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Tumor Cell Attachment to Laminin Promotes Degradation of the Extracellular Matrix and Cell Migration in High-metastatic Clone Cells of RCT Sarcoma *in vitro*

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We investigated the roles of extracellular matrix proteins, laminin and fibronectin, in promoting invasiveness through the extracellular matrix in high-metastatic [RCT(+)] clone cells established from poorly differentiated murine RCT sarcoma in C3H/He mice. Laminin stimulated the type IV collagenolytic activity of RCT(+) cells. After more than 6 h of incubation, the type IV collagenolysis of the cell-conditioned medium was significantly higher in laminin-treated groups compared with the control. The migration activity of RCT(+) cells was stimulated by laminin. However, fibronectin did not influence the type IV collagenolysis or cell migration in this clone cell. The amino acid sequence YIGSR, which is derived from laminin, inhibited the laminin-mediated cell attachment and the laminin-promoted type IV collagenolysis, as well as cell migration of RCT(+) cells. RGD derived from fibronectin did not influence the cell attachment to laminin or Matrigel in this clone. In the invasion assay employing a Matrigel coated filter in a Boyden chamber, YIGSR showed greater inhibition of invasion through the Matrigel than did RGD with RCT(+) cells. YIGSR might inhibit the promoted-matrix degradation and cell migration in response to the cell attachment to laminin by competing with laminin for binding to cell surface laminin receptor. We suggest that laminin-mediated cell attachment to the extracellular matrix may play a role in promoting the matrix degradation and cell migration during metastatic cascades.

Key words: Laminin — Fibronectin — Invasion — Extracellular matrix — Murine sarcoma

During metastatic cascades, tumor cells must pass through the extracellular matrix and basement membrane as they initially invade the lymphatic or blood vessels, and when they penetrate into the target organ. Following tumor cell attachment to the extracellular matrix, tumor cells secrete proteolytic enzymes that cause the degradation of the matrix, thereby enabling the cells to migrate into the region of the matrix modified by proteolysis and enter the circulation.[1–3] The interaction of tumor cells with the extracellular matrix is a critical step in tumor metastasis.

Laminin, the major noncollagenous component of the basement membrane, is an important promoter of cell-matrix interactions. In various tumor cell lines, the level of laminin receptor expression is higher in metastatic tumor cells than in nonmetastatic tumor cells,[4–6] and laminin-adherent cells selected *in vitro* were shown to be more malignant *in vivo* than unattached cells.[7] A site on the B1 chain of laminin, the pentapeptide YIGSR, has been shown to promote cell attachment to laminin and to block lung metastasis, suggesting that laminin promotes metastasis by facilitating attachment of tumor cells to the extracellular matrix.[8, 9] Furthermore, it has been suggested that binding of tumor cells to extracellular matrix proteins such as laminin and fibronectin might induce matrix-degrading proteases.[10, 11] We have found that exogenous laminin stimulates type IV collagenolytic activity of high-metastatic clone cells of RCT (Radiological, Chiba and Toyama) sarcoma *in vitro*.[12] These findings suggested a possible role of tumor cell attachment to laminin in the degradation of the extracellular matrix.

In the present study, we investigated the role of tumor cell attachment to the extracellular matrix proteins laminin and fibronectin, which are known to have an adhesive function, in promoting both the degradation of the extracellular matrix and cell migration, using high-metastatic clone cells of RCT sarcoma. We also evaluated the inhibitory effect of the amino acid sequence YIGSR derived from laminin or RGD derived from fibronectin on the invasiveness of this clone into the extracellular matrix.

MATERIALS AND METHODS

Cell culture High-metastatic [RCT(+)] clone cells of RCT sarcoma obtained by the combination of lung passage and the limiting-dilution method were cultured in a 25 cm^2 plastic flask (MS-20050, Sumitomo Bakelite, Tokyo).[13–15] The clone cells were cultured in RPMI 1640 (Flow Laboratories, Inc., Irvine, Scotland), supplemented with 100 μg/ml streptomycin (Meiji Seika, Tokyo), 100 U/ml penicillin (Meiji Seika) and 10% fetal bovine

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serum (FBS; Bocknek Ltd., Toronto, Canada) at 37°C under a humidified atmosphere (5% CO_2 and 95% air).

Attachment assay Assay was performed in the culture dishes (diameter: 35 mm) as described previously.[12] Laminin (20.0 μg/dish, Collaborative Research, Inc., Bedford, MA), fibronectin (20.0 μg/dish, UCB-Bioproducts S. A., Belgium) or reconstituted basement membrane (5.0 μg/dish, Matrigel: Collaborative Research Inc.) was precoated on the culture dishes. Unbound surfaces were blocked with 200 μl of 1% bovine serum albumin (BSA; Sigma Chemical, St. Louis, MO) for 30 min at 25°C.

Subconfluent cultures of RCT(+) cells were prelabeled with 18.5 kBq/ml ^{3}H-thymidine (74 kBq/mmol, ICN, Costa Mesa, CA) for 24 h in the culture medium. The cells were washed twice with cold phosphate-buffered saline (PBS) to remove free radioisotope followed by resuspension in RPMI with 0.1% BSA. The labeled cells (1×10^5/dish) were placed on the coated dishes and incubated at 37°C. As the control, cells were incubated on uncoated culture dishes.

For assessment of the effect of YIGSR (Bachem Inc., Torrance, CA) or RGD (Sigma) on cell attachment to laminin, fibronectin or Matrigel, the cells (1×10^5/dish) suspended in RPMI with 0.1% BSA were incubated with YIGSR (20.0 μg/ml) or RGD (20.0 μg/ml) on the coated dishes. After 30 min of 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, which was then dried overnight in a laminar air flow apparatus. For assay of the type IV collagenolytic activity induced by cell attachment to laminin or fibronectin, cells (1×10^5/dish) suspended in RPMI containing 0.1% BSA were placed on culture dishes coated with laminin (20.0 μg/ml) or fibronectin (20.0 μg/ml) and incubated at 37°C for 2, 4, 6, 8, 10 and 12 h. As the control, cells were incubated on uncoated culture dishes. At the end of each incubation, the cell-conditioned medium was added to a 96-well tissue culture plate precoated with ^{3}H-labeled type IV collagen. After 24 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 radioactivity in the supernatant, and expressed as the net amount (ng) of degraded type IV collagen.[16, 17] For the study of the effect of YIGSR or RGD on the laminin-promoted or fibronectin-promoted type IV collagenolysis of RCT(+) cells, the cells (1×10^5/dish) suspended in RPMI containing 0.1% BSA were incubated with YIGSR (20.0 μg/ml) on laminin-coated dishes, or with RGD (20.0 μg/ml) on fibronectin-coated dishes at 37°C for 8 h.

Migration assay To investigate the migration activity mediated by laminin of fibronectin, a Boyden chamber with a built-in uncoated filter was used in the migration assay.[12, 18] Polyvinyl-pyrrolidone-free polycarbonate filters (8 μm pore size; Nucleopore, CA) were placed in the Boyden chambers. Cells (1×10^5/chamber) suspended in RPMI containing 0.1% BSA were added to the upper chamber and incubated with laminin (20.0 μg/ml) or fibronectin (20.0 μg/ml) at 37°C for 8 h. As the control, cells were incubated on the filter alone. After the 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. Each assay was performed in triplicate, and repeated three times.

In the study of the effect of YIGSR or RGD on laminin-promoted or fibronectin-promoted cell migration, the cells (1×10^5/chamber) suspended in RPMI containing 0.1% BSA were incubated with laminin in the presence of YIGSR (20.0 μg/ml) or with fibronectin in the presence of RGD (20.0 μg/ml) at 37°C for 8 h.

Invasion assay The invasiveness of RCT(+) cells was assayed according to the method reported by Albini *et al.*[19] Polycarbonate filters (8 μm pore size) were coated with 50 μg/filter of Matrigel. 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 and incubated for 8 h at 37°C in 5% CO_2 and 95% air. After each incubation, the procedure was the same as that for the migration assay.

To examine the effect of YIGSR or RGD on tumor cell invasion, the cells (1×10^5) suspended in RPMI containing 0.1% BSA were incubated with or without various concentrations (1.0–50.0 μg/ml) of YIGSR or RGD in the upper compartment of the 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.

Statistical analysis Values were expressed as means ± standard deviations (SD). Student's *t* test was used to analyze the results, and the criterion of statistical significance was $P<0.05$.

RESULTS

Cell attachment At 30 min of incubation, the values of the mean percentage of RCT(+) cell attachment to dishes coated with laminin, fibronectin and Matrigel were 81%, 63% and 72%, respectively. The ability of RCT(+) cells to attach to laminin, fibronectin or Matrigel was significantly higher than that of the control (Fig. 1).

YIGSR significantly inhibited the attachment of RCT(+) cells to the laminin-coated as well as the Matrigel-coated dishes. However, the ability of the cells to attach to fibronectin-coated dishes was not affected by YIGSR. RGD inhibited the attachment of the cells to the fibronectin-coated dishes, whereas there was no significant difference in the ability to attach to laminin-coated or to Matrigel-coated dishes between the RGD-treated group and the control group (Table I).

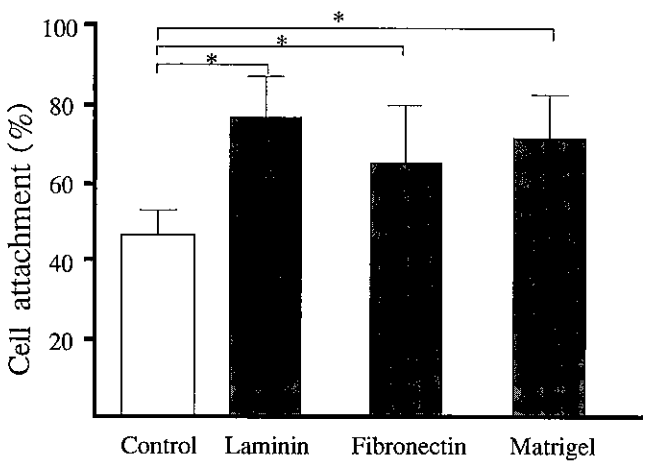

Fig. 1. The ability of RCT(+) cells to attach to laminin, fibronectin or Matrigel. Columns and bars show the mean± standard deviations (n=9 each). *: $P<0.05$.

Table I. Effect of YIGSR and RGD on RCT(+) Cell Attachment to Extracellular Matrix Components

Group	Number of attached cells×10^{-4} cells (mean±SD)		
	Laminin (20 μg/ml)	Fibronectin (20 μg/ml)	Matrigel (5 μg/ml)
Control	5.36±0.52	4.78±0.86	5.79±1.12
YIGSR (20 μg/ml)	1.86±0.48	4.35±0.68	2.26±0.72
RGD (20 μg/ml)	4.87±0.68	2.35±0.58	5.12±0.78

*: $P<0.05$. **: $P<0.01$.

Type IV collagenolytic activity mediated by cell attachment to laminin or fibronectin The mean amount of degraded type IV collagen increased with time in both the laminin-treated and control groups. After more than 6 h of incubation, the type IV collagenolytic activity of RCT(+) cell-condition medium was significantly higher in laminin-treated groups than in the control groups (Fig. 2).

In the presence of YIGSR, laminin did not affect the degradation of type IV collagen in RCT(+) cells. Regardless of the presence of RGD, there was no significant difference in type IV collagenolytic activity between the fibronectin-treated group and the control group (Table II).

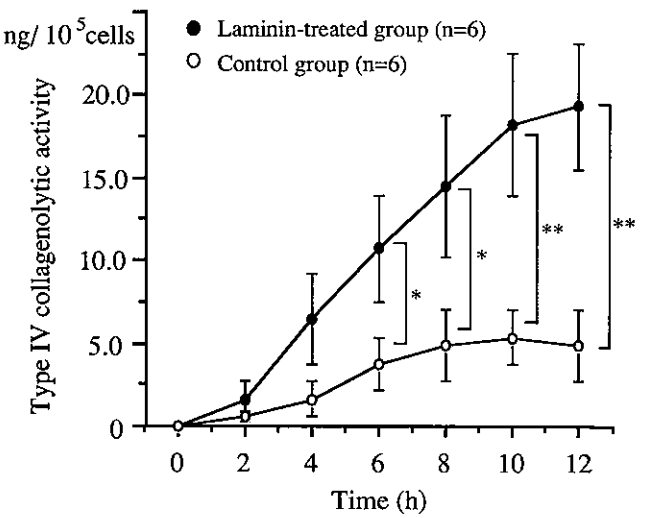

Fig. 2. Time course of laminin effect on type IV collagenolysis. Each point represents the mean from six cultures. Bars indicate standard deviations. *: $P<0.05$. **: $P<0.01$.

Table II. Type IV Collagenolytic and Migration Activities Mediated by Laminin or Fibronectin

Group	Type IV collagenolytic activity (ng/10^5 cells)	Number of migrating cells/10^5 cells (mean±SD)
Control	5.46±2.16	42±12
Laminin (20 μg/ml)	15.36±3.57	68±18
YIGSR (20 μg/ml)	6.12±2.09	39±13
YIGSR (20 μg/ml) +Laminin (20 μg/ml)	5.89±1.86	46±11
Fibronectin (20 μg/ml)	5.98±2.46	41±11
RGD (20 μg/ml)	6.12±1.89	38±8
RGD (20 μg/ml) +Fibronectin (20 μg/ml)	5.87±1.92	44±12

*: $P<0.05$.

Table III. Effects of YIGSR and RGD on Cell Invasion in RCT(+) Cells

Group	Concentration (μg/ml)	Number of invading cells/field (Matrigel: 50 μg/filter) (mean±SD)
Control		31±8
YIGSR	1.0	28±9
	5.0	18±6*
	10.0	13±5*
	20.0	7±3**
	50.0	8±4**
RGD	1.0	32±6
	5.0	27±5
	10.0	24±9*
	20.0	19±8*
	50.0	20±7*

*: $P<0.05$. **: $P<0.01$.

Migration activity mediated by laminin or fibronectin In the presence of laminin, the number of RCT(+) cells which migrated through the filter was significantly increased compared with the control. In the presence of YIGSR, the number of the cells which migrated through the filter showed no significant laminin-induced change (Table II). In the presence or absence of RGD, there was no significant difference in the number of cells passing through the filter between the fibronectin-treated group and the control group (Table II).

Inhibitory effect of YIGSR or RGD on invasiveness of tumor cells into Matrigel YIGSR inhibited the invasiveness of RCT(+) cells into Matrigel compared with the control in a concentration-dependent manner. The number of cells which invaded the Matrigel was significantly decreased at the YIGSR concentration of 5.0 μg/ml. Maximal inhibition of cell invasion was obtained with a YIGSR concentration of 20.0 μg/ml. RGD also inhibited cell invasion through the Matrigel in a concentration-dependent manner. The cells showed maximal inhibition by RGD at the same concentration as YIGSR (20.0 μg/ml) (Table III). However, in terms of the number of the cells invading the Matrigel, the mean inhibition ratio induced by RGD (38%) was lower than that by YIGSR (78%) at the concentration of 20.0 μg/ml.

DISCUSSION

RCT(+) cells were isolated in our department as a high-metastatic clone from RCT sarcoma, inducing spontaneous pulmonary metastasis at 2 to 3 weeks after subcutaneous implantation.[15] Tumor cell invasion of the extracellular matrix involves three crucial steps, i.e., tumor cell attachment to, degradation of, and migration into the extracellular matrix.[2] We previously reported that *in vitro* invasiveness is correlated with metastatic potential,[20] and that the attachment of the cells to the extracellular matrix and the degradation of type IV collagen by the cells are significantly greater in the case of high-metastatic RCT(+) cells than low-metastatic clone cells of RCT sarcoma, whereas there is no significant difference in the migration of these clone cells into the extracellular matrix.[12] These findings suggested that tumor cell attachment and degradation of the extracellular matrix are important factors in the invasiveness of RCT sarcoma cells.

Our previous work showed that type IV collagenolytic activity of RCT(+) cells is stimulated by exogenous laminin in the culture medium.[12] To elucidate whether tumor cell attachment to laminin and fibronectin promotes the degradation of the matrix, type IV collagenolytic activity of RCT cell-conditioned medium, which was obtained by the incubation of the cells on laminin- or fibronectin-precoated dishes, was compared with that of the control cell-conditioned medium obtained on uncoated dishes. RCT(+) cell-conditioned medium from laminin-coated dishes showed a significantly higher level of type IV collagenolysis than the control. However, there was no significant difference in type IV collagenolytic activity between fibronectin-treated cells and the control cells. These findings suggested that the attachment of RCT(+) cells to extracellular matrix protein, especially laminin, induced the secretion of type IV collagenase by the cells. Type IV collagen is composed of a polygonal network which forms the main structure of the extracellular matrix and acts as a tissue barrier in the metastatic cascade. It has been reported that the metastatic potential of tumor cells is correlated with the enzymatic degradation of type IV collagen by metalloproteases.[17, 21–23] The present data provide evidence that the triggering of extracellular matrix degradation might be mediated by tumor cell attachment to laminin in the extracellular matrix.

Haptotactic migration is important for extravasation once tumor cells have come into contact with the extracellular matrix following endothelial cell retraction or in regions of exposed basement membrane. It has been reported that laminin and fibronectin promote the haptotactic migration of melanoma cells.[8, 18] Here, in RCT(+) cells, the migration activity was significantly stimulated in the presence of laminin. However, the laminin-promoted migration was not a result of haptotactic response to laminin. The accelerated migration of the cells might be due to stimulated cell motility in response to laminin. Fibronectin had no effect on the cell migration of RCT(+) cells. The results of the present

study suggest that type IV collagenolytic and migration activities of tumor cells might be accelerated following the attachment of tumor cells to laminin in the extracellular matrix.

YIGSR derived from laminin and RGD derived from fibronectin have been used to inhibit laminin-mediated and fibronectin-mediated cell attachment, respectively.[8, 24, 25] Here, YIGSR inhibited the attachment of RCT(+) cells to not only laminin but also Matrigel, whereas RGD only inhibited the cell attachment to fibronectin. RGD did not influence cell attachment of this clone to Matrigel. Matrigel, which consists of laminin, fibronectin and type IV collagen, has been used as a reconstituted basement membrane in *in vitro* invasion assay. Thus, the above findings suggested that cell attachment to the extracellular matrix might be mainly mediated by laminin via cell surface laminin receptors in RCT(+) cells.

YIGSR significantly inhibited the increased type IV collagenolytic and migration activities of RCT(+) cells in response to cell attachment to laminin. YIGSR and RGD significantly inhibited the invasiveness of the cells through Matrigel. The inhibition potential of YIGSR was approximately two-fold that of RGD in the case of RCT(+) cells. YIGSR might inhibit not only the laminin-mediated cell attachment to the matrix but also the type IV collagenolysis- and cell migration-promoting activities in response to tumor cell attachment to laminin by competing with laminin for binding to tumor cell surface laminin receptors. These findings suggest that the laminin-induced matrix degradation and cell migration were mediated via a common sequence of the cell-binding site on the laminin receptor. Thus, the use of YIGSR might offer a promising therapeutic approach for the prevention of tumor invasion and metastasis.

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