Enhanced Expression of Type IV Collagen-binding Protein (p29) in *Fyn*-transfected Murine Fibrosarcoma Cells

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We investigated the mechanism of the enhancement of metastatic potential induced by transfection of the *fyn* gene, a member of the src family. We employed two murine *fyn* cDNA-transfected clones, ML-SN1 and ML-SN2, which were previously established from an ML-01 low-metastatic clone of Meth A sarcoma of BALB/c mice and were proven to have higher metastatic ability than ML-01 and the mock-transfected clone ML-MT-neo (Takayama *et al.*, 1993). Our present investigation revealed that the two transfectants showed higher metastatic ability and higher rates of adherence to type IV collagen than ML-MT-neo. However, no difference was found in *in vitro* or *in vivo* growth rates, attachment to laminin or endothelial cells or cell motility through a reconstituted basement membrane. Analysis of surface membrane proteins labeled with ^{125}I on SDS-PAGE showed that a 29 kD band specifically bound to type IV collagen-coupled beads was more intense in ML-SN2 than in ML-MT-neo. Genistein, a protein tyrosine kinase inhibitor, dramatically reduced protein tyrosine kinase (PTK) activity of ML-SN2 in a dose-dependent fashion, corresponding to the reduction of adhesiveness to type IV collagen. The expression of the type IV collagen-binding protein (p29) of ML-SN2 was also reduced significantly by genistein treatment. These results suggested that the *fyn* product in Meth A cells augments the expression of a type IV collagen-binding protein through elevation of the PTK activity of the membrane fraction and thus facilitates the metastasis of Meth A.

Key words: *fyn* — Attachment — Type IV collagen — Metastasis — Meth A

Metastasis characterizes a distinctive malignant phenotype of neoplasm. The formation of metastases consists of multiple steps of tumor cell behavior such as detachment from the primary lesion, invasion into the surrounding extracellular matrix, penetration into the circulation, lodging in the vascular bed in a distant organ, reinvasion through the vascular basement membrane into the surrounding tissue and proliferation to form metastatic foci. Better understanding of factors which modulate the metastatic processes could ultimately lead to the development of effective therapeutic modalities for cancer patients. However, the mechanisms of metastasis formation have not been completely elucidated because of the huge variety of host and tumor cell properties.[1]

We have previously established a high-metastatic clone (MH-02) and a low-metastatic clone (ML-01) from a BALB/c mouse-derived 3-methylcholanthrene-induced A sarcoma cell line (Meth A)[2] and identified a 29 kD type IV collagen-binding protein that was expressed four-fold more on the surface of MH-02 than on ML-01.[3] We further found that the expression of the *fyn* oncogene, which belongs to the non-receptor type tyrosine kinase family,

was four times higher in MH-02 than in ML-01[4] and that the metastatic ability of two *fyn*-transfected clones was significantly increased compared to that of the mock-transfected clone.[4] On the basis of the above observations, in the present investigation we attempted to elucidate the metastatic properties of *fyn*-transfectants, with special reference to their expression of the 29 kD type IV collagen-binding protein and tyrosine kinase activity, and compared them to those of mock transfectant.

MATERIALS AND METHODS

Cell lines The two *fyn*-transfected clones, ML-SN1 and ML-SN2, were obtained by co-transfecting the fyn expression vectors pMT-SN and pSV2-neo into ML-01, followed by G418 selection as described earlier.[4] The mock transfectant ML-MT-neo was also obtained by co-transfecting the vector DNAs, pMT and pSV2-neo, into ML-01.[4]

Cell culture and proliferation assay *in vitro* ML-01 and its transfectants were maintained in RPMI 1640 media (GIBCO BRL, Grand Island, NY) with 10% fetal calf serum (FCS) (GIBCO BRL), 100 units/ml penicillin-G and 100 mg/ml streptomycin using culture flasks (Costar #3050, Cambridge, MA) in 5% CO_2/95% air in a humidified atmosphere at 37°C. Since pMT-SN contains a dex-

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amethasone responsive element (mouse mammary tumor virus long-terminal repeats (LTR)), the transfectants were pre-treated with 1 mM dexamethasone for 48 h in order to induce fyn expression. For the cell proliferation assay, viable cells were counted at 24, 48 and 72 h of cultivation.

Zymography Gelatinase, one of the matrix metalloproteinases, secreted by each clone was detected by zymography as described by Heussen and Dowdle.[5] Cells at late log-phase in 75-cm^2 culture flasks (1.5×10^7 cells) were washed with serum-free RPMI 1640 medium and incubated with 15 ml of RPMI 1640 medium for 12 h to remove fetal bovine serum (FBS), after which the medium was replaced by 10 ml of fresh serum-free medium. After an additional 24 h incubation, the conditioned medium was collected and centrifuged (110g×5 min). The supernatants were further centrifuged (4000g×20 min), concentrated 50-fold in a Centricon 10 (Amicon, Danvers, CO) and applied to the zymography gel. The substrate, 0.1% gelatin (Wako, Osaka) was incorporated into the matrix of a 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel. After the gel was electrophoresed at 4°C, SDS was removed from the gel by incubating the gel in 0.05 M Tris-HCl (pH 7.5) containing 2.5% Triton X-100 for 30 min. Then the gel was incubated at 37°C in the presence of 0.5 mM CaCl$_2$ and 0.02% NaN$_3$ overnight. The gel was stained with Coomassie brilliant blue R250 (0.25%, w/v) and destained in a mixture of methanol, acetic acid and water (50:10:40). The proteolytic activity was detected as unstained bands. Some gels were incubated overnight in the presence of 10 mM EDTA to determine whether metalloproteinases were responsible for the gelatinase activity.

Metabolic radiolabeling of tumor cells The transfectants were metabolically labeled with ^{125}I-iodo-2′-deoxyuridine (^{125}I-IUDR, Amersham, Aylesbury, UK) as described by Fidler.[6] Briefly, cells (1.0×10^5/ml) were cultured in RPMI 1640 supplemented with 10% FCS containing 0.1 mCi/ml of ^{125}I-IUDR for 36 h at 37°C. Cells were then washed three times in RPMI 1640 medium to remove the free ^{125}I-IUDR before they were used for the experiments.[3, 7]

Motility assay and transmembrane invasion assay ^{125}I-IUDR-labeled tumor cells (1.0×10^5 cells/ml) in RPMI 1640 were placed in the upper chamber of a "Transwell" double chamber well with 6.5 mm diameter and 8 μm pores (Costar #3422). A conditioned medium obtained by incubating confluent NIH3T3 cells for 24 h in serum-free IMDM medium (GIBCO BRL) was poured into the lower chamber. After incubation for 12 h at 37°C in humidified 5% CO$_2$, the cells in the lower chamber were lysed with 1 N NaOH and the radioactivity was measured with a gamma counter. The motility of tumor cells was expressed by the ratio of the number of cells in the lower chamber to that of cells in the upper chamber. Transmembrane invasion assay through a reconstituted basement membrane "Matrigel" (Becton Dickinson Labware, Bedford, MA)

was assayed by the same method except that the "Transwell" filters were precoated with "Matrigel" (100 mg/well).

Attachment assay Tumor cell adhesiveness to type IV collagen (Cell matrix type IV, 3 mg/ml, purity: 95%, Nitta Gelatin, Inc., Tokyo) and laminin (Sigma, St. Louis, MO) were assayed by the methods of Tao and Johnson.[8] Plates coated with type IV collagen or laminin were prepared by evaporating distilled water containing the protein (0.3 mg/ml) at 37°C in culture plates (Costar #3512, Becton Dickinson Labware). One milliliter of ^{125}I-IUDR-labeled cells (1.0×10^5/ml) was added to each well of the protein-coated tissue culture plates. The culture plates were centrifuged at 200g for 5 min in a plate centrifuge (Model CD-60R, Tomy Seiko Co., Tokyo). Fifteen minutes later, non-adherent cells were removed by gentle aspiration. Adherent cells were washed gently three times with RPMI 1640 medium and solubilized in 1 ml of 1 N NaOH to measure the radioactivity in an autogamma counter. For the assay of the effect of anti β1-integrin antibody on cell attachment, an experiment was performed by the same method except that cells were pre-treated with mouse mAb JB1(anti-human β1-integrin, IgG1, 0.2 mg/ml, CHEMICON International, Inc., Temecula, CA) for 30 min before the cells were added to the wells of a culture plate.

FACS analysis of cell-surface β1-integrin subunits Tumor cells were washed with growth medium and incubated in fresh growth medium for 30 min at 37°C in 5% CO$_2$. Subsequently the cells were washed three times in ice-cold PBS (phosphate-buffered saline, pH 7.2) supplemented with bovine serum albumin (BSA; 0.1%, w/v) and, for fluorocytometric analysis, NaN$_3$ (0.1%, w/v). The washed cells (5×10^5 in 100 μl volumes for analysis) were incubated for 30 min on ice with primary antibody (final dilutions: 1:1, hybridoma supernatants; 1:100, ascites). After two washes, FITC-conjugated rabbit-anti-mouse IgG (RAM-FITC; DAKO, Carpinteria, CA) was added to the resuspended cells (1:40 final dilution) for 30 min on ice, and the cells were then washed three more times. Analysis was performed by fluorocytometry on a FACSCAN analyser (Becton Dickinson, Mountain View, CA) with Consort 30 software. Background fluorescence was determined by omitting the primary antibody from the treatment of the negative control sample.

Treatment of cells with genistein ML-MT-neo and ML-SN2 cells (1.5×10^5/ml) were radiolabeled with ^{125}I-IUDR as described earlier.[6] Then the radiolabeled transfectants were treated for 3 h in RPMI 1640 with different concentrations of genistein (Extrasynthese Laboratories, Lyon, France) (3.0, 5.0 or 25.0 μg/ml) at 37°C in 5% CO$_2$. Genistein was dissolved first in dimethyl sulfoxide (DMSO) and diluted with RPMI 1640 to certain concentrations. In all experiments, the final concentration of DMSO was 1% (v/v). After the genistein treatment, the

cells were washed with RPMI 1640 three times and assayed *in vitro* for cell proliferation, artificial metastasis or attachment to type IV collagen as described elsewhere.[2, 3, 9]

Preparation of cell membrane protein Membrane protein was prepared by the method of Usui *et al.*[10] ML-01 and ML-SN2 cells (1.0×10^8) were washed once with RPMI 1640 and washed three times with 137 mM NaCl, 2.7 mM KCl, 1.47 mM KH$_2$PO$_4$, 0.84 mM NaH$_2$PO$_4$, 0.5 mM PMSF and 1 mM EDTA (pH 7.4). All subsequent procedures were carried out with samples maintained in an ice bath. The cells were homogenized with 5 volumes of 0.02 M boric acid, 0.5 mM phenylmethylsulfonyl fluoride (PMSF) and 1 mM EDTA (pH 10.2) in a Dounce homogenizer (loose fitting, 60 strokes). The majority of nuclei were pelleted by centrifugation at 1000g for 45 s. The supernatants were removed and centrifuged for 30 min at 20 000g to pellet mitochondria and plasma membrane vesicles. The pellet was resuspended in PBS and layered on top of PBS containing 35% sucrose. The membrane protein suspension was floated on the 35% sucrose by centrifugation at 40 000g for 45 min, removed from the top of the sucrose layer, diluted 3-fold with PBS and centrifuged for 30 min at 75 000g. The pellet was resuspended at a concentration of 3–4 mg of protein/ml in 20 mM Hepes, pH 7.4, quick-frozen and stored in aliquots in liquid nitrogen.

Protein tyrosine kinase assay of membrane fractions Protein tyrosine kinase (PTK) activity was assayed as described by Usui *et al.*[10] Briefly, the reaction was carried out in a 60 μl reaction mixture containing [γ-^{32}P]ATP (920 cpm/pmol) at a final concentration of 15 mM and 20 mM Hepes (pH 7.4), 3 mM MnCl$_2$, 10 mM ZnCl$_2$, 30 mM NaVO$_3$, 0.5 mM dithiothreitol, 0.2% Nonidet P-40 (NP-40), 0.1 mg/ml tyrosine-glutamate (1:4) copolymers (Mr 28 000, Sigma) as a substrate, and 1.4 mg of membrane protein. After 10 min at 30°C, a 50 μl aliquot was spotted on Whatman 3MM paper, washed in 10% trichloroacetic acid containing 8% sodium pyrophosphate twice, and then washed in 99.9% ethanol twice. The paper was dried and the radioactivity was quantitated with a liquid scintillation counter.

Inhibition of PTK activity by genistein The inhibitory effect of genistein on membrane-bound PTK activity was assayed as described by Mustelin *et al.*[11] Briefly, the membrane protein was incubated in different concentrations of genistein dissolved in DMSO (final concentration 1% (v/v), mixed with assay buffer (20 mM Hepes (pH 7.4), 3 mM MnCl$_2$, 10 mM ZnCl$_2$, 30 mM NaVO$_3$, 0.5 mM dithiothreitol, 0.2% NP-40)) containing [γ-^{32}P]ATP (920 cpm/pmol) at a final concentration of 15 mM, and 0.1 mg/ml tyrosine-glutamate (1:4) copolymers (Mr 28 000) as a substrate for 10 min in an ice bath. After 10 min at 30°C, a 50 μl aliquot was spotted onto Whatman 3MM paper,

washed in 10% trichloroacetic acid containing 8% sodium pyrophosphate twice and then washed in 99.9% ethanol twice. The paper was dried and the radioactivity was quantitated with a liquid scintillation counter.

Preparation of ^{125}I-labeled membrane proteins of tumor cells Surface membrane proteins of ML-SN2, ML-MT-neo and ML-01 cells were radioiodinated by an immobilized lactoperoxidase-glucose oxidase method according to the manufacturer's directions using "Enzymobeads" (Bio-Rad, Richmond, CA). Cells (2.0×10^7) suspended in 1 ml of PBS were treated with 200 μl of 5 mg/ml β-D-glucose, 200 μl of "Enzymobeads" reagent and 1 mCi of sodium-^{125}I (100 mCi/ml, Daiichi Radioisotope Labs., Ltd., Tokyo). The suspension was incubated for 30 min at room temperature with constant shaking, and the enzymatic reaction was terminated by washing the cells five times with PBI buffer (137 mM NaI, 2.68 mM KCl, 8.32 mM Na$_2$PO$_4$•12H$_2$O, 1.47 mM KH$_2$PO$_4$, pH 7.4, 2 mM PMSF). The cells were then lysed at 4°C in 1.2 ml of the lysis buffer (1% NP-40, 0.15 M NaCl, 50 mM Tris (pH 8.0), 5 mM EDTA, 2 mM PMSF, 1 mg/liter pepstatin (Boehringer Mannheim, Ridgefield, NJ), 1 mg/liter leupeptin (Boehringer Mannheim), and sonicated in a sonicator Model W225R (Waken Yakuhin K.K., Tokyo) at 20 watts for 3 min on ice. Cell debris was pelleted by centrifugation for 30 min at 100 000g at 4°C. The supernatant was used as solubilized ^{125}I-labeled membrane proteins.

Extraction of type IV collagen-binding protein from tumor cells Type IV collagen was coupled to CNBr-activated Sepharose 4B beads (Pharmacia) according to the manufacturer's directions.[12] Two hundred microliters of solubilized ^{125}I-labeled membrane proteins (containing equivalent amounts of radioactivity) was incubated for 2 h at 4°C with 50 μl of type IV collagen-coupled Sepharose 4B beads in NET buffer (1% NP-40, 0.4 M NaCl, 50 mM Tris (pH 8.0), 5 mM EDTA, 2 mM PMSF). The beads then were washed twice in the NET buffer and once with washing buffer (100 mM Tris buffer, pH 6.8). The beads were boiled for 5 min with an equal volume of 2× SDS sample buffer, and centrifuged briefly. The supernatant was analyzed by SDS-polyacrylamide gradient gel (4–20%) electrophoresis. The gel was fixed, dried, and subjected to autoradiography.

***In vivo* tumor growth** Female BALB/c mice of 6 weeks of age were purchased from CLEA Japan, Inc. (Sendai). Animals were maintained according to the institutional guidelines for care and use of laboratory animals. Tumor cells (1.0×10^6/100 μl) were inoculated into the right abdominal flank of BALB/c mice. At day 7, day 14 and day 21, tumor dimensions were measured. Tumor volumes were calculated by using the formula $0.5 \times ab^2$, where a is the larger and b the smaller of the two dimensions.[2]

Experimental pulmonary metastasis Experimental pulmonary metastasis was prepared as described earlier.[3]

BALB/c mice were injected via the tail vein with 1.0×10^4 genistein-treated or non-treated ML-MT-neo or ML-SN2 cells suspended in 0.1 ml of RPMI 1640 medium and were sacrificed 14 days later. The numbers of metastatic nodules on the surface of the lungs were determined under a dissecting microscope.[3]

Statistical analysis Statistical analysis for comparison of *in vitro* assay was performed by the use of Student's *t* test. The Mann-Whitney test was used for statistical analyses of *in vivo* assays. All calculations were performed on a Macintosh microcomputer using SigmaPlot (Jandel Scientific Software, Chicago, IL). The criterion for statistical significance was $P<0.05$.

RESULTS

Growth of *fyn*-transfectants Since the growth rate of tumor cells is known to affect their metastatic ability, we first examined the *in vitro* and *in vivo* growth of each transfectant. The *in vitro* proliferation assay revealed no significant difference in the doubling time of the *fyn*-transfectants, ML-SN1, ML-SN2 clones or their parental cells, ML-01 or its mock transfectant or ML-MT-neo (data not shown). The *in vivo* growth assay of ML-SN2, ML-SN1 and ML-MT-neo cells in syngeneic BALB/c mice also showed no significant differences (Fig. 1). The results indicated that transfection of *fyn* did not affect the proliferation of the cells.
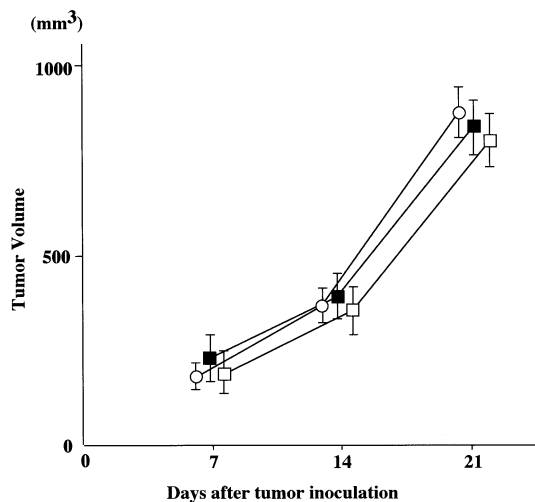

Fig. 1. *In vivo* growth of *fyn*-transfectants. Tumor cells ($1.0 \times 10^6 / 100 \ \mu$l) were inoculated into the right abdominal flank of BALB/c mice. At day14, tumor dimensions were measured and the tumor volumes were calculated by using the formula $0.5 \times ab^2$, where *a* is the larger and *b* the smaller of the two dimensions.[3] The results are the mean±SD (*n*=5). ∗ Statistically not significant compared with ML-MT-neo cells. □ ML-SN2, ■ ML-SN1, ○ ML-MT-neo.

Invasiveness of *fyn*-transfectants through reconstituted basement membrane We next examined the invasiveness of two *fyn*-transfectants, ML-SN1 and ML-SN2 clone, through an artificial basement membrane ("Matrigel"). The results, as shown in Fig. 2, disclosed that the invasiveness of both ML-SN1 and ML-SN2 through "Matrigel" was significantly higher than that of ML-MT-neo clone.

Secretion of gelatinase activity The gelatinase activity of tumor cells is well known to correlate with the invasiveness of tumor cells or metastatic ability.[13] Therefore, the conditioned media of transfectants were next analyzed for the activity of gelatinase. Gelatin zymography of Meth
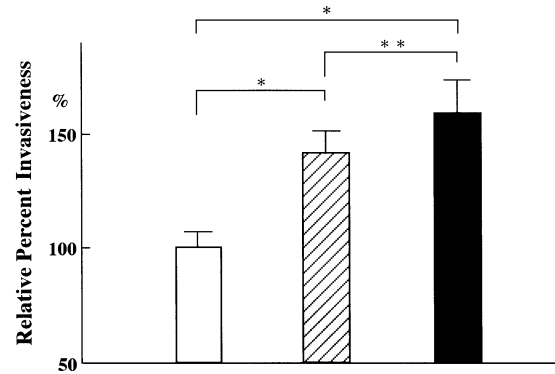

Fig. 2. Invasiveness of *fyn*-transfectants through reconstituted basement membrane. Transmembrane invasion assay was carried out as described in "Materials and Methods" using a "Transwell" double chamber precoated with "Matrigel" (100 μg/well). Values represent the means of triplicate experiments±SD. ∗ Statistically significant ($P<0.05$) differences compared with ML-MT-neo cells. □ ML-MT-neo, ▨ ML-SN1, ■ ML-SN2.
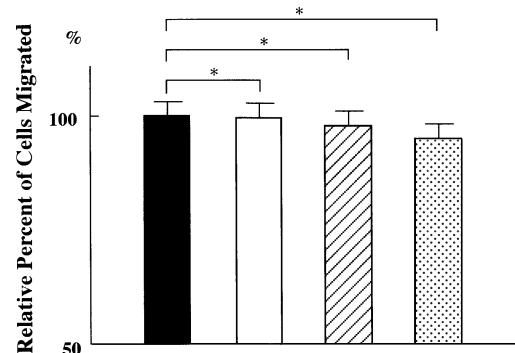

Fig. 3. Cell motility of *fyn*-transfectants. Cell motility of *fyn*-transfectants was assayed as described in "Materials and Methods" using a non-treated "Transwell" double chamber. Values represent the means of triplicate experiments±SD. ∗ Statistically not significant compared with ML-01 cells. ■ ML-01, □ ML-MT-neo, ▨ ML-SN1, ▦ ML-SN2.

A cells revealed two distinct bands with molecular weights of 92 kD and 64 kD, of which the former was believed to correspond to metallomatrixproteinase-9 (MMP-9) and the latter to MMP-2.[14] The intensity of the two bands of

ML-01, ML-MT-neo, ML-SN1 and ML-SN2 cells showed no essential difference. All the gelatinolytic bands were totally effaced by EDTA (data not shown). These results indicated that the gelatinases secreted from the *fyn*-transfectants can not account for the enhanced invasiveness of *fyn*-transfectants.

Cell motility assay Cell motility is also a factor which modulates the invasiveness of tumor cells.[15] Cell motility assays through micropore membranes were then performed using "Transwell" chambers. As shown in Fig. 3, no difference in migration was found between ML-01, ML-MT-neo, ML-SN1 and ML-SN2.

Attachment of *fyn*-transfectants to type IV collagen, laminin and fibronectin We then examined the adhesiveness of ML-SN1 and ML-SN2 cells to type IV collagen, laminin and fibronectin, which are major components of the extracellular matrix and vascular basement membrane. Both ML-SN1 and ML-SN2 cells showed higher rates of adherence to type IV collagen than did ML-MT-neo cells (Fig. 4). However, no difference in adhesiveness to laminin and fibronectin was found among these cells (Fig. 4). We also examined the adhesiveness of the three clones to calf pulmonary endothelial cells (CPAE) (American Culture Tissue Collection, Rockville, MO). However, there was no difference between the three clones (data not shown).
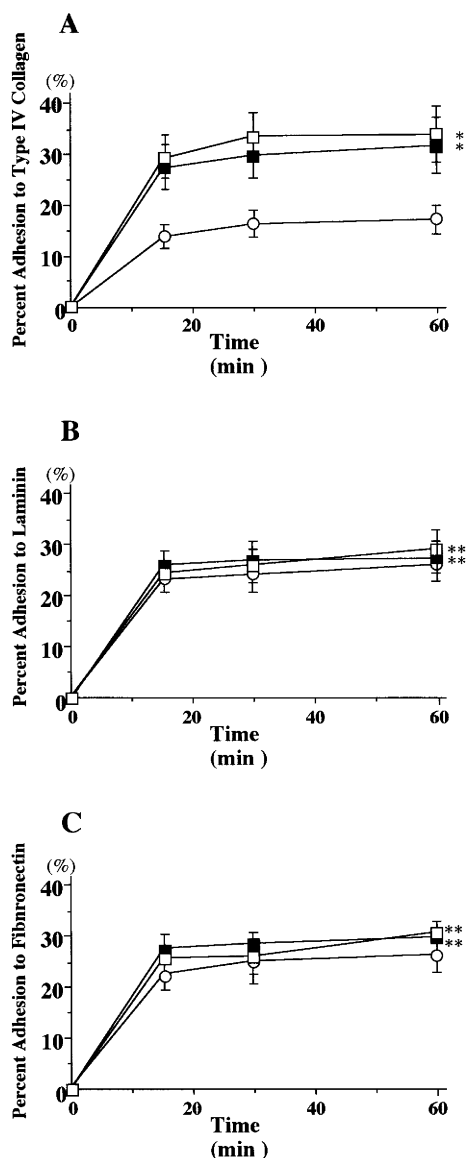

Fig. 4. Attachment of radiolabeled *fyn*-transfectants to type IV collagen, laminin and fibronectin. Tumor cell adhesiveness to type IV collagen (A), laminin (B) and fibronectin (C) were assayed using plates coated with type IV collagen or laminin or fibronectin and ^{125}I-IUDR-labeled cells. Radioactivity of adherent cells was counted in an autogamma counter. Values represent the means of triplicate experiments±SD. ∗ Statistically significant (*P*<0.01) differences compared with ML-MT-neo cells. ∗∗ Statistically not significant compared with ML-MT-neo cells. □ ML-SN2, ■ ML-SN1, ○ ML-MT-neo.
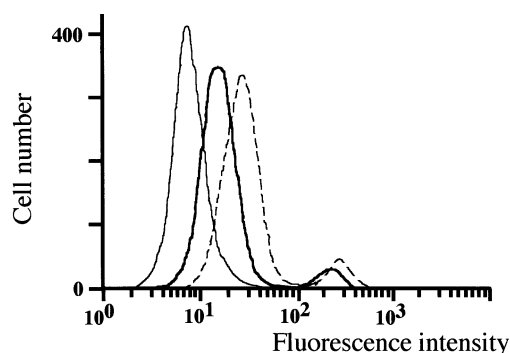

Fig. 5. Expression of β1 integrin subunit on *fyn*-transfected cells as analyzed by flow cytometry. Cells were washed with growth medium and incubated in fresh growth medium for 30 min at 37°C in 5% CO_2. Subsequently, cells were washed three times in ice-cold PBS (pH 7.2) supplemented with BSA (0.1%, w/v) and, for fluocytometric analysis, NaN_3 (0.1%, w/v). Cells (5×10^5 in 100 μl volumes for analysis) were incubated for 30 min on ice with primary antibody (final dilutions: 1:1, hybridoma supernatants; 1:100, ascites). After two washes, FITC-conjugated rabbit-anti-mouse IgG (RAM-FITC; DAKO) was added to the resuspended cells (1:40 final dilution) for 30 min on ice, and the cells were then washed 3 more times. Analysis was performed by fluorocytometry on a FACSCAN analyzer (Becton Dickinson) with Consort 30 software. Background fluorescence was determined by omitting the primary antibody from the treatment of the negative control sample. — ML-MT-neo, — ML-SN1, ······· ML-SN2.

Table I. Effect of Anti β1-integrin Antibody on Attachment to Type IV Collagen of *Fyn*-transfectants

Clone	Percent of cells attached			
	β1-integrin antibody (final concentration; μg/ml)			
	0	2	10	50
ML-MT-neo	15.2±1.1	12.9±0.9	8.7±1.8	8.4±0.6 (44.7)[a]
ML-SN2	31.4±1.4	25.5±1.6	19.8±1.8	18.4±1.0 (40.8)[a]

a) % reduction rate.
The assay for the blocking effect of anti β1-integrin antibody on cell attachment was performed by the same method as that of tumor cell adhesiveness to type IV collagen, except that cells were pre-treated with anti β1-integrin antibody for 30 min before the cells were added to the wells of culture plates.
Values represent the means of triplicated experiments±SE.

From the above results, we concluded that enhanced attachment to type IV collagen is one of the best candidate factors contributing to the augmented invasiveness of *fyn*-transfectants.

Effect of anti β1-integrin antibody on the cell attachment of *fyn*-transfectants It is well known that the β1 subunit of integrin, in combination with various types of α subunit, plays a major role in tumor cell attachment to basement membrane, which mainly consists of type IV collagen.[16] Therefore, we examined the possibility that the elevated attachment of *fyn*-transfectants to type IV collagen was due to the increment of β1-integrin expression on their cell membranes. FACS analysis demonstrated a higher expression level of β1-integrin subunits in *fyn*-transfectant cells than that of ML-MT-neo (Fig. 5).

By treatment with anti β1-integrin Ab, the percentage of both ML-SN2 and ML-MT-neo cells which attached to type IV collagen was inhibited dose-dependently, and the rate of the inhibition for each cell type was almost the same (Table I).

There were, however, appreciable amounts of both ML-MT-neo and ML-SN2 cells remaining undetached even after antibody treatment. In particular, a significantly higher proportion of ML-SN2 cells remained on the collagen plate after antibody treatment than in the case of ML-MT-neo cells, suggesting the presence of β1-integrin-unrelated type IV collagen-binding molecules on the cell membranes.

Expression of type IV collagen-binding proteins in *fyn*-transfectants We then examined whether the *fyn*-transfectants express increased type IV collagen-binding proteins other than β1-integrin. Membrane proteins bound to type IV collagen were extracted according to the method described in "Materials and Methods" and were visualized by autoradiography as several bands (Fig. 6). Among the
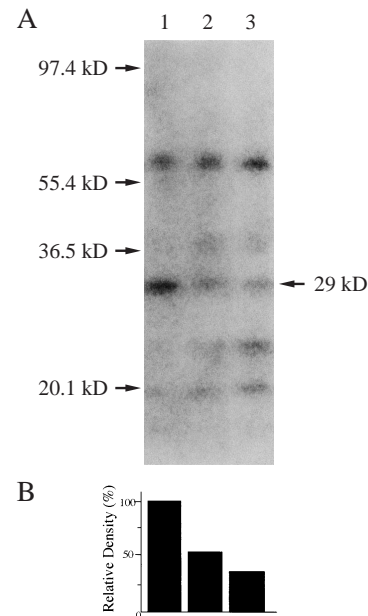

Fig. 6. Expression of type IV collagen-binding proteins in *fyn*-transfectants. (A) Autoradiography of the SDS-PAGE analysis of ^{125}I-labeled *fyn*-transfectants and the parent clone is shown. Cell membrane proteins were radioiodinated as described in "Materials and Methods." Two hundred microliters of solubilized ^{125}I-labeled membrane proteins (containing equivalent amounts of radioactivity) was incubated for 2 h at 4°C with 50 μl of type IV collagen-coupled Sepharose 4B beads in NET buffer. Then the beads were washed and boiled for 5 min with an equal volume of 2× SDS sample buffer and centrifuged briefly. The supernatant was analyzed on an SDS-polyacrylamide gradient gel (4–20%). The gel was fixed, dried and subjected to autoradiography. (B) The closed bar graphs show the relative 29 kD protein level as determined by densitometric analysis when the level of ML-SN2 was taken as 100%. Lane 1, ML-SN2; lane 2, ML-MT-neo; lane 3, ML-01.

Table II. Effect of Genistein on Membrane PTK Activity of *Fyn*-transfectants

Clone	PTK activity (pmol/mg protein)			
	Genistein conc. (μg/ml)			
	0	3	5	25
ML-MT-neo	19.8±4.1	13.5±4.8	11.9±6.9	9.7±4.5
(% suppression)	(100±20.7)	(88.8±24.2)[a]	(60.1±34.8)[a]	(48.9±22.7)[a]
ML-SN2	134.5±5.3	74.9±5.9	33.8±5.2	18.3±4.9
(% suppression)	(100±3.9)	(55.7±4.3)[b]	(25.1±3.9)[b]	(13.6±3.6)[c]

Total membrane-bound tyrosine kinase activity of *fyn*-transfected cells was treated for 3 h in RPMI 1640 with different concentrations of genistein. Then the total membrane-bound PTK activity was assayed as described in the "Materials and Methods" section.
Values represent the means of triplicate experiments±SE.
a) Inhibition was statistically significant with $P<0.01$.
b) Inhibition was statistically significant with $P<0.005$.
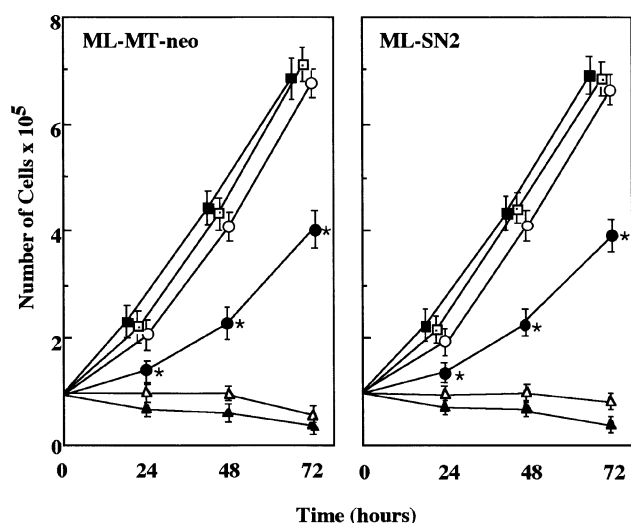*c*) Inhibition was statistically significant with $P<0.002$.

Fig. 7.   Effect of genistein treatment on cell growth. The effect of genistein on cell growth was assayed. Cells were grown in the presence of DMSO or in the presence of different concentrations of genistein (1.0–50 μg/ml). At the indicated time, the number of cells was counted. Values represent the means of triplicate experiments±SD. ∗ Statistically significant ($P<0.01$) differences compared with control values. Genistein conc. (μg/ml): □ 0, ■ 1.0, ○ 5.0, ● 10.0, △ 20.0, ▲ 50.0.

Fig. 8.   Suppression of attachment to type IV collagen by genistein. The ^{125}I-IUDR-labeled cells were treated for 3 h in RPMI 1640 with different concentrations of genistein at 37°C in 5% CO_2. After the genistein treatment, the cells were washed with RPMI 1640 three times and assayed for attachment to type IV collagen as described. ∗ Statistically significant ($P<0.01$) differences compared with control values. ∗∗ Not significant. □ control, ▨ 3.0 μg/ml genistein, ■ 5.0 μg/ml genistein.

bands, the one with a molecular weight of 29 kD was clearly more intense in ML-SN2 cells than in ML-MT-neo. The same band was seen on the ML-01 lane, which corresponded to the parent clone of the two transfectants.[3] There was no difference in the intensity of the 29 kD band between the ML-MT-neo and ML-01 lanes. This result indicated that the *fyn* product in Meth A cells could augment the expression of the type IV collagen-binding protein p29.
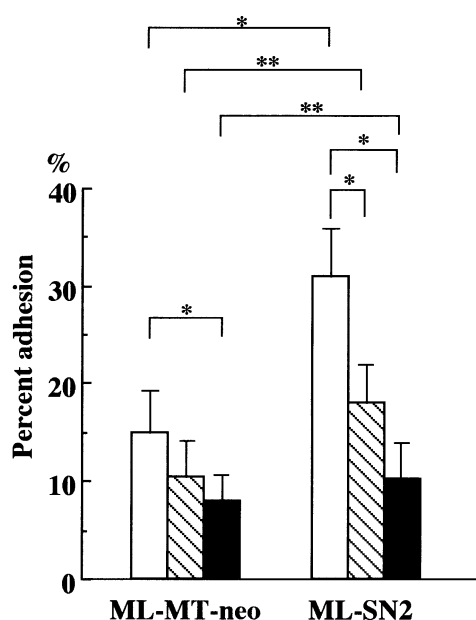
Effect of genistein on attachment of *fyn*-transfectants to type IV collagen   We next employed genistein, a PTK inhibitor,[11, 17, 18] to examine whether the increased adhesiveness of the *fyn*-transfectants to type IV collagen was related to the PTK activity of the *fyn* product. The PTK
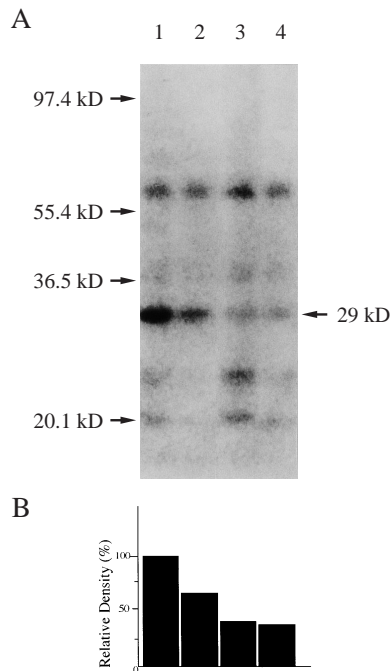
A

B

Fig. 9. Suppressed expression of type IV collagen-binding proteins of *fyn*-transfectants by genistein. (A) Cells (1×10^7/ml) were treated with genistein at concentrations of 3.0 μg/ml or 5.0 μg/ml for 3 h. Cells were rinsed three times with warm RPMI 1640 and examined for the expression of the type IV collagen-binding protein as described in Fig. 6. (B) The closed bar graphs show the relative 29 kD protein level as determined by densitometric analysis, when the level of ML-SN2 treated with genistein 0 μg/ml was taken as 100%. Lane 1, ML-SN2 treated with genistein 0 μg/ml; lane 2, ML-SN2 treated with genistein 3.0 μg/ml; lane 3, ML-SN2 treated with genistein 5.0 μg/ml; lane 4, ML-01.

Table III. Effect of Genistein on Experimental Metastasis of *Fyn*-transfectants

Cells	Genistein conc. (μg/ml)	Average number of pulmonary metastatic nodules[a]	Metastatic incidence
ML-01	0	42.4±5.9 ⎤ b)	6/6
	5.0	40.3±5.7 ⎦	6/6
ML-MT-neo	0	45.7±6.7 ⎤ b)	6/6
	5.0	41.3±6.2 ⎦	6/6
ML-SN2	0	103.8±13.5 ⎤ c)	6/6
	5.0	64.6±11.3 ⎦	6/6

a) Tumor cells were treated without or with genistein at concentration of 5.0 μg/ml for 3 h. Cells were rinsed in RPMI 1640 medium and pipetted gently to dissociate all cell clumps. Cells were injected into the tail vein of BALB/c mice at 8.0×10^4/0.1 ml PBS/mouse. On day 14, the mice were killed and metastatic nodules on the surface of the lungs were counted. Data represent the means±SE.
b) Not significant.
c) Statistically significant ($P<0.01$) differences.

activity of ML-SN2 cells was significantly higher than that of ML-MT-neo (Table II). When the effect of genistein on PTK activity was assayed, genistein at concentrations between 3 μg/ml and 25 μg/ml significantly reduced the elevated PTK activity of ML-SN2 in a dose-dependent manner. Treatment of ML-01 cells or ML-MT-neo with genistein, on the other hand, resulted in a lesser degree of suppression of PTK activity (Table II). We also confirmed that a genistein concentration of less than 5 μg/ml did not influence the viability of Meth A cells *in vitro* (Fig. 7). Therefore, we used a concentration of less than 5 μg/ml for the cell attachment experiment. The attachment assay revealed that the treatment of ML-MT-neo cells and ML-SN2 cells with genistein (3.0, 5.0 μg/ml) reduced their adhesiveness to type IV collagen in a dose-dependent manner (Fig. 8). Unlike the effect of anti-β1 antibody, suppression of adhesiveness by genistein on ML-SN2 was such that the adhesion percentage of ML-SN2 cells treated with 5.0 μg/ml of genistein was almost the same as that of ML-MT-neo cells treated with 5.0 μg/ml genistein. These results supported the notion that elevated PTK activity due to fyn over-expression enhances the cell attachment to type IV collagen.

Effect of genistein on expression of type IV collagen-binding proteins of *fyn*-transfectants We then investigated whether the elevated PTK activity of the *fyn*-transfectants was in fact related to the augmented expression of the type IV collagen-binding protein at the cell membrane. The treatment of ML-SN2 cells with genistein at concentrations of 3.0 μg/ml and 5.0 μg/ml significantly reduced the expression of the type IV collagen-binding protein (Fig. 9). The *fyn* product was thus considered to augment the expression of the type IV collagen-binding protein by increasing the PTK activity.

Effect of genistein on experimental pulmonary metastasis of *fyn*-transfectants We therefore attempted to investigate whether the reduction of PTK activity can lead the suppression of metastasis. Tumor cells were injected intravenously after pretreatment with genistein (5.0 μg/ml) for 3 h, and the numbers of pulmonary metastatic nodules were counted after 14 days. As shown in Table III, ML-SN2 cells pretreated with genistein formed significantly fewer pulmonary nodules than untreated ML-SN2 cells. Treatment of ML-01 cells or ML-MT-neo with genistein, on the other hand, did not result in decreased metastatic ability.

DISCUSSION

In the present investigation, we proved that transfection of a fyn expression vector into ML-01, a low-metastatic clone of Meth A, increased the adhesiveness to type IV collagen. Experiments using anti-β1 antibody disclosed that not only *fyn*-transfectants, but also neogene transfec-

tants showed some adhesiveness to type IV collagen through β1 integrin-collagen interaction (Table I). However, some portion of the adhesiveness was unrelated to this interaction in both transfectants. The integrin-unrelated adhesion was more prominent in *fyn*-transfectant, indicating induction of a non-integrin collagen-adhesive molecule by *fyn* transfection. Since we have previously observed that the 29 kD membrane protein is highly expressed in MH-02, a high-metastatic clone of Meth A cells, we then examined the expression of the 29 kD type IV collagen-binding protein and found that the *fyn*-transfectants exhibited elevated expressions of this particular protein. Both adhesiveness of the *fyn*-transfectants to type IV collagen and expression of the 29 kD type IV collagen binding protein were lowered by genistein treatment to the levels of adhesiveness and expression of 29 kD protein of control cells. We therefore deduced that fyn enhances the adhesiveness and metastatic ability of Meth A cells by increasing the expression of the type IV collagen-binding protein through tyrosine kinase activity. Thus, we were able to demonstrate a linked sequence of events in Meth A cells beginning with the augmented expression of fyn, leading to activation of tyrosine kinase enhanced expression of 29 kD protein, increased attachment to type IV collagen and an enhanced metastastic property.

Fyn was originally identified by Semba *et al.*[19] and Kawakami *et al.*[20] as a gene which is homologous to fgr and yes. The *fyn* gene, whose expression is high in the brain and the thymus,[21] encodes an src-like 59 kD protein which is associated with the internal portion of the plasma membrane. Little is known about the exact function of fyn, although as fyn physically associates with the CD3 complex in T cells, it has been suggested that fyn plays crucial roles in T-cell receptor-mediated signal transduction.[22, 23]

Recently, the stable association of phosphorylated p59[fyn] and the focal adhesion-associated protein tyrosine kinase, pp125[FAK], in chicken embryo cells was reported.[24, 25] Fyn and fak have also been reported to cooperate in CD4+CD8+ T-cell development in a unique fashion.[26] Because FAK colocalizes with integrins in cellular focal adhesions, it may be reasonable to speculate that there is a close relation between fyn and adhesion molecules like p29. Further investigation of the mechanism by which fyn augments the expression of p29 is needed.

Several molecules have been reported as type IV collagen binding proteins, however, ours, with a molecular weight of 29 kD, is the smallest ever reported.[7, 27, 28] The molecules mediating cell-cell or cell-extracellular matrix interactions are so far classified into the cadherin,[29] integrin,[16, 30] immunoglobulin[31] or selectin superfamilies.[32] Some adhesion molecules, such as fibronectin and laminin, are known to utilize specific amino acid sequences as binding sites.[2, 33, 34] Therefore, it would be intriguing to identify the superfamily to which our 29 kD protein belongs, and what amino acid sequence the protein uses as a binding site. We are now working on further characterization of 29 kD.

In conclusion, our results indicated that the increased expression of fyn endowed tumor cells (Meth A cells) with enhanced metastatic potential by increasing the expression of an adhesion molecule 29 kD through PTK activity. Further investigation into the relationship between fyn and tumor cell adhesion may lead to the development of new antimetastatic agents including specific inhibitors to 29 kD protein or to PTK.

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