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Advances in Brief

Multimeric Forms of Tyr–Ile–Gly–Ser–Arg (YIGSR) Peptide Enhance the Inhibition of Tumor Growth and Metastasis

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Abstract

The Tyr–Ile–Gly–Ser–Arg (YIGSR) peptide derived from the laminin B1 chain has been shown to decrease tumor growth and metastasis. Utilizing the multimeric antigen peptide system assembled on a branched lysine core, we synthesized several sizes of multimeric YIGSR, (CH_3CO–Tyr–Ile–Gly–Ser–Arg–Gly)$_{16}$–Lys_8–Lys_4–Lys_2–Lys–Gly [(Ac-YIGSRG)$_{16}$ $K_8K_4K_2KG$] (designated Ac-Y16), (Ac-YIGSRG)$_8K_4K_2KG$ (Ac-Y8), and (Ac-YIGSRG)$_4K_2KG$ (Ac-Y4), and related peptides, Ac-(YIGSRG)$_4$-NH_2 (Ac-Y4L) and Ac-YIGSR-NH_2 (Ac-Y1) and evaluated their biological activities in inhibiting tumor growth and metastasis. Coinjection of 0.2 mg/mouse of Ac-Y16 i.v. with B16-F10 mouse melanoma cells inhibited lung colony formation by 97%, whereas 0.2 mg/mouse of Ac-Y1 inhibited by 50%. The larger the peptide (Ac-Y16 > Ac-Y8 > Ac-Y4 > Ac-Y1), the more inhibitory effect there was on lung metastasis. Ac-Y16 also inhibited the growth of s.c.-injected B16-F10 tumors. These data demonstrate that the multimeric YIGSR peptides strongly enhanced the activity of YIGSR in inhibiting tumor growth and metastasis and suggest that these compounds are potentially useful for clinical applications.

Introduction

Laminin is a large heteromeric glycoprotein (M_r 900,000) located in the basement membrane extracellular matrix (1, 2). Laminin has multiple biological activities including promotion of cell adhesion, growth, differentiation, migration, neurite outgrowth, tumor metastasis, and collagenase IV induction. Several active sites on laminin chains were identified using synthetic peptides or proteolytic fragments (3–6). The YIGSR[2] sequence located on the B1 chain (positions 929–933) has been shown to promote cell adhesion and migration and to inhibit angiogenesis (3, 7–9). The peptide has also been shown to reduce experimental metastasis and s.c. tumor growth (8–13). Recently, a mouse melanoma B16-F10 variant, which was established by selection on YIGSR-coated dishes and adhered more strongly to YIGSR, was shown to form more lung colonies after i.v. injection and larger tumors after s.c. injection than the parent B16-F10 cells (14). These findings suggest that the YIGSR sequence on laminin may regulate tumor growth due to its effects on both angiogenesis and direct tumor cell interactions.

The YIGSR peptide is a potential candidate for development of anticancer and antimetastasis agents, and many modifications of YIGSR peptides have been reported to enhance its activity. Polymerized YIGSR peptides were shown by Murata *et al.* (12) to more effectively inhibit experimental metastasis than the monomeric peptides. The activity of YIGSR has been also increased by coupling to polyethylene glycol (13). These results suggest that molecular weight of the compound containing the active peptides might be important for enhancement of its activity. On the other hand, the cyclic YIGSR was shown to have increased effectiveness (15), and conformational studies by nuclear magnetic resonance and computer modeling suggest that the turn structure of the peptide may be an important criterion for activity (16). It is not yet clear whether these modifications directly enhance the potency of YIGSR and/or maintain a longer half-life in the circulatory system.

Recently, Tam *et al.* (17, 18) established the MAP system in which the antigen peptide is assembled on a lysine tree. The branched core lysine structure is located in the interior of the molecule allowing numerous active site peptides on the surface to be accessible for interactions. The MAP approach is suitable for direct preparation of high molecular weight products which can be used as an immunogen without further conjugation procedures. For example, the MAP method has been used successfully for development of many vaccines (18). In addition, MAP peptides seem to possess favorable molecular shapes as synthetic macromolecular mimics.

Using the MAP method, we designed multimeric YIGSR peptides, (CH_3CO–Tyr–Ile–Gly–Ser–Arg–Gly)$_{16}$–Lys_8–Lys_4–Lys_2–Lys–Gly[(Ac-YIGSRG)$_{16}$$K_8K_4K_2KG$] (designated Ac-Y16), (H-YIGSRG)$_{16}$$K_8K_4K_2KG$ (H-Y16), (Ac-YIGSRG)$_8K_4K_2KG$ (Ac-Y8), and (Ac-YIGSRG)$_4K_2KG$ (Ac-Y4), to explore the potentiation of the antitumor effects of this peptide.

Materials and Methods

Synthesis of Peptides. The linear peptides [Ac-(YIGSRG)$_4$-NH_2 and Ac-Y1] were synthesized by the solid-phase method using an Applied Biosystems 431 automated peptide synthesizer on *tert*-butyloxycarbonyl strategy. Deprotection and cleavage from the resin were achieved by treatment with anhydrous HF, and the crude peptides were purified by gel filtration and reverse phase high performance liquid chromatography.

The branched peptides (Ac-Y16, Ac-Y8, Ac-Y4, H-Y16, and Ac-R16) were synthesized manually by 9-fluorenylmethyloxycarbonyl strategy on Wang-type resin (9-fluorenylmethyloxycarbonyl–Gly–resin, 0.1 mmol/g) using successive diisopropylcarbodiimide-*N*-hydroxybenzotriazole coupling and N^{α}-deprotection with 20% (v/v) piperidine/dimethylformamide. Deprotection and cleavage from the resin were achieved by treatment with trimethylsilyl bromide-thioanisole in trifluoroacetic acid (0°C, 2 h) (19). The crude peptides were purified by either gel filtration or reverse phase high performance liquid chromatography. The branched peptides were examined by socium dodecyl sulfate-polyacrylamide gel electrophoresis and a major band of the expected molecular size was observed. The identity of the peptides was confirmed by amino acid analysis. The peptides used in this paper are summarized in Fig. 1*a*, and the structure of Ac-Y16 is schematically shown in Fig. 1*b*.

Cell Culture. B16-F10 mouse melanoma cells (20) (a gift of Dr. I. J. Fidler, M. D. Anderson Hospital, Houston, TX) were cultured in Eagle's minimum essential medium (GIBCO Laboratories, Grand Island, NY) containing 9% (vol/vol) fetal calf serum (HyClone, Logan, UT), 100 units/ml penicillin, and 100 μg/ml streptomycin (GIBCO).

Experimental Metastasis. For the experimental metastasis assay, Versene-detached B16-F10 cells (1×10^5) in 0.2 ml of minimum essential medium containing 0.2 mg of the synthesized peptide were injected in the tail vein of syngenic C57BL6/N mice. Five mice were used for each group. Seventeen days later, mice were sacrificed. Lungs were removed, and the number of surface colonies was counted.

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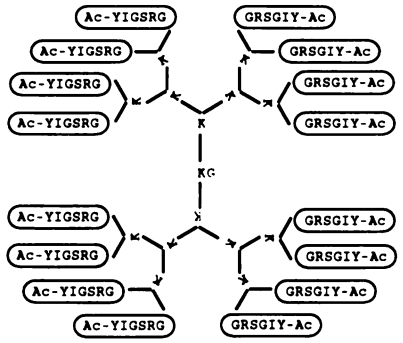
[2] The abbreviations used are: YIGSR, Tyr-Ile-Gly-Ser-Arg; MAP, multimeric antigen peptide; Ac-Y16, (Ac-YIGSRG)$_{16}$$K_8K_4K_2KG$, ($CH_3CO$-Tyr-Ile-Gly-Ser-Arg-Gly)$_{16}$-Lys_8-Lys_4-Lys_2-Lys-Gly-OH; Ac-Y1, Ac-YIGSR-NH_2; GRGDS, Gly-Arg-Gly-Asp-Ser.

(a)

Ac-Y16 : (Ac-YIGSRG)$_{16}$K$_8$K$_4$K$_2$KG-OH
H-Y16 : (Ac-YIGSRG)$_{16}$K$_8$K$_4$K$_2$KG-OH
Ac-Y8 : (Ac-YIGSRG)$_8$K$_4$K$_2$KG-OH
Ac-Y4 : (Ac-YIGSRG)$_4$K$_2$KG-OH
Ac-Y4L : Ac-(YIGSRG)$_4$-NH_2
Ac-Y1 : Ac-YIGSR-NH_2
Ac-R16 : (Ac-GRDSG)$_{16}$K$_8$K$_4$K$_2$KG-OH

(b)



Ac-Y16

Fig. 1. List of synthetic peptides and multimeric YIGSR peptide (*a*) List of synthetic peptides used. Ac-Y16. (*b*) Schematic representation of Ac-Y16.

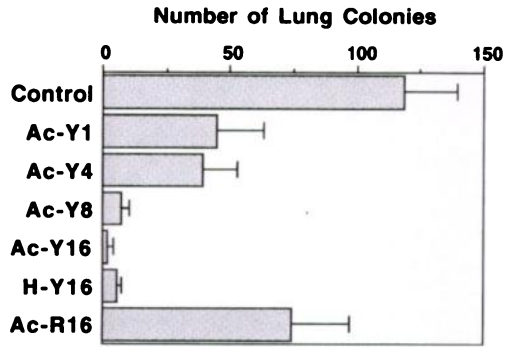


Fig. 2. Inhibitory effect of synthetic peptides on the formation of lung tumors. B16-F10 cells (1×10^5) and a synthetic peptide (0.2 mg) were coinjected into the tail vein of a mouse (C57BL6/N). Seventeen days later, the number of B16-F10 cell colonies in the lung was counted. The number was an average from 5 mice. *Bars*, SD.

Tumor Growth Assay. B16-F10 cells (1×10^6) were injected s.c. into the right lower backs of C57BL6/N mice (day 0). On day 1 to day 9, each peptide (0.5 mg/mouse/day) was injected i.p. daily. On day 10, mice were sacrificed, and the tumors were weighed.

Results and Discussion

Three sizes of multimeric YIGSR peptide containing 4, 8, and 16 YIGSR sequences with either free NH_2-terminal amino or NH_2-terminal acetyl group were synthesized and assembled on a lysine tree using the MAP method (17, 18). A glycine residue was used as a spacer between the active sequence and the lysine tree. The Ac-Y16 has a high molecular weight (M_r 12,554) and is highly branched, most probably with a globular shape (Fig. 1). For comparison with the multimeric YIGSR peptides, GRGDS (21) were prepared using the MAP method [(Ac-GRGDSG)$_{16}$K$_8$K$_4$K$_2$KG (Ac-R16)].

We examined the effect of the multimeric peptides on experimental metastasis (Fig. 2). Ac-Y16 inhibited experimental tumor metastasis in a dose-dependent manner (Fig. 3). Coinjection of only 0.2 mg/mouse of Ac-Y16 reduced the number of lung colonies by more than 97%. In contrast, Ac-Y1 (monomeric YIGSR) reduced the number of lung colonies by 50% at the same dose. The increase in the number of YIGSR sequences in the peptides paralleled the inhibitory effect on lung metastasis. A much larger multimeric peptide containing 32 YIGSR sequences, however, showed activity comparable to that of Ac-Y16 (data not shown). Therefore, Ac-Y16 seems to have a sufficient number of YIGSR sequences to be maximally effective. The NH_2-terminal acetylated and free multimeric peptides, Ac-Y16 and H-Y16, showed similar inhibitory activity suggesting that the NH_2-terminal group of multimeric molecules (CH_3CO- or H-) did not affect this activity. Ac-R16 showed a weak inhibitory effect on tumor metastasis when compared to Ac-Y16. This result is identical to a previous study using a GRGDS peptide (8).

Next we studied the antitumorigenic activity of Ac-Y16 and Ac-Y1 in a s.c. tumor model. Ac-Y16 significantly inhibited the s.c. growth of B16-F10 melanoma cells when 0.5 mg/mouse of this peptide was injected i.p. each day for 9 days (Fig. 4). However, Ac-Y1 did not show a significant inhibitory effect at this concentration. Previously, Cys–Asp–Pro–Gly–Tyr–Ile–Gly–Ser–Arg–NH_2 (CDPGYIGSR-NH_2) was shown to inhibit the growth of solid tumors when it was injected at 3 mg/day for 4 days (10). Ac-Y1, therefore, can significantly inhibit the growth of solid tumor if greater amounts are injected. It was previously suggested that this inhibition of tumor growth was due to the antiangiogenic effect of the peptide (10). The enhanced antitumor effect of this multimeric YIGSR peptide is also likely caused in part

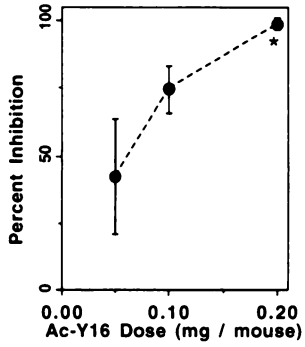


Fig. 3. Inhibitory effect of Ac-Y16 at various concentrations on the formation of lung tumors. Various amounts of Ac-Y16 were coinjected with B16-F10 cells (1×10^5) into the tail vein of a mouse and the number of the tumor colonies was counted as described in Fig. 2. *Bars*, SD. *, values at 0.2 mg/mouse differ significantly from 0.1 mg/mouse ($P < 0.05$) and 0.05 mg/mouse ($P < 0.01$).

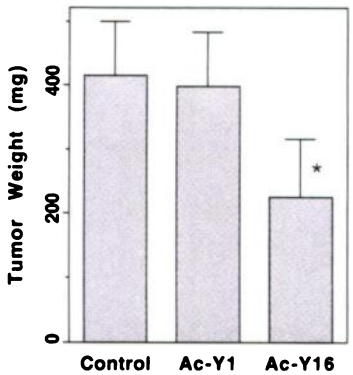


Fig. 4. Effect of Ac-Y16 and Ac-Y1 on growth of tumor after s.c. injection. B16-F10 cells (1×10^6) were injected s.c. into the right low back of the mice (C57BL6/N). Each peptide (0.5 mg) was injected i.p. every day from day 1 to day 9. On day 10, mice were sacrificed and the tumor was weighed. *Bars*, SD. *, values of Ac-Y16 differ significantly from control ($P < 0.01$) and Ac-Y1 ($P < 0.05$).

by an inhibition of vessel formation. In related work, we have also reported that this peptide binds directly to certain highly malignant tumor cells (14), suggesting additional activities of this peptide on tumor cells.

When the multimeric YIGSR peptides were tested for cell attachment activity using B16-F10 cells, they were more active than the monomeric peptide (Ac-Y1; data not shown). Multimeric peptide, Ac-Y16, also had enhanced attachment activity compared to Ac-Y8, Ac-Y4, and Ac-Y1. Fassina *et al.* (22) reported that a multimeric complementary peptide, which was synthesized using the MAP method, could enhance the binding affinity with the target native peptides by several orders of magnitude.

In this paper, we have described multimeric YIGSR peptides prepared by the MAP method and found that these peptides have greater antitumor activities than the original monomeric YIGSR peptide. The MAP approach is advantageous, because the synthetic process is brief and the molecular size is easily controlled. One potential drawback is that the MAP structures may be antigenic but the idea has not been tested yet for the YIGSR peptide. Recently, many active sequences have been identified within large intercellular matrix proteins (*e.g.*, laminin, fibronectin, collagen, thrombospondin, entactin, etc.) (23, 24). Unfortunately, the biological activities of synthetic peptides of active site segments have been often shown to be weak and sometimes too low to be recognized in *in vivo* experiments. The branched multimeric peptide approach described here is useful to enhance the activities of short cell adhesive peptides and to clarify their *in vitro* and *in vivo* activities. Furthermore, the multimeric YIGSR compounds described here are potentially useful for therapeutic applications as antitumor growth and metastasis agents.

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