Jpn. J. Cancer Res. 84, 558–565, May 1993

# Antimetastatic Activity of Polymeric RGDT Peptides Conjugated with Poly(ethylene glycol)

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**Polymeric peptides containing defined repetitive or cyclic structures of RGDT sequence, $(RGDT)_n$ (n=1 to 11) and cyclo$(RGDT)_n$ (n=2 to 4), at a dose of 500 μg exhibited an inhibitory effect on experimental lung metastasis upon co-injection with tumor cells and the magnitude of the effect increased in parallel with the increase of degree of repetition of the RGDT sequence. The conjugation of $(RGDT)_n$ (n=1, 5, 11) with poly(ethylene glycol), PEG as a polymeric carrier led to enhanced inhibition of lung metastasis in proportion to the degree of RGDT sequence repetition and in a dose-dependent manner. Multiple i.v. administrations of PEG-$(RGDT)_{11}$, at 2-day and 3-day intervals before the excision of primary tumors, effectively inhibited spontaneous lung metastasis by s.c. inoculation of tumors, whereas $(RGDT)_{11}$ exhibited inhibition of lung metastasis only when given at 2-day intervals. This indicates that the conjugation of PEG with $(RGDT)_n$ allowed the prolongation of administration interval, implying a sustained inhibitory effect on tumor metastasis. In support of this supposition, a decrease in the arrest of radiolabeled tumor cells in the lungs was observed when PEG-$(RGDT)_{11}$ was co-injected i.v. with tumor cells, or injected i.v. one day before tumor inoculation. In contrast, $(RGDT)_{11}$ significantly inhibited the tumor cell arrest in the lungs only upon co-injection with tumor cells. We also noted that $(RGDT)_n$, cyclo$(RGDT)_n$ and PEG-$(RGDT)_{11}$ inhibited tumor cell invasion into Matrigel in a concentration-dependent manner and in proportion to the degree of RGDT sequence repetition, indicating that the peptide-mediated antimetastatic effect is partly associated with the anti-invasive potential. Thus, the conjugation of anti-cell adhesive and anti-metastatic RGDT peptide with PEG might provide a therapeutically promising basis for the prevention of cancer metastasis ("anti adhesion therapy").**

Key words: Metastasis — Invasion — RGDT peptide — Drug carrier

During the metastatic cascade, tumor cells interact with various host cells (endothelial cells, platelets or lymphocytes) and/or extracellular matrix (ECM) and basement membrane components (laminin, fibronectin, etc.).[1–4] Such adhesive interaction may lead to the enhancement of survival, arrest, or invasiveness of tumor cells and is one of the most important events in the metastatic process.[4–7] Therefore, understanding the controlling mechanism of metastasis (in particular, the adhesive interaction with tumor cells) may assist in the development of antimetastatic or anti-adhesive therapies.

Extensive studies of the interactions of the cells with ECM have led to elucidation of the primary structures of some cell adhesive proteins such as fibronectin, laminin and vitronectin, and their cell surface receptors including integrins have been identified.[8–11] A characteristic Arg-Gly-Asp (RGD) sequence within fibronectin and other related adhesion molecules has been demonstrated to be a recognition site for adhesion, spreading and motility of the cells.[12–15] The RGD-containing peptides have been used to inhibit fibronectin-mediated cell adhesion and spreading *in vitro*, platelet aggregation, and the migration of neural crest cells.[12–15] Various attempts have been made to regulate cell adhesion during the metastatic process, and RGD peptide has been reported to inhibit experimental lung metastasis when co-injected i.v. with B16 melanoma cells.[16,17] However, a high dose of RGD oligopeptides was needed to obtain a sufficient effect because of rapid clearance of the peptides from the circulation or low affinity with their cell surface receptors, and so on. Therefore, it seems important to find superior peptide analogues and improved methods to control tumor metastasis.

We have previously reported that poly(RGD), which contains a repetitive RGD sequence, inhibited experimental and spontaneous tumor metastasis of different tumors, as well as cell-adhesive properties, more effectively than RGD-containing oligopeptides, and we showed that radiolabeled polymeric peptide was biphasically cleared from the circulation following i.v. injection more slowly than the oligopeptide.[18–20] This suggested that polymerization of RGD sequence was able to augment the inhibition of tumor metastasis and that the inhibitory effect was partly due to slower clearance and

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slower decomposition. Other attempts, including cyclization of RGD peptide, have also been made to enhance the inhibitory effect on tumor metastasis and platelet aggregation.[21-25]

In order to suppress the toxic side effects of anticancer drugs and to improve their activity against malignant tumors, conjugation of the drugs to polymeric carriers is a promising approach, which is expected to provide certain advantageous features such as better distribution of drugs to the designated tissue, prolonged half-lives of drugs in plasma, controlled drug release, etc. Several kinds of polymers have been extensively investigated as carriers, such as poly(ethylene glycol),[26] poly(divinyl ether-co-maleic anhydride),[27] poly[N-2-(hydroxypropyl) methacrylamide],[28] and dextran,[29] etc. In particular, poly(ethylene glycol), which is referred to as PEG hereafter, is a useful, nontoxic and nonimmunogenic water-soluble polymer. In the present study, we synthesized polymeric peptides containing defined repetitive or cyclic structures of Arg-Gly-Asp-Thr (RGDT) sequence i.e. $(RGDT)_n$ or $cyclo(RGDT)_n$ and their PEG conjugates, and we examined the effect of these polymeric peptides on experimental and spontaneous lung metastasis in mice.

## MATERIALS AND METHODS

**Mice** Specific pathogen-free female C57BL/6 mice, 7–10 weeks old, were purchased from Japan SLC, Inc., Hamamatsu. Mice were maintained in the Laboratory for Animal Experiment, Institute of Immunological Science, Hokkaido University, under laminar air-flow conditions. All mice used in this study were sex-matched.

**Cells** Highly metastatic B16-BL6 melanoma cells, obtained by an *in vitro* selection procedure for invasion,[30] were kindly provided by Dr. I. J. Fidler, M.D. Anderson Cancer Center, Houston, TX. The cells were maintained as monolayer cultures in Eagle's minimal essential medium (MEM) supplemented with 7.5% fetal bovine serum (FBS), vitamin solution, sodium pyruvate, nonessential amino acids, and L-glutamine.

**Synthesis of peptide analogues** Synthetic linear and cyclic peptide analogues possessing repetitive units of an RGDT sequence derived from fibronectin were prepared by a systematic liquid-phase method in which the peptides were synthesized by repeated condensation of a protected Asp-Thr-Arg-Gly (DTRG) unit. *t*-Butoxycarbonyl (Boc), phenacyl (Pac) and benzyl (Bzl) groups were utilized at the protecting groups for $\alpha$-amino and $\alpha$-carboxyl groups, and *p*-toluenesulfonyl (Tos), cyclohexyl (cHex) and Bzl groups were used for guanidino, $\beta$-carboxyl and hydroxyl groups. Coupling reactions of these peptide compounds were performed by the standard method employing 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide and 1-hydroxybenzotriazole. For the preparation of cyclic peptide analogues, cyclization reactions of the protected linear peptides were carried out by the active ester method in the presence of a large excess of pyridine. Removal of protecting groups from the resulting sequential linear and cyclic peptide analogues was accomplished by treatment with hydrogen fluoride. After deprotection, the crude peptides were purified by RP-HPLC to afford the desired products. All final products were confirmed by FAB-MASS spectrometry, amino acid analysis and amino acid sequence analysis. All the amino acids used in this study were of the L-form. Boc-amino acids and coupling reagents were purchased from Peptide Institute, Inc., Japan.

Hybridization of linear peptides was carried out with two types of PEG derivatives in 0.1 *M* borate buffer (pH 8.21). Complete modification of the linear peptides was confirmed by RP-HPLC and high-performance gel filtration chromatography (GPC). The obtained crude PEG-conjugated peptides were purified by RP-HPLC to give the final products, which were confirmed by amino acid analysis. The structures of these products are indicated in Fig. 1. PEG was purchased from Nippon Oils & Fats K.K., Japan.

**Assay for experimental and spontaneous lung metastases of melanoma cells** C57BL/6 mice were given i.v. injection of B16-BL6 melanoma cells ($5\times10^4$) with or without polymeric RGDT peptide analogues. Fourteen days after the inoculation of tumor cells, mice were killed and the number of lung tumor colonies was recorded (experimental metastasis). In a spontaneous lung metastasis assay, mice were given s.c. injections of B16-BL6 melanoma cells ($5\times10^5$) into the right hind footpad. Polymeric RGDT peptide analogues were administered i.v. on various days before the surgical excision of primary tumors on day 21. Mice were killed 14 days after surgery. The lungs were fixed in Bouin's solution and the lung tumor colonies were counted under a dissecting microscope.

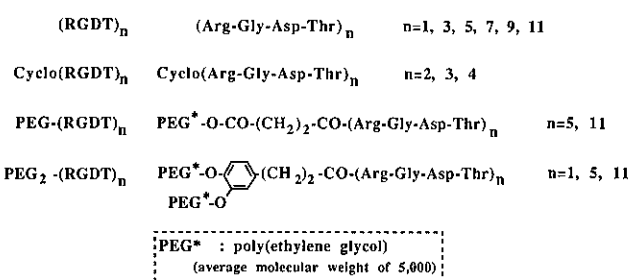


Fig. 1. Chemical structures of synthetic linear and cyclic peptide analogues containing repetitive RGDT sequence and the PEG-conjugates.

**Invasion assay** The invasive activity of tumor cells was assayed according to the method reported previously.[31] Polyvinylpyrrolidone-free polycarbonate filters with an 8.0-$\mu$m pore size (Nucleopore, Pleasanton, CA) were precoated with 5 $\mu$g of fibronectin in a volume of 50 $\mu$l on the lower surface and dried overnight at room temperature. Then Matrigel was diluted to 100 $\mu$g/ml with cold PBS, applied to the upper surface of the filters (5 $\mu$g/filter), and dried at room temperature under a hood. The coated filters were washed extensively in PBS and then dried immediately before use. The filters thus prepared were designated Matrigel/fibronectin-coated filters. Log-phase cell cultures of tumor cells were harvested with 1 mM EDTA in PBS, washed 3 times with serum-free MEM, and resuspended to a final concentration of $2\times10^6$/ml in MEM with 0.1% BSA. Cell suspensions (100 $\mu$l) with or without agents were added to the upper compartment and incubated for an appropriate number of hours at 37°C in a 5% $CO_2$ atmosphere. The filters were fixed with methanol and stained with hematoxylin and eosin. The cells on the upper surface of the filter were removed by wiping with cotton swabs. The cells that had invaded through the filter to various areas of the lower surface were manually counted under a microscope at a magnification of ×400, and each assay was performed in triplicate.

**Organ retention of radiolabeled tumor cells** B16-BL6 melanoma cells in the exponential growth phase were labeled with [$^{125}$I]iododeoxyuridine ($^{125}$I-UdR, specific activity: 200 mCi/mmol, New England Nuclear, Boston, MA). $^{125}$I-UdR-labeled tumor cells ($1\times10^5$) in a volume of 0.2 ml were co-injected i.v. with polymeric RGDT peptide analogues or injected i.v. into C57BL/6 mice that had received i.v. injection of the peptides one day before. Mice were killed at times ranging from 30 min to 8 h after the injection. The lungs, liver, spleen, kidneys and blood were collected from each mouse, and rinsed in 70% ethanol. The radioactivity in each organ was measured in a gamma counter.

**Statistical analysis** The statistical significance of differences between the groups was determined by applying Student's two-tailed $t$ test.

## RESULTS

**Effect of polymeric RGDT analogues on tumor metastasis of B16-BL6 melanoma** We first investigated the effect of polymeric RGDT analogues with defined degree of RGDT sequence repetition, $(RGDT)_n$ or cyclo-$(RGDT)_n$, on the experimental lung metastasis produced by i.v. injection of B16-BL6 melanoma. Peptides dissolved in PBS were admixed with B16-BL6 melanoma cells and the mixtures were immediately injected i.v. into C57BL/6 mice. Mice were killed 14 days after the co-injection. Fig. 2A shows that $(RGDT)_n$ peptides (n=1 to 11) at a dose of 500 $\mu$g resulted in significant inhibition of lung metastasis in proportion to the degree of RGDT sequence repetition and that such inhibitory effects of linear-type peptides with more than 5 RGDT sequences were maximum under the experimental conditions used. $(RGDT)_1$, i.e. RGDT tetrapeptide did not exhibit any significant inhibitory effect, whereas $(RGDT)_5$, $(RGDT)_7$, $(RGDT)_9$ and $(RGDT)_{11}$ exhibited dramatic inhibition of lung metastasis. As shown in Fig. 2B, similar results of antimetastatic activity were obtained by the co-injection of cyclo$(RGDT)_n$ (n=2, 3, 4) with tumor cells as the case of $(RGDT)_n$. However, $(RGDT)_5$ was more effective at inhibiting lung metastasis than cyclo-$(RGDT)_4$. These results indicated that the peptide-mediated inhibition of lung metastasis was positively associated with the increase of degree of RGDT repetition.

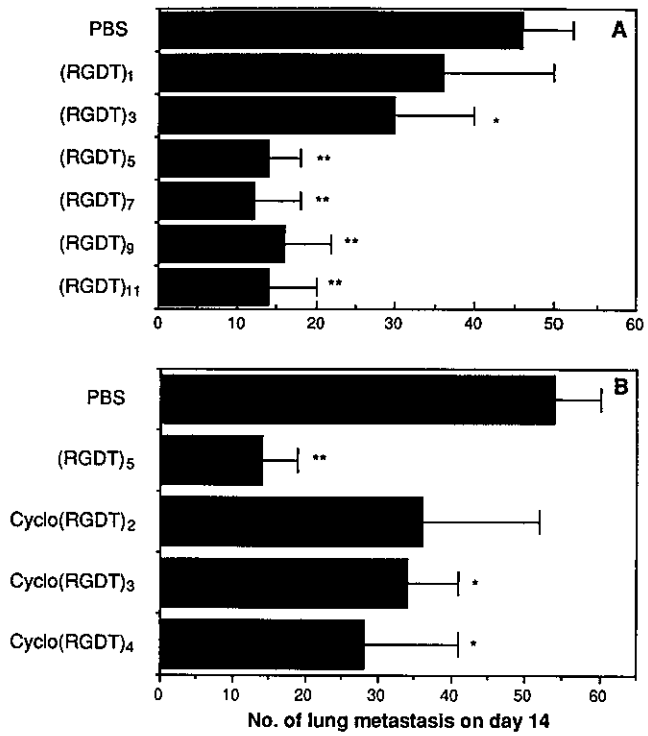


Fig. 2. Effect of polymeric RGDT peptides on experimental lung metastasis produced by i.v. injection of B16-BL6 melanoma cells. Five C57BL/6 mice per group were injected i.v. with B16-BL6 cells ($5\times10^4$) admixed with or without 500 $\mu$g of $(RGDT)_n$ (n=1 to 11) (A) or cyclo$(RGDT)_n$ (n=2 to 4) (B). Mice were killed 2 weeks after tumor inoculation and lung tumor colonies were measured. *: $P<0.05$, **: $P<0.001$ compared with untreated control by Student's two-tailed $t$ test.

**Inhibition of tumor metastasis by $(RGDT)_n$ conjugated with PEG** The above results demonstrated that polymeric peptides $(RGDT)_n$ (n=5 to 11) dramatically reduced the formation of B16-BL6 melanoma colonies in the lungs. To augment further the peptide-mediated antimetastatic effect, we next tried to conjugate $(RGDT)_n$ to PEG as a representative polymeric carrier and examined the effect of $PEG_2$-$(RGDT)_n$ (n=1, 5, 11) on experimental lung metastasis by i.v. injection of B16-BL6 melanoma. Table I shows that all the PEG conjugates, $PEG_2$-$(RGDT)_1$, $PEG_2$-$(RGDT)_5$ and $PEG_2$-$(RGDT)_{11}$ significantly inhibited lung metastasis upon i.v. co-injection

Table I. Effect of $PEG_2$-$(RGDT)_n$ Conjugates on Experimental Lung Metastasis Produced by i.v. Injection of B16-BL6 Melanoma

| Administered i.v. with: | No. of lung metastasis on day 14 Mean±SD (Range) | |
|---|---|---|
| Untreated (PBS) | 241±8 (230–250) | |
| $(RGDT)_{11}$ | 143±33 (92–185) | * |
| $PEG_2$-$(RGDT)_1$ | 108±6 (104–112) | * |
| $PEG_2$-$(RGDT)_5$ | 3±3 (0–6) | * |
| $PEG_2$-$(RGDT)_{11}$ | 0 | * |
| $PEG_2$ (1000 $\mu$g) | 196±28 (167–232) | |

Five C57BL/6 mice per group were inoculated i.v. with B16-BL6 ($3\times10^4$) with or without $PEG_2$-$(RGDT)_n$ conjugates (1000 $\mu$g peptide/mouse). Mice were killed 2 weeks after tumor inoculation and tumor colonies in the lungs were counted. *: $P<0.001$.

Table II. Effect of PEG-conjugated $(RGDT)_n$ on Experimental Lung Metastasis Produced by i.v. Injection of B16-BL6 Melanoma

| Administered i.v. with: | RGDT /mouse ($\mu$g) | No. of lung metastasis on day 14 Mean±SD (Range) | |
|---|---|---|---|
| Untreated (PBS) | | 190±47 (48–85) | |
| $(RGDT)_{11}$ | 1000 | 10±5 (0–14) | * |
| | 200 | 127±28 (101–168) | |
| | 40 | 156±34 (126–194) | |
| $PEG_2$-$(RGDT)_{11}$ | 1000 | 0 | * |
| | 200 | 50±12 (38–64) | * |
| | 40 | 118±43 (70–172) | |
| PEG-$(RGDT)_{11}$ | 1000 | 0 | * |
| | 200 | 68±8 (57–78) | * |
| | 40 | 125±11 (125–140) | |
| $PEG_2$ (3000 $\mu$g) | | 112±36 (78–145) | |

Five C57BL/6 mice per group were inoculated i.v. with B16-BL6 ($3\times10^4$) with or without PEG-conjugated $(RGDT)_n$. Mice were killed 2 weeks after tumor inoculation and tumor colonies in the lungs were counted. *: $P<0.001$.

with B16-BL6 cells as compared with the untreated control, and the inhibitory effect was in parallel with the increase of degree of RGDT sequence repetition. In particular, $PEG_2$-$(RGDT)_5$ and $PEG_2$-$(RGDT)_{11}$ were significantly more effective for the inhibition of lung metastasis than either $PEG_2$ or $(RGDT)_{11}$ alone. We also investigated the dose-response relation of two kinds of PEG-conjugated $(RGDT)_{11}$ on experimental lung metastasis of B16-BL6 melanoma (Table II). Lung tumor colonization of B16-BL6 cells was inhibited by $(RGDT)_{11}$ and the PEG-conjugates in a dose-dependent manner. $PEG_2$-$(RGDT)_{11}$ and PEG-$(RGDT)_{11}$ at the doses of 200 and 1000 $\mu$g showed significant inhibition of lung metastasis as compared with $(RGDT)_{11}$ alone. However, $PEG_2$ alone did not exhibit any significant effect. These results clearly indicated that the conjugation of $(RGDT)_{11}$ with PEG led to augmentation of the antimetastatic activity of $(RGDT)_{11}$. In the next set of experiments, we examined the therapeutic effect of multiple administrations of $(RGDT)_n$ or PEG-conjugated $(RGDT)_n$ on spontaneous lung metastasis caused by s.c. inoculation of B16-BL6 melanoma. Peptide analogues were administered i.v. into the lateral tail vein of mice after tumor inoculation. Expt. 1 of Table III shows that seven i.v. administrations of $(RGDT)_{11}$ at intervals of 2 days caused a marked decrease of lung tumor colonies. Multiple administrations of $(RGDT)_5$ were less effective for the inhibition of spontaneous lung metastasis than those of $(RGDT)_{11}$, although $(RGDT)_5$ at a dose of 500 $\mu$g was as active in inhibiting experimental lung metastasis produced by the i.v. co-injection with tumor cells as $(RGDT)_{11}$ (Fig. 2). On the other hand, administrations of PEG-$(RGDT)_5$ and PEG-$(RGDT)_{11}$ also exhibited a significant inhibition of spontaneous lung metastasis. In this experiment, i.v. administrations of these polymeric peptides and their PEG-conjugates did not affect the primary tumor growth (size) at the time of excision (day 21). As shown in Expt. 2 of Table III, when PEG-$(RGDT)_5$ or PEG-$(RGDT)_{11}$ was administered i.v. seven times at intervals of 3 days, a significant reduction of lung tumor colonization was observed. However, $(RGDT)_5$ and $(RGDT)_{11}$ did not exhibit any inhibitory effects in this treatment modality (3-day interval). These results indicate that PEG-$(RGDT)_n$ (n=5, 11) conjugates effectively inhibit spontaneous lung metastasis upon intermittent i.v. administrations at both 2-day and 3-day intervals, but $(RGDT)_{11}$ showed antimetastatic activity only with the 2-day treatment interval. This suggests that the conjugation of PEG with $(RGDT)_n$ allows prolongation of the administration interval by providing a sustained inhibitory effect on tumor metastasis. Multiple administrations of PEG alone were not effective for the inhibition of spontaneous lung metastasis of B16-BL6 cells, although Table I shows that the i.v. co-injection of

Table III. Effect of PEG-$(RGDT)_n$ on Spontaneous Lung Metastasis Caused by Intrafootpad Injection of B16-BL6 Melanoma Cells

| Administered i.v. with: | No. of lung metastasis on day 35 Mean±SD (range) | P |
|---|---|---|
| Expt. 1 | | |
| Untreated (PBS) day 7, 9, 11, 13, 15, 17, 19 | 73±12 (62–86) | |
| $(RGDT)_5$ | 49±24 (48–24) | |
| $(RGDT)_{11}$ | 11±6 (6–19) | <0.01 |
| PEG-$(RGDT)_5$ | 34±12 (18–45) | <0.01 |
| PEG-$(RGDT)_{11}$ | 28±10 (14–36) | <0.01 |
| Expt. 2 | | |
| Untreated (PBS) day 7, 10, 13, 16, 19, 22, 25 | 74±14 (61–89) | |
| $(RGDT)_5$ | 55±9 (43–66) | |
| $(RGDT)_{11}$ | 64±22 (48–96) | |
| PEG-$(RGDT)_5$ | 20±20 (8–43) | <0.01 |
| PEG-$(RGDT)_{11}$ | 30±19 (9–49) | <0.01 |
| PEG | 74±22 (31–102) | |

Five C57BL/6 mice per group were inoculated i.v. with PEG-$(RGDT)_n$ conjugates (100 $\mu$g peptide/mouse) at the indicated days after intrafootpad injection of B16-BL6 melanoma cells ($5\times10^5$). Primary tumors were surgically removed on day 21 and mice were killed 2 weeks after tumor excision.

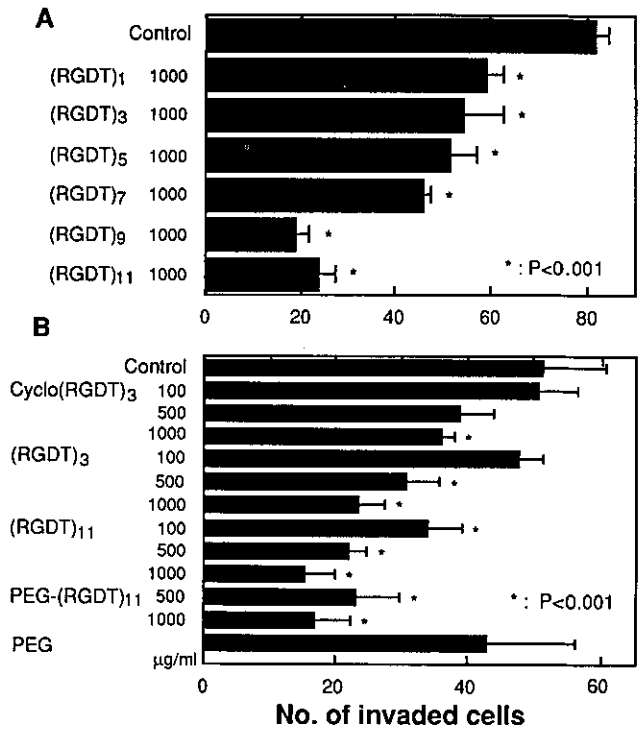


Fig. 3. Effect of polymeric RGDT peptide and the PEG-conjugates on the invasion of B16-BL6 melanoma cells into reconstituted basement membrane Matrigel. Filters were precoated with 5 $\mu$g of fibronectin on their lower surfaces, and with 5 $\mu$g of Matrigel on their upper surfaces. B16-BL6 cells ($2\times10^5$/well) in 0.1% BSA-medium were seeded with or without RGDT peptides (A and B) into the upper compartment of a Transwell cell culture chamber. After a 6-h incubation, the invaded cells on the lower surface were counted visually.

tumor cells with PEG caused a slight inhibition of experimental lung metastasis. This slight effect may be attributable to the physicochemical properties of PEG such as its high viscosity at high concentration.

**Effect of RGDT peptide analogues and PEG-$(RGDT)_{11}$ on tumor cell invasion** Tumor cell invasion into extracellular matrices and basement membranes is crucial in the complex multistage process of metastasis.[1-5,32] We therefore examined the effect of these antimetastatic RGDT peptides on tumor cell invasion of reconstituted basement membrane, Matrigel. Fig. 3A shows that the addition of 1000 $\mu$g/ml of $(RGDT)_n$ into the upper compartment of the chamber significantly inhibited the invasion of tumor cells through the Matrigel/fibronectin-coated filters in parallel with the increase of degree of RGDT sequence repetition. As shown in Fig. 3B, all the peptides and the PEG-conjugate used in this study inhibited tumor cell invasion into Matrigel in a concentration-dependent manner. It also seems likely that $(RGDT)_3$ is more active in inhibiting tumor invasion than cyclo$(RGDT)_3$ at concentrations ranging from 100 to 1000 $\mu$g/ml, although both peptides have the same molecular weight. Since our preliminary study indicated that cyclo$(RGDT)_3$ was much more effective for the inhibition of platelet aggregation elicited by ADP or collagen than linear $(RGDT)_3$ (data not shown), antimetastatic effects mediated by $(RGDT)_n$ or cyclo-$(RGDT)_n$ may be partly associated with the anti-invasive effect of the peptides. PEG-$(RGDT)_{11}$ showed similar inhibitory effects on tumor invasion to $(RGDT)_{11}$, but PEG alone did not exhibit any effect.

**Organ localization and retention of B16-BL6 melanoma cells co-injected with $(RGDT)_{11}$ and PEG-$(RGDT)_{11}$** We have previously demonstrated that poly(RGD) led to a decrease in the arrest and retention of tumor cells in the lungs after its co-injection with radiolabeled tumor cells,[20] and also that the clearance rate of the radiolabeled polypeptide from the circulation was approximately 6 times slower than that of a small RGD-containing peptide, i.e., GRGDS.[18] To investigate the mechanism responsible for the effective inhibition of tumor metastasis by conjugating $(RGDT)_n$ to PEG, we tested the organ retention and distribution of $^{125}$I-UdR-labeled tumor cells to see whether or not the injection of PEG-conjugates can lead to a decrease in the arrest of labeled melanoma cells in the capillary bed of the chosen organ. Labeled tumor cells were co-injected i.v. with $(RGDT)_{11}$ or PEG-$(RGDT)_{11}$, or injected i.v. into the

mice that had received i.v. injection of these peptides one day earlier. Mice were killed at various intervals following the injection, and their visceral organs were collected and monitored for radioactivity in a gamma counter. As shown in the upper panel of Fig. 4, significantly lower values were found in the lungs of mice at 4 h after the co-injection with $(RGDT)_{11}$ or PEG-$(RGDT)_{11}$. In contrast, significant reduction of tumor cell arrest in the lungs was observed in the mice which had been pretreated with PEG-$(RGDT)_{11}$ one day earlier, but not in the $(RGDT)_{11}$-pretreated mice. On the other hand, there was no significant difference between the control and either peptide-pretreated or -coinjected mice in the arrest and retention of labeled tumor cells in liver, kidneys (lower panel of Fig. 4), spleen and blood after tumor injection.

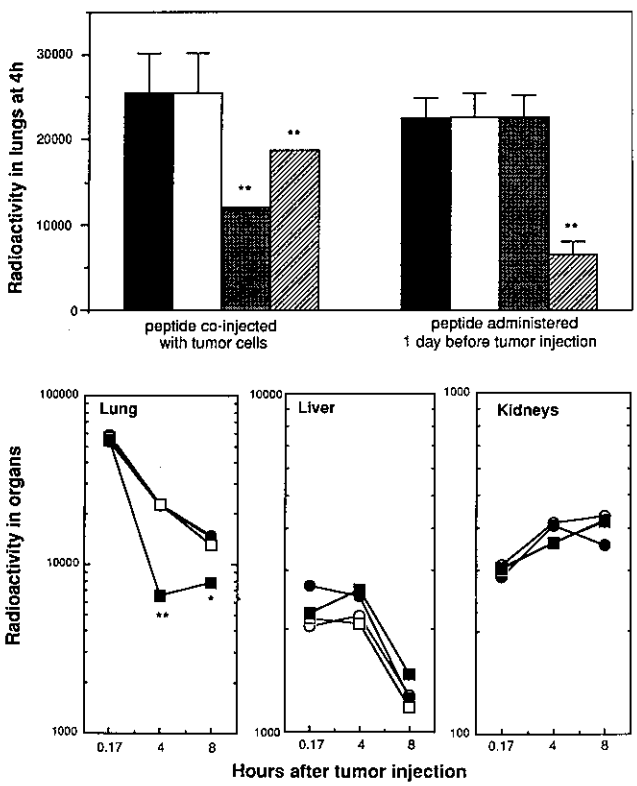


Fig. 4. Lung retention of $^{125}$I-UdR-labeled B16-BL6 melanoma cells co-injected i.v. into mice or mice pre-injected i.v. with peptides on day before. $^{125}$I-UdR-labeled B16-BL6 cells ($10^5$ per mouse) were co-injected i.v. with or without 1000 $\mu$g of $(RGDT)_{11}$ or PEG-$(RGDT)_{11}$ peptide (upper panel), or injected i.v into the mice which had received i.v. injection of 1000 $\mu$g of the peptides on the day before (upper and lower panels). At the indicated times, mice were killed and radioactivity retained in various organs was measured. Results are mean cpm of three mice per group. PBS (■, ○), PEG (□, ●), $(RGDT)_{11}$ (▨, □), PEG-$(RGDT)_{11}$ (▨, ■). *: $P<0.02$, **: $P<0.001$.

## DISCUSSION

Attempts have been made to control the mechanisms involved in cell functions such as adhesion, migration and invasion of tumor cells during the metastatic process. We have previously shown that poly(RGD), which contains a repetitive RGD sequence derived from fibronectin, with a relatively sharp molecular weight distribution (approximately 5,000 to 10,000), inhibited experimental and spontaneous lung metastasis through interference with cellular adhesive interactions between tumor and host, and brought about a prolongation of the survival of mice when it was administered i.v. after tumor inoculation.[18-20] Similar studies have shown that cyclization of RGDS derived from fibronectin or YIGSR derived from laminin effectively enhanced the inhibition of tumor metastasis and platelet aggregation.[21, 22, 24, 25] Therefore, the development of better peptide analogues and improved administration methods may provide a means for the prevention of cancer metastasis.

To extend our previous study on the antimetastatic effect of our original polymeric RGD peptides, we synthesized polymeric or cyclic RGDT peptides with defined sequence repetition, $(RGDT)_n$ (n=1 to 11) or cyclo-$(RGDT)_n$ (n=2 to 4), for examination of their inhibitory effects on lung metastasis of melanoma cells. The use of $(RGDT)_n$ and cyclo$(RGDT)_n$ caused significant inhibition of lung metastasis upon co-injection with melanoma cells in proportion to the increase of RGDT sequence repetition, and did so in a dose-dependent manner (Fig. 2 and Table II). Although $(RGDT)_5$ and $(RGDT)_{11}$ at the dose of 500 $\mu$g showed a similar inhibition of experimental lung metastasis (Fig. 1), multiple administrations of $(RGDT)_{11}$ were more effective for the inhibition of spontaneous lung metastasis than those of $(RGDT)_5$ (Expt. 1 of Table III). The antimetastatic effect of linear-type $(RGDT)_n$ clearly supported our previous findings using poly(RGD) with a relatively homogeneous molecular weight distribution.[18-20] The tumor cell invasion into Matrigel-coated filters *in vitro* was also inhibited by $(RGDT)_n$ in parallel with the increasing number of RGDT repeats and in a concentration-dependent fashion (Fig. 3). These results obviously indicate that the antimetastatic and anti-invasive effects of the polymeric peptides are positively associated with increasing repetition of RGDT sequence. We have previously reported that poly(RGD)-mediated inhibition of tumor metastasis may be related to the relatively (approximately 6-fold) slow clearance rate in the circulation as compared with the small RGD peptide.[18] Therefore, this may be a factor in the prominent effect of linear-type $(RGDT)_n$.

Most peptides, such as RGDS-containing peptides, as well as lymphokines and some anticancer drugs, have a

very short half-life in the circulation, which may result in a decrease of their therapeutic and biological potential *in vivo*. The control of drug release *in vivo* may lead to greater effectiveness in the expression of their biological effects. For this purpose, polymeric carriers including PEG have been utilized to achieve sustained delivery of drugs, proteins and macromolecules. To augment further the $(RGDT)_n$-mediated antimetastatic effect, we therefore attempted to conjugate $(RGDT)_n$ to nontoxic and nonimmunogenic water-soluble PEG as a representative carrier, and examined the inhibitory effect of the conjugates on tumor metastasis and invasion. PEG-conjugates of $(RGDT)_n$ (n=1, 5, 11) inhibited lung metastasis upon co-injection with melanoma cells effectively than $(RGDT)_n$ or carrier alone, and such an inhibitory effect was in proportion to the RGDT sequence repetition and was dose-dependent (Tables I and II). PEG-$(RGDT)_{11}$ and $PEG_2$-$(RGDT)_{11}$ were notably effective for the inhibition of experimental lung metastasis. PEG-$(RGDT)_{11}$, however, showed a similar inhibitory effect on *in vitro* tumor cell invasion into Matrigel to $(RGDT)_{11}$ (Fig. 3). These results indicated that the conjugation of $(RGDT)_n$ with PEG can augment the peptide-mediated antimetastatic effect *in vivo*.

In the spontaneous lung metastasis produced by s.c. inoculation of melanoma cells, intermittent i.v. administrations of PEG-$(RGDT)_{11}$ at 2-day and 3-day intervals resulted in the significant inhibition of lung metastasis before the primary tumor excision (Table III). In contrast, $(RGDT)_{11}$ showed significant inhibition of lung metastasis only in the treatment modality with 2-day intervals. This indicates that the conjugation of $(RGDT)_{11}$ with PEG may allow the prolongation of treatment interval, suggesting a sustained antimetastatic action of $(RGDT)_{11}$. The i.v. co-injection of $(RGDT)_{11}$ or PEG-$(RGDT)_{11}$ with radiolabeled melanoma cells resulted in a marked reduction of the tumor cell arrest in the lung (but not in other organs) (Fig. 4). Significantly lower values in the lungs were also observed when PEG-$(RGDT)_{11}$ was injected i.v. one day before the i.v. injection of labeled tumor cells, but this was not the case with $(RGDT)_{11}$. These results support the inhibitory effect on spontaneous lung metastasis of even intermittent treatments with PEG-conjugated peptide at 3-day intervals, and indicate that the enhancement of antimetastatic effect by conjugation with PEG and the polymerization of RGDT sequence may be associated with sustained release of the peptide from the PEG-conjugates, slow clearance from the circulation and delayed decomposition, leading to the prolongation of the peptide action. Further study will be needed to examine in detail the mechanism of the controlled release from PEG-peptide conjugate as well as the inhibition of tumor metastasis.

In conclusion, we have demonstrated that polymeric peptides containing defined repetitive structure of RGDT sequence, $(RGDT)_n$ (n=1 to 11) are able to inhibit tumor lung metastasis in experimental and spontaneous (therapeutic) metastasis models, in proportion to the number of RGDT repeats. The conjugation of $(RGDT)_{11}$ with PEG augmented the ability to inhibit experimental and spontaneous lung metastasis.

## ACKNOWLEDGMENTS

This work was supported in part by Grants-in-Aid for Cancer Research from the Japanese Ministry of Education, Science and Culture, for the Comprehensive 10-Year Strategy for Cancer Control from the Japanese Ministry of Health and Welfare, and for Scientific Research from the Japanese Ministry of Education, Science and Culture, as well as a grant from the Osaka Foundation for Promotion of Clinical Immunology, Grants-in-Aid for Special Project Research from Hokkaido University, Japan, and a grant from NHK Public Welfare Organization, Japan. We thank Ms. M. Sato for typing the manuscript.

*(Received December 5, 1992/Accepted February 23, 1993)*

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