Increased expression of integrin α3β1 in highly brain metastatic subclone of a human non-small cell lung cancer cell line

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To clarify the roles of integrin and extracellular matrix (ECM) in the process of non-small cell lung cancer (NSCLC) brain metastasis, we established an *in vivo* model of brain metastasis of human NSCLC cell line EBC-1/original in athymic mice, and established highly brain metastatic subclone EBC-1/brain and highly bone metastatic subclone EBC-1/bone. Integrin expression of these subclones was evaluated by flow cytometry. *In vitro* cell attachment, migration and proliferation assays with ECMs were performed using these subclones. Expression of integrin α3 subunit was higher in EBC-1/brain than in both EBC-1/original and EBC-1/bone. *In vitro* cell attachment, migration, and proliferation assays revealed that EBC-1/brain had higher affinity and higher reactivity to laminin than EBC-1/original and EBC-1/bone. Blocking of integrin α3β1 significantly ($P<0.05$) decreased brain metastasis by EBC-1/brain. Interaction of integrin α3β1 and laminin plays important roles in the process of brain metastasis of non-small cell lung cancer. (Cancer Sci 2004; 95: 142–148)

Lung cancer patients often suffer from brain metastasis. Indeed, clinical data in Japan show that more than half of metastatic brain tumors originates from lung cancer,[1] implying that most lung cancers have high potential for metastasis to brain.

Cancer metastasis is known to depend on the affinity of cancer cells for target organs. Target organs of metastasis are characterized by their own proportions of the components of extracellular matrix (ECM). Integrins are well-known receptors of ECM,[2] and the process of cancer metastasis is regulated by the interaction of integrins and ECMs.[3,4] The brain is a unique environment which includes limited components of ECM.[5] However, it is known that ECM and integrin play important roles in the progression of brain tumors.[6] Progression of glioma in the brain is correlated with the laminin distribution. Glioma cells recognize laminin through the cell surface integrin α3β1. Similar mechanisms may exist in the brain metastasis of lung cancer.

To clarify the mechanism of brain metastasis of lung cancer, we established an *in vivo* brain metastatic model using human non-small cell lung cancer (NSCLC) cell lines and athymic mice, since no model suitable for this purpose had been established. After sequential transplantation in our model, a highly brain metastatic subclone was obtained, and used for the principal investigation focused on integrin α3β1 and laminin.

Materials and Methods

Materials. Seven human NSCLC cell lines, EBC-1, LK-2, PC-3, VMRC-LCD, LC-1/sq, PC-14, and IA-5, were obtained from cell banks. EBC-1, LK-2, PC-3, and VMRC-LCD were provided by the Japanese Collection of Research Bioresources Cell Bank. LC-1/sq, PC-14, and IA-5 were provided by the Riken Cell Bank. Eleven anti-integrin subunit antibodies, α1 (Clone HP2B6; Life Technologies Oriental, Inc., Japan), α2 (Clone AK7; Novocastra Laboratories, Ltd., UK), α3 (Clone P1B5; Life Technologies Oriental, Inc.), α4 (Clone P4C2; Life Technologies Oriental, Inc.), α5 (Clone P1D6; Life Technologies Oriental, Inc.), α6 (Clone GoH3; Immunotech, France), αv(Clone AMF7; Life Technologies Oriental, Inc.), β1 (Clone SG/19; Seikagaku Corp., Japan), β3 (Clone SZ21; Immunotech), β4 (Clone 3E1; Life Technologies Oriental, Inc.), and β5 (Clone P4C2; Life Technologies Oriental, Inc.), were used for flow-cytometric analysis. Laminin (LN) was purchased from Becton Dickinson, USA. Fibronectin (FN) and type I collagen (COL) were purchased from Nitta Gelatin, Japan. Bovine serum albumin (BSA) was purchased from COSMO BIO, Japan. Four-week-old female athymic mice were provided by CLEA, Japan. All animals were treated in accordance with the guidelines of our institutional laboratory animal center.

Cell cultures. EBC-1, PC-3, and VMRC-LCD were cultured in Dulbecco's minimum essential medium (DMEM; Life Technologies Oriental, Inc.) supplemented with 10% heat-denatured fetal calf serum (FCS; Life Technologies Oriental, Inc.), 100 U/ml penicillin and 100 μg/ml of streptomycin (Life Technologies Oriental, Inc.). LK-2, LC-1/sq, PC-14, and IA-5 were cultured in RPMI-1640 (Sigma-Aldrich, Japan) supplemented with 10% FCS, 100 U/ml penicillin, and 100 μg/ml of streptomycin. For routine subculturing, cells were detached from the culture flask with 0.05% trypsin and 0.53 m*M* EDTA (Life Technologies Oriental, Inc.).

Flow cytometry. Aliquots (100 μl) containing 1×10^5 single cell suspensions were subjected to indirect immunofluorescence staining for expression of surface integrins using 100 μg/ml of monoclonal antibodies. The cell suspensions were placed on ice and the following procedure was conducted at 4°C. Control cells (no monoclonal antibody added) were prepared for evaluation of nonspecific fluorescence intensity. After 30-min incubation and 2 subsequent washes with phosphate buffer solution (PBS), the cells were resuspended in 1:30-diluted FITC-conjugated second antibody (Dako, Denmark), and incubated for 30 min. After 2 washes with PBS, cells were applied to a flow cytometer (FACS Calibur system; Becton Dickinson). The levels of expression of integrin subunits were evaluated using net fluorescence intensity, which was calculated as the geometric mean fluorescence intensities of stained cells minus that of control cells. Fluorescence intensities of stained cells and those of control cells in each sample were compared using Kolomogorov-Smirnov statistical analysis. When the fluorescence intensity of the sample did not reach statistical significance ($P<0.05$), integrin subunit expression was regarded as negative.

Investigation of abilities of 7 NSCLC cell lines to metastasize to brain: preliminary experiment. To investigate whether 7 NSCLC

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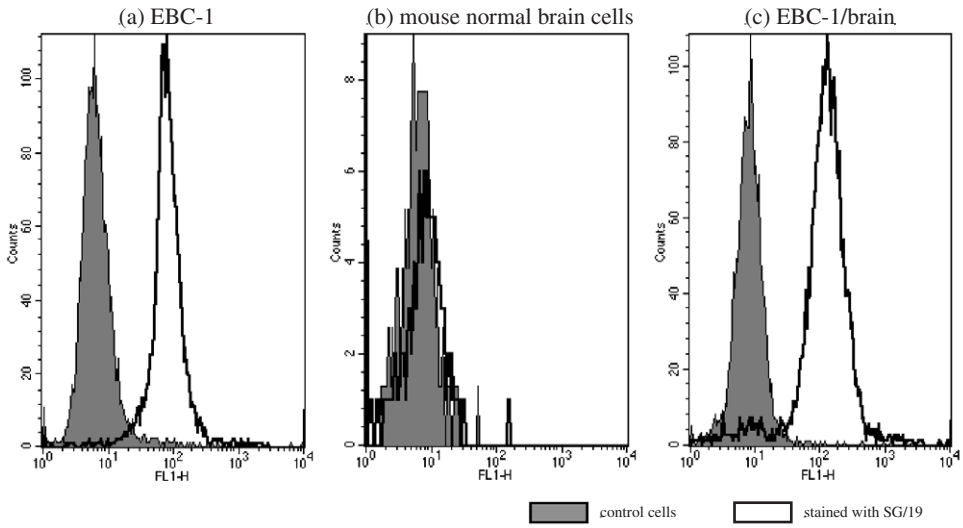

Fig. 1. Result of flow cytometry for (a) EBC-1, (b) cultured mouse normal brain cells, and (c) cancer cells obtained from mouse brain (EBC-1/brain). Staining by anti-human integrin β1 subunit antibody was found in EBC-1 and cancer cells obtained from mouse brain (EBC-1/brain), but cultured mouse normal brain cells were not stained at all.

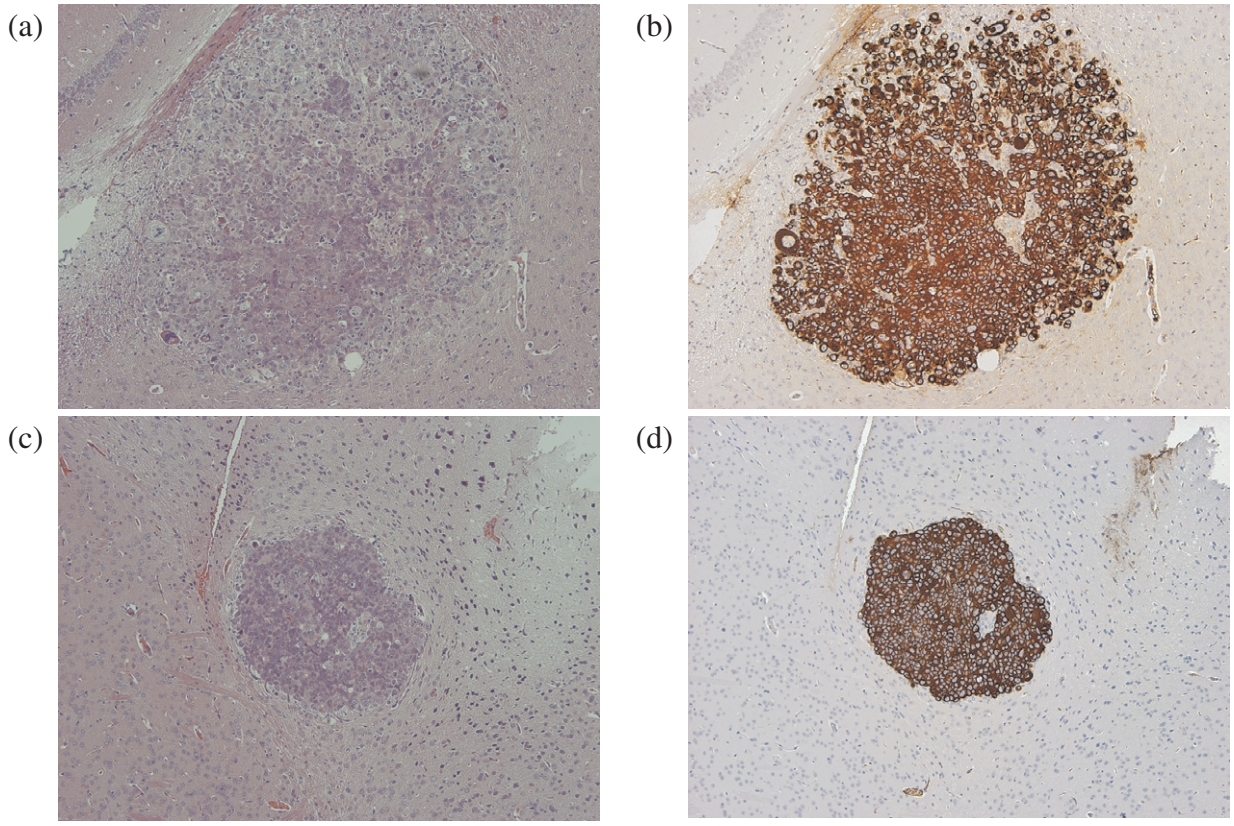

Fig. 2. Microscopic findings of brain metastatic lesion. (a) EBC-1/original, HE staining ×100, (b) EBC-1/original, cytokeratin staining ×100, (c) EBC-1/brain, HE staining ×100, (d) EBC-1/brain, cytokeratin staining ×100. Metastatic lesions were detected by both HE staining and immunohistochemical staining.

cell lines have the ability to metastasize to brain, each NSCLC cell line was transplanted into athymic mice. A total of 1×10^6 cells of each cell line was transplanted by 2 different methods, intravenous inoculation through a tail vein and intra-left ventricular inoculation. When mice became severely cachectic, or at 3 months after transplantation, they were sacrificed. Whole brain was extracted and minced. Brain tissue was cultured with 18 ml of culture medium in 75-cm^2 cell culture flasks. After 3-day incubation, brain tissue was discarded from the flask, and the cells attached to the bottom of the flask were collected. After several culture passages, the cells were stained with human specific monoclonal antibody (anti-integrin β1 subunit, clone SG/19) and measured by flow cytometry. Since all the NSCLC cells used in this study have integrin β1 subunit, brain metastasis was verified by detection of expression of human integrin β1 subunit (Fig. 1).

Establishment of highly brain metastatic subclone, EBC-1/brain. A NSCLC cell line, EBC-1 (EBC-1/original), was selected for establishment of a highly brain metastatic subclone. A total of 1×10^6 EBC-1/original cells was transplanted by left-ventricu-

lar inoculation. One or 2 months after inoculation, when mice became severely cachectic, they were sacrificed. Cancer cells were obtained in the same manner as described previously. After 5 cycles of *in vivo* serial passage of the cells in the mouse brain metastatic lesions, a highly brain metastatic subclone EBC-1/brain was established.

Establishment of highly bone-metastatic subclone, EBC-1/bone: control experiment. EBC-1/original also exhibited bone-metastatic ability when inoculated into athymic mice through the intra-left ventricular route. A total of 1×10^6 EBC-1/original cells were transplanted by left-ventricular inoculation. One or 2 months after inoculation, when bone metastatic lesions became macroscopically observable, the animals were sacrificed. Metastatic bone tumors were resected and minced. They were cultured in flasks in the same manner as for EBC-1/brain. After 5 cycles of *in vivo* serial passage of the cells in the mouse bone-metastatic lesions, a highly bone-metastatic subclone EBC-1/bone was established.

Evaluation of ability to metastasize to brain of EBC-1/original, EBC-1/brain, and EBC-1/bone. A total of 1×10^6 EBC-1/original, EBC-1/brain, or EBC-1/bone cells was transplanted through the intra-left ventricular route. Three weeks after transplantation, the mice were sacrificed. Whole brains were extracted and minced. Brain tissue of each mouse was cultured in 75-cm^2 cell culture flasks. After 14 days of culture, the flasks were examined by phase-contrast inverted microscopy. Brain metastasis was considered to have occurred when cancer cells were detected in the flask.

To confirm the existence of brain metastasis, histological examinations were also done with EBC-1/original and EBC-1/brain. Two months after transplantation, whole brains were extracted and fixed with formalin. Four horizontal sections were prepared in each mouse. Brains were examined microscopically by both hematoxylin-eosin staining and immunohistochemical staining for cytokeratin to detect the brain metastases.

Evaluation of integrin subunit expression of EBC-1/original, EBC-1/brain, and EBC-1/bone. Expression of integrin subunits of EBC-1/original, EBC-1/brain, and EBC-1/bone was investigated by flow cytometry.

Cell attachment assays on ECM. Substrates for the attachment assay were prepared by dry-coating with 1 μg/100 μl of test ligand (i.e., LN, COL, FN, or 1 mg/100 μl of BSA as a control) in 96-well assay plates at room temperature (RT) overnight. The plates were rinsed with PBS, followed by a quench with 1% BSA in PBS at RT for 2 h. A 100 μl of single cell suspension (2×10^5 cells/ml) of EBC-1/original, EBC-1/brain, or EBC-1/bone in DMEM was applied to each well in quadruplicate and incubated at 37°C for 1 h. Cells were stained with 1% crystal violet, and plates were gently rinsed twice with 100 μl of PBS to remove non-adherent cells. Attached cells were dissolved in 100 μl/well dimethylsulfoxide (DMSO), and the absorbance of the solution was read at 595 nm on a plate reader (Model 550; BIO RAD, Japan). Cell attachment capacity was evaluated as a percentage absorbance (*A*) of each ligand divided by the average *A* of control wells.

***In vitro* migration assays with ECM.** The chemotactic activity of EBC-1/original, EBC-1/brain, and EBC-1/bone for ECM was assayed using Chemotaxicell chambers (Kurabou, Japan). Chemotaxicell chambers with 8-μm diameter pored membranes were inserted in 24-well cell culture plates. A 4×10^4 single cell suspension in DMEM supplemented with 1% BSA was applied to the Chemotaxicell chamber. Ten micrograms per milliliter of LN, COL, or FN diluted with DMEM was applied outside the chamber. DMEM supplemented with 1% BSA was used as a control. The plates were incubated at 37°C for 12 h. The number of cells migrating outside the Chemotaxicell chamber through the pored membrane was measured using MTT assay. Forty microliters of MTT solution (0.2% of MTT and 0.05 *M* sodium succinate diluted with PBS) was added to each well and incubated for 3 h. The culture medium was removed, the formazan was dissolved in 400 μl/well DMSO, and the absorbance of the solution was read at 570 nm on the plate reader. Cell migration capacity was evaluated as percentage *A* of each ligand divided by the average *A* of control wells.

Table 1. Abilities of 7 NSCLC cell lines to metastasize to brain

NSCLC cell lines	Route of inoculation	
	Tail vein	Left ventricle
Squamous cell carcinoma		
EBC-1	0/3	1/2
LK-2	0/3	0/1
LC-1/sq	0/5	—
Adenocarcinoma		
PC-14	0/4	1/5
PC-3	0/4	2/2
VMRC-LCD	0/4	0/2
Large cell carcinoma		
IA-5	0/5	0/2

Values show the number of mice (brain metastasis (+)/evaluable ones).

Cell proliferation assays on ECM. Substrates for the attachment assay were prepared by wet-coating with 1 μg/100 ml of test ligand (i.e., LN, COL, FN, or 1 mg/100 μl of BSA as a control) in 96-well cell culture plates at room temperature overnight. The plates were rinsed with PBS, followed by a quench with 1% BSA in PBS at room temperature for 2 h. A 100 μl of single cell suspension (2×10^3 cells/ml) of EBC-1/original, EBC-1/brain, or EBC-1/bone in DMEM supplemented with 10% FCS was applied to each well in quadruplicate and incubated at 37°C for 48 h. Thereafter, 10 μl of MTT solution was added to each well and incubated for 3 h. Culture medium was removed, formazan was dissolved in 100 μl/well DMSO, and the absorbance of the solution was read at 570 nm on the plate reader. Cell proliferation was evaluated as percentage *A* of each ligand divided by the average *A* of control wells.

Blocking of integrin α3 subunit in EBC-1/brain. A 1×10^6 cells/ml single cell suspension of EBC-1/brain was incubated with 100 mg/ml of blocking antibody for integrin α3 subunit (clone P1B5) at 4°C. After 30-min incubation and 2 subsequent washes with PBS, the cells were resuspended in DMEM. The same procedure was conducted with purified mouse IgG instead of blocking antibody, as a control.

A total of 1×10^6 cells of integrin α3-blocked EBC-1/brain, or EBC-1/brain treated as a control was transplanted into athymic mice through the intra-left ventricular route. Three weeks after transplantation, the mice were sacrificed and formation of brain metastasis was checked for using the same method as described previously.

Cell viability after the blocking procedure was confirmed by routine cell culture of these cells. Integrin α3-blocked EBC-1/brain could proliferate as well as control cells in the flask. Blocking of integrin α3 was confirmed by loss of stretching capacity on LN (Fig. 2).

Statistics. All values are given as means±SD. ANOVA and the χ^2 test were employed to evaluate the significance of differences between the data. *P* values less than 0.05 were taken to indicate statistical significance.

Results

Abilities of 7 NSCLC cell lines to metastasize to brain. Brain metastasis was detected in EBC-1/original, PC-14, and PC-3 by inoculation through the left-ventricular route (Table 1). We decided to use EBC-1/original with inoculation through the left-ventricular route to obtain a highly brain metastatic subclone.

Evaluation of ability to metastasize to brain of EBC-1/original, EBC-1/brain, and EBC-1/bone. The results are summarized in Table 2. EBC-1/original yielded brain metastasis in 1 of 5 mice. EBC-1/brain yielded brain metastasis in 6 of 6, and EBC-1/bone in none of 5 mice. EBC-1/brain exhibited higher brain metastatic potential than EBC-1/original ($P<0.05$) and EBC-1/bone ($P<0.01$).

Microscopic examination revealed brain metastasis in 1 of 8 mice with EBC-1/original, and 3 of 8 mice with EBC-1/brain. Photographs of the metastatic lesions are shown in Fig. 3. A solitary brain metastatic lesion was seen in EBC-1/original, while the lesions were multiple in 3 mice with EBC-1/brain.

Attachment and morphology of EBC-1/original, EBC-1/brain, and EBC-1/bone on different ECM. The cells were incubated in 5% CO_2 at 37°C on FN, COL, LN, and plastic as a control for 48 h. Photographs were taken under phase-contrast inverted microscopy (Fig. 4). All 3 types of cells attached and stretched on plastic, FN, and COL. On LN, only EBC-1/brain exhibited stretched morphology.

Integrin subunit expression of EBC-1/original, EBC-1/brain, and EBC-1/bone. Integrin subunit expression in EBC-1/original, EBC-1/brain, and EBC-1/bone, evaluated by flow cytometry, is summarized in Fig. 5. These cells commonly expressed integrin subunits α1, α2, α3, α5, α6, αv, β1, β4, and β5. The most marked change in EBC-1/brain compared with EBC-1/original was an increase in α3 subunit expression ($P<0.0001$ by Kolomogorov-Smirnov statistical analysis). Integrin α3 subunit was not increased in EBC-1/bone.

Table 2. Abilities to metastasize to brain

EBC-1/original	1/5	(20%)
EBC-1/brain	6/6	(100%)
EBC-1/bone	0/5	(0%)

Values show the number of mice (brain metastasis (+)/evaluable ones).

Integrin α3 subunit expression of 7 NSCLC cell lines. Expression of integrin α3 subunit in 7 NSCLC cell lines evaluated by flow cytometry is summarized in Fig. 6. The expression levels in EBC-1/original, PC-14, and PC-3, which exhibit brain metastatic ability, were higher than those in the other cell lines except for IA-5.

***In vitro* assays to ECM.** EBC-1/brain was more strongly attached to FN ($P<0.05$), COL ($P<0.05$), and LN ($P<0.01$) than was EBC-1/original. EBC-1/bone was more strongly attached to FN than was EBC-1/original ($P<0.05$) (Fig. 7a). *In vitro* migration assays revealed that the number of cells which had migrated on LN was larger for EBC-1/brain than for EBC-1/original and EBC-1/bone ($P<0.05$) (Fig. 7b). EBC-1/brain exhibited higher cell proliferation capacity than EBC-1/original and EBC-1/bone in COL ($P<0.05$) and LN ($P<0.01$). Proliferation of EBC-1/original and EBC-1/bone on LN was inhibited compared with that on BSA, while proliferation of EBC-1/brain was not inhibited on LN compared with BSA (Fig. 7c).

Inhibition of brain metastasis of EBC-1/brain blocked with integrin α3 subunit antibody. The results are summarized in Table 3. EBC-1/brain yielded brain metastasis in 6 of 6 mice when it was treated with mouse IgG (control). EBC-1/brain yielded brain metastasis in 1 of 6 mice when it was treated with integrin α3 blocking antibody. The brain metastatic capacity of EBC-1/brain was significantly inhibited by treatment with integrin α3 blocking antibody ($P<0.05$).

Discussion

Mouse melanoma cells are frequently used for experimental investigation of brain metastasis of cancer.[7,8] Since patterns of integrin expression of melanoma cells differ from those of human NSCLC cells,[9] melanoma cells could not be used for investigation for brain metastasis of NSCLC. Glioma cells, which are widely used as an experimental model of brain tumor, also could not be used in this study, since their integrin expression

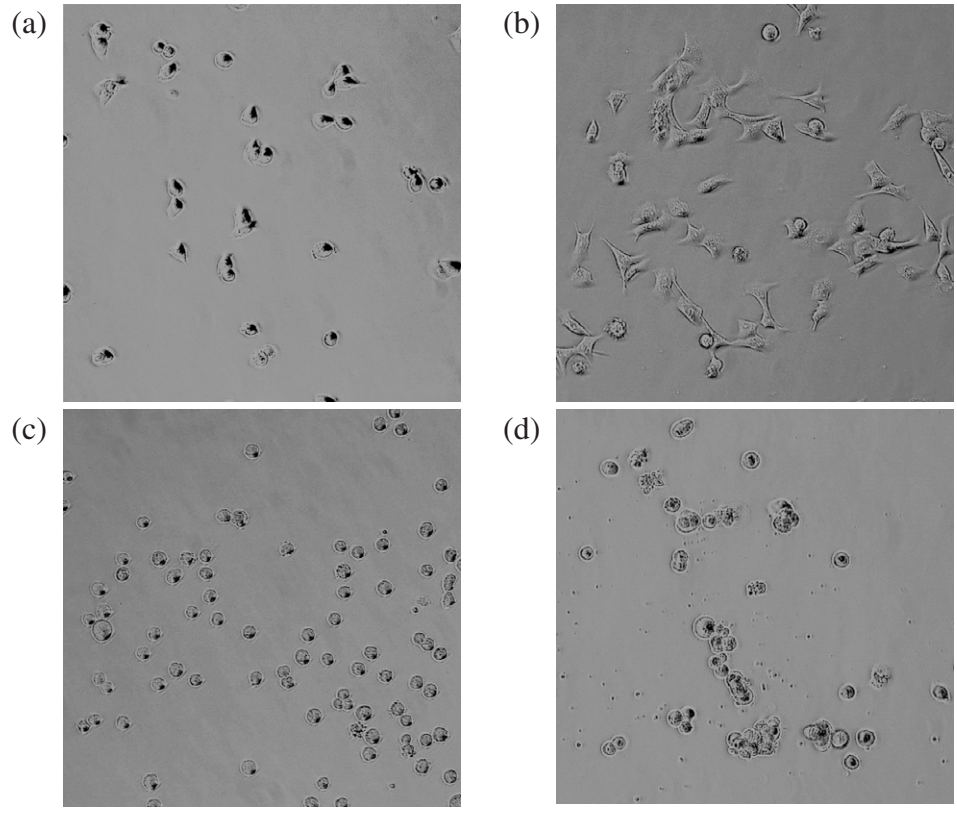

Fig. 3. EBC-1/brain treated with integrin α3 subunit blocking antibody were cultured in cell culture flask (plastic) (a, b), and in laminin-coated cell culture flask (c, d). Photographs were taken under phase-contrast inverted microscopy after 6-h (a, c) and 48-h (b, d) cell culture. Cell proliferation was observed on both plastic and laminin. Cells were stretched on plastic but not at all on laminin.

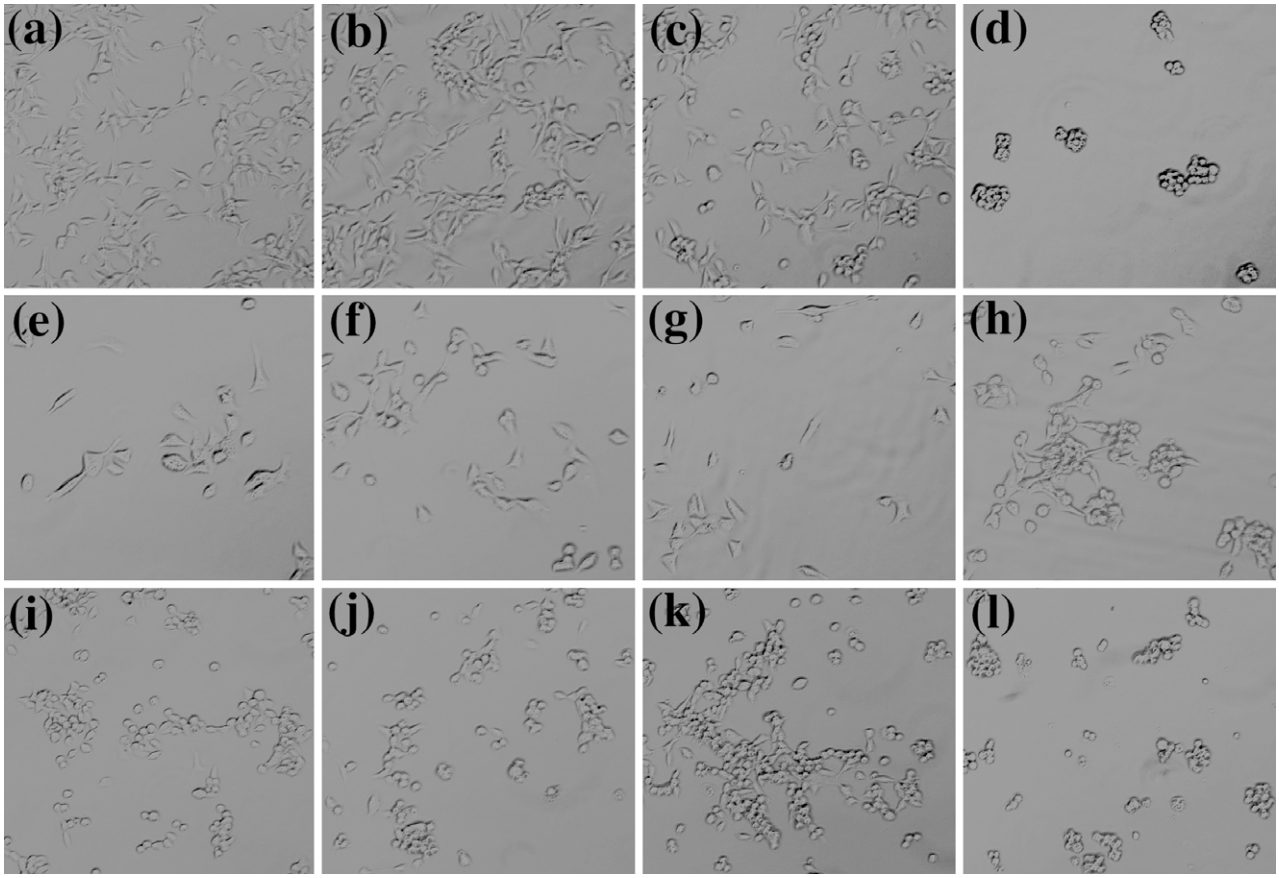

Fig. 4. Attachment and morphology of EBC-1/original (a, b, c, d), EBC-1/brain (e, f, g, h), and EBC-1/bone (i, j, k, l) on untreated cell culture plate (a, e, i), FN (b, f, j), COL (c, g, k), and LN (d, h, l). The cells were incubated in 5% CO_2 at 37°C for 48 h. Photographs were taken under phase-contrast inverted microscopy.

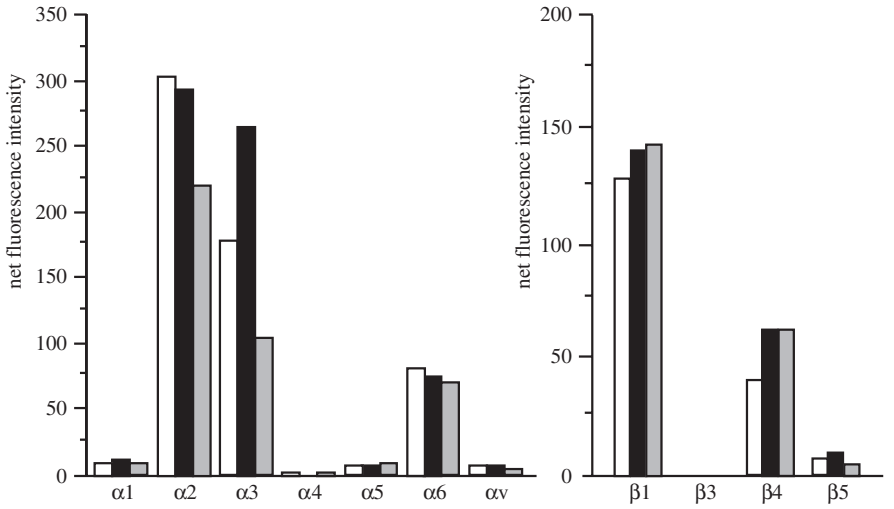

Fig. 5. Integrin subunit expression of EBC-1/original, EBC-1/brain, and EBC-1/bone. Values are net fluorescence intensities. These cells commonly expressed integrin subunit α1, α2, α3, α5, α6, αv, β1, β4, and β5. The most marked change in EBC-1/brain compared with EBC-1/original was an increase in α3 subunit expression.

differs from that of human NSCLC cells, as well.[6, 10, 11] Human NSCLC cell lines were therefore used. Because no experimental model of brain metastasis of human NSCLC had been developed, we needed initially to establish such a model using athymic mice.

Establishment of brain metastasis in athymic mice using human NSCLC cells presented several problems. Brain metastasis easily becomes lethal. It is therefore difficult to decide when mice should be sacrificed to obtain cancer cells from brain metastasis. Another problem is how to judge the formation of brain metastasis. Brain metastasis cannot grow to macroscopic size in mice, and microscopic examination is somewhat complicated. Brain metastases of melanoma cells are usually pigmented and easily detected macroscopically, but brain metastatic lesions of NSCLC cells are not pigmented and are invisible macroscopically. The final problem is the low frequency of brain metastasis of human NSCLC in athymic mice.

We inoculated various human NSCLC cell lines to find cell

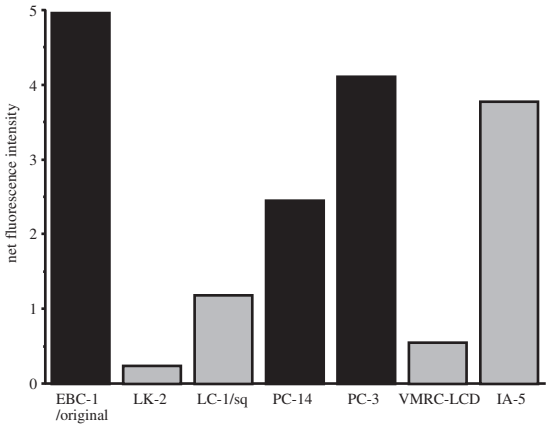

Fig. 6. Integrin α3 subunit expression of 7 NSCLC cell lines. Values are net fluorescence intensities. Expression of integrin α3 subunit in EBC-1/original, PC-14, and PC-3, in which cells brain metastastatic ability was observed, is higher than those in the other 4 cell lines. Settings of the flow cytometer were not identical with those in Fig. 5.

lines with the ability to metastasize to the brain in athymic mice. Cancer cells from primary lesions of lung cancer easily drain into the left ventricle through the pulmonary vein. We therefore inoculated tumor cells through both a tail vein and the left ventricle.

To detect invisible brain metastatic lesions with certainty, we used a cell culture method. Whole brain was extracted from cachectic mice and minced, and the brain tissue was cultured in cell culture flasks. This technique enabled detection of invisible brain metastasis in 3 cell lines in the group with inoculation through the left ventricle.

This result implies that the anatomical location of lung cancer is an important reason why brain metastasis often occurs in lung cancer. Lung cancer cells easily drain into arterial blood through the pulmonary vein and the left ventricle. Therefore, they escape capture by the pulmonary capillary bed.

The technique of *in vivo* sequential selection of cancer cells in experimental metastatic lesions is commonly used for the investigation for cancer metastasis.[12–15] Generally, newly selected subclones are more advantageous for formation of metastasis than parental clones. In our study, we intended to investigate what changes in the selected clone in the brain are specific for brain metastasis. We therefore decided to generate another subclone from the same parental cell line selected in an organ other than brain, and to compare these 3 subclones. A preliminary experiment revealed that EBC-1/original also has the ability to metastasize to bone. We therefore decided to use EBC-1/original in this study, and produced EBC-1/brain and EBC-1/bone.

EBC-1/brain did not form bone metastasis during 5 *in vivo* selections, EBC-1/bone did not form brain metastasis during 5 *in vivo* selections. Moreover, EBC-1/brain had significantly a higher ability to metastasize to the brain than did EBC-1/original and EBC-1/bone. These 3 subclones were considered to be suitable for the investigation of NSCLC brain metastasis.

EBC-1/brain cells are larger than the cells of the other 2 subclones. The diameters of EBC-1/original, EBC-1/brain, and EBC-1/bone were 23.9±0.3 μm, 31.5±0.3 μm, and 21.3±0.3 μm, respectively. However, we could not explain why EBC-1/brain was larger than other 2 subclones.

The most obvious alteration in integrin expression of EBC-1/brain compared with EBC-1/original was an increase in α3 subunit. On the other hand, expression of integrin α3 subunit was decreased in EBC-1/bone compared with EBC-1/original. Expression levels of integrin α3 subunit in EBC-1/original, PC-14, and PC-3, which showed brain metastastatic ability, were higher than those in the other cell lines except for IA-5. The increase of integrin α3 subunit in EBC-1/brain appears to be specific to brain metastasis.

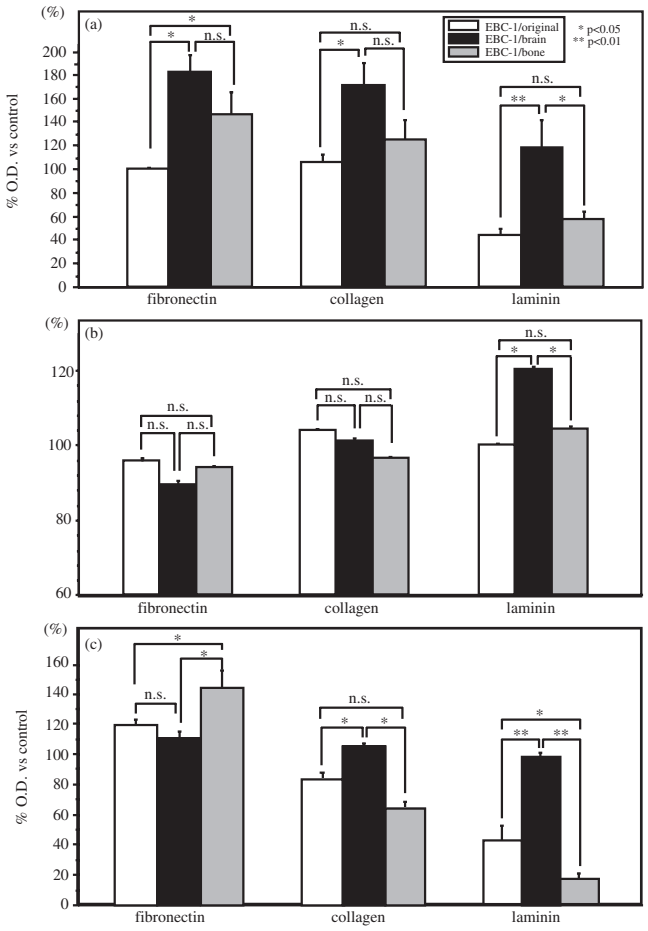

Fig. 7. Results of *in vitro* assays of (a) cell attachment, (b) migration, and (c) cell proliferation. EBC-1/brain was more strongly attached on FN ($P<0.05$), COL ($P<0.05$), and LN ($P<0.01$) than EBC-1/original. EBC-1/bone was more strongly attached on FN than EBC-1/original ($P<0.05$) (a). The number of cells that had migrated on LN was larger for EBC-1/brain than for EBC-1/original or EBC-1/bone ($P<0.05$) (b). EBC-1/brain exhibited higher cell proliferation capacity than EBC-1/original or EBC-1/bone on COL ($P<0.05$) and LN ($P<0.01$) (c).

Table 3. Inhibition of brain metastasis by P1B5

Control	6/6	(100%)
Treated with P1B5	1/6	(17%)

Values show the number of mice (brain metastasis (+)/evaluable ones).

Integrin molecules are heterodimers of an α subunit and a β subunit, and the α3 subunit yields only integrin α3β1.[2] We concluded that expression of integrin α3β1 is higher in EBC-1/brain than those in EBC-1/original and EBC-1/bone.

We also have some data about the gene transfection of integrin α3 subunit. CHO-B2 cells, which do not have integrin α3β1, do not have brain metastatic ability. Expression of integrin α3β1 was observed after transfection of integrin α3 subunit (α3-CHO-B2), and brain metastasis was observed in 36% of α3-CHO-B2-inoculated athymic mice. There was no significant difference in metastatic abilities to other organs between CHO-B2 and α3-CHO-B2 (manuscript submitted for publica-

tion). These results also indicate that integrin α3β1 is important in brain metastasis.

Integrin α3β1 is a laminin receptor. Expression of integrin α3β1 has been widely observed in various cancer cells.[16] Recent studies revealed that integrin α3β1 plays important roles in invasion and metastasis of cancer.[6, 17–24] Increased expression of integrin α3β1 was observed in metastatic lesions and peritoneal dissemination of gastric cancer and colorectal cancer.[17–19] *In vitro* experiments have revealed that adhesion of integrin α3β1 to laminin leads to migration and invasion by cancer cells.[20–22] It is also known that integrin α3β1 is related to the regulation of matrix metalloproteases.[17, 23]

It has already been reported that glioma invades and proliferates in the brain on laminin distributed in the brain.[6, 24] Glioma cells recognize laminin through cell surface integrin α3β1. Similar mechanisms may operate in the brain metastasis of lung cancer. However, the role played by integrin in the progression of metastatic brain tumors has not yet been determined.

In our study, increased expression of integrin α3β1 was observed in EBC-1/brain compared with EBC-1/original. Only EBC-1/brain among the 3 subclones stretched on laminin. Cell adhesion, migration, and proliferation capacity on laminin were higher in EBC-1/brain than in EBC-1/original or EBC-1/bone. Blocking of α3β1 made EBC-1/brain lose stretching capacity on laminin and strongly suppressed metastasis to the brain. The higher affinity and higher reactivity for laminin of EBC-1/brain appeared to be related to increased expression of α3β1.

The brain provides a unique environment in which ECM is rarely present. However, laminin plays important roles in glioma progression and development of neurons in the brain.[6, 24–27] Although we cannot fully explain why brain metastasis is frequent only for lung cancer, the results of our study suggest that laminin plays important roles in the process of brain metastasis of NSCLC through interaction with integrin α3β1.

Conclusion

To investigate the roles of integrins and extracellular matrices in the process of brain metastasis by NSCLC, we established an *in vivo* model of experimental brain metastasis in athymic mice using the human non-small cell lung cancer cell line EBC-1/original, and established the highly brain metastatic subclone EBC-1/brain. Expression of integrin α3β1 was higher in EBC-1/brain than in EBC-1/original or EBC-1/bone. Higher affinity and higher reactivity for laminin were observed in EBC-1/brain than in EBC-1/original or EBC-1/bone. The ability of EBC-1/brain to metastasize to brain was significantly depleted by the blocking of integrin α3β1. The results of our study suggest that interaction of integrin α3β1 and laminin plays important roles in the process of brain metastasis of NSCLC.

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