Chapter 3

In vivo analysis of sialyl Lewis X on canine mammary gland tumors

Introduction

SLe(x) is a ligand of E-selectin, one of the selectin families. This ligand is expressed on leukocytes and implicated in the adhesion to E-selectin on the vascular endothelial cells in acute inflammatory process [30-32]. The expression of this carbohydrate antigen was reported to be limited in normal tissues such as the mucosa and glands of the esophagus, the proximal tubules and descending loops of Henle of the kidney, the limited part of some deep crypts of the colon, alveolar macrophages, some acinar cells of the pancreas, hepatic cells and Kupffer cells in the liver and the ureter [33, 34].

Recently it was reported that sLe(x) was frequently expressed on human cancer cells and that it might play an important role in the hematogenous metastasis of the cancer, where it mediates the initial adhesion step of tumor cells to the distal vascular endothelial cells by sLe(x)-E-selectin adhesion [18, 83]. In some human cancers, the degree of expression of sLe(x) on the surface of cancer cells was shown to be well correlated with frequency of hematogenous metastasis and prognostic outcome of cancer patients [35, 36, 84, 85]. In human breast cancers, the expression of sLe(x) in both primary and metastatic lesions was reported and the relationship between its expression and the metastatic behavior of the tumor was implied [37, 38, 86].

Some cell adhesion molecules were reported to be detected not only on the tumor tissues but in the sera of patients with cancer [87, 88]. The expression of sLe(x) was reported

in human breast, lung, ovarian and gastrointestinal cancers [89, 90]. The level of sLe(x) was found to elevate in the sera of patients with metastatic breast cancers and suggested to be a useful tumor marker of human breast cancers. However, there has been no investigation reported yet on the *in vivo* expression of sLe(x) and its clinical significance of sLe(x) in CMGT.

In the previous chapter, this carbohydrate antigen was detected on the cell surface of CMGT cell lines which showed the adhesive ability to HUVECs and it was suggested that the sLe(x) expression might be involved in hematogenous metastasis of CMGT, though the number of the cell lines evaluated was limited. To investigate whether the expression of sLe(x) correlated with the behavior of CMGT such as metastasis and prognosis, in this study I examined the sLe(x) expression on the CMGT tissues surgically removed from the spontaneous patients and performed a preliminary study on measuring the serum concentration of sLe(x) in dogs including CMGT patients.

Materials and Methods

Tissue samples:

CMGT tissues were obtained from 56 dogs undergoing surgical resection at Veterinary Medical Center, the University of Tokyo, between April 2000 and March 2002. Normal mammary gland tissues were obtained from 5 dogs without bearing any tumors. The medical records of these CMGT patients were also reviewed. Age, history of spaying before the first estrus, breed, body weight, histopathological diagnosis, tumor size, regional lymph node involvement, distant metastasis and prognostic outcome were obtained from the medical records or telephone interview to the owner or referral practitioners. Tumor size was calculated as the maximum diameter of the largest mass of the patients. The regional lymph node involvement was confirmed by histopathological examination, and the distant metastasis was confirmed by thoracic radiography. The WHO clinical stage was used for classification of CMGT staging [91](Table. 3-1). Survival time was defined as the time from surgery to death.

Serum samples:

Between January 2003 and October 2004, serum samples were collected from 82 dogs bearing tumor including 31 CMGT patients and 89 dogs with nonneoplastic diseases referred to the Veterinary Medical Center, the University of Tokyo. Sixteen healthy dogs were also included in this study. The diagnosis of CMGT was carried out by histological

examination. After blood sampling, serum was separated and stored at -20°C until analysis. The medical records of these patients were also reviewed; Age, sex, breed, diagnosis, blood examination.

Antibody:

For the immunohistochemical analysis, anti-sLe(x) monoclonal antibody (clone: CSLEX1) was obtained from BD Transduction Laboratories (Lexington, KY, USA), the same one as used in Chapters 1 and 2. The working solution was diluted at 1:1000 with 1% BSA-TBS.

Immunohistochemistry:

The expression of sLe(x) on CMGT tissues was analyzed by immunohistochemistry. Mammary tissues were fixed in 10% neutral buffered formalin and embedded in paraffin. A series of 4 μm-thick sections were used for immunohistologic and light microscopic examination. Immunohistochemical protocol was performed using a DAKO ENVISION+ kit/HRP (DAB)(DAKO) similar to that used in Chapter 2. Briefly, endogenous peroxidase activity was abolished by treatment with 0.03% hydrogen peroxide containing sodium azide. For the staining of sLe(x), sections were reacted with the primary antibody against sLe(x) at room temperature for 2 hours, and incubated with polymer solution containing HRP conjugated antibody against mouse Ig at room temperature for 30 minutes. Sections were visualized with DAB/hydrogen peroxide solution and counterstained with hematoxylin before

observation. For light microscopy, sections were stained with hematoxylin and eosin (HE staining). All sections were assessed in a blind fashion without knowledge of the patient's outcome or clinicopathological features. Degree of sLe(x) antibody reactivity in individual tissue sections was defined as positive (+), (++) and (+++) if unequivocal staining of the membrane or cytoplasm was observed in more than 5%, 20% and 40% of tumor cells, respectively. If it was less than 5%, the case was considered as negative (Fig. 3-1).

Assay of sLe(x) concentration in the blood:

The concentrations of sLe(x) in the blood samples were determined by sandwich enzyme immunoassay using the N-test EIA plate CSLEX kit (Nittobo Medical, Tokyo, Japan). Briefly, serum samples were reacted with first anti-sLe(x) monoclonal antibodies (clone: CSLEX1) immobilized on the wells of plates at 37°C for 2 hours. These antibodies bound the antigen to the solid phase if sLe(x) was present in a patient's blood sample. Then second anti-sLe(x) monoclonal antibodies labeled with HRP were added to the wells and incubated for 2 hours at 37°C. HRP-labeled antibodies bound to antigens fixed to the well by first antibodies and formed sandwich complexes. The bound enzyme reacted with the substrate ortho-phenylenediamine (OPD) for 10 minutes at room temperature and optical densities were measured at 492 nm with a microplate reader (MPR A4; Tosoh Co., Tokyo, Japan).The concentration of sLe(x) was calculated in U/ml.

Statistical analysis:

In immunohistochemical analysis, comparison of variables among the groups according to the degree of sLe(x) expression was made by Fisher's exact test or the chi-squared test. Survival curves in the groups according to the expression of sLe(x) were obtained by the Kaplan-Meier method and were compared by the log-rank test to evaluate statistical significance. Statistical analysis of serum sLe(x) concentrations among groups divided with their disease was made by Fisher's exact test. In comparison between serum sLe(x) concentrations and variables, the Student's *t*-test was performed. A probability of less than 5% ($P<0.05$) was considered significant in all the analyses.

Results

Clinical and pathological features of CMGT patients for immunohistochemistry:

A total 56 CMGT patients were used in this study. The mean age of the dogs at surgery was 10.7 years old (range: 3.6-15.3 years). All dogs were female. No dogs had a history of spaying before their first estrus and only 8.9% (5 dogs) had underwent ovariohysterectomy after their first estrus. The range of body weight was 1.9-34.3kg. The breed distribution was as follows: 11 toy poodles, 9 malteses, 8 mixed breed dogs, 4 Yorkshire terriers, 4 miniature dachshunds, 4 Shih-tzues and 16 various other breeds. Histologically, tumor tissues consisted of 16 adenomas, 15 adenocarcinomas, 22 benign mixed tumors and 3 malignant mixed tumors. The clinicopathological features of CMGT patients grouped by their histological diagnosis were shown in Table 3-2. Numbers of the patients of each clinical stage were 23 dogs with stage I, 20 with stage II, 4 with stage III and 9 with stage IV, respectively. All of 9 patients with stage IV had metastatic lesions. Two dogs were diagnosed as inflammatory carcinoma.

Expression of sLe(x) on CMGT tissues:

Table 3-3 shows the expression of sLe(x) in tissues of CMGT patients and healthy dogs. The expression of sLe(x) was observed in 29 of 56 dogs bearing MGT (51.8%) with various degree (Fig. 3-1). The rates of cases with sLe(x)-positive expression in adenomas, adenocarcinomas, benign and malignant mixed tumors were 50.0%, 53.3%, 50.0% and 66.6%,

respectively. There was a tendency to increase the total positive rate of sLe(x) related to histological malignancy, but the difference was not significant. The expression of sLe(x) was observed in cancerous tissues and no expression was found in non-cancerous tissues of CMGT patients. In normal mammary gland tissues of 5 healthy dogs, the expression of sLe(x) was not detected (Fig. 3-1).

Relationship between sLe(x) expression and clinicopathological features:

The clinicopathological features of CMGT patients grouped by their expression of sLe(x) were shown in Table 3-4. The correlation of sLe(x) expression to clinicopathological features in CMGT was also examined. No statistically significant difference was found among the groups exhibited sLe(x)-positive ((+), (++) and (+++)) and -negative expression in each variables. Long term follow-up was obtained in 38 patients (Table 3-5). Comparison of the survival times among the groups exhibited sLe(x)-positive ((+), (++) and (+++)) and -negative expression of CMGT patients was shown in Fig. 3-2. Two years survival rates of sLe(x)-positive ((+), (++) and (+++)) and -negative groups were 76.2%, 50.0%, 40.0% and 72.2%, respectively. Among sLe(x)-positive groups, 2 years survival rate was low according to the degree of sLe(x) expression, but that of sLe(x)-negative group was lower than sLe(x)-(+) positive group. Using the log-rank test, statistically significance was not found in survival time among the groups.

Clinical and pathological features of dogs:

A total 187 dogs were used in this study and their clinical features were shown in Table 3-6. Blood sampling was undergone before surgery in CMGT patients and at their first referral in patients with other disease. The mean age of all dogs was 7.8 years old (range: 0.5-14.8 years). There were 69 intact males, 22 castrated males, 55 intact females and 41 spayed females. Breed distributions were as follows: in CMGT patients; 5 Shih-tzues, 4 Yorkshire terriers, 3 pomeranians, 3 mixed breed dogs and 11 various other breeds: in patients with other tumors; 19 golden retrievers, 5 mixed breed dogs, 4 Shetland sheepdogs, 3 Siberian husky, 3 Shih-tzues and 13 various other breeds: and in patients of nonneoplastic disease; 19 miniature dachshunds, 7 labrador retrievers, 7 mixed breed dogs, 5 Shih-tzues, 5 golden retrievers, 5 French bulldogs, 5 Cavalier King Charles spaniels and 24 various other breeds. Healthy dogs were consisted with 9 beagles and 7 labrador retrievers. Histologically, CMGT patients were diagnosed as 10 adenomas, 11 adenocarcinomas, 8 benign mixed tumors and 2 malignant mixed tumors. Various tumors were included in patients of other tumors such as mast cell tumors (n=4), lymphomas (n=4) and osteosarcomas (n=3). In nonneoplastic disease, the most frequently encountered disease was intervertebral disk disease (n=14). Subsequently to the intervertebral disk disease, there were many orthopedic disease (n=12), epilepsy (n=5) and hernia (n=5).

Serum sLe(x) concentration in dogs:

Serum sLe(x) concentrations of CMGT patients, other tumor patients, nonneoplastic

disease patients and healthy dogs were shown in Table 3-7. As for mean values of serum sLe(x) concentration, the group with other tumors exhibited the highest concentration and the group of healthy dogs was lowest. In CMGT patients, the mean value was higher in benign tumors (adenomas and benign mixed tumor) than in malignant tumors (adenocarcinomas and malignant mixed tumors). But the range of values in all groups was wide and there was no significant difference among groups. In comparison with clinical data (age, sex and several values of blood examination including WBC), serum sLe(x) concentrations showed no correlation. The wide range of sLe(x) concentrations was also observed among their breeds (Fig. 3-3).

Discussion

In this study, I examined the expression of sLe(x) on the MGT tissues of canine patients and normal mammary tissues of healthy dogs. A half of neoplastic tissues were found to express sLe(x) in all CMGT patients, whereas both non-neoplastic tissues of CMGT patients and normal mammary gland tissues of healthy dogs exhibited negative. The rates of sLe(x) positive cases were slightly higher in adenocarcinomas and malignant mixed tumors than those in adenomas and benign mixed tumors, however there was no significant difference. According to the specific sLe(x) expression on neoplastic tissues of CMGT patients, the expression of this ligand might be related to tumorigenesis of CMGT.

The relationship between the expression of sLe(x) and variables of clinicopathological features was evaluated, but no statistically significant difference was observed in any variables. In comparison of survival curves among sLe(x)-positive ((+),(++) and(+++)) and -negative groups by Kaplan-Meier method, sLe(x)-(+++) positive group had the lower survival rate by 2 years than other sLe(x)-positive groups. However that of sLe(x)-negative group was lower than sLe(x)-(+) positive group and there was no significant difference in all the groups investigated.

In this study, population of CMGT cases investigated was small and numbers of each histopathologic type were fewer than 20 except benign mixed tumors. In addition, many cases were lost in the follow up investigation. In addition, this information was obtained by

telephone interview to owners or private practitioners and accurate information could not be obtained, such as latent lymphatic invasion and metastasis. In the present study, I investigated only on the primary lesion of CMGT for the purpose of evaluating the efficacy of sLe(x) as a diagnostic tool. In the previous chapter, this carbohydrate antigen was detected on the cell surface of CMGT cells derived from metastatic lesions. Evaluating its expression on the metastatic lesion of CMGT may clarify the relationship to distant metastasis, though tissue specimens of distant metastatic lesions such as pulmonary metastasis were difficult to obtain.

Some cell adhesion molecules including sLe(x) were reported to be detected not only on the tumor tissues but in the sera of patients with cancer [87-90, 92]. It was reported that the mean serum sLe(x) concentration of 225 healthy human was 2.53 U/ml and the upper limit of the reference range was 8.0U/ml [92]. The level of sLe(x) was found to elevate significantly in the sera of breast cancer patients with distant metastasis compared to those without metastasis (87.5±142.1 and 28.8±18.1, respectively) and suggested to be useful as a tumor marker of human breast cancers.

In this study, serum sLe(x) concentrations in patients with CMGT, other tumors and nonneoplastic diseases, and healthy dogs were examined. Measured values were various among patients. Healthy dogs showed lower concentrations than any patients, however no significant difference was found. Significant correlation to clinical data was not observed in this study. Although some of the breeds showed significant differences in serum sLe(x)

concentrations, the influence of breeds on the serum sLe(x) concentration was unclear. Investigations using a larger number of dogs with a sufficient number of breeds may reveal the relationship between the serum sLe(x) concentration and breeds.

Blood sampling was performed only once in each patients in this preliminary study. Various sLe(x) concentrations in all groups and possibility of the influence of breed may represent the need to estimate baseline of serum sLe(x) concentrations in each dogs or breeds. In human breast cancers, the level of sLe(x) in the sera of patients was reported to elevate with tumor progression such as metastasis and recurrence [89, 90]. Continuous evaluation in the same patients for a longer-term period may clarify the clinical significance of serum sLe(x) in CMGT.

Conclusion

In this study, the specific expression of sLe(x) on CMGT tissues was observed, while the expression was not detected in non-neoplastic CMGT tissues and normal mammary tissues of healthy dogs. This may suggested that sLe(x) is a tumor-associated antigen in CMGT. However its expression of primary lesions seemed not to correlate with malignancy and prognosis of CMGT. The serum sLe(x) concentration was revealed to be various in dog patients. Significant correlation was not found between the level of serum sLe(x) and limited clinical features. A large prospective study should be needed to clarify the relation ship between sLe(x) expression and the tumor behavior.

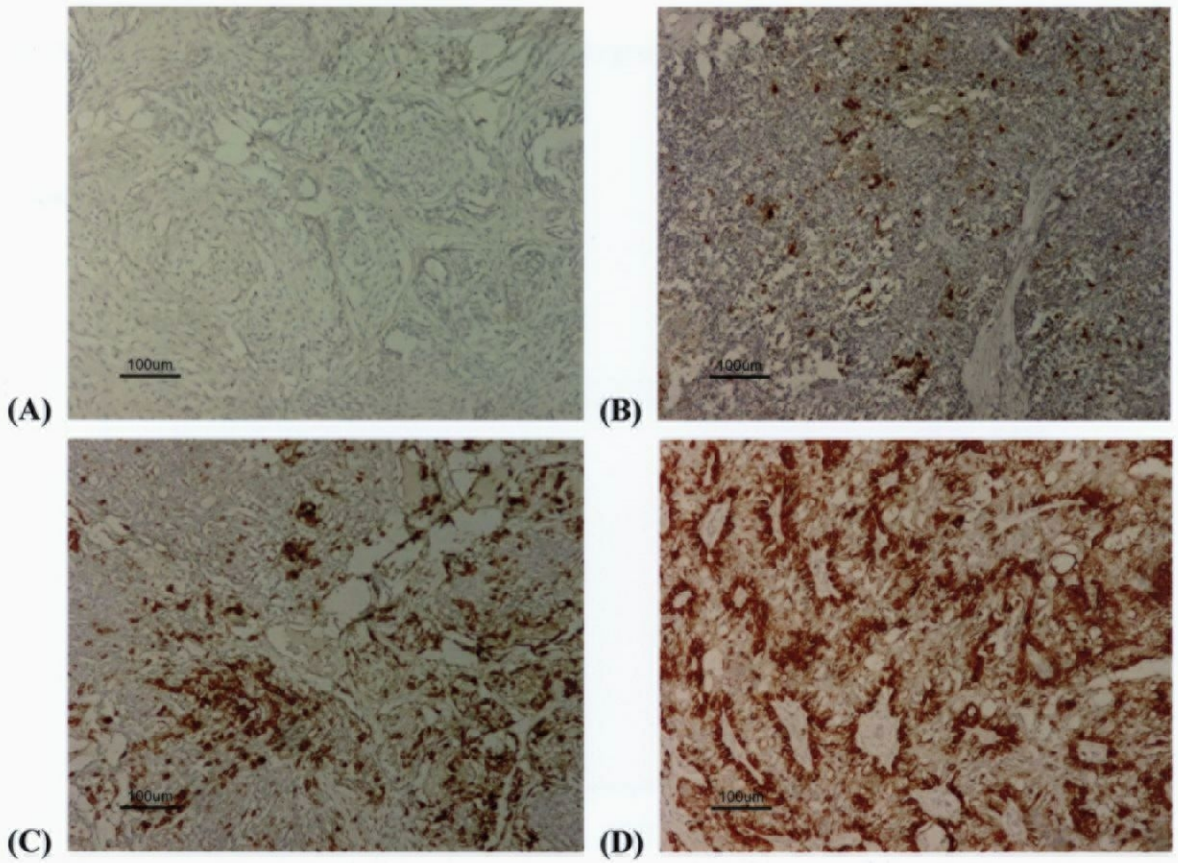

Fig. 3-1 Immunohistochemical analysis of sLe(x) on tissues of CMGT patients and healthy dogs with anti-sLe(x) antibody (magnification x100). Degree of sLe(x) antibody reactivity in individual tissue sections was defined as positive (+)(B), (++)(C) and (+++)(D) if unequivocal staining of the membrane or cytoplasm was observed in more than 5%, 20% and 40% of tumor cells, respectively. If it was less than 5%, the case was considered as negative (A).

Fig. 3-2 Survival curve of CMGT patients grouped by the degree of sLe(x) expression.

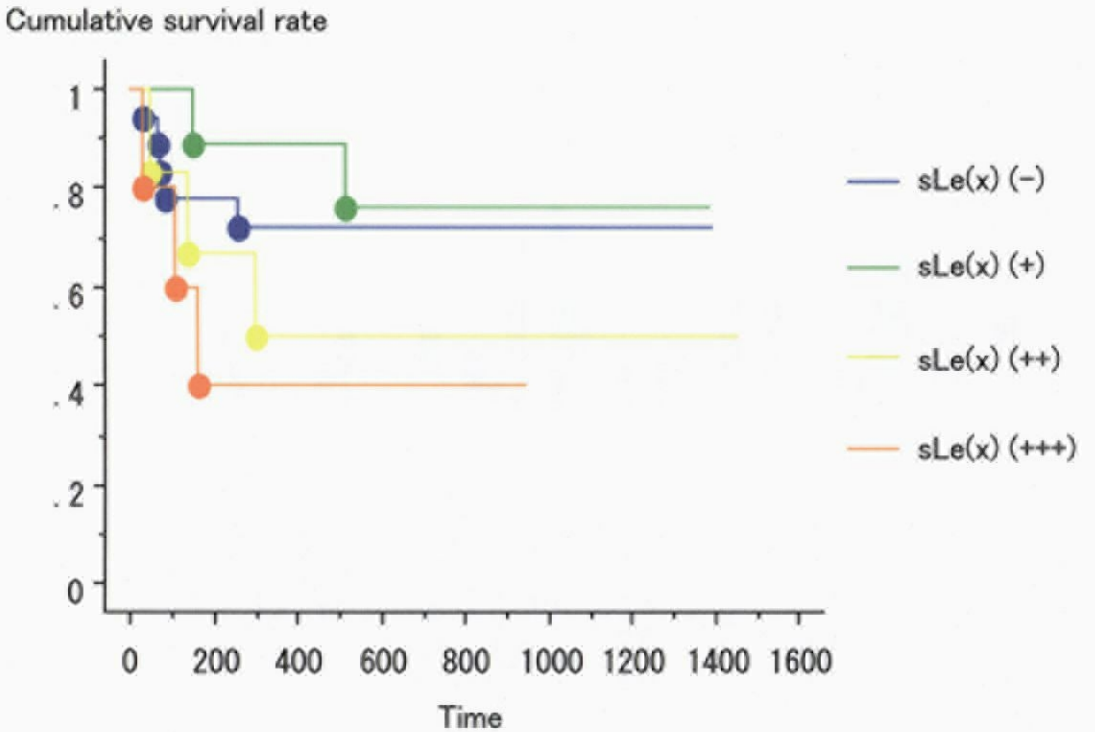

Fig. 3-3 Serum sLe(x) concentrations grouped by their breeds.

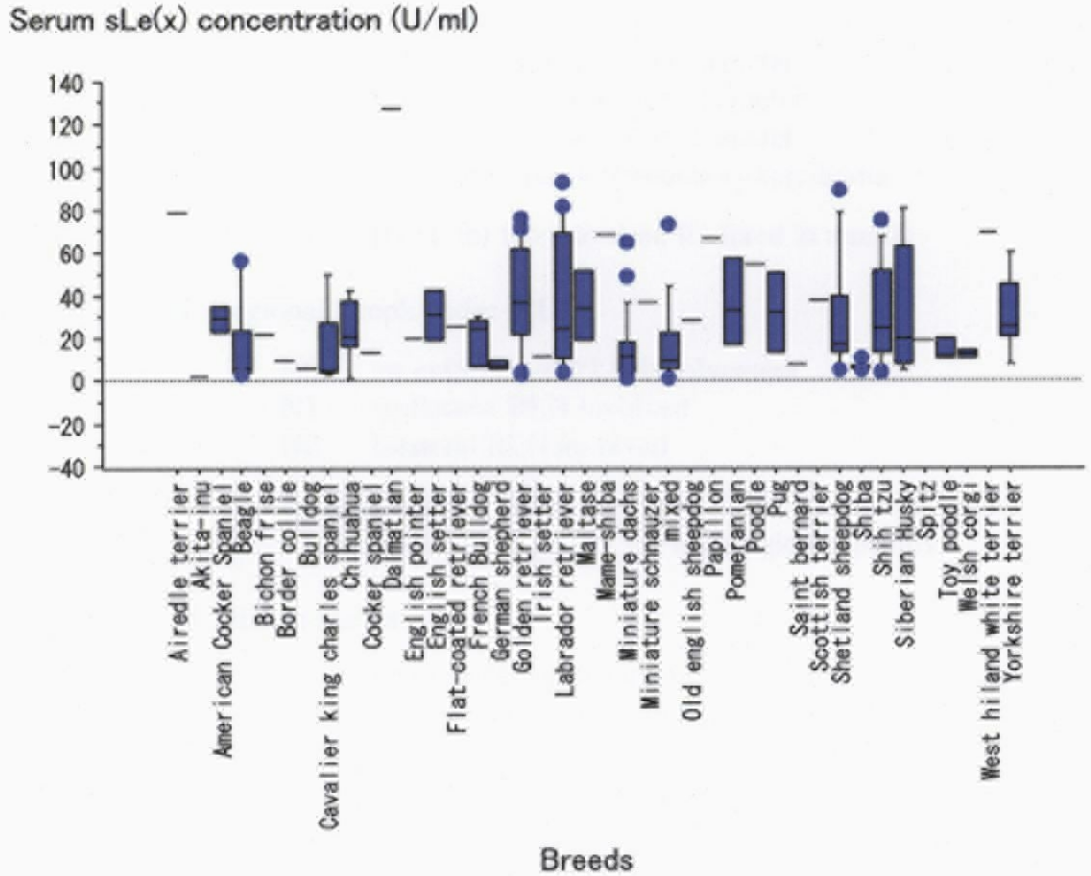

Table 3-1 The World Health Organization classification system for CMGT.

TNM classification

T: Primary tumor

T0	no evidence of tumor
T1	tumor <3cm. maximum diameter
T2	tumor 3-5cm. maximum diameter
T3	tumor >5cm. maximum diameter
T4	tumor any size, inflammatory carcinoma

(a) not fixed, (b) fixed to skin, (c) fixed to muscle

N: Regional lymph nodes (RLN)

N0	no evidence of RLN involvement
N1	ipsilateral RLN involved
N2	bilateral RLN involved

(a) not fixed, (b) fixed
(-) histologically negative, (+) histologically positive

M: Distant metastasis

M0	no evidence of distant metastasis
M1	distant metastasis including distant nodes

Clinical stage grouping

	TNM classification		
Clinical stage	T	N	M
I	T1a,b,c	N0(-),N1a(-),N2a(-)	M0
II	T0 T1a,b,c T2a,b,c	N1(+) N0(+),N1(+),N1a(+)	M0
III	any T3,T4 any T	any N any Nb	M0
IV	any T	any N	M1

Table 3-2 Clinical and pathological features of CMGT patients.

Variables	Adenoma (n=16)	Adeno-carcinoma (n=15)	Benign mixed tumor (n=22)	Malignant mixed tumor (n=3)
Clinical features				
Age (years)				
Mean ± SD	9.3±2.5	11.5±1.3	11.2±2.3	11.4±1.7
(Range)	(3.6-13.0)	(9.5-13.9)	(7.0-15.3)	(9.7-13.0)
Ovarian status				
intact	15	13	21	2
spayed	1	2	1	1
Weight (kg)				
Mean ± SD	10.5±8.7	10.9±10.3	6.4±4.8	2.5±0.7
(Range)	(2.1-27.4)	(3.1-34.3)	(2.6-21.5)	(1.9-3.3)
Clinical stage				
I	9	1	13	0
II	5	7	7	1
III	2	2	0	0
IV	0	5	2	2
Pathological features				
Tumor size (cm)				
Mean ± SD	3.0±2.2	4.4±1.3	2.9±2.5	3.8±1.9
(Range)	(0.5-7.5)	(1.5-6.0)	(0.8-11.0)	(2.5-6.0)
<3	9	2	14	1
3-5	5	8	7	1
>5	2	5	1	1
Regional lymph node involvement				
Positive	0	4	0	2
Negaive	16	11	22	1
Distant metastasis				
Positive	0	5	2	2
Negaive	16	10	20	1

Table 3-3 Expression of sLe(x) on tissues of CMGT patients and healthy dogs.

Histopathological diagnosis	Number of cases	sLe(x) -negative	sLe(x)-positive		
			+	++	+++
CMGT patients	56	27 (48.2%)	14 (25.0%)	9 (16.1%)	6 (10.7%)
Adenoma	16	8 (50.0%)	5 (31.3%)	3 (18.8%)	0
Adenocarcinoma	15	7 (46.7%)	3 (20.0%)	3 (20.0%)	2 (13.3%)
Benign mixed tumor	22	11 (50.0%)	5 (22.7%)	3 (13.6%)	3 (13.6%)
Malignant mixed tum	3	1 (33.3%)	1 (33.3%)	0	1 (33.3%)
Healthy dogs	5	5 (100%)	0	0	0
Mammary gland	5	5 (100%)	0	0	0

Table 3-4 Relationship between sLe(x) expression and clinicopathological features in CMGT.

Variables	sLe(x) expression				*P* value
	- (n=27)	+ (n=14)	++ (n=9)	+++ (n=6)	
Clinical features					
Age (years)					0.15
Mean ± SD	10.8±2.3	9.6±2.5	11.3±2.1	12.0±1.2	
(Range)	(6.1-15.3)	(3.6-14.0)	(6.8-14.0)	(10.3-13.3)	
Ovarian status					0.67
intact	24	13	9	5	
spayed	3	1	0	1	
Weight (kg)					0.41
Mean ± SD	10.2±8.5	6.9±6.4	8.6±10.1	5.1±2.4	
(Range)	(1.9-34.3)	(2.6-26.7)	(2.5-30.0)	(2.2-9.0)	
Clinical stage					0.54
I	14	5	3	1	
II	9	5	3	3	
III	1	1	2	0	
IV	3	3	1	2	
Pathological features					
Tumor size (cm)					0.58
Mean ± SD	2.8±1.7	3.6±2.7	3.1±2.6	3.9±1.7	
(Range)	(0.5-7.0)	(0.6-11.0)	(1.0-7.5)	(1.5-6.0)	
<3	14	6	4	2	0.95
3-5	10	5	3	3	
>5	3	3	2	1	
Regional lymph node involvement					0.94
Positive	3	1	1	1	
Negaive	24	13	8	5	
Distant metastasis					0.56
Positive	3	3	1	2	
Negaive	24	11	8	4	

Table 3-5 Prognostic outcome of CMGT patients grouped by both their histological diagnosis and degree of sLe(x) expression.

Outcome	Adenoma (n=7)	Adeno-carcinoma (n=11)	Benign mixed tumor (n=17)	Malignant mixed tumor (n=3)
Alive	7	1	10	0
Dead of disease	0	9	1	3
Dead of other causes	0	1	6	0

Outcome	sLe(x)-negative (n=18)	sLe(x)-positive + (n=9)	++ (n=6)	+++ (n=5)
Alive	9	5	3	1
Dead of disease	5	2	3	3
Dead of other causes	4	2	0	1

Table 3-6 Clinical features of dogs examined in serum sLe(x) concentration analysis.

Variables	Patients			Healthy dogs
	CMGTs (n=31)	Other tumors (n=51)	Nonneoplastic diseases (n=89)	(n=16)
Age (years)				
Mean ± SD	9.6±2.5	10.1±2.3	6.5±3.6	2.7±1.9
(Range)	(5.0-13.8)	(4.8-14.8)	(0.5-14.4)	(1.2-6.0)
Sex				
Male	0	30	50	11
(Castrated)	(0)	(11)	(9)	(2)
Female	31	21	39	5
(Spayed)	(6)	(12)	(19)	(4)
Blood examination				
WBC ($x10^3/\mu L$)	12.1±6.4	16.0±12.5	13.6±8.0	9.1±2.1
RBC ($x10^6/\mu L$)	7.12±0.81	6.37±1.28	6.67±1.18	6.58±0.71
Hb (g/dL)	16.4±1.8	14.7±3.0	15.7±2.9	15.0±1.3
Ht (%)	45.2±4.6	41.2±7.7	44.0±7.0	43.7±3.9
Plt ($x10^3/\mu L$)	406±199	326±142	332±161	232±57

Table 3-7 Serum sLe(x) concentrations of CMGT patients, other tumor patients, non-neoplastic disease patients and healthy dogs.

Diagnosis	Number of cases	Serum sLe(x) concentration	
		Mean ± SD	(Range)
Patients	171	28.8±24.9	(1.2-127.6)
CMGT	31	24.1±20.2	(1.4-81.9)
Adenoma	10	27.6±22.4	(6.0-81.9)
Adenocarcinoma	11	17.0±17.8	(1.4-50.4)
Benign mixed tumor	8	30.7±22.3	(5.3-66.0)
Malignant mixed tumo	2	20.2±1.6	(19.1-21.3)
Other tumor	51	36.3±29.9	(1.2-127.6)
Nonneoplastic disease	89	26.1±22.4	(1.2-92.8)
Healthy dogs	16	13.7±14.7	(3.7-56.7)
Total	187	27.5±24.5	(1.2-127.6)

Chapter 4

Analysis of sialyl Lewis X on CMGT-xenografted immunodeficient mice

Introduction

Distant metastasis defines malignancy of the tumor and is a major cause of tumor morbidity and mortality [93, 94]. Multiple pathogenic steps are involved in tumor metastasis. These include proliferation and detachment of tumor cells from the primary lesion, invasion to the surrounding extracellular matrix, angiogenesis, vascular or lymphatic dissemination, and eventually, homing of the tumor cells and proliferation at the distant organ [95]. Due to its complexity, the mechanism of metastasis remains unclear.

Experimental animal models are the useful tool to reveal the cellular and molecular changes associated with tumor behaviors in the living environment and have a potential for understanding disease mechanisms. Several models have been designed for the analysis of tumor metastasis including subcutaneous transplantation models [96], tail vein injection models[97], and implant models to several organs [98, 99].

CMGTs are the most common tumors in intact female dogs and reported to account for approximately 50% of all tumors in female dogs [2]. Tumor invasion to the surrounding tissues and metastasis to distal organs such as the lung are the most significant prognostic factors in malignant tumors [9-11]. There have been some reports of CMGT xenograft experimental animal models for the evaluation of drug and therapy [100, 101] and a few reports to investigate on the molecular mechanism of CMGT metastasis [102].

In the previous chapter, sLe(x) was detected on the cell surface of CMGT cell line

which had the adhesive ability to HUVECs. However, the correlation between the expression of sLe(x) and the behavior of CMGT such as metastasis and prognosis was not obscure in the previous study, though the sLe(x) expression was clear both on the tissues and in sera of CMGT patients.

The purpose of this study was to produce the experimental animal model of CMGT metastasis to the lung using severe combined immunodeficient (SCID) mice as model hosts and to evaluate the expression of sLe(x) in mice and cells with high potential of distant metastasis by pulmonary selection.

Materials and Methods

Cell line:

The CHMp cell line, derived from the primary lesion of a CMGT patient with pulmonary metastasis, was used in this study. This cell line was found to exhibit negative expression of sLe(x) in the previous chapter. The cells were cultured in RPMI1640 medium supplemented with 10% fetal bovine serum, 5 mg/L gentamicin sulfate and 6 mg/L fungizone and maintained at 37°C in a humidified atmosphere of 5% CO_2.

Mice:

Six- to seven-week-old female C.B-17 SCID-BEIGE mice (C.B-*Igh-1*^{b} GbmsTac-*Prkdc*^{scid}-*Lyst*^{bg} N7; Taconic, Germantown, NY, USA) were used as model hosts for the production of experimental pulmonary metastasis of CMGT. The mice were maintained in specific pathogen-free conditions: temperature of 24±1°C, humidity of 40-70% and a 12hr-light and dark cycle. Two or three mice were put in one cage (mouse clean S TPX; Clea Japan Inc., Tokyo, Japan) and the cages were set in a laminar flow rack housing system (ICM Inc., Ibaragi, Japan). Animals were fed with sterilized food (CL-2; Clea Japan) and water *ad libitum*.

Production of experimental pulmonary metastasis model:

CHMp cells were suspended in saline at a dose of 1 x10^{7} cells/200μl and subcutaneously injected into the right flank of SCID-BEIGE mice using a 21-gauge needle.

After the transplantation, the size of each tumor was measured using calipers. Tumor volume (V) was calculated according to the formula:

$$V=a \times b^2 \times 1/2 \ (mm^3)$$

with a and b being the tumor length and width (in mm), respectively. Mice were sacrificed at 6 weeks after CMGT cell injections. Masses produced at the site of transplantation and major organs were collected and measured their weight. The lung surface of each animal was inspected for the total number of metastatic tumor foci. Blood samples of each mouse were also collected for the measurement of serum sLe(x) concentration.

Primary culture of the cells collected from the primary tumor masses and lungs of mice was performed. Briefly, tissues were treated overnight with PBS containing 50 mg/L gentamicin sulfate. Then tissues were cut into small pieces and suspended in RPMI 1640 medium supplemented with 20% fetal bovine serum, 5 mg/L gentamicin sulfate and 6 mg/L fungizone, and incubated at 37°C in a humidified atmosphere of 5% CO_2. After the second passage, when cell growth appeared to be stable, the concentration of FBS in the culture medium was decreased from 20% to 10%. Cultured cells derived from metastatic lesion of the lung were collected and injected back to new mice for the next passage of pulmonary selection to establish highly lung metastatic cell lines of CMGT.

Western blot analysis:

The expressions of sLe(x) on the cells derived from mice in each passage of

pulmonary selection was evaluated by Western blotting analysis similar to the method in Chapter 1.

Immunohistochemistry:

Immunohistochemical analysis was performed to evaluate the expression of sLe(x) on primary tumor masses and the lung excised from mice. Tissues were fixed in 10% neutral-buffered formalin and embedded in paraffin. A series of 4 μm-thick sections were cut for immunohistologic and light microscopic examination. Immunohistochemical protocol was performed using a DAKO ENVISION+ kit/HRP (DAB)(DAKO) similar to that used in Chapters 2 and 3.

Assay of sLe(x) concentration in the blood:

The concentrations of sLe(x) in the blood samples were determined by sandwich enzyme immunoassay using the N-test EIA plate CSLEX kit (Nittobo Medical) similar to that used in Chapter 3.

Statistical analysis:

Comparison of serum sLe(x) concentrations between mice with different passages was made by Fisher's exact test. A probability of less than 5% ($P<0.05$) was considered significant in all the analyses.

Results

CMGT cell xenograft in SCID-BEIGE mice:

Six female SCID-BEIGE mice were used in each passage of pulmonary selection. All mice were sacrificed 6 weeks after transplantation of CMGT cells except for 4 mice in the third passage. Two mice were dead of tumor 37 and 38 days after transplantation in the third passage. Another two mice were euthanized for severe respiratory problems caused by pulmonary metastasis 36 and 38 days after transplantation. Growth of the tumor mass at the transplanted site was observed in all mice (Fig. 4-1). Ulceration of primary masses was observed in all mice after the second passage, whereas it was not observed in 4 of 6 mice in the first passage. In the third passage, necrosis and fluid collection was observed within the tumor masses, although the masses were solid till the second passage. Calculation of the tumor volumes in mice of the second passage could not be obtained. Therefore those in mice of the first and third passages were performed and shown in Fig. 4-2. The tumor volume increased significantly ($P<.0001$) in the third passage compared to that in the first passage.

Macroscopic findings of the lung were also shown in Fig. 4-3. Small foci on the surface of the lung were also observed in all mice and were confirmed histologically to be distant metastatic lesions of CMGT cells (Fig. 4-4). The number of metastatic foci observed on the lung surface increased according to the passages of pulmonary selection (Fig. 4-3). Metastases to the liver were observed after the second passage and the number of their foci

increased according to the passages (1 focus in 1/6 mice of the second passage versus 1, 5 and 8 foci in 3/6 mice of the third passage, respectively) (Fig. 4-3). The lymph node involvement was also observed in all passages (Fig. 4-3). The weights of whole body and major organs of CMGT xenografted mice were shown in Table 4-1. The weight of the lung increased due to metastasis in the second and third passage compared to that in the first passage significantly (P=0.026 and 0.006, respectively).

Established CMGT cell lines with high metastatic potential to the lung:

In an attempt to establish CMGT cell lines with high metastatic potential, I obtained 6 new cell lines, CHMp-p1/-m1, -p2/-m2 and -p3/-m3, through 3 passages of pulmonary selection. These cell lines were established from both primary and metastatic lesions of mice in each passage and showed stable growth in RPMI medium. The letter "-p" or "-m" indicates a primary or metastatic lesion and the number indicates the passages of pulmonary selection. These 6 cell lines were morphologically different from its parental CHMp in culture. These cells appeared to be more round in shape and tended to aggregate together compared to the parental cells (Fig. 4-5).

Analysis of sLe(x) in cell lines and tissues:

The expression of sLe(x) on these cell lines and tissues obtained from SCID-BEIGE mice transplanted with CMGT cells was evaluated. Cell lysates were extracted from CHMp-p1/-m1, -p2/-m2 and -p3/-m3 cells of the third passage for Western blot analysis.

Among 6 cell lines, no positive reaction was observed similar to that in their parental cell line; CHMp (Fig. 4-6).

Tissue samples obtained from both the primary mass and metastatic lesion of the lung were also examined by immunohistochemical analysis (Fig. 4-6). Similarly to the results of Western blot analysis, neither primary tumors nor metastatic foci were stained positively with sLe(x) on immunohistochemistry.

Serum sLe(x) concentration in xenotransplanted mice:

Blood samples were collected from sacrificed mice in each passage except 2 mice dead of tumor. Serum sLe(x) concentration of mice in the first, second and third passages were 0.003±0.00, 0.021±0.039 and 0.006±0.001, respectively. In comparison among passages, no significant difference was found.

Discussion

In the course of distant metastasis, several steps are thought to be involved and many factors were entangled intricately in each step [12, 13]. For the molecular analysis to understand the metastasis mechanism, *in vivo* experimental animal models are quite valuable to obtain the similar situation occurred in the tumor patients [96-99]. In this study, I tried to produce the experimental animal model of lung metastasis caused by CMGT cells. CHMp cells derived from the primary lesion of a CMGT patient with pulmonary metastasis showed rapid growth at the transplanted site and metastasized to the lung of T, B and NK cell deficient SCID-BEIGE mice. Metastatic tumor cells were cultured with stable growth. According to the passages of pulmonary selection, the weight of the lung increased significantly and more metastatic foci were observed macroscopically. This may be due to the metastasis of transplanted tumor cells. In addition, proliferation of transplanted tumor cells seemed to be accelerated by the findings such as the increased tumor volume, ulceration and necrosis within the center of masses. In the third passage, 4 mice were dead or euthanized with severe pulmonary metastasis. From these findings, 6 cell lines derived from both primary and metastatic lesions of CMGT-xenografted mice were thought to have different metastatic potential and malignancy according to the passages of pulmonary selection.

The expression of sLe(x) on the tissue of transplanted mice was examined by immunohistochemical analysis. Both primary masses and metastatic lesions in the lung

exhibited negative expression in all passages. Similar to the result of immunohistochemistry, no positive reaction was detected in Western blot analysis. Serum sLe(x) concentrations were lower than the limit of detection in all samples.

The mechanism of sLe(x) induction associated with tumor invasion and metastasis was not fully understood. In one study, it was reported that the expression of sLe(x) on tumor cells was induced under the condition of hypoxia [103]. Hypoxic conditions in the tumor mass were known to be observed in the rapid proliferation of tumor cells *in vivo*. In this study, tumor necrosis was observed within the center of masses in the third passage of the model, further repeat of pulmonary selection may lead to express sLe(x) in these CMGT cell lines.

For the understanding of complicated factors and their interactions in tumor metastasis, methods for detection of the cellular and molecular changes in cells and tissues have been performed. In this experimental animal model of CMGT metastasis, lung metastasis was observed in all transplanted mice after the short period of time. This model can provide useful samples with the same genomic background having different potential of distant metastasis. The role of sLe(x) in CMGT metastasis might be revealed by further investigations on the alternation and interaction of many factors including sLe(x) such as differential display and cDNA expression microarray analysis.

Conclusion

In this study, I successfully established 6 CMGT cell lines with distant metastatic potential from their parental cell line CMHp by 3 passages of pulmonary selection. As the xenograft in mice was repeated, CMGT cells showed higher metastatic potential and poorer prognosis *in vivo*. In these 3 passages, the expression of sLe(x) was not detected in cell lines and tissues. Although further pulmonary selections may induce the expression of sLe(x), the role and significance of sLe(x) in the metastasis were not clear in those 3 passages of the experimental metastasis model. Exhaustive analyses on the difference among these cell lines and parental cell line could provide the beneficial data for understanding the mechanism of CMGT metastasis.

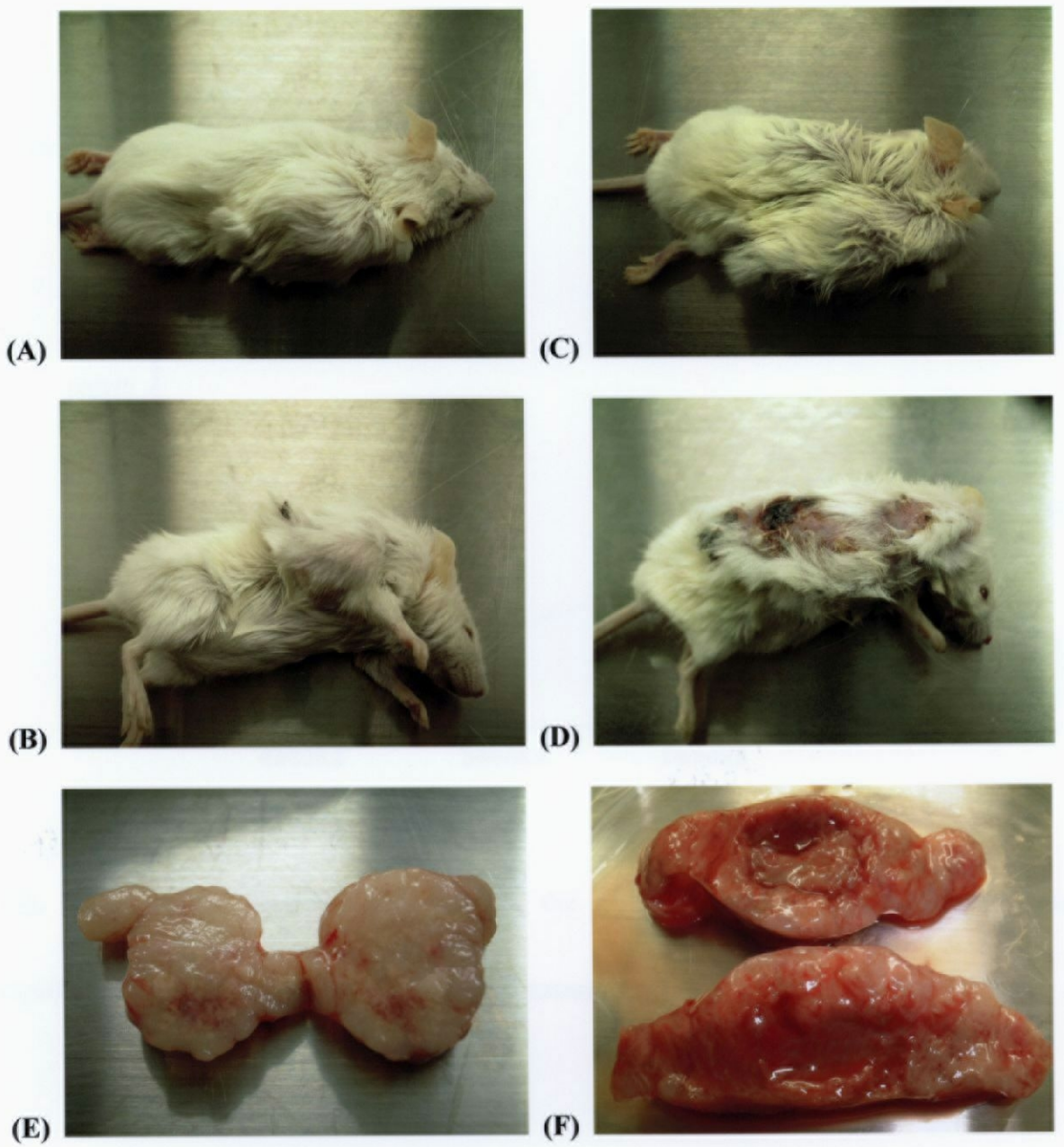

Fig. 4-1 Macroscopic findings of CMGT xenograft mice. The large tumor mass was formed at the transplanted site of SCID-BEIGE mice (mice of the first (A)(B) and third (C)(D) passages were shown). The cut surfaces of the tumor mass of the first and third passaged mice were shown in Fig. 4-1(E) and (F), respectively. Necrosis at the center of the mass with fluid collection was found in all mice after the third cycle.

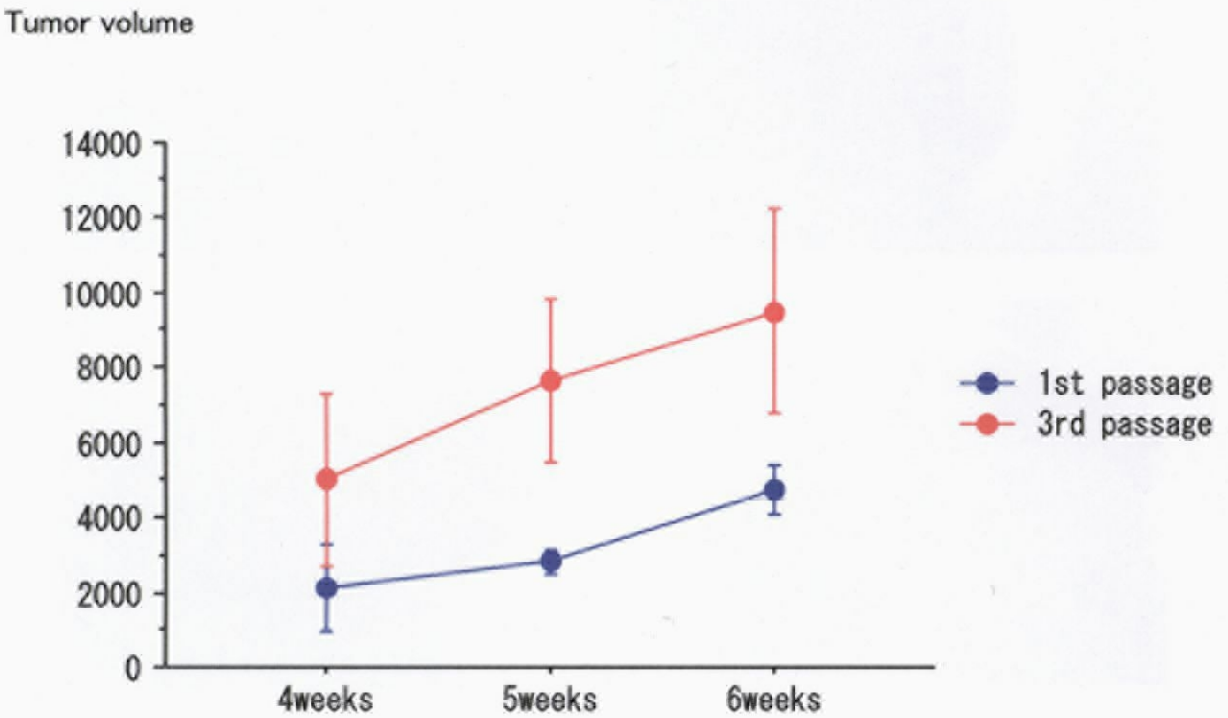

Fig. 4-2 Calculated tumor volume of the first and third passages. Tumor volume significantly ($P<.0001$) increased in the third passage compared to that in the first passage.

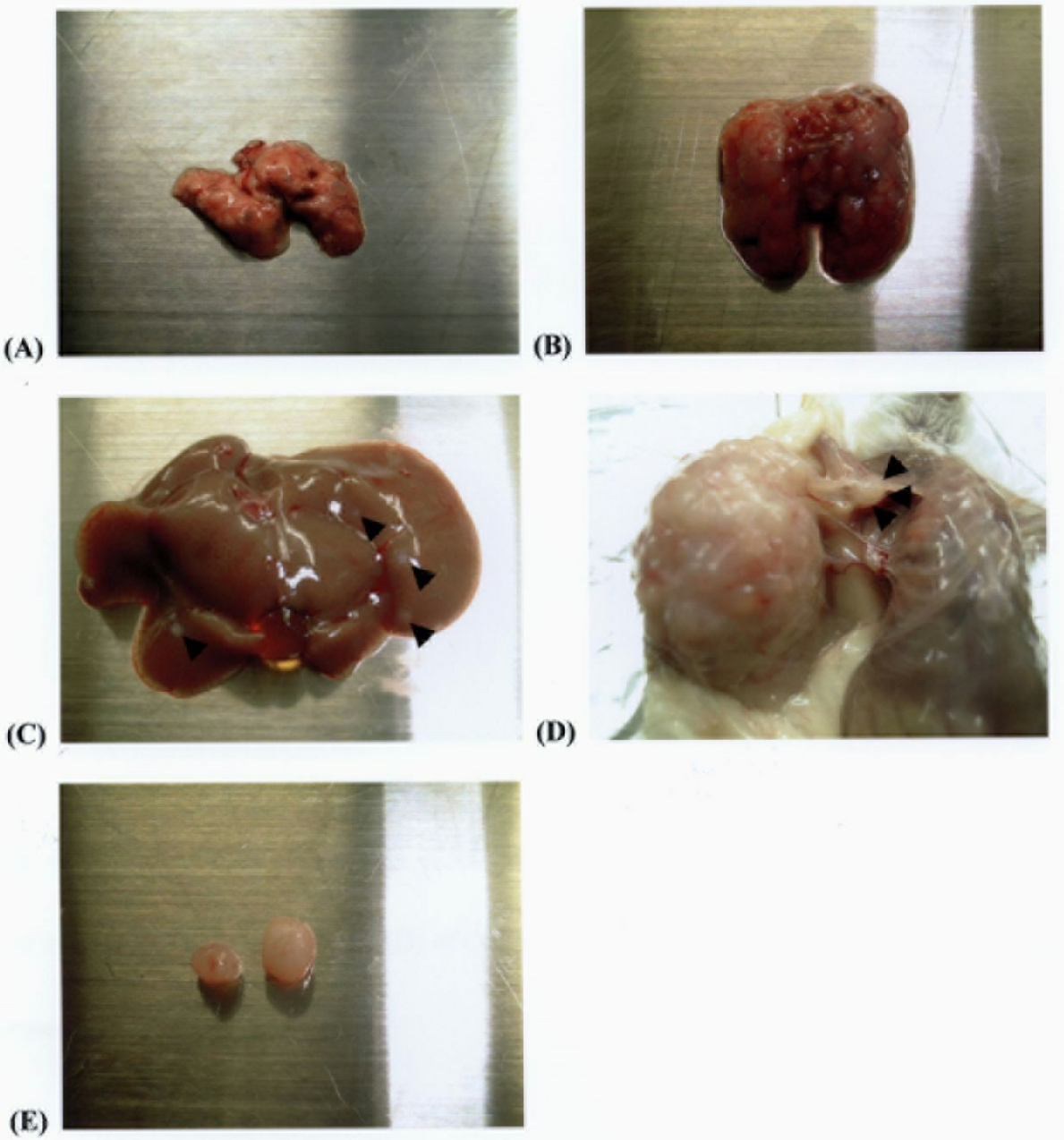

Fig. 4-3 Macroscopic findings of organs excised from CMGT xenograft mice. Numerous small foci were observed on the surface of the lung in all mice and the number was increased according to the passage of pulmonary selections (the lung of a mouse in the first (A) and third (B) passage was shown). Metastasis to the liver (C: arrow heads) and involvement of the regional lymph node (D: arrow heads)(E) was also observed.

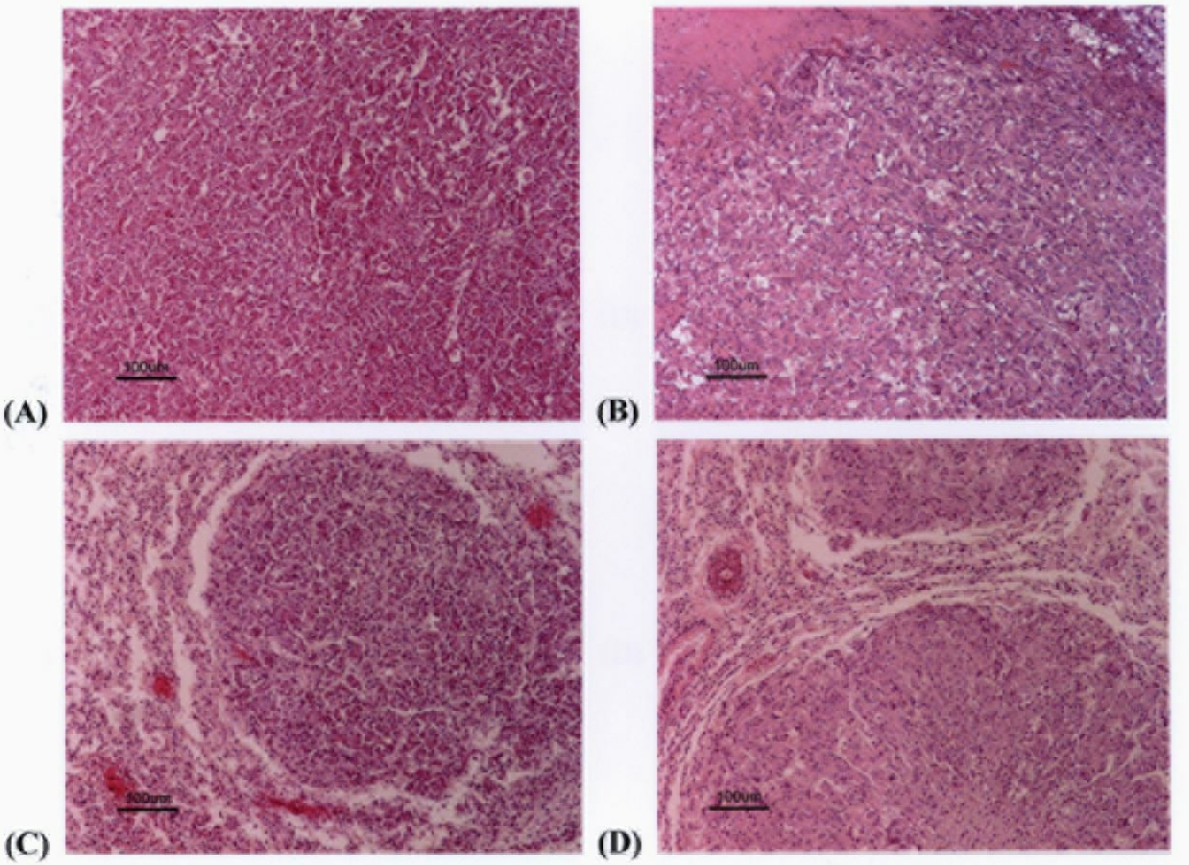

Fig. 4-4 Pathological findings of the masses developed at the transplanted site (A)(B) and metastatic foci in the lung (C)(D) of CMGT xenograft mice of the first and third passages, respectively (magnification x100).

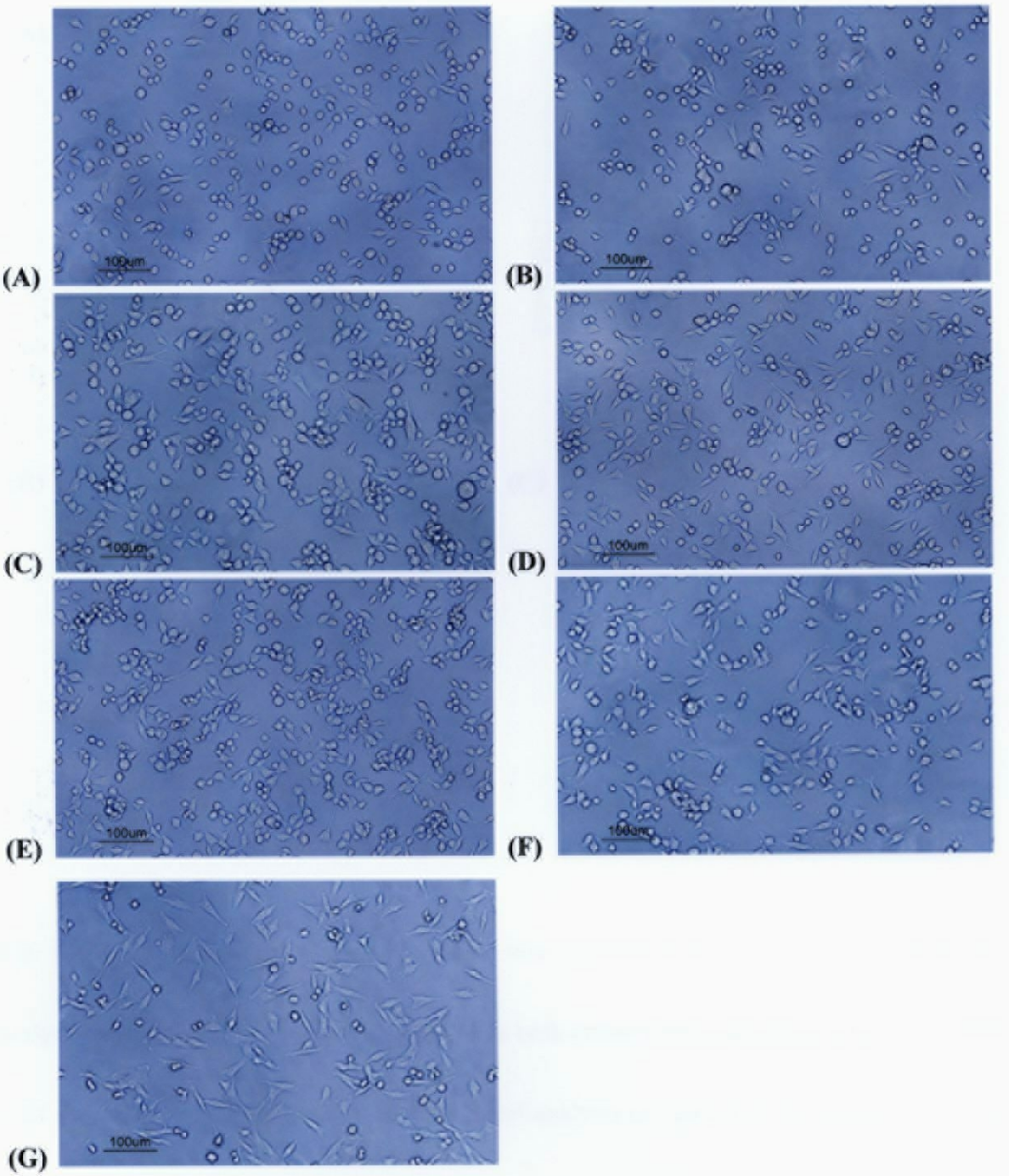

Fig. 4-5 Microscopic findings of new 6 cell lines, CHMp-p1 (A) /-m1 (B), -p2 (C) /-m2 (D) and -p3 (E) /-m3 (F), established from both primary and metastatic lesions of mice through the 3 passages of pulmonary selections. These cells appeared to be more round in shape and tended to aggregate together compared to the parental cells, CHMp (G) in culture.

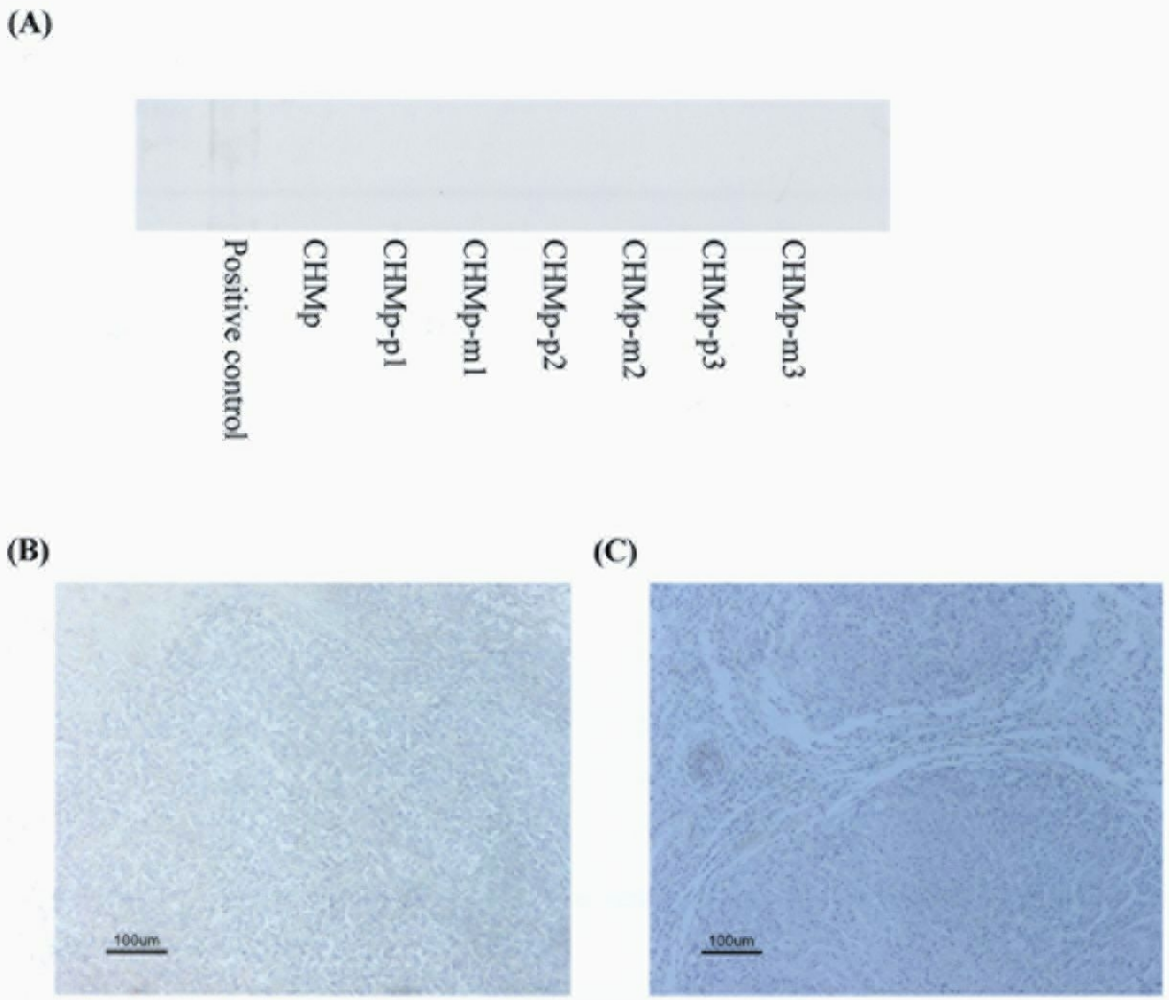

Fig. 4-6 Analysis of sLe(x) on the *in vivo* experimental mice model. Western blot analysis on the 6 cell lines newly derived from both primary and metastatic lesions of CHMp cells xenograft mice (A). Immunohistochemical analysis on tissues of the primary mass and the metastatic lung was also performed. Figures of the primary mass (B) and the lung (C) of a mouse of the third passage were shown (magnification x100). In both analyses, there was no positive reaction with sLe(x).

Table 4-1 The weights of whole body and major organs of CMGT xenograft SCID-BEIGE mice.

	1st passage	2nd passage	3rd passage
Body Weight	24.82±2.13	25.32±0.31	24.58±3.11
Tumor mass	5.75±1.41	7.31±1.45	7.12±1.81
Organs			
Lung	0.18±0.03	0.70±0.38 *	0.70±0.24 **
Heart	0.10±0.01	0.12±0.04	0.13±0.02
Liver	1.53±0.28	1.46±0.05	1.45±0.29
Spleen	0.20±0.06	0.32±0.18	0.24±0.11
Kidney	0.17±0.03 0.18±0.03	0.17±0.01 0.19±0.02	0.19±0.02 0.19±0.02

Data were expressed as mean ± SD (g)

* Significantly difference against the 1st passage (P=0.026)

** Significantly difference against the 1st passage (P=0.006)

Conclusion

Many factors have been found to be related to tumor invasion and metastasis through *in vitro* and *in vivo* studies on cancers. In several steps of tumor invasion and metastasis, several factors are thought to be involved such as cell adhesion molecules, like cadherin, selectin, integrin and other families [12, 14, 15]. In tumor cell proliferation, cell cycle and growth regulators [19-21], apoptosis-related molecules [22] and scatter folding factors [23] are supposed to be involved. Some receptors show overexpression in the course of tumorigenesis and represent a degree of differentiation of tumor cells [24, 25]. The identification and analysis of these factors has helped to clarify the mechanisms of tumorigenesis and malignant formation in human cancers, and some of them have been applied to clinical diagnosis and treatment [26-28]. However, basic information on the expression of these factors in CMGTs has been limited. Based on these backgrounds, a series of studies was carried out to investigate the factors associated with biological behaviors of CMGT, especially distant metastasis.

As a first step of this study, the expression of several oncological factors in 6 CMGT cell lines—3 pairs of cells derived from a primary and a metastatic lesion of 3 dog patients (CHMp/m, CIPp/m and CNMp/m)—was evaluated by Western blot analysis. Among 24 factors, the levels of sLe(x), 14-3-3 sigma, cyclinD1 and Rb, differed between the pairs. Especially, sLe(x) showed strong expression only in CHMm cell line derived from distant metastatic lesion. Though the number of cell lines used was limited, this result suggested that

sLe(x) might be related to distant metastasis in CMGTs and the pair of CHMp/m cells might be meaningful tools.

From the result of Chapter 1, I focused on this carbohydrate antigen, sLe(x) which adheres to E-selectin [30] and has been implicated in their adhesion to vascular endothelial cells in the acute inflammation process [31, 32]. The cell-cell adhesion mediated by the sLe(x)-E-selectin binding is also supposed to be involved in hematogenous metastasis of the cancer as the first adhesional step of tumor cells to the distal vascular endothelial cells prior to the integrin and immunoglobulin families [18, 33, 34, 79]. In human breast cancers, the expression of sLe(x) in both primary and metastatic lesions was reported and the relationship between its expression and the metastatic behavior of the tumor was implied [37, 38, 86]. The expression of sLe(x) was also reported in the sera of human breast cancer patients and the level of sLe(x) was found to elevate with metastatic breast cancers [89, 90].

In Chapter 2, I investigated the expression and localization of sLe(x) on the cultured cells and its adhesional function to blood vessel epithelial cells. On immunohistochemistry, only CHMm cells showed strong expression of sLe(x) on their cell surface among 6 CMGT cell lines and this result supported that of the western blotting analysis in the previous chapter. Using cell lines of CHM pair, I evaluated the adhesional function of sLe(x) on CMGT cells by the cell adhesion assay in 96-well plates and the flow through chamber adhesion assay. The number of attached CHMm cells to HUVECs which expressed E-selectin by the stimulus

of rhTNF-α was significantly increased under conditions with calcium, which is necessary for the binding activity of E-selectin. Under flow conditions, I could see the process of cell-cell adhesion, called rolling. This phenomenon was reported in the adhesion between leukocytes and blood vessel endothelial cells by the sLe(x)-E-selectin adhesional interaction [31, 32, 76]. From these results, CHMm cell line was revealed to have the adhesional ability to blood vessel endothelial cells by the sLe(x)-E-selectin binding and might be related to the distant metastasis of CMGTs.

To investigate whether the expression of sLe(x) correlated with the behavior of CMGT such as metastasis and prognosis, I examined the sLe(x) expression on the CMGT tissues surgically removed from the spontaneous patients and performed a preliminary study on the serum concentration of sLe(x) in dogs including CMGT patients in Chapter 3. A half of neoplastic tissues were found to express sLe(x) in CMGT patients, whereas both non-neoplastic tissues of CMGT patients and normal mammary gland tissues of healthy dogs exhibited negative on immunohistochemical analysis. Serum sLe(x) concentrations in patients of CMGT, other tumor and nonneoplastic disease and healthy dogs were also examined. Measured values were various in all groups with no significant difference. The specific expression of sLe(x) on CMGT tissues may suggested that sLe(x) is a tumor-associated antigen in CMGT. However its expression of primary lesions and its level in sera seemed not to correlate with malignancy and prognosis of CMGT.

In the course of distant metastasis, several steps are thought to be involved and several factors were entangled intricately in each step. To obtain the similar situation occurred in the tumor patients, *in vivo* experimental animal models are quite valuable. In Chapter 4, I tried to produce the experimental animal model of lung metastasis caused by CMGT cells and examined the expression of sLe(x) in this model. By pulmonary selection, 6 new cell lines derived from both primary and metastatic lesions of CMGT-xenografted mice were obtained and they were thought to have different metastatic potential and malignancy according to the passages of selection. On immunohistochemical analysis, both primary masses and metastatic lesions in the lung exhibited negative expression of sLe(x) in all passages and no positive reaction was also detected in Western blot analysis. Serum sLe(x) concentrations were lower than the limit of detection in all samples. Although further pulmonary selections may induce the expression of sLe(x), the role and significance of sLe(x) in the metastasis were not clear in those 3 passages of this experimental metastasis model.

In this series of the present study, I focused on sLe(x) and investigated its expression and the relationship to the behavior of CMGT *in vitro* and *in vivo*. From the result of this study, the expression of this ligand might be related to tumorigenesis of CMGT and was thought to have the potential to metastasize via blood vessel. However, the relationship between its expression and behavior of CMGTs such as clinicopathological features, distant metastasis and prognosis was not found in this study. The mechanism of sLe(x) expression is

still controversy and further *in vitro* studies might be needed to understand its roles through the investigations on glycosyltransferases which synthesize sLe(x). To clarify the relation ship between sLe(x) expression and the tumor behavior *in vivo*, a large prospective study should be needed including the examination of the sLe(x) expression on the metastatic lesion of CMGT and the serial evaluation of serum sLe(x) concentrations of the same patient for the longer period. Experimental animal model obtained in this study can provide useful samples with the same genomic background having different potential of distant metastasis. The role of sLe(x) in CMGT metastasis might be revealed by further investigations on the alternation and interaction of many factors including sLe(x) such as differential display and cDNA expression microarray analysis.

Acknowledgements

I wish to express my gratitude to my mentor, Professor Nobuo Sasaki, Laboratory of Veterinary Surgery, Graduate School of Agricultural and Life Sciences, the University of Tokyo, for his continuous supervision, support, guidance and challenge he provided along the way. I would like to thank Associate Professor Ryohei Nishimura and Assistant Professor Manabu Mochizuki, Laboratory of Veterinary Surgery, the University of Tokyo for their precious consultation, advice and discussion during the study. I would also like to thank Professor Hiroyuki Ogawa, Laboratory of Veterinary Emergency Medicine, the University of Tokyo for his kind advice and contributions to my study. I also thank Professor Sumio Sugano and Dr. Manabu Watanabe, Laboratory of Functional Genomics, Graduate School of Frontier Sciences, the University of Tokyo for their advice, support and discussion during this study.

I appreciate Drs. Emi Ohashi and Noriko Kanaya for cordial support and continuous encouragement during this study. My thanks are also due to all the member of Laboratory of Veterinary Surgery, the University of Tokyo. I am indebted to everyone who contributed to this study. I am thankful for the assistance from many people associated with this project.

References

1. Dorn, C.R., et al., *Survey of animal neoplasms in Alameda and Contra Costa Counties, California. II. Cancer morbidity in dogs and cats from Alameda County.* J Natl Cancer Inst, 1968. **40**(2): p. 307-18.

2. Brodey, R.S., M.H. Goldschmidt, and J.R. Roszel, *Canine mammary gland neoplasms.* J Am Anim Hosp Assoc, 1983. **19**: p. 61-90.

3. Moe, L., *Population-based incidence of mammary tumours in some dog breeds.* J Reprod Fertil Suppl, 2001. **57**: p. 439-43.

4. Withrow, S.J. and E.G. MacEwen, *Tumors of the mammary gland.* 3rd ed. ed. Small animal clinical oncology, ed. S.J. Withrow and E.G. MacEwen. 2001, Philadelphia: WB Saunders Co. 455-477.

5. Gilbertson, S.R., et al., *Canine mammary epithelial neoplasms: biologic implications of morphologic characteristics assessed in 232 dogs.* Vet Pathol, 1983. **20**(2): p. 127-42.

6. Hellmen, E., *The pathogenesis of canine mammary tumors.* Cancer J, 1996. **9**: p. 282-286.

7. Gartner, F., et al., *DNA measurement and immunohistochemical characterization of epithelial and mesenchymal cells in canine mixed mammary tumours: putative evidence for a common histogenesis.* Vet J, 1999. **158**(1): p. 39-47.

8. Susaneck, S.J., T.A. Allen, and J. Hoopes, *Inflammatory mammary carcinoma in the dog.* J Am Anim Hosp Assoc, 1983. **19**(7): p. 971-976.

9. Kurzman, I.D. and S.R. Gilbertson, *Prognostic factors in canine mammary tumors.* Semin Vet Med Surg (Small Anim), 1986. **1**(1): p. 25-32.

10. Shofer, F.S., et al., *Histopathologic and dietary prognostic factors for canine mammary carcinoma.* Breast Cancer Res Treat, 1989. **13**(1): p. 49-60.

11. Hahn, K.A., R.C. Richardson, and D.W. Knapp, *Canine malignant mammary neoplasia: Biologic behavior, diagnosis and treatment alternatives.* J Am Anim Hosp Assoc, 1992. **28**: p. 251-256.

12. Tang, D.G. and K.V. Honn, *Adhesion molecules and tumor metastasis: an update.* Invasion Metastasis, 1994. **14**(1-6): p. 109-22.

13. Woodhouse, E.C., R.F. Chuaqui, and L.A. Liotta, *General mechanisms of metastasis.* Cancer, 1997. **80**(8 Suppl): p. 1529-37.

14. Crawford, H.C. and L.M. Matrisian, *Tumor and stromal expression of matrix metalloproteinases and their role in tumor progression.* Invasion Metastasis, 1994. **14**(1-6): p. 234-45.

15. Bikfalvi, A., *Significance of angiogenesis in tumour progression and metastasis.* Eur J Cancer, 1995. **31A**(7-8): p. 1101-4.

16. Frixen, U.H., et al., *E-cadherin-mediated cell-cell adhesion prevents invasiveness of*

human carcinoma cells. J Cell Biol, 1991. **113**(1): p. 173-85.

17. Vleminckx, K., et al., *Genetic manipulation of E-cadherin expression by epithelial tumor cells reveals an invasion suppressor role.* Cell, 1991. **66**(1): p. 107-19.

18. Turner, G.A., *Surface properties of the metastatic cell.* Invasion Metastasis, 1982. **2**(4): p. 197-216.

19. Narita, T., et al., *Proliferating cell nuclear antigen immunostaining in breast cancer and its relation to prognosis.* Jpn J Clin Oncol, 1993. **23**(1): p. 20-5.

20. Sherr, C.J., *Cancer cell cycles.* Science, 1996. **274**(5293): p. 1672-7.

21. Howe, L.R. and A.M. Brown, *Wnt signaling and breast cancer.* Cancer Biol Ther, 2004. **3**(1): p. 36-41.

22. Sawan, A., et al., *Retinoblastoma and p53 gene expression related to relapse and survival in human breast cancer: an immunohistochemical study.* J Pathol, 1992. **168**(1): p. 23-8.

23. Dubois, T., et al., *Structure and sites of phosphorylation of 14-3-3 protein: role in coordinating signal transduction pathways.* J Protein Chem, 1997. **16**(5): p. 513-22.

24. Slamon, D.J., et al., *Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene.* Science, 1987. **235**(4785): p. 177-82.

25. Issemann, I. and S. Green, *Activation of a member of the steroid hormone receptor superfamily by peroxisome proliferators.* Nature, 1990. **347**(6294): p. 645-50.

26. Narita, T., et al., *Serum and immunohistochemical studies of NCC-ST-439 in breast cancer.* J Surg Oncol, 1993. **54**(1): p. 5-8.

27. Gion, M., et al., *Comparison of the diagnostic accuracy of CA27.29 and CA15.3 in primary breast cancer.* Clin Chem, 1999. **45**(5): p. 630-7.

28. Ross, J.S. and J.A. Fletcher, *The HER-2/neu oncogene in breast cancer: prognostic factor, predictive factor, and target for therapy.* Stem Cells, 1998. **16**(6): p. 413-28.

29. Uyama, R., et al., *Establishment of four pairs of canine mammary tumor cell lines derived from primary and metastatic origin and their E-cadherin expression.* Vet Comp Oncol, (*under submission*).

30. Phillips, M.L., et al., *ELAM-1 mediates cell adhesion by recognition of a carbohydrate ligand, sialyl-Lex.* Science, 1990. **250**(4984): p. 1130-2.

31. Lowe, J.B., et al., *ELAM-1--dependent cell adhesion to vascular endothelium determined by a transfected human fucosyltransferase cDNA.* Cell, 1990. **63**(3): p. 475-84.

32. Berg, E.L., et al., *A carbohydrate domain common to both sialyl Le(a) and sialyl Le(X) is recognized by the endothelial cell leukocyte adhesion molecule ELAM-1.* J Biol Chem, 1991. **266**(23): p. 14869-72.

33. Fukushima, K., et al., *Characterization of sialosylated Lewisx as a new tumor-associated antigen.* Cancer Res, 1984. **44**(11): p. 5279-85.

34. Fukushi, Y., et al., *Location and distribution of difucoganglioside (VI3NeuAcV3III3Fuc2nLc6) in normal and tumor tissues defined by its monoclonal antibody FH6.* Cancer Res, 1985. **45**(8): p. 3711-7.

35. Sugiyama, K., et al., *Tumor-associated carbohydrate antigens in primary pulmonary adenocarcinomas and their metastases.* Hum Pathol, 1992. **23**(8): p. 900-4.

36. Nakagoe, T., et al., *Immunohistochemical expression of sialyl Lex antigen in relation to survival of patients with colorectal carcinoma.* Cancer, 1993. **72**(8): p. 2323-30.

37. Renkonen, J., T. Paavonen, and R. Renkonen, *Endothelial and epithelial expression of sialyl Lewis(x) and sialyl Lewis(a) in lesions of breast carcinoma.* Int J Cancer, 1997. **74**(3): p. 296-300.

38. Nakagoe, T., et al., *Immunohistochemical expression of ABH/Lewis-related antigens in primary breast carcinomas and metastatic lymph node lesions.* Cancer Detect Prev, 1998. **22**(6): p. 499-505.

39. Oka, H., et al., *Expression of E-cadherin cell adhesion molecules in human breast cancer tissues and its relationship to metastasis.* Cancer Res, 1993. **53**(7): p. 1696-701.

40. Yoshida, R., et al., *The loss of E-cadherin, alpha- and beta-catenin expression is associated with metastasis and poor prognosis in invasive breast cancer.* Int J Oncol, 2001. **18**(3): p. 513-20.

41. Spieker, N., et al., *E-cadherin expression and in vitro invasion of canine mammary tumor cells.* Eur J Cell Biol, 1995. **68**(4): p. 427-36.

42. Restucci, B., et al., *E cadherin expression in normal and neoplastic canine mammary gland.* J Comp Pathol, 1997. **116**(2): p. 191-202.

43. Reis, A.L., et al., *Immunohistochemical study of the expression of E-cadherin in canine mammary tumours.* Vet Rec, 2003. **152**(20): p. 621-4.

44. Hellmen, E. and A. Lindgren, *The expression of intermediate filaments in canine mammary glands and their tumors.* Vet Pathol, 1989. **26**(5): p. 420-8.

45. Jones, T.C., R.D. Hunt, and N.W. King, *Mammary gland.* 6th ed. ed. Veterinary Pathology, ed. T.C. Jones, R.D. Hunt, and N.W. King. 1997, Baltimore: Williams & Wilkins. 1190-1198.

46. Moll, R., *Cytokeratins in the histological diagnosis of malignant tumors.* Int J Biol Markers, 1994. **9**(2): p. 63-9.

47. Herwig, S. and M. Strauss, *The retinoblastoma protein: a master regulator of cell cycle, differentiation and apoptosis.* Eur J Biochem, 1997. **246**(3): p. 581-601.

48. Barbareschi, M., et al., *Cyclin-D1-gene amplification and expression in breast carcinoma: relation with clinicopathologic characteristics and with retinoblastoma gene product, p53 and p21WAF1 immunohistochemical expression.* Int J Cancer, 1997. **74**(2): p. 171-4.

49. Yamashita, H., et al., *Analysis of oncogenes and tumor suppressor genes in human breast cancer.* Jpn J Cancer Res, 1993. **84**(8): p. 871-8.

50. Jares, P., et al., *Cyclin D1 and retinoblastoma gene expression in human breast carcinoma: correlation with tumour proliferation and oestrogen receptor status.* J Pathol, 1997. **182**(2): p. 160-6.

51. Hirohashi, S. and Y. Kanai, *Cell adhesion system and human cancer morphogenesis.* Cancer Sci, 2003. **94**(7): p. 575-81.

52. Gopalakrishna, R., et al., *Differential distribution of protein phosphatase 2A in human breast carcinoma cell lines and its relation to estrogen receptor status.* Cancer Lett, 1999. **136**(2): p. 143-51.

53. Friedrichs, K., et al., *Overexpression of p53 and prognosis in breast cancer.* Cancer, 1993. **72**(12): p. 3641-7.

54. Rungsipipat, A., et al., *Immunohistochemical analysis of c-yes and c-erbB-2 oncogene products and p53 tumor suppressor protein in canine mammary tumors.* J Vet Med Sci, 1999. **61**(1): p. 27-32.

55. Haga, S., et al., *Overexpression of the p53 gene product in canine mammary tumors.* Oncol Rep, 2001. **8**(6): p. 1215-9.

56. Lee, C.H., et al., *Mutation and overexpression of p53 as a prognostic factor in canine mammary tumors.* J Vet Sci, 2004. **5**(1): p. 63-9.

57. Aitken, A., et al., *The role of specific isoforms of 14-3-3 protein in regulating protein kinase activity in the brain.* Biochem Soc Trans, 1992. **20**(3): p. 607-11.

58. Vercoutter-Edouart, A.S., et al., *Proteomic analysis reveals that 14-3-3sigma is down-regulated in human breast cancer cells.* Cancer Res, 2001. **61**(1): p. 76-80.

59. Urano, T., et al., *Efp targets 14-3-3 sigma for proteolysis and promotes breast tumour growth.* Nature, 2002. **417**(6891): p. 871-5.

60. Slamon, D.J., et al., *Studies of the HER-2/neu proto-oncogene in human breast and ovarian cancer.* Science, 1989. **244**(4905): p. 707-12.

61. Sugano, K., et al., *Sandwich radioimmunometric assay with murine monoclonal antibody, NCC-ST-439, for serological diagnosis of human cancers.* Jpn J Cancer Res, 1988. **79**(5): p. 618-25.

62. Yamaguchi, A., et al., *Clinical significance of tumor marker NCC-ST 439 in large bowel cancers.* Dis Colon Rectum, 1991. **34**(10): p. 921-4.

63. Anan, K., et al., *Postoperative follow-up of patients with early breast cancer: reappraisal of serum tumor markers.* Surg Today, 2002. **32**(1): p. 13-8.

64. Rutteman, G.R., et al., *Prolactin binding in benign and malignant mammary tissue of female dogs.* Anticancer Res, 1986. **6**(4): p. 829-35.

65. Mueller, E., et al., *Terminal differentiation of human breast cancer through PPAR gamma.* Mol Cell, 1998. **1**(3): p. 465-70.

66. Kitamura, S., et al., *Peroxisome proliferator-activated receptor gamma induces growth arrest and differentiation markers of human colon cancer cells.* Jpn J Cancer Res, 1999. **90**(1): p. 75-80.

67. Yoshimura, R., et al., *The effect of peroxisome proliferator-activated receptor-gamma ligand on urological cancer cells.* Int J Mol Med, 2003. **12**(6): p. 861-5.

68. Keshamouni, V.G., et al., *Peroxisome proliferator-activated receptor-gamma activation inhibits tumor progression in non-small-cell lung cancer.* Oncogene, 2004. **23**(1): p. 100-8.

69. Motomura, W., et al., *Inhibition of cell invasion and morphological change by troglitazone in human pancreatic cancer cells.* J Gastroenterol, 2004. **39**(5): p. 461-8.

70. Rosen, S.D., et al., *Involvement of sialic acid on endothelial cells in organ-specific lymphocyte recirculation.* Science, 1985. **228**(4702): p. 1005-7.

71. Rosen, S.D. and C.R. Bertozzi, *The selectins and their ligands.* Curr Opin Cell Biol, 1994. **6**(5): p. 663-73.

72. Somers, W.S., et al., *Insights into the molecular basis of leukocyte tethering and rolling revealed by structures of P- and E-selectin bound to SLe(X) and PSGL-1.* Cell, 2000. **103**(3): p. 467-79.

73. Kaila, N. and B.E.t. Thomas, *Design and synthesis of sialyl Lewis(x) mimics as E- and P-selectin inhibitors.* Med Res Rev, 2002. **22**(6): p. 566-601.

74. Paulson, J.C. and K.J. Colley, *Glycosyltransferases. Structure, localization, and control of cell type-specific glycosylation.* J Biol Chem, 1989. **264**(30): p. 17615-8.

75. Fukuda, M., et al., *Structure of sialylated fucosyl lactosaminoglycan isolated from human granulocytes.* J Biol Chem, 1984. **259**(17): p. 10925-35.

76. Lawrence, M.B. and T.A. Springer, *Leukocytes roll on a selectin at physiologic flow rates: distinction from and prerequisite for adhesion through integrins.* Cell, 1991. **65**(5): p. 859-73.

77. Lasky, L.A., *Lectin cell adhesion molecules (LEC-CAMs): a new family of cell adhesion proteins involved with inflammation.* J Cell Biochem, 1991. **45**(2): p. 139-46.

78. Vestweber, D. and J.E. Blanks, *Mechanisms that regulate the function of the selectins and their ligands.* Physiol Rev, 1999. **79**(1): p. 181-213.

79. Berman, A.E. and N.I. Kozlova, *Integrins: structure and functions.* Membr Cell Biol, 2000. **13**(2): p. 207-44.

80. Nakamori, S., et al., *Involvement of carbohydrate antigen sialyl Lewis(x) in colorectal cancer metastasis.* Dis Colon Rectum, 1997. **40**(4): p. 420-31.

81. Nemoto, Y., et al., *Comparison of 16 human colon carcinoma cell lines for their expression of sialyl LeX antigens and their E-selectin-dependent adhesion.* Clin Exp Metastasis, 1998. **16**(6): p. 569-76.

82. Kurahara, S., et al., *Immunohistochemical study of sialyl Le(a) and sialyl Le(x) antigen in oral squamous cell carcinoma: the association of sialyl Le(a) expression with the metastatic potential.* Head Neck, 1999. **21**(4): p. 330-7.

83. Hakomori, S., *Aberrant glycosylation in cancer cell membranes as focused on glycolipids: overview and perspectives.* Cancer Res, 1985. **45**(6): p. 2405-14.

84. Sakamoto, S., et al., *Expression of Lewisa, Lewisb, Lewisx, Lewisy, siayl-Lewisa, and sialyl-Lewisx blood group antigens in human gastric carcinoma and in normal gastric tissue.* Cancer Res, 1989. **49**(3): p. 745-52.

85. Farmer, R.W., W.J. Richtsmeier, and R.L. Scher, *Identification of sialyl Lewis-x in squamous cell carcinoma of the head and neck.* Head Neck, 1998. **20**(8): p. 726-31.

86. Narita, T., et al., *Association of expression of blood group-related carbohydrate antigens with prognosis in breast cancer.* Cancer, 1993. **71**(10): p. 3044-53.

87. Ferdeghini, M., et al., *Preoperative serum intercellular adhesion molecule-1 (ICAM-1) and E-selectin (endothelial cell leukocyte adhesion molecule, ELAM-1) in patients with epithelial ovarian cancer.* Anticancer Res, 1995. **15**(5B): p. 2255-60.

88. Lim, A.G., et al., *Soluble E-selectin and vascular cell adhesion molecule-1 (VCAM-1) in primary biliary cirrhosis.* J Hepatol, 1995. **22**(4): p. 416-22.

89. Kannagi, R., et al., *Quantitative and qualitative characterization of human cancer-associated serum glycoprotein antigens expressing fucosyl or sialyl-fucosyl*

type 2 chain polylactosamine. Cancer Res, 1986. **46**(5): p. 2619-26.

90. Matsuura, N., et al., *Increased level of circulating adhesion molecules in the sera of breast cancer patients with distant metastases.* Jpn J Clin Oncol, 1997. **27**(3): p. 135-9.

91. Owen, L.N., *Classification of Tumors in Domestic Animals*. 1st ed ed. 1980, Geneva: World Health Organization.

92. 吉野浩之, *乳癌における血清sialyl Le^X 測定の臨床的意義.* 東京女子医科大学雑誌, 1993. **63**(9): p. 1008-1024.

93. Kohn, E.C. and L.A. Liotta, *Molecular insights into cancer invasion: strategies for prevention and intervention.* Cancer Res, 1995. **55**(9): p. 1856-62.

94. Hanahan, D. and R.A. Weinberg, *The hallmarks of cancer.* Cell, 2000. **100**(1): p. 57-70.

95. Liotta, L.A. and E.C. Kohn, *The microenvironment of the tumour-host interface.* Nature, 2001. **411**(6835): p. 375-9.

96. Fidler, I.J., *Rationale and methods for the use of nude mice to study the biology and therapy of human cancer metastasis.* Cancer Metastasis Rev, 1986. **5**(1): p. 29-49.

97. Price, J.E., et al., *Injection of cells and monoclonal antibodies into mice: comparison of tail vein and retroorbital routes.* Proc Soc Exp Biol Med, 1984. **177**(2): p. 347-53.

98. Aamdal, S., O. Fodstad, and A. Pihl, *Human tumor xenografts transplanted under the*

renal capsule of conventional mice. Growth rates and host immune response. Int J Cancer, 1984. **34**(5): p. 725-30.

99. Nagamachi, Y., et al., *Orthotopic growth and metastasis of human non-small cell lung carcinoma cell injected into the pleural cavity of nude mice.* Cancer Lett, 1998. **127**(1-2): p. 203-9.

100. Visonneau, S., et al., *Adoptive therapy of canine metastatic mammary carcinoma with the human MHC non-restricted cytotoxic T-cell line TALL-104.* Oncol Rep, 1999. **6**(6): p. 1181-8.

101. Yamashita, A., et al., *Experimental chemotherapy against canine mammary cancer xenograft in SCID mice and its prediction of clinical effect.* J Vet Med Sci, 2001. **63**(8): p. 831-6.

102. Okano, F. and K. Yamada, *Canine interleukin-18 induces apoptosis and enhances Fas ligand mRNA expression in a canine carcinoma cell line.* Anticancer Res, 2000. **20**(5B): p. 3411-5.

103. Kannagi, R., *Molecular mechanism for cancer-associated induction of sialyl Lewis X and sialyl Lewis A expression-The Warburg effect revisited.* Glycoconj J, 2004. **20**(5): p. 353-64.