsams and resins, 30% waxes, 10% oils and 5% pollen. Characteristic components of propolis are many kinds of flavonoid aglycones, *i.e.*, flavones, flavanones and the related compounds such as phenolic acids, some of which have been known to show certain biological activities ; *e.g.*, antibacterial, antiviral, antifungal, anti-inflammatory, anti-ulceratious, cicatrizing, analgesic, narcotic, antispastic and antitumor effects.[1-4] In the present study, we have investigated the effects of propolis on skin-tumorigenesis and development of skin-tumors induced by repeated application of 7,12 - dimethylbenz(a)anthracene (DMBA) in mouse back skin.

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Materials and Methods

Animals and treatments : ICR male mice (Sankyo Laboratory Service Co. Ltd., Tokyo, Japan) were used in this work. All animals were kept in plastic cages, 5 each, in an animal room, that was air-conditioned (21-22 °C and 50-60% relative humidity) and lighted (14 hours of light from 5 : 00 to 19 : 00 h). Laboratory standard diet (CE-2 : CLEA Japan Co., Ltd., Tokyo, Japan) and tap water were provided *ad libitum*. At 7 weeks of age, the fur was shaved from the test area of the mice with electric clippers and a depilatory cream for ladies. Beginning at 8 weeks of age, 100 μl of a 0.3 per cent solution of DMBA (Sigma Chemical Co., St. Louis, U.S.A.) in acetone was applied to the mid-dorsal region of each mouse twice a week for 10 weeks. Each mouse was weighed weekly and inspected for incidence and growth of tumors.

Crude drugs : The propolis used in the present study was the crude extract in two forms : Forms I and II. Form I was prepared by an ethanol extraction. A hundred volumes of 98 % ethanol was added to the propolis and vigorously shaken. Subsequently ethanol was removed by rotary evaporation, and the residual dark brown extract was dissolved in small quantities of dimethylsulfoxide followed by dilution with glycerol. The propolis constituents were contained around 2.0% in the extract, which was prepared for a direct application to the mouse skin. Form II extract was a gift from Nihon Propolis Co., Ltd., Tokyo, Japan ; micro-capsulated propolis extract was prepared by micelle method of extraction using self-catalytic reaction. It is easily digested and absorbed in the intestine, and has the advantage being rapidly contenteffective without minimum waste. The detailed method of extraction is based on Japanese patent No. 1775886. The propolis content in the extract was approximately 5 %. This extract was diluted with 9 volumes of glycerol, *i.e.* 0.5 % emulsion of propolis, and prepared for an oral administration into mice using a gastric tube.

Experimental schedule : Biginning at 8 weeks of age, the animals were divided into 3 groups of 15 mice each. Two experimental groups were given the skin-application of 2.0% emulsion of propolis (Form I) and the oral administration of 0.5% emulsion of propolis (Form II), respectively, 4 times a week for 10 weeks between 8 and 18 weeks of age, in combination with the DMBA application. Control group was given both the skin-application and oral administration of glycerol alone by the same procedure as that of the experimental group. Beginning at 25 weeks of age, the animals prepared by DMBA application were divided into 3 groups of 12 mice each. Control and two experimental groups were treated with glycerol, Forms I & II, respectively, by the same procedure as that of the former experiment, for 6 weeks between 25 and 31 weeks of age. All animals were killed by cervical dislocation under light ether anesthesia at the end of each experiment, followed by a removal of skin tumors.

Enzyme preparation and assay : All specimens of tumors removed and stored in each mouse were pulverized with an autopulverizer under liquid nitrogen and homogenized with 10 volumes of 5 mM Tris-HCl buffer, pH 7.5, containing 0.1 mM EDTA, 1mM mercaptoethanol and 0.25 M sucrose, at final concentration, at 0°C .The homogenate was centrifuged for 1 hour at 4°C at 105,000×g, and the supernatant was used as the crude enzyme preparation.

As previously reported,[5, 6] activities of thymidylate synthetase (TS) and thymidine kinase (TK) were determined by the methods of Dunlap *et al.*[7] and Taylor *et al.*,[8] respectively. Enzyme activity was normalized to tissue protein content and expressed as fmol/mg protein/minute. Values were means of duplicate assays.

Statistical analyses : The statistical significance of difference among groups was evaluated by Student's *t*-test or Duncan's new multiple range test ; $p < 0.05$ was considered significant.

Results

No significant difference in body growth was observed among groups between 8 and 31 weeks of age ; however, the mice to which propolis (Form I) was applied were livelier than untreated controls with a finer fur and less ulcer and wound given by biting and / or scratching on the clipped skin (data not shown).

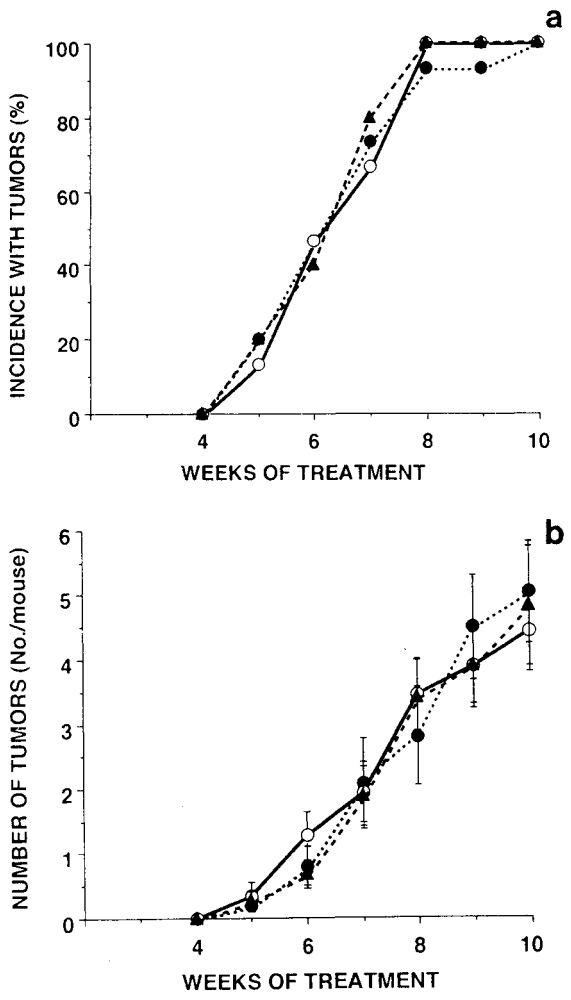

Fig. 1 Incidence (a) and number (b) of squamous cell papilloma induced by DMBA application in each group (mean±SEM).
···●··· ; control group without propolis treatment,
—○— ; experimental group applied propolis (Form I),
---▲--- ; experimental group given oral administration of propolis (From II) by gastric tube.

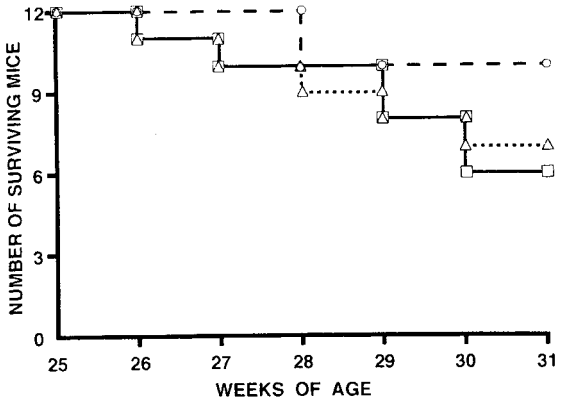

Fig. 2 Survival times of mice with squamous cell papillomas between 25 and 31 weeks of age.
□——□ ; control group without propolis treatment,
△----△ ; experimental group applied propolis (Form I),
○----○ ; experimental group given oral administration of propolis (Form II) by gastric tube.
Significance was calculated by Duncan's new multiple range test.

Table I TS and TK activities in papillomas per group at 31 weeks of age.

Groups	(n)	Enzyme activity (fmol/mg protein/minute) TS	TK
Control	(6)	443.7±32.5	8.26±1.74
Form I	(7)	373.4±20.0	6.27±0.19
Form II	(10)	320.1±22.6**	4.49±0.48

mean±SEM n, number of mice ; Form I, mice applied propolis ; Form II, mice given propolis by gastric tube ; TS, thymidylate synthetase ; TK, thymidine kinase.
**Significantly different from the control at $p<0.01$ by Student's t-test.

All skin tumors induced by DMBA application were histologically squamous cell papillomas. The mice of each group developed papillomas rapidly beginning 5 weeks after the initial application of DMBA, and tumor incidence peaked the 8 th week, *i. e.* nearly 100% of mice (15/15) had papillomas at 16 weeks of age (Figure 1a). The average number and region of papillomas were approximately 5 per mouse and 60 mm² per mouse (data not shown), respectively, at 18 weeks of age ; there was little difference among groups (Figure 1b). The life-span of mice with papillomas induced with DMBA was significantly prolonged by oral administration of propolis (Form II) ($p<0.05$ by Duncan's new multiple range test) (Figure 2). Survivors in the group given Form II propolis were more (83%) than that of the control (50 %) at 31 weeks of age.

There was little difference in number and volume of papillomas among groups for 6 weeks between 25 and 31 weeks of age (data not shown). TS activity in the group given oral administration of propolis (Form II) was markedly suppressed to 72.1% of that in the control ($p < 0.01$ by Student's t-test) (Table I). Although the difference was not statistically signifi-

cant, TK activity in the propolis-oral administration group (Form II) was reduced to 54.4 % of that in the control.

Discussion

An application of DMBA to mouse skin increases the incidence and the development of skin tumors[9, 10]; *i.e.* papilloma, squamous cell carcinoma and subcutaneous sarcoma.

Skin-application or oral administration of propolis, on the bases of the present data, did not affect the incidence and development of papillomas which were histologically benign tumors with poor dysplasia but were biologically suspect for preneoplastic transformation, *i.e.* there was little difference in the incidence, the multiplicity and the growth of papillomas. The activities of tissue TS and TK, key enzymes in the *de novo* and salvage pathways for pyrimidine nucleotide synthesis, respectively was markedly reduced in the tumors in mice given oral administration of propolis (Form II), followed by the prolongation of life-span in this group compared with the control. The mice applied with propolis (Form I) were all lively with a fine fur, less ulcer and wound given by biting or scratching on the clipped skin. These findings suggest that propolis has antibacterial, anti-inflammatory and anti-ulcerative activities.

In conclusion, our data suggests that propolis has an anti-inflammatory activity on the skin and a prolongation activity on the life-span in skin tumor-bearing mice. Further study is required concerning propolis-extraction method, extract-administration method and dose in the near future.

和文抄録

プロポリスはミツバチが種々の花や樹皮から集めてきた樹脂状物質をその唾液とともに練り合わせた粘着性で複雑な組成のミツバチ生産物の一つである。化学発癌剤で誘発したマウス皮膚乳頭腫に与えるプロポリスの影響を検討した。プロポリスの経口投与により，マウス皮膚乳頭腫では，DNA 合成が有意に抑制され，皮膚乳頭腫保有マウスの生存日数を延長した。また，プロポリスを連日塗布したマウスでは，行動が活発で，皮膚創傷が少なく，良好な毛並みを有することが観察された。

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