[CANCER RESEARCH 49, 821–825, February 15, 1989]

Correlation between Mammary Tumor and Blood Glucose, Serum Insulin, and Free Fatty Acids in Mice[1]

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ABSTRACT

The blood glucose level and serum levels of insulin, glucagon, and free fatty acids were examined in 7- to 8-mo-old female SHN mice with or without spontaneous mammary tumors (MT). Blood glucose levels in the females with MT were significantly higher than in those without MT, rising in proportion to the increase in size of MT up to 30 mm in diameter. In 4-mo-old male SHN and 11-mo-old female C57BL mice bearing mammary tumor grafts (MTg), the blood glucose level was significantly higher than in mice without MTg. Serum insulin and free fatty acids in female SHN mice with MT rose to higher levels than in mice without MT, whereas serum glucagon levels were unaltered. In 50% of mice with MT, pancreatic islets contained a large number of pyknotic cells. Livers of mice with MT or MTg were significantly heavier than those of mice without MT or MTg. In both female SHN mice with spontaneous MT and male SHN and female C57BL mice with MTg, the total number of hepatocytes and the total amount of liver DNA increased significantly compared with values from corresponding controls without MT or MTg. These findings suggest that MT or MTg induce a hyperglycemic state and an enhanced production of free fatty acids and insulin, which may in turn stimulate the growth of mammary tumors and the liver.

INTRODUCTION

The relationships between hormones and mammary tumor growth have been extensively studied in rats and mice. Estrogen is regarded as an initiator as well as a promoter of mammary tumorigenesis, while PRL[3] is a promoter of the tumor but not an initiator. Progesterone stimulates the formation of mammary hyperplastic alveolar nodules in mice when the blood PRL and estrogen are unchanged (1).

Recent studies have demonstrated that the diabetic state stimulates the growth of several tumors: the growth rates of Morris hepatoma 7288CTC and Jensen sarcoma *in vivo* are dependent on free fatty acids in adult rats with streptozotocin-induced diabetes (2); the growth rate of the R3230AC mammary adenocarcinoma transplanted into rats with streptozotocin-induced diabetes was reduced by insulin administration (3). These studies suggest that hyperglycemia promotes tumor growth. No other studies on blood glucose levels in tumor-bearing animals have been encountered. We report herein that mammary tumors cause a rise in blood glucose level concurrently with the promotion of liver growth in mice.

MATERIALS AND METHODS

Seven- to 8-mo-old female mice of the high-mammary-tumor SHN/Mei strain, 3-mo-old male SHN/Mei mice, and 10-mo-old female mice of the low-mammary-tumor C57BL/Tw strain were used in the present experiments. All mice were kept on a 12-h light/12-h dark regimen at 23–25°C. A spontaneously developed MT from a normal female SHN/Mei mouse was maintained in our laboratory by successive s.c. transplantations into virgin females; the tenth to 12th generations of MT grafts were used in the present study. A spontaneous MT occurring in a female C57BLcfC3H/Tw mouse was also maintained by transplantation into virgin female C57BL/Tw mice; the eighth to tenth generations were used. A piece of MT (approximately 5.0 mm^3) taken from SHN or C57BLcfC3H/Tw donors was transplanted under the abdominal skin of 3-mo-old intact male SHN or 10-mo-old intact female C57BL/Tw mice, respectively.

BGLs were measured in 7- to 8-mo-old female SHN mice with varying sizes of MT. In male SHN mice, the BGL was measured immediately before and on the tenth, 20th, and 30th day after transplantation. Serum levels of insulin, glucagon, and free fatty acids were measured in 7-mo-old female SHN mice with or without MT. The BGL was measured by the glucose oxidase method (4); serum insulin and glucagon levels were determined by the enzyme immunoassay method (Insulin B-test and Glucagon Test; Wako, Tokyo, Japan) (5); serum free fatty acid levels were measured by the acyl CoA oxidase method (NEFAC-test; Wako).

Seven- to 8-mo-old female SHN mice with MT, and 4-mo-old male SHN and female C57BL mice, into which MT pieces had been transplanted 30 days before, were sacrificed by cervical dislocation. Livers, pancreata, and adrenals were weighed and fixed in Bouin's solution, embedded in paraffin, and sectioned at 8 μm thickness. Sections of livers and adrenals were stained with Delafield's hematoxylin and eosin. Sections of the pancreas were stained by the aldehyde fuchsin trichrome method (6). Percentages of areas of the pancreatic islet occupied by A- and B-cells were calculated by using a CIA-102 color image analyzer (Olympus, Tokyo, Japan), respectively. The number of hepatocytes per mm^2 was also counted on the histological sections by the analyzer. Polynuclear hepatocytes comprising 2.4 to 2.8% of the liver cell population were excluded from the total count of hepatocytes. The number of hepatocytes per mm^3 (n) was estimated by Abercrombie's method (7); the total number of hepatocytes was estimated by the following formula: $n \times$ liver volume (mm^3). The volume was estimated as liver weight/liver gravity (1.04). At sacrifice, pieces of the liver, approximately 100 mg each, were stored at −70°C for DNA assay by the method of Labarca and Paigen using Hoechst 33258 dye (Calbiochem, La Jolla, CA) (8).

RESULTS

Changes in Liver and Pancreas and in Levels of Blood Glucose and Serum Insulin in Female SHN Mice Bearing Spontaneous Mammary Tumors. The female SHN mice died within 2 mo after the occurrence of palpable MT. The tumors were adenocarcinomas, showing a basic acinar structure with some compact masses of cells. The mean body weight of 8-mo-old female SHN mice bearing MTs was significantly heavier than that of age-matched female SHN mice without MT; however, the net body weight (less the MT weight) was not significantly different between SHN mice with and without MT. In MT-bearing SHN mice, weights of pancreas and adrenals, calculated per 20-g net body weight, were not significantly different from those in mice without MT (Table 1).

In pancreatic islets of 8-mo-old female SHN mice without MT, cells stained with Ponceau-fuchsin mixture (A-cells) were located in the peripheral region, while cells stained with alde-

Received 8/22/88; revised 11/14/88; accepted 11/16/88.

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[1] Supported by Grants-in-Aid for Scientific Research and for Encouragement of Young Scientists from the Ministry of Education, Science, and Culture of Japan.

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[3] The abbreviations used are: PRL, prolactin; MT, mammary tumor; BGL, blood glucose level; NBW, net body weight; MTg, mammary tumor graft(s).

Table 1 *Blood glucose and organ weights in 8-mo-old female SHN mice with or without spontaneous mammary tumors*

	No. of mice	Body wt (g)	Tumor wt (g)	Blood glucose (mg/dl)	Organ wt/20-g NBW		
					Pancreas (mg)	Adrenals (mg)	Liver (g)
MT−	7	30.3 ± 0.83[a]		140 ± 4.4	229 ± 15.2	8.3 ± 0.44	1.1 ± 0.04
MT+	10	42.5 ± 1.60[b]	9.2 ± 0.72	174 ± 4.3[b]	232 ± 9.0	8.1 ± 0.41	1.8 ± 0.05[b]
		33.4 ± 1.21[c]					

[a] Mean ± SE.
[b] $P < 0.001$ (Student's t test).
[c] Body weight after subtraction of tumor weight (net body weight).

hyde fuchsin (B-cells) were in the central region. The islets in 50% of the mice with MT were histologically similar to those of mice without MT. However, in the other 50% of the mice with MT, all islets contained a large number of pyknotic cells which could not be identified as to cell type. The mean area of the islets was significantly larger in the last (pyknotic) group than in the islets histologically similar to those in MT-free mice. There were no significant differences in the percentage of respective pancreatic islet areas occupied by A- and B-cells between MT-bearing and MT-free mice (Table 2). No histological changes were observed in adrenals between the mice with and without MT.

In 7- to 8-mo-old female SHN mice, BGLs rose in proportion to the increase in maximum diameter of MT (Fig. 1); however, BGL fell when MT exceeded 30 mm in diameter. The BGL in a group of 8-mo-old, female MT-bearing SHN mice was significantly higher than that in the age-matched MT-free females (Table 1). In another group of 7-mo-old female SHN mice with MT, the mean value of serum insulin levels was also significantly higher than in MT-free females at the same age, although their pancreatic islets were not examined histologically; serum levels of free fatty acids in the MT-bearing females were significantly higher than those in the females without MT. There was no significant difference in serum glucagon levels between SHN mice with and without MT (Table 3).

Livers of 8-mo-old female SHN mice with MT were significantly increased in weight over those of the females without MT (Table 1). There was a positive correlation between tumor weight and liver weight in MT-bearing female SHN mice (Fig. 2). Both the total number of hepatocytes and the number of hepatocytes per 20-g net body weight were significantly greater in mice with MT (Fig. 3). There was no significant difference in the amount of DNA per g of liver between mice with and without MT. Both the total amount of DNA of the liver and the amount of liver DNA per 20-g NBW in mice with MT were significantly increased over those of mice without MT (Fig. 3).

Changes in Liver, Pancreas, and Blood Glucose Level in 4-mo-old Male SHN and 11-mo-old Female C57BL Mice Bearing Mammary Tumor Grafts. The male SHN and female C57BL mice bearing MTg died within 2 mo after transplantation. The tumor grafts showed adenocarcinomas with a basically acinar structure. Four-mo-old male SHN and 11-mo-old female C57BL mice bearing MTg were significantly increased in body weight compared to the age-, sex-, and strain-matched mice without MTg (Table 4). Only in male SHN mice with MTg was the net body weight (minus the graft weight) greater than that of mice without MTg. However, there was no significant difference in NBW between MTg-bearing and MTg-free C57BL mice. In male SHN and female C57BL mice bearing MTg, weights of pancreas and adrenals per 20-g NBW were not

Table 2 *Changes in pancreatic islets of 8-mo-old female SHN mice with (MT+) or without (MT−) spontaneous mammary tumors*

	No. of mice	Mean area of islets[a] (× $10^3/\mu m^2$)	% of islet area occupied by	
			A-cells	B-cells
MT−	7	19.4 ± 1.9[b]	10.3 ± 0.7	80.7 ± 1.1
MT+	10 (5)[c]	22.1 ± 2.3	11.3 ± 0.8	82.8 ± 3.3
	(5)[d]	32.3 ± 7.1[e]		

[a] One hundred islets randomly examined.
[b] Mean ± SE.
[c] Number of mice having normal islet cells.
[d] Number of mice having pyknotic islet cells.
[e] $P < 0.05$ (Student's t test).

Table 3 *Blood glucose and serum insulin, glucagon, and free fatty acids in 7-mo-old female SHN mice with or without spontaneous mammary tumors*

	No. of mice	Glucose (mg/dl)	Insulin (microunits/ml)	Glucagon (pg/ml)	Free fatty acids (μeq/ml)
MT−	10	134 ± 5.0[a]	19.3 ± 5.15	85.3 ± 7.01	0.61 ± 0.044
MT+	10	152 ± 5.9[b]	36.3 ± 7.29[b]	79.2 ± 9.40	0.84 ± 0.067[b]

[a] Mean ± SE.
[b] $P < 0.05$ (Student's t test).

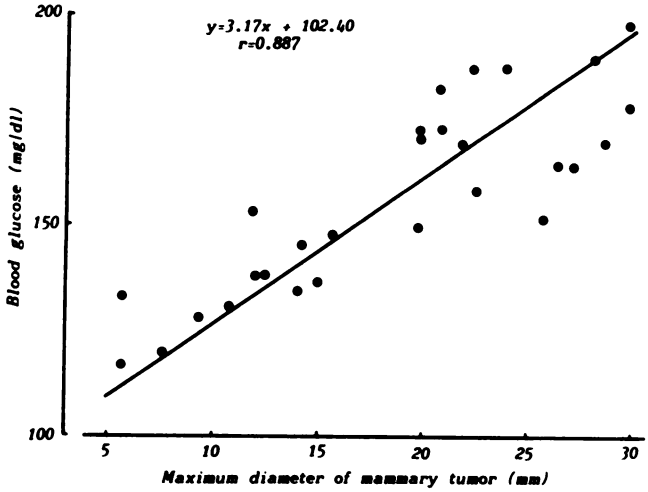

Fig. 1. Correlation between blood glucose levels and spontaneous mammary tumor size in 7- to 8-mo-old female SHN mice.

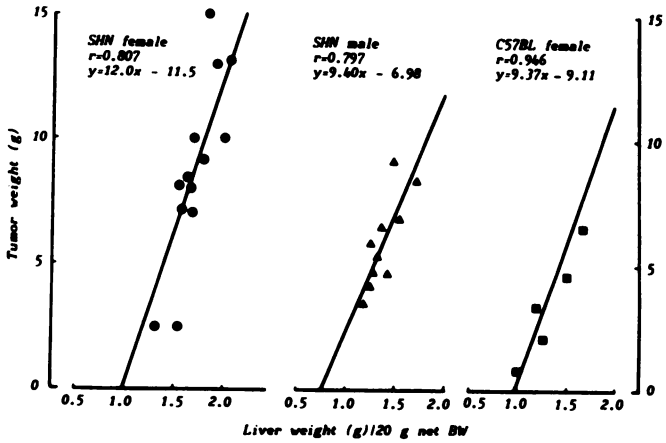

Fig. 2. Correlation between mammary tumor weight and liver weight in female SHN, male SHN, and female C57BL mice.

Table 4 *Blood glucose and organ weights in male SHN and female C57BL mice with or without MTg*

Strains, sex, and age	Presence of MTg	No. of mice	Net body wt (g)	Tumor wt (g)	Blood glucose (mg/dl)	Organ wt/20-g net body wt		
						Pancreas (mg)	Adrenals (mg)	Liver (g)
SHN male, 4 mo	−	7	26.5 ± 0.40[a]		129 ± 6.3	208 ± 11.3	4.8 ± 0.37	1.0 ± 0.01
	+	11	31.9 ± 1.04	5.8 ± 0.63	172 ± 4.7[b]	197 ± 5.8	4.3 ± 0.11	1.3 ± 0.04[b]
C57BL female, 11 mo	−	5	18.5 ± 1.01		130 ± 11.5	208 ± 7.7	5.7 ± 0.52	1.0 ± 0.04
	+	5	17.3 ± 0.60	3.8 ± 0.70	165 ± 11.2[c]	191 ± 11.9	5.4 ± 0.65	1.4 ± 0.07[b]

[a] Mean ± SE.
[b] $P < 0.005$ *versus* −MTg (Student's *t* test).
[c] $P < 0.05$.

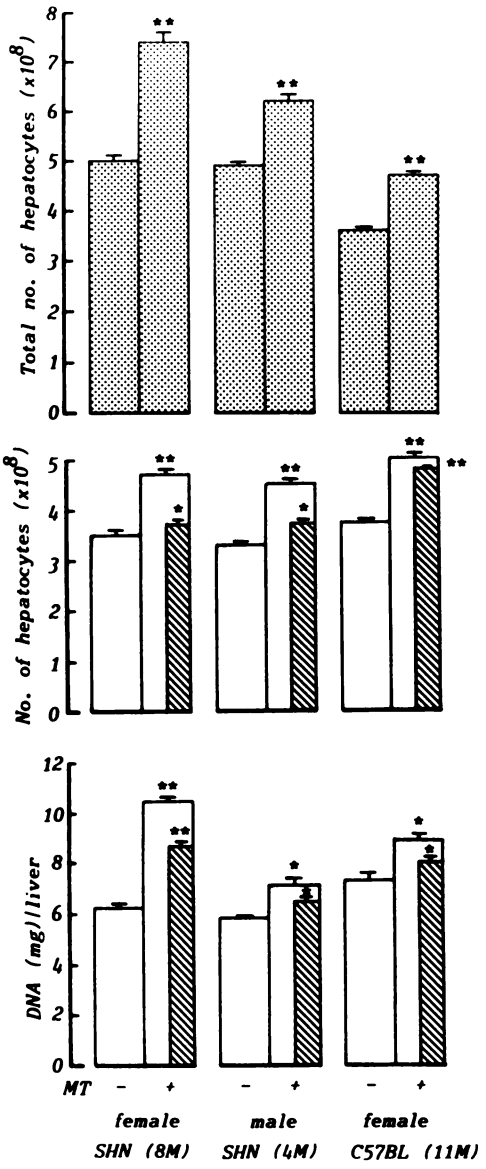

Fig. 3. Number of hepatocytes and amount of DNA per liver in mice with or without mammary tumors. Spontaneous MT, SHN female; grafted MT, SHN male and C57BL female. □, estimated per 20-g NBW; ■, estimated per 20-g body weight with MT. *, $P < 0.05$; **, $P < 0.005$ *versus* controls (−MT or −MTg) (Student's *t* test).

different from those in mice without MTg (Table 4). In male SHN and female C57BL mice bearing MTg, pancreas and adrenals were not significantly different in weight per 20-g NBW from those in the sex- and strain-matched mice without MTg.

In male SHN and female C57BL mice with MTg, livers were significantly increased in mean weight per 20-g NBW over that in comparables without MTg (Table 4).

No histological changes in adrenals were found between mice with and without MTg in both strains of mice. MT from female SHN donors grafted into male SHN hosts grew similarly to that in the females until 30 days after transplantation. The BGL in the male hosts with MTg gradually rose until the 20th day after transplantation (Fig. 4). In male SHN and female C57BL mice with MTg, liver weights were significantly higher than in mice without MTg (Table 4). There was a positive correlation between tumor weight and liver weight in male SHN and female C57BL mice bearing MTg (Fig. 2). The number of hepatocytes in the liver per 20-g NBW in mice with MTg was significantly larger than that in mice without MTg. There was no significant difference in the amount of DNA per g of liver between mice with and without MTg. Both total amount of liver DNA and the amount of DNA per 20-g NBW were significantly greater in mice with MTg than those in mice without MTg (Fig. 3).

DISCUSSION

The present study demonstrated that BGLs in SHN and C57BL mice bearing spontaneous (MT) or transplanted mammary tumors (MTg) are significantly higher than in mice without MT or MTg. In female SHN mice, there was no difference in weight of the pancreas between mice with and without MT, although 50% of the females with MT showed pyknosis in their islets. A previous study showed that cells of the islets became pyknotic in hyperglycemic diabetic rats (9), suggesting that the pyknosis in the present study was caused by the hyperglycemia of mice with MT. There were no differences in weight and

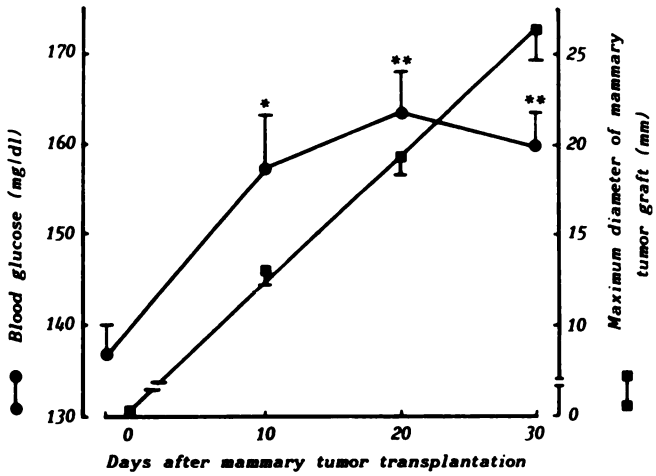

Fig. 4. Alteration of blood glucose levels and growth of mammary tumor grafts after mammary tumor transplantation in 3-mo-old male SHN mice. *, $P < 0.01$; **, $P < 0.001$ (Student's *t* test).

histology of adrenals between female SHN mice with and without MT, thus suggesting that the high blood glucose levels of mice with MT are not induced by functional changes in the adrenals.

In male SHN and female C57BL mice, there was no difference in the weight of the pancreas between mice with and without MTg. In the group of MT-bearing female SHN mice whose islet cells underwent pyknosis, the islets were significantly increased in mean area over those in females without MT.

The present study showed that the BGL rose in proportion to the growth of MT or MTg in mice. When MT (1.35 ± 0.47 g, $n = 5$) was removed from MT-bearing female SHN mice at 7 to 8 mo of age, 7 days later the BGL (180.5 ± 7.3 mg/dl, $n = 5$) fell significantly to match that in the females bearing no MT (132.2 ± 10.85, $n = 5$, $P < 0.05$, Student's t test).[4] Furthermore, in female SHN mice bearing MT, serum insulin levels were significantly higher than those in the females without MT. These high insulin levels (37.3 ± 5.40 microunits/ml, $n = 5$) also fell significantly to the levels close to those in the MT-free females (20.5 ± 5.58 microunits/ml, $n = 5$) when examined 7 days after the MT removal (11.2 ± 2.75 microunits/ml, $n = 5$, $P < 0.005$, Student's t test).[4] No significant difference in the insulin level was found between the MT-free females and the females after the MT removal. However, there was no difference in serum glucagon levels between female SHN mice with and without MT. These findings raise the possibility that the hyperglycemic state in the MT-bearing animals is caused by a MT-derived hyperglycemic factor(s) different from glucagon. Peak plasma insulin values and the return of insulin secretion to baseline were delayed in breast cancer patients undergoing a glucose tolerance test (10). In the present study, it was found that MT presence is accompanied by hyperglycemia, resulting in a hyperinsulinemic state. Insulin stimulates DNA synthesis and cell proliferation in the epithelial component of the mammary tumor tissue *in vitro* (11, 12). Alloxan-induced diabetes produces rapid regression in 90% of mammary carcinomas induced by dimethylbenz(*a*)anthracene in rats (13). Approximately 60% of the dimethylbenz(*a*)anthracene-induced rat mammary tumors regressed in streptozotocin-induced diabetes, and tumor growth was reversed by the administration of insulin to these diabetic rats (14). These studies suggest that MT has a stringent requirement for insulin for *in vivo* growth. In our studies, therefore, it is suggested that there is a "vicious cycle" between MT and pancreatic islets mediated by the high BGL.

The growth rate of Morris hepatoma 7288CTC and Jensen sarcoma is dependent on the amount of free fatty acids in diabetic rats (2). Unsaturated fatty acids are required for the growth of a rat mammary tumor cell line (15), and dietary polyunsaturated fatty acids have a growth-promoting effect on mammary tumors (16). In the present study, serum levels of free fatty acids in female SHN mice with MT were significantly higher than in mice without MT. Accordingly, it seems probable that an increased level of free fatty acids resulting from the hyperglycemic state caused by MT contributes to the promotion of MT growth.

In male and female SHN and female C57BL mice bearing MT or MTg, the present study showed that the mean weight of livers was significantly greater than that in mice without MT or MTg. The liver weight increase was positively correlated with both the increase in tumor weight and the rise in BGL in mice with MT or MTg. It is suggested, therefore, that the growth of livers in MT- or MTg-bearing mice is stimulated by the presence of mammary tumor, resulting in an increase in BGL. In the present study, the number of hepatocytes in the liver per net 20-g body weight of mice with MT or MTg was significantly increased over that of mice without MT or MTg; the total amount of liver DNA and the amount per 20-g net body weight in mice with MT were also greater than in mice without MT or MTg. The possibility, therefore, cannot be excluded that hepatocyte proliferation in mammary tumor-bearing mice is stimulated by putative hyperglycemic factor(s) released from MT or MTg, although other factors relating to cell proliferation such as insulin-like growth factors I and II, epidermal growth factor, and transforming growth factor α in the sera of MT-bearing mice have not yet been examined in the present study.

The cytoplasmic level of liver fatty acid-binding protein is markedly elevated in hepatocytes during mitosis, the liver fatty acid-binding protein being the mitosis-associated protein target of carcinogen in rats (17). This protein has been thought to be involved in the metabolism of free fatty acids. Since in the present study, serum levels of free fatty acids in MT-bearing mice are significantly elevated, the elevated free fatty acids may also stimulate the proliferation of hepatocytes in MT-bearing mice. In addition, administration of insulin to alloxan-induced severely diabetic rats leads to a marked proliferation of liver cells (18). However, the mean liver weight in MT-unbearing male C57BL and SHN mice treated with insulin was not significantly altered from that in control mice,[4] suggesting that insulin has a stimulatory effect on proliferation of hepatocytes only when blood glucose levels are high. In summary, we suggest that MT may release hyperglycemic factor(s) followed by a blood glucose increase, resulting in an enhanced production of free fatty acids and insulin, which in turn stimulate the growth of both mammary tumor and liver.

ACKNOWLEDGMENTS

We thank Professor Howard A. Bern of the Cancer Research Laboratory at the University of California, Berkeley, and Guest Professor at Yokohama City University for his valuable advice and critical reading of this manuscript, and Professor Hiroshi Nagasawa of the Experimental Animal Laboratory at Meiji University for his kind supply of the SHN/Mei strain of mice.

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Cancer Res 1989;49:821-825.