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Inhibitory Effect of Recombinant Fibronectin Polypeptides on the Adhesion of Liver-metastatic Lymphoma Cells to Hepatic Sinusoidal Endothelial Cells and Tumor Invasion

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We have investigated the inhibitory mechanism of the initial arrest of L5178Y-ML25 lymphoma cells in a target organ (liver) by using recombinant fibronectin fragments with cell- and/or heparin-binding domains (C-274, H-271 or the fusion fragment CH-271). Pretreatment of hepatic sinusoidal endothelial (HSE) cell monolayers with lymphoma cells or their conditioned medium for 4 to 6 h resulted in the enhancement of lymphoma cell adhesion to HSE cell monolayer. The increased tumor adhesiveness was completely abolished by preincubation of the conditioned medium with anti interleukin-1β monoclonal antibody (mAb). Synthetic sialyl Le^x (SLe^x) as a ligand for endothelial cell leukocyte adhesion molecule-1 (ELAM-1) adhesion receptor and anti ELAM-1 mAb blocked the conditioned medium-induced enhancement of tumor-endothelial cell interaction, while pretreatment of the activated HSE cell monolayer with anti vascular cell adhesion molecule-1 (VCAM-1) mAb did not affect the enhanced tumor cell adhesion. These results indicate that tumor cell interaction with the stimulated HSE cells is mediated by ELAM-1 molecules on HSE cells. However, the expression of SLe^x and SLe^a on the tumor surface was not observed by flow cytometric analysis. ELAM-1-mediated enhancement of tumor cell adhesion to HSE monolayer was also inhibited in a concentration-dependent manner by CH-271 fusion polypeptide or the sulfated chitin derivative sulfated carboxymethyl-chitin, which can bind to the heparin-binding domain of CH-271. In addition, CH-271 inhibited not only tumor-endothelium interaction but also tumor cell invasion into reconstituted basement membrane Matrigel *in vitro*.

Key words: Recombinant fibronectin fragment — Cell adhesion — Lymphoma — Endothelial cell — Liver metastasis

A complex series of steps is required to allow the successful establishment of tumor metastasis.[1-3] Tumor arrest initiated by tumor cell-endothelial cell contact followed by extravasation are important steps for the success of hematogenous metastasis.[4,5] Following the arrest, tumor cells must establish stable contacts with the endothelium, induce endothelial cell retraction, migration, and attach to the subendothelial matrix. Finally, they must proteolytically degrade the subendothelial matrix, extravasate, and proliferate. The adhesion of tumor cells to host endothelial cells in target organs for metastasis formation appears to involve multiple molecular interactions.[6,7]

Recently, there are many reports that cytokines such as IL-1[8],[6] and TNF[9] produced by tumor cells activate and induce endothelial cells to express ICAM-1,[10,11] ELAM-1[12] or VCAM-1[13,14] which normally mediate the adhesion or recruitment of neutrophils and lymphocytes to the stimulated vascular endothelium in the processes of tissue damage or inflammation. This suggests an interesting analogy for tumor metastasis.[15] Cytokines may affect the adhesion of malignant tumor cells to endothelium in a manner similar to that observed in leukocyte-endothelium interaction. Therefore, these adhesion molecules can serve as receptors for the circulating tumor cells and

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[6] The abbreviations used are: IL-1β, interleukin-1β; BSA, bovine serum albumin; DMEM, Dulbecco's modified Eagle's medium; EDTA, disodium ethylenediamine tetraacetate; ELAM-1, endothelial cell leukocyte adhesion molecule-1; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; HSE, hepatic sinusoidal endothelial; ICAM-1, intercellular adhesion molecule-1; IFN-γ, interferon-γ; IgG, immunoglobulin G; SLe^a, sialylated lacto-*N*-fucopentaose II; SLe^x/SSEA-1, sialylated lacto-*N*-fucopentaose III/stage-specific embryonic antigen; LFA-1, lymphocyte function-associated antigen-1; mAb, monoclonal antibody; PBS, phosphate-buffered saline; SCM-chitin, sulfated carboxymethyl-chitin; TNF-α, tumor necrosis factor-α; VCAM-1, vascular cell adhesion molecule-1; VLA-5, very late activated antigen-5.

promote the metastatic spread of tumors, probably by increasing tumor cell-endothelial cell adhesion.[16, 17]

Several attempts have been made to regulate the mechanism involved in tumor cell interaction with host cells and extracellular matrices during the metastatic process. Synthetic peptides derived from extracellular matrix components including fibronectin[18-20] and laminin,[21] or mAbs against a new variant form of CD44[22] or against cell surface glycoconjugate Fucα1-2Galβ1-R[23] have been used to inhibit experimental tumor metastasis through interference with tumor-host interaction and metastasizing functions such as attachment and motility. We have previously reported that recombinant fusion polypeptide of fibronectin containing cell- and heparin-binding domain (referred to as CH-271) was more active in inhibiting liver metastasis of L5178Y-ML25 lymphoma cells than polypeptide with the cell-binding domain (C-274), polypeptide with heparin-binding domain (H-271) or their mixture (C-274+H-271) when they were coinjected with tumor cells or separately injected after tumor inoculation.[24] We found that CH-271 led to a decrease in the arrest and retention of lymphoma cells in the liver (target organ) over 8 h after its coinjection with radiolabeled tumor cells, and also inhibited tumor cell adhesion to subendothelial matrix including fibronectin and laminin.[20]

In the present study, we focused on the tumor cell-endothelium interaction in the metastatic cascade, and investigated the effect of recombinant fibronectin polypeptides on tumor cell interaction with endothelium followed by tumor cell invasion, to clarify the mechanism of the inhibition of tumor cell arrest.

MATERIALS AND METHODS

Cells Liver metastatic L5178Y-ML25 lymphoma cells (partially metastasizing to the spleen) were maintained in RPMI1640 supplemented with 7.5% FBS and L-glutamine. Murine HSE cells were kindly provided by Dr. G. L. Nicolson, M. D. Anderson Cancer Center, Houston, TX. HSE cells[25] were maintained in 1% gelatin-coating plastic tissue-culture plates containing a 1:1 mixture of DMEM:F12 (GIBCO, Grand Island, NY) supplemented with 7.5% FBS and 0.1 mg/ml endothelial mitogen (Biomedical Technologies, Stoughton, MA).

Recombinant fibronectin fragments and other reagents We prepared three kinds of recombinant fragments of human fibronectin (C-274, H-271 and CH-271; shown in Fig. 5A) by expressing cDNA of human fibronectin in *Escherichia coli*, using an expression vector pUC118N/119N first described by Maki *et al.*[26] C-274 and H-271 correspond to cell- and heparin-binding domains of fibronectin, respectively, while another fragment, CH-271, is a fusion protein with both cell- and heparin-binding domains. All protein sequences are numbered by the system of Kornblihtt *et al.*[27] and all nucleotide sequences are numbered as in the EMBL data-bank file HUMFNMC of Kornblihtt. Two cDNA clones, pLF5 and pLF2435[28] were used for the construction of expression plasmids. The cell-binding polypeptide C-274 was expressed through a recombinant plasmid pTF7221 which had been constructed mainly from pLF5 and pUC119N. The plasmid pTF7221 was derived from pTF7121, which expresses a cell-binding polypeptide C-279 with five additional amino acids at the carboxyl terminus of C-274. The heparin-binding polypeptide H-271 was expressed by use of a recombinant plasmid pHD101 constructed from pLF2435 and pUC118N. The fusion protein CH-271 was expressed by use of a recombinant plasmid pCH101 constructed from pLF2435 and pTF7121. Detailed accounts of these constructions and expressions will be given elsewhere.[29] The recombinant fragment C-274 expressed in *E. coli* was purified from the cell extract by DEAE ion-exchange chromatography followed by gel filtration. Fragments H-271 and CH-271 were purified by carboxymethyl ion-exchange chromatography followed by affinity chromatography with heparin as a ligand. The purity of these polypeptides was verified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The amino-terminal sequence was checked with an automated peptide sequencer, model 477A (Applied Biosystems, Inc., Foster City, CA). The carboxy-terminal amino acid was also determined by use of carboxypeptidase P (Takara Shuzo Co., Ltd., Kyoto). These polypeptides were dissolved in Ca^{2+}- and Mg^{2+}-free PBS before use.

SLe^x [NeuAc2-3Gal1-4(Fuc1-3)GlcNAc-Gal-GlcNAc-] was chemically synthesized as described previously.[30] Basement membrane Matrigel (containing laminin, collagen type IV, heparan sulfate proteoglycan and entactin) was obtained from Collaborative Research Inc., MA. Anti mouse α5 mAb HMα5-1 was generously provided by Dr. Hideo Yagita (Juntendo University School of Medicine, Tokyo). Anti mouse LFA-1 mAb KBA was from Dr. Koji Nishimura (Tokai University School of Medicine, Kanagawa). Anti mouse CD44 mAb KM81 and anti mouse VCAM-1 mAb M/K-1 were provided by Dr. Kensuke Miyake (Saga Medical School, Saga). Anti Le^x mAb FH-6 and anti Le^a mAb 2D3 were kindly provided by Dr. Reiji Kannagi (Aichi Cancer Center Research Institute, Nagoya). Anti mouse ELAM-1 mAb 21KC10[31] was kindly provided by Dr. Dietmar Vestweber (Max-Planck Institute for Immunology, Freiburg). Anti heparan sulfate mAb HepSS-1 was from Seikagaku Kogyo Co., Ltd., Tokyo. Anti mouse IL-1α mAb, anti mouse TNF-α mAb and anti mouse IFN-γ were purchased from Genzyme Corporation, Cambridge, MA. Anti mouse IL-1β mAb was purchased from

Chemicon International Inc., CA. Anti mouse IFN-α mAb was obtained from Yamasa Shoyu Co., Ltd., Chiba. Mouse recombinant IFN-γ, TNF-α and IL-1β were purchased from Genzyme Corporation, Cambridge, MA. All the reagents and media in this study were endotoxin-free (approximately <1.0 ng/ml) as determined by a colorimetric assay (Pyrodick, Seikagaku Kogyo Co., Ltd.).

Preparation of the conditioned medium The conditioned medium was prepared from confluent cultures of tumor cells in RPMI-1640 containing 7.5% FBS in T-75 culture flasks for 16 h at 37°C. After incubation, the medium was collected and filtered through a millipore filter (pore size, 0.22 μm) to remove cells and cell debris. Thus prepared conditioned medium was used at the concentration of 100% for the assays.

Invasion assay The invasive activity of tumor cells was assayed in Transwell cell culture chambers according to previously described methods with some modifications.[32, 33] Matrigel (100 μg/ml) in a volume of 10 μl was applied to the lower surface of polyvinylpyrrolidone-free polycarbonate filters with 8 μm pore size (Nucleopore, Pleasanton, CA), and kept for 30 min in a 5% CO_2 atmosphere. L5178Y-ML25 cells in an exponential growth phase were incubated for 24 h in RPMI-1640 containing 7.5% FBS supplemented with 0.3 μCi/ml [^{125}I]iododeoxyuridine ([^{125}I]IUdR) (specific activity, 200 mCi/mmol, New England Nuclear, Boston, MA). The cells were washed twice with PBS to remove unbound radiolabels. Labeled tumor cells (2×10^5) with or without agents in a volume of 100 μl were added to the upper compartment of the chamber, and incubated for an appropriate number of hours at 37°C in a 5% CO_2 atmosphere. After incubation, the remaining tumor cells on the upper surface of filters were removed by wiping with cotton swabs, and then Matrigel containing the invading cells on the lower surface was wiped and absorbed on cotton swabs. The cells that had invaded through the filter into the Matrigel were calculated from the radioactivity by γ-counting, and each assay was performed in triplicate.

Tumor cell-endothelial cell adhesion The adhesive activity of tumor cells to endothelium was assayed according to the method reported by Takada *et al.*[34] with some modifications. Briefly, the adhesion assay was carried out in 24-well tissue culture plates (Costar 3422, Cambridge, MA). Confluent monolayers of HSE cells were washed twice with 1 ml of serum-free medium. HSE cell monolayers were preincubated with the conditioned medium (1:1) for various times. L5178Y-ML25 cells (5×10^5/ml) suspended in DMEM containing 1% BSA were added to the wells of confluent HSE cell monolayers and incubated at room temperature for up to 120 min with shaking on a reciprocal shaker (30 cycles/min). At various times the wells were washed four times with PBS to remove unattached cells. The remaining monolayer-bound tumor cells were manually counted under a microscope in 3 predetermined fields at a magnification of 200, and each assay was performed in triplicate. The data represent the number of attached cells per field.

Flow cytometric analysis Tumor cells was washed with PBS containing 0.2% BSA and 0.1% NaN_3, and suspended in the same buffer. Cells (1×10^6/30 μl) were then incubated with saturating concentrations of mAbs for 30 min at 4°C, and washed 3 times with PBS containing 0.1% NaN_3. The mAb-treated cells were incubated with appropriate FITC-conjugated antibodies for 30 min at 4°C. After washing, cells were analyzed by using FACScan flow cytometer (Becton-Dickinson Immunocytometry Systems, Mountain View, CA).

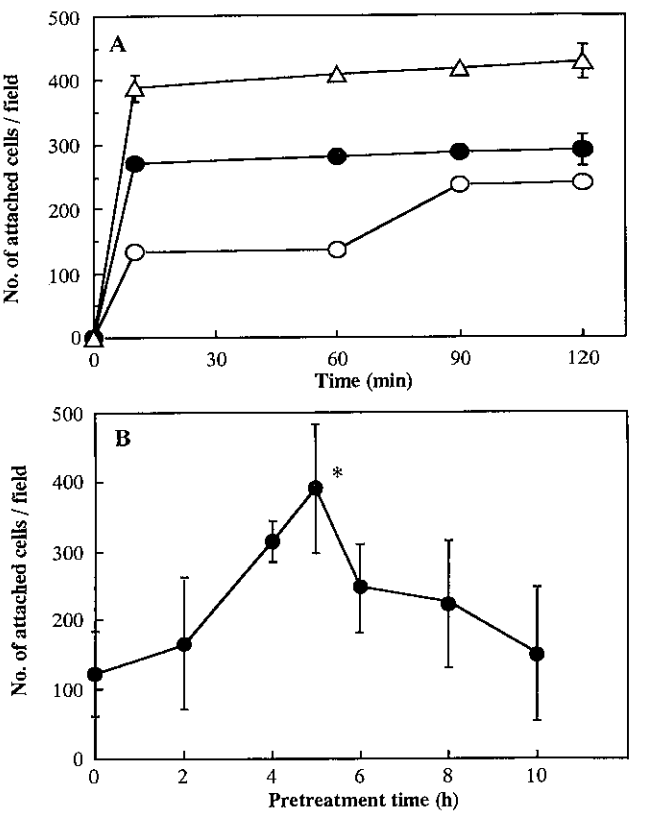

Fig. 1. Time course of L5178Y-ML25 lymphoma cell adhesion to HSE cell monolayers. (A) Various cell numbers of L5178Y-ML25 lymphoma cells (1×10^5; ○, 2×10^5; ●, 5×10^5; △) were added to confluent monolayers of HSE cells in 24-well culture plates and incubated for various time periods on shakers. Remaining attached cells were counted under a microscope. (B) Lymphoma cells (5×10^5) were added to HSE cell monolayers which had been preincubated with or without tumor conditioned medium (1:1, ●) for various time periods, and incubated for 20 min. *; $P<0.05$.

Statistical analysis The significance of differences between groups was calculated by applying Student's two-tailed t test.

RESULTS

Adhesion of L5178Y-ML25 lymphoma cells to endothelial cell monolayer Initial arrest to the circulating tumor cells in the target organ has been characterized in terms of endothelial cell-tumor cell contact.[4,5] We first investigated tumor cell adhesion to a confluent monolayer of HSE cells *in vitro*. L5178Y-ML25 lymphoma cells at various cell concentrations were added to HSE cell monolayers and incubated for appropriate times under shaking. Fig. 1A shows that the adhesion of tumor cells to HSE cell monolayers was cell number-dependent and the maximal cell adhesion was noted after more than 10 min incubation. In a separate experiment, we investigated whether pretreatment of the HSE monolayer with tumor conditioned medium can influence the subsequent tumor cell adhesion to the cell monolayer (Fig. 1B). When the cell monolayers were preincubated with the conditioned medium, the ability of lymphoma cells to adhere to the treated monolayers increased in a time-dependent manner, reached the maximal level after 4 to 6 h and thereafter decreased. These results indicate that the adhesive interaction between tumor cells and endothelial cells occurred rapidly during coincubation, and that the pretreatment of an endothelial cell monolayer with

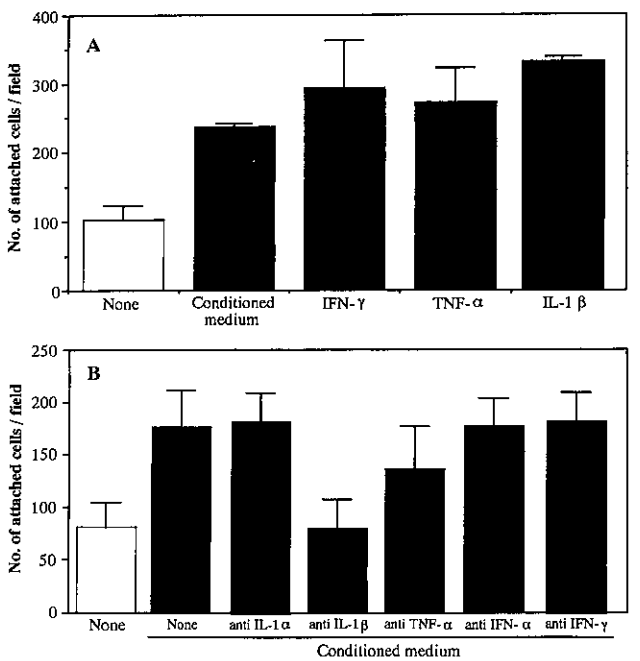

Fig. 2. Adhesion of L5178Y-ML25 lymphoma cells to HSE cell monolayers pretreated with tumor-conditioned medium. (A) L5178Y-ML25 cells (5×10^5/ml) were added to HSE cell monolayers which had been pretreated with the tumor-conditioned medium (1:1), IFN-γ (44 U/ml), TNF-α (1 ng/ml) or IL-1β (100 pg/ml) for 4 h, and incubated for 20 min with shaking. Remaining attached cells were counted under a microscope. (B) The conditioned medium was preincubated for 30 min with anti IL-1α (10 μg/ml), anti IL-1β (10 μg/ml), anti TNF-α (100 μg/ml), anti IFN-α (10 μg/ml) or anti IFN-γ (10 μg/ml) mAb before being added to the monolayers. Lymphoma cells (5×10^5/ml) were incubated for 20 min with HSE cell monolayers which had been pretreated with mAb-treated conditioned medium for 4 h. Remaining attached cells were counted under a microscope.

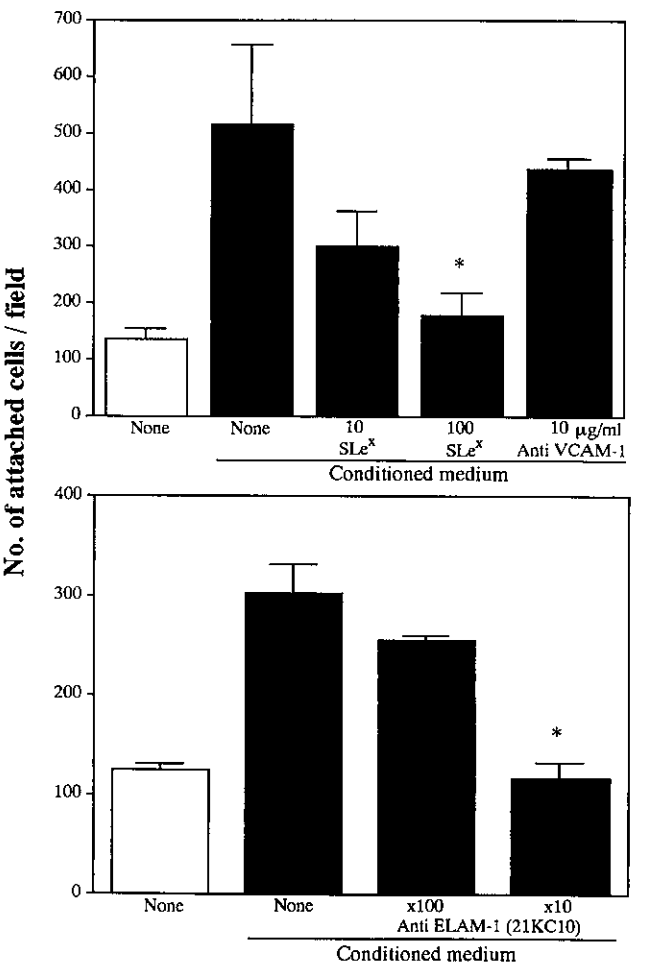

Fig. 3. Inhibitory effect of synthetic SLe^x, anti VCAM-1 or anti ELAM-1 mAbs on the adhesion of L5178Y-ML25 lymphoma cells to HSE cell monolayers. Lymphoma cells were added to conditioned medium-stimulated HSE cell monolayers which had been preincubated with synthetic SLe^x, anti VCAM-1 mAb (upper panel) or anti ELAM-1 mAb (lower panel) for 30 min at room temperature, and incubated for 20 min with shaking. Remaining attached cells were counted under a microscope. *; $P<0.01$.

tumor conditioned medium resulted in enhancement of the adhesiveness of the tumor cells to the cell monolayers. As shown in Fig. 2A, preincubation of HSE cell monolayers with IFN-γ, IL-1β or TNF-α as well as the conditioned medium of lymphoma cells for 4 h caused enhancement of adhesiveness of tumor cells to the cell monolayers. We therefore investigated the effect of tumor conditioned media which had been pretreated with or without mAbs against cytokines on tumor cell adhesion to HSE monolayers (Fig. 2B). The pretreatment of an HSE monolayer with the untreated conditioned medium enhanced tumor cell adhesion to the monolayer. The preincubation of the conditioned medium with anti IL-1β mAb before adding it to the HSE cell monolayers abolished the enhancement of tumor cell adhesiveness to HSE monolayers, while the anti IL-1α, anti TNF-α, anti IFN-α and anti IFN-γ mAbs did not affect the enhanced adhesiveness of tumor cells to HSE cell monolayers. The above results indicate that the enhancement of tumor cell adhesion to HSE cell monolayers may be due to the activation of the HSE cell monolayers by IL-1β produced by tumor cells or contained in the conditioned medium.

Effect of SLe^x and various antibodies on the enhanced lymphoma cell adhesion to HSE cell monolayers induced by tumor conditioned medium Since inflammatory cytokines such as IL-1[8] and TNF[9] can induce the expression of adhesion molecules such as ELAM-1 and VCAM-1 on endothelial cells, we investigated whether the enhanced tumor cell adhesiveness to endothelial cell monolayers is mediated by the expression of such cell adhesion molecules. Enhancement of tumor cell adhesion to HSE cell monolayers was completely inhibited by the addition of synthetic SLe^x (100 μg/ml) as a ligand for ELAM-1,[35,36] and anti mouse ELAM-1 mAb 21KC10 (1:10), while the pretreatment of conditioned medium-stimulated HSE cell monolayers with anti VCAM-1 mAb did not have any inhibitory effect on the tumor adhesion (Fig. 3). We also examined the expression of adhesion molecules including SLe^x and SLe^a on the surface of lymphoma cells by flow cytometry after immunofluorescence labeling with various mAbs. Fig. 4 shows that lymphoma cells expressed CD44, LFA-1 and heparan sulfate glycosaminoglycan at the surface, but SLe^x, SLe^a and VLA-5 were not detected on the cell surface.

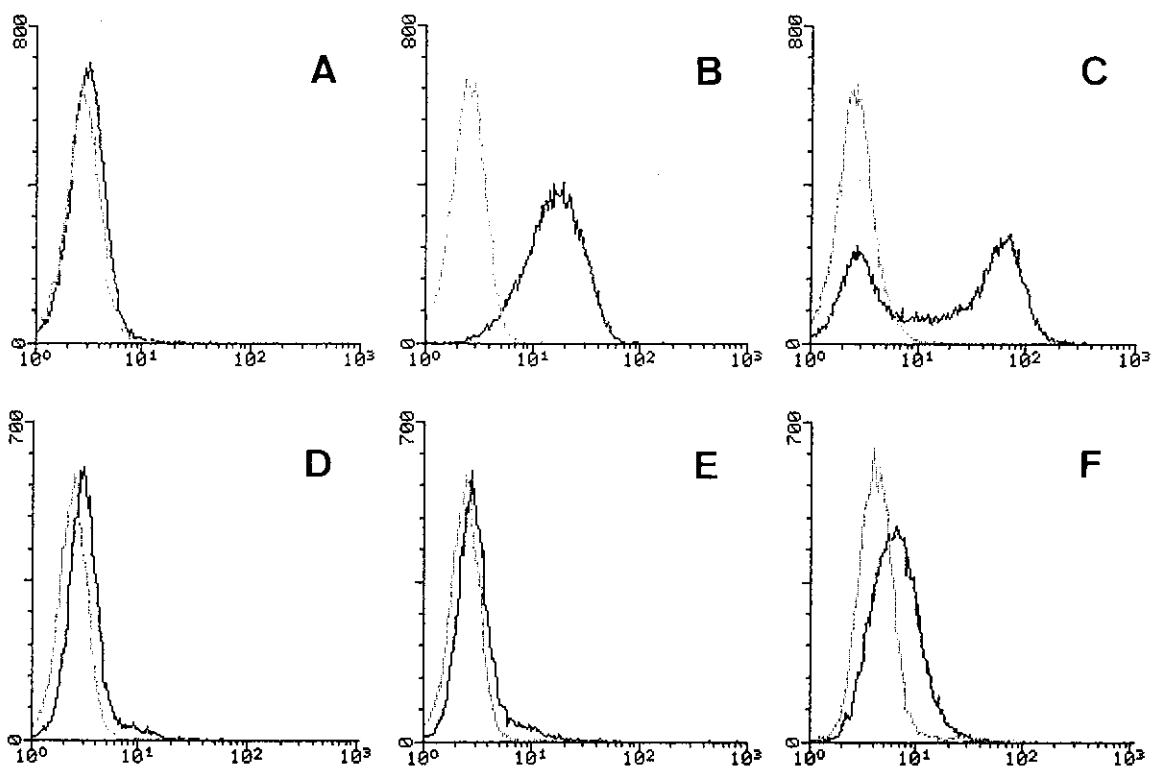

Fig. 4. Cell surface expression of some adhesion molecules on L5178Y-ML25 cells evaluated by flow cytometric analysis. The cells were treated with saturating concentrations of the following mAbs for staining and analyzed by FACScan: A; HMα5.1 (anti-α5), B; KBA (anti-LFA-1), C; KM81 (anti-CD44), D; FH-6 (anti-SLe^x), E; 2D3 (anti-SLe^a), and F; HepSS-1 (anti-heparan sulfate). The solid lines represent cell staining with (——) or without (——) the above primary antibodies, respectively.

Effect of recombinant fibronectin polypeptides on tumor cell adhesion to HSE cell monolayers Our previous studies have shown that coinjection of lymphoma cells with fibronectin-derived fusion polypeptide CH-271 containing a cell-binding domain (C-274) and a heparin-binding domain (H-271) led to a decrease in the arrest of tumor cells in the capillary bed of the chosen organ (liver) up to 8 h after the injection, but coinjection with a mixture of C-274 and H-271 (similar molar ratio to CH-271) did not affect the arrest and retention of labeled tumor cells in any organ as compared with the untreated control.[20, 24] We therefore examined the effect of recombinant fibronectin polypeptides (Fig. 5A) on lymphoma cell adhesion to HSE cell monolayers. CH-271 fusion polypeptide inhibited L5178Y-ML25 cell adhesion to the conditioned medium-activated HSE cell monolayers in a concentration-dependent manner, while tumor cell adhesion to unstimulated HSE cell monolayers was only slightly inhibited by CH-271 and the effect was not statistically significant (Figs. 5B and 6). Similarly, CH-271 was able to inhibit tumor cell adhesion to IL-1β-activated HSE cell monolayers (data not shown). On the other hand, H-271 polypeptide with the heparin-binding domain at the high concentration of 10 μg/ml inhibited tumor cell adhesion to the activated HSE monolayer, but C-274 polypeptide with the cell-binding domain did not exhibit any inhibitory effect at concentrations ranging from 0.1 to 10 μg/ml (Figs. 5C and 6). These results clearly indicated that the linkage of the cell-binding domain with the heparin-binding domain contributes to the augmentation of the inhibitory effect. Furthermore, the inhibitory effect may be attributed to the adhesive interaction between tumor cells and the heparin-binding domain in CH-271. SCM-chitin, which has been shown to possess antimetastatic and anti-adhesive effects partly mediated by its binding to the heparin-binding domains of laminin and fibronectin,[37, 38] inhibited tumor cell adhesion to a conditioned medium-stimulated HSE cell monolayer in a concentration-dependent manner (Fig. 5C). As shown in Fig. 7, anti ELAM-1 mAb (1:50) inhibited tumor cell adhesion to a conditioned medium-stimulated HSE monolayer by approximately 50%, whereas the antibody (1:10) or CH-271 (0.1 μg/ml) potently inhibited the adhesion (Figs. 3 and 5). CH-271 in combination

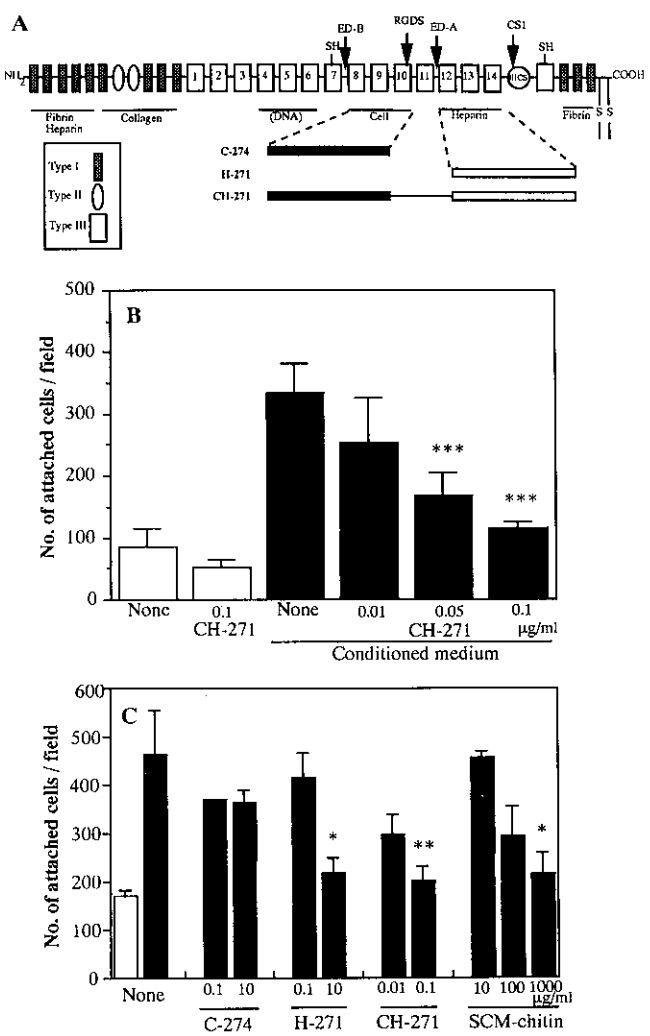

Fig. 5. Schematic diagram of recombinant fibronectin fragments and their effect on L5178Y-ML25 cell adhesion to HSE cell monolayers pretreated with tumor conditioned medium. (A) Locations of the fragments are shown by open and closed bars. The cell-binding polypeptide C-274 (Pro1239-Asp1512, the sequence numbered by the system of Kornblihtt *et al.*[27]) covers three units of type III homology at the cell-binding domain. The heparin-binding polypeptide H-271 (Ala1690-Thr1960) covers the complete region of the heparin-binding domain. The fusion polypeptide CH-271 (Pro1239-Ser1515-Met-Ala1690-Thr1960) contains both cell- and heparin-binding domains. The boxes at the top represent the locations of the type I, II and III homology repeats. The vertical arrow indicates the RGDS and CS1(EILDV) sites (↓). ED-A and ED-B indicate that extra domains arise from alternative splicing, respectively. (B) L5178Y-ML25 cells (5×10^5/ml) were added to HSE cell monolayers, which had been pretreated with or without tumor conditioned medium for 4 h, in the absence or presence of CH-271 polypeptide. After 20 min incubation with shaking, remaining attached cells were counted under a microscope. (C) L5178Y-ML25 cells (5×10^5/ml) were incubated with conditioned medium-activated HSE cell monolayers in the absence or presence of fibronectin-derived polypeptides (C-274, H-271, or CH-271) or a sulfated chitin derivative (SCM-chitin). After 20 min incubation with shaking, remaining attached cells were counted under a microscope. *; $P<0.05$, **; $P<0.01$, ***; $P<0.001$.

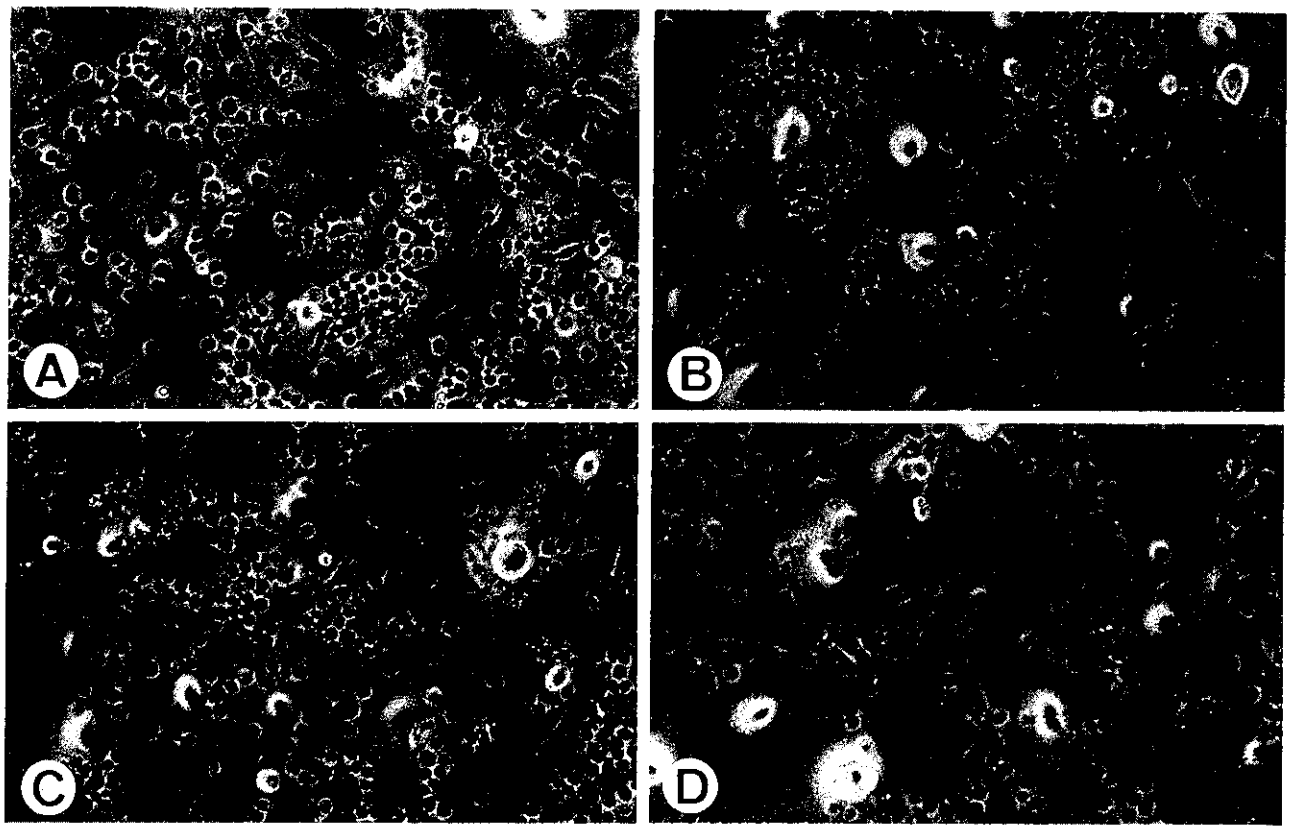

Fig. 6. Phase-contrast microscopy of L5178Y-ML25 cell adhesion to HSE cell monolayers. L5178Y-ML25 cells (5×10^5/ml) were added to the untreated HSE cell monolayers (A). L5178Y-ML25 cells were added to HSE cell monolayers pretreated with tumor conditioned medium for 4 h (B). L5178Y-ML25 cells were added to the conditioned medium-stimulated HSE cell monolayers in the presence of 10 μg/ml C-274 polypeptide (C) or 0.1 μg/ml CH-271 polypeptide (D).

with anti ELAM-1 mAb (1:50) augmented the mAb-mediated inhibitory effect and abolished the activity to a level similar to that of tumor cell adhesion to unstimulated endothelium. In contrast, although CH-271 (0.1 μg/ml) or anti ELAM-1 (1:10) completely inhibited the enhanced adhesion of tumor cells to the activated endothelium, the combination of CH-271 and anti ELAM-1 mAb did not show any additional inhibitory effect (data not shown).

Effect of fibronectin polypeptides on tumor cell invasion into the reconstituted basement membranes We next examined whether antimetastatic peptide CH-271 is able to inhibit the invasion of L5178Y-ML25 cells into reconstituted basement membrane Matrigel *in vitro*. Labeled tumor cells (2×10^5) were seeded onto the filters precoated with Matrigel on the lower surface, and incubated for various time periods at 37°C. Matrigel in which tumor cells invaded through the filter was wiped and monitored for radioactivity. Fig. 8 (A and B) and Fig. 9 show that lymphoma cells substantially penetrated through the filter into Matrigel on the lower surface both time- and cell number-dependently, and maximal invasive activity into Matrigel was observed after 12 h incubation. Approximately 10% of the cells that had invaded the Matrigel were recovered from the lower compartment of the chamber over 12 h after incubation and thereafter the level of recovered cells increased to 24% during a 24-h incubation (data not shown). The invasion of lymphoma cells into Matrigel was significantly inhibited by the addition of CH-271 into the upper compartment of the chamber in a concentration-dependent manner (Fig. 8C). In contrast, a mixture of C-274 and H-271 at a molar ratio similar to CH-271 did not inhibit tumor cell invasion into Matrigel.

DISCUSSION

To clarify the mechanism of tumor cell arrest in the target organ, we focused our attention on tumor cell-endothelium interaction, which is considered to be an

important step in the metastatic cascade, and we also investigated the effect of recombinant polypeptide of fibronectin on tumor cell adhesion to endothelium followed by invasion into subendothelial matrix *in vitro*. The preincubation of HSE cell monolayers with tumor conditioned medium for 4 to 6 h enhanced the adhesiveness to HSE cell monolayers (Fig. 1). Similar results were observed when radiolabeled tumor cells were added to HSE cell monolayers which had been preincubated for 4 h with 10^3 unlabeled tumor cells, in place of the conditioned medium (data not shown). Such enhanced adhesion of L5178Y-ML25 cells to the conditioned medium-stimulated HSE cell monolayer was specifically abolished by the pretreatment of the conditioned medium with anti IL-1β mAb, but not with mAbs specific for other cytokines (Fig. 2). Takeda *et al.*[39] have shown that culture supernatant of L5178Y-ML25 cells containing IL-1 activity induced IL-6 production in the lymphoid

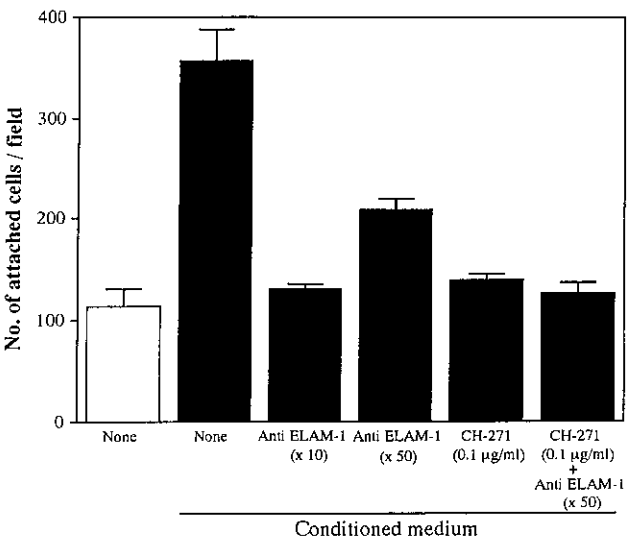

Fig. 7. Effect of CH-271 and anti ELAM-1 mAb in combination on L5178Y-ML25 cell adhesion to HSE cell monolayers pretreated with tumor conditioned medium. L5178Y-ML25 cells (5×10^5/ml) were added to conditioned medium-stimulated HSE cell monolayers which had been pretreated with or without anti ELAM-1 mAb for 30 min, in the absence or presence of CH-271 polypeptide. After 20 min incubation with shaking, remaining attached cells were counted under a microscope.

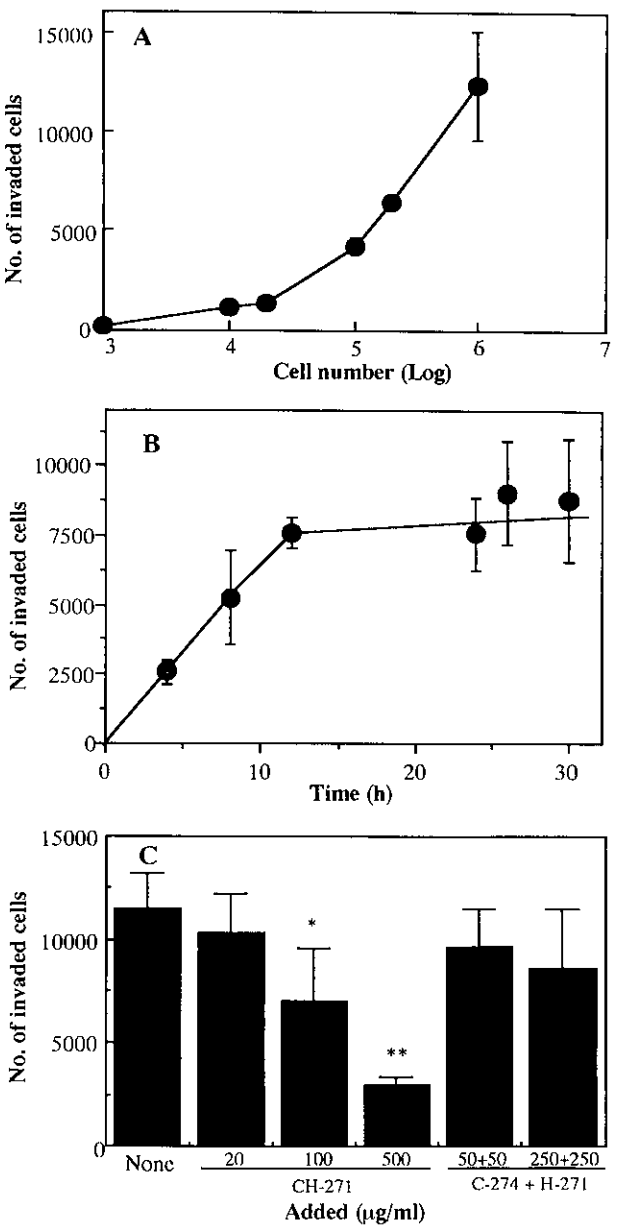

Fig. 8. Inhibitory effect of recombinant fibronectin-derived polypeptide on the invasion of L5178Y-ML25 cell into Matrigel. (A) Dose-dependence of L5178Y-ML25 cells on the invasion into Matrigel. Various concentrations of [^{125}I]IUdR-labeled lymphoma cells were added to the upper compartment of Transwell chambers and incubated for 6 h. Invaded cells contained in Matrigel were counted with a γ-counter. (B) Time course of L5178Y-ML25 cell invasion into Matrigel. Labeled lymphoma cells (2×10^5/0.1 ml) were added to the upper compartment of Transwell chambers and incubated for various time periods. The cells which had invaded the Matrigel were counted with a γ-counter. (C) Effect of recombinant polypeptides on the invasion of L5178Y-ML25 cells into Matrigel. Labeled lymphoma cells (2×10^5/0.1 ml) were added to the upper compartment of Transwell chambers in the absence or presence of the polypeptides and incubated for 6 h. Invaded cells were counted with a γ-counter. *; $P<0.05$, **; $P<0.01$.

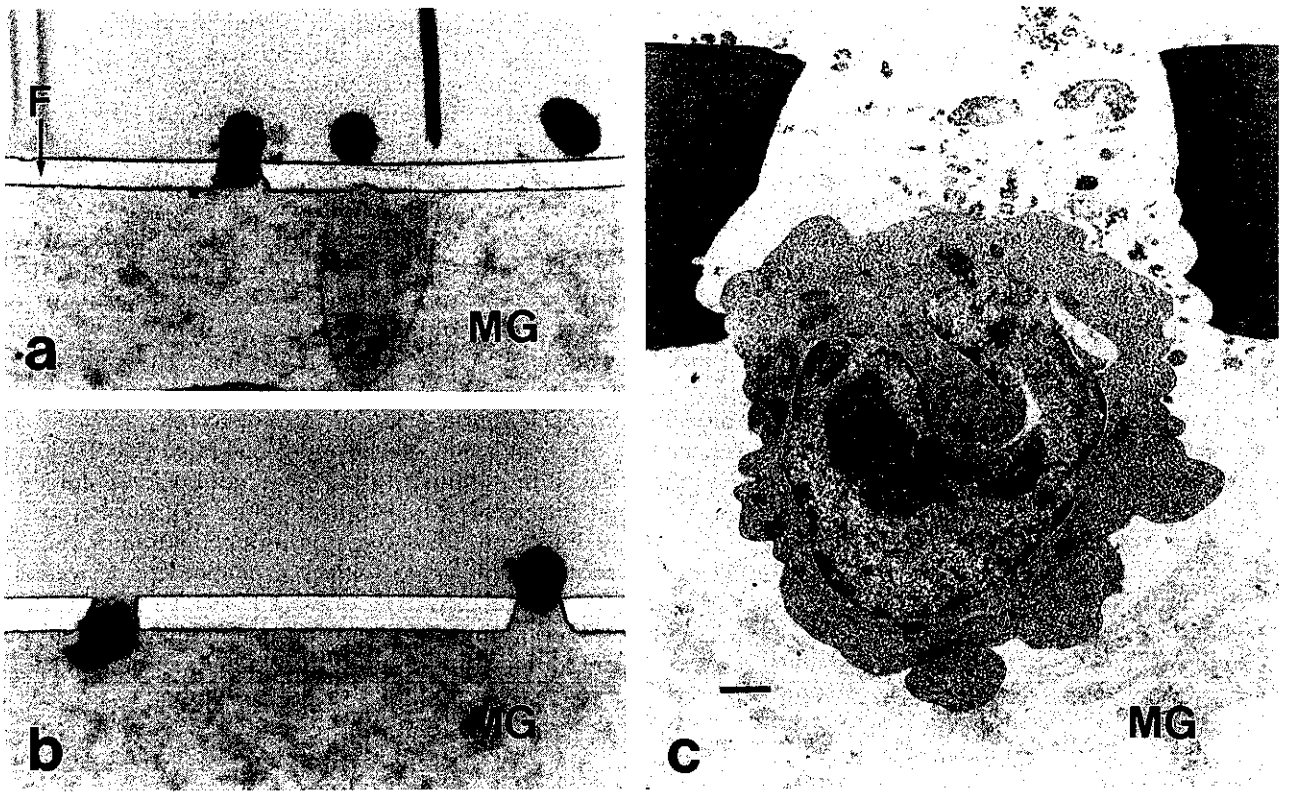

Fig. 9. Phase contrast and electron micrographs showing L5178Y-ML25 cell invasion into Matrigel. Shown are L5178Y-ML25 lymphoma cells which have been invading reconstituted basement membrane Matrigel (MG) during 4 h incubation in Transwell chambers. Vertical sections of polycarbonate filters with 8.0-μm pores (F) precoated with Matrigel on the lower surface are shown in a, b ($\times$100) and c (bar; 1 μm).

tissues of mice. We also observed that the tumor conditioned medium possessed IL-1 activity in the proliferation assay using mouse thymocytes *in vitro* (data not shown).

There have been many reports that inflammatory cytokines such as IL-1 and TNF can induce the expression of ELAM-1,[12] ICAM-1[10, 11] and VCAM-1[13, 14] adhesion molecules on the endothelial cell surface several hours after stimulation, resulting in increased adhesiveness of tumor cells as well as leukocytes to the endothelium. The enhanced tumor cell adhesion to conditioned medium-stimulated HSE cell monolayers was completely inhibited by the addition of anti mouse ELAM-1 mAb and synthetic SLe^x, which is well known as a ligand for ELAM-1 or GMP-140.[40] In contrast, the pretreatment of the activated HSE cell monolayer with anti VCAM-1 mAb did not have any inhibitory effect (Fig. 3). In our tumor-endothelial cell adhesion assay under shaking, pretreatment of HSE cell monolayers with anti ICAM-1 mAb KAT-1, or of L5178Y-ML25 cells with anti LFA-1 mAb KBA did not affect the enhanced tumor cell adhesion to HSE monolayers (data not shown). Recent studies have raised the possibility that some human tumors express SLe^x on the surface and can utilize ELAM-1 molecules to adhere to stimulated endothelial cells.[17, 41] These results indicated that the adhesive interaction between lymphoma cells and the activated endothelial cells is mediated by ELAM-1 molecules on the endothelial cell surface. This suggests that the initial attachment of a few circulating tumor cells to the endothelium and tumor-derived IL-1β can actually lead to the activation of the endothelial cells and subsequently to the enhancement of tumor cell adhesion to the activated cell monolayers.

Recombinant polypeptides containing the heparin-binding domain (CH-271 and H-271) were active in inhibiting the enhanced tumor cell adhesion to the monolayers, whereas C-274 was not able to inhibit the adhesion (Figs. 5 and 6). CH-271 in combination with anti mouse ELAM-1 mAb augmented the mAb-mediated inhibitory effect on tumor cell adhesion to the activated endothelium, but the optimal combination of CH-271 and anti mouse ELAM-1 mAb was unable to inhibit the adhesion to below the level of the control or either

treatment alone (Fig. 7). We have also shown that the pretreatment of CH-271 with IST-1 mAb, specific for the heparin-binding domain (H-271) of fibronectin, resulted in the suppression of the CH-271-mediated antimetastatic activity, but that anti cell-binding domain mAbs FN 12-8 and FN30-8 did not affect the activity.[20] SCM-chitin, which structurally mimics heparin and heparan sulfate,[37,38] also inhibited tumor cell adhesion to the activated HSE cell monolayers in a concentration-dependent manner. These results indicate that the heparin-binding domain of CH-271 is responsible for the inhibition of ELAM-1-mediated tumor cell adhesion to conditioned medium-activated HSE cell monolayers (Fig. 5).

However, the expression of SLe^x and SLe^a (as a ligand for ELAM-1) on the L5178Y-ML25 cell surface was not detected by flow cytometric analysis (Fig. 4). We recently observed that fibronectin and its heparin-binding domain are not able to bind to SLe^x in a thin-layer chromatogram overlay assay (data not shown). Yuen *et al.*[42] demonstrated that not only the fucooligosaccarides of Le^a or Le^x/SSEA-1 type and their sialyl analogues, but also sulfated oligosaccharides which present a negative charge in a similar spatial orientation, although differing in molecular detail, can form high-affinity ELAM-1/E-selectin ligands, and, furthermore, they suggested that biosynthetically distinct E-selectin ligands may exist in various tissues and cells. Therefore, sulfated polysaccharides or sulfatides on the surface of lymphoma cells may serve as ligands for ELAM-1 on the endothelial surface, and CH-271 may interfere with such an interaction through binding of these ligands to the heparin-binding domain of CH-271. Further study will be needed to identify the surface molecules involved in the tumor-endothelial cell interaction and to determine the inhibitory mechanism by CH-271 polypeptide in detail.

Following endothelial cell retraction, tumor cell invasion of subendothelial matrix and basement membrane is also an important step in stabilizing tumor cell arrest at a secondary site. Several investigators have reported that injury resulting from surgical or mechanical trauma, exposure to chemicals, and treatment with radiation[43] and anticancer drugs[44] can induce vascular endothelial damage and exposure of the subendothelial matrix to blood flow, which may result in the enhancement of tumor invasion and metastasis. CH-271 polypeptide inhibited the invasion of L5178Y-ML25 cells into reconstituted basement membrane Matrigel precoated on the lower surface of the filters (Fig. 8). However, the tumor invasion into Matrigel was not significantly inhibited by a mixture of C-274 and H-271 at a similar molar ratio to CH-271.

In conclusion, we have demonstrated that IL-1β produced from initially attached tumor cells can induce the enhancement of subsequent tumor cell adhesiveness to endothelium. However, various kinds of tumor cells may produce different cytokines to induce the activation of endothelium. Antimetastatic CH-271 also inhibited not only the tumor-endothelium interaction, but also tumor invasion of the basement membrane *in vitro*.

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