Original

Spontaneous Tumors in Hypocatalasemic C3H/ C_s^b/Gen and C3H Mice

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Abstract: Hypocatalasemic mice (C_s^b) were examined for their life span and compared to C3H mice with reference to oxidase enzyme activity and spontaneous tumor development. N¹-methylnicotinamide oxidase, xanthine oxidase (XO), xanthine dehydrogenase (XDH), and XO+XDH activities in 8 week-old male C_s^b mice were decreased as compared to the C3H counterparts. On the other hand, benzaldehyde oxidase in both sexes and xanthine dehydrogenase activity in females were significantly elevated as compared to the C3H. The total tumor incidence, number of tumors per mouse, liver tumors, and other tumor induction were significantly greater in as compared with until 499 days group in both sexes (P<0.01). The total incidence of tumors and incidence of liver tumors were not significantly different between the two strains in both sexes. The number of tumors per animal in C3H females and incidence of sarcoma in both sexes were significantly decreased as compared with the C_s^b case but the numbers of liver tumor per animal and ovarian tumors were significantly greater. (J Toxicol Pathol 2004; 17: 25–30)

Key words: spontaneous tumor, oxidase enzyme, hypocatalasemic mice, C3H mice

Introduction

The relationship between cancer and oxygen radicals, produced by many chemicals included in the daily diet, is the subject of continuing debate^{1–3}. In experimental studies in animals, oxygen radicals have been found to play a role in tumor promotion, with phorbol ester-type tumor promoters for example, being shown to activate their production in phagocytes and non-phagocytes^{4–6}. The enzymes catalase, Mn- and Cu/Zn-superoxide dismutase (SOD) and glutathione peroxidase (GPx) are known to protect cells against oxidative stress. SOD has attracted particular attention in connection with tumor progression⁷.

We have been maintaining a hypocatalasemic mouse (C_s^b) strain, which is a radiation-induced mutant of the C3H/He mouse. Compared with the parent, this mouse strain has a decreased catalase activity in most solid tissues and especially in the blood whereas superoxide dismutase (SOD) activity is elevated^{8–10}. Shaffer *et al.* reported that the

hypocatalasemic state in C_s^b is not due to alteration within the catalase structural gene or the level of gene transcription or mRNA stability, but rather to the level of mRNA translation and/or catalase protein turnover^{11,12}. In previous studies, C_s^b was shown to be sensitive to induction of duodenal tumors by hydrogen peroxide¹³, and signet ring cell carcinomas in the glandular stomach were observed after X-irradiation in males^{14,15}. It is of interest whether other oxidase activities might differ between C_s^b and C3H mice^{16–19}. The present study was therefore performed to examine oxidative enzyme activities as well as spontaneous tumor development in long term surviving animals for differences between the two strains.

Materials and Methods

Animals

C3H/C_s^b/Gen mice (C_s^b) were propagated by sibling mating in our laboratory and 6 weeks old C3H/HeNCrj mice (C3H) were purchased from Charles River Japan, Inc., (Hino). The animals were housed in polycarbonate cages, eight per cage, and kept under constant conditions of temperature (24 ± 2°C) and humidity (50 ± 10%) with a 12 h light/12 h dark cycle. The mice were maintained according to the Guide for Care and Use of Laboratory Animals established by Hiroshima University and fed a commercial diet MF (Oriental Yeast Co., Ltd., Tokyo, Japan) and tap

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water *ad libitum*.

Experimental animals

Two experiments in C_s^b were carried out, one short term (403–480 days) and one long-term (700–790 days). For tumorigenesis the animals were killed and autopsied when they became moribund and all remaining mice were killed at 403–480 days in C_s^b strain only (short term experiment) and at 700–790 days in both strains for survival and tumor induction experiment. There were no tumors during 481 to 499 days, so in C_s^b strain tumor inductions were separately shown until 499 days and over 500 days. All major organs and tissues were resected, fixed in 10% neutral formalin and processed routinely to paraffin-embedded sections stained with hematoxylin and eosin.

For enzyme assay 8 weeks old mice of both strains and 1 year old C_s^b strain were used.

Assay of XO (xanthine oxidase) and XDH (xanthine dehydrogenase) activities

XO and XDH activities in liver cytosol were measured by the oxidation of 1-methylxanthine to 1-methyluric acid. Each incubation mixture consisted of 0.2 μ mol of 1-methylxanthine and 0.1 ml of liver cytosol (equivalent to 20 mg liver wet weight; 1.1–1.5 mg protein) in a final volume of 0.5 ml of 0.1 M K,Na-phosphate buffer (pH 7.4). In the case of XDH activity, 0.27 μ mol of NAD^+ was added to the incubation mixture. The incubation was performed at 37°C for 10 min, then 0.5 ml of methanol and 50 μ g of acetaminophen as an internal standard were added. After mixing, the solution was centrifuged. An aliquot of the supernatant was analyzed by HPLC in a Hitachi L-6000 chromatograph (Tokyo, Japan) fitted with a Mightysil RP-18 column (150 $\times$ 4.6 mm, Kanto Chemical Co., Inc., Tokyo, Japan). The mobile phase was 0.5% acetic acid - methanol (92:8, v/v). The chromatograph was operated at a flow rate of 0.5 ml at a wavelength of 280 nm. The elution times of 1-methyluric acid, 1-methylxanthine, and acetaminophen were 11.5, 15.0, and 18.5 min, respectively.

Assay of aldehyde oxidase activity

The assay was performed using benzaldehyde or N^1 -methylnicotinamide as a substrate. Benzaldehyde oxidase activity was measured according to the method of Johns¹⁹. The decrease in absorption at 249 nm was monitored in 0.165 M phosphate buffer (pH 7.8). In this study, 0.1 ml of liver cytosol (about 2 mg protein) was used for the assay.

N^1 -metylnicotinamide oxidase activity was measured according to the slightly modified method of Ohkubo and Fujimura²⁰. N^1 -methylnicotinamide was incubated with liver cytosol (11–13 mg protein) in a final volume of 0.5 ml of 0.1 M Na,K-phosphate buffer (pH 7.4). The incubation was performed at 37°C for 10 min. After incubation, the oxidation products, N^1 -metyl-2-pyridone-5-carboxamide (2-pyridone) and N^1 -metyl-4-pyridone-3-carboxamide (4-pyridone), were determined by HPLC.

Statistical analysis

The significance of differences in numerical data was evaluated with the chi-squared test, t-test and the Dunnett multiple test.

Results

In the long term experiment in C_s^b strain, body weight, survival, and tumor induction were studied sequentially as well as in C3H strain. In the C_s^b strain, male body weights increased until 6 months after birth, remained at a plateau until 18 months, and then decreased. In female an increase was observed until 12 months and then decreased. Body weights were the same in both sexes from 15 months (until 14 months significantly different) (Fig. 1). In C3H strain body weights were smaller than in C_s^b strain (both sexes $P < 0.01$). Body weights were increased until 6 months, remained at a plateau until 14 months and then decreased. In female C3H the body weight was increased until 13 months after birth and then decreased.

Survival rates are shown in Fig. 2. In male C_s^b the survival was 95% at 11 months, 92% at 18 months, and finally was 65%. In females, 100% survival continued until 13 months, then 93% until 18 months, and the final value was 76%. In male C3H, the survival gradually decreased until 49% at 17 months of age. At 24 months, the male C3H survival was significantly decreased as compared with male C_s^b mice. In C3H females 97% survival was shown until 14 month, then decreased to a final value of 71%.

Table 1 summarizes body and organ weights. C_s^b animals were separated and summed up until 499 days and over 500 days. In C_s^b males over 500 days the liver and adrenal were heavier than in the group of 499 days, but body weights were decreased. In C_s^b females, kidney and adrenal weights in the 500 day group were significantly increased as compared with the 499 days group. The relative weight of kidney and adrenal in male, and adrenal and spleen in female group of 500 days were significantly increased as compared with the group of 499 days. Liver weights in male C_s^b were significantly low as compared with male C3H. In female C_s^b kidney and spleen weights were significantly greater than those in C3H but the ovary weight was lower. Relative weights of adrenals ($P < 0.01$) in male C_s^b and of kidneys ($P < 0.05$) and ovaries ($P < 0.01$) in females were significantly lower than in C3H mice.

The first tumor, a hepatoma, appeared on day 375 days after birth in male C_s^b and an ovarian tumor was observed on day 403 in a female C_s^b . On the other hand, in C3H mice the first neoplasias were a skin tumor on day 458 in male and a Harderian gland tumor on day 546 in a female. The cumulative tumor incidence of autopsied animals is shown in Fig. 3. The tumors were moderately increased at 18 months after birth in both strain males and at 21 months in female groups.

In the 499 days group, total tumor incidences were 35/90 (39%) in males and 21/112 (19%) in females and numbers of tumor per mouse were 0.4 ± 0.6 and 1.2 ± 0.5 , respectively

Fig. 1. Body weight

—□—: C3H Male, —■—: C^b male, —○—: C3H female, —●—: C^b female

Fig. 2. Animal survival

—□—: C3H Male, —■—: C^b male, —○—: C3H female, —●—: C^b female

Table 1. Body and Organ Weight

	No	BW	Brain	Liver	Kidney	Ov/Testis	Adrenal	Spleen
Male C ^b _s								
Until 499	91	38 ± 4	0.458 ± 0.021	2.002 ± 0.879	0.533 ± 0.098	0.138 ± 0.014	0.005 ± 0.002	0.178 ± 0.082
Over 500	60	35 ± 6**		2.732 ± 1.383**	0.599 ± 0.153	0.130 ± 0.073	0.008 ± 0.002	0.334 ± 0.049
Female C ^b _s								
Until 499	112	33 ± 6	0.472 ± 0.019	1.370 ± 0.192	0.351 ± 0.046	0.020 ± 0.027	0.006 ± 0.001	0.151 ± 0.040
Over 500	49	35 ± 6**		1.762 ± 0.928	0.387 ± 0.058**	0.015 ± 0.020b	0.009 ± 0.002	0.496 ± 0.091**
Male C3H	101	31 ± 6	0.523 ± 0.019	2.870 ± 1.375	0.580 ± 0.124	0.122 ± 0.027	0.008 ± 0.003	0.327 ± 0.474
Female C3H	100	29 ± 3	0.533 ± 0.018	1.503 ± 0.612	0.355 ± 0.056	0.119 ± 0.183b	0.007 ± 0.005	0.328 ± 0.511

** : significantly different from C3H (P<0.01 by Student *t*-test).

Table 2. Relative Weight

	Brain	Liver	Kidney	Ov/Testis	Adrenal	Spleen
Male C _s ^b						
Until 499	12.02 ± 1.18	52.22 ± 23.60	13.93 ± 2.51	3.62 ± 0.40	0.12 ± 0.05	4.63 ± 2.15
Over 500		79.79 ± 44.83	17.39 ± 5.32	3.74 ± 2.18	0.23 ± 0.07**	9.25 ± 12.96
Female C _s ^b						
Until 499	14.62 ± 2.50	41.73 ± 4.94	10.78 ± 1.95	0.58 ± 0.70	0.17 ± 0.05	4.62 ± 1.34
Over 500		50.43 ± 23.20	11.30 ± 2.22*	0.43 ± 0.52**	0.26 ± 0.07	13.94 ± 23.85
Male C3H	16.52 ± 2.45	94.04 ± 48.90	8.70 ± 3.61	3.90 ± 0.75	0.29 ± 0.14	6.39 ± 8.43
Female C3H	12.07 ± 2.27	50.94 ± 19.94	12.07 ± 2.21	19.36 ± 22.58	0.24 ± 0.16	0.01 ± 5.11

*: Significantly different from C3H (P<0.05 by Student *t*-test). **: Significantly different from C3H (P<0.01 by Student *t*-test).

Fig. 3. Cumulative tumor incidence

—□—: C3H Male, —■—: C_s^b male, —○—: C3H female, —●—: C_s^b female

Table 3. Incidence of Tumor

	No	Total tumor	No of tumor	Double	Triple	Liver			Other tumors	Metastasis
						Incidence	No of tumor	Size		
Male C _s ^b										
Until 499	90	35 (39)**	0.4 ± 0.6**	0**	0	35 (39)**	0.5 ± 0.7	5.9 ± 13.5**	1 (1.1)**	0
Over 500	61	51 (84)	1.2 ± 0.8	15 (25)	1 (2)	46 (75.4)	1.5 ± 1.1**	15.2 ± 11.7	13 (21.3)	2
Female C _s ^b										
Until 499	112	21 (19)**	1.2 ± 0.5	3 (3)**	0	4 (3.6)*	1.0 ± 0.1	1.1 ± 0.8	19 (16.9)**	0
Over 500	49	24 (49)	0.7 ± 0.9	8 (16)	1 (2)	7 (14)	0.1 ± 0.4	1.3 ± 3.7	21 (42.9)	1
Male C3H	98	78 (80)	1.0 ± 0.1	22 (22)	27 (28)	72 (74)	2.1 ± 1.7**	18.6 ± 15.0	26 (26.5)	0
Female C3H	89	49 (24)	0.4 ± 0.2*	21 (24)	21 (24)	20 (22)	0.3 ± 0.5*	1.4 ± 3.3	24 (27.0)	0

*: Significantly different from over 500 days value in C_s^b strain (P<0.05 by χ^2 -test or Dunnett method).

**: Significantly different from over 500 days value in C_s^b strain (P<0.01 by χ^2 -test or Dunnett method).

Table 4. Oxidative Enzyme Activity in 8 Weeks Old and 1 Year Old Mice

Group	Body weight	N ¹ -methylnicotinamide oxidase activity pyridone formed (nmol/ min/mg protein)		
		4-pyr	2-pyr	total pyridones
C _s ^b male	26.8 ± 1.7	0.179 ± 0.025	0.198 ± 0.023	0.377 ± 0.048
C _s ^b female	20.5 ± 1.4	0.048 ± 0.006	0.056 ± 0.006	0.103 ± 0.012
1 year old C _s ^b male		0.191 ± 0.050	0.328 ± 0.076	0.519 ± 0.125
1 year old C _s ^b female		0.223 ± 0.120	0.265 ± 0.140	0.488 ± 0.257
C3H male	22.2 ± 0.8**	0.222 ± 0.025**	0.248 ± 0.026**	0.469 ± 0.050**
C3H female	20.2 ± 0.7	0.066 ± 0.019	0.076 ± 0.019	0.143 ± 0.038

	Benzaldehyde oxidase (nmol/min/mg protein)	Xanthine oxidase (XO) nmol/min/mg protein	Xanthine dehydrogenase (XDH)	XO+XDH
C _s ^b male	6.72 ± 0.69	0.372 ± 0.041	1.76 ± 0.31	2.17 ± 0.35
C _s ^b female	3.39 ± 0.43	0.510 ± 0.044	3.24 ± 0.45	2.74 ± 0.42
1 year old C _s ^b male	3.73 ± 0.70	0.769 ± 0.091	3.82 ± 0.62	4.60 ± 0.70
1 year old C _s ^b female	2.14 ± 0.99	0.548 ± 0.125	2.49 ± 0.45	3.11 ± 0.50
C3H male	3.64 ± 0.20**	0.607 ± 0.068**	2.35 ± 0.20*	2.96 ± 0.25**
C3H female	1.45 ± 0.31**	0.510 ± 0.034	2.47 ± 0.14**	2.92 ± 0.16

*: Significantly different from C_s^b value P<0.05 by Dunnett method.

**: Significantly different from C_s^b value P<0.01 by Dunnett method.

(Table 3). The main organs were liver (53%) in males and ovaries (17%) in females. In the group over 500 days old, the total tumor incidence was 51/61 (84%) in males and 24/49 (49%) in females, numbers of tumors per mouse being 1.54 ± 1.02 and 0.76 ± 0.92 , respectively. The total tumor incidence in both sexes and number of tumors per mouse in male were significantly increased as compared with until 499 days group in both sexes (P<0.01).

The total incidence of tumors was not significantly different between the strains in both sexes. The number of tumors per animal in C3H females and incidence of sarcoma in both sexes were significantly lower than in the C_s^b strain but the number of liver tumors per animal and ovarian tumors were significantly higher.

In 8-week old mice 4-pyr-, 2-pyr and total N¹-methylnicotinamide oxidase, xanthine oxidase (XO), xanthine dehydrogenase (XDH), and XO+XDH activities in male C_s^b were significantly lower than in their C3H counterparts (Table 4). XDH activity in females and benzaldehyde oxidase in both sexes were higher than in C3H. In one year old C_s^b mice, 2-pyridone was increased as compared with at 8-weeks in both sexes, and XO and XDH in males, while benzaldehyde oxidase was decreased.

Discussion

When planning and conducting long-term animal studies, it is important to know to what extent deaths caused by spontaneous tumors might influence the results and their valuation. In the present experiment we compared several factors in two substrains, C3H and C3H/ C_s^b /GEN. Especially, the body weights and incidence of sarcomas in C_s^b were significantly higher than in C3H, while the number of liver tumors was significantly smaller. Liver aldehyde

oxidase, xanthine oxidase, and xanthine dehydrogenase activities were higher in C3H than in C_s^b but the opposite was the case for benzaldehyde oxidase. Some enzymes changed with aging. Previously we reported that catalase activity in C_s^b mice is low as compared with the level in C3H but SOD and glutathione peroxidase activities are higher^{6,18}. Hypocatalasemic mice have low catalase activity but other oxidative enzymes may compensate. It can be considered that the animal survival and tumor incidence except sarcomas are almost similar in both strains under normal conditions. Further investigations are required to explain the relationship between tumorigenesis and enzyme activities.

Previously we reported the induction of duodenal tumors in C_s^b by oral administration of hydrogen peroxide¹³, increased skin tumorigenesis by the promotion of 10-*O*-tetradecanoylphorbol-13-acetate in C_s^b or benzoyl peroxide in C3H¹⁸, and high sensitivity to hepatotumorigenesis in C_s^b mice exposed to low doses of ²⁵²Cf fission neutrons and ⁶⁰Co gamma-rays¹⁶. Lipid peroxide concentration was increased to threefold in 24 month old C_s^b²¹. In the near the future we may have enzyme assay of various ages in both strains and mechanisms. Recently we found that 0.1 ml injection of 50% of carbon tetrachloride in C_s^b caused severe liver damage and mortality whereas C3H counterparts could survive (Watanabe, unpublished data). In the case of such damage antioxidant enzymes may act to rescue the animals. Thus the C_s^b stain may be a suitable model to examine the role of oxidative stress.

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