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Augmented Expression of a Type IV Collagen-binding Protein in a Highly Metastatic Murine Fibrosarcoma Clone

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The adhesive properties of highly and weakly metastatic murine sarcoma (Meth A) clones were investigated. A highly metastatic clone, MH-02, preferentially adhered to type IV collagen-coated plastic dishes and to bovine pulmonary arterial endothelial cell-coated plastic dishes as compared to a weakly metastatic clone, ML-01. Pretreatment of MH-02 and ML-01 cells with antisera against MH-02 cells resulted in almost equivalent adhesiveness to type IV collagen. Preincubation of ^{125}I-radiolabeled tumor cells with the antisera against MH-02 significantly reduced the arrest of MH-02 cells in the lung, but ML-01 cells were not affected. The number of pulmonary metastatic nodules of MH-02 cells was reduced to the same level as that of ML-01 cells by preincubation of the tumor cells with the antisera in an experimental metastasis experiment. These results indicated that the high metastatic ability of MH-02 can be attributed to its preferential adhesiveness to type IV collagen. The type IV collagen-binding proteins of MH-02 and ML-01 were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography. Among several proteins which bound to type IV collagen, expression of a protein with a molecular weight of 29 kD was significantly greater in MH-02 than in ML-01. These results suggest that the greater adhesion of highly metastatic MH-02 cells to type IV collagen is due to enhanced expression of the type IV collagen-binding 29 kD protein.

Key words: Meth A sarcoma — Highly metastatic clone — Type IV collagen-binding protein

The blood-borne metastasis of cancer involves various processes such as release of cancer cells from the primary lesion, intravasation, dissemination via vessels, lodging at a distant organ, extravasation into surrounding tissue and proliferation.[1-4] During these processes, tumor cells come into contact with various host cells and extracellular matrices. When tumor cells are circulating in blood vessels, they contact lymphocytes,[5] macrophages,[6] platelets[7,8] and other blood components.[9,10]

Once tumor cells lodge at the target organ, they come into contact with endothelial cells[3,11,12] and subsequently with basement membranes.[13-16] According to previous reports, certain highly metastatic cells exhibit increased adhesiveness to endothelial cells and basement membrane components such as laminin,[17,18] fibronectin,[17,19] and type IV collagen.[20,21] However, there have been only a few analyses of the increased adhesiveness at the molecular level.[22-24] We have established several clones from murine methylcholanthrene-induced fibrosarcoma A (Meth A) which show different metastatic potentials in experimental models.[25,26] Our previous studies revealed that metastatic potentials were closely related to platelet aggregating activities, since the lodging of the tumor cells in the pulmonary capillary was facilitated by platelets.[26] However, the difference in metastatic potential of these clones could not be attributed solely to the difference in platelet aggregating activity, because the administration of prostacyclin, a potent antiplatelet agent, did not completely neutralize the metastatic diversity.[26] These facts led us to conclude that certain factors other than platelet aggregating activity must be involved in determining the metastatic capabilities of these clones.

In this report, we compare the adhesive properties of highly and weakly metastatic clones of Meth A to endothelial cells and to the components of basement membranes. The results indicated that the highly metastatic clone exhibits higher adhesiveness to both endothelial cells and type IV collagen, the main components of basement membranes, and that a type IV collagen-binding membrane protein with a molecular weight of 29 kD is expressed in greater amounts in the highly metastatic clone than in the weakly metastatic clone.

MATERIALS AND METHODS

Materials Fetal bovine serum (FBS) was obtained from Flow Laboratories Inc.; pepsin (3200 U/mg) was from Sigma; ^{125}I-deoxyuridine was from Amersham. DEAE-Sephacel, CNBr-activated Sepharose 4B and Sephadex G75 were from Pharmacia and type IV collagen (Cell-

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matrix type IV, 3 mg/ml, purity: 95%) was obtained from Nitta Gelatin Inc., Tokyo.

Cell culture A highly metastatic clone (MH-02) and a weakly metastatic clone (ML-01), established from 3-methylcholanthrene-induced fibrosarcoma (Meth-A) in BALB/c mice[26] were cultured in RPMI 1640 media (GIBCO) containing 10% FBS, 100 units/ml penicillin G and 100 mg/ml streptomycin using culture flasks (Costar #3050) in humidified 5% CO_2 at 37°C.

Bovine pulmonary arterial endothelial cells (CPAE cells) were from ATCC (American Type Culture Collection, USA). The cells were cultured in RPMI1640 medium containing 20% fetal bovine serum in humidified 5% CO_2 at 37°C. The cells were maintained in monolayer culture and subcultured every 3 days.

Preparation of anti MH-02 antibody and $F(ab')_2$ Antisera to MH-02 cells were raised in male rabbits by injecting the cells (5×10^7 cells) into the auricular vein a total of 10 times (every two days), and a booster immunization was given 2 weeks after the last immunization.

Blood samples were drawn from the rabbit 14 days after the booster immunization. Antisera were obtained from the blood, made up to 40% saturation with ammonium sulfate, dialyzed against 0.0175 *M* sodium phosphate buffer (pH 6.3) and applied to a DEAE-Sephacel column (0.5×25 cm) which had been equilibrated in the same buffer. A part of the pass-through fraction (IgG fraction) from the column was concentrated in a CENTRICON30 (Amicon, USA) to a protein concentration of 15 mg/ml. The concentrated IgG fraction was used as anti-MH-02 antibody. The rest of the pass-through fraction was dialyzed against 0.5 *M* sodium acetate buffer (pH 4.5) and incubated with 0.5 mg/ml pepsin (Sigma) at 37°C for 24 h. After dialysis against PBS, the pepsin-digested IgG preparation was fractionated on Sephadex G75 columns (1.5×50 cm) to obtain pure $F(ab')_2$ fragments, which were eluted in the void volume. The purity of $F(ab')_2$ was greater than 80% as determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) with Coomassie blue staining. Then, 1 ml of the $F(ab')_2$ fragment (15 mg/ml) and 0.1 ml of anti MH-02 antibody were incubated with 1×10^7 and 1×10^6 ML-01 cells, respectively, for 30 min at 4°C, and centrifuged at 2,100*g* for 10 min to recover the supernatant. The procedure was repeated four times and the supernatants were used for subsequent experiments as anti-MH-02 $F(ab')_2$ and anti MH-02 antibody preabsorbed with ML-01 cells respectively. Preimmune $F(ab')_2$ was made by the same procedure from preimmune rabbit serum.

Metabolic radiolabeling of tumor cells Tumor cells were metabolically labeled with ^{125}I-deoxyuridine as described by Fidler.[27] Briefly, 1×10^5/ml of MH-02 and ML-01 cells were cultured with 0.1 μCi/ml ^{125}I-deoxyuridine in RPMI 1640 medium containing 10% FBS for 24 h. Cells were then washed three times with RPMI 1640 medium and resuspended in the same medium before they were used for the *in vitro* or *in vivo* experiments described in this paper.

Measurements of tumor cell adhesiveness to endothelial cells and basement membrane components Tumor cell adhesiveness to endothelial cells and type IV collagen, fibronectin and laminin were assayed by the methods of Tao and Johnson with slight modifications.[18]

Type IV collagen, fibronectin, and laminin-coated plates (Costar #3512) were prepared by drying protein solution in water (0.3 mg/ml) at 37°C. Completely confluent CPAE cells in the same culture plates were washed three times with warm RPMI medium. One ml of ^{125}I-deoxyuridine-labeled MH-02 or ML-01 cells (1×10^5/ml) was added to each well of the protein-coated tissue culture plates or CPAE cells-coated culture plates. The culture plates were centrifuged at 200*g* for 5 min in a plate centrifuge machine (Model CD-60R, Tomy Seiko Co., Tokyo) to allow tumor cells to attach to the bottom of the plates. The plates were then shaken for 10 s at 100 rpm by rotary shaker TAIYO R-II (Taiyo Scientific Co., Tokyo) and non-adherent cells were removed by gentle aspiration. Adherent cells were gently washed three times with RPMI 1640 medium and solubilized in 1 ml of 1 *N* NaOH to measure the radioactivity in an autogamma counter.

Measurement of tumor cells arrested in the lung The experimental procedures to measure the tumor cell arrest in the lung was essentially based on the method of Fidler.[27] Briefly, ^{125}I-deoxyuridine-labeled MH-02 and ML-01 cells were preincubated with anti-MH-02 $F(ab')_2$ or preimmune $F(ab')_2$ (1.0 mg/ml) at 37°C for 30 min. Then cells were washed twice and pipetted gently to dissociate all cell clumps. Each BALB/c mouse was given an injection of the treated tumor cells (1×10^5 cells/0.1 ml RPMI 1640 medium) via the tail vein and killed at a selected time point. The lungs were immediately removed, fixed in 70% ethanol, and rinsed three times every 12 h in the same solution. Radioactivity in each organ was measured with an autogamma counter. Tumor cell arrest in the lungs was assessed in terms of the percentage of radioactivity compared to the total radioactivity of injected ^{125}I-deoxyuridine-labeled cells.

Experimental pulmonary metastasis Experimental pulmonary metastasis was induced as described earlier.[25, 26] BALB/c mice were injected with 1×10^4 MH-02 or ML-01 cells suspended in 0.1 ml of RPMI 1640 medium via the tail vein and were killed 14 days after injection. The numbers of metastatic nodules on the surface of the lungs were determined under a dissecting microscope.

Preparation of ^{125}I-labeled membrane protein of tumor cells Cell membrane proteins were radioiodinated by an

immobilized lactoperoxidase-glucose oxidase method according to the manufacturer's directions (Enzymobeads™, Bio Rad). MH-02 or ML-01 cells (2×10^7) suspended in 1 ml of PBS were treated with 200 μl of 5 mg/ml D-glucose. The suspension was incubated for 30 min at room temperature with constant shaking, then the enzymatic reaction was terminated by washing the cells five times with PBI buffer (137 m*M* NaI, 2.68 m*M* KCl, 8.32 m*M* $Na_2HPO_412H_2O$, 1.47 m*M* KH_2PO_4, pH 7.4) containing 2 m*M* phenylmethylsulfonyl fluoride (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 m*M* Tris (pH 8.0), 2 m*M* EDTA-Na_2, 1 m*M* PMSF, 1 mg/liter leupeptin (Boehringer Mannheim), 1.0 mg/liter pepstatin (Boehringer Mannheim)], and sonicated by a sonicator Model W225R (Waken Yakuhin K.K., Tokyo) at 20 W for 3 min on ice. Cell debris was pelleted by centrifugation for 30 min at 100,000*g* 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 (Pharmacia) beads according to the manufacturer's directions. Two hundred μl of solubilized ^{125}I-labeled membrane proteins (containing equivalent amounts of radioactivity) from MH-02 and ML-01 cells were 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 m*M* Tris (pH 8.0), 2 m*M* EDTA-Na_2, 1 m*M* PMSF]. Then the beads were washed twice in the NET buffer and once with washing buffer [100 m*M* Tris (pH 6.8)]. The beads were boiled for 5 min with equal volume of 2×SDS sample buffer, and centrifuged briefly. The supernatant was analyzed by SDS-PAGE in 12% gel. The gel was fixed, dried and subjected to autoradiography.

Immunoprecipitation of radiolabeled membrane proteins by anti MH-02 antibody Two hundred μl of solubilized ^{125}I-labeled membrane proteins from MH-02 or ML-01 cells was treated with 5 μl of anti MH-02 antibody, and immunoprecipitated for 2 h at 4°C. Immunoprecipitates were collected by absorption on 20 μl of protein A-Sepharose (Pharmacia) for 2 h at 4°C, and washed three times in TBS (10 m*M* Tris-HCl, pH 8.0, 150 m*M* NaCl) containing 0.1% NP-40. Immunoprecipitates were analyzed by 12% SDS-PAGE and autoradiography.

RESULTS

Adhesiveness of MH-02 and ML-01 to type IV collagen, laminin, fibronectin and endothelial cells Differences between highly metastatic cells (MH-02) and weakly metastatic cells (ML-01) in adhesiveness to vascular wall components were examined. Fig. 1 shows the kinetics of adherence of these tumor cells to laminin, fibronectin, type IV collagen, and endothelial cell-coated plastic dishes. MH-02 cells showed significantly higher rates of adherence to endothelial cells and type IV collagen than ML-01 cells. The difference of adhesion was most apparent against type IV collagen. On the other hand, there was no difference in adhesiveness of MH-02 and ML-01 to laminin or fibronectin. From these results, it was suggested that the high metastatic ability of MH-02 cells may be attributed to the preferential adhesiveness to type IV collagen or endothelial cells. The adhesiveness to type IV collagen of the two clones was therefore investigated further.

Effect of anti MH-02 F(ab′)$_2$ on tumor cell adhesion to type IV collagen In order to characterize the difference of cell surface properties of MH-02 and ML-01 cells, we raised anti-MH-02 antibodies in a rabbit. The antibody might block surface molecules on MH-02 cells which are relevant to the adhesiveness of MH-02 cells. MH-02 and ML-01 cells were pretreated with the anti-MH-02 F(ab′)$_2$ or preimmune F(ab′)$_2$ and their adhesiveness to type IV collagen was examined. As shown in Fig. 2, adhesiveness of MH-02 cells to type IV collagen was suppressed to the same level as that of ML-01 cells by the anti-MH-02 F(ab′)$_2$ treatment. The same treatment of ML-01 cells did not affect their adhesiveness to type IV collagen. This result suggests that MH-02 cells possess cell surface molecules which promote tumor cell attachment to type IV collagen, and whose function is inhibited by anti-MH-02 F(ab′)$_2$ treatment.

Effect of anti MH-02 F(ab′)$_2$ on the arrest of tumor cells in the lung and spleen ^{125}I-Deoxyuridine-labeled tumor cells that were pretreated with the F(ab′)$_2$ were injected intravenously into mice and the radioactivity in the lung and spleen were measured at intervals. As shown in Fig. 3, MH-02 cells exhibited higher arresting ratios in the lung than ML-01 cells even after treatment with the preimmune F(ab′)$_2$ throughout the experimental time period. At 5 min and 10 min after injection of the tumor cells, preimmune F(ab′)$_2$-treated MH-02 cells showed significantly higher arresting ratio than preimmune F(ab′)$_2$-treated ML-01 cells. This result suggested that the highly metastatic MH-02 cells adhere to pulmonary vasculature more quickly than ML-01 cells, and the arrest ratios of the two clones in the lung were not affected by the preimmune F(ab′)$_2$ treatment. On the other hand, anti-MH-02 F(ab′)$_2$ treatment dramatically decreased the adhesion of MH-02 cells to the same level as that of ML-01 cells treated in the same manner. These results demonstrate an inhibiting effect of the anti MH-02 F(ab′)$_2$ on lodging of the highly metastatic clone in the lung capillaries. They also indicate that higher adhesiveness of the MH-02 cells can be attributed to the cell surface components which are blocked with the anti MH-02 F(ab′)$_2$. The arrest of MH-02 cells and ML-01 cells in

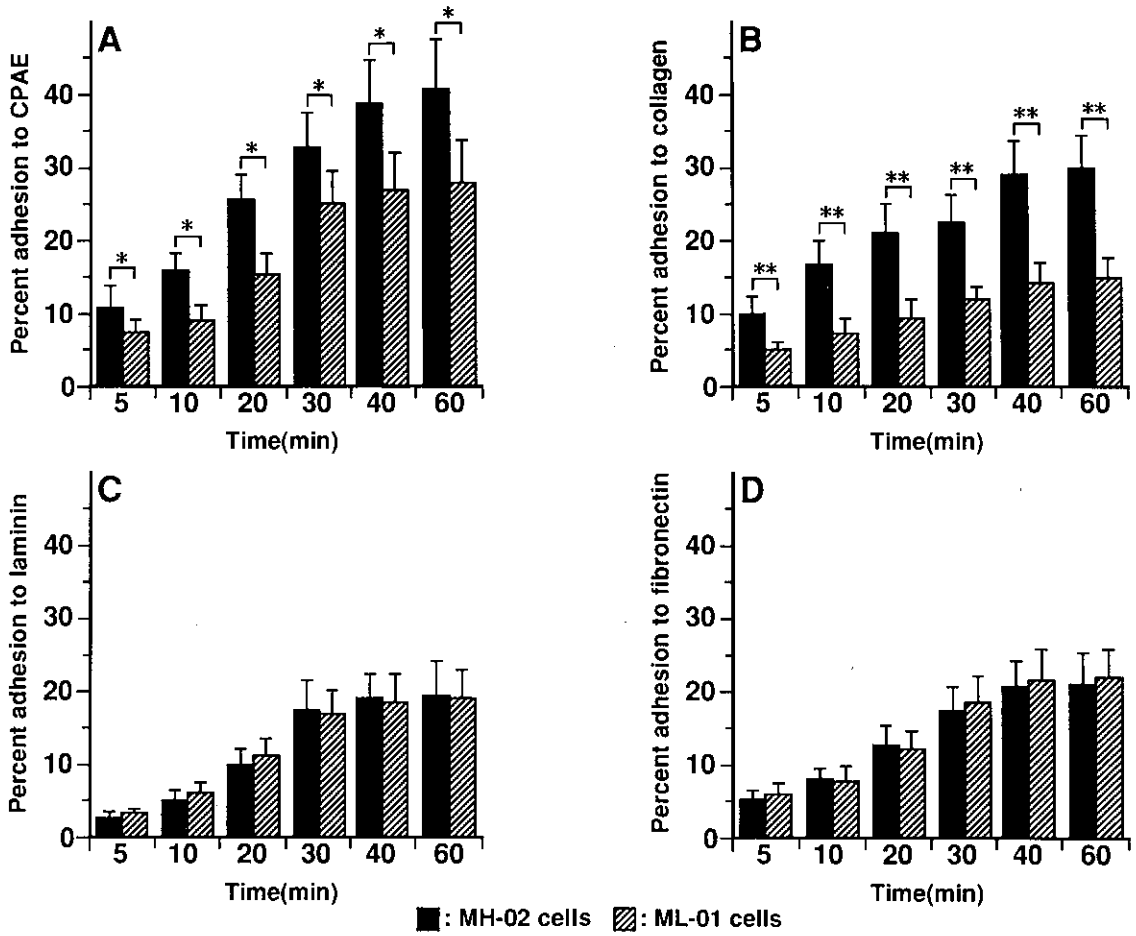

Fig. 1. Attachment of radiolabeled MH-02 and ML-01 cells to laminin, fibronectin, type IV collagen and CPAE cells *in vitro*. Cells were radiolabeled with ^{125}I-deoxyuridine, washed three times with RPMI 1640 medium and resuspended in the same medium to give a final concentration of 1.0×10^5 cells/ml. Cells (1.0×10^5) were then aliquoted into the wells of plastic dishes coated with CPAE (A), type IV collagen (B), laminin (C) or fibronectin (D), and settled to the bottom of the dishes by centrifugation (200*g*, 5 min). After an incubation period sufficient to allow the cells to adhere to the dishes, the dishes were agitated using a rotary shaker (100 rpm) and washed three times with RPMI 1640 medium in order to remove the non-adherent cells. Adhered cells were lysed with 1 *N* NaOH and their radioactivities were counted. The percent of cells attached was calculated from the ratio of the counts of adherent cells to the total cells in a well. All data represent the means of at least three experiments ± SEM. *: $P<0.05$ by Wilcoxon's test. ** $P<0.025$ by Wilcoxon's test.

the spleen under the same conditions did not show any significant difference, suggesting that the $F(ab')_2$ treatment did not affect the trapping of tumor cells by the spleen (data not shown). Treatment of MH-02 and ML-01 cells with the anti MH-02 $F(ab')_2$ affected neither the platelet aggregating activities nor the growth rates of those clones (data not shown).

Effect of anti MH-02 $F(ab')_2$ on experimental pulmonary metastasis by MH-02 and ML-01 cells The ability of anti-MH-02 $F(ab')_2$ to decrease pulmonary metastases *in vivo* was also examined. Tumor cells were injected intravenously after pretreatment with anti MH-02 $F(ab')_2$ or preimmune $F(ab')_2$ and the numbers of pulmonary metastatic nodules were counted after 14 days. MH-02 cells pretreated with anti MH-02 $F(ab')_2$ gave significantly fewer pulmonary nodules than preimmune $F(ab')_2$-treated cells (Table I). Treatment of ML-01 cells with the anti-MH-02 $F(ab')_2$, on the other hand, did not decrease their metastatic ability. These data indicate that anti-MH-02 antibody has the capacity to decrease not only tumor cell adhesion to type IV collagen but also metastatic nodule formation.

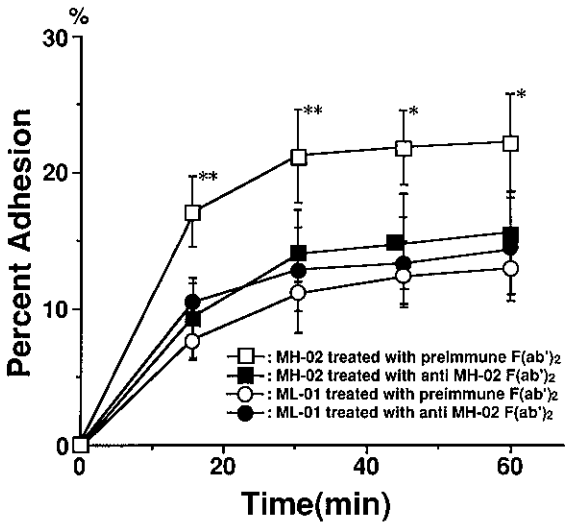

Fig. 2. Effects of anti-MH-02 $F(ab')_2$ on attachment of MH-02 and ML-01 cells to type IV collagen. Cells were radiolabeled with ^{125}I-deoxyuridine, and washed with RPMI 1640 medium three times. Then the cells were treated with either preimmune $F(ab')_2$ or anti-MH-02 $F(ab')_2$ (1.0 mg/ml) for 30 min. Cells were again washed three times with RPMI 1640 medium and resuspended in the medium to give a final concentration of 1.0×10^5/ml. Cells (1.0×10^5) were aliquoted into the wells of type IV collagen-coated plastic plates, then settled to the bottom of the dishes by centrifugation (200*g*, 5 min). The cells were allowed to adhere to type IV collagen-coated plates for 15, 30, 45 or 60 min, then the dishes were agitated by a rotary shaker (100 rpm) and washed three times with RPMI 1640 medium to remove the non-adherent cells. Adhered cells were lysed with 1 *N* NaOH and their radioactivities were counted. The percent of cells attached was calculated from the ratio of the counts of adherent cells to the total cells in a well. All data represent the means of at least three experiments±SEM.

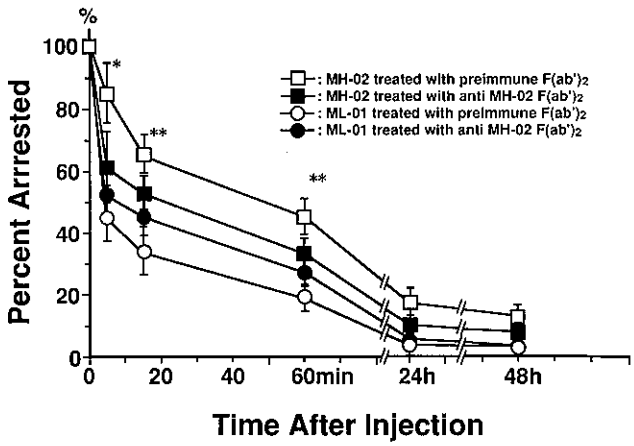

Fig. 3. Effect of anti-MH-02 $F(ab')_2$ on the arrest of radiolabeled MH-02 and ML-01 cells in the lung. Cells were radioiodolabeled, treated with either preimmune $F(ab')_2$ or anti-MH-02 $F(ab')_2$ (1.0 mg/ml) for 30 min and injected into the tail vein of BALB/c mice. The animals were killed at various times thereafter and the lungs were removed. After treatment with 70% ethanol, radioactivities of the lungs were counted. The percent of cells arrested was calculated from the ratios of the radioactivities of the lungs to the radioactivities of total cells injected. All data represent the means of at least three experiments±SEM. *: $P<0.025$ by Wilcoxon's test. **: $P<0.05$ by Wilcoxon's test.

Table I. Antimetastatic Effect of Anti-MH-02 $F(ab')_2$ on Pulmonary Metastases of ML-01 and MH-02 Cells[a]

Cell	Pulmonary metastases: Treatment	Number of metastatic nodules	
ML-01	preimmune $F(ab')_2$ (n=10)	17.2±7.1	[b]
	postimmune $F(ab')_2$ (n=10)	21.7±9.3	
MH-02	preimmune $F(ab')_2$ (n=10)	96.4±17.3	[c]
	postimmune $F(ab')_2$ (n=10)	27.6±6.7	

a) ML-01 and MH-02 cells (1.0×10^4/ml) were injected into the tail vein of BALB/c mice after pretreatment with anti-MH-02 $F(ab')_2$ (1.0 mg/ml) for 30 min at 37°C.
b) No significant difference by Wilcoxon's test.
c) $P<0.01$ by Wilcoxon's test.

Analysis of type IV collagen-binding proteins of MH-02 cells From the above results, it was suggested that the increased adhesive capacity of MH-02 cells to type IV collagen is relevant to MH-02 cells' high metastatic ability. Since the adhesiveness of MH-02 cells to type IV collagen can be attributed to some cell surface molecules, we attempted to analyze the surface proteins of MH-02 cells and ML-01 cells which bind to type IV collagen.

As shown in Fig. 4A, membrane proteins bound to type IV collagen appeared as a few bands on the lanes of MH-02 cells and ML-01 cells. The intensity of the bands was compared by densitometric analysis, and the band with the molecular weight of 29 kD of MH-02 cells was five times more intense than that of ML-01 cells.

Immunoprecipitation of radiolabeled membrane proteins by anti MH-02 antibody was also carried out. As shown in Fig. 4B, anti-MH-02 antibody precipitated several bands, though a band with the size of 29 kD can be seen on the lanes of both MH-02 and ML-01. In addition, the intensity of the band on MH-02 is stronger than that of ML-01. From these results, we concluded that the 29 kD protein which binds to type IV collagen not only is expressed more highly in MH-02 than in ML-01 but also is recognized by anti MH-02 antibody. The results also suggest that the increased expression of collagen-binding proteins, especially the 29 kD protein, is related to the increased metastatic ability of MH-02 cells.

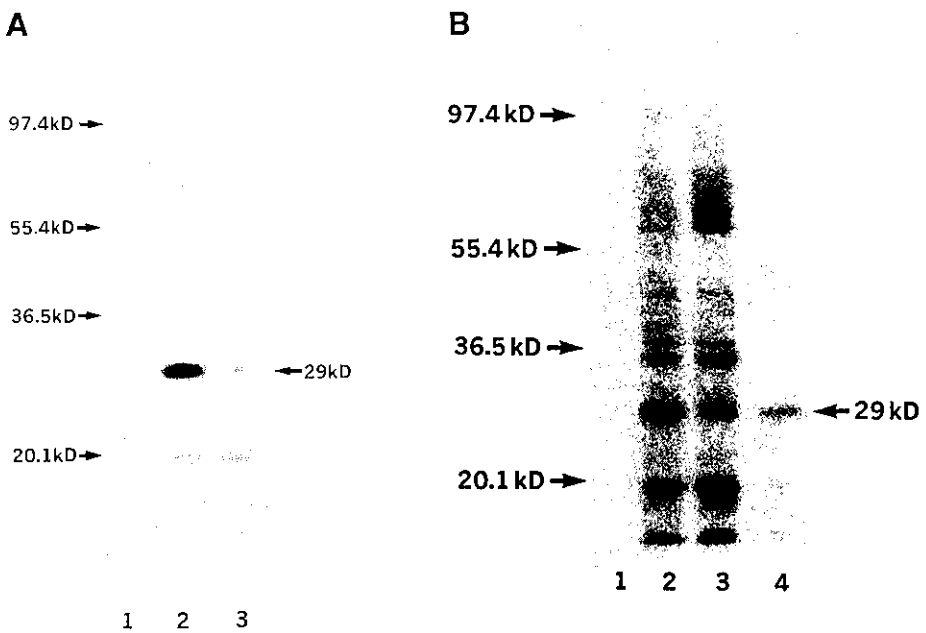

Fig. 4. Autoradiographs of SDS-PAGE analysis of ^{125}I-labeled MH-02 membrane proteins bound to type IV collagen-Sepharose 4B and immunoprecipitated by anti-MH-02 antibody. A: Cell membrane proteins of MH-02 and ML-01 cells were radiolabeled with Na^{125}I and solubilized in NP-40-Tris-buffered saline. Solubilized membrane proteins with equal amounts of radioactivity were incubated with Sepharose 4B coupled to type IV collagen. The column was washed extensively with washing buffer (see "Materials and Methods"). Membrane proteins bound to the Sepharose 4B were analyzed on SDS-PAGE and autoradiography. Lane 1; membrane proteins of MH-02 cells bound to Sepharose 4B. Lane 2; membrane proteins of MH-02 cells bound to type IV collagen-Sepharose 4B. Lane 3; membrane proteins of ML-01 cells bound to type IV collagen-Sepharose 4B. B: Two hundred μl of solubilized ^{125}I-labeled membrane proteins from each of MH-02 and ML-01 cells was treated with 5 μl of anti-MH-02 antibody and immunoprecipitated for 2 h at 4°C. Immunoprecipitates were collected by absorption on protein A-Sepharose for 2 h at 4°C, and washed three times in TBS containing 0.1% NP-40. Immunoprecipitates with equal amounts of radioactivity were analyzed by 12% SDS-PAGE and autoradiography. Lane 1; membrane proteins of MH-02 cells bound to protein A-Sepharose. Lane 2; membrane proteins of MH-02 cells immunoprecipitated by anti-MH-02 antibody. Lane 3; membrane proteins of ML-01 cells immunoprecipitated by anti-MH-02 antibody. Lane 4; membrane proteins of MH-02 cells bound to type IV collagen-Sepharose 4B.

DISCUSSION

Lodging of tumor cells in the microvasculature represents one of the most important events in blood-born metastasis.[3, 17, 18] The wall of microvessels consists of a layer of endothelial cells and subendothelial basement membrane and extracellular matrices. There have been several reports on the adhesiveness of tumor cells to endothelial cell surfaces.[28–31] In the majority of those reports, a high rate of tumor cell-endothelial cell adhesion was found to be correlated with high metastatic ability.[17, 18, 32–34]

We therefore first analyzed the adhesiveness of highly and weakly metastatic clones of Meth A cells to endothelial cells, and found that highly metastatic MH-02 cells adhere to the endothelial monolayers with a higher rate than weakly metastatic ML-01 cells. However, in the murine fibrosarcoma system, Wang *et al.* reported that there was no clear relationship between metastatic properties and the rate of adhesion of tumor cells to endothelial monolayers.[35] This fact suggests that tumor cell-endothelial cell adhesion is not always a key factor for the formation of metastasis. In addition to the endothelial cells, metastatic tumor cells also adhere to the subendothelial basement membrane.

The adhesion of tumor cells to basement membrane is another important process in blood-born metastasis.[12–14] The subendothelial basement membrane is sometimes a better substrate for tumor cells to adhere to than the endothelial cell surface.[19, 36] In the metastatic 13762 NF rat mammary carcinoma cell system, only the attachment rate of tumor cells to subendothelial matrix was related to the metastatic phenotype of tumor cell clones with different metastatic abilities.[37]

The components of subendothelial basement membranes which have been reported as tumor cell adhesion molecules include components such as laminin,[16, 38] fibronectin,[19, 39] type IV collagen,[20, 21, 40] heparan sulfate proteoglycan,[21, 41] vitronectin,[41] elastin,[21] and hyaluronic acid.[41] Adhesive properties of tumor cells with high metastatic potential to the subendothelial basement membranes have also been well documented and characterized. Integrin family complexes comprising receptors for laminin, fibronectin and/or collagen, have been identified on tumor cell membranes.[38, 40, 42, 43] In the mammary carcinoma system, the metastatic properties reportedly correlate with the numbers of laminin receptors on tumor cell surfaces.[38, 44] The integrin receptor family also includes the VLA protein family such as VLA-1[43] and VLA-2,[45] which bind not only to laminin, but also to type IV collagen.

Membrane molecules which adhere solely to type IV collagen, but not to laminin or fibronectin, as found in our highly metastatic clone, however, have not been previously reported. Our highly metastatic clone MH-02, exhibited higher adhesiveness to type IV collagen as compared to the weakly metastatic clone ML-01. Evidence that the higher adhesiveness can be attributed to membrane components was obtained from both *in vitro* and *in vivo* experiments. The antitumor cell surface antibody suppressed the adhesiveness of MH-02 to type IV collagen-coated dishes to roughly the same level as that of the weakly metastatic ML-01. The same antibody was also able to decrease both the initial trapping in mouse lungs and the number of pulmonary metastasis of MH-02, but not of ML-01. These results demonstrate that the preferential adhesion is determined by components at the tumor cell surface.

The cell surface molecule which may bind to type IV collagen was examined by using type IV collagen-coated Sepharose beads. By SDS-PAGE and autoradiography, several proteins specifically bound to type IV collagen were identified in both the highly and the weakly metastatic clones. Among them, a 29 kD protein was significantly increased in the highly metastatic clone. The protein is also immunoprecipitated by anti MH-02 antibody, supporting the results of adhesion and metastasis suppression experiments. Subsequent analysis of highly metastatic MH-02 cells revealed that they were associated with increased expression of *fyn* gene, a *src* family oncogene,[46, 47] as compared to that of weakly metastatic clone ML-01 cells (data not shown). It was also shown that both adhesiveness to type IV collagen and experimental metastatic potential were enhanced by transfection of cells with *fyn* gene, and the *fyn*-transfected ML-01 cells exhibited augmented expression of the type IV collagen binding proteins, including the 29 kD protein described here (submitted). Those results strongly support the idea that the expression level of the 29 kD protein is relevant to higher adhesiveness to type IV collagen, and the high metastatic potential of MH-02.

The 29 kD protein apparently has a different molecular weight from the previously reported type IV collagen specific binding proteins, including colligin of 47 kD,[48] 67 kD protein of neutrophils,[49] and 38 kD-87 kD-102 kD proteins of HeLa cells.[50] VLA-1 and VLA-2, members of the integrin receptor family, which bind to both laminin and type IV collagen, reportedly have molecular weights of 200/130 kD and 160/135 kD, respectively. Anchorin CII (31 kD) reportedly binds to type II collagen.[51] A 30 kD integrin protein was found in an osteosarcoma cell line, but the protein binds only to type I collagen.[50] We are now attempting to characterize the protein to verify whether the 29 kD protein is a novel type IV collagen-binding protein.

There may be several explanations for the fact that the antibody against MH-02 inhibited adhesiveness to endothelial cells as well as to type IV collagen. One possibility is that endothelial cells and type IV collagen share similar epitopes. Another possibility is that epitopes reactive to these vascular components are dissimilar, but are reactive to the polyclonal antibody used in this series of experiments. The third possibility is that endothelial cells and type IV collagen possess both common and distinctive epitopes. Analysis using monoclonal antibodies to each membrane component may be used to elucidate these possibilities.

In conclusion, we have demonstrated that a highly metastatic clone, MH-02, possesses augmented adhesiveness to endothelial cells and type IV collagen, presumably due to higher expression of type IV collagen-binding proteins. The metastatic process consists of several distinct steps, and it would be a great advantage for metastatic tumor cells to possess multiple properties favorable for these steps. In this context, it seems noteworthy that such a "pure" cell line as cloned MH-02 is furnished with multiple properties leading to increased metastatic potential. Both increased platelet aggregating activity as previously reported[26] and adhesive properties of tumor cells to endothelial and type IV collagen are considered to be favorable for metastasis because they conceivably could synergistically enhance the tumor cell arrest in the target organ.

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