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# EFFECT OF CYCLIC RGD PEPTIDE ON CELL ADHESION AND TUMOR METASTASIS

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Several kinds of cyclic peptides containing an L-arginine-glycine-L-aspartic acid RGD sequence were synthesized by the liquid phase method, and we investigated their effects on the attachment of mouse B16 melanoma cells onto fibronectin-coated well. Cyclo(GRGDSPA) inhibited the cell attachment at a 20-fold lower concentration than the linear form. The cell adhesion was inhibited by the synthetic peptides with the following relative order of activity: cyclo(GRGDSPA)>>cyclo(GRGD)> cyclo(RGDS), cyclo(GRGDSP)> cyclo(GRGDS)>cyclo(RGDSP), cyclo(RGDSPA). Cyclo(GRGDSPA) was more effective at inhibiting cell attachment to vitronectin than it was at competing with fibronectin attachment, as reported in the case of GRGDSP. Moreover, cyclo(GRGDSPA) significantly reduced the formation of colonies in mice injected with B16-FE7 melanoma cells. 

The metastatic process is comprised of a complex series of events that can be subdivided into a number of steps involving the attachment and degradation of the extracellular matrix(1,2). Among these events in metastatic process, one of the most important events is the attachment of tumor cells to the extracellular matrix, which is composed of macromolecules that include fibronectin, laminin and collagen (3). Cell adhesion and interactions with the extracellular matrix have been analyzed extensively using a model system that monitors the cell attachment and spreading onto fibronectin and other extracellular matrix protein-coated wells.

These analysis demonstrated that a tripeptide, L-arginyl-glycyl-L-aspartic acid (RGD) located within the fibronectin molecule is the recognition site for the attachment of cells to this protein(4). The RGD peptide can inhibit fibronectin mediated cell attachment and spreading in vitro, and it also has been used as a probe to study the adhesive properties of

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not only fibronectin but also other extracellular matrix proteins (5,6). The RGD peptide has been demonstrated to block gastrulation in vivo, neuronal crest cell migration and platelet function, and also reduce the formation of lung colonies in mice injected with B16 melanoma cells(7,8,9). High concentration of RGD peptides, however, was needed to inhibit these functions because of low affinity of RGD peptides with their receptors. The examination of obtaining peptides with improved affinities has been investigated(10,11,12,13). Many peptides have been synthesized to investigate the contribution of individual amino acids in and around the RGD tripeptide. It has been reported previously that in RGDS sequence, R,G and D residues cannot be replaced with the chemically similar residues with retention of activity. That the RGD peptide can not form a fixed conformation is a possibility that must be considered.

In this report, we used a series of novel synthetic RGD-containing peptide to obtain information about the structure onto fibronectin substrates, and demonstrate that cyclic form of GRGDSPA peptide is effective 10-fold as much as a linear form. The cyclo(GRGDSPA) recognized vitronectin receptor more than fibronectin receptor, as previously reported for RGD peptide.

We conclude that cyclo(GRGDSPA) mimics the fixed structure giving a property for the recognition of RGD receptor.

## MATERIALS AND METHODS

Synthetic peptides and proteins

Peptides were synthesized by the solid phase(14) or liquid phase method. After treating the synthesized peptides by HF, they were purified by HPLC using a YMC ODS column (YMC, Tokyo). Peptide solutions were neutralized with the acylizer (Asahi Chemical Co. Tokyo) and then lyophilized and stored at 4℃ until use.

Fibronectin, vitronectin and laminin were obtained from Iwaki Glass Co. Ltd (Tokyo).

Animals

C57BL/6j female mice (6week old) were purchased from Sankyo Laboservice, Inc., Tokyo, and housed in plastic cages.

Cell attachemnt asssy

A431 and B16 melanoma cells(15)(high metastatic cell line, FE7, a kind gift from Dr. H. Tanaka, Osaka Adult Disease Center) were maintained in D-MEM(Gibco, Chagrin Falls, OH) supplemented with heat-inactivated 10% fetal bovine serum(Boehringer Manheim), penicillin and streptomycin(Gibco). After the cells were grown, they were washed with PBS and detached with a

solution of 0.25% trypsin(Gibco) plus EDTA. The cells were then pelleted by low speed centrifugation and resuspended in D-MEM containing bovine serum albumin(Sigma).

The adhesion of A431 cells or B16 melanoma cells to fibronectin, vitronectin or laminin substrates was assayed as described previously (16). Fibronectin or vitronectin was coated onto plastic tissue culture wells for 3hr at 37℃, and then 0.1% BSA solution was added to each culture well and incubation continued for 1 hr more at 37℃. Cells ($1x10^5$) were added to the wells and incubated for an additional 1 hr at 37℃ in 95% air and 5% $CO_2$. Nonattached cells after 1 hr incubation were removed by washing with PBS. Attached cells were trypsinized and counted after centrifugation. The ability of the peptide analogs of the cell attachment site of fibronectin to inhibit the adhesion was assayed by including the soluble peptides in the incubation medium at a vaious concentrations.

Peptide Mapping

RGD peptides (1mg/ml) were treated by trypsin(5 μg/ml) for 2-16 hr. After stopping the trypsin digestion by the addition of 1N HCl, the reaction was fractionated over a C18 column using a gradient from 0% to 30% acetonitryl in 0.1% TFA.

Experimental metastasis assay(17)

The B16FE7 cells at exponential growth phase were harvested by trypsin treatment, washed twice and resuspended in serum-free RPMI1640. The cells alone or cells mixed with RGD-containing peptide were injected into the lateral vein of a C57BL/6 mouse. The number of tumor cells iv-challenged in a mouse was $1.2x10^5$ in a volume of 200 ul. After 14 days, the mice were killed and the number of metastatic foci on the lung surface was counted under a dissecting microscope.

## RESULTS

Synthesis and purification of cyclic RGD peptides

Several kinds of synthetic peptide analogs of a crucial cell binding sequence in fibronectin were compared for relative activity in inhibiting cell adhesion to fibronectin coated substrates.

We reasoned that the relatively low apparent affinity of the short synthetic peptides for the adhesive receptors might be due to their high degree of flexibility. By the liquid phase method, we synthesized the following cyclic peptides:cyclo(Gly-Arg-Gly-Asp-Ser-Pro-Ala) (cyclo(GRGDSPA)), cyclo(Gly-Arg-Gly-Asp-Ser-Pro)(cyclo(GRGDSP)), cyclo(Gly-Arg-Gly-Asp-Ser) (cyclo(GRGDS)), cyclo(Gly-Arg-Gly-Asp) (cyclo(GRGD)), cyclo(Arg-Gly-Asp-Ser-Pro-Ala) (cyclo(RGDSPA)), cyclo(Arg-Gly-Asp-Ser-Pro)(cyclo(RGDSP)) and cyclo(Arg-gly-Asp-Ser) (cyclo(RGDS)).

Figure 1 shows the HPLC purification of linear GRGDSPA and cyclo(GRGDSPA) using a gradient of 0% to 30% acetonitrile in 0.1% TFA. The purified peptide was confirmed to be a monomeric cyclic peptide by

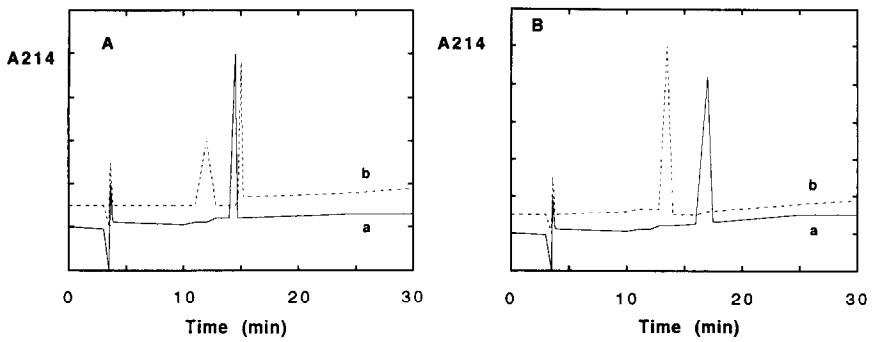


**Figure 1. Purification of a linear and cyclic RGD peptide.**
A linear peptide (GRGDSPA) (A) or a cyclic peptide (cyclo(GRGDSPA)) (B) was incubated with(b) or without(a) 5 μg/ml trypsin for 16hr. After the addition of HCl, the peptide digest was applied to a C18 column and eluted with a gradient of 0% to 30% acetonitrile in 0.1% trifluoroacetate.

peptide mapping using trypsin and mass spectroscopy. When cyclo-(GRGDSPA) was treated by trypsin, the (Arg-Gly) bond was cleaved to yield GDSPAGR, corresponding to the single peak obtained in the HPLC pattern of tryptic digest (Fig. 1A-b). In contrast, chromatography of linear GRGDSPA after the trypsin treatment yielded two peaks corresponding to GR and GDSPA (Fig. 1B-b). We also confirmed for the peptide to be a cyclic form by mass specroscopy (data not shown).

Like cyclo(GRGDSPA), other cyclic peptides were purified using the same method and confirmed to be the respective cyclic forms by peptide mapping or mass spectroscopy.

Effect of cyclic peptides on cell adhesion onto fibronectin wells

The results in Fig. 2 show that cyclo(GRGDSPA) effectively inhibited the attachment of A431 human epidermal carcinoma cells to fibronectin-coated wells as compared with a linear peptide containing the same sequence. Cyclo(GRGDSPA) also inhibited efficiently the adhesion of B16FE7 mouse melanoma cells to fibronectin-coated plates (Fig. 3).

Synthetic peptide analogs were compared for relative activities in inhibiting the adhesion of B16 mouse melanoma cells to fibronectin substrates. The inhibitory effectiveness of the cyclic peptides were in the following relative order of activity: cyclo(GRGDSPA)>>cyclo(GRGD)> cyclo(RGDS), cyclo(GRGDSP)> cyclo(GRGDS)> cyclo(RGDSP), cyclo (RGDSPA) (Table I).

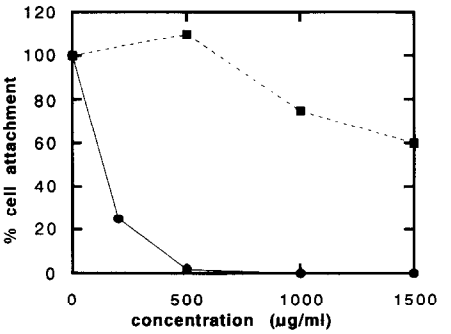


**Figure 2. Inhibitory effect of cyclic RGD peptide on the adhesion of A431 adenocarcinoma cells onto fibronectin coated wells.**
Plastic tissue culture wells were coated with 1 μg/ml fibronectin. Cell adhesion in the presence of the indicated concentration of each synthetic peptide was determined as described in Materials and Methods.
(●) cyclo(GRGDSPA) and (■) linear GRGDSPA.
Attachment is expressed as a percentage with 100% being the attachment in the absence of peptides.

Effect of cyclic peptides on cell adhesion onto vitronectin and laminin wells

As reported previously, RGD peptides more effectively inhibited attachment to vitronectin than attachment to fibronectin. The similar result was obtained for the cyclic peptide cyclo(GRGDSPA) (Fig. 3).

Laminin can serve as an attachment protein for A431 cells. Neither the linear form nor the cyclic form of GRGDSPA inhibited the cell adhesion significantly even at the high concentration of 1 mg/ml (data not shown). This result suggests that cell attachment on laminin is less sensitive to the inhibition by cyclo(GRGDSPA) than those on fibronectin and vitronectin.

Table I. Inhibitory effect of various kinds of cyclic RGD peptides on the adhesion of B16 melanoma cells to fibronectin-coated wells

| Peptides | $IC_{50}/IC_{50}$ of cyclo(GRGDSPA) |
|---|---|
| cyclo GRGDSPA | 1.0 |
| cyclo GRGDSP | 10.5 |
| cyclo GRGDS | 14 |
| cyclo GRGD | 8.2 |
| cyclo RGDS | 10 |
| cyclo RGDSP | >20 |
| cyclo RGDSPA | >20 |

B16FE7 melanoma cells ($10^5$ cells) were added onto fibronectin-coated wells with various kinds of synthetic peptides and incubated for 1hr at 37 C in 95% air and 5% CO2. After non-attached cells were washed out wirh PBS, the number of attached cells were counted. The $IC_{50}$ of cyclo(GRGDSPA) was 10-20 μM.

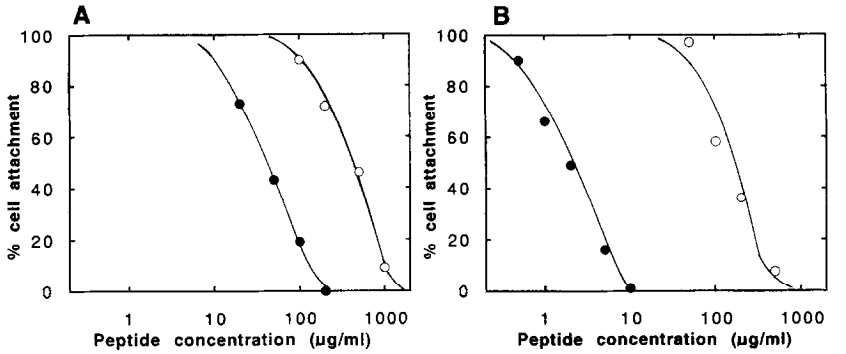


**Figure 3. Inhibition of cell adhesion on fibronectin-coated or vitronectin-coated substrate by cyclic RGD peptide**
(A) Inhibitory effect of GRGDSPA(○) or cycol(GRGDSPA)(●) on the attachmeent of B16FE7 melanoma cells onto fibronectin-coated wells.
(B) Inhibitory effect of GRGDSPA(○) or cyclo(GRGDSPA)(●) on the cell attachment to vitronectin-coated wells.
Cell adhesion in the presence of the indicated concentration of each peptide was determined as described in Materials and Methods. 100% cell attachment (without peptide) equals about $0.7 \times 10^5$ cells.

Effect of cyclo(GRGDSPA) on pulmonary metastasis of tumor cells

Since the cell adhesion with the extracellular matrix would be closely related to tumor metastasis, we examined the effect of cyclo(GRGDSPA) on this phenomenon. Simultaneous injection of cyclo(GRGDSPA) with the tumor cells (B16 FE7 melanoma) into the tail vein of mice resulted in a reduction of colony formation on the surface of the lung (Table II).

Table II. Effects of synthetic RGD peptides on lung colonization of B16 melanoma cells (B16FE7) injected to mice

| Treatment | | Mean No. of metastatic foci in lung |
|---|---|---|
| control | | 34 |
| GRGDSPA | 0.5mg | 39 |
| | 1.0mg | 20 |
| cyclo GRGDSPA | 0.1mg | 2 |

B16FE7 melanoma cells($5 \times 10^5$ cells) were injected iv into mice with or without peptides. After 14 days, the mice were killed, and the number of metastatic foci on the lung surface was counted under a dissecting microscope.

DISCUSSION

The understanding of the molecular interactions that result in cell adhesion has progressed very rapidly. The RGD sequence serves as the cell recognition site of many adhesive proteins including fibronectin, vitronectin, osteopontin, collagens, thrombospondin, fibrinogen and von Wilbrand factor, which are present in the extracellular matrix and the blood. Although the RGD adhesion system appears to play roles in many cellular functions, high concentration of RGD peptides were needed to inhibit these functions, which suggested to us that peptide conformation may be a determinant of their effectiveness.

Our approach to address the contribution of peptide conformation to the cell adhesion-inhibiting activity of the RGD peptides was to cyclize these peptides. Here we demonstrated that a cyclic synthetic peptide, cyclo(GRGDSPA) had high capacity to interfere with the cell adhesion to fibronectin- and vitronectin-coated substrates. One reason why cyclo (GRGDSPA) has higher affinity towards the RGD receptors than the parent peptide is that cyclization of the peptide allows it to closely mimic the structure within the adhesive proteins.

There are numerous approaches for designing peptides that will have higher affinity toward the receptor than the parent peptide. Our peptide analogue studies resulted in synthesis of cyclo(GRGDSPA) with high affinities toward both the fibronectin receptor and the vitronectin receptor. Some successes were also achieved by Pierschbacher and Ruoslahti (10) or Saiki et al (12). Pierschbacher and Ruoslahti investigated the contribution of individual amino acids in and around the RGD peptide. They synthesized many peptides having the structure of GRGD XaaPC in which each of the peptides had the Xaa position substituted with one of the 20 natural L-amino acidws. It was found that a peptide containing Asn at the position of Xaa (GRGDNPC) was six times more effective at inhibiting cell attachment to fibronectin than the prototype serine-containing peptide (GRGDSPC). They also found that the

cyclized peptide Gly-Pen-Arg-Gly-Asp-Ser-Pro-Cys-Ala (where Pen is penicillamine) inhibits the attachment predominantly to vitronectin and not to fibronectin. These analyses will make it possible to obtain a peptide with improved affinities toward their receptors or with selectivity towards an individual receptor. With regard to the ligand binding properties of the adhesion receptors, it is important to elucidate the structure of the attachment site of the adhesive proteins that serve as the ligands to derive the conformation of their RGD sequences. Such information may be derived from the NMR analysis of the cyclic RGD peptides described here and may allow us to design peptides or non-peptide compounds that more closely mimic the structure of this sequence within the adhesive proteins, and thereby may have higher selectivity and affinity for the individual receptors (described elsewhere).

The RGD adhesion system appears to play a role in phenomena such as tumor metastasis, platelet aggregation, host-parasite relations. Humphries et al (7) also found that RGD-containing peptides also inhibited tumor metastasis by B16 melanoma cells in the lung when the peptides was injected together with the tumor cells into mice. The peptides may be useful for counteracting the invasion of tumor cells through the connective tissue matrix, leading to possible future therapeutic roles for these peptides. Cyclo (GRGDSPA) was found to reduce the colony formation in mice injected with B16 melanoma (FE7) at a 10-fold lower concentration than the parent peptide. The cyclic peptide also effectively inhibited the platelet aggregation induced by ADP or collagen (described elsewhere). Our present results suggest that the cyclo RGD peptide would be used for clinical applications such as tumor metastatic and thrombolytic therapy.

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