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VLA-4 Molecules on Tumor Cells Initiate an Adhesive Interaction with VCAM-1 Molecules on Endothelial Cell Surface

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To elucidate the role of VLA-4 ($\alpha 4\beta 1$ integrin) in tumor metastasis, we have transfected cDNA coding $\alpha 4$ subunit into human fibrosarcoma (HT1080) cells. VLA-4-overexpressing HT-VC1 cells exhibited increased ability to interact with known ligands for VLA-4, such as CS1 peptide and VCAM-1 (vascular cell adhesion molecule-1). In addition, the *in vitro* invasive ability of HT-VC1 cells was augmented and the mRNA for type IV collagenase was increased in HT-VC1 cells. The induction of VCAM-1 molecules on lung endothelial cells of nude mice by tumor necrosis factor-α treatment resulted in augmentation of *in vivo* HT-VC1 cell adhesion to the lung endothelial cells. Thus, the VLA-4 molecules on tumor cells initiate an adhesive interaction with VCAM-1 molecules on endothelial cells, that is important for hematogenous metastasis.

Key words: Integrin — VLA-4 — cDNA transfection — Human sarcoma cell — Metastasis

Hematogenous metastasis requires a series of interactions of tumor cells with extracellular matrix (ECM) components or with other cells.[1-3] Since almost all of these adhesive interactions are dependent on cell surface receptors, they are likely to be important regulators of tumor invasion and metastasis. Some of these receptors belong to the integrin family.[4-7] The integrins are heterodimers consisting of noncovalently associated α and β subunits, and have been divided into three major subfamilies. Members of the $\beta 1$ subfamily (called very late antigen; VLA) each contain the $\beta 1$ subunit in association with one of at least six different α subunits. VLA-1[8,9] and VLA-2[10] interact with both collagen and laminin. VLA-3 binds to fibronectin, collagen and laminin, and also binds to a novel glycoprotein, epiligrin, which is an ECM component of human foreskin keratinocytes.[11-13] VLA-5[14] and VLA-6[15] are receptors for fibronectin and laminin, respectively.

VLA-4 is unique among VLA proteins because of its functional versatility.[16] In contrast to other VLA proteins, VLA-4 is involved in both cell-matrix and cell-cell adhesion. VLA-4 recognizes the CS1 region of fibronectin,[17] and mediates cell adhesion to vascular endothelial cells via vascular cell adhesion molecule-1 (VCAM-1) which appears on the cell surface after cytokine-activation.[18] In addition, *in vitro* inhibition assays using various anti-VLA-4 monoclonal antibodies (mAbs) have indicated that VLA-4 is involved in both homotypic and heterotypic aggregations of lymphocytes.[19,20] Thus, VLA-4 is considered to play important roles in *in vivo* inflammatory response.

In addition, VLA-4 has been proposed to have a role in tumor metastasis, since some malignant melanoma cell lines adhere to cytokine-activated human umbilical vein endothelial cells (HUVECs) via VLA-4/inducible cell adhesion molecule 110 (INCAM-110) interaction (INCAM-110 is identical to VCAM-1).[21] In support of this suggestion is a previous demonstration[22] that the number of melanoma lung colonies is increased in interleukin-1 (IL-1)- and tumor necrosis factor (TNF)-treated nude mice. However, the role of VLA-4 in hematogenous metastasis has not been confirmed directly *in vivo*.

Our recent study demonstrated that only VLA-4 was expressed on 100% of osteogenic sarcoma cells among VLA-1 to VLA-6 integrins, and the strong expression of VLA-4 remained unchanged on tumor cells in the primary lesion, the invasive portion, and the pulmonary metastatic foci (S. Kawaguchi and T. Uede, submitted for publication). Thus VLA-4 can be postulated to play a pivotal role in pulmonary metastasis of osteogenic sarcoma cells. We in this study introduced a gene coding for VLA-4 α subunit into HT1080 cells, which originally express a small amount of endogenous VLA-4. With the use of the VLA-4-overexpressing clone, we analyzed in detail the function of VLA-4 in the hemotogenous metastasis of human sarcoma cells.

MATERIALS AND METHODS

Animals, cells, and reagents Five-week-old female Balb/c-*nu/nu* mice were purchased from Japan Clea Inc.

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(Tokyo). The mice were maintained in the Laboratory for Animal Experiments, Sapporo Medical College, under a specific pathogen-free condition. HUVECs were purchased from Kurabo Inc. (Osaka) and were cultured in MCDB 131 medium containing 2% fetal calf serum (FCS), hydrocortisone (1 μg/ml), and endothelial growth factor (10 ng/ml). HT1080 (human fibrosarcoma) cells were obtained from the Japanese Cancer Research Resources Bank and maintained in Eagle's minimal essential medium (MEM) supplemented with 10% FCS. Fibronectin (human), type I collagen (bovine) and laminin (murine) were purchased from Koken, Inc. (Tokyo). Basement membrane Matrigel (containing laminin, type IV collagen, heparan sulfate proteoglycan, and entactin) was obtained from Collaborative Research Inc. (Bedford, MA). The synthetic peptides, GRGDSP and CS1 (CDELPQLVTLPNLHGPEILDVPST), and the peptide-protein conjugates, GRGDSP-bovine serum albumin (BSA) and CS1-BSA, were purchased from Iwaki Glass Inc. (Tokyo). ^{125}I and ^{32}P were from NEN Research Products (Boston, MA). mAbs used throughout this study are as follows: 8H3 (control) was obtained as described previously.[23] Anti-α1 mAb TS2/7[24] and anti-α4 mAb B5G10[25] were gifts of Dr. Martin Hemler (Dana-Farber Cancer Institute, MA). Anti-α2 mAb 12F1[26] was from Dr. Virgil Wood (University of California, San Diego, CA). Anti-α3 mAb J143[27] and anti-β1 mAb AJ-2[28] were provided by Dr. Tony Albino (Memorial Sloan-Kettering Cancer Center, NY). Anti-α4 mAb HP2/1[29] was from Dr. Sanchez-Madrid (Hospital de la Princesa, Madrid, Spain). Anti-α5 mAb BIIG 2[30] was obtained from Dr. Caroline Damsky (University of California at San Francisco, San Francisco, CA). Anti-α6 mAb GoH3[31] was from Dr. A. Sonnenberg (Red Cross Blood Transfusions Services, Amsterdam, The Netherlands). Anti-mouse VCAM-1 mAb M/K-1[32] was from Dr. Kensuke Miyake (Saga Medical School, Saga). Anti-human VCAM-1 mAb was purchased from Cosmo Bio, Tokyo. Fluorescein isothiocyanate (FITC)-conjugated rabbit anti-mouse immunoglobulin and FITC-conjugated goat anti-rat immunoglobulin were obtained from Jackson Immunoresearch Laboratories, Inc. (West Grove, PA) and Kirkegaard and Perry Laboratories, Inc. (Maryland), respectively.

Transfection of α4 cDNA Assembly of the entire α4 coding region[20] into the mammalian cell expression vector pFneo[33] was accomplished as described previously.[18] HT1080 cells (1.2×10^6), seeded on the previous day in a six-well plate (Corning, 25810), were transfected with 10 μg of α4-pFneo construct by using the calcium phosphate coprecipitation procedure.[34] Transfectants were isolated by selection with 1 mg/ml geneticin (G418 sulfate, Gibco Co.), and tested for cell surface expression of α4 by flow cytometry using anti-α4 mAb, B5G10. The transfectant which had expressed the highest level of α4 was subcloned by limiting dilution and was designed as HT-VC1.

Flow cytometry HT1080 or HT-VC1 cells were seeded in culture dishes (Falcon, 10 cm diameter) at a cell density of 1×10^6/10 ml/dish, and incubated for 24 h at 37°C in 5% CO_2. Cells were harvested with 0.05% trypsin/0.02% ethylenediaminetetraacetic acid (EDTA) in phosphate-buffered saline (PBS) and then incubated for an additional 1 h. After washing with PBS, cells were treated with saturating concentrations of mAbs recognizing VLA α (α1–6) and β1 for 45 min at 4°C. Cells were washed with PBS, then cells treated with anti-α1–4 and anti-β1 mAbs or with anti-α5 and α6 mAbs were incubated with FITC-rabbit anti-mouse immunoglobulin and FITC-goat anti-rat immunoglobulin for 45 min at 4°C, respectively. After washing, cells were analyzed by using a fluorescence-activated cell sorter (FACS; Becton Dickinson Co., Sunnyvale, CA).

Cell labeling and immunoprecipitation Cells were externally labeled with ^{125}I by using lactoperoxidase according to the method previously described.[35] After iodination, cells were washed three times with PBS by centrifugation, and the cell membrane was disrupted by adding lysis buffer (0.05 *M* Tris-HCl buffer, pH 7.4, containing 0.14 *M* NaCl, 0.5% Nonidet P-40, 1 m*M* phenylmethylsulfonyl fluoride, 1 μg/ml of pepstatin, 0.05% sodium azide, 0.2 TIU/ml of aprotinin, 8 m*M* iodoacetamide, and 5 m*M* EDTA) for 1 h at 4°C. Aliquots of the labeled cell membrane were then incubated with *Staphylococcus aureus* Cowan 1 strain (The Enzyme Center, Maiden, MA) for 8 h at 4°C. After centrifugation, the resulting supernatant was incubated with control mAb, anti-α4 mAb, or anti-β1 mAb for 8 h at 4°C, followed by addition of protein A-Sepharose (Pharmacia, Uppsala, Sweden) and further incubation overnight at 4°C. The antigen-antibody complexes were washed four times with buffer containing 0.05 *M* Tris-HCl, pH 7.4, 0.14 *M* NaCl, and 0.1% Nonidet P-40, and then dissociated by boiling in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (10% SDS/10% glycerol/0.01% bromphenyl blue/62.5 m*M* Tris-HCl, pH 7.0) without 5% 2-mercaptoethanol for 5 min. Samples were analyzed by electrophoresis in a gradient SDS-polyacrylamide gel (Daiichi Pure Chemicals Co., Tokyo). The radioactivity in the slab gel was visualized by autoradiography with a Cornex intensifying screen (Dupont Instruments, Wilmington, DE).

Cell spreading assay Interaction with CS1 region in fibronectin was assessed by cell spreading assay as described by Guan and Hynes.[36] Briefly, 96-well plates (Falcon, 3072) were coated with 1% BSA or various concentrations of CS1-BSA conjugates overnight at 4°C and then blocked with PBS containing 1% heat-denatured BSA.

Cells were resuspended in MEM with 1% BSA and added to the CS1-coated wells (1×10^4 cells/well). After 2 h of incubation at 37°C, the plates were fixed in 3.7% formaldehyde in PBS. Photographs were taken using a Nikon inverted phase-contrast microscope. Quantitation of the percentage of cells exhibiting spreading was determined by counting four fields at random.

Cell adhesion to HUVEC monolayers Confluent monolayers of HUVECs at second passage were prepared in eight-well LAB-TEK chamber/slides (Nunc, Inc., Naperville, IL). HUVECs were incubated for 24 h with TNF-α (10 ng/ml, Genzyme Co., Boston, MA). HT1080 and HT-VC1 cells were resuspended in MEM with 1% BSA and plated on top of HUVEC monolayers (4×10^4 cells/well). After 1 h of incubation at 37°C, nonattached cells were removed by washing the chambers gently with PBS. The chambers were fixed in methanol and cells were stained with Diff-Quik (Kokusai Reagent Co., Kobe), which is a modification of the Wright and Giemsa staining method. Attached cells were counted visually under a microscope in five random fields at a magnification of $\times100$. For inhibition experiments, cells were preincubated in MEM with 1% BSA and control mAb 8H3 (1:400 dilution of ascites), anti-α4 mAb HP2/1 (1:10 dilution of supernatant), synthetic GRGDSP peptide (100 μg/ml), or CS1 peptide (100 μg/ml) for 30 min at room temperature, and then added directly to the HUVEC monolayers.

Immunoperoxidase staining Lungs were removed from nude mice which had been given i.v. injections of TNF-α (1 μg). Lungs were snap-frozen in liquid nitrogen and kept at –70°C until cryostat sectioning. Cryostat sections were placed on albumin-coated slides, fixed with cold acetone for 10 min, incubated with M/K-1 (anti-mouse VCAM-1 mAb) for 1 h at room temperature, washed three times with PBS, and again reacted with anti-rat κ-chain immunoglobulin for 30 min. After washing with PBS, the sections were stained by using avidin-biotin-peroxidase complex (Nichirei, Inc., Tokyo).

Cell adhesion to ECM and GRGDSP peptide Adhesive ability to ECM proteins and GRGDSP peptide was examined as described by Dedhar and Saulnier.[37] Briefly, 96-well plates (Falcon, 3072) were coated with increasing concentration of fibronectin, type I collagen, laminin, and GRGDSP-BSA conjugate overnight at 4°C. The plates were blocked with PBS supplemented with 1% heat-denatured BSA for 30 min at room temperature. After harvesting and an additional 1 h incubation in MEM with 10% FCS, cells were resuspended in MEM with 1% BSA and plated in matrix-precoated wells (5×10^4 cells/well) for 15 min (ECM-coated plates) or 2 h (peptide-coated plate) at 37°C. Nonattached cells were washed away with PBS and the attached cells were fixed with 3.7% paraformaldehyde, stained with 0.5% toluidene blue, and washed again. The absorbance at 490 nm was measured to determine relative cell number. Each assay was performed in triplicate. The statistical significance of differences between HT1080 and HT-VC1 cells was determined by using Student's two-tailed t test.

***In vitro* migration and invasion assays** The ability of HT1080 and HT-VC1 cells to migrate through ECM was assayed in ECM-coated chemotaxis chambers containing polycarbonate filters with 5 μm pores (Chemotaxicell; Kurabo, Osaka). The assay was modified from that described by Saiki *et al.*[38] Briefly, type I collagen, fibronectin or laminin (5 μg in total) was used to coat the lower surface of the polycarbonate filters, which were allowed to dry overnight. Cells were harvested, incubated for an additional 1 h, and then resuspended at a cell density of 2×10^5/ml in MEM with 10% FCS. Cell suspensions (500 μl) were placed in chemotaxicells containing ECM-coated filters, and the chemotaxicells were placed in a 24-well plate filled with MEM containing 10% FCS. After incubation for an appropriate time at 37°C in 5% CO_2, the filters were fixed with methanol, and stained with Diff-Quik. For quantification, the cells which had migrated to the lower surface were counted visually in five predetermined fields at a magnification of $\times400$. Each assay was performed in triplicate.

To quantify the ability of these cells to invade through basement membranes, reconstituted basement membrane, Matrigel, was used according to the methods described by Albini *et al.*[39] Briefly, the lower surface of the filters was precoated with type I collagen, as described above. The Matrigel was diluted with cold PBS, applied to the upper surface of filters (50 μg in total), and allowed to dry overnight. The subsequent procedures were the same as those of the migration assay. The statistical significance of differences in these assays was determined by using Student's two-tailed t test.

RNA isolation, blotting, and hybridization Total RNA was isolated from cultured HT1080 and HT-VC1 cells by the guanidinium-CsCl method.[40] RNA (20 μg) from each cell line was electrophoresed in 1% agarose gels containing formaldehyde and was transferred to nylon membranes (NEN Research Product). cDNA probe (bp 1–1824) for VLA-4 α chain was isolated by *Eco*RI digestion. Probes for human 72 kd type IV collagenase (bp 122–1747)[41] and 92 kd type IV collagenase (bp 381–1247)[42] were kind gifts from Dr. Motoharu Seiki (Cancer Research Institute, Kanazawa University, Kanazawa), and the probe for β-actin was purchased from Wako Pure Chemical Industries (Osaka). They were labeled with ^{32}P using Klenow polymerase and then hybridized with RNA-transferred membranes at 42°C for 12 h as previously described.[43] Membranes were washed with 2×, 1×SSC at 65°C. Radioactivity of the membranes was visualized as described for immunoprecipitation.

***In vivo* tumor cell adhesion to lung endothelial cells** Mice were given intravenous (i.v.) injection of 1 μg of TNF-α (R & D Systems Inc., Minneapolis, MN) 1 h before tumor cell injection according to Giavazzi *et al.*[22] In some experiments, HT-VC1 and HT1080 cells were pretreated with either a saturating amount of mAb reacting with α4, HP2/1 or human MHC class I, HH-1[35] for 30 min at 4°C. The tumor cells were washed and i.v. injected into nude mice. At various times after tumor cell inoculation (1×10^6), the lungs were removed, sections were made and the number of tumor foci adhering to lung endothelial cells were counted under a microscope in five randomly selected fields at a magnification of $\times100$ as described previously.[44] The statistical significance of differences was determined by using Student's two-tailed *t* test.

Experimental metastasis analysis Nude mice were pretreated with TNF-α (1 μg/mouse) at 1 h prior to tumor cell injection. HT1080 as well as HT-VC1 (1×10^5 in 0.1 ml of PBS) cells were injected into the lateral vein of nude mice. After 4 weeks, the mice were killed, the lungs were removed, and the numbers of metastatic foci in separated lung lobes were counted under a microscope as described previously.[45]

RESULTS

Expression of VLA proteins on α4-transfectant As shown in Fig. 1, HT1080 cells expressed various VLA proteins (dashed line). The α3, α5, and α6 were expressed at comparably high levels, and α2 was present at a moderate level, whereas α1 and α4 were present in lower amounts. Transfection of α4-cDNA resulted in

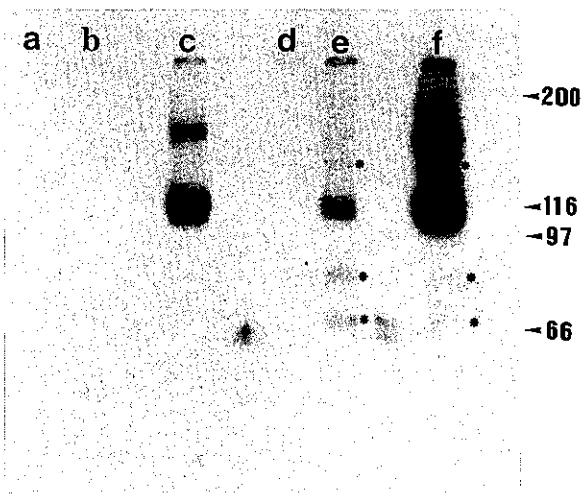

Fig. 2. Immunoprecipitation analysis of surface VLA proteins. HT1080 (lanes a to c) and HT-VC1 cells (lanes d to f) that were labeled with ^{125}I were solubilized by lysis buffer. Cell extracts were immunoprecipitated with mAb 8H3 as a negative control (lanes a and d), anti-α4 mAb B-5G10 (lanes b and e), and anti-β1 mAb AJ-2 (lanes c and f). Precipitated samples were then analyzed by SDS-PAGE under nonreducing conditions. * indicates the 140 kd, 80 kd, and 70 kd components of α4 subunit.

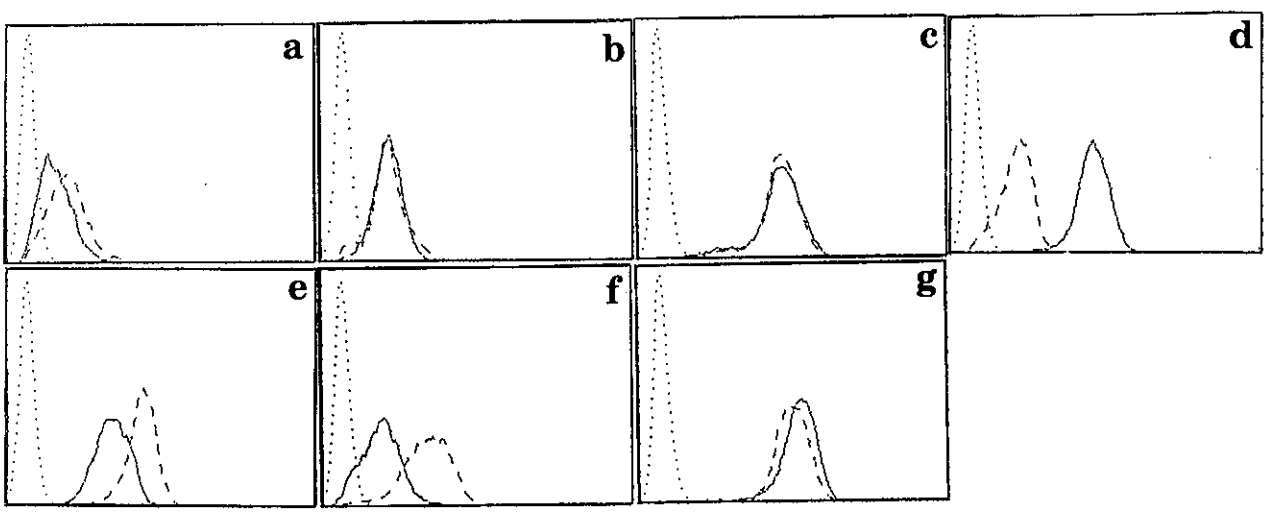

Fig. 1. Cell surface expression of VLA proteins on HT1080 cells and α4-transfectants (HT-VC1 cells). Abscissa; logarithm of fluorescence intensity. Ordinate; relative cell number. Flow cytometric analysis was performed on HT1080 (dashed line) and HT-VC1 cells (solid line). Cell samples were stained with the following mAbs and analyzed by FACS: TS2/7 (anti-α1) (a), 12F1 (anti-α2) (b), J143 (anti-α3) (c), B-5G10 (anti-α4) (d), BIIG2 (anti-α5) (e), GoH3 (anti-α6) (f), and AJ-2 (anti-β1) (g). The dotted line indicates cell samples where the primary antibody is omitted.

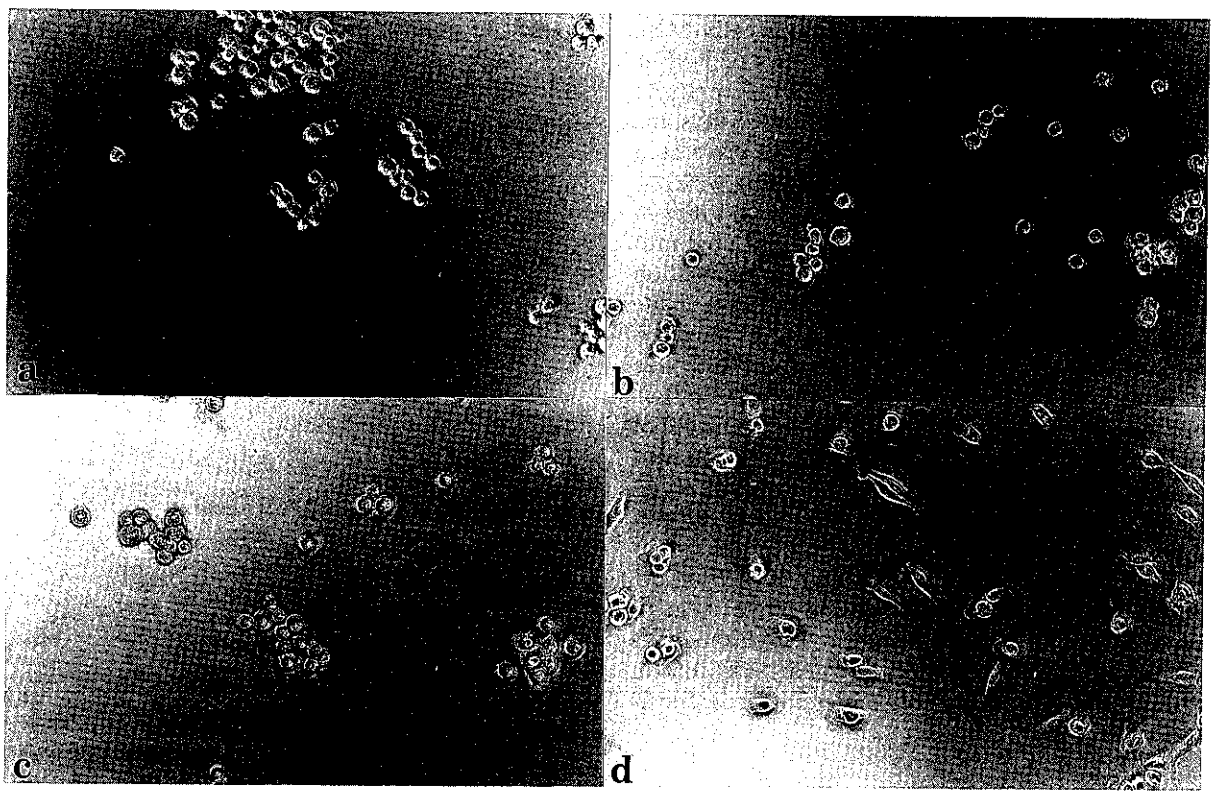

Fig. 3. Ability of HT1080 and HT-VC1 cells to spread on synthetic CS1 peptide. HT1080 (a and c) or HT-VC1 (b and d) cells were seeded in BSA (1%)-coated wells (a and b) or CS1 peptide (30 μg/ml)-coated wells (c and d). After 2 h incubation at 37°C, the plates were photographed (magnification ×170).

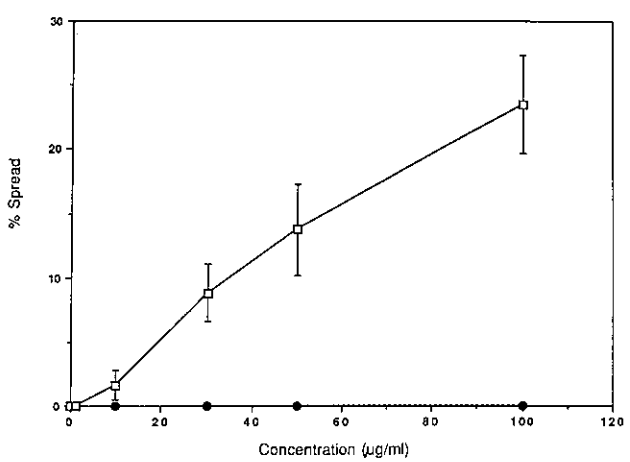

Fig. 4. The plates were precoated with CS1 peptide at different concentrations. The spreading of HT1080 (●) and HT-VC1 cells (□) was quantitated by counting four randomly selected fields under the phase contrast microscope. Approximately 200 to 300 spreading and non-spreading cells were detected per field. Results are indicated as the percentage of spreading cells per added cells with standard deviations.

several alterations in VLA expression (solid line). There were not only a great increase of $\alpha 4$ subunit but also notable decreases in $\alpha 5$ and $\alpha 6$, and a slight decrease in $\alpha 1$. $\beta 1$ was expressed at a slightly increased level, and $\alpha 2$ and $\alpha 3$ at the same levels. RNA blotting analysis also revealed that $\alpha 4$-transfectant (termed HT-VC1) produces a high level of $\alpha 4$ mRNA transcript compared with HT1080 cells (data not shown).

In order to determine whether $\alpha 4$ gene product is associated with the endogenous $\beta 1$ subunit of HT1080 cells, an immunoprecipitation experiment was performed. Fig. 2 showed that the $\alpha 4$ subunit was precipitated as a heterodimer with $\beta 1$ (lane e) from HT-VC1 cells; the 140 kd component was $\alpha 4$, the 110 kd component was $\beta 1$, and breakdown products derived from $\alpha 4$ were detected at Mr 80 kd and 70 kd under non-reducing conditions, as is characteristic of $\alpha 4$ subunit.[25] In contrast, $\alpha 4$ was not detected in HT1080 cells after the same exposure time as used for HT-VC1 cells (lane b). Similarly, anti-$\beta 1$ mAb coprecipitated $\beta 1$ as well as $\alpha 4$ subunit from HT-VC1 cells (lane f), whereas the same antibody precipitated $\beta 1$, but not $\alpha 4$ from HT1080 cells (lane c).

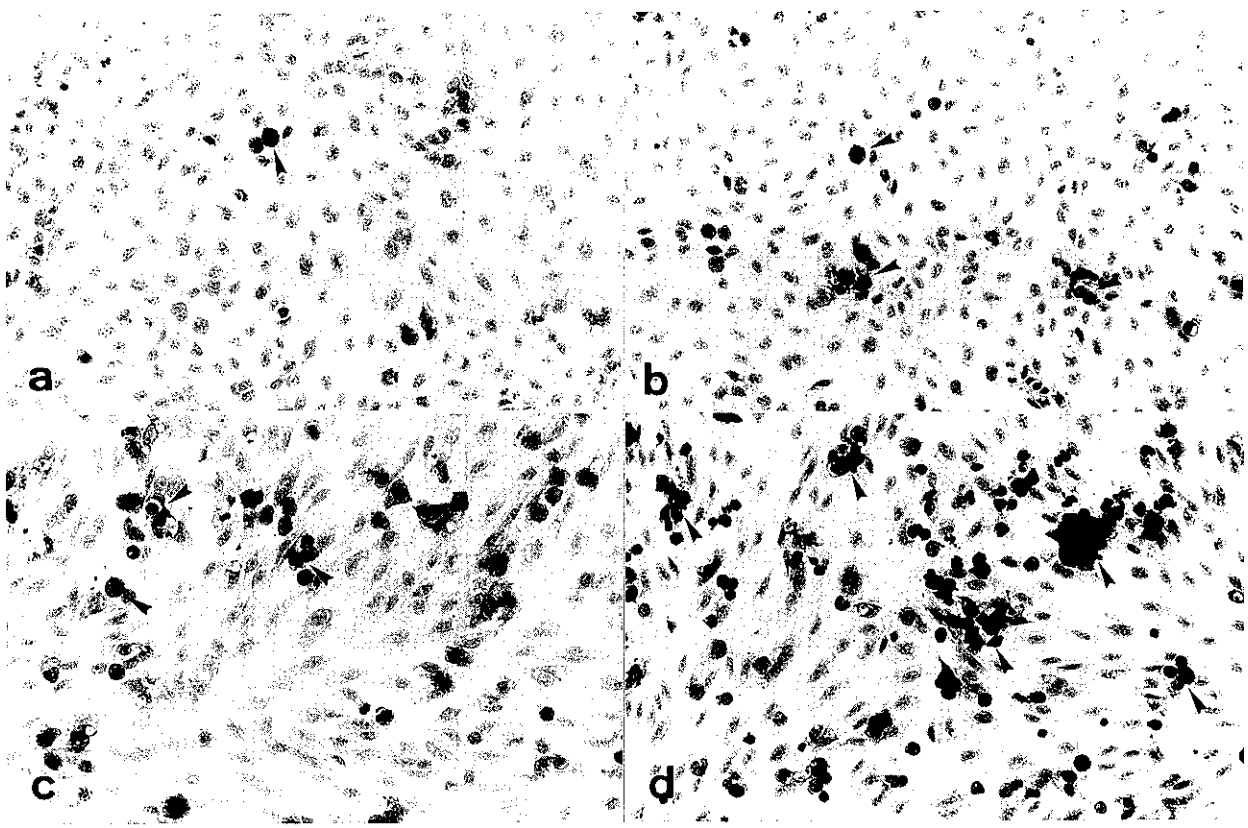

Fig. 5. Adhesion of HT1080 and HT-VC1 cells to HUVEC monolayers. HT1080 (a and c) or HT-VC1 cells (b and d) were plated on monolayers of HUVEC with (c and d) or without (a and b) prior treatment by TNF-α (10 ng/ml, 24 h) as described in "Materials and Methods." After 1 h incubation at 37°C, the chambers were washed with PBS to remove unbound cells and then were photographed (magnification $\times 170$). Arrowheads indicate tumor cells adhered to endothelial cells.

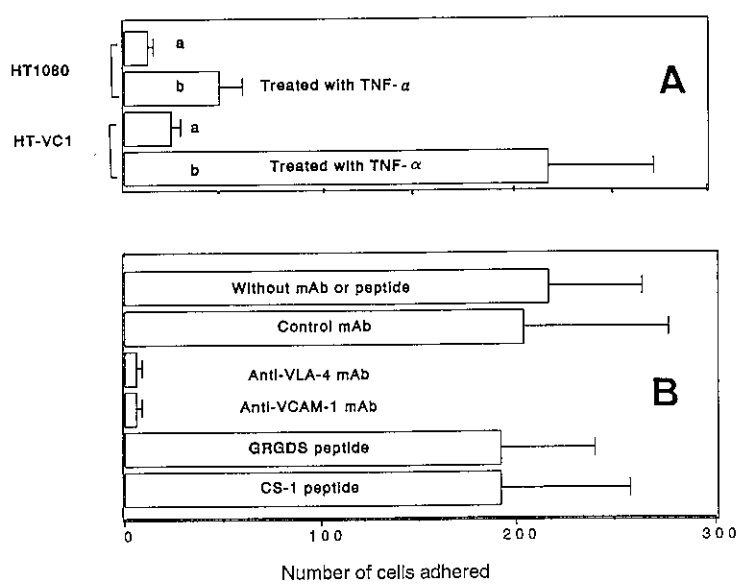

Fig. 6. (A) HT1080 or HT-VC1 cells which had adhered to untreated (a) or TNF-α-treated (b) HUVEC monolayers were counted visually under the phase contrast microscope in five randomly selected fields. The average number of adhered cells is shown with standard deviation. (B) HT-VC1 cell adhesion to TNF-α-activated HUVEC monolayers was examined in the presence of mAb 8H3 (control), anti-VLA4 (HP2/1), anti-VCAM-1, synthetic GRGDSP peptide (100 μg/ml), or CS1 peptide (100 μg/ml). Specific adhesion of HT-VC1 cells was analyzed as indicated in "Materials and Methods."

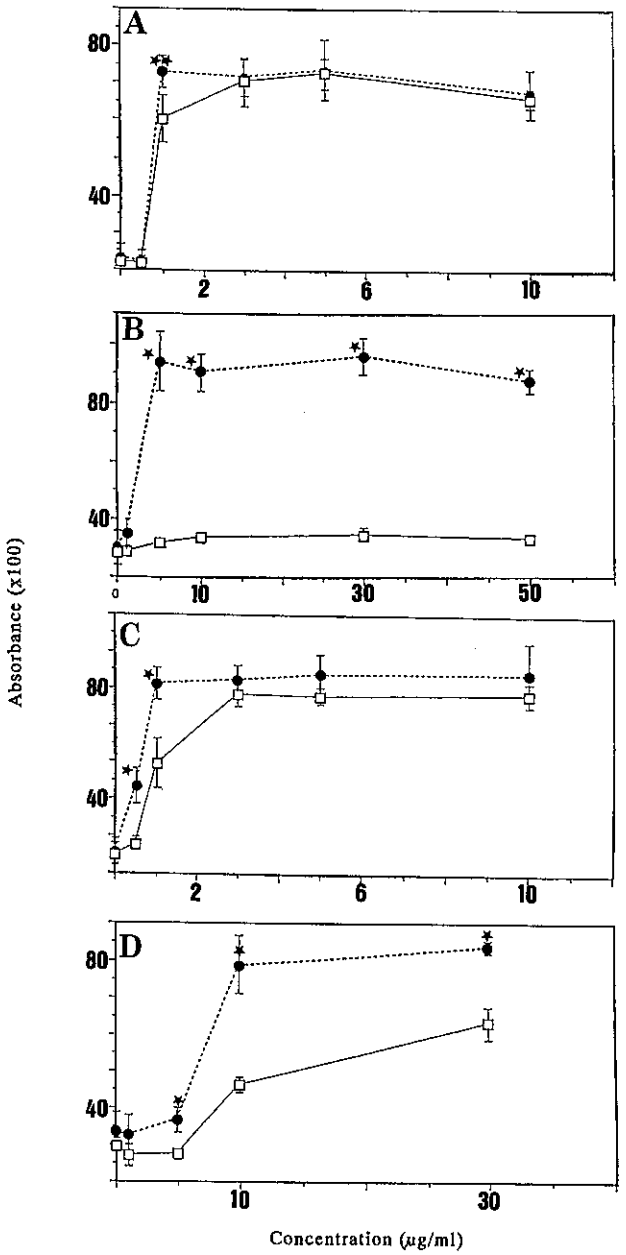

Fig. 7. Adhesion of HT1080 and HT-VC1 cells to ECM proteins and RGD-containing peptide. Microtiter plates were coated with increasing concentrations of fibronectin (A), laminin (B), type I collagen (C), and GRGDSP-BSA conjugates (D). HT1080 (●) and HT-VC1 cells (□) were plated in ECM-coated plates for 15 min or peptide-coated plates for 2 h at 37°C. Nonattached cells were washed away with PBS and the attached cells were fixed and then stained with 0.5% toluidine blue. After washing of the cells, the absorbance at 490 nm was measured to determine relative bound cell number. The average absorbance of six wells at each concentration is shown with standard deviation. ★ and ★★ indicate that the difference observed between HT1080 and HT-VC1 cells is significant (★; $P<0.0001$ and ★★; $P<0.003$).

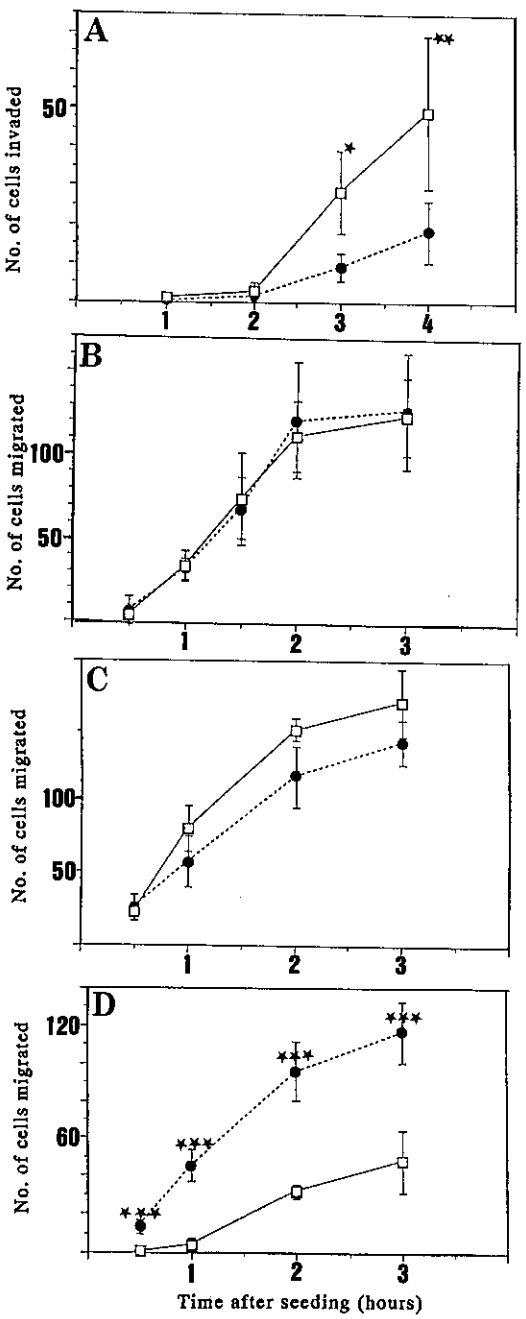

Fig. 8. Comparison of invasive and migratory capacity. HT1080 (●) or HT-VC1 cells (□) were seeded into the upper component of the chemotaxicells. For migration assay, filters in the chamber were precoated with 5 μg/ml of type I collagen (B), fibronectin (C), or laminin (D) on the lower surface. For invasion assay (A), Matrigel (50 μg) was coated on the upper surface of filters in addition to the type I collagen-precoated lower surface, and cells were counted visually in five predetermined fields. The average number is shown with standard deviation. ★, ★★ and ★★★ indicate that the difference observed between HT1080 and HT-VC1 cells was statistically significant (★; $P<0.005$, ★★; $P<0.02$ and ★★★; $P<0.0001$).

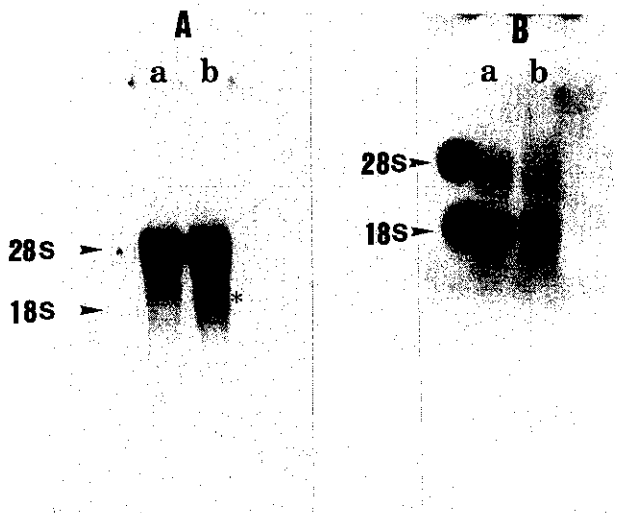

Fig. 9. RNA blotting analysis of 92 kd type IV collagenase. Total RNA (20 μg) isolated from HT1080 cells (lanes a) or HT-VC1 cells (lanes b) was separated on agarose gels, transferred to nylon membrane, and then hybridized with ^{32}P-labeled cDNA encoding 92 kd type IV collagenase (A) or β-actin (B). Migration points of 28S and 18S ribosomal RNA are indicated. * indicates the positions of gene transcripts.

Fig. 10. Expression of VCAM-1 molecules in the lungs of nude mice. Nude mice were intravenously injected with PBS (a) or TNF-α (1 μg/mouse) (b). Lungs were removed 1, 3, 6, 12, 24, 36, 48, and 72 h after treatment and frozen sections were made. Each group consisted of three nude mice. The expression of VCAM-1 molecules was examined by means of the immunoperoxidase technique. a; lung tissues were obtained 1 h after PBS injection. b; lung tissues were obtained 1 h after TNF-α injection. Open and closed arrowheads indicate the position of endothelial cells.

Other α subunits at Mr 150–160 kd were also detected and were associated with β1 subunit (lanes c and f).

***In vitro* interaction with known VLA-4-ligand** As can be seen in Fig. 3, HT-VC1 cells (d) exhibited spreading on CS1 peptide-coated wells at 2 h incubation, whereas HT1080 cells (c) did not. Neither cell line could spread on BSA-coated wells (a and b). The CS1 peptide promoted the spreading of HT-VC1 cells in a dose-dependent manner, but HT1080 cells could not spread even at a concentration of 100 μg/ml (Fig. 4). The interaction with VCAM-1 molecules was evaluated by using cultured HUVECs as described previously.[18] The adhesive ability of HT1080 and HT-VC1 cells to HUVEC monolayers before and after TNF-α-stimulation is shown in Fig. 5. Both cell lines exhibited low levels of adhesion to unstimulated HUVEC monolayers (Fig. 5a and b). In contrast, pretreatment of HUVECs with TNF-α (10 ng/ml, 24 h), which presumably induces VCAM-1 on HUVEC surfaces, resulted in a marked increase in the adhesion of HT-VC1 cells (Fig. 5d), but only a slight increase in the case of HT1080 cells (Fig. 5c). To examine whether the increased adhesion observed here is dependent upon the VLA-4 overexpression, the inhibitory effects of mAbs and synthetic peptides on this adhesion were assessed. As depicted in Fig. 6, anti-VLA-4 mAb HP2/1 as well as anti-human VCAM-1 mAb strongly inhibited the adhesion of HT-VC1 cells to TNF-α-treated HUVECs. However, control mAb as well as one of the VLA-4 ligands, CS1 peptide, and GRGDSP peptide (as a control peptide) failed to inhibit this adhesion.

Adhesion to ECM proteins and RGD-containing peptide The adhesive ability to fibronectin (Fig. 7A) and type I collagen (Fig. 7C) was similar in HT1080 and HT-VC1 cells when the matrix was precoated over 3 μg/ml, whereas the binding to low concentrations of fibronectin (1 μg/ml) or type I collagen (0.5 μg/ml and 1 μg/ml) was decreased significantly in HT-VC1 cells (fibronectin, $P<0.003$; type I collagen, $P<0.0001$). A notable difference was seen in the capacity for adhering to laminin (Fig. 7B). HT-VC1 cells were unable to adhere to laminin in a 15-min binding time, and that finding appears to correlate with a great decrease in expression of VLA-6 (laminin receptor) on the cell surface. The adhesion

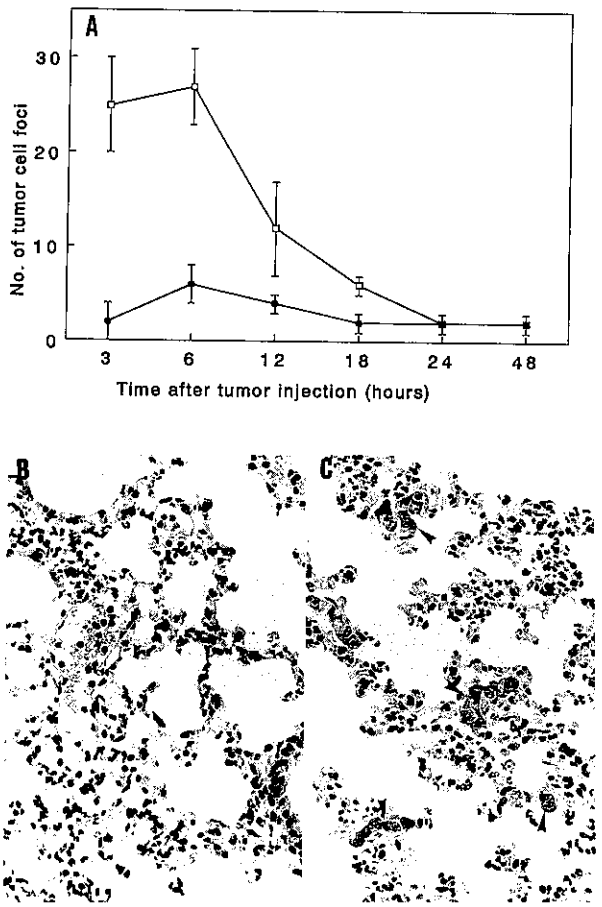

Fig. 11. The augmentation of tumor cell retention in the lung induced by TNF-α. Nude mice (5 mice per group) were i.v. injected with TNF-α (1 μg/mouse) 1 h prior to 1×10^6 HT1080 or HT-VC1 cell injection. Three, 6, 12, 18, 24, and 48 h after tumor cell injection, the lungs were removed and sections were made so that the biggest longitudinal plane was obtained. The numbers of tumor cell foci in sections of HT1080 (●) and HT-VC1 (□) injected mice were counted (A) as described in "Materials and Methods." Lung tissues were also obtained from nude mice 3 h after HT1080 (B) or HT-VC1 (C) injection. Arrowheads indicate tumor cell foci.

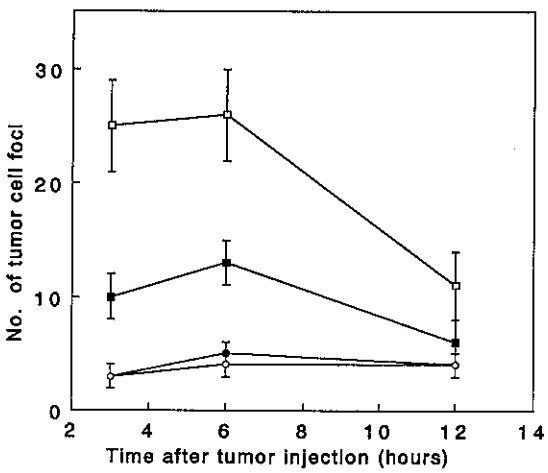

Fig. 12. The reduction of tumor cell foci by anti-α4 treatment. Nude mice (5 mice per group) were i.v. injected with TNF-α (1 μg/mouse). Then at 1 h, HT1080 (● and ○) or HT-VC1 (□ and ■) cells (1×10^6) either treated with anti-α4 mAb (○ and ■) or control mAb (● and □) were injected into mice. Three, 6, and 12 h after tumor injection, lungs were removed and sections were made. The number of tumor cell foci was counted by the same method as described in the legend to Fig. 11.

to GRGDSP peptide was also significantly decreased in HT-VC1 cells (Fig. 7D), which is consistent with the reduced expression of VLA-5 (fibronectin receptor recognizing RGD sequence).

***In vitro* invasive and migratory capacity** The invasive capacity of HT1080 and HT-VC1 cells was compared by means of an *in vitro* invasion assay through basement mambrane (Matrigel) and interstitial matrix (type I collagen). The results shown in Fig. 8A indicate that HT-VC1 cells are more invasive than HT1080 cells. The observed differences were reproducible in three different experiments. In addition, the migratory capacities of these cell lines through filters precoated with various ECM proteins were compared. The migratory capacity through type I collagen (Fig. 8B) was similar for HT1080 and HT-VC1 cells. A slight difference was observed with fibronectin (Fig. 8C). In contrast, migration through laminin was much less in HT-VC1 cells (Fig. 8D). The *in vitro* growth rates of the cell lines were comparable (data not shown).

Expression of type IV collagenases We used RNA blotting analysis to compare type IV collagenase transcripts in RNA extracted from HT1080 and HT-VC1 cells. As shown in Fig. 9A, the expression of mRNA transcript of the 92 kd type IV collagenase in HT-VC1 cells (lane b) was higher than that in HT1080 cells (lane a). Hybridization of the same blot with a cDNA probe for human β-actin (Fig. 9B) gave comparable signals in both cell lines. Expression of the 72 kd type IV collagenase mRNA was indistinguishable in the two cell lines (data not shown).

Effects of TNF-α on the expression of VCAM-1 by lung endothelial cells and tumor cell-binding The expression of VCAM-1 molecules in the lungs of nude mice was examined immunohistochemically before and after TNF-α injection. As shown in Fig. 10a, VCAM-1 molecules were not detected on endothelial cells of lung before TNF-α injection. However, VCAM-1 molecules were

clearly induced on endothelial cells from 1 h through 48 h after TNF-α injection. Very faint expression of VCAM-1 molecules was detected at 72 h. The expression of VCAM-1 at 1 h after TNF-α injection is illustrated in Fig. 10b. Nude mice were pretreated with TNF-α and then at 1 h tumor cells (1×10^6/mice) were i.v. injected into the mice. A considerable number of HT-VC1 cells were detected in the lungs at 3 h after tumor injection (Fig 11C), whereas very few HT1080 cells were detected (Fig. 11B). However, the time course study shown in Fig. 11A demonstrated that rather few HT-VC1 tumor cell foci remained adhering to lung endothelium at 12 h, and thereafter.

Effect of anti-VLA-4 mAb treatment on tumor cell binding to lung endothelium of nude mice In order to confirm that VLA-4 molecules on tumor cells are responsible for the interaction of HT-VC1 cells with lung endothelium *in vivo,* HT-VC1 as well as HT1080 cells were pretreated with anti-VLA-4 mAb prior to injection. As shown in Fig. 12, the binding of tumor cell foci to lung endothelium was strongly inhibited in mice receiving HT-VC1 cells pretreated with anti-VLA-4 mAb, but not with control mAb.

Metastatic ability of HT1080 and HT-VC1 cells At 4 weeks after i.v. tumor cell injection into TNF-α-treated nude mice, seven out of seven HT1080-injected mice had lung metastasis, while eight out of eight HT-VC1-injected mice developed lung metastasis. The mean numbers of metastatic foci were 12.1 (range, 3 to 22) and 10.5 (range, 2 to 18) in HT1080-injected and HT-VC1-injected mice, respectively.

DISCUSSION

The anchorage of circulating tumor cells to the vascular endothelium, the first step of extravasation, is thought to be critical for the establishment of hematogenous metastasis.[2, 46, 47] Adhesive interactions between tumor cells and endothelial cells are mediated through a variety of cell surface proteins.[21, 46, 48, 49] Among these proteins we have focused on the VLA-4 molecule, because our previous study raised the possibility that VLA-4 molecules play a particularly important role in the pulmonary metastasis of osteogenic sarcoma cells (S. Kawaguchi and T. Uede, submitted for publication). VLA-4 is the receptor for the VCAM-1 molecule, which is a cytokine-inducible surface protein of endothelial cells.[18] Thus, the interaction of VLA-4 on tumor cells and VCAM-1 on endothelial cells is possibly a key event in extravasation. This hypothesis is supported by several previous findings: (i) pretreatment of nude mice with IL-1 and TNF-α augmented melanoma lung colonization,[22] (ii) those cytokines can induce VCAM-1 expression on lung endothelial cells of nude mouse,[21] and (iii) *in vitro* adhesive ability of melanoma cells to activated endothelial cells correlates with VLA-4 expression.[48] However, there is no direct evidence so far that VLA-4/VCAM-1 interaction is essential for the first step of hematogenous metastasis *in vivo.* The expression of VLA-4 is limited to a few tumor cell types and lymphocytes[17, 25, 50] in spite of the fact that many malignant tumors are indeed capable of hematogenous metastasis. Furthermore, the possibility can not be ruled out that augmentation of lung colonization by cytokine treatment depends on other cytokine-inducible molecules expressed on endothelial cells.

Thus, in this study, we attempted to overexpress VLA-4 in human fibrosarcoma cells (HT1080) to determine the role of VLA-4-dependent adhesive events during tumor cell extravasation *in vivo.* Flow cytometry and immunoprecipitation experiments have demonstrated that introduction of a cDNA coding for VLA-4 α subunit results in a great increase in surface expression of $\alpha4\beta1$ (VLA-4) complex due to association of endogenous $\beta1$ and exogenous $\alpha4$ subunit (Figs. 1 and 2). The VLA-4-overexpressing clone, designated as HT-VC1 cell line, exhibited enhanced ability to interact with the CS1 region of fibronectin and more importantly, exhibited increased binding to TNF-α-treated endothelial cells (Figs. 3, 4, and 5). The binding of HT-VC1 cells to TNF-α-treated endothelial cells was inhibited by mAb against VLA-4 as well as VCAM-1, but not CS1 peptide, indicating that binding of HT-VC1 cells involves VLA-4/VCAM-1 interaction (Fig. 6).

The extravasation phase of hematogenous metastasis consists of at least four steps[47]; the first step is tumor cell attachment to vascular endothelial cells of the target organ; the second step involves tumor cell attachment to subendothelial basement membrane; the third step is local degradation of the basement membrane by tumor cell-associated proteases; and the fourth step involves cell migration through basement membrane and interstitial matrix. Thus, increased ability of HT-VC1 cells to interact with endothelial cells through VCAM-1 molecules would be expected to augment the metastatic capacity *in vivo.* We first examined VCAM-1 expression in the lung of nude mice. In human lung, endothelial cells express VCAM-1 molecules.[21] In a patient with malignancy, endothelial cell activation with increased expression of adhesion molecules might result from host cell production of TNF-α or from concurrent infection or sepsis.[21, 44, 51] In nude mice, however, VCAM-1 molecules were expressed on lung endothelial cells only after TNF-α injection (Fig. 10).

A considerable number of HT-VC1 cells were retained in the lungs of TNF-α-treated nude mice until 6 h after injection (Fig. 11), whereas very few HT1080 cells were retained in the lungs of TNF-α-treated nude mice. Furthermore, the number of HT-VC1 cell foci in the lung

was considerably reduced by pretreatment of tumor cells with anti-α4 mAb (Fig. 12). Therefore, it is clear that VLA-4 molecules on tumor cells initiate the adhesion to endothelial cells through interaction with VCAM-1 molecules on lung endothelial cells *in vivo*.

An unexpected finding was that the number of HT-VC1 cell foci adhering to lung endothelium was very small by 12 h after tumor cell injection (Fig. 11A) in spite of the fact that the expression of VCAM-1 on lung endothelium persisted until 48 h after TNF-α injection. Furthermore, our data demonstrated that there was no difference in pulmonary metastasis in TNF-α-treated nude mice between HT-VC1 and HT1080 cells after 4 weeks, indicating that adhesion of tumor cells to the endothelial cell surface is only a first step and other factors determine whether the tumor cells can form metastatic foci. Among various factors, laminin receptor on tumor cells might determine the next step of hematogenous metastasis, which is the binding of tumor cells to subendothelial basement membrane as suggested by Fidler.[47] It was shown recently that VLA-2-overexpressing tumor cells not only exhibited enhanced binding to collagen and laminin, but also formed more metastatic colonies.[45] Indeed, HT-VC1 cells exhibited impaired ability to adhere *in vitro* to laminin (Fig. 7B), which is a major component of basement membrane.[2] It is also possible that HT-VC1 cells had impaired ability to degrade the basement membrane as well as to migrate, factors that determine the third and fourth steps of hematogenous metastasis, respectively.[47] However, invasive assay designed to measure the ability of cells to invade through Matrigel (reconstituted basement membrane) clearly demonstrated that HT-VC1 cells were more invasive than HT1080 cells. In addition, there was increased production of mRNA transcript of 92 kd type IV collagenase in HT-VC1 cells (Fig. 9). Furthermore, the migratory capacity of HT-VC1 cells through laminin was stronger than that shown by HT1080 cells. The enhanced invasive and migratory abilities of HT-VC1 cells are not necessarily due to overexpression of α4, but may be influenced by other factors. In this regard it should be noted that the expression of α5 decreased in HT-VC1 cells (Fig. 1). Previously it was shown that overexpression of α5 suppressed transformed phenotype of Chinese hamster ovary cells, such as migratory ability and growth rate.[52] Thus, it is possible that the invasive capacity of HT-VC1 cells is due to alteration in the expression of α5. Another possible explanation is that HT-VC1 cells were eliminated by natural killer cells. It was shown that the expression of VLA-2 and other VLAs correlated with increased susceptibility to lysis mediated by natural killer cells.[53] However, the susceptibilities of HT-VC1 and HT1080 cells to lysis by natural killer cells were not significantly different from each other (data not shown). Taken together, the results suggest that the main reason for the reduced number of tumor cell foci after 12 h may be the inability of HT-VC1 cells to bind with laminin, a major component of basement membrane. These results have led us to conclude that the interaction between VLA-4 molecules on the tumor cell surface and VCAM-1 molecules on the endothelial cell surface initiates extravasation, but the subsequent adhesive event to subendothelial basement membrane is also critical for the establishment of metastatic foci. We recently obtained a subclone of HT-VC1, designated HT-VL2. This subclone exhibited high laminin-binding ability compared to HT-VC1, and comparable binding ability to TNF-α-treated lung endothelial cells. By using these two clones we are attempting to clarify the significance of the laminin receptor, together with VLA-4 molecules, for the establishment of hematogenous metastasis.

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