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A POTENT ANTI-METASTATIC ACTIVITY OF TUMOR INVASION-INHIBITING FACTOR-2 AND ALBUMIN CONJUGATE[1]

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SUMMARY: Tumor invasion-inhibiting factor-2 (IIF-2) peptide was chemically conjugated to albumin with 1-ethyl-3(3-dimethylaminopropyl)-carbodiimide hydrochloride. The molar ratio of the covalently linked IIF-2 peptide and albumin in the purified conjugate was 1.4 ± 0.4, as determined with a radioactive peptide. The conjugate inhibited the invasion of HT1080 and B16FE7 cells *in vitro* at 40- to 60-fold lower concentrations than IIF-2 peptide. Scatchard analysis of binding data demonstrated that the IIF-2-albumin conjugate bound to HT1080 and B16FE7 cells with Kd values of 240 and 340 nM, respectively. The conjugate suppressed the lung colonization of B16FE7 cells more effectively than the IIF-2 peptide in an experimental metastasis. These results indicate that the covalent linkage of the IIF-2 peptide to a carrier macromolecule provides a structure that enables this peptide to exhibit increased inhibitory action in cancer cell invasion and metastasis, and that IIF-2 exerts its inhibitory action on invasion by binding to a specific binding site on the tumor cell surface.

Tumor invasion is one of the most characteristic steps to occur during the course of cancer metastasis. Thus, blocking of this step would be expected to be useful in the prevention of cancer metastasis. Invasion consists of sequential steps, including (a) attachment of tumor cells to the extracellular matrix (ECM), (b) degradation of the ECM by various hydrolases, and (c) tumor cell migration into the parenchyma of the target organ (1-3). Many studies have shown that inhibition of any one of the steps in this cascade succeeds in preventing tumor cell colonization in the target organ (4-9).

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Abbreviations: IIF, invasion-inhibiting factor; EDC, 1-ethyl-3(3-dimethylaminopropyl)-carbodiimide hydrochloride; IC_{50}, half-maximum inhibitory concentration; FBS, fetal bovine serum; MSA, mouse serum albumin; HPLC, high performance liquid chromatography; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; PBS, Dulbecco's phosphate-buffered saline; ECM, extracellular matrix.

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In our previous study (10), using an *in vitro* invasion assay system developed by Akedo et al.(11), we purified two types of tumor invasion-inhibiting factors (IIF), designated IIF-1 and IIF-2, from bovine liver by successive chromatography. Recently (12), we determined the primary structure of IIF-2 and revealed that it was a novel anti-invasive substance which might be a specific inhibitor of cell migration. Moreover, we demonstrated that IIF-2 prevented lung metastasis in highly metastatic B16 melanoma FE7 (B16FE7) and Lewis lung carcinoma cells, possibly by impairing extravasation of tumor cells *in vivo* (12).

In the present study, we attempted to enhance the anti-invasive activity of IIF-2 in an investigation of its inhibitory action on tumor invasion, and in an attempt to raise its usefulness in the control of cancer metastasis. We report here the construction of the IIF-2-albumin conjugate which had potent inhibitory action on the invasion and metastasis of tumor cells.

MATERIALS AND METHODS

Animal and Cells. Specific pathogen-free female C57BL/6j mice (7 weeks old) were purchased from Sankyo Labservice Inc., Tokyo, Japan, and maintained in a plastic cage. Human HT1080 fibrosarcoma cells (13), obtained from Japanese Cancer Research Resource Bank, were cultured in Dulbecco's modified medium (DMEM; Gibco Laboratory, Chagrin Falls, OH) supplemented with 10 % fetal bovine serum (FBS; Boehringer Mannheim, Mannheim, Germany). B16FE7 cells were maintained as described previously (12).

Peptide Synthesis. IIF-2 was synthesized and purified as described previously (12). Tyrosyl-IIF-2 (Y-IIF-2) was also synthesized so that radioiodination could be performed. The purity of each peptide was more than 98 %, as demonstrated by analytical C18 reverse phase chromatography on HPLC. The anti-invasive activity of Y-IIF-2 was confirmed to be identical to that of the IIF-2 peptide in an *in vitro* invasion assay (data not shown).

Production of Polyclonal Anti-IIF-2 Antibody. IIF-2 was coupled to keyhole limpet hemocyanin (KLH; Calbiochem Corp., La Jolla, CA, USA) with glutaraldehyde (14). Antibody to IIF-2 was raised in a rabbit by five immunizations with 500 μ g of IIF-2-KLH complex in Freund's complete adjuvant. The IgG was purified by a procedure involving fractionation with ammonium sulfate and DEAE-Sepharose CL-6B (Pharmacia LKB Biotechnology, Uppsala, Sweden) column chromatography.

Preparation of IIF-2-albumin Conjugate. IIF-2 was coupled to mouse serum albumin (MSA; Organon Teknika N.V., West Chester, PA, USA), using 1-ethyl-3(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC; Pierce Chemicals Co., Rockford, IL, USA) as a coupling reagent, essentially as described previously (15), with some modifications. Thirty μ mole of IIF-2 (60mg) was mixed with 1 μ mole MSA (65mg) in 60 ml of PBS. The pH of the mixture was adjusted to 7.4 by adding 1 N NaOH. The reaction was initiated by slow mixing of 60 mg EDC dissolved in PBS. The mixture was incubated for 2 h at 37 ℃ and then overnight at 4 ℃. The IIF-2-albumin was purified by a semi-preparative C18 column on HPLC, using a gradient of 0 % to 80 % acetonitrile with 0.1 % trifluoroacetic acid in water. The complete separation of free IIF-2 was confirmed by analytical C18 column on HPLC. To determine the molar ratio of IIF-2 and albumin in the purified conjugate, a trace amount of radioiodinated Y-IIF-2 (see below) was added to the reaction mixture and the radiolabeled conjugate was purified as described above.

Immunoblotting. MSA and the purified IIF-2-albumin conjugate were subjected to SDS-PAGE and transferred to a nitrocellulose membrane (Bio-Rad Laboratories, Richmond, CA, USA).

Immunoblotting was performed with anti-IIF-2 IgG and peroxidase-conjugated goat anti-rabbit IgG antibody (Organon Teknika). The positive band was visualized with an ECL Western blotting detection kit (Amersham International plc., Bucks, UK).

Chemoinvasion Assay. The chemoinvasion assay was performed as described previously (12). Briefly, HT1080 and B16FE7 cells were labeled by culture for 24 h in complete medium supplemented with 1.85 MBq/ml 5-[^{125}I]Iodo-2'-deoxyuridine (74 TBq/mmole; Amersham), after which they were resuspended, to a final concentration of 2 x 10^5 cells/ml, in DMEM containing 10 mg/ml MSA. The cell suspension (200 μ l) was added to the upper compartment of a Chemotaxicell chamber (Kurabo, Inc., Osaka, Japan) fitted with a filter coated with Matrigel (Collaborative Research, Inc., Bedford, MA, USA). The radioactivity associated with the migrated cells under the filter was determined with a gamma-counter, Model ARC-300 (Aloka Co. Ltd., Tokyo, Japan).

Iodination of Y-IIF-2 Peptide, IIF-2-albumin, and Type IV Collagen. Labeling of Y-IIF-2, IIF-2-albumin conjugate, and type IV collagen (Iwaki Co. Ltd., Tokyo, Japan) with Na[^{125}I] (564 MBq/μg; Amersham) was performed with Iodo-Beads (Pierce) according to the manufacture's protocol. Specific activities of 1.9 x 10^5, 1.7 x 10^5, and 5 x 10^4 cpm/μg were obtained for Y-IIF-2, IIF-2-albumin conjugate, and type IV collagen, respectively.

Binding Assay. The cells were collected by EDTA-treatment and resuspended in serum-free DMEM containing 10 mg/ml of MSA; the cell number was adjusted to 1 x 10^6 cells/ml. One hundred μl of [^{125}I]IIF-2-conjugate, at various dilutions in DMEM supplemented with 10 mg/ml MSA, and 100 μl of cell suspension, were mixed (in triplicate experiments) and incubated for 2 h at 4℃. Non-specific binding was determined by carrying out parallel incubations with 100-fold excess amounts of the nonlabeled ligand. After the reaction was completed, unbound radioactivity was removed by three washings with DMEM containing MSA (10mg/ml), and the cell-bound radioactivity was measured in a gamma-counter. Saturation data, fitted by least squares analysis, were presented as a Scatchard plot.

Experimental Metastasis. B16FE7 cells suspended in PBS containing 10 mg/ml MSA with or without IIF-2-albumin conjugate were injected into the lateral tail vein of a C57BL/6 mouse. The number of tumor cells challenged in each mouse was 1.0 x 10^5, or 1.5 x 10^5, in a volume of 200 μl. On the 14th day after injection, the number of metastatic colonies on the lung surface was counted under a dissecting microscope.

Other Biological Assays. The effects of IIF-2-albumin conjugate on the proliferation, adhesion to the ECM, and protease activity of the tumor cells were investigated as described previously (12). The type IV collagenase activity in the medium obtained from the Chemotaxicell chamber was measured by the solid phase radioactivity method (16). Briefly, a solution of the radioiodinated type IV collagen was coated onto 96-well microtiter plates, and media from the chemoinvasion assay were added to the wells. After incubation for 24 h at 37 ℃, the released radioactivity was measured.

Other Methods. Peptide and protein concentrations were measured by amino acid analysis and BCA protein assay (Pierce), respectively. The statistical significance of differences between groups was determined by Student's *t*-test or the Mann-Whitney *U*-test.

RESULTS

Construction of IIF-2-albumin conjugate. We attempted to enhance the anti-invasive activity of IIF-2 by linking it to albumin. It was expected that the linkage of IIF-2 on the surface of the albumin molecule might fix its structure to favor its inhibitory action in tumor invasion. Excess amounts of IIF-2 peptides were added to albumin to avoid its inter-molecular coupling. After this reaction was completed, the IIF-2-albumin conjugate was purified by semi-preparative C18 reverse phase chromatography on HPLC. Under the conditions used in this experiment, the molar ratio of IIF-2 and

albumin in the conjugate was estimated to be 1.4 ± 0.4, in three separate experiments, using radiolabeled IIF-2 as described in "Materials and Methods". There was no contamination of free IIF-2 peptide, as demonstrated by C18 reverse phase chromatography on HPLC (Fig. 1 A) and by analytical gel-filtration on HPLC (results not shown). SDS-PAGE and Western blotting showed that the purified conjugate fraction did not contain albumin oligomers (Fig. 1 B).

Effects of IIF-2-albumin conjugate on invasion. Invasion of HT1080 and B16FE7 cells through Matrigel were significantly inhibited by the IIF-2-albumin conjugate with IC_{50} of 7 and 8 μ M, respectively (Fig.2). In contrast, the half-maximum inhibition of HT1080 and B16FE7 cell invasion produced by IIF-2 peptide occurred at concentrations of 300 and 500 μ M (Fig. 2 and *ref.* 12), respectively. Thus, the anti-invasive activity of the conjugate was increased 40- to 60-fold of that of free IIF-2. This result clearly indicates that the linkage of IIF-2 peptide to albumin enhances its anti-invasive activity. The addition of excess amounts of MSA (200 μ M) to the upper compartment of the chamber did not affect cell invasiveness (data not shown), indicating that the IIF-2 moiety in the IIF-2-albumin complex could exhibit invasion inhibitory action.

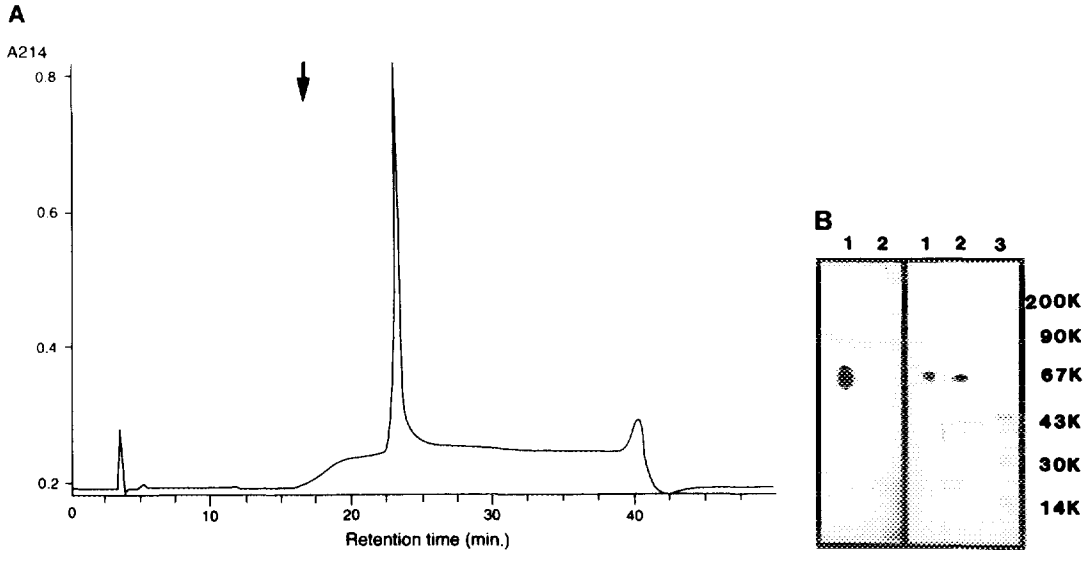

Figure 1.Analysis of IIF-2-albumin conjugate by C18 column chromatography on HPLC and by Western blotting. A, Purified IIF-2-albumin conjugate was applied to a C18 column on HPLC and eluted with a gradient of 0 % to 80 % acetonitrile in the presence of 0.1 % trifluoroacetate. The arrow indicates the position at which free IIF-2 was eluted. B, MSA and purified conjugate were subjected to SDS-PAGE and transferred to a nitrocellulose membrane. Immunoblotting was performed with anti-IIF-2 antibody and peroxidase-conjugated goat anti-rabbit IgG antibody. *Left*, Immunoblotting; *Right*, SDS-PAGE; Lane 1, Purified conjugate; Lane 2, MSA; Lane 3, Molecular weight marker.

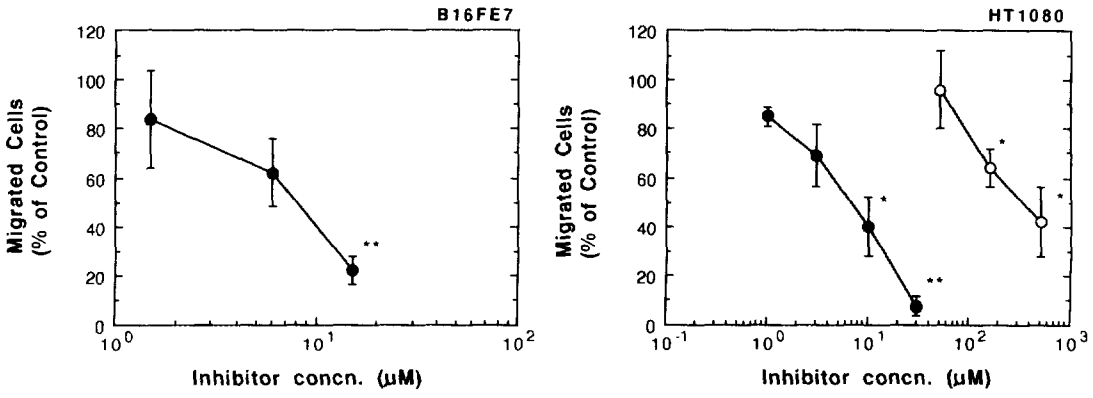

Figure 2.Effects of IIF-2-albumin conjugate on chemoinvasion of tumor cells. [^{125}I]-labeled B16FE7 and HT1080 cells were added to the upper compartment of a Chemotaxicell with a Matrigel-coated filter. After 20-h incubation at 37℃, cells on the upper surface of the filter were removed and the radioactivity associated with the cells under the filter was counted. The radioactivity associated with migrated cells in the absence of inhibitor was assigned a value of 100 % as a control. *Left*, B16FE7; *Right*, HT1080; Points, mean of triplicate determinations; bars, SD; ●, IIF-2-albumin conjugate; ○, IIF-2 peptide. The IC$_{50}$ of the IIF-2 peptide for invasion of B16FE7 cells was found to be 500 μ M in the previous work (12). *, P<0.05; **, P<0.01, compared with control by Student's *t* test.

We have already shown that IIF-2 peptide impairs the chemotaxis of highly invasive cells but does not affect their growth, adhesion to the ECM, or the activity of type IV collagenase (gelatinase) (12). The IIF-2-albumin conjugate also had no inhibitory effect on cell growth or adhesion, or on gelatinase (data not shown). When type IV collagenase activity in the media obtained from the Chemotaxicell chamber after the invasion assay was measured, we found no significant differences between the control and conjugate-treated groups (data not shown). This result shows that the conjugate did not suppress both the activity and secretion of the enzyme from the tumor cells.

Binding assay. We estimated the binding affinity of the IIF-2-albumin conjugate to explore the existence of IIF-2-binding sites on the cells. The conjugate bound specifically to HT1080 and B16FE7 cells, with Kd values of 240 and 340 nM, respectively (Fig.3). The albumin moiety did not affect the binding of the conjugate to the cells, since an excess amount of MSA was included in the reaction mixture to eliminate non-specific binding. A 100-fold excess of cold conjugate inhibited the binding of the labeled conjugate by more than 90 %. In contrast, free IIF-2 competed partially (less than 50 %) for the binding of the conjugate to B16FE7 cells (data not shown).

Effect of IIF-2-albumin conjugate on lung metastasis of tumor cells. It was of interest to examine whether the IIF-2-albumin conjugate might suppress tumor metastasis more

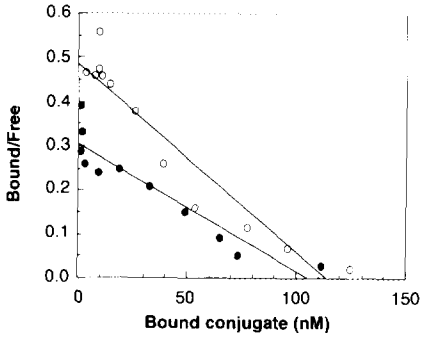

Figure 3. Binding assay of IIF-2-albumin conjugate. B16FE7 and HT1080 were incubated in the presence of increasing amounts of [^{125}I]IIF-2-albumin conjugate for 2 h at 4 ℃. Non-specific binding was estimated by adding a 100-fold excess of unlabeled materials. After the reaction, unbound radioactivity was removed by washing and cell-bound radioactivity was measured in a gamma-counter. Saturation data were presented as a Scatchard plot. ●, B16FE7; ○, HT1080.

effectively than the peptide. As shown in Table 1, the conjugate greatly reduced B16FE7 cell lung colonization in mice. More than 70 % inhibition of metastasis was observed at a dose of 15 nmole conjugate/mouse (Table 1). On the other hand, 150 nmole IIF-2/mouse inhibited the lung metastasis of B16FE7 cells by only about 60 % (12). This shows clearly that the increased anti-invasive activity of the IIF-2-albumin conjugate *in vitro* resulted in the enhancement of its anti-metastatic activity.

Table 1.Inhibitory effects of IIF-2-albumin conjugate on pulmonary metastasis of B16FE7 cells

Dose (nmole/mouse)	Number of metastatic foci/lung (mean ± SEM)	% Inhibition
Experiment I		
0	176.3 ± 43.7	-
10	72.1 ± 14.4	59.1*
30	23.1 ± 7.4	86.9**
Experiment II		
0	47.2 ± 6.2	-
5	39.2 ± 13.5	16.9
15	14.0 ± 4.2	70.3*

B16FE7 cells were mixed with IIF-2-albumin conjugate and injected into the lateral tail vein of C57BL6 mice. The numbers of injected cells were 1.5 x 10^5 and 1.0 x 10^5 /mouse for experiments I and II, respectively. On the 14th day after injection, mice were killed and the numbers of metastatic foci on the lung surfaces were counted under a dissecting microscope. * $P<0.05$, ** $P<0.01$ as compared with control by Mann-Whitney *U* test.

DISCUSSION

We generated a potent inhibitor of cancer metastasis with IIF-2-mediated anti-invasive activity. Although IIF-2 itself is a novel inhibitor of cell migration, relatively high concentrations of this inhibitor (50 - 500 μ M) were required to exhibit significant inhibition of B16FE7 cell invasion through Matrigel in a modified Boyden chamber (12). In the present study, we attempted to modify IIF-2 to increase its anti-invasive activity, with the idea both of expanding the potential of this motility-inhibitor as an anti-metastatic agent, and of exploring the mechanisms by which it suppressed invasion and metastasis. It was expected that immobilization of the IIF-2 peptide on albumin might extend its stability *in vivo*. Taking into consideration that *in vivo* experiments would be conducted in murine systems, we used albumin purified from mice to exclude any antigenic effects of the carrier molecule *in vivo*.

The molar ratio of IIF-2 and albumin in the purified conjugate was 1.5, i.e., one mg conjugate contained approximately 40 μ g IIF-2 peptide. We found that more than 30-fold excess amounts of the peptide were necessary to avoid conjugation between albumin molecule. The purified conjugate did not contain free IIF-2 peptide or albumin oligomers, as demonstrated by reverse phase chromatography on HPLC and SDS-PAGE analysis.

The linkage of IIF-2 to albumin resulted in enhancement of its anti-invasive activity. The conjugate inhibited *in vitro* invasion of tumor cells at 40- to 60-fold lower concentrations than the peptide (Fig. 2). Invasion consists of several steps, including adhesion, degradation of the ECM, and migration (1-3, 17, 18). The IIF-2-albumin conjugate like the IIF-2 peptide did not affect cell adhesion onto the ECM nor inhibit the secretion and activity of type IV collagenase. These results indicate that the conjugate is a potent inhibitor of cell motility. Although the mechanism by which IIF-2 exerts its activity is unknown, the binding assay revealed the existence of IIF-2 binding sites on the cell surface. We are now trying to identify the binding site on the cell surface. We speculate that IIF-2 binds to the cell membrane via specific binding sites, and that this is followed by impairment of cell motility.

The conjugate inhibited lung colonization of B16FE7 cells more efficiently than the corresponding free peptide. This enhanced anti-metastatic activity may be a result of increased activity of IIF-2 *in vitro*. Moreover, it appears that the albumin moiety in the conjugate plays a role in preserving the stability of the conjugate *in vivo*.

In conclusion, the IIF-2 conjugate with albumin appeared to be a potent inhibitor of cell motility and could lead to the development of an anti-metastatic drug.

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