3-1) Usefulness of Medium-Term Bioassay Determining Formations of
Pulmonary Adenoma in NIH(GP) Mice for Finding
Anticarcinogenic Agents from Natural Products

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Summary

Various long-term bioassay methods have been used to determine the carcinogenicity of chemical substances. Among them, a long-term method developed by Toth (1968), which scores the incidence of pulmonary adenoma formation at 28 to 56 weeks following subcutaneous administration of chemical carcinogens to new-born mice is used widely. This particular long-term bioassay method, while it takes long periods, has proved useful in determining the anticarcinogenic effect of ginseng extracts against the pulmonary adenoma formation inducible with dimethylbenzanthracene, urethane, and aflatoxin B1. More recently, in order to shorten the assay durations to 9 weeks (medium-term), we have modified the existing method by adjusting the doses of carcinogens to be administered to the new-born mice. We have established a modified method in which 40-50% of mice were found to develop pulmonary adenoma 9 weeks after a subcutaneous injection of 0.5 mg/kg to new-born mice of NIH(GP) strain and this modified medium-term bioassay system was found useful in the screening of cancer preventive agents among natural products, such as ginseng and caffeine.

Introduction:

Since the mid 1970s, it was learned that the incidence of cancers could be reduced either by eliminating the carcinogens from environment or by modifying the metabolism of cancer initiators with inducers of drug metabolism (1). This method of reducing tumor incidence is called chemoprevention and cancer prevention has attracted great interest of cancer researchers (2). Studies of cancer

prevention is divided into various stages and the primary prevention can be divided into five categories: 1) identification of cancer-producing agents and their removal from environment, 2) modulation of in vivo drug metabolizing enzyme systems so that the rate of formation of ultimate carcinogens would be retarded, 3) enhancement of metabolic defenses so that the cancer-producing agents would not reach the critical macromolecules in target cells, 4) enhancement of cellular immune systems to produce the reversal of early stages of neoplasia, and 5) cancer screening of human and animal populations. However, there have not been adequate methods to study these various categories of cancer prevention in a comprehensive manner. An experimental method which allows accurate determinations of carcinogenicity of chemicals as well as that allows accurate scoring of reductions of basal carcinogenicity of chemicals was necessary.

In 1959, Pietra et al (3) have observed that, when new-born mice were given subcutaneous injection of 7,12-dimethylbenz(a)anthracene (DMBA) within 24 hrs after their birth, there were very high incidences of tumor formation. The usefulness of new-born mice in experimental carcinogenesis has been noticed by many scientists. In 1968, Toth (4) has summarized the results of experiments which had been reported by then, and explained why when carcinogens were given to new-born mice rather than adult mice had caused higher incidences of tumor formations. As explained, the first reason is that there is weak or inactive metabolic detoxification capacity in new-born mice. The second reason is that the cells of new-born mice proliferate rapidly and the rapidly-proliferating cells are subject to carcinogenesis more easily. The third reason is that the immunological defense mechanism is poorly developed in new-born mice. Furthermore, Roe and Rowson (5) have reported that, in experiments using new-born mice, the duration of experiment may be shortened and thus, animals are used more economically. They have also noted that the incidence of lung adenoma formation was particularly high. Because of this high susceptibility of the lung to carcinogenic chemicals, the incidence of lung adenoma formation may be used as the model system to test the carcinogenicity of many chemicals. However, the developed method required sufficiently long durations and was not adequate for quick economical identification of cancer preventive agents. Thus, it was necessary to develop a

shorter-term bioassay method with higher sensitivity so that cancer preventive agents could be identified.

Since the 1980s, a rapidly increasing number of cancer researchers have utilized this experimental carcinogenesis model systems employing small rodents and studied the inhibitory effect of various compounds such as retinol, beta-carotene, folic acid, vitamin B12, vitamin C, vitamin E, fibers, calcium, piroxicam, 4-hydroxyphenylretinamide, minerals, trans-retinoic acid, retinyl acetate, 13-cis retinoic acid, and selenium against the induction of cancers inducible with wide variety of environmental chemical carcinogens (2). However, as many of these synthetic cancer preventive substances such as vitamin A analogs have serious toxicity of their own, we have focused our attention to identify the cancer preventive agents among natural products.

Materials and Methods:

Experimental Animals:

Sets of inbred and outbred strains of mice received from various sources such as the Jackson Laboratory were grown at the experimental animal quarters of Korea Cancer Center Hospital and were used in this study. Mice donated from the animal quarters were then kept in temperature and light controlled rooms and were given either pelletized solid NIH-7 open formula diet or various experimental diets containing natural products thought to have chemopreventive actions. These include fresh ginseng, red ginseng, carrot, B-carotene, soybean lecithin, spinach, *Sesamum indicum*, *Ganoderma lucidum* and caffeine (6,7).

Roots of fresh ginseng of four years old were ground in a mixer to make a suspension of 12.9 mg/ml and was administered through drinking water. The Korean red ginseng extract powder (spray dried) was obtained from the Korean Office of Monopoly and was dissolved in tap water at 1 mg/ml and was administered through drinking water ad libitum from the date of weaning to the time of sacrifice. Fresh carrots were ground and mixed in the diet at 25% and then given to mice ad libitum. B-carotene (Sigma, USA) was mixed with diet at 500 mg/kg and was fed ad libitum. Soybean lecithin granules (as 95% phosphatides and obtained from Elite Co.,

Japan) were solubilized in tap water at 25 mg/ml and were administered through drinking water. Fresh spinach was ground and mixed with diet at 25% and was given to mice ad libitum. Ground leaves of *Sesamum indicum* were mixed with diet to make up 5% by weight and the dried *Ganoderma lucidum* was ground and mixed with diet to make up 25% by weight. These diets were given to mice ad libitum. Caffeine (BDH Chemicals, England) was dissolved in tap water at 2 mg/ml for administration via drinking.

Administration of Carcinogens

All chemical carcinogens were used within 1 hr after emulsification or solubilization in 1% aqueous gelatin (DIFCO Labs., Detroit, USA). Each new-born mouse less than 24 hr of age was given 0.02 ml of either this vehicle or the vehicle containing various concentrations of carcinogens. Dimethylbenzanthracene (DMBA) was obtained from Calbiochem (USA) and 30 ug was given to each new-born mice by single subcutaneous injection at subscapular region. Similarly, 1 mg of urethan (Fisher, USA) or 8 ug of Aflatoxin B-1 (Calbiochem, USA) were given to each new-born mice by subcutaneous injections (8,9).

Autopsy and Histological Examinations.

Mice belonging to either experimental or control diet groups were autopsied at 9 through 56 weeks after their birth. Animals were sacrificed by pressing their neck and various organs were inspected to detect for the presence of tumors. After counting the exact number of whitish-pink adenoma nodules which have developed in the lung, the organ and tumors were excised, weighed using chemical balance, fixed in 10% formalin, stained with hematoxylin-eosin, and then observed under a light microscope (8,9).

Statistical Analysis:

Chi-square test was used to determine the statistical differences between the control and experimental groups.

Results and Discussion

In a concerted effort to improve the existing in vivo carcinogenicity testing method, we had first to establish

the conventional method in our laboratory. One of the first question that we had to deal was to determine whether the experimental mice could survive the long incubation periods (up to 56 weeks) after the subcutaneous injection of chemical carcinogens or their respective injection vehicles to new-born mice within 24 hr after their birth. Results shown in Table 1 indicate that, except when the strong hepatocarcinogen aflatoxin B-1 (AFB) was given, most of the new-born ICR strain of mice withstood the subcutaneous injection of vehicles or chemical carcinogens. Furthermore, most of these experimental mice which have survived through weaning could last 26 through 56 weeks, at which time the autopsy was performed. Thus, in future experiments, we have used the weaning-survivors as the initial starting mouse number.

Table 1. Survival rates at weaning of new-born ICR mice when injected with various chemical carcinogens subcutaneously

substances	Dose	initial number	weaned survivors	% survivors
1% gelatin	0.02 ml	199	194	97.5
DMSO	0.01 ml	200	192	96.0
DMBA	30 ug	210	204	97.1
Urethan	1.0 mg	200	186	93.0
Aflatoxin B1	8.0 ug	200	104	52.0

After establishment of the soundness of our experimental approach, we have injected a well known standard lung carcinogen, urethan, to new-born ICR mice and 28 weeks later, scored for the formation of pulmonary adenomas (Table 2). Results show that untreated control, gelatin (vehicle for urethan) injected control, and red-ginseng injected control mice did not develop lung cancers even after 28 weeks of incubation period. However, the injection of urethan has produced lung adenomas in most of the surviving mice (94%). When red-ginseng, a natural plant product which is suspected of having anticancer effect for centuries in the Orient, was given together with urethan, there was a significant reduction in the lung adenoma formation at 28 wks. Thus, this experiment has demonstrated that urethan was highly tumorigenic and red-ginseng had significant chemo-preventive effect. However, the required experimental duration was too long to be efficient or economic for quick screening of chemical carcinogens or anticancer agents.

Table 2..Lung adenoma formations induced with subcutaneous injection of urethan (1.0 mg) to new-born ICR mice and autopsied at 28th week.

substances	weaned initial	28 week survivors	survivors with adenomas
none	60	59 (98.3)	0
1% gelatin	70	69 (98.6)	0
ginseng	80	74 (92.5)	2
urethan	36	34 (94.4)	32 (94.1)
urethan + ginseng	34	30 (88.2)	22 (73.3)

The next question that we have selected to address was whether the scoring of lung adenoma formation could be applied to other carcinogens known to have affinity for organs other than the lung. Therefore, we have selected a well known and strong hepatocarcinogen AFB and injected subcutaneously to the new-born ICR mice (Table 3). When autopsy was conducted at 28 weeks, as have been done after

Table 3. Induction of lung adenomas and hepatomas with subcutaneous injection of Aflatoxin B-1 (8ug AFB) to new-born ICR mice and autopsied at 56th week.

Substances	Weaned survivors	56 week survivors (%)	Survivors with adenomas (%)
normal	40	27 (67.5)	3
DMSO	192	118 (61.5)	4
ginseng	120	89 (74.1)	10 (11.2)
AFB	50	38 (76.0)	9 (23.6)
AFB + ginseng	47	29 (61.7)	5 (17.2)

Table 4. Induction of lung adenomas with subcutaneous injection of benzo(a)pyrene (0.5 and 1.0 mg BP) to various strains of new-born mice and autopsied at 9th week.

Substances	NIH(GP) (%)	A/J (%)	C57BL/6J	C57BR/cdJ
1% gelatin	2/79 (2.5)*	3/76 (3.9)	0/80	0/18
0.5 mg BP	37/79 (46.8)	65/75 (86.7)	1/78	----
1.0 mg BP	37/68 (54.4)	53/60 (89.8)	0/74	0/47

* Numbers indicate the number of mice with lung adenomas at 9th week per weaned survivors and the number in parenthesis indicate their percentages.

the urethan injection, while the 28 week survival rates were found equivalent to those seen earlier (]90% survival rate at 28 wk), the lung adenoma or hepatoma formations were hardly detectable (data not shown) and thus, autopsy was conducted at 56 wks. The 56 wk survival rate was considerably lower (=70%) and in the mice given AFB injection, lung adenoma and hepatoma formations were detected only in 24% and 11% of 56 wk surviving mice, respectively. Contrary to expectations, when AFB was injected to new-born mice subcutaneously, the rate of lung adenoma formation was even higher than the rate of hepatoma formations. This result suggested that, depending on the route of carcinogen administration and the age of experimental animals at which a specific carcinogen is administered, the target organ may change. Results shown in Table 3 also indicate that, again, red-ginseng had significant chemopreventive action against the AFB inducible lung adenoma and hepatoma formations. In any case, the detection of lung adenoma formation after subcutaneous injection of carcinogen to new-born mice has proved to be a sound method for in vivo testing of chemical carcinogenesis. However, again, the required durations to demonstrate lung adenoma formation with AFB was found too long to be of any value.

Next, we have determined the suitability of lung adenoma scoring model of carcinogenicity testing with benzo(a)pyrene (BP), one of the most common and well known environmental carcinogens. As the objective of our project was to develop a sensitive, efficient, economical and quick standardized method of in vivo screening test for chemical carcinogens, we have tried to shorten the duration of experiment by finding a sensitive mouse strain. Pilot tests with new-born ICR mice given BP have indicated that at least 20 weeks were required to detect any lung adenoma formations (data not shown). Again, pilot tests in which the inbred strain of A/J mice were injected with 1.0 mg BP and autopsied at earlier weeks have indicated that 9-week tumor developmental period was most suitable (data not shown). Therefore, we have selected several strains of new-born mice such as those of A/J, NIH(GP), C57BL/6j, and C57BR/cdj strains and gave a single subcutaneous injection of either 0.5 and 1.0 mg BP (arbitrarily selected dose). Results shown in Table 4 indicate that both the A/J and NIH(GP) strains of mice have developed lung adenomas by 9 weeks after the BP injection. However, lung adenoma formations were not detected in the

C57BL/6j and C57BR/cdj strains of mice by 9 week autopsy assay. In particular, the A/J strain of mice were super-sensitive to the BP dependent lung adenoma formations.

By employing the A/J and NIH(GP) strains of new-born mice, we have then tried to establish a carcinogen dose dependent relations by detecting the formation of lung adenomas at 9 week autopsy assay (Table 5). Results show that the lung adenoma formation response with BP in the inbred strain of A/J mice was extremely sensitive and we could not establish the carcinogen and cancer formation dose response relationship. However, for the out-bred strain of NIH(GP) mice, the rate of lung adenoma formations had clear carcinogen dose response relations.

Table 5. Induction of lung adenomas with varying doses of benzo(a)pyrene given to new-born mice of A/J and NIH(GP) strains and autopsied at 9th week.

Substances	A/J mice (%)	NIH(GP) mice (%)
1% gelatin	1/31 (3.2)*	0/80 (0.0)
40 ug BP	22/31 (71.0)	5/80 (6.3)
63 ug BP	24/31 (77.4)	13/80 (16.3)
125 ug BP	42/45 (93.3)	25/73 (34.2)
250 ug BP	35/39 (89.7)	27/77 (37.3)
500 ug BP	40/45 (88.9)	39/79 (49.4)
1.0 mg BP	26/29 (89.7)	43/80 (53.8)

* Numbers indicate the number of mice with lung adenomas at 9th week per weaned survivors and the number in parenthesis indicate their percentage.

In selecting the proper dose of BP to be given to the new-born NIH(GP) strain of mice, we have chosen the 50% lung adenoma forming BP dose (0.5 mg/new-born mouse). The underlying reason for selecting this 50% adenoma forming BP dose was to evaluate not only to employ the developed methodology in the screening of anticancer agents but also in the screening of tumor promoters in the future. Therefore, finally, the use of new-born NIH(GP) strain of mice and 0.5 mg BP injection dose was found to be most suitable for the 9-week medium-term bioassay of lung adenoma formations.

By employing such established bio-assay carcinogenicity testing method, we have examined various natural products for their anticancer effects (Table 6). Results show that all tested natural products, except for fresh-ginseng and

carrot, had significant anticancer effects. Particularly notable anticancer effects were observed with the feeding of *Ganoderma lucidum* and caffeine. At least in our experimental model system, caffeine was not carcinogenic by itself and produced rather strong anticancer effect against the BP dependent lung adenoma formations.

Table 6. Anticancer effects of natural products tested with 0.5 mg BP given to new-born NIH(GP) mice subcutaneously and autopsied at 9th week.

Substances	1% Gelatin (%)	0.5 mg BP (%)	Comments
control	2/79 (2.5)*	37/79 (46.8)	control
Ascorbic Acid	0/80	23/78 (29.5)	anticancer
Fresh Ginseng	0/77	33/78 (42.3)	no effect
Red Ginseng	2/78 (2.6)	22/80 (27.5)	anticancer
Carrot	0/78	32/76 (41.0)	no effect
B-carotene	0/78	30/80 (28.8)	anticancer
Soy Lecithin	0/79	18/80 (22.5)	anticancer
Spinach	1/65 (1.5)	22/68 (32.4)	anticancer
Sesamum indicum	3/50 (6.0)	5/80 (6.3)	anticancer
<i>Ganoderma lucidum</i>	1/73 (1.4)	-----	
Caffeine (75 ug)	1/80 (1.3)	-----	
Caffeine (100 ug)	0/78	15/80 (18.8)	anticancer
Caffeine (1.0 mg)	0/60	4/78 (5.1)	anticancer
Caffeine (2.0 mg)	0/59		

* Numbers indicate the number of mice with lung adenomas at 9th week per weaned survivors and the number in parenthesis indicate their percentage.

In conclusion, by undergoing the tedious but systematic selection processes, we have established a suitable in vivo carcinogenicity testing system that utilizes the new-born NIH(GP) strain of mice, 0.5 mg of BP injected subcutaneously per mouse, and 9 week autopsy schedule for detection of lung adenoma formations. By employing this newly established medium-term bioassay method, we have begun to screen for effective anticancer agents from natural products.

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