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Differences in Chemotaxis to Fibronectin in Weakly and Highly Metastatic Tumor Cells

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We have examined the chemotactic ability of tumor cell lines with different metastatic potential to plasma fibronectin in Transwell chamber assay. Human renal carcinoma cells with highly metastatic potential, SN12 C-2, chemotactically migrated to fibronectin (10 μg/ml) about three-fold more strongly than weakly metastatic SN12 C-4 cells. Similarly, murine melanoma B16-BL6 cells (highly metastatic) showed higher motility to soluble fibronectin in comparison with weakly metastatic B16-F1 cells. Anti-VLA-α_3 and β_1 antibodies potently blocked the chemotaxis of both highly and weakly metastatic cells (SN12 C-2 and C-4) to fibronectin. This implies that the migration of both C-2 and C-4 cells to fibronectin is basically mediated by VLA-3 receptor. In contrast, the anti-VLA-α_5 antibody and RGDS peptide significantly inhibited the chemotaxis of SN12 C-2 cells to fibronectin, but did not affect weakly metastatic SN12 C-4 cells. These results suggest that the chemotactic ability to fibronectin positively correlates with the metastatic potential in SN12 and B16 cell lines, and that VLA-5 receptor is concerned in the motility of highly metastatic SN12 C-2 cells to soluble fibronectin.

Key words: Metastasis — Chemotaxis — Fibronectin — Very late antigen

The formation of tumor metastasis is composed of a characteristic series of steps.[1,2] Tumor invasion of surrounding tissue is required at many stages of the metastasis. Liotta[3] proposed a three-step hypothesis of tumor cell invasion of the extracellular matrix: 1) tumor cell attachment to matrix components, 2) local degradation of the matrix by tumor cell-associated proteases, and 3) tumor cell locomotion into the region of the matrix modified by proteolysis. Concerning the third step, several studies have been reported on whether or not a relationship exists between the degree of motility and metastatic potential. Hosaka *et al.*[4] have demonstrated that highly invasive rat ascites hepatoma cells showed active locomotion, while a weakly metastatic variant did not. Raz and Ben-Ze'ev[5] have reported that highly metastatic cells migrated more potently than weakly metastatic counterparts. Furthermore, Partin *et al.*[6] have shown that rat prostatic cells with initially low metastatic ability acquired the metastatic phenotype, such as increased membrane ruffling, pseudopodal extension, and translocation, upon transfection with v-Harvey-*ras* oncogene.

Many factors derived from host tissue have been implicated in the stimulation of cell motility, including organ-derived chemoattractants,[7] insulin-like growth factors,[8] platelet-derived growth factors,[9] scatter factor,[10,11] and autocrine motility factor.[12,13] Extracellular matrix components, such as laminin,[14,15] type IV collagen,[16] thrombospondin,[17] vitronectin,[18] and fibronectin,[14,19] also mediate the migration of malignant cells.

Fibronectin is a glycoprotein which is widely distributed in plasma and tissues, and consists of similar subunits of 220 kD linked by disulfide bonds into dimers and polymers.[20] Fibronectin mediates the adhesion, spreading and migration of cells through a characteristic sequence in this molecule.[21] Such a sequence is specifically recognized by cell surface receptors (integrins) which are heterodimers of noncovalently linked α and β subunits (called very late antigen; VLA).[22] The integrin receptors which bind to fibronectin include at least three members of the β_1 subfamily, VLA-3 ($\alpha_3\beta_1$), VLA-4 ($\alpha_4\beta_1$), and VLA-5 ($\alpha_5\beta_1$).[22] Recently, a correlation between $\alpha_5\beta_1$ integrin and tumorigenicity has been described. Elevated expression of $\alpha_5\beta_1$ integrin is associated with lowered tumorigenicity.[23] Oncogenically transformed rodent cells showed reduction of $\alpha_5\beta_1$ expression.[24] In contrast, oncogenic transformation of human cells affects the rate of biosynthesis and the distribution of $\alpha_5\beta_1$ without increasing its overall expression.[25] These reports indicate that malignant transformation can affect the expression levels and distribution of fibronectin receptor ($\alpha_5\beta_1$). On the other hand, although fibronectin stimulates the motility of both normal and malignant cells, little is known about the relationship between the degree of motility towards fibronectin and metastatic potential, or about the regulatory mechanism of fibronectin receptor.

In this study, we examined whether or not a correlation exists between the cell motility to fibronectin in

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Transwell chamber assay and the metastatic potential of tumor cells. We also analyzed the specificity of chemotaxis to fibronectin and the expression levels of fibronectin receptors on highly and weakly metastatic SN12 carcinoma lines.

MATERIALS AND METHODS

Cell lines Human renal carcinoma cell lines SN12 C-2 and SN12 C-4 were kindly provided by Dr. I. J. Fidler (M.D. Anderson Cancer Center, Houston, TX). SN12 C-2 is a subline with high metastatic potential and SN12 C-4 is a weakly metastatic variant of the SN12 parental cells.[26] Murine melanoma cell lines B16-BL6 (highly metastatic) and B16-F1 (weakly metastatic), derived from the parental B16, were also provided by Dr. I. J. Fidler. All cell lines were maintained as monolayer cultures in Eagle's minimal essential medium (EMEM) (M.A. Bioproducts, Walkersville, MD) supplemented with 7.5% fetal bovine serum (FBS), vitamin solution, sodium pyruvate, nonessential amino acid and L-glutamine.

Reagents Human plasma fibronectin was purchased from Biomedical Technologies Inc. (Stoughton, MA). Mouse plasma fibronectin was purchased from Seikagaku Kogyo Co. Ltd. (Tokyo). RGDS tetrapeptide was purchased from Bachem Feinchemikauen AG (Switzerland). CS1 peptide (DELPQLVTLPHPNLHGPEILDVPST), which is a partial sequence of type III connecting segment (IIICS) of human fibronectin, was kindly provided by Takara Shuzo Co. Ltd. (Kyoto). Monoclonal antibodies (mAbs) against human VLA receptors were as follows: AIIB2 (anti-β_1) was kindly provided by Dr. C. Damsky (University of California, San Francisco, CA); PIC5 (anti-α_3) was purchased from Telios Pharmaceutical Inc. (San Diego, CA); CD_w49d (anti-α_4) and CD_w49e (anti-α_5) were purchased from Immunotech S.A. (Marseille, France); G0H3 (anti-α_6) was kindly provided by Dr. A. Sonnenberg (Netherlands Cancer Institute, Amsterdam, Holland). All the reagents and media used in this study were endotoxin-free (<0.1 ng/ml), as determined by a colorimetric assay (Pyrodick, Seikagaku Kogyo, Co. Ltd.).

Cell migration assay The chemotactic migration of tumor cells was measured by using Transwell cell culture chambers (Costar 3422, Cambridge, MA) as described previously.[27] Polyvinylpyrrolidone-free polycarbonate filters with 8.0-μm pore size (Nucleopore, Pleasanton, CA) were coated with 50 μl of 100 μg/ml gelatin on the lower surface and dried overnight at room temperature. The coated filters were washed extensively in Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline (PBS) and then dried immediately before use. Log-phase cultures of tumor cells were harvested with 1 m*M* EDTA in PBS, washed 3 times with serum-free EMEM, and resuspended to a final concentration of 2×10^6 cells/ml in EMEM containing 0.1% bovine serum albumin (BSA). Cell suspensions (2×10^5 cells/100 μl) were added to the upper compartment of the chamber, and various concentrations of fibronectin were added to the lower compartment, then the chamber was incubated at 37°C in a 5% CO_2 atmosphere. After a 16-h incubation, the filters were fixed with methanol and stained with hematoxylin and eosin. The cells on the upper surface of the filters were removed by wiping with cotton swabs. The cells that had migrated to various areas of the lower surface were manually counted under a microscope at $\times400$, and each assay was performed in triplicate. Tumor cell migration along a gradient of substratum-bound fibronectin (haptotactic migration) was assessed in Transwell cell culture chambers according to the methods reported by McCarthy *et al.*[28] with some modifications.[29] The polycarbonate filters were precoated with 50 μl of 100 μg/ml fibronectin on the lower surface. Tumor cell suspensions (2×10^5 cell/100 μl) were added to the upper compartment and incubated at 37°C for 4 h. The following procedures were the same as those of the chemotactic migration assay.

Flow cytometry SN12 cells (C-2 and C-4) were washed with PBS containing 0.2% BSA and 0.1% NaN_3, and suspended in the same buffer. Tumor cell suspensions (1×10^6 cells/30 μl) were then preincubated individually with mAbs specific for VLA-α (α_3-α_5) and VLA-β_1 for 30 min at 4°C. Cells were washed 3 times with PBS, incubated with rabbit anti-mouse IgG coupled to FITC (Medical Biology Laboratories, Nagoya) for anti-VLA-α (α_3-α_5) mAb-treated cells, or fluorescein isothiocyanate (FITC)-conjugated goat anti-rat IgG (TAGO Inc., Burlingame, CA) for anti-VLA-β_1 mAb-treated cells for 30 min at 4°C, washed 3 times as above, and finally resuspended in PBS containing 2% FBS and 0.1% NaN_3. FITC-labeled cells were analyzed using a FACScan apparatus (Becton Dickinson & Co., Oxnard, CA).

RESULTS

Chemotactic response of tumor cells to fibronectin In the first set of experiments, we examined the chemotactic response of tumor cells towards soluble fibronectin. The upper panel of Fig. 1 shows that soluble fibronectin stimulated the motility of both human SN12 cell lines (C-2 and C-4) in a concentration-dependent manner. Highly metastatic SN12 C-2 cells migrated approximately three-fold more strongly than SN12 C-4 cells (weakly metastatic) at a concentration of 10 μg/ml of fibronectin. In the case of murine B16 melanoma cell lines (BL6 and F1), highly metastatic B16-BL6 cells showed higher levels of migration to soluble fibronectin in comparison

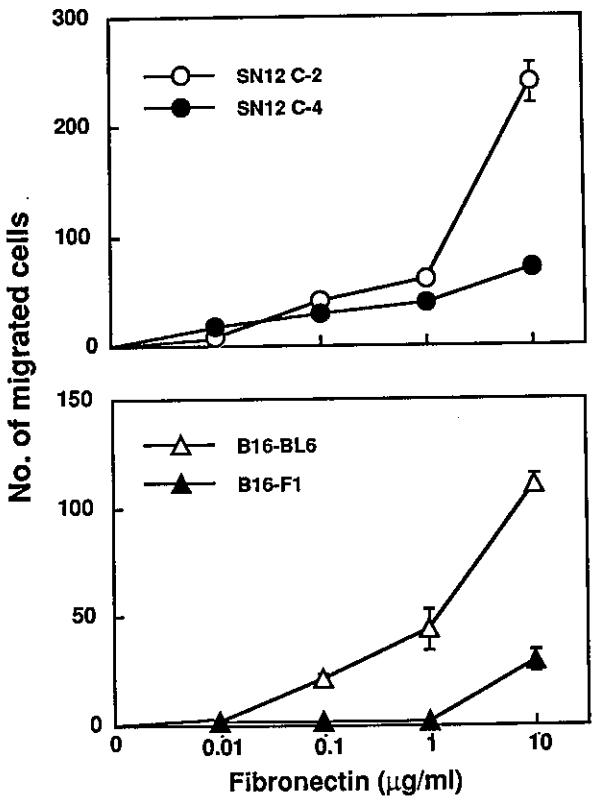

Fig. 1. Chemotaxis of SN12 and B16 cell lines to soluble fibronectin. SN12 or B16 cell lines (2×10^5) were seeded onto the filters which were precoated with 5 μg of gelatin on the lower surface, and various concentrations of plasma fibronectin were added to the lower compartment in Transwell chambers. After a 16-h incubation, the migrated cells on the lower surface were visually counted.

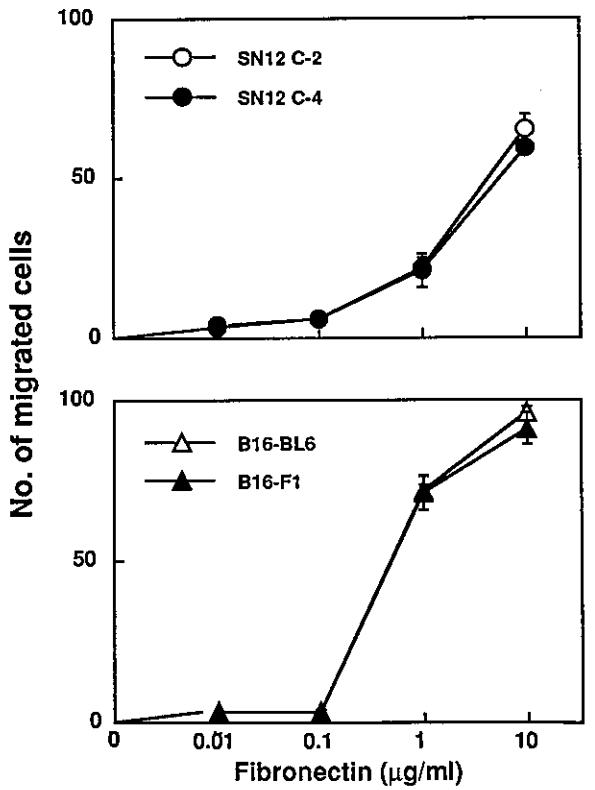

Fig. 2. Haptotaxis of SN12 and B16 cell lines to immobilized fibronectin. SN12 or B16 cell lines (2×10^5) were seeded onto the filters, which had been precoated with 5 μg of fibronectin on the lower surface, in Transwell chambers. After a 4-h incubation, the migrated cells on the lower surface were visually counted.

with weakly metastatic B16-F1 cells (Fig. 1, lower panel). No migration of either cell line was observed when tumor cells were seeded on filters precoated with gelatin in the absence of fibronectin (data not shown). These results indicate that chemotactic ability of tumor cells to fibronectin may closely correlate with their metastatic potential.

Haptotactic migration of tumor cells to immobilized fibronectin We next examined the tumor cell migration along a gradient of substratum-bound fibronectin (haptotaxis) for comparison with the chemotaxis. SN12 C-2 and C-4 cells migrated in response to increasing concentrations of immobilized fibronectin (Fig. 2, upper panel). There was no difference between C-2 and C-4 cell migration in response to increasing amounts of fibronectin precoated on the lower surface of filters. A similar result was obtained by using B16-BL6 and -F1 cells (Fig. 2, lower panel). These results indicate that haptotaxis of SN12 or B16 cell lines to the immobilized fibronectin did not correlate with metastatic potential, suggesting that chemotaxis and haptotaxis of tumor cells to fibronectin, occur through different mechanisms.

Specificity of tumor chemotaxis to fibronectin Interaction of tumor cells with fibronectin is mediated through the recognition of characteristic sequences such as RGD and/or CS1 sequences within fibronectin by integrin receptors on the cell surface.[30] To characterize the chemotaxis of tumor cells to fibronectin, we tested the effect of fibronectin-derived peptides (RGDS and CS1) on chemotactic migration of tumor cells to soluble fibronectin. Fig. 3 shows that RGDS peptide significantly inhibited chemotaxis of highly metastatic SN12 C-2 cells to fibronectin in a concentration-dependent manner, whereas the chemotaxis of weakly metastatic SN12 C-4 cells was not inhibited by RGDS peptide at any concentration used. On the other hand, CS1 peptide had no effect on either C-2 or C-4 cells. SN12 C-2 cells as well as

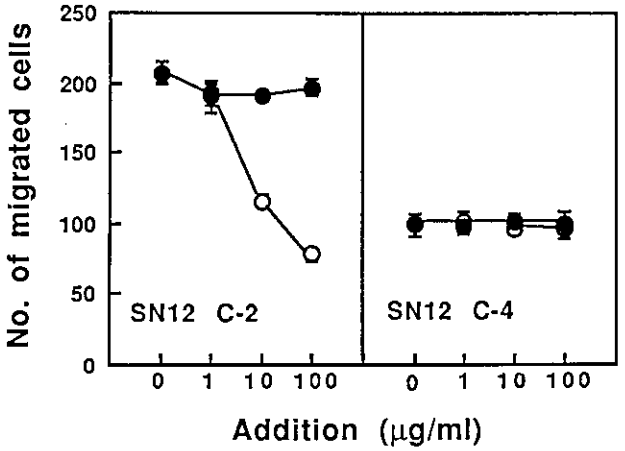

Fig. 3. Effect of RGDS and CS1 peptides on SN12 cell chemotaxis to fibronectin. SN12 C-2 (left panel) or C-4 (right panel) cells (2×10^5) were pretreated with various concentrations of RGDS (○), or CS1 (●) peptides at 37°C for 30 min. The cells were then seeded on filters which had been precoated with gelatin on the lower surface, and 10 μg/ml fibronectin was added to the lower compartment in Transwell chambers. After a 16-h incubation, the migrated cells on the lower surface were visually counted.

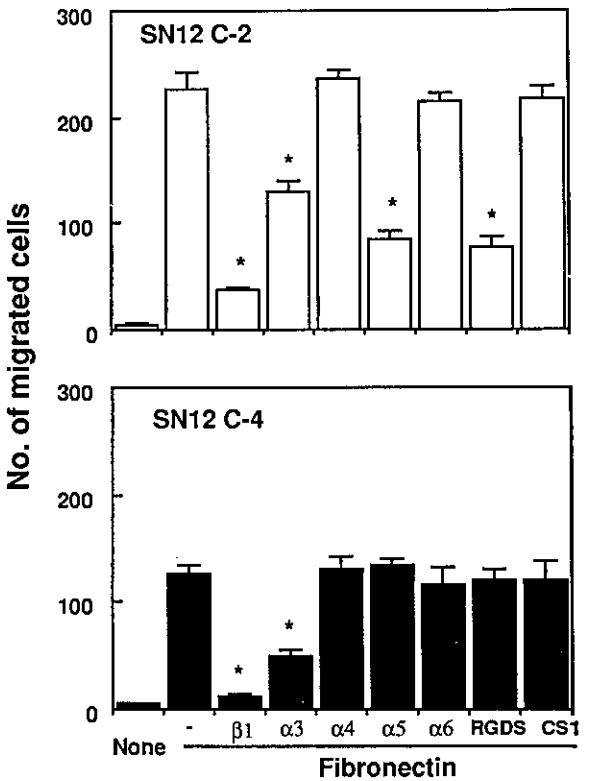

Fig. 4. Effects of anti-VLA antibodies on SN12 cell chemotaxis to fibronectin. SN12 C-2 (upper panel) or C-4 (lower panel) cells (2×10^5) were pretreated with an appropriate concentration of anti-VLA-β_1 (×10), -α_3 (×20), -α_4 (10 μg/ml), -α_5 (10 μg/ml), or -α_6 (×10) antibodies, or RGDS (100 μg/ml) or CS1 (100 μg/ml) peptide at 37°C for 30 min. The chemotaxis assay was the same as described in "Materials and Methods." * $P<0.001$ compared with the chemotaxis untreated with antibodies by Student two-tailed t test.

C-4 cells, however, did not show chemotactic migration to RGDS and CS1 peptides when the peptides, in place of fibronectin, were added to the lower compartment of the chamber (data not shown). These results indicate that the chemotaxis of highly metastatic SN12 C-2 cells to fibronectin is attributable to the RGDS-dependent mechanism.

To determine which integrins on the cell surface might mediate SN12 cell chemotaxis to fibronectin, we performed a tumor cell chemotaxis inhibition study by using anti-integrin antibodies. Fig. 4 shows that anti-VLA-β_1 mAb (AIIB2) inhibited chemotaxis of both C-2 and C-4 cells to fibronectin by 80–90%, indicating that the chemotactic migration of SN12 cells to fibronectin was mainly mediated by β_1 integrin subfamilies. Anti-VLA-α_3 mAb (PIC5) also inhibited that chemotaxis of both C-2 and C-4 cells by 42% and 60%, respectively. In contrast, mAb against VLA-α_5, which recognizes RGD sequence in fibronectin, blocked the migration of highly metastatic C-2 cells (by 63%), but did not affect weakly metastatic C-4 cell migration. The mAb against VLA-α_4, which interacts with CS1 region in fibronectin, and anti-VLA-α_6 mAb (G0H3) did not show any inhibitory effect on the chemotaxis of either C-2 or C-4 cells. These results suggest that VLA-5 receptor is associated with the enhancement of chemotoxis of highly metastatic SN12 C-2 cells to fibronectin, although the migration of both C-4 and C-2 cells to fibronectin may basically be mediated by VLA-3 receptor.

Cell surface expression of VLA receptors To examine the quantitative expression of VLA molecules on the cell surface, SN12 cells were immunofluorescently labeled and analyzed by FACS. Fig. 5 shows that VLA-β_1 was highly expressed on C-2 and C-4 cells. Both cell lines expressed moderate amounts of VLA-α_3. No difference was noted in the expression of VLA-α_4 between C-2 and C-4 cells (data not shown). In contrast, VLA-α_5 was poorly expressed on the highly metastatic C-2 cells in comparison with C-4 cells, although Fig. 4 shows that anti-VLA-α_5 mAb significantly inhibited the C-2 cell chemotaxis to fibronectin. These results indicate that expression levels of VLA-5 on highly metastatic C-2 cells might not be positively correlated with the high motility of the cells to soluble fibronectin.

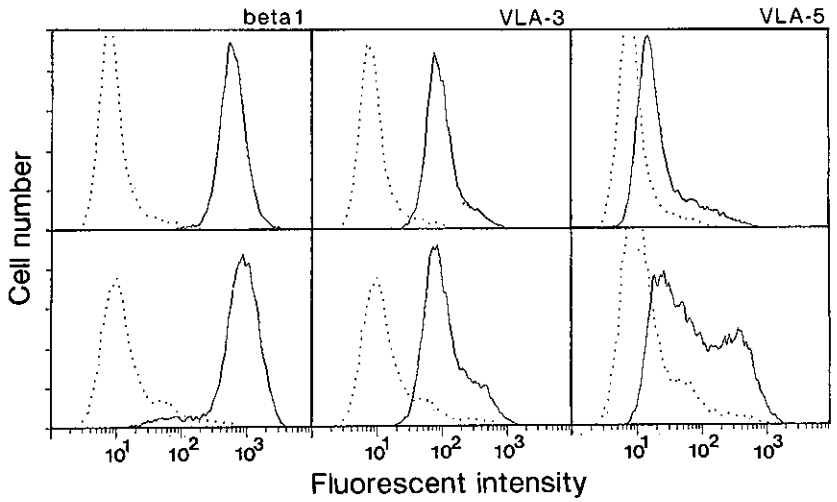

Fig. 5. Cell surface expression of VLA receptors on SN12 cells. Flow cytometric analysis was performed on SN12 C-2 (upper panel), and C-4 (lower panel) cells. The cells were treated with saturating concentrations of the following monoclonal antibodies for staining: AIIB2 (anti-β_1), PIC5 (VLA-3, anti-α_3), and CD_w49_e (VLA-5, anti-α_5). The solid or dotted lines represent cell staining with or without the above primary antibodies, respectively.

DISCUSSION

Metastasizing tumor cells in the blood stream could be arrested at the vascular bed in a distant organ. Tumor cells then penetrate through the basement membrane and underlaying interstitial stroma. Although tumor cells degrade the basement membrane by utilizing proteolytic enzymes such as type IV collagenase,[31] the factor which determines the direction of tumor cells towards interstitial stroma is not well defined. We recently found that highly metastatic B16-BL6 melanoma cells successfully invaded reconstituted basement membrane (Matrigel) in the presence of fibroblasts in the lower compartment of a Transwell chamber, but did not invade without fibroblasts (manuscript in preparation). In addition, B16-BL6 cells stimulated the production of soluble fibronectin on coculture with fibroblasts without cell-cell contact. Furthermore, highly metastatic 16-BL6 cells caused the Matrigel invasion and chemotaxis towards fibroblasts more potently than weakly metastatic B16-F1 cells (manuscript in preparation). These results encouraged us to study the correlation between the migratory ability of tumor cells to fibronectin and the metastatic potential.

Previous studies have indicated that soluble fibronectin stimulated the chemotactic responses of human A2058 melanoma cells.[32] Similarly, murine Y1 carcinoma cells chemotactically migrated to fibronectin in Boyden chamber assay.[33] Our present study showed that human SN12 carcinoma cell lines as well as murine B16 melanoma lines chemotactically migrated to soluble fibronectin in a concentration-dependent manner in Transwell chamber assay (Fig. 1). Interestingly, anti-VLA-α_3 and -β_1 antibodies blocked the chemotaxis of both C-2 (highly metastatic) and C-4 (weakly metastatic) cells, derived from the same parental SN12 carcinoma cells, to soluble fibronectin in the lower compartment of the chamber (Fig. 4). In addition, a similar flow cytometric profile was observed in the expression of VLA-α_3 and -β_1 receptors on both C-2 and C-4 cells (Fig. 5). These results suggest that VLA-3 acts as one of the common receptors of both highly and weakly metastatic variants of SN12 lines for chemotaxis to soluble fibronectin.

We have demonstrated that highly metastatic SN12 C-2 cells chemotactically migrated to fibronectin (10 μg/ml) about three-fold more strongly than weakly metastatic SN12 C-4 cells (Fig. 1). A similar difference was noted between highly and weakly metastatic B16 lines (Fig. 2). These results are in good agreement with the study of Fridman *et al.*, who found a positive correlation between the chemotactic response to fibronectin and metastatic potential in murine Y1 carcinoma cell lines.[33] We also found that RGDS peptide and anti-VLA-α_5 antibody specifically inhibited the chemotaxis of highly metastatic SN12 C-2 cells to fibronectin, but did not affect that of weakly metastatic SN12 C-4 cells (Figs. 3 and 4). This implies that enhanced migration of SN12 C-2 cells may be associated with $\alpha_5\beta_1$ receptor on the cell surface. However, it seems unlikely that elevated levels of $\alpha_5\beta_1$ lead to higher motility of C-2 cells to fibronectin,

since VLA-α_5 is poorly expressed on C-2 cells in comparison with C-4 cells (Fig. 5).

The exact mechanism of enhanced chemotaxis of SN12 C-2 cells to fibronectin is still unknown. One possible explanation is that qualitative and functional alteration of VLA-α_5 receptors on highly metastatic cells leads to the enhancement of chemotaxis. Since the chemotaxis of tumor cells to certain factors is mediated by specific receptors coupled to GTP-binding protein,[34] disorder of the transmembrane or cytoplasmic domain of VLA-receptors may affect the following signal transduction pathway. Further investigation of the intracellular events is needed.

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