Studies on factors associated with metastasis of canine mammary gland tumor

（犬乳腺腫瘍の転移関連因子に関する研究）

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Contents

General Introduction

Canine mammary gland tumors (CMGTs) are the most common tumors in intact female dogs. CMGT was reported to account for approximately 50% of all tumors in female dogs[1-3]. Though the prevalence of CMGTs has been reduced because of ovariohysterectomy at an early age [4], the incidence of CMGTs is still high and malignant CMGTs are still an important neoplasia in dogs. Histologically, 50-60% of CMGTs are considered malignant [1, 2, 5]. Most of malignant CMGTs are the epithelial tumors or carcinomas and pure sarcomas (fibrosarcoma, osteosarcoma and sarcomas of other types) are rare. The second common type of malignant CMGT composed of cells morphologically resembling the epithelial and connective tissue components—both of which are malignant—and that is termed carcinosarcoma or malignant mixed tumor. Among benign tumors, adenoma and benign mixed tumor, which is containing an epithelial component and a mesenchymal component of cartilage and/or bone, are relatively common types [6, 7]. Inflammatory carcinoma, a specific type of malignant CMGT, is a poorly differentiated carcinoma with extensive evidence of both mononuclear and polymorphonuclear cellular infiltrates and often edema. Clinically, neoplasms of this type grow and metastasize with extreme rapidity and invade lymphatics and the skin, resulting in marked edema and inflammation [8].

Several prognostic factors have been identified in dogs with mammary gland tumors including the tumor size, histologic type, malignancy grade, degree of nuclear differentiation,

degree of invasion, intravascular growth, lymph node involvement, presence of distant metastasis, steroid hormone receptor activity, S-phase fraction as an index of proliferation, DNA aneuploidy, and number of silver-stained nucleolar organizer regions (AgNORs) [4]. Of all prognostic factors, distant metastasis is the most significant factor and it was reported that dogs with distant metastasis have a worse prognosis than dogs with negative staging or only regional lymph node involvement [9-11].

It has been shown that there are several steps in the mechanism of tumor infiltration to the surrounding tissues and formation of metastatic lesion. These steps consist of detachment of the tumor cells from the primary lesion, invasion to the surrounding tissues and blood vessels, adhesion and invasion to the endothelial cells of distant blood vessels, and proliferation in the metastatic site [12, 13]. Through *in vitro* and *in vivo* studies on cancers, many factors have been found to be related to the biological behavior of tumors such as invasion and metastasis and various factors are known to be involved in each step, such as cell adhesion molecules, matrix metalloproteinase (MMP), vascular endothelial growth factors (VEGF) and others [12, 14, 15]. Cell adhesion molecules are classified into several families according to their structures, such as cadherin, integrin, selectin, immunoglobulin and other families. Researches on these factors have been conducted in CMGT, but information on expression of these factors or the relationship to the biological behavior of CMGT is still limited.

Based on these backgrounds, a series of studies was carried out in this thesis to investigate the factors associated with the biological behavior of CMGT, especially distant metastasis. In Chapter 1, expression of several oncological factors in 6 CMGT cell lines was evaluated to see the molecular character of CMGT cells and to identify factors associated with tumor progression or metastasis of CMGT. As a result, sialyl Lewis X [sLe(x)], which is a ligand of E-selectin, was detected strongly only in a cell line which derived from the distant metastatic lesion. Therefore, I focused on this carbohydrate antigen, sLe(x), and confirmed its expression on the cultured cells and its adhesional function to blood vessel epithelial cells (Chapter 2). In Chapter 3, I examined the expression of sLe(x) on the tumor tissues and sera from spontaneous CMGT patients and evaluated the correlation with their clinicopathological features. Further more, I tried to obtain the CMGT model with high lung metastasis in immuno-deficient mice by transplanting a CMGT cell line and evaluated the change in expression of sLe(x) on the cell lines before transplantation and those derived from this animal model (Chapter 4).

Chapter 1

Expressions of oncological factors in six canine mammary gland tumor cell lines

Introduction

Canine mammary gland tumor is the most common neoplasia in female dogs, accounting for approximately 50% of all tumors [1, 2, 4]. Histologically, 50-60% of CMGTs are considered malignant [1, 2, 5]. Tumor invasion to the surrounding tissues and metastasis are the most significant prognostic factors in malignant tumors [9-11].

Through *in vitro* and *in vivo* studies on cancers, several factors have been found to be related to tumor invasion and metastasis. Cell adhesion molecules, including cadherin, selectin, integrin and other families are thought to be involved in several steps of tumor invasion and metastasis [12, 14, 15]. The cell-cell adhesion is controlled by the connection of E-cadherin/β-catenin complex and the reduction in these factors is thought to induce detachment of cells from the primary lesion [16, 17]. When tumor cells in blood flow attach to vessel endothelial cells, selectin and integrin families are supposed to play important roles [12, 18]. 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. The cell cycle is found to regulate mainly by cyclins, Rb, and cdks [20]. Abnormalities in Wnt signaling pathway or p53-mediated apoptosis are known to be associated with the tumor cell progression [21, 22]. 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. For example, NCC-ST-439 and CA15-3 have been used as markers to diagnose and evaluate the prognosis of human breast cancers [26, 27]. In addition, herceptin (Trastuzumab) has been used as an antibody therapy for human breast cancer patients with c-erbB2 (HER2/neu)-positive tumors [28]. However, basic information on the expression of these factors in CMGTs has been limited.

Previously, 6 canine mammary gland tumor cell lines from both primary and metastatic lesions of three animals were established in our laboratory [29]. In this study we characterized these CMGT cells by exhaustive step protein expression analysis to identify factors associated with the tumor progression or metastasis of CMGT.

Materials and Methods

Cell lines:

Six CMGT cell lines, CHMp, CHMm, CIPp, CIPm, CNMp and CNMm, were used in this study. Three pairs of these cell lines were established from both primary and metastatic lesions of 3 patients with spontaneous mammary adenocarcinomas [29]. The letters CHM, CIP, and CNM indicate the patient, and the letter "p" or "m" indicates a primary or metastatic lesion. The breed, age, source of cells, method of collection, TNM classification and clinical staging of these animals are shown in Table 1-1. Cells were maintained in RPMI1640 medium supplemented with 10% 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. Growth patterns of these epithelial cells were shown in Fig.1-1. The passage numbers of the cell lines used in this study were 52/52, 71/74 and 46/82, respectively.

Antibodies:

The primary antibodies used for Western blot evaluation were obtained from the following sources: sLe(x), E-cadherin, Rb, proliferating cell nuclear antigen (PCNA), glycogen synthase kinase-3β (GSK-3β), β-catenin and protein phosphatase 2A (PP2A)(BD Transduction Laboratories, Lexington, KY, USA), vimentin (Nichirei, Tokyo, Japan), cyclinD1, p53 and peroxisome proliferator-activated receptor-γ (PPAR-γ)(Santa Cruz. Biotech, CA, USA), 14-3-3 all, 14-3-3 beta, 14-3-3 gamma, 14-3-3 epsilon, 14-3-3 zeta,

14-3-3 eta, 14-3-3 sigma and 14-3-3 tau (Immuno-Biochemical Laboratories, Gunma, Japan), c-erbB2 (DAKO, Glostrup, Denmark), prolactin receptor (Affinity BioReagents, Golden, CO, USA), NCC-ST-439 (Nihonkayaku, Tokyo, Japan), α-tubulin and β-actin (NeoMarker, Fremont, CA, USA)(internal controls). The dilutions of each antibody were shown in Table 1-2.

Western blot analysis:

Sub-confluent cultured cells were lysed in RIPA buffer (10 mM Tris-HCl, 1% NP40, 0.1% sodium deoxycholate, 0.1% SDS, 0.15 M NaCl, 1 mM EDTA, 10 μg/ml aprotinin, 0.1 mM Na_2MoO_4, 2 mM Na_3VO_4, 1 mM PMSF, 10 mM NaF, 25 mM sodium β-glycerophosphate, 10 mM sodium pyrophosphoric acid and 1 mM EGTA). Protein concentrations were measured using the BCA protein assay reagent (Pierce, Rockford, IL). Cell lysates were boiled for 5 minutes in 2X SDS sample buffer and resolved by SDS-PAGE at about 10 μg per lane in a gel containing an appropriate concentration of acrylamide. The concentration was dependent on the weight of proteins (5, 7.5, 10 and 12%). Lysates of other species known to stain positive with each primary antibody were used as a positive control (data not shown). After electrophoresis, separated proteins were blotted onto a polyviniylidene difluoride (PVDF) membrane (Bio-rad, Hercules, CA). The blots were then incubated for 16 hours at 4°C with PBS-T buffer containing 5% non-fat milk. The blots were incubated with appropriate primary antibodies for 2 hours at room temperature, and

subsequently incubated with horseradish peroxidase-conjugated antibodies against mouse Ig or rabbit Ig (Amersham Bioscience, Piscataway, NJ, USA) for 1 hour at room temperature with vigorous agitation. Signals were visualized with diaminobenzidine (DAB)/hydrogen peroxide solution (WAKO, Osaka, Japan).

Measurement of signal intensity:

To evaluate differences in the expression of these factors accurately, the signal intensity was measured by using an ATTO Light Capture system (AE-6962; ATTO, Tokyo, Japan). The expression levels of each factor were normalized with that of α-tubulin.

Statistical analysis:

Comparisons between cells of primary and metastatic origin were made by ANOVA. A probability of less than 5% ($P<0.05$) was considered significant.

Results

Western blot analysis:

Among proteins analyzed, the levels of expression of E-cadherin, vimentin, cyclinD1, Rb, PCNA, GSK-3b, p53, 14-3-3 sigma, c-erbB2, NCC-ST-439 and sLe(x) differed either among the CMGT cell lines or between cell lines of primary and metastatic origin in an individual animal. Strong expression of sLe(x) was only detected in CHMm cells, while others were negative. CNMm showed lower expression of E-cadherin than CNMp, while CHMm showed higher expression of E-cadherin than CHMp. In CIPp and CIPm cell lines, E-cadherin expression was almost similar. Vimentin is known to be a constituent part of cytoskeletal fiber and a marker of mesenchymal cells. In the present study, vimentin was expressed in all the cell lines despite of different levels (Fig. 1-2(A)); CHMm and CIPp showed very slight expression whereas strong signals were observed in CIPm and CNMp.

Expression of cyclinD1, a positive regulator of the cell cycle in G1 phase, varied among the cell lines. In the CIP pair, CIPp showed higher expression than CIPm, while the opposite result was obtained in other pairs. Rb, a negative regulator of the cell cycle in G1 phase, showed strong expression in CNMm cells, while there was little expression in the CHMp and CIP pair. Expression of PCNA, synthesized in G1-S phase and used as a marker for cell proliferation, and GSK-3β, a kinase playing roles such as resolution of β-catenin [21] or induction of degradation of cyclinD1 [20], differed slightly, but not significantly, among

the cell lines (Fig. 1-2(B)).

Expression of p53, a tumor suppressor factor associated with DNA repair, cell cycle arrest and apoptosis, was observed in all cell lines. In the CHM pair, CHMm showed higher expression than CHMp, while in the CIP and CNM pairs the expression was similar between cell lines of primary and metastatic origin (Fig. 1-2(C)).

Among 14-3-3 protein and its 7 isoforms, which amplify or suppress the signaling pathway, 14-3-3 sigma exhibited various levels of expression in CMGT cell lines. In the CIP pair, CIPp showed lower expression than CIPm, while in the CNM pair CNMm showed lower expression than CNMp (Fig. 1-2(D)).

Expression of c-erbB2, one of the membrane proteins and the target of antibody therapy in human breast cancers, was observed in all CMGT cell lines, but its expression varied randomly without any consistent tendency. NCC-ST-439, a carbohydrate antigen used in diagnosis and evaluation of human breast cancers, was also observed in all cell lines. CHMm and CNMm cells expressed this antigen more strongly than other cells (Fig. 1-2(E)).

Beta-catenin, PP2A, 14-3-3 protein and the 6 other isoforms of 14-3-3 proteins (beta, gamma, epsilon, zeta, eta and tau), prolactin receptor and PPAR-γ were detected in all cell lines investigated with almost the similar intensity.

Measurement of signal intensities:

The levels of expression of these factors were standardized based on the levels of

α-tubulin by using an ATTO Light Capture system. The calculated values of each signal are shown in Fig. 1-3. The differences in calculated values were comparable to those evaluated visually. Differences in calculated values ranged within 0.9-57.3% of α-tubulin intensities where no differences were observed visually.

Discussion

In this study, I evaluated the expression of various factors associated with differentiation and tumorigenesis in 6 CMGT cell lines. The expressions of sLe(x), E-cadherin, vimentin, cyclinD1, Rb, PCNA, GSK-3b, p53, 14-3-3 sigma, c-erbB2 and NCC-ST-439 were found to differ among CMGT cell lines or between the cell lines of primary and metastatic origin in each pair. Among these factors, sLe(x) showed the most interesting expression—namely, only CHMm was positive for sLe(x).

SLe(x) is a carbohydrate ligand which adheres to E-selectin [30]. This ligand is expressed on granulocytes and monocytes, and has been implicated in their adhesion to vascular endothelial cells in the acute inflammation process [31, 32]. SLe(x) is also reported to be expressed on human cancer cells and is suggested to play important roles in hematogenous metastasis of the cancer, in which it mediates the initial adhesional step of tumor cells to the distal vascular endothelial cells by sLe(x)-E-selectin adhesion [18, 33, 34]. The degree of expression of the ligand at the surface of cancer cells has been shown to be well correlated with the frequency of hematogenous metastasis and poor prognostic outcome of patients with cancers [35-38]. Only CHMm cells with strong expression of sLe(x) in this study were derived from the distant metastatic lesion (pleural effusion). Other cell lines were derived from the primary lesion (CHMp, CIPp and CNMp) or metastatic lesion of the lymph node (CIPm and CNMm). This result suggested that the acquisition of sLe(x) expression may

be an important factor of hematogenous metastasis in CMGT, though this was observed in only one CMGT cell line. Further studies will be needed to clarify the role of sLe(x) in the metastasis of CMGT.

In other molecules investigated in this study, their expressions were various and not consistent between cells of primary or metastatic origin. E-cadherin functions as a cell-cell adhesion molecule, and reduction in its expression is thought to be related with tumor malignancy through the loss of cell interaction [16, 17]. In human breast cancers, decreased expression of E-cadherin might be associated with the acquisition of invasiveness, metastasis and poor prognosis [39, 40]. Similar to human breast cancers, down regulation of E-cadherin may be associated with poor differentiation and malignancy in CMGT [41-43]. In this study, E-cadherin was not consistently expressed among 6 CMGT cell lines. In the CNM pair, CNMm showed lower expression of E-cadherin than CNMp, while CHMm showed higher expression than CHMp in the CHM pair. In the CIP pair, the expression was almost the same.

Half of CMGT tissues show proliferation of myoepithelial cells or formation of cartilage, and has been reported that they consist of both epithelial and mesenchymal cells [44, 45]. In this study, we detected the signal of vimentin in all cell lines, but the level of expression varied. The expression was very low in CHMm and CIPp, whereas it was quite high in CIPm and CNMp. On the other hand, expression of the cytokeratin 8, 9, and 19 epithelial markers [46] could not be detected in any of the cell lines. Further investigation on

the expression of cytokeratin and vimentin might be needed to clarify the character and origin of these cells.

CyclinD1 and Rb are important proteins which regulate the cell cycle in G1 phase. CyclinD1 is a major positive regulator of the G1 restriction point, which binds to cdk and promotes inactivation of Rb by phosphorylation [20, 47]. High expression of cyclinD1 has been reported in up to 50% of human primary breast cancers, and cyclinD1 has been identified as one of the most common oncogenes in human breast cancers [48]. In contrast, absent or low-level Rb expression has been correlated with proliferation of human breast cancers [49, 50]. In this study, I observed the increased cyclinD1 expression in CIPp/m and CNMm cells and the loss of Rb expression in CIPp/m and CHMp cells. There have been few reports on cyclinD1 and Rb in CMGTs. The CIP pair, which showed high expression of cyclinD1 and loss of Rb, appeared to be useful for analyzing cell-cycle abnormalities in the G1/S phase.

Both GSK3β and β-catenin are involved in the Wnt-signaling pathway [21]. Beta-catenin is also an important molecule in cell-cell adhesion as an integral component of the cadherin-adhesion protein complex [51]. In this study, the expression level of GSK-3β varied slightly among the cell lines, and β-catenin was detected with similar intensity in all cell lines. Since the location of β-catenin was not evaluated in this study, the significance of the Wnt-signaling pathway in CMGTs was not clear.

The expression of PCNA, a marker for cell proliferation [19], and PP2A, an abundant serine/threonine phosphatase involved in many cellular events [52], was shown to be similar in all cell lines. There has been little information on these factors in CMGT, and further research may be needed to reveal their relationship to CMGT.

The p53 protein regulates the expression of a wide variety of genes involved in cell cycle arrest and apoptosis in response to genotoxic or cellular stress. Numerous studies have reported that loss of p53 protein, mutation of the p53 gene and overexpression of the nonfunctional p53 product were related to tumor progression in several cancers, including human breast cancers and CMGTs [22, 53-56]. In the CHM pair, CHMm showed higher expression than CHMp, while in the other pairs, cell lines of primary and metastatic origin showed similar levels of expression.

14-3-3 proteins play important roles in signal transduction pathways. There are many isoforms that can form both homo- and heterodimers which contribute to protein phosphorylation and protein-protein interactions [23, 57]. Among the 14-3-3 proteins and 7 isoforms, only 14-3-3 sigma showed different levels of expression among the cell lines in this study. Its expression was lower in CNMm and CIPp than in CNMp and CIPm, respectively. One of the major functions of 14-3-3 sigma is a negative regulator of the cell cycle in G2/M phase, and this isoform has also been associated with cell proliferation in human breast cancers [58, 59]. This present results suggested that the 14-3-3 sigma protein may be

associates with the proliferation of CNMm and CIPp.

Tumor markers for human breast cancers, c-erbB2 and NCC-ST-439, or prolactin receptor and PPAR-γ, were also evaluated. C-erbB2 is a transmembrane protein with intrinsic tyrosine kinase activity, and its high expression has been implicated in human breast cancers [24, 60]. NCC-ST-439 has high sensitivity as a tumor marker for breast cancers, and its serum level is thought to reflect tumor progression or metastasis [26, 61-63]. Prolactin is an important regulator of mammary gland differentiation and growth [64]. PPAR-γ is an isotype of peroxisome proliferator-activated receptors [25]. This receptor indicates the level of differentiation of tumor cells [65, 66]. Troglitazone, a selective ligand for PPAR-γ, has antitumor activity in various cancer models and is thought to be effective in the treatment of cancer as an adjuvant therapy [67-69]. The expression of these molecules in CMGT cell lines in this study varied, but the detection of PPAR-γ in CMGT cells suggested the possibility of the application of PPAR-γ target therapy for CMGTs.

Conclusion

In this study, I measured the expression of various factors related to malignancy in 6 CMGT cell lines. Results of this study should provide useful information on the characteristics of these cell lines, although differences in their expression were not consistent within the cell lines of primary or metastatic origin. Some of the factors, such as sLe(x), 14-3-3 sigma, cyclinD1 and Rb showed different levels of expression between the pairs. These cell pairs might be meaningful tools for *in vitro* tumor analysis, because they were established from the same animal and may have the similar genomic background. Especially, sLe(x) showed strong expression only in CHMm cell line derived from distant metastatic origin. This result suggested that sLe(x) might be related to distant metastasis in CMGTs though the number of cell lines used was limited.

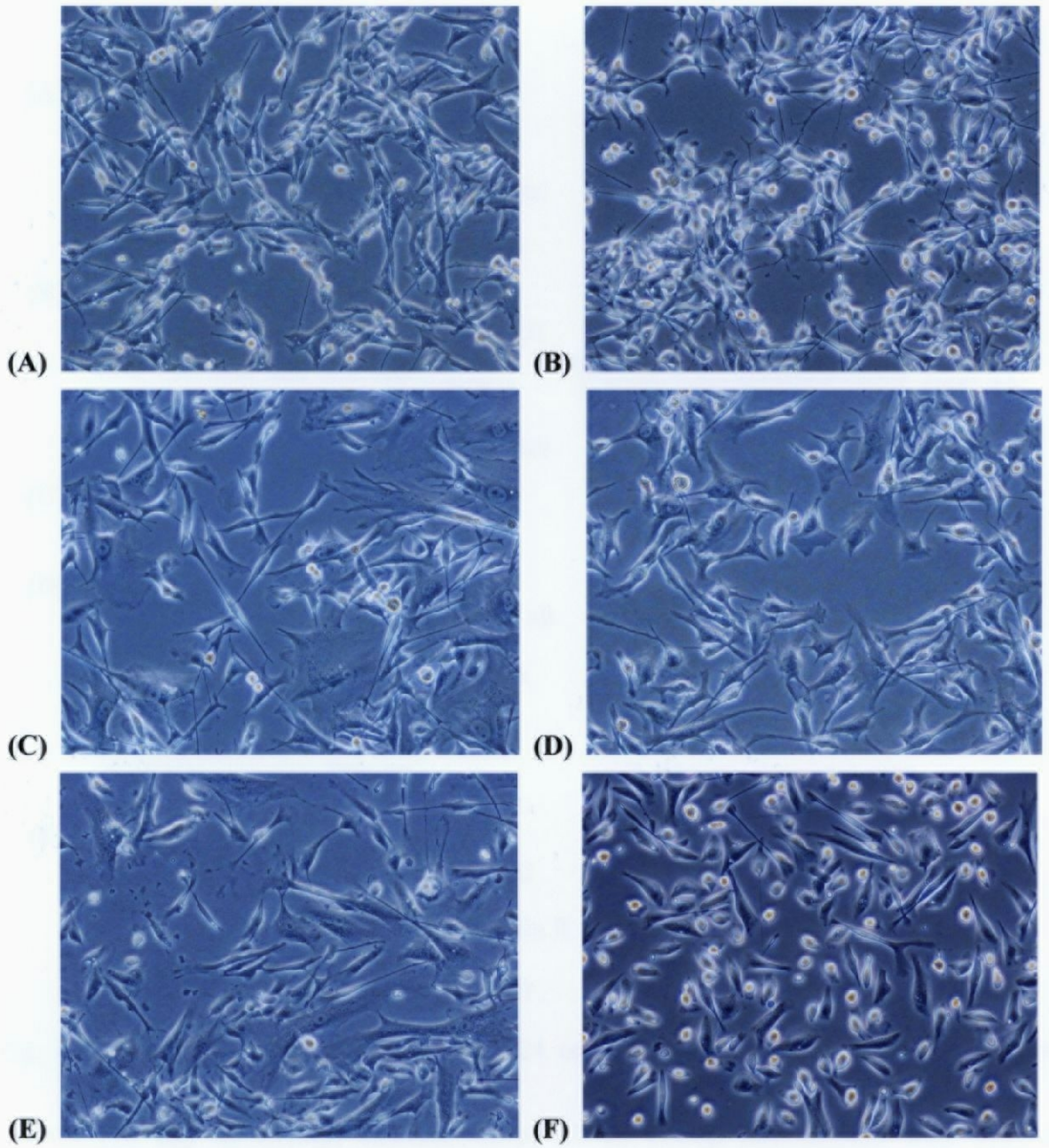

Fig. 1-1 Microscopic findings of 6 CMGT cell lines, CHMp (A), CHMm (B), CIPp (C), CIPm (D), CNMp (E) and CNMm (F). Different growth pattern was seen between the pairs, especially in CHM pair, where the shape of cells was different between primary and metastatic origin.

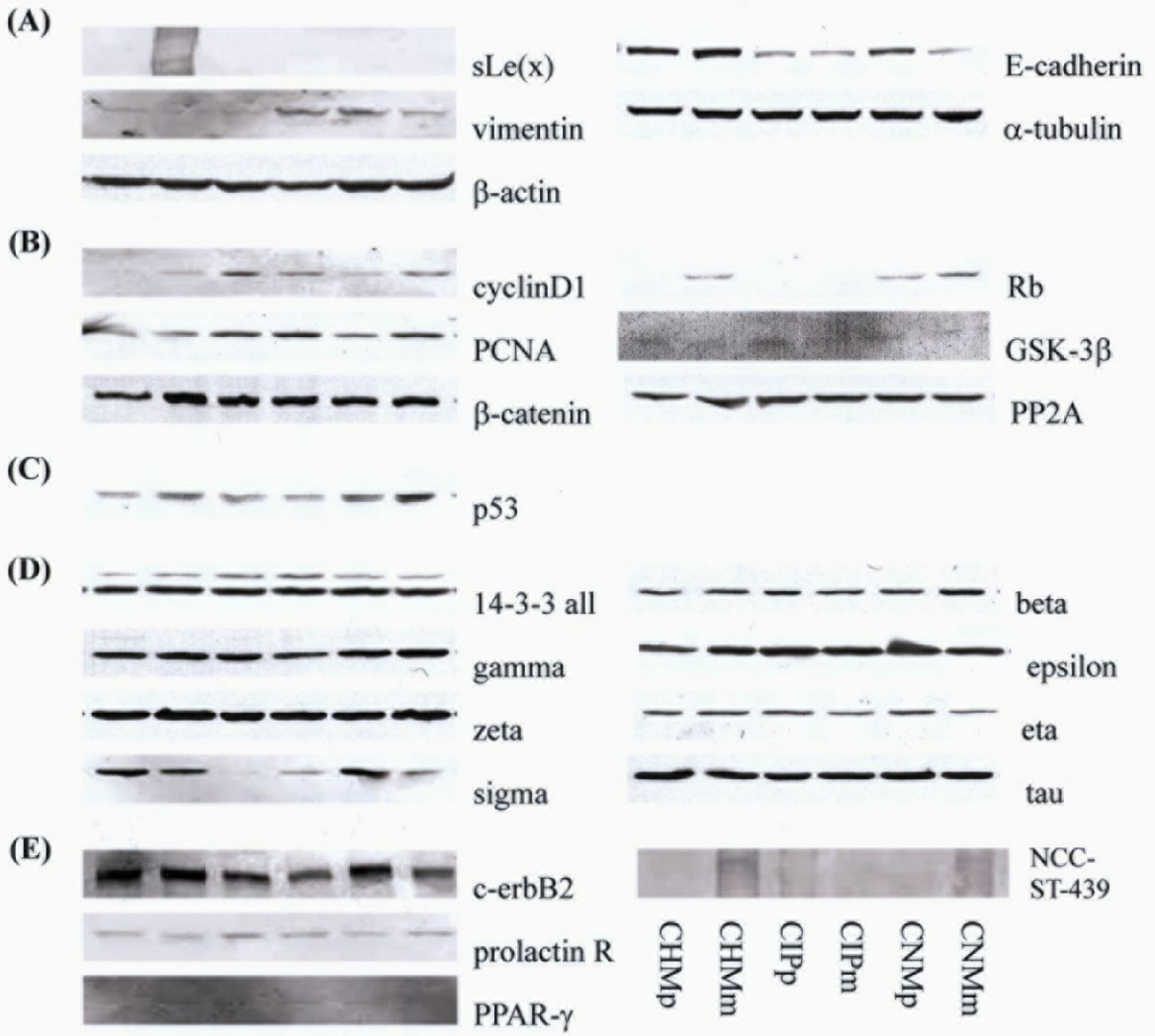

Fig. 1-2 Western blotting analysis for 24 oncological factors of 6 CMGT cell lines. Lysates (10 μg) of CHMp, CHMm, CIPp, CIPm, CNMp and CNMm were applied sequentially from the left. Factors analyzed were categorized as follows: A; cell adhesion and structure molecules, B; cell cycle factors and growth regulators, C; apoptosis-related molecule, D; scatter folding factors and E; receptors and glycoproteins.

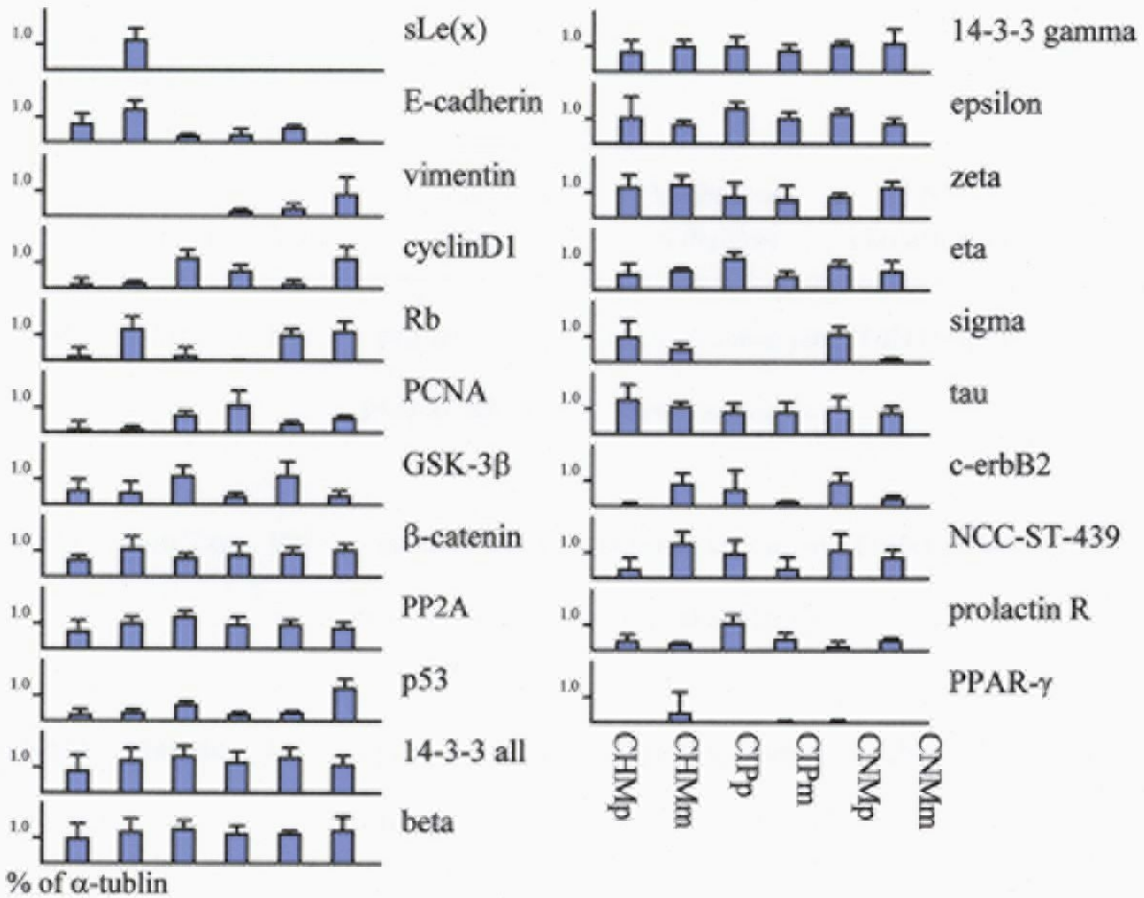

Fig. 1-3 Signal intensities measured by the ATTO Light Capture system. The expression levels of the factors were standardized with the value of α-tubulin by CS Analyzer software.

Table 1-1 Clinical data of dogs with mammary adenocarcinomas from which the cell lines were established.

Cells	Breed	Age	The source of cells	Method of collection	TNM classification	Clinical stage
CHMp /m	Mix	12y	primary mass pleural effusion	surgical specimen thoracocentesis	T4N1(+)M1	IV
CIPp /m	Shih Tzu	10y	primary mass metastatic regional lymph node	surgical specimen surgical specimen	T1cN1(+)M1	IV
CNMp /m	Maltese	11y	primary mass metastastic regional lymph node	surgical specimen surgical specimen	T1cN1(+)M0	II

Table 1-2 The dilution of each antibody used in Western blot analysis.

Antibodies	Dilutions
Cell adhesion and structure molecules	
sLe(x)	1:1000
E-cadherin	1:400
vimentin	1:500
α-tubulin	1:1000
β-actin	1:1000
cell clycle factors and growth regulators	
cyclinD1	1:200
Rb	1:500
PCNA	1:1000
GSK-3β	1:2500
β-catenin	1:500
PP2A	1:5000
apoptosis-related molecule	
p53	1:500
scatter folding factors	
14-3-3 all	1:100
14-3-3 beta	1:70
14-3-3 gamma	1:70
14-3-3 epsilon	1:100
14-3-3 zeta	1:70
14-3-3 eta	1:70
14-3-3 sigma	1:100
14-3-3 tau	1:100
receptors and glycoproteins	
c-erbB2	1:100
NCC-ST-439	1:70
prolactin receptor	1:500
PPAR-γ	1:200

Chapter 2

In vitro analysis of sialyl Lewis X on canine mammary gland tumor cell lines

Introduction

SLe(x) is a type 2 sugar chain, which has a type 2 structure (Galβ1,4GlcNAc) in the backbone region and important determinants, the Fuc residue and the terminal sialic acid (NeuNAc), required for selectin binding (sLe(x): NeuNAcα2,3Galβ1,4(Fucα1,3)GlcNAc) [70-73]. The carbohydrate chain is elongated by glycosyltransferases adding monosaccharides at their ends and most sugar addition takes place within the Golgi apparatus or endoplasmic reticulum lumen [74]. This sugar chain is mainly observed on the surface of neutrophils [75] and serves as a carbohydrate ligand which adheres to E-selectin [30].

E-selectin, one of the selectin families, is a cell-cell adhesion molecule involved in leukocyte-endothelial cell adhesive interaction [76]. Selectins belong to the C-type (calcium-dependent) lectin family and have a single C-type lectin domain at their extracellular amino termini, followed by an epidermal growth factor (EGF)-like domain, several complement regulatory domains, transmembrane domain and short cytoplasmic tail [77].

In the acute inflammation, the expression of E-selectin on vascular endothelial cells is induced by proinflammatory factors such as TNF-α and interleukin 1β [78]. Rapid and transient binding and dissociation between E-selectin on blood vessel endothelial cells and sLe(x) on leukocytes make leukocytes slow down at the site of inflammation [31, 32]. This process, called rolling, was originally reported in an *in vitro* flow chamber experiment using

microscopic videometry [76]. After this rolling process, adhesion of leukocytes to endothelial cells is firmed by adhesion molecules such as integrin and immunoglobulin families [79]. Similar process is supposed to be involved in hematogenous metastasis of the cancer, in which the sLe(x)-E-selectin binding mediates the initial adhesional step of tumor cells to the distal vascular endothelial cells [18, 33, 34].

In the previous study, I observed the strong expression of sLe(x) only in the cell lysate of CHMm cell line which was derived from the distant metastatic lesion, while the expression of other cell lines derived from primary and lymphatic metastatic lesions was negative. The purpose of this study was to confirm the expression and localization of sLe(x) on CMGT cells and to evaluate the ability of adhesional function of sLe(x) to endothelial cells.

Materials and Methods

Cell lines:

Six CMGT cell lines, CHMp, CHMm, CIPp, CIPm, CNMp and CNMm, were used in this study. These CMGT cells were maintained in RPMI1640 medium supplemented with 10% fetal bovine serum, 5 mg/L gentamicin sulfate and 6 mg/L fungizone. Human umbilical vein endothelial cells (HUVECs) were grown in EBM-2 medium supplemented with 2% fetal bovine serum. All of the cell lines were incubated at 37°C in a humidified atmosphere of 5% CO_2.

Antibodies:

Anti-sLe(x) monoclonal antibody (clone: CSLEX1) was obtained from BD Transduction Laboratories (Lexington, KY, USA), the same one as used in Chapter 1. Anti-E-selectin monoclonal antibody (clone: 1.2B6) was obtained from Sigma-Aldrich Corp. (St. Louis, MO, USA). The working solution was diluted at 1:1000 and 1:400 with 1% BSA-TBS, respectively.

Immunohistochemistry:

The expression of sLe(x) on CMGT cells was analyzed by immunohistochemistry. Each CMGT cell was cultured on Lab-Tek II chamber slides (Nalge Nunc International, Rochester, NY, USA) to sub-confluent growth and fixed in 10% neutralized formalin. Immunohistochemical procedures were performed using a DAKO ENVISION+ kit/HRP

(DAB)(DAKO). Briefly, endogenous peroxidase activity was abolished by treatment with 0.03% hydrogen peroxide containing sodium azide. For the staining of sLe(x), samples 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.

Cell adhesion assay to HUVECs in 96-well plates:

HUVECs were cultured in 96-well plates (Sumitomo Bakelite, Tokyo, Japan) to confluent growth. The cells were incubated for 4 hours at 37°C with/without recombinant human TNF-α (rhTNF-α) (20ng/ml; PeproTech, Rocky Hill, NJ, USA), to induce the expression of E-selectin. E-selectin expression was confirmed by immunohistochemical staining with anti-E-selectin antibody. The protocol of this immunohistochemistry was similar to that above mentioned. CHMp and CHMm cells were labeled with calcein AM (25ng/ml; Dojindo Laboratories, Kumamoto, Japan), which is well retained within live cells and produces an intense uniform green fluorescence. Calcein-labeled viable tumor cells were added to HUVECs at 1 x 10^4 cells/well in TBS with/without 1mM $CaCl_2$ buffer and were left for 30 minutes under gently shaking (100r/min). Then each well was washed 3 times with PBS buffer to exhaust unattached cells and analyzed by fluorescence inverted phase-contrast microscopy (IX51, Olympus Corp., Tokyo, Japan) with a digital camera (DXM1200F, Nikon

Corp., Tokyo, Japan). The number of attached CMGT cells was calculated from the digital pictures of each well using Scion Image software. (version 4.0.2β, Scion Corp., Frederick, MD, USA).

Flow adhesion assay to HUVECs:

HUVECs were cultured on the flow through chamber, the ibidi cell culture μ-slide I collagen IV-coated (NIPPON Genetics, Tokyo, Japan), to confluent growth and activated with/without rhTNF-α, the same as in 96-well plate assay. Calcein-labeled CHMm cells were prepared in 1mM $CaCl_2$-TBS buffer at 5 $x10^4$ cells/ml. The flow through chamber in which HUVECs were cultured was mounted on the stage of a fluorescence inverted phase-contrast microscope (IX51, Olympus Corp.) with a digital camera (DXM1200F, Nikon Corp.). CHMm cells suspension was perfused in the flow through chamber with a syringe pump attached to the outlet side via the flow through kit (NIPPON Genetics). A single field of view was monitored and videotaped to observe the interaction of CHMm cells with HUVECs (PV130, Canon Inc., Tokyo, Japan).

Statistical analysis:

Data were expressed as mean ± SEM. Statistical significance of differences between means was determined by two-factor factorial ANOVA. A probability of less than 5% ($P<0.05$) was considered significant.

Results

Immunohistochemistry:

To evaluate the expression and localization of sLe(x) in CMGT cells, 6 CMGT cell lines were examined by immunohistochemistry using anti-sLe(x) antibody. Among these cell lines, only CHMm cells showed strong expression of sLe(x) and there was no positive reaction in the other 5 cell lines (Fig.2-1). In CHMm cells, the cell surface was strongly stained and the cytoplasm was also stained, while there was little positive reaction in the nucleus (Fig.2-1).

Cell adhesion assayf to HUVECs in 96-well plates:

CHMp and CHMm cells, which showed negative and strong positive expression of sLe(x), respectively, were used as samples to evaluate the adhesional function of sLe(x) on CMGT cells. Binding function of sLe(x) to E-selectin was evaluated by cell adhesion assay using HUVECs cultured on 96-well plates. By rhTNF-α stimulation for 4 hours, the expression of E-selectin was well induced on HUVECs. Positive and negative expressions of E-selectin were confirmed by immunohistochemical analysis on rhTNF-α stimulated and non-stimulated HUVECs, respectively (Fig.2-2). The cell adhesion assay was performed using TBS buffer with/without calcium, which is required for the binding of calcium-dependent lectin, E-selectin. Images of each well were obtained under fluorescence microscopy (Fig. 2-3 and 2-4), and the numbers of attached CMGT cells to HUVECs were

calculated (Fig. 2-5). The number of attached CHMm cells to rhTNF-α stimulated HUVECs was significantly (P<0.0001) higher than that to non-stimulated HUVECs under the condition with calcium (37.20±9.00 and 2.44±0.51 cells/mm^2, respectively). When the adhesion assay was performed without calcium, significant difference was not observed between numbers of CHMm cells to non-/rhTNF-α stimulated HUVECs (1.59±0.38 and 3.83±2.08, respectively). The number of attached CHMp cells to non-/rhTNF-α stimulated HUVECs under without/with calcium were 3.38±0.70, 3.11±0.82, 4.70±0.87 and 12.40±2.85, respectively. Among all experiments the number of CHMp cells showed no significant difference. In comparison between CHMp and CHMm, the attached cell number of CHMm increased significantly by the rhTNF-α stimulation to HUVECs than that of CHMp (P=0.054).

Adhesional assay on flow through chamber:

I used a flow through chamber system to evaluate the adhesion of CHMm cells to HUVECs under flow conditions. Expressions of E-selectin were obtained successfully only on rhTNF-α stimulated HUVECs, the same as in 96-well plate assay (data not shown). The adhesive interaction of CHMm cells to rhTNF-α stimulated HUVECs was observed in the flow through chamber adhesion assay. Serial images of CHMm cells' adhesion to HUVECs—slow down with transient binding and dissociation, and attach to activated HUVECs under flow conditions—were shown in Fig. 2-6.

Discussion

SLe(x) is a carbohydrate ligand which adheres to E-selectin [30] and has been implicated in their adhesion to vascular endothelial cells in the acute inflammation process [31, 32]. This sugar chain was synthesized within the Golgi apparatus and endoplasmic reticulum lumen and expressed on the cell surface [74]. In this study, I evaluated the expression and localization of sLe(x) using 6 CMGT cell lines. The adhesional function of CMGT cells to E-selectin on HUVECs was also examined. Among 6 CMGT cell lines, only CHMm showed strong expression of sLe(x) on immunohistochemistry and this result supported that of the western blotting analysis in the previous study. In positively reacted cells of CHMm cell line, the cell surface was stained strongly. CHMp cell line, which was derived from the primary lesion of the same patient with CHMm cells, showed negative expression of sLe(x). Therefore, the CHM pair was thought to be a useful tool for the analysis of sLe(x) in CMGT.

I evaluated the adhesional function of sLe(x) on CMGT cells using cell lines of CHM pair by the cell adhesion assay in 96-well plates. Under conditions with calcium, the number of attached CHMm cells to rhTNF-α-stimulated HUVECs was significantly increased compared with that to non-stimulated HUVECs. However there was no significant difference between those to activated and non-activated HUVECs in the assay performed without calcium, which is necessary for the binding activity of C-type (calcium-dependent)

lectin family. The number of CHMp cells showed no significant difference among all experiments. In comparison between CHMp and CHMm, the attached cell number of CHMm increased significantly by the rhTNF-α stimulation to HUVECs than that of CHMp.

The attachment of CHMm cells to HUVECs was also observed in the flow through chamber adhesion assay which substitutes for blood vessels. Under flow conditions, I could see the process of cell-cell adhesion in which the flow speed of CHMm cells was slowed down with transient binding and dissociation to the rhTNF-α-stimulated HUVECs, and finally adhere to the HUVECs on the flow through chamber slide. This phenomenon, which is called rolling, was reported in the adhesion between leukocytes and blood vessel endothelial cells, and revealed to be mediated by the adhesional interaction of sLe(x) on leukocytes and E-selectin on endothelial cells [31, 32, 76]. From these results, CHMm cell line was revealed to have the adhesional ability to blood vessel endothelial cells and this ability was supposed to be mediated by the sLe(x)-E-selectin binding.

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]. SLe(x) expression of cell lines derived from several human cancers was evaluated in the past reports [80-82]. In the study of human colorectal cancer, metastatic abilities of 2 cell lines, which were selected for their high or low cell surface levels of sLe(x),

were analyzed. Cells of higher sLe(x) expression were more adhesive to activated HUVECs and more likely to metastasize to the liver in nude mice than cells of lower sLe(x) expression [80]. CHMm cell line, which expressed sLe(x) antigen on the cell surface strongly and showed adhesive ability to HUVECs in this study, was derived from the distant metastatic lesion (pleural effusion). It was supposed that the sLe(x) expressed on CHMm cells might be related to the lung metastasis in the patients.

Conclusion

In this study, expression of sLe(x) on the cell surface of CMGT cell line was confirmed and the sLe(x) expressed CMGT cells were found to have the adhesive ability to HUVECs. This carbohydrate antigen was only detected in the cell line which was derived from the distal metastatic lesion and this result suggested that the sLe(x) expression might be involved in hematogenous metastasis of CMGT, though the number of the cell lines evaluated were limited. These cell lines, CHM pair, were thought to be useful tools for the study of sLe(x) in CMGT and further *in vitro* and *in vivo* studies will be needed to clarify the role of sLe(x) in the metastasis of CMGT.

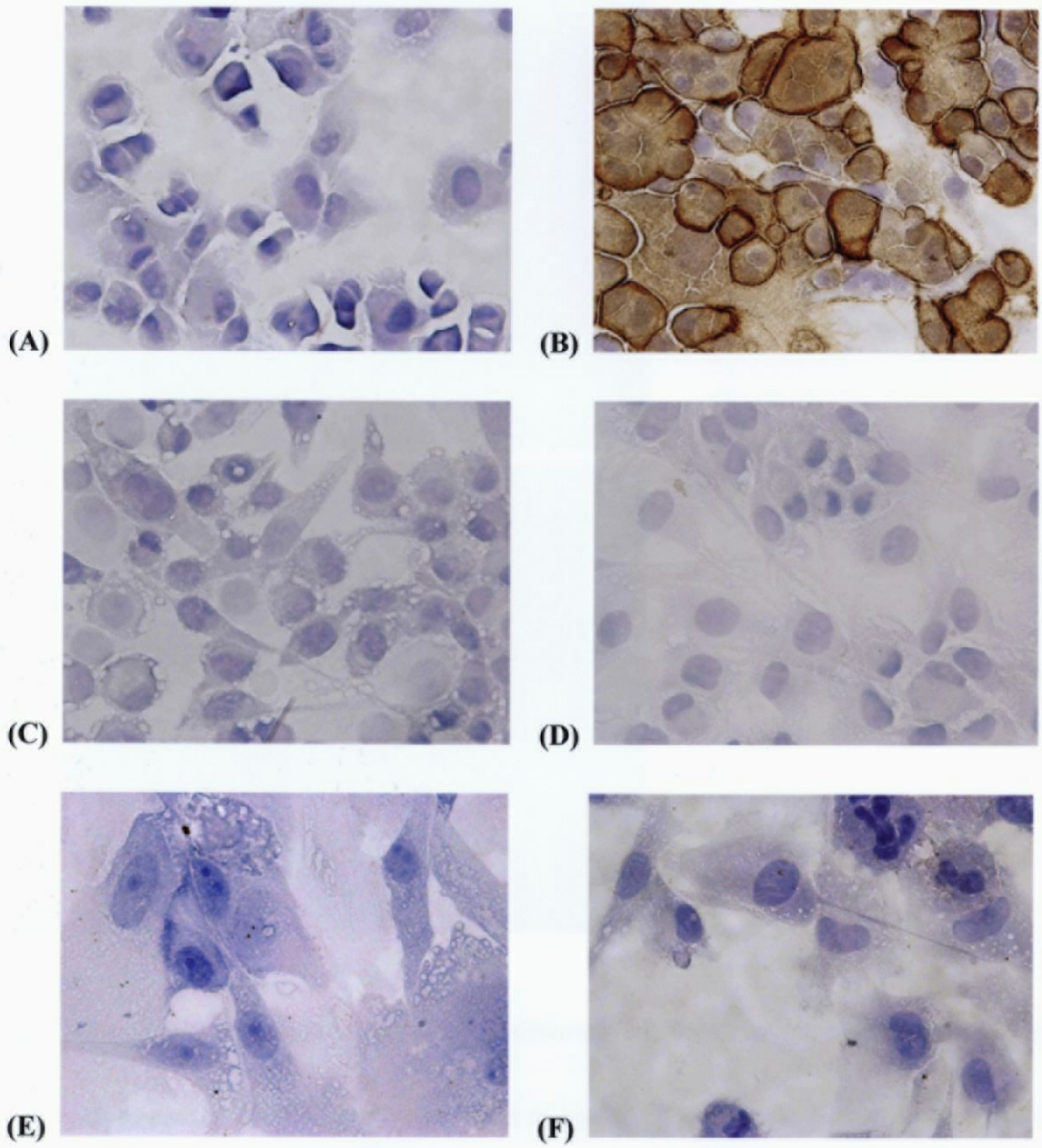

Fig. 2-1 Immunohistochemical analysis of CMGT cells. Cells were cultured to sub-confluence and reacted with anti-sLe(x) antibody. CHMm (B) cells exhibited positive staining for sLe(x), while CHMp (A), CIPp (C), CIPm (D), CNMp (E) and CNMm (F) exhibited negative reactions.

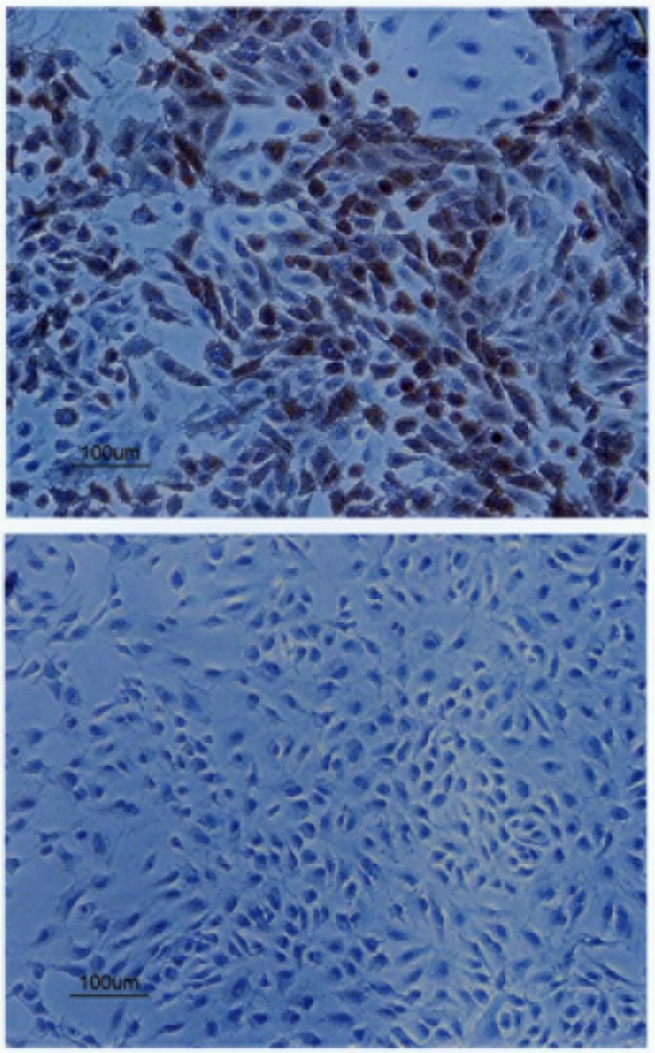

Fig. 2-2 Expressions of E-selectin detected by immunohistochemistry using anti-E-selectin antibody. HUVECs were cultured to confluent growth on 96-well plates and flow through chambers. After the rhTNF-α stimulation for 4hours 37°C, HUVECs were activated to express E-selectin (A), while there was no expression of E-selectin observed in non-stimulated HUVECs (B).

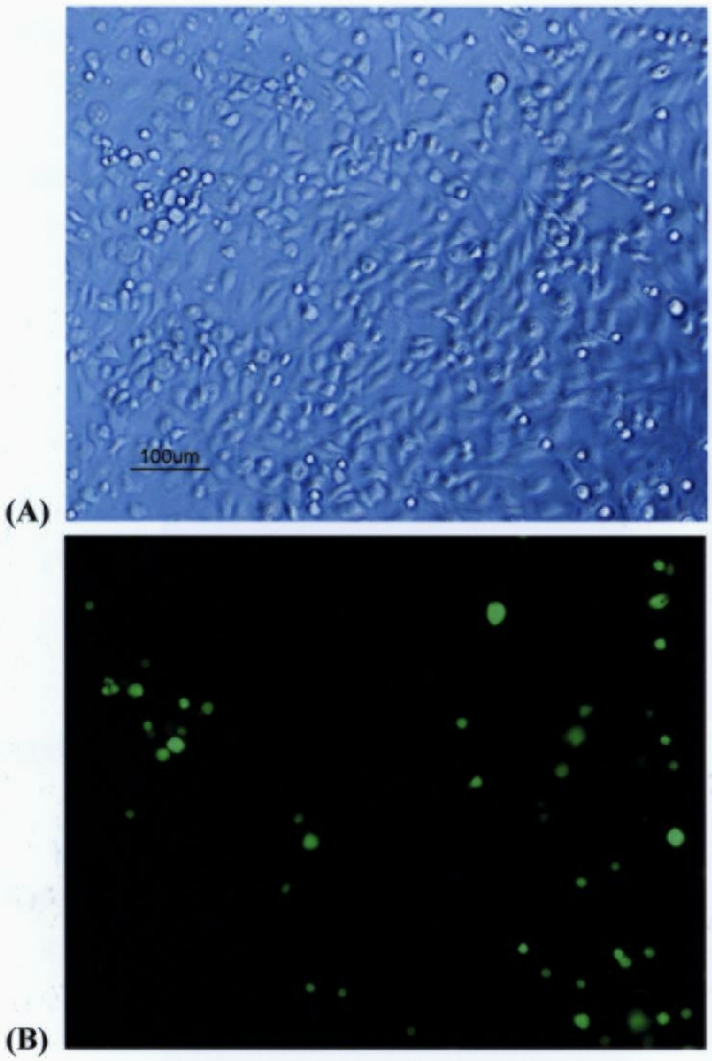

Fig. 2-3 Images of adhered CMGT cells to HUVECs were obtained by fluorescence inverted phase-contrast microscope with a digital camera. CMGT cells were labeled with calcein AM to distinguish from HUVECs. On phase-contrast microscopy, CMGT cells which showing round shapes were found to attach to HUVECs with confluent growth to formed monolayer (A). Under fluorescence excitation, calcein labeled CMGT cells were only detected, whereas HUVECs exhibited negative (B).

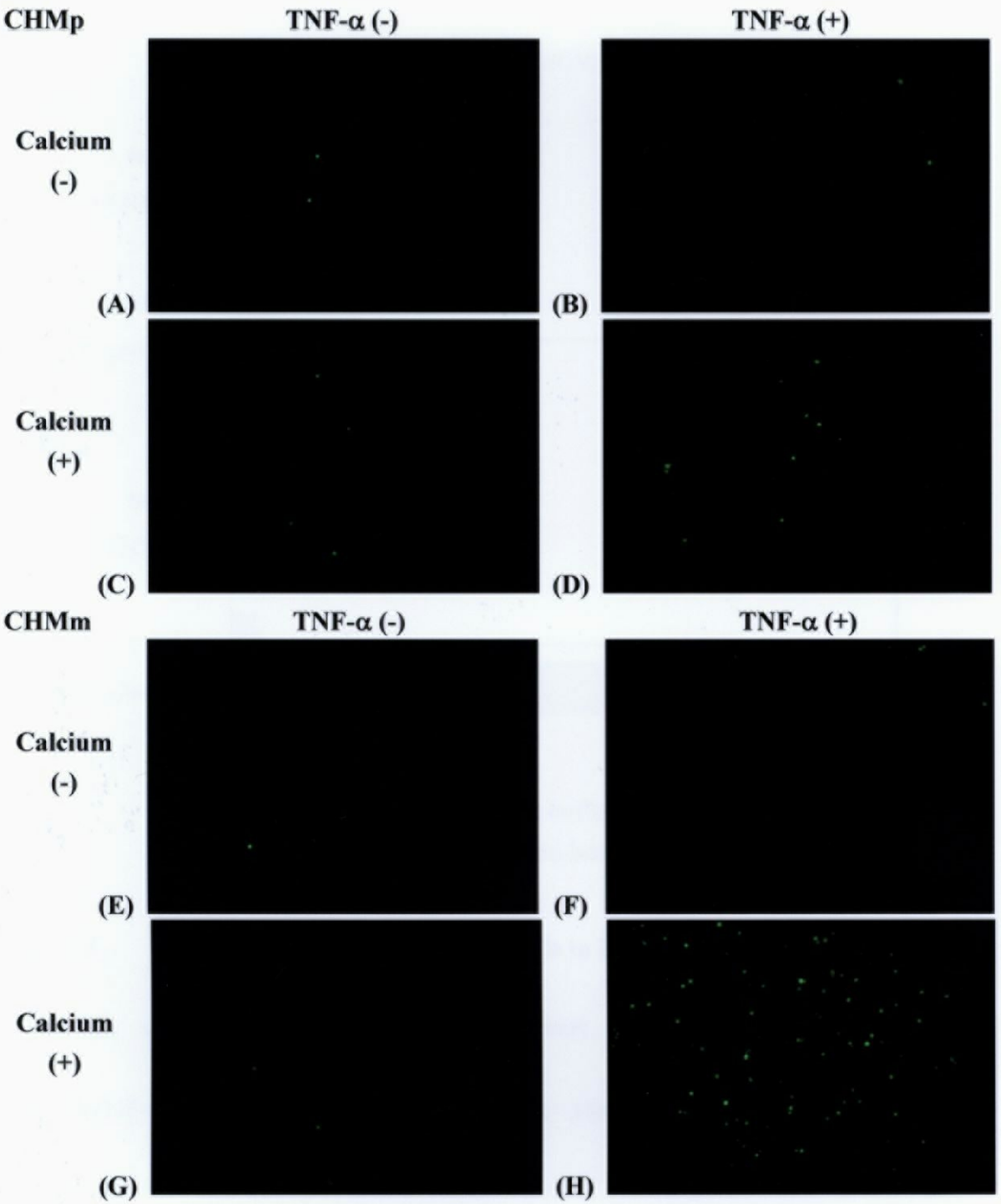

Fig. 2-4 Findings of cell adhesion assay to HUVECs in 96-well plates. The attached cells of CHMp (A-D) and CHMm (E-H) showed green fluorescence on inverted phase-contrast fluorescence microscopy.

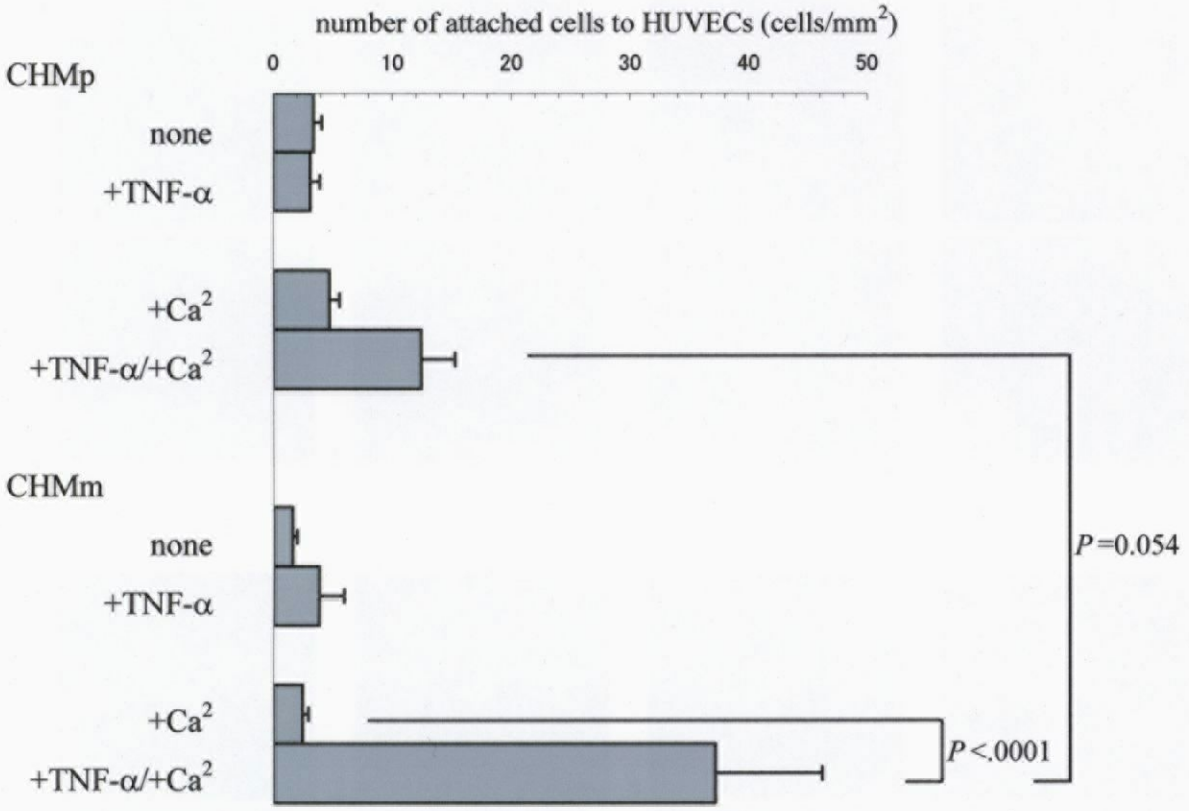

Fig. 2-5 The number of attached CMGT cells to HUVECs (cells/mm^2). Significant difference was observed between the numbers of CHMm cells adhered to non-/rhTNF-α–stimulated HUVECs under the condition containing calcium. In comparison between CHMp and CHMm, the attached cell number of CHMm increased significantly by the rhTNF-α stimulation to HUVECs than that of CHMp.

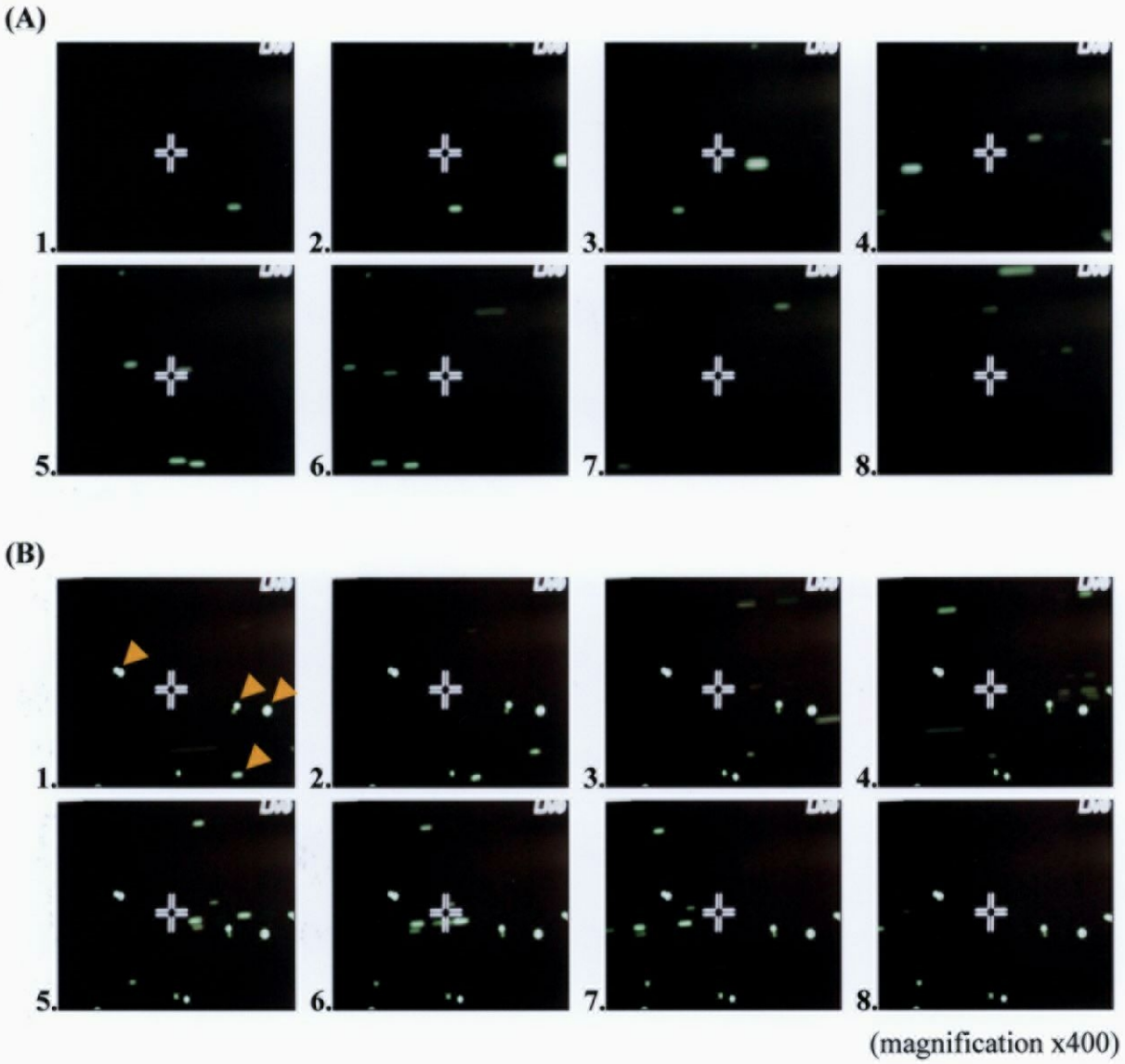

Fig. 2-6 Serial images obtained by the flow through chamber adhesion assay with non-stimulated (A) and TNF-α stimulated (B) HUVECs. Rolling of fluorescent labeled CHMm cells on activated HUVECs (B: arrow heads) was observed; slow down with transient binding and dissociation, and attach to activated HUVECs under flow conditions. The time interval between images was 1/2 second.