Jpn. J. Cancer Res. 85, 63–71, January 1994

Effects of Epidermal Growth Factor on Invasiveness through the Extracellular Matrix in High- and Low-metastatic Clones of RCT Sarcoma *in vitro*

Kazuo Yudoh,[1] Hisao Matsui, Masahiko Kanamori, Akio Maeda, Kazuo Ohmori and Haruo Tsuji

Department of Orthopaedic Surgery, Toyama Medical and Pharmaceutical University, Faculty of Medicine, 2630 Sugitani, Toyama 930-01

We investigated the invasiveness of tumor cells through the extracellular matrix and the influence of epidermal growth factor (EGF) on tumor cell invasion using *in vitro* systems in high-[RCT(+)] and low-metastatic [RCT(−)] clones established from poorly differentiated murine RCT sarcoma in C3H/He mice. In the invasion assay using a filter coated with reconstituted basement membrane (Matrigel) in a Boyden chamber, RCT(+) cells were more invasive than RCT(−) cells. The attachment of RCT(+) cells to extracellular matrix components and the degradation of type IV collagen by the cells were significantly greater than with RCT(−) cells. However, there was no significant difference in the migration of cells to the extracellular matrix components between cultured RCT(+) and RCT(−) cells. These findings suggested that the different invasiveness of these clone cells was associated with the difference in the ability of attachment to and degradation of the matrix. The level of laminin receptor expression in RCT(+) cells was about four-fold that in RCT(−) cells and laminin stimulated the type IV collagenolytic activity of RCT(+) cells, suggesting that RCT(+) cell attachment to laminin via laminin receptor on the cell surface induced the production of type IV collagenase by the tumor cells. EGF did not affect the invasiveness of RCT(−) cells. In RCT(+) cells, EGF stimulated the invasiveness through Matrigel, the attachment to extracellular matrix components and the degradation of type IV collagen through high-affinity EGF receptors (EGFR), with K_d of p*M* order, while the migration to the matrix was not influenced by EGF. These findings suggest that the stimulatory effect of EGF on invasion is related to the acceleration of cell adhesion, and the degradative cascade of the extracellular matrix and high-affinity EGFRs play an important role in the effect of EGF on *in vitro* invasiveness in this tumor.

Key words: Tumor invasion — Metastasis — Murine sarcoma — Extracellular matrix — Epidermal growth factor

In the process of metastasis formation, tumor cells must pass through basement membranes and extracellular matrices prior to invasion into and exit from the lymphatic or blood vessels. Tumor cell invasion of the extracellular matrix involves three crucial steps: 1) tumor cell attachment to the extracellular matrix components via cell surface receptors for matrix glycoproteins such as laminin, 2) secretion by tumor cells of proteolytic enzymes that cause the degradation of the matrix, 3) tumor cell migration into the region of the matrix modified by proteolysis.[1-10] Based on these findings, *in vitro* models have been developed to study mechanisms of tumor cell interactions with extracellular matrix barriers. Using an *in vitro* invasion assay system, it is well known that malignant tumor cells have high invasive potential to penetrate extracellular matrix barriers, and tumor cell invasiveness *in vitro* may be correlated with malignant potential *in vivo*.[6, 8, 11-14] Furthermore, a correlation between invasiveness *in vitro* and the metastatic potential has been reported in cell lines with different metastatic potential derived from a single tumor.[15] Systematic analysis of extracellular matrix invasion in clones with different metastatic potential established from a single tumor is quite important to determine the mechanism of metastasis.

Epidermal growth factor (EGF) and epidermal growth factor receptor (EGFR) have been implicated in malignant behavior of various human neoplasms. The EGFR is often overexpressed in tumor cells, and significantly higher EGFR levels have been found in several malignant tumors than in normal or benign tumors.[16-18] The level of EGFR expression might be associated with prognostic factors such as the stage of tumor, histological malignancy and metastasis, suggesting that the level of EGFR expression could be a useful marker of tumor invasion and metastasis.[19-22] In the RCT (Radiological, Chiba and Toyama) sarcoma cell line, we have found that the level of EGFR expression in high-metastatic [RCT(+)] clone cells was higher than that in low-metastatic clone [RCT(−)] cells, and pulmonary metastasis of RCT(+) cells was inhibited by treatment with anti-EGFR monoclonal antibody *in vivo*.[23] It has been reported that EGF stimulated growth, migration and invasion of brain tumor cells into the host tissue *in vitro*.[24, 25] It is suggested that the regulation of extracel-

[1] To whom correspondence should be addressed.

lular matrix invasion is closely linked with growth factors such as EGF.[26, 27]

In the present study, we investigated the characteristics of high- and low-metastatic clone cells established from RCT sarcoma in terms of invasiveness through the extracellular matrix using an *in vitro* system. We also evaluated the effect of EGF on the invasion process in these clone cells.

MATERIALS AND METHODS

Cell culture High-metastatic [RCT(+)] and low-metastatic [RCT(−)] clone cells of RCT sarcoma obtained by the combination of lung passage and the limiting-dilution method[28] were cultured in 25 cm^2 plastic flasks (MS-20050, Sumitomo Bakelite, Tokyo).[29, 30] RCT(+) and RCT(−) cells before the 20th generation of *in vitro* passage were used in the present study. These cells were cultured in RPMI 1640 (Flow Laboratories, Inc., Irvine, Scotland), supplemented with 100 mg/ml streptomycin (Meiji Seika, Tokyo), 100 U/ml penicillin (Meiji Seika) and 10% fetal bovine serum (FBS; Bocknek Ltd., Toronto, Canada) at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. The specificity of the monoclonal antibody (MoAb, 528IgG_2; Oncogene Science, Manhasset, NY) to EGFR used in this study has been established by many authors.[31-34] When the anti-EGFR monoclonal antibody was used in each assay system, normal mouse IgG (ICN ImmunoBiologicals, Lisle, IL) and ascites from immunized mice were used as the negative control and the positive control, respectively. We have previously found that anti-EGFR MoAb (528 IgG_2) selectively blocks EGF binding to high-affinity EGFR in RCT sarcoma cells.[23] To observe the effect of EGF (Sigma Chemical, St. Louis, MO) on each assay in the presence of anti-EGFR MoAb, cells were preincubated with MoAb (20.0 μg/ml) for 2 h prior to EGF addition to the culture medium.

***In vitro* invasion assay** The invasiveness of RCT sarcoma cells was assayed according to the method reported by Albini *et al.*[11] Polyvinyl-pyrrolidone-free polycarbonate filters (8 μm pore size; Nucleopore, Pleasanton, CA) were coated with 50 μg/filter of reconstituted basement membrane (Matrigel: Collaborative Research Inc., Bedford, MA). The Matrigel, diluted to 100 mg/ml with cold phosphate-buffered saline (PBS), was applied to the upper surface of the filters and dried at room temperature under a hood. The coated filters were placed in Boyden chambers. Cells (1×10^5) were suspended in RPMI containing 0.1% bovine serum albumin (BSA; Sigma Chemical), added to the upper chamber and incubated for 2, 4, 6, 8, 10 and 12 h at 37°C in 5% CO_2 and 95% air. At the end of each incubation, the cells on the upper surface of the filter were completely removed by wiping with a cotton swab. The filter was fixed in methanol and stained with hematoxylin and eosin. The cells that had migrated to the lower surface of the filter were counted under 400-fold magnification.

In the study of the effect of EGF on tumor cell invasion, cells (1×10^5) suspended in RPMI containing 0.1% BSA were incubated with or without EGF (1.6×10^{-3} to 16.0 n*M*) in the upper compartment of a Boyden chamber. After 8 h of incubation, the number of cells which had penetrated through the Matrigel was counted according to the above-mentioned method. Each assay was performed in triplicate, and repeated three times.

Attachment assay Assay was performed according to the method reported by Voldavsky *et al.*[35] or Iwamoto *et al.*[36] Subconfluent cultures of RCT(+) and RCT(−) cells were prelabeled with 18.5 kBq/ml ^{3}H-thymidine (74 kBq/mmol, ICN) for 24 h in the culture medium. The cells were washed twice with cold PBS to remove free radioisotope followed by resuspension in RPMI with 0.1% BSA. The culture dishes (Diameter: 35 mm) were coated with Matrigel (5 μg/ml), laminin (20 μg/ml, Collaborative Research, Inc.) or fibronectin (20 μg/ml, UCB-Bioproducts S. A., Belgium). After that, each dish was blocked with 1 mg/ml of BSA for 30 min at 25°C. The labeled cells (1×10^5) were placed on the coated dishes and incubated at 37°C. For assessment of the effect of EGF on cell attachment, the cells (1×10^5) suspended in RPMI with 0.1% BSA were incubated with EGF (1.6×10^{-3} to 0.8 n*M*) on the coated dishes. After 30-min incubation, the dishes were carefully washed three times with PBS to remove unattached cells. The attached cells were dissolved in 0.1 *N* NaOH, and the radioactivity was measured using a liquid scintillation counter (Aloka, LSC903, Tokyo). The attachment capacity was determined relative to the radioactivity of seeded cells (1×10^5) which was considered to be 100%. Each assay was performed in triplicate, and repeated three times.

Type IV collagenolysis assay An aliquot of ^{3}H-proline-labeled type IV collagen (6.0 μg/well; 0.011 GBq/mg, New England Nuclear, Boston, MA) dissolved in 0.5 *M* acetic acid was added to each well of a 96-well tissue culture plate (n=9) which was then dried in a laminar air flow apparatus overnight. Cells (1×10^4) suspended in 200 μl of RPMI containing 0.1% BSA were incubated on a 96-well tissue culture plate precoated with ^{3}H-collagen. For the study of the effect of EGF on type IV collagenolytic activity, cells (1×10^4) suspended in RPMI containing 0.1% BSA were incubated with EGF (1.6×10^{-3} to 16.0 n*M*) in ^{3}H-collagen-coated wells. After 8 h of incubation, the supernatant was withdrawn, and undigested materials were precipitated by mixing with 100 μl of ice-cold 10% trichloroacetic acid and 0.5% tannic acid, followed by centrifugation at 18,000*g* for 10 min. Type IV collagenolytic activity was calculated from the radioac-

tivity in the supernatant, and expressed as the net amount (ng) of degraded type IV collagen.[10, 13]

Laminin or fibronectin receptor expression and effect of laminin or fibronectin on type IV collagenolysis The level of laminin or fibronectin receptor expression in cultured RCT(+) and RCT(−) cells was analyzed by the Scatchard method. The confluent cells were incubated with various concentrations of ^{125}I-laminin (0.01 to 10.0 ng/ml, 3.7 kBq/μg, Amersham International plc, Buckinghamshire, England) in the presence or absence of a large excess of unlabeled laminin (2.0 μg/ml) in a final volume of 1.0 ml of RPMI-BSA (RPMI 1640 supplemented with 0.1% BSA) for 90 min at 18°C in the culture dishes. Cells were dissolved in 1.0 ml of 1 *N* NaOH. Radioactivity of the solutions was measured using a gamma counter (Aloka, ARC-2000, Tokyo), and Scatchard analysis was subsequently performed.[23] The level of fibronectin receptor expression was also assayed according to the above-mentioned method using ^{125}I-fibronectin.

To verify the effect of laminin or fibronectin on the collagenolytic activity, cells (1×10^4) suspended in RPMI containing 0.1% BSA were incubated with and without laminin (20.0 μg/ml) or fibronectin (20.0 μg/ml) in a 96-well tissue culture plate coated with ^{3}H-labeled type IV collagen (n=9 each). Type IV collagenolytic activity was measured by a procedure similar to that described above for type IV collagenolysis assay.

Haptotactic migration assay To investigate the haptotactic activity (directed migration toward a gradient of substratum-bound attractant), a Boyden chamber with a built-in laminin or fibronectin-coated filter with no Matrigel was used.[10, 37] Polycarbonate filters (8 μm pore size) were precoated with either 5.0 μg/filter of laminin or fibronectin on the lower surface of the filter, and dried overnight at room temperature. The coated filters were placed in Boyden chambers. Cells (1×10^5) suspended in RPMI containing 0.1% BSA were added to the upper chamber of the Boyden chamber, and incubated for 8 h. To examine the effect of EGF on cell migration, the assay was performed using EGF (1.6×10^{-3} to 1.6 n*M*). The subsequent procedure was the same as for the invasion assay. Each assay was performed in triplicate, and repeated three times.

Statistical analysis Values were expressed as means ± SD. Student's *t* test was used to evaluate the significance of differences between groups, and the criterion of statistical significance was taken as $P<0.05$.

RESULTS

Invasiveness of tumor cells through Matrigel The number of cells which penetrated to the lower surface of the Matrigel-coated filter increased with time with both RCT(+) and RCT(−) cells. After more than 6 h of incubation, the number of RCT(+) cells which had migrated through the Matrigel was significantly greater than that of RCT(−) cells (Fig. 1).

EGF stimulated the invasiveness of RCT(+) cells through the Matrigel compared with the control in a concentration-dependent manner (Fig. 2). The number of RCT(+) cells which invaded the Matrigel was significantly increased at the EGF concentration of 40 p*M*. As the EGF concentration was raised to 0.8 n*M*, the number of RCT(+) cells penetrating the Matrigel reached a plateau. In contrast, EGF did not influence the number of RCT(−) cells passing through the Matrigel (Fig. 2). EGF did not influence the number of RCT(+) cells penetrating the Matrigel in the presence of anti-EGFR MoAb (528IgG$_2$). The ability to invade the Matrigel was not affected by anti EGFR MoAb alone in either clone (Table I).

Cell attachment The mean percentages of RCT(+) cells which became attached to dishes coated with Matrigel, laminin and fibronectin were 74%, 82% and 61%, respectively. The corresponding values of RCT(−) cells were 51%, 48% and 42%, respectively. The ability of RCT(+) cell attachment to Matrigel, laminin or fibronectin was significantly higher than that of RCT(−) cells (Fig. 3).

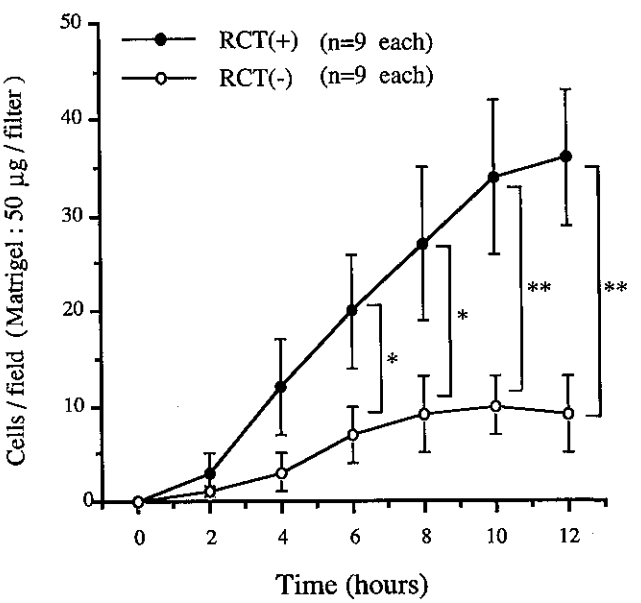

Fig. 1. Invasiveness of RCT(+) and RCT(−) cells. The number of cells which penetrated through the Matrigel-coated filter increased with time in both clones. Six hours after incubation, RCT(+) cells showed a significant level of invasion. *: $P<0.05$, **: $P<0.01$).

EGF stimulated the attachment of RCT(+) cells to the extracellular matrix components. The attachment of RCT(+) cells to Matrigel-, laminin- or fibronectin-coated dishes was significantly increased at the EGF concentration of 80 p*M* or higher (Table II). In RCT(−) cells with EGF, however, the attachment did not significantly differ from the control. In the presence of anti-

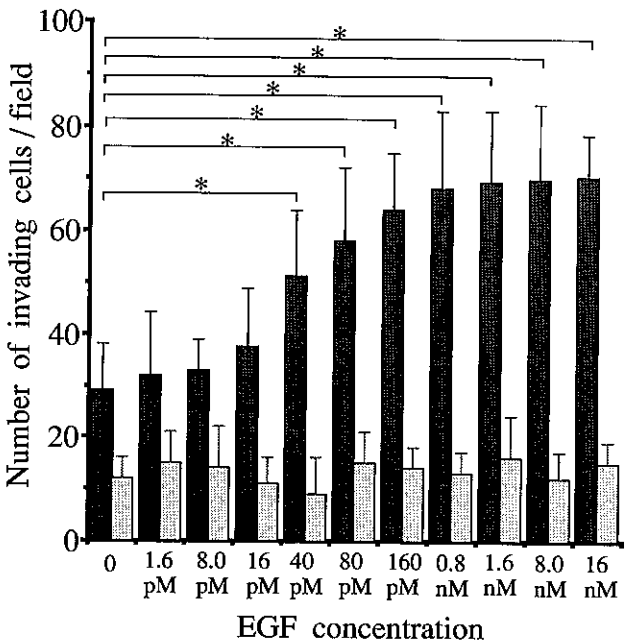

Fig. 2. EGF effects on extracellular matrix invasion. For RCT(+) cells, the number of cells which invaded the Matrigel was significantly increased at an EGF concentration of 40 p*M* or higher. For RCT(−) cells, EGF did not influence the invasiveness. ■ RCT(+) (n=9 each). ▨ RCT(−) (n=9 each). ∗: $P<0.05$.

Table I. Effects of EGF on Cell Invasion in RCT(+) and RCT(−) Cells

Group	Concentration	Number of invading cells/field (Matrigel): 50 μg/filter) RCT(+) (mean±SD)	RCT(−) (mean±SD)
Control		29±9	10±6
EGF[a]	1.6 p*M*	28±11	12±7
	40 p*M*	48±8	9±4
	0.8 n*M*	68±7	13±5
Anti-EGFR MoAb[b]	20.0 μg/ml	29±7	12±6
Anti-EGFR MoAb +EGF	20.0 μg/ml 0.8 n*M*	36±8	14±7

a) EGF: epidermal growth factor.
b) Anti EGFR MoAb: anti EGF receptor monoclonal antibody.
∗: $P<0.05$.

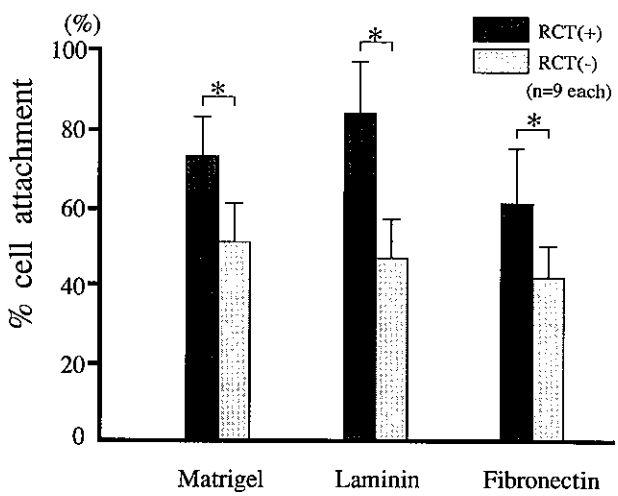

Fig. 3. Attachment ability to extracellular matrix components. RCT(+) cells showed a significant attachment ability to Matrigel, laminin or fibronectin. ∗: $P<0.05$.

Table II. Effect of EGF on RCT(+) Cell Attachment to Extracellular Matrix Components

Group	Concentration	Number of attached cells×10^{-4} cells Matrigel (5 μg/ml) (mean±SD)	Laminin (20 μg/ml) (mean±SD)	Fibronectin (20 μg/ml) (mean±SD)
Control		5.45±0.37	6.26±0.48	5.99±0.69
EGF[a]	1.6 p*M*	5.96±0.46	6.58±0.39	6.25±0.54
	80 p*M*	6.54±0.40	8.54±0.79	7.46±0.61
	0.8 n*M*	7.43±0.45	8.43±0.73	7.67±0.36
Anti-EGFR MoAb[b]	20.0 μg/ml	5.03±0.68	5.99±0.58	6.34±0.49
Anti-EGFR MoAb +EGF	20.0 μg/ml 0.8 n*M*	5.36±0.57	5.89±0.68	5.32±0.56

a) EGF: epidermal growth factor.
b) Anti EGFR MoAb: anti EGF receptor monoclonal antibody.
∗: $P<0.05$. ∗∗: $P<0.01$.

EGFR MoAb, EGF did not affect the attachment to Matrigel, laminin or fibronectin in either clone (Table II).

Type IV collagenolytic activity In this assay, the type IV collagenolytic activity of RCT(+) cells was found to be significantly higher (1.35 ± 0.16 $\mu g/10^4$ cells at 8 h incubation) than that of RCT(−) cells (0.44 ± 0.12 $\mu g/10^4$ cells at 8 h incubation) (Table III).

EGF stimulated the type IV collagenolysis compared with the control in RCT(+) cells. The collagenolytic activity of RCT(+) cells was significantly enhanced by 80 p*M* EGF, and reached a plateau at an EGF concentration of 0.8 n*M* or higher (Fig. 4). In contrast, type IV collagenolytic activity of RCT(−) cells showed no significant EGF-induced change. In the presence of anti-EGFR MoAb, EGF did not affect the degradation of type IV collagen in either clone (Table III).

Laminin or fibronectin receptor expression and effect of laminin or fibronectin on type IV collagenolysis The average numbers of laminin receptors in RCT(+) and RCT(−) cells were 1.1×10^5 and 0.3×10^5 per cell, respectively. There was no significant difference in the K_d value of laminin receptors between RCT(+) ($K_d=1.45$ n*M*) and RCT(−) ($K_d=1.38$ n*M*). Laminin significantly stimulated the type IV collagenolysis of RCT(+) cells (2.45 ± 0.39 $\mu g/10^4$ cells at 8 h incubation) compared with the control (1.34 ± 0.44 $\mu g/10^4$ cells at 8 h incubation) (Table IV). There was no significant acceleration of the type IV collagenolitic activity in RCT(−) cells induced by laminin (laminin-treated: 0.62 ± 0.29 $\mu g/10^4$ cells, control: 0.44 ± 0.12 $\mu g/10^4$ cells at 8 h incubation). The average numbers of fibronectin receptors in RCT(+) and RCT(−) cells were 4.8×10^5 and 2.6×10^5 per cell, respectively. There was no significant difference in the K_d value of fibronectin receptors between RCT(+) ($K_d=1.33$ μM) and RCT(−) ($K_d=1.29$ μM). However, fibronectin did not influence the type IV collagenolysis in either clone (data not shown).

Haptotactic migration assay There was no significant difference in the migration to a laminin-precoated or fibronectin-precoated filter between RCT(+) and RCT(−) cells. In each clone, the migration ability was not influenced by EGF, irrespective of the presence or absence of anti-EGFR MoAb (Table V).

DISCUSSION

RCT sarcoma induces pulmonary metastasis 2 to 3 weeks after subcutaneous implantation in C3H/He mice.[29] RCT(+) and RCT(−) were isolated as high-

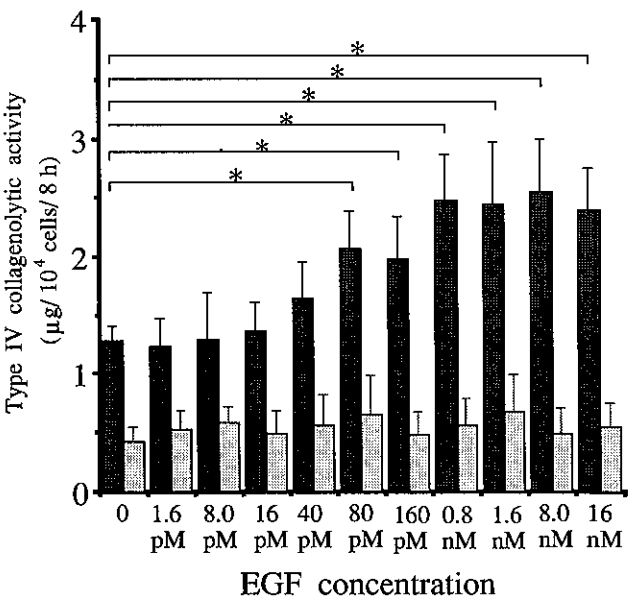

Fig. 4. Effect of EGF on the degradation of tritiated type IV collagen. In RCT(+) cells, type IV collagenolytic activity significantly increased at an EGF concentration of 80 p*M* or higher. In RCT(−) cells, the activity showed no significant EGF-induced change. ■ RCT(+) (n=9 each). ▨ RCT(−) (n=9 each). *: $P<0.05$.

Table III. Type IV Collagenolytic Activity in 8-h Incubation

Group	Concentration	Type IV collagenolytic activity ($\mu g/10^4$ cells) RCT(+) (mean±SD)	RCT(−) (mean±SD)
Control		1.35±0.16	0.44±0.12
EGF[a]	1.6 p*M*	1.54±0.24	0.56±0.18
	80 p*M*	2.17±0.45 *	* 0.62±0.22
	0.8 n*M*	2.59±0.56	0.48±0.14
Anti-EGFR MoAb[b]	20.0 μg/ml	1.28±0.36	0.62±0.26
Anti-EGFR MoAb +EGF	20.0 μg/ml 0.8 n*M*	1.44±0.39	0.57±0.32

a) EGF: epidermal growth factor.
b) Anti EGFR MoAb: anti EGF receptor monoclonal antibody.
*: $P<0.05$.

Table IV. Effect of Laminin on Type IV Collagenolysis in 8-h Incubation

Group	Concentration	Type IV collagenolytic activity ($\mu g/10^4$ cells) RCT(+) (mean±SD)	RCT(−) (mean±SD)
Control	−	1.34±0.44 *	0.52±0.22
Laminin	20.0 μg/ml	2.45±0.39 *	0.62±0.29

*: $P<0.05$.

Table V. Haptotactic Migration Assay

Group	Concentration	Number of migrating cells/10^5 cells (mean ± SD)			
		Laminin (20 μg/ml)		Fibronectin (20 μg/ml)	
		RCT(+)	RCT(−)	RCT(+)	RCT(−)
Control	−	44±6	52±12	48±7	49±9
EGF	1.6 p*M*	52±9	51±8	46±9	48±11
	80 p*M*	48±7	48±6	51±10	52±7
	1.6 n*M*	46±6	54±8	52±9	50±8
Anti-EGFR MoAb	20.0 μg/ml	49±11	56±7	45±8	49±7
Anti-EGFR MoAb +EGF	20.0 μg/ml 1.6 n*M*	51±12	48±5	46±6	52±10

EGF: epidermal growth factor.
Anti EGFR MoAb: anti EGF receptor monoclonal antibody.

metastatic and low-metastatic clones in our department.[30] We have reported that the stromal stickiness and membrane glycoprotein of cells are closely related to the difference in the metastatic potential of RCT sarcoma cells.[29, 30] Using the three-dimensional culture invasion assay with RCT(+) and RCT(−) clones, we previously found a correlation between the metastatic potential *in vivo* and the invasion ability of cells into the host tissue *in vitro*.[38]

Extracellular matrix components such as laminin, fibronectin, type IV collagen, and other glycoproteins act as a barrier in the process of tumor cell invasion and metastasis formation.[3] The penetration of tumor cells through the extracellular matrix is necessary for metastasis formation. In the present study, an *in vitro* invasion assay employing a reconstituted basement membrane (Matrigel) coated on the filter of a Boyden chamber was performed to elucidate the invasiveness of RCT(+) and RCT(−) cells through the extracellular matrix. This assay provides a quantitative evaluation of the invasiveness of tumor cells through the extracellular matrix.[10, 11] Using this invasion assay system, Albini *et al.* reported that malignant tumor cells show increased invasiveness compared to normal cells or benign tumor cells, suggesting a correlation between invasiveness through Matrigel and malignant behavior.[11] In the present study, the invasiveness of RCT(+) cells through the Matrigel was significantly greater than that of RCT(−) cells. The *in vitro* invasiveness through the extracellular matrix might be correlated with the *in vivo* metastatic potential of this tumor.

In order to analyze the invasive process into the extracellular matrix, we investigated RCT(+) and RCT(−) cell attachment, degradation and migration into extracellular matrix components. The attachment ability of RCT(+) cells to Matrigel or to extracellular matrix components such as laminin and fibronectin was greater than that of RCT(−) cells. Tumor cell attachment to the extracellular matrix has been shown to be mainly mediated by cell surface laminin receptors.[1-3, 5] The level of laminin receptor expression in metastatic tumor cells might be higher than that in nonmetastatic cells,[39, 40] and laminin-adherent cells selected *in vitro* have been shown to be more malignant *in vivo* than either the parent cells or unattached cells.[41] In the current study, the amount of laminin and fibronectin receptor in RCT(+) cells was about four-fold and two-fold that in RCT(−) cells, respectively. These findings suggested that the attachment ability of cells to the matrix is associated with the level of laminin or fibronectin receptor expression. Metalloproteases capable of degrading type IV collagen have been recognized to play an important role in tumor invasion and metastasis formation.[8, 10, 13, 14, 42] Furthermore, an increased type IV collagenase production is associated with progression of tumor cell growth and is a good prognostic marker of malignant tumors.[43] In the present study, the type IV collagenolytic activity of RCT(+) cells was significantly greater than that of RCT(−) cells. In contrast, there was no significant difference in the migration ability to the extracellular matrix components between RCT(+) and RCT(−) cells. These findings *in vitro* suggested that the different levels of invasiveness between RCT(+) and RCT(−) cells might be due to the differences in the ability of attachment to the extracellular matrix components and matrix degradation. No correlation was found between the migration *in vitro* and the metastatic potential *in vivo* in these clone cells.

It has been reported that extracellular matrix potein might signal through a protease system, leading to increased matrix synthesis.[2, 44, 45] In the present study, the type IV collagenolytic activity was stimulated by exogenous laminin in cultured RCT(+) cells. Fibronectin did not influence the type IV collagenolysis in either clone.

These findings suggest that the tumor cell attachment to the extracellular matrix, especially to laminin via the cell surface laminin receptor, may induce production of type IV collagenase from RCT(+) cells. In RCT(−) cells, type IV collagenolysis is not influenced by laminin. Also, the difference in laminin-induced activation of type IV collagenolysis might be related to the level of laminin receptor expression. The present results may support a link between the tumor cell attachment and degradation of the extracellular matrix. Furthermore, this suggests that the triggering of extracellular matrix degradation might be mediated by the tumor cell attachment to laminin in the extracellular matrix.

We have observed that the high- and low-affinity EGFRs were expressed in both RCT(+) and RCT(−) cells, and the level of EGFR expression in RCT(+) cells was significantly higher than that in RCT(−) cells. In RCT(+) cells, EGF-induced phosphorylation of EGFR and pulmonary metastasis were inhibited by anti-EGFR monoclonal antibody, which could block EGF binding to the high-affinity EGFR.[23] These finding suggested that EGFR activation has a functional role in the metastatic property of RCT sarcoma cells. In the present study, the invasiveness of RCT(+) cells through Matrigel was significantly stimulated by EGF at the p*M* level. With n*M* concentrations of EGF, there was no further stimulation or inhibition of the invasion. These findings suggest that high-affinity EGFR with a K_d of p*M* order is related to the stimulatory effect of EGF on matrix invasion, whereas low-affinity EGFR with a K_d of n*M* order was not involved in a stimulatory or inhibitory effect of EGF. In the invasion process, EGF stimulated RCT(+) cell attachment to the extracellular matrix and degradation of type IV collagen, while the migration was not affected by EGF. Liotta *et al.* reported that autocrine motility factor (AMF) may play a major role in the invasive behavior of tumor cells and the activity of AMF was not replaced or blocked by growth factors such as EGF.[46] Thus, the stimulatory effect of EGF on the matrix invasion *in vitro* system might be due to the acceleration of cell adhesion and the degradative cascade of the matrix; the stimulatory effect observed at EGF concentration of p*M* order suggests that high-affinity receptors are involved in the stimulatory effect of EGF on tumor cell attachment and matrix degradation. The amount of high-affinity receptors per cell in RCT(+) cells was about four-fold that in RCT(−) cells.[23] The sensitivity to the EGF effect on the invasion process might be associated with the level of high-affinity EGFR expression in RCT sarcoma cells. In RCT(+) cells, the average number of laminin receptors or fibronectin receptors per cell showed a tendency to be increased by EGF (data not shown). These findings suggested that the influential effect of EGF on cell attachment to laminin or fibronectin might be due to a change of expression of its receptor by EGF.

EGFR expression has been evaluated in many human neoplasms, and might be an indicator or poor prognosis.[19–22] *In vitro*, EGF induces the expression of mRNAs for extracellular matrix degradative enzymes such as stromelysin and type IV collagenase.[27] Here, when high-affinity receptors in RCT(+) cells were blocked by anti-EGFR MoAb, EGF did not influence the invasiveness through Matrigel, tumor cell attachment to the extracellular matrix components or degradation of type IV collagen. These results also suggest that EGF binding to high-affinity EGFR mediates the signal transmission for the stimulatory effect of EGF on the matrix invasion in RCT(+) cells. In metastatic tumor cells, it is suggested that EGFR expression is useful not only as a marker of malignancy such as tumor invasion and metastasis, but also as a target of anti-metastatic therapies.

In conclusion, the present study has provided several findings to suggest that tumor cell attachment and degradation of the extracellular matrix are important factors in the invasiveness of RCT sarcoma cells. EGF stimulates the invasiveness of high-metastatic clone [RCT(+)] cells *in vitro*. The stimulatory effect of EGF on invasion might be closely related to the acceleration of cell adhesion and the degradative cascade of the extracellular matrix.

ACKNOWLEDGMENTS

This work was supported in part by a Grant-in-Aid for Scientific Research B (1992-1993, #04454373) from the Japanese Ministry of Education, Science and Culture.

(Received August 5, 1993/Accepted October 12, 1993)

REFERENCES

1) Liotta, L. A. Tumor invasion and metastasis: role of the basement membrane. *Am. J. Pathol.*, **117**, 339–348 (1984).
2) Liotta, L. A., Rao, C. N. and Wewer, U. N. Biochemical interactions of tumor cells with the basement membrane. *Ann. Rev. Biochem.*, **55**, 1037–1057 (1986).
3) Liotta, L. A. Tumor invasion and metastases — role of the extracellular matrix: Rhodes memorial award lecture. *Cancer Res.*, **46**, 1–7 (1986).
4) Gehlsen, K. R., Argraves, W. S., Pierschbacher, M. D. and Ruoslahti, E. Inhibition of tumor cell invasion by Arg-Gly-Asp-containing synthetic peptides. *J. Cell Biol.*, **106**, 925–930 (1988).

5) Terranova, V. P., Liotta, L. A., Russo, R. G. and Martin, G. R. Role of laminin in the attachment and metastasis of murine tumor cells. *Cancer Res.*, **42**, 2265–2269 (1982).
6) Volk, T., Geiger, B. and Raz, A. Motility and adhesive properties of high- and low-metastatic neoplastic cells. *Cancer Res.*, **44**, 811–824 (1984).
7) Mignatti, P., Robbins, E. and Rifkin, D. B. Tumor invasion through the human amniotic membrane: requirement for a proteinase cascade. *Cell*, **47**, 487–498 (1986).
8) Liotta, L. A., Tryggvason, K., Garbisa, S., Hart, I., Foltz, C. M. and Shafie, S. Metastatic potential correlates with enzymatic degradation of basement membrane collagen. *Nature*, **284**, 67–68 (1980).
9) Davis, G. E. and Martin, B. M. A latent Mr 94,000 gelatin-degrading metalloprotease induced during differentiation of HL-60 promyelocytic leukemia cells: a member of the collagenase family of enzymes. *Cancer Res.*, **50**, 1113–1120 (1990).
10) Yoneda, J., Sasaki, I., Fujii, H., Abe, F., Kojima, Y. and Azuma, I. Inhibition of tumor invasion and extracellular matrix degradation by ubenimex (bestatin). *Clin. Exp. Metastasis*, **10**, 49–59 (1992).
11) Albini, A., Iwamoto, Y., Kleinmam, H. K., Martin, G. R., Aaronson, S. A., Kozlowski, J. M. and McEwan, R. N. A rapid *in vitro* assay for quantitating the invasive potential of tumor cells. *Cancer Res.*, **47**, 3239–3245 (1987).
12) Kurt, R. G., Davis, G. E. and Sriramarao, P. Integrin expression in human melanoma cells with differing invasive and metastatic properties. *Clin. Exp. Metastasis*, **10**, 111–120 (1992).
13) Nakajima, M., Welch, D. R., Belloni, P. N. and Nicolson, G. L. Degradation of basement membrane type IV collagen and lung subendothelial matrix by rat mammary adenocarcinoma cell clones of differing metastatic potentials. *Cancer Res.*, **47**, 4869–4876 (1987).
14) Ura, H., Bonfil, R. D., Reich, R., Reddel, R., Pfeifer, A., Harris, C. C. and Klein-Szanto, A. J. P. Expression of type IV collagenase and procollagen genes and its correlation with the tumorigenic, invasive, and metastatic abilities of oncogene-transformed human bronchial epithelial cells. *Cancer Res.*, **49**, 4615–4621 (1989).
15) Sadano, H., Taniguchi, S. and Baba, T. Newly identified type of β actin reduces invasiveness of mouse B16-melanoma. *FEBS Lett.*, **271**, 23–27 (1990).
16) Vambutas, A., Di Lorenzo, T. P. and Steinberg, B. M. Laryngeal papilloma cells have high levels of epidermal growth factor receptor and respond to epidermal growth factor by a decrease in epithelial differentiation. *Cancer Res.*, **53**, 910–914 (1993).
17) Neal, D. E., Marsh, C., Bennett, M. K., Abel, P. D., Hall, R. R., Sainsbury, J. R. C. and Harris, A. L. Epidermal growth factor receptors in human bladder cancer: comparison of invasive and superficial tumors. *Lancet*, **i**, 366–368 (1985).
18) Smith, K., Fennelly, J. A., Neal, D. E., Hall, R. R. and Harris, A. L. Characterization and quantitation of epidermal growth factor receptors in invasive superficial bladder tumors. *Cancer Res.*, **49**, 5810–5815 (1989).
19) Fitzpatrix, S. L., Brighywellm, J., Wittlif, J. L., Barrows, G. H. and Schultz, G. S. Epidermal growth factor binding by breast tumor biopsies and relationship to estrogen receptor and progestin receptor levels. *Cancer Res.*, **44**, 3448–3458 (1984).
20) Tahara, E. Human epidermal growth factor in gastric carcinoma as a biologic marker of high malignancy. *Jpn. J. Cancer Res.*, **77**, 145–152 (1986).
21) Tsuda, H., Hirohashi, S., Shimosato, Y., Hirota, T., Tsugane, S., Yamamoto, H., Miyajima, N., Toyoshima, K., Yamamoto, T., Yokota, J., Yoshida, T., Sakamoto, H., Terada, M. and Sugimura, T. Correlation between long-term survival in breast cancer patients and amplification of two putative oncogene-coamplification units: hst-1/int-2 and erbB2/ear-1. *Cancer Res.*, **49**, 3104–3108 (1989).
22) Maurizi, M., Scambia, G., Panici, P. B., Ferrandinam, G., Almadori, G., Paludetti, G., Vincenzo, R. D., Distefano, M., Brinchi, D., Cadoni, G. and Mancuso, S. EGF receptor expression in primary laryngeal cancer: correlation with clinico-pathological features and prognostic significance. *Int. J. Cancer*, **52**, 862–866 (1992).
23) Yudoh, K., Matsui, H., Kanamori, M., Maeda, A., Ohmori, K. and Tsuji, H. Inhibitory effect of monoclonal antibody to epidermal growth factor receptor on tumor growth and metastasis in high- and low-metastatic clones of murine RCT sarcoma. *J. Exp. Clin. Cancer Res.*, in press.
24) Lund-Johansen, M., Bjerkvig, R., Humphrey, P. A., Bigner, S. H., Bigner, D. D. and Laerum, O. D. Effect of epidermal growth factor on glioma cell growth, migration, and invasion *in vitro*. *Cancer Res.*, **50**, 6039–6044 (1990).
25) Engebraaten, O., Bjerkvig, R., Pedersen, P. H. and Laerum, O. D. Effects of EGF, bFGF, NGF and PDGF-(bb) on cell proliferative, migratory and invasive capacities of human brain-tumor biopsies *in vitro*. *Int. J. Cancer*, **53**, 209–214 (1993).
26) Mizoguchi, H., Komiyama, S., Matsui, K., Hamanaka, R., Ono, M., Kiue, A., Kobayashi, M., Shimizu, N., Welgus, H. G. and Kuwano, M. The response to epidermal growth factor of human maxillary tumor cells in terms of tumor growth, invasion and expression of proteinase inhibitors. *Int. J. Cancer*, **49**, 738–743 (1991).
27) Yoshida, K., Kuniyasu, H., Yasui, W., Kitadai, Y., Toge, T. and Tahara, E. Expression of growth factors and their receptors in human esophageal carcinomas: regulation of expression by epidermal growth factor and transforming growth factor α. *J. Cancer Res. Clin. Oncol.*, **119**, 401–407 (1993).
28) Fidler, I. J. Selection of successive tumor lines for metastasis. *Nature*, **242**, 148–149 (1973).
29) Matsui, H. Experimental study on prophylactic whole lung irradiation for the metastases of murine RCT sarcoma with special reference to the effectiveness and the side effects. *J. Jpn. Soc. Cancer Ther.*, **21**, 786–796 (1986).
30) Matsui, H., Tatezaki, S., Tsuji, H. and Ochiai, H. Isola-

tion and characterization of low- and high-metastatic clones from murine RCT (Radiological, Chiba and Toyama) sarcoma. *J. Cancer Res. Clin. Oncol.*, **115**, 9–16 (1989).
31) Kawamoto, T., Satom, J. D., Le, A., Polikoff, J., Sato, G. H. and Mendelsohn, J. Growth stimulation of A431 cells by epidermal growth factor: identification of high-affinity receptors for epidermal growth factor by an anti-receptor monoclonal antibody. *Proc. Natl. Acad. Sci. USA*, **80**, 1337–1341 (1983).
32) Ennis, B., Valverius, E., Bates, S., Lippman, M., Bellot, F., Kris, R., Schlessinger, J., Masui, H., Goldenberg, A., Mendelsohn, J. and Dickson, R. Anti-epidermal growth factor receptor antibodies inhibit the autocrine-stimulated growth of MDA-468 human breast cancer cells. *Mol. Endocrinol.*, **3**, 1830–1838 (1989).
33) Sunda, H., Yu, P., Peacock, J. and Mendelsohn, J. Modulation of tyrosine, serine and threonine phosphorylation and intracellular processing of the epidermal growth factor receptor by anti-receptor monoclonal antibody. *J. Cell Physiol.*, **142**, 284–292 (1990).
34) Chau-Jye, F., Sherwood, E. R., Mendelsohn, J., Lee, C. and Kozlowski, J. M. Epidermal growth factor receptor monoclonal antibody inhibits constitutive receptor phosphorylation, reduces autonomous growth, and sensitizes androgen-independent prostatic carcinoma cells to tumor necrosis. *Cancer Res.*, **52**, 5887–5892 (1992).
35) Voldavsky, I., Ariav, Y., Atzmon, R. and Fukus, Z. Tumor cell attachment to the vascular endothelium and subsequent degradation of the subendothelial extracellular matrix. *Exp. Cell Res.*, **140**, 149–159 (1982).
36) Iwamoto, Y., Fujita, Y. and Sugioka, Y. YIGSR, a synthetic laminin peptide, inhibits the enhancement by cyclophosphamide of experimental lung metastasis of human fibrosarcoma cells. *Clin. Exp. Metastasis*, **10**, 183–189 (1992).
37) McCarthy, J. B. and Furcht, L. T. Laminin and fibronectin promote the haptotactic migration of B16 mouse melanoma cells *in vitro*. *J. Cell Biol.*, **98**, 1474–1480 (1984).
38) Makiyama, N., Matsui, H., Tsuji, H. and Ichimura, K. Attachment and invasion of high- and low-metastatic clones of RCT sarcoma in a three-dimensional culture system. *Clin. Exp. Metastasis*, **9**, 411–415 (1991).
39) Varani, J., Lovett, E. J., McCoy, J. P., Shibata, S., Maddox, D. E., Goldstein, I. J. and Wicha, M. Differential expression of a laminin-like substance by high- and low-metastatic tumor cells. *Am. J. Pathol.*, **111**, 27–34 (1983).
40) Albini, A., Aukerman, L., Ogle, R. C., Noonan, D. M., Fridman, R., Martin, G. R. and Fidler, I. J. The *in vitro* invasiveness and interactions with laminin of K-1735 melanoma cells. Evidence for different laminin-binding affinities in high and low metastatic variants. *Clin. Exp. Metastasis*, **7**, 437–451 (1989).
41) Barsky, S. H., Rao, C. N., Williams, J. E. and Liotta, L. A. Laminin molecular domains which alter metastasis in a murine model. *J. Clin. Invest.*, **74**, 843–848 (1984).
42) Turpeenniemi-Hujanen, T., Thorgeirsson, V. P., Hart, I. R., Grant, S. S. and Liotta, L. A. Expression of collagenase IV (basement membrane collagenase) activity in murine tumor cell hybrids that differ in metastatic potential. *J. Natl. Cancer Inst.*, **75**, 99–103 (1985).
43) Mark, E. S. and Min, W. Type IV collagenase (Mr 72,000) expression in human prostate: benign and malignant tissue. *Cancer Res.*, **53**, 878–883 (1993).
44) Werb, Z., Tremble, P. M., Behrendtsen, O., Growley, E. and Damsky, C. H. Signal transduction through the fibronectin receptor induces collagenase and stromelysin gene expression. *J. Cell Biol.*, **109**, 877–889 (1989).
45) Turpeenniemi-Hujanen, T., Thorgeirsson, U. P., Rao, C. N. and Liotta, L. A. Laminin increases the release of type IV collagenase from malignant cells. *J. Biol. Chem.*, **261**, 1883–1889 (1986).
46) Liotta, L. A., Mandler, R., Murano, G., Katz, D. A., Gordon, R. K., Chiang, P. K. and Schiffmann, E. Tumor cell autocrine motility factor. *Proc. Natl. Acad. Sci. USA*, **83**, 3302–3306 (1986).