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Molecular Basis of Organospecific Carcinogenesis by Chemical Carcinogens–Study with Breast Cancer Specific Carcinogens: DMBA as an Indirect-Acting Carcinogen and NMU as a Direct-Acting Cancinogen.

Chong-Young Park#, Seung Won Kimm, Sang Chul Park*

Department of Biochemistry, College of Medicine,
Seoul National University, 28 Yun Kun Dong, Chong-No Ku, 110-744
Seoul, Korea

**Corresponding author*

#Present address: Department of Pediatrics, College of Medicine, Hallym University

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To study the selective organospecific carcinogenesis by the specific chemical carcinogens, the breast cancer induction model by oral administration of 7,12-dimethylbenzanthracene (DMBA) or by intravenous injection of N-methylnitrosourea(NMU) on female rats was analyzed. In the present experiment, we compared the effects of ages on the chemical mammary carcinogenesis by studying the metabolic system of the carcinogenic activation, detoxification or DNA damage and repair. The breast tumor incidence was significantly higher in the young rats of 50 days old than in those of one year old rats. As an index of organospecific DNA damage or repair, the *in vivo* covalent binding index (CBI) of the specific organs by the specific chemical carcinogens was monitored. And for the analysis of carcinogenic activation, the quantity of cytochrome P450's was determined with the respective type-specific monoclonal antibody, while the detoxication capacity was deduced by the activity monitoring of glutathione S-transferase (GST) and peroxidase. The skin tissues of the mammary region had the highest CBI with both of DMBA and NMU at 50 days of age. And there were contrasting differences in the contents of carcinogenic activation and detoxication system: that is, the content of T.C.D.D.-inducible cytochrome P450 was high, while the activities of GST and peroxidase was low in the mammary skin tissues at tumor prevalent age. These results led us to conclude that the molecular organospecific carcinogenesis, as illustrated with mammary carcinogenesis by DMBA and NMU, is operated probably through the differential capacity of the target tissues in the high carcinogenic activation, low detoxication and the low DNA repair function.

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INTRODUCTION

The molecular mechanism of carcinogenesis by chemical carcinogens should be illustrated in order to develop the preventive or the therapeutic policy against the cancer, especially in the present era of the tremendously environmentally-polluted world. The chemical carcinogens from the varying sources, would rather be increasing in numbers and amounts. The epidemiological study showed that 60 to 90% of the human cancers are caused by the chemical carcinogens from the environmental contamination (Higginson, 1969), which indicated the importance of the research on the chemical carcinogenesis. Nonetheless, the basic molecular mechanism of the chemical carcinogenesis is unclear yet.

The carcinogenic mechanism by the chemicals should include their DNA-damaging or genetic modulation effects (Doull *et al.*, 1980, Brookes & Lowley, 1964; Janss *et al.*, 1972; Janss & Ben, 1978; Weinstein, 1984; Moschel *et al.*, 1977). The evidences that the chemical carcinogens are essentially genotoxic and mutagenic can be summarized as follows: 1. Most of chemical carcinogens are ultimately electrophiles, which can interact with the nucleic acids; 2. Skin cancer incidences are significantly high in the genetic disorders of DNA-repair ability as Xeroderma pigmentosum; 3. Most of carcinogens are mutagens; 4. Most of cancers have the chromosomal anomaly. For the chemicals to act as genotoxic agents, they should be metabolically activated as the ultimate carcinogens at the target cell. The metabolic fate of these chemicals can be summarized as Fig. 1. The chemically inert compounds, predominant in the nature, should be bio-transformed into the active carcinogens (indirect-acting carcinogens), while the reactive compounds by themselves can play the DNA damaging effects without metabolic intervention (direct-acting carcinogens) (Lutz, 1979). The metabolic activation system for the chemical carcinogens

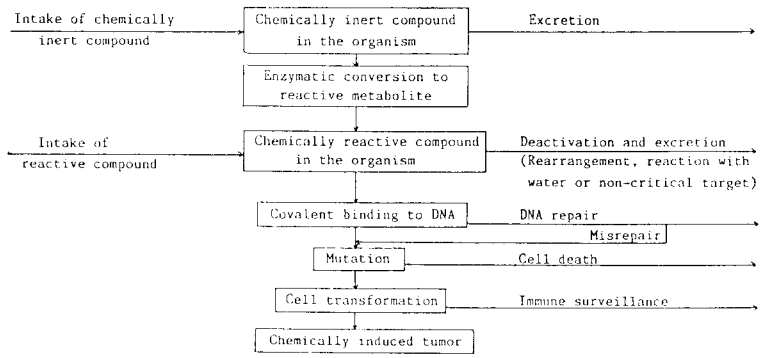

Figure 1. Sequence of events in the chemical induction of a tumor. From the left to the center: Intake of the chemical. From the center to the right: Reactions of the chemical or of the organism which do not lead to heritable damage or a tumor.

is represented by the cytochrome P450 complex, which comprises many different isozymes with independent inducible properties. The next choice of the metabolic fates of the active carcinogens is either the attacking the macromolecules such as DNA and proteins or the inactivation and excretion by the conjugation system such as glutathione S-transferase, UDP-glucuronyl transferase or sulfotransferase (Hogson & Guthrie, 1980). And the ultimate DNA damaging effect by the chemical carcinogens would be finally modified by the DNA repair function. The net result of these complex mechanism in the target tissues would explain the organospecificity of the specific carcinogens.

The specific induction of murine mammary tumors by oral administration of DMBA or by I.V. injection of NMU is well reported, which is modified by the ages of the host (Dao, 1969; Janss & Ben, 1978). Therefore, the clear organospecificity with the aging effect of the murine mammary carcinogenesis by the certain chemicals could be the good candidate as a cancer model for the basic research on molecular chemical carcinogenesis.

In the present experiment, we intended to illustrate the molecular mechanism of chemical carcinogenesis by the comparison study of murine mammary carcinogenesis by the administration of direct-acting and indirect-acting carcinogens and by the differences of the host ages through the analysis of *in vivo* and *in vitro* CBI, amount of cytochrome P450's and activities of detoxification enzymes.

MATERIALS AND METHODS

Animals and materials

The female Sprague-Dawley rats of different age groups were obtained from Seoul National University Animal Breeding House. The chemicals were purchased from varying sources: 7,12-dimethylbenzanthracene (DMBA), N-methylnitrosourea (NMU), glutathione, dinitrochlorobenzene (DNCB), 0-dianisidine, phenylmethylsulfonyl fluoride (PMSF) were from Sigma Co. (St. Louis, Mo. USA); radiolabelled DMBA (dimethyl C^{14}-100 mCi/mmol) and NMU (methyl C^{14}, 20 mCi/mmol), from New England Nuclear Co. (Boston, Ma, USA) (Fig. 2); alkaline phosphatase-conjugated goat anti-mouse IgG, and goat anti-rabbit IgG, from Kirkegaard & Perry Laboratories Inc. (Gaithersberg, Md, USA) and other chemicals of analytical grade were obtained from the commercial sources. And the type-specific monoclonal antibodies to cytochrome P450's were kindly donated by Dr. Sang Shin Park from National Cancer Inslitute (Bethesda, Md, USA).

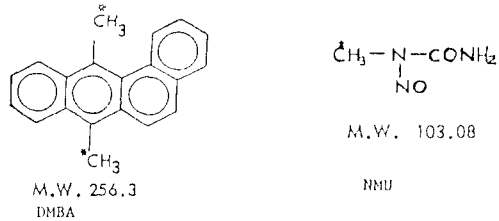

Figure 2. Structures of 7,12-Dimethylben-zanthracene (DMBA) and N-methylnitrosourea (NMU)

Induction of breast cancer

The mammary cancers of female Sprague-Dawley rats were induced either by oral administrantion of DMBA (20 mg/2 ml corn oil per head or by intravenous injection of NMU 5 mg/100 gm body weight) for once (Gullino *et al.*, 1975; Huggins *et al.*, 1961; Dao, 1969). To analyze the age-dependence of mammary carcinogenesis, the two different age groups of rats, 50 days of age and 1 year old, were simultaneously treated. And 6 month after the treatment, the rats were sacrificed and the tumor tissues were divided into two fractions: one fraction was rapidly frozen in liquid nitrogen tank and stored in the deep freezer at -70°C until biochemical analysis, while the other fraction was immersed into the formalin solution for the histological and the immunohistochemical analysis.

Preparation of samples

The samples were homogenized with polytron homogenizer (Biotron, Swiss) in 5 volumes of Tris-HCl buffer (pH 7.4, 10 mM). The homogenates were centrifuged at 10,000 x g for 30 min at 4°C and the supernatants were subjected to the recentrifuge at 105,000 x g for 1 hour at 4°C with Beckman L-8 ultracentrifuge. The supernatants were divided into 100 μl aliquots to use as cytosol fraction for GST and peroxidase analysis. The precipitates were suspended in potassium phosphate buffer (pH 7.4, 0.15 M, 0.1 mM PMSF), added glycerol to 20% solution to use as microsomal fraction for the determination of cytochrome P450's.

Determination of covalent binding index

The *in vivo* CBI by DMBA and NMU was calculated by monitoring the radioactivity in the extracted DNA from the organs such as skin and liver after the time function following the administration of radiolabelled chemical carcinogens (50 μCi/head) (Lutz, 1979). The *in vitro* CBI was determined by analyzing the incorporation of the radiolabelled carcinogens into the exogenous DNA in the presence of microsomal fraction; that is, 10 μg of thymic DNA was dissolved in TMK buffer (Tris 100 mM pH 7.4, $MgCl_2$ 10 mM, KCl 10 mM), where 10 μg protein of microsomal fraction and 10 mM NADPH were included. By addition of DMBA (0.1 μCi) the reaction was started. After two hours of incubation, the reaction was stopped by DNA isolation with phenol/chloroform method. The quantity of covalent DNA-adduct was calculated by monitoring the radioactivity incorporation in the final DNA preparation.

Determination of enzyme activities

The activities of GST and peroxidase in the samples were determined by the respective standard procedure (Habig *et al.*, 1974 a,b; Klebanoff, 1965). And the protein concentration was determined by Lowry method (1951).

Enzyme-linked-immunoassay for cytochrome P450's

Each 10 μg protein of the microsomal fractions from the varying sources was applied on the 96 well plate (Costar). After four hours incubation, the nonabsorbed samples were washed out three times with potassium phosphate buffer (pH 7.4, 0.1 M). The monoclonal antibodies to the type-specific cytochrome P450's, such as T.C.D.D.-inducile and Pb-inducible P450's were 200 fold diluted and each 100 μl of antibody solution was added to each well and the mixture was incubated for the next 4 hours. Then the well plate was washed again with phosphate buffered saline (PBS) three times. Finally the alkaline phosphatase-conjugated second antibody solution (1/1,000 dilution) was added and incubated overnight. After overnight incubation, the plates were washed now with Tris-HCl buffer (pH 7.4, 0.1 M). The alkaline phosphatase activity in the washed plate well was visualized with enzyme analysis kit, containing p-nitrophenyl phosphate and the absorbances at 540 nm were determined.

DNA preparation

DNA's were isolated essentially after Marmur's method (1961) with the slight modification such as the three times extraction with phenol/chloroform and RNase treatment of the final ethanol precipitates. The DNA solution for CBI analysis should have absorbance ratio (A260/A280) higher than 1.8.

RESULTS

Age dependence of mammary carcinogenesis

Six months after the carcinogen administration, the female Sprague-Dawley rats were compared for the incidence of mammary carcinoma in the 50 days old group and 1 year old group. As shown in Table 1, the fractions of tumor-bearing rats were 88% by DMBA and 79% by NMU treatment in the 50 day-old group, while the tumor-positive rate in the one year old group was less than 36%. The significant difference in the tumor incidence between the age groups indicated the strong age-dependence of mammary cancers. The histolo-

Table 1. Comparative incidence of breast cancers by application of DMBA or NMU to the female Sprague-Dawley rats of different ages. (6 months after treatment)

Carcinogen/Age	50 day-old rats	1 year-old rats
DMBA	22/25 (88.0%)	8/22 (36.4%)
NMU	15/19 (78.9%)	5/22 (20.8%)

DMBA : $X^2 = 11.4$ d.f. $= 1$ $P < 0.001$

NMU : $X^2 = 12.5$ d.f. $= 1$ $P < 0.001$

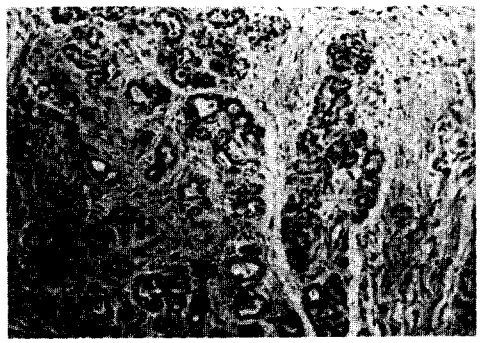

Figure 3. Microscopic finding of DMBA-induced mammary carcinoma in the rat.

gical analysis showed that most of the induced mammary tumors were of the adenomatous type (Fig. 3).

Organ difference of CBI

The *in vivo* CBI after 20 hours of DMBA administration into 50 day-old rats was 337 ± 58 at the mammary skin tissues, while 110 ± 5 at the liver tissues. The *in vivo* CBI by NMU was calculated respectively at 2 hours and 10 days after the carcinogen administration in both tissues of skin and liver. Two hours after injection, the CBI of NMU was 583 ± 22 at mammary skin tissues and 780 ± 14 at liver tissues in contrast to the CBI's of 295 ± 19 at skin tissues and 195 ± 17 at liver tissues following 10 days after injection (Table 2). The *in vitro* CBI by DMBA was compared for mammary gland and liver tissues with age differences. A shown in Table 3, *in vitro* CBI by DMBA showed the highest

Table 2. Comparison of *in vivo* covalent binding index of NMU and DMBA to the mammary gland and liver tissues in 50 day-old female Sprague-Dawley rats.

1. 7, 12-dimethylbenzanthracene (50 microcurie/head, oral route)

Tissues/Time after application	20 hours
Mammary gland	337 ± 58*
Liver	110 ± 5

2. N-methylnitrosourea (50 microcurie/head, intravenous route)

Tissues/Time after application	2 hours	10 days
Mammary gland	583±22	295±19
Liver	780±14	195±17

* Mean ± S.D. of 3 experiments.

Table 3. Comparison of *in vitro* covalent binding index of DMBA to the exogenous thymic DNA, affected by microsomal fractions from the mammary gland and liver tissues of the female Sprague-Dawley rats with different ages.

Source of microsomal fraction	50 day-old rats	1 year-old rats
Mammary gland	171	60
Liver	65	114

score with the microsomal fraction of mammary skin tissues of 50 day-old rats.

Metabolic activation of carcinogens

The quantitative changes of cytochrome P450's by aging were monitored with the type-specific monoclonal antibodies in the mammary skin tissues. As shown in Table 4, the amounts of T.C.D.D. (tetrachlorodibenzo-P-dioxin) inducible cytochrome P450 in the mammary skin tissues were shown to be decreased with the increase of the host ages: that is, one year-old rat had only

Table 4. Comparison of cytochrome P-450 contents of T.C.D.D.-dependent and of phenobarbital-dependent types in the microsomal fractions from the mammary gland tissues in the each age group of female Sprague-Dawley rats, analyzed by Elisa assay.

Age of rats/Class of P-450	T.C.D.D. P-450[a]	Pb P-450[b]
50 day-old rats	0.554±0.005*	0.814±0.039
3 month-old rats	0.360±0.026	0.579±0.024
6 month-old rats	0.408±0.148	0.612±0.044
1 year-old rats	0.208±0.015	0.716±0.020

a) Tetrachlorodibenzo-P-dioxin

b) Phenobarbital-dependent cytochrome P-450

*Mean ± S.D. of 3 experiments. The values represent the absorbances at 540 nm

Table 5. Comparison of cytoplasmic activities of glutathione S-transferase and peroxidase in the mammary gland and liver tissues from each age group of female Sprague-Dawley rats.

Age of rats	Enzyme activities	Glutathione S-transferase		Peroxidase	
		Mammary gland	liver	Mammary gland	liver
50 day-old rats		N.D.*	59.0±4.0**	7.5±0.5	41.0±1.0
3 month-old rats		N.D.	82.0±4.0	8.0±1.0	48.5±4.5
6 month-old rats		N.D.	140.0±2.5	9.0±0.5	40.0±1.0
1 year-old rats		N.D.	86.0±3.5	8.5±0.5	47.0±3.0

* N.D. = not detectable

**Mean ± SD of 3 experiments. Specific enzyme activities are expressed in mU/mg protein.

the half of T.C.D.D.-P450 compared to that of 50 day-old rat. The amounts of Pb-P450 in the skin tissues were shown to have the similar pattern of decrease by age in amounts.

Inactivation of carcinogens

As indices for the inactivation of chemical carcinogens, we determined the activities of glutathione S-transferase and peroxidase in the tissues of skin and liver at different ages. The results were summarised in Table 5. In case of GST, the skin tissues had the very low activities in the not-detectable range, while the liver tissues had the high activities, especially in the 6 month-old rat groups. In case of peroxidase, both tissues of skin and liver did not show any significant age-dependent changes, though liver tissues had the higher specific activities than the skin tissues.

DISCUSSION

The standard procedures to induce the mammary carcinomas in the rats were well established by the oral administration of DMBA (20 mg per head) or by I.V. injection of NMU (5 mg per 100 gm weight) (Huggins *et al.*, 1961; Dao 1969; Gullino *et al.*, 1975). As shown in the present experiment (Table 1), the adenomatous mammary tumors were selectively induced regardless of the carcinogenic class and administration route, whose incidence was modulated by the age of the host; that is, the young rats of 50 day old were more susceptible, while the old rats of one year old were more resistent to the mammary carcinogens. The molecular mechanism to explain the above organospecific carcinogenesis of age-dependence was not thoroughly illustrated except the high incidence of mammary cancer by administration of DMBA, NMU and MC (methylcholanthrene) at the age of 50 ± 5 days (Dao, 1969; Janss & Ben, 1978; Huggins *et al.*, 1961) and the simultaneous high activity of arylhydrocarbon hydroxylase (Greiner *et al.*, 1980) and so on.

Therefore in the present study, we determined the *in vivo* and *in vitro* CBI of the target organs by the mammary specific carcinogens as a integrative marker for the DNA damaging and repair capacity in relation with the carcinogenesis. Actually, CBI indicates the degree of DNA damages by the administered genotoxic agents, representing the carcinogenic effeciency and target-specific affinity (Lutz, 1979). For example, the strength of hepatocarcinogenic agents is well correlated with the respective CBI. In case of DMBA, we monitored its CBI at 20 hours after the administration, since the tissue arylhydrocarbon hydroxylase, major metabolic activator for the polyaromatic hydrocarbons such as DMBA and MC, showed its peak activity at the same time interval after MC administration (Janns *et al.*, 1972; Janss & Ben, 1978; Bast *et al.*, 1981). The CBI of DMBA to the mammary skin tissues was three fold higher than that to the liver tissues at 50 days old group, which illustrated the higher susceptibility of mammary DNA to the exogenous carcinogens at the specific develop-

mental stage. In contrast to DMBA, the indirect-acting cancinogen, the mode of metabolic fate of NMU, the direct-acting carinogen, is different in the sense that it does not require activation (Miller, 1978). Therefore, CBI of NMU was monitored at 2 hours after intravenous injection, when the direct-acting carcinogens were most reactive (Pegg, 1977) and also at 10 days after the administration, when most of the damaged DNA could have been repaired. As shown in Table 2, its immediate CBI in the liver was slightly higher than that in the skin tissues, but at later stage, the mammary tissue showed the higher CBI to NMU than the liver tissue. The age-dependent relatively high CBI's of NMU and DMBA at the mammary tissues illustrated the organospecificty of these chemicals, which suggested the presence of the probable higher activity of carcinogenic activation and lower activity of DNA repair function in the mammary skin tissues at the specific developmental stage of the rats.

In order to analyze the difference of CBI by age and target tissues, we compared the *in vitro* CBI of DMBA by changing the microsomal preparation from the respective tissue under the assumption that the CBI of the same DMBA to the identical exogenous DNA's would be modulated by the capacity of metabolic activation (Miller, 1978; Gelboin, 1980). As summarized in Table 3, the *in vitro* CBI of DMBA was three fold higher by the microsomal fraction from the mammary skin tissues than that from the liver tissues, of the 50 days old groups of rats, in contrast to the lower CBI in the mammary tissues than in the liver tissues from the one year old groups. The above result explained that the organospecificty of the carcinogens and the differential CBI, are at least in part due to the differential metabolic activation of the target tissues.

In the metabolic activation of chemical carcinogens, cytochrome P450's play the major roles. There are many different types of P450's more than 10 gene families (Lu & West, 1980; Nebert & Gonzales, 1987). For the specific activation of DMBA, the arylhydrocarbon hydroxylase (AHH) activity of the P450 especially T.C.D.D.-inducible P450, was concerned (Alvares *et al.*, 1967; Fagan *et al.*, 1982; Park *et al.*, 1984). In order to compare the quantitative differences of the cytochrome P450's in the tissues, we performed the ELISA assay with the type-specific monoclonal antibodies such as MAb 1-7-1 for T.C.D.D.-inducible P450 and MAb 2-66-3pj for Pb-inducible P450 on the microsomal fractions of the skin and liver tissues from 50 days, 3 months, 6 months and one year old rats (Park *et al.*, 1982; 1984; Fujino *et al.*, 1982; 1984). The relative amount of T.C.D.D.-inducible P450 was highest in the microsomal fraction of the skin tissues from 50 days old rats with the decreasing tendency with aging (Table 4). And the amount of Pb-inducible P450 was also high in the young aged rats. These results would emphasize the probable role in carcinogenesis of the higher metabolic activation to convert the inert carcinogens into ultimate reactive carcinogens in the mammary skin tissues of 50 days old rats.

In addition to the metabolic activation capacity of the target tissue, the detoxication function should be taken into consideration to understand the molecular carcinogenesis, because the high detoxifying potential would eradicate the reactive radicals, formed by the complex activation mechanism. The

relation of these contrasting effects of activation and detoxification should be analyzed simultaneously. The detoxifying potential of the tissues against the reactive carcinogens includes many different types of conjugation systems such as transfer of glutathione, sulfate, UDP-glucuronidation and peroxidation (Morgenstern *et al.*, 1981; Worholm *et al.*, 1983; Mannervik, 1985). In the present study, we also monitored the effect of developmental change on the enzyme activities of glutathione S-transferase (GST) and peroxidase in the skin and liver tissues. In case of GST, the skin tissues showed the activities of non-detectable range in comparison with the high activities in the liver tissues, the GST activity was highest at 6 months of age (Table 5). Since GST plays the major detoxifying role against most of the xenobiotic toxic chemicals, its low activity would suggest the high susceptibility to those insults. Recently, the novel type of GST isozyme was identified in the skin tissues with different Km and substrate specificity (Kwak & Park, unpublished data). Moreover, peroxidase would serve as one of the radical scavengers in the tissues. But the activity monitoring of peroxidase did not reveal any age dependent change in the skin and liver tissues.

From these results, it could be summarized that the organospecificity of the specific carcinogens and its age-dependence would be illustrated in terms of *in vivo* CBI and its molecular mechanism is related with functional difference in carcinogenic activation through cytochrome P450's and detoxification via conjugation system as well as the repair efficiency of the damaged DNA in the target tissues.

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화학적 발암원의 조직 특이성 암유발기전 – DMBA와 NMU의 선택적 유암 발생기전을 중심으로.

박종영#, 김승원, 박상철*

*서울대학교 의과대학 생화학교실
#한림대학교 의과대학 소아과교실

특정 발암원의 조직특이성 암유발기전을 연구하기 위하여 DMBA의 구강투여 또는 NMU의 동맥주입에 의하여 유암이 유도되는 실험모델을 대상으로 선택하였다.

본 실험에서는 화학적 발암원의 유암유발기전에 미치는, 숙주인 흰쥐의 연령효과를 아울러 비교분석하였으며, 특히 발암원의 조직내 활성화, 불활화 및 해독 그리고 DNA 손상과 수선등의 변화를 구명하였다. 유암의 발생율은 1년생 흰쥐보다 생후 50일 흰쥐에서 현저하게 높았다. 특정조직의 선택적 발암기전을 설명하는 기전의 일환으로 조직 DNA의 특정 발암원에 의한 공유결합성 지표 (covalent binding index, CBI)를, 발암원의 활성화 기전 지표로는 cytochrome P450의 함량을, 반면 불활화의 지표로는 glutathione S-transferase와 peroxidase의 활성을 비교하였다. 조직의 CBI는 생후 50일군의 유선조직이 DMBA나 NMU에 대하여, 간조직보다 유의하게 높았으며 시험관내 CBI 실험에서는, 생후 50일군 유선조직의 microsome 분획의 발암원 활성화능이 보다 높음을 관찰하였다. 또한 T. C. D. D. 의존성 cytochrome P450 함량도 생후 50일군에서 가장 높았다. 그러나 불활화 효소들은 연령 변화에 따라 유의한 변화를 보여주지 않았다. 상기의 결과들은 DMBA나 NMU와 같은 발암물질이 특정조직, 특히 유선조직에 생후 50일군에서 유암을 선택적으로 유발하는 기전은 표적조직의 높은 발암원 활성화능, 낮은 불활화능 그리고 효율이 낮은 DNA 수선능이 연계적으로 작동함으로써 이루어지고 있음을 보여주고 있다.