Tumor Invasion-inhibiting Factor 2: Primary Structure and Inhibitory Effect on Invasion in Vitro and Pulmonary Metastasis of Tumor Cells

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INTRODUCTION

There is currently great interest in prevention of cancer metastasis for improving the prognosis of cancer patients. Cancer metastasis is known to consist of a cascade of multiple steps including (a) release of tumor cells from the primary site, (b) cell invasion to surrounding tissues and vasculature, (c) arrest of the circulating cells in the microvasculature of target organs, (d) cell invasion to the parenchyma of the organ and growth in the secondary sites (1-3). It is expected that blocking of either one of the steps in this cascade is clinically useful to prevent cancer metastasis.

We have developed the in vitro invasion system for estimating the ability of tumor invasion (4). In this system, highly invasive rat ascites hepatoma cells, which were inoculated onto the mesothelial cell monolayer, penetrated and grew to form colonies underneath the monolayer. The number of penetrated cells and colonies shows the invasive ability of tumor cells. This model led us to study the mechanisms of tumor cell invasion by evaluating various substances which influence the potential of tumor invasion (5-7). In a previous study, we purified two types of tumor invasion-inhibiting factors, designated as IIF-1 and IIF-2, from the extract of bovine liver by successive chromatographies (8). We report here the complete sequence of purified IIF-2 and the possible mechanism by which the synthetic IIF-2 inhibits invasion and pulmonary metastasis of tumor cells in mice, in addition to its invasion inhibitory activity.

MATERIALS AND METHODS

Animals. Specific-pathogen-free female C57BL/6J mice (6 weeks old) were purchased from Sankyo Labo service Inc., Tokyo, Japan, and housed in sterilized plastic cages. All mice were provided water and gamma lay-sterilized mouse diet ad libitum (FR-2; Sankyo). Mice between 7 and 8 weeks of age were used in the experimental metastasis.

Cells and Cell Culture. The highly invasive Cl-30 cells that had been cloned from rat ascites hepatoma AH130 cells were cultured in Dulbecco's modified Eagle's medium (GIBCO Laboratory, Chargrin Falls, OH) supplemented with 10% FBS (Boehringer Mannheim, Mannheim, Germany). Murine B16 melanoma (B16FE7) cells with highly metastatic potential was kindly provided from Dr. Hajime Tanaka, The Center for Adult Diseases, Osaka, Japan, and maintained in RPMI 1640 (GIBCO) supplemented with 10% FBS. Mesothelial cells were isolated from normal rat mesentery and cultured in 35-mm plastic plates as described previously (4). Mesothelial cells in 10% FBS-Dulbecco's modified Eagle's medium grew to a confluent monolayer after 6-7 days (M-cell sheet).

Purification of Tumor IIF-2. IIF-2 was purified by methods described previously (8). Briefly, the HCl-ethanol extract of bovine liver was subjected to ultrafiltration using an Amicon PM10 membrane. The filtrate was further purified by ion-exchange, gel filtration, and reverse phase (C18) chromatographies. Purified IIF-2 was confirmed to be homogeneous by an analytical µBondasphere C18-300A column (waters Associates, Milford, MA) on HPLC.

Determination of Amino Acid Sequence of IIF-2. The amino acid sequence of IIF-2 was determined with an Applied Biosystems 477A liquid phase sequencer on-lined with 120A PTH amino acid analyzer (Applied Biosystems, Inc., Foster City, CA). Purified IIF-2 (500 pmol) was directly applied to a filter and subjected to 25 cycles of NH2-terminal sequencing.

Peptide Synthesis. IIF-2 and its fragment (residue [1-11]) were chemically synthesized according to the primary structure by the solid-phase method (9) on a Milligen automated peptide synthesizer model 9050 (Milligen, Bedford, MA). After deprotection and cleavage from the resin, peptides were purified by a semi-preparative C18 column on HPLC. Purity of each peptide was above 98% demonstrated by an analytical C18 column on HPLC, and the identity was confirmed by amino acid analysis.

Monolayer Invasion Assay Using Cl-30 Cells. In vitro invasive capacity of Cl-30 cells was estimated as described previously. The Cl-30 cells (1.5 x 105 cells/plate) were seeded on an M-cell sheet and incubated for 20 h at 37°C. The number of penetrated tumor cells and colonies formed underneath the M-cell sheet was counted under a phase contrast microscope.

Chemotaxis and Invasion Assay Using B16FE7 Cells. The chemotactic migration of B16FE7 cells was assayed in a Chemotaxis cell chamber with an 8-µm pore size polycarbonate filter (Kuraray, Inc., Osaka, Japan) essentially according the methods of McCarthy and Furcht (10). The chambers were adjusted in a 24-well culture plate. Ten µg/ml of human fibronectin (Iwaki Glass Co., Ltd., Tokyo, Japan) diluted with serum-free RPMI in the lower compartment were used as a chemoattractant.
In some experiments, 25 μg/ml of human laminin (GIBCO) were used in place of fibronectin. B16F11 cells were labeled by culture for 48 h at 37°C in 10% FBS-RPMI supplemented with 1 μCi/ml [methyl-3H] thymidine (80 Ci/mmol; Amersham Japan Co., Tokyo, Japan). After washing in serum-free RPMI, cells at log-phase were harvested by 0.25% trypsin and 0.02% EDTA (trypsin-EDTA) treatment and resuspended in serum-free RPMI containing 0.1% BSA. The cells (5 × 10⁴ in 200 μl) were seeded onto the upper compartment with or without IIF-2. After 4 h incubation at 37°C, the cells on the upper surface of the filter were removed with a cotton swab. The filter was treated with tissue solubilizer, NCS (Amersham), and cell-associated radioactivity was determined in a Beckman LS 5000TD liquid scintillation counter (Beckman Instruments, Inc., Palo Alto, CA). The invasion assay was performed essentially according to the methods of Albini et al. (11). The filter was coated with 50 μg of Matrigel (Collaborative Research, Inc., Bedford, MA), which is the mixture of basement membrane components such as fibronectin, type IV collagen, laminin, vitronectin, and proteoglycans. [³H]Thymidine-labeled cells (1 × 10⁵) in 200 μl in 0.1% BSA-RPMI with or without IIF-2 were added to the upper compartment and incubated for 20 h at 37°C. The following procedures were the same as those of chemotaxis assay. Preliminary experiments demonstrated that [³H]thymidine-labeled cells migrated in a manner identical to the unlabeled cells.

Cell Growth Assay. B16F11 cells were harvested by trypsin-EDTA solution and inoculated (1.5 × 10⁴ cells/well) into 96-well culture plates with or without IIF-2 in 10% FBS-RPMI. The cells were incubated for 24 h at 37°C. After the incubation, the number of viable cells was detected by the colorimetric method (12) using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrasodium bromide assay kit (Chemicon International, Inc., Temecula, CA).

Cell Adherence Assay. Twenty-four-well culture plates were coated with 2.5 μg/ml of human fibronectin or 25 μg/ml of Matrigel. After treating the plate with 5% BSA in Dulbecco's phosphate-buffered saline, B16F11 cells suspended in 0.1% BSA-RPMI were added to the wells (5 × 10⁴ cells/well) and incubated for 30 min at 37°C. At the end of the incubation, the wells were washed 5 times with serum-free RPMI to remove unadhered cells. The adhered cells were harvested by trypsin-EDTA solution and counted with a hemocytometer.

Type IV Collagenase Assay (Zymographic Analysis). A M<sub>W</sub> 90,000 type IV collagenase (gelatinase) in the serum-free conditioned medium of human schwannoma (YST-3) cells was prepared by Dr. Kaoru Miyazaki, Kihara Institute for Biological Research, Yokohama City University, Yokohama, Japan. The type IV collagenase was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis on the substrate (1 mg/ml)-containing gel. Sodium dodecyl sulfate was removed from the gel after the electrophoresis, and the gel was incubated in the reaction buffer (50 mM Tris-HCl, pH 7.5, containing 10 mM CaCl<sub>2</sub> and 0.02% NaN<sub>3</sub>) with or without 1 mg/ml of IIF-2. After the incubation, the gel was stained with Coomassie brilliant blue and destained. The protease activity was visualized as a nonstained band of lysis against a blue background.

Experimental Metastasis Assay. The B16F11 and 3LL cells at exponential growth phase were harvested by a brief trypsin-EDTA treatment, washed twice, and resuspended in serum-free RPMI. The cells were mixed with or without IIF-2 and injected in the lateral tail vein of a C57BL/6 mouse. The number of tumor cells challenged i.v. in each mouse was 1.2 × 10⁶ for B16F11 cells and 2.5 × 10⁶ for 3LL in a volume of 200 μl. After i.v. injection, the cell viability was assessed by the dye exclusion test and found to be above 95%. At 14 days, mice were killed and the number of metastatic foci on the lung surface was counted under a dissecting microscope.

Statistical Analysis. The statistical significance of differences between groups was determined by Student's two-tailed t test or the Mann-Whitney U test (nonparametric rank sum test).

RESULTS

Primary Structure of IIF-2. We previously reported two types of tumor invasion-inhibiting factors from bovine liver with the molecular weight of about 5000 and 2000 and named them IIF-1 and IIF-2, respectively (8). In the present study, the primary structure of IIF-2 was determined. The predominant sequence was :NH₂-Ala-Glu-Asp-Gly-Asp-Ala-Lys-Thr-Asp-Gln-Ala-Glu-Lys-Ala-Glu-Gly-Ala-Gly-Asp-Ala-Lys-COOH. This sequence was found to be identical to that of the COOH-terminal region of HMG17 protein, by a computer search of the National Biomedical Research Foundation protein data bank. To confirm whether or not this peptide could inhibit tumor invasion, we chemically synthesized the above peptide and compared the extent of its invasion-inhibitory activity with that of purified IIF-2, which has been obtained in the previous experiment (8). As shown in Fig. 1, the synthetic peptide showed similar inhibitory activity as that of purified IIF-2. The amino acid composition and analytical pattern on C<sub>18</sub> chromatography of synthetic peptide were identical to those of the purified IIF-2 (cf. Ref. 8). From these results, we have concluded that IIF-2 which has a sequence identical to that of the COOH-terminal region [69-89] of HMG17 is the entity of the tumor invasion-inhibitory activity.

Inhibitory Effect of IIF-2 on Tumor Cell Migration and Invasion through Matrigel. To confirm the effect of IIF-2 on tumor cell invasion using in vitro models other than our monolayer system, we examined the effects of the synthetic IIF-2 on the chemotaxis of highly metastatic B16F11 melanoma cells to fibronectin and on the invasion through Matrigel using a modified Boyden chamber. When IIF-2 was incubated with B16F11 cells at various concentrations from 1 to 1000 μg/ml in the upper compartment of the chemotaxis chamber, it inhibited the chemotactic migration to fibronectin and invasion by tumor cells through Matrigel in a dose-dependent manner (Fig. 2), although less effectively than its effect on Cl-30 cells measured

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4 H. Yasumitsu, K. Miyazaki, F. Umenishi, N. Koshikawa, and M. Umeda. Comparison of extracellular matrix-degrading activities between 64-kDa and 90-kDa gelatinases purified in inhibitor-free forms from human schwannoma cells, manuscript in preparation.
IIF-2 inhibits tumor invasion and metastasis

Fig. 2. Effect of synthetic IIF-2 on tumor cell chemotaxis to fibronectin and invasion through Matrigel in a modified Boyden chamber. Left, cell motility assayed in a Chemotaxiscell chamber with an 8-μm pore size filter. Ten μg/ml of fibronectin in the lower compartment were used as a chemotactic agent. Right, invasion assayed by using the filter coated with 50 μg of Matrigel. Thymidine-labeled B16F1E7 cells (5 × 10⁴/200 μl) were inoculated with or without the synthetic IIF-2 in the upper compartment and incubated for 4 h at 37°C. After the incubation, the cells on the upper surface of the filter were removed and cell-associated radioactivity was measured as described in “Materials and Methods.” Points, mean of triplicate determinations; bars, SD. All experiments were repeated three to five times. +, P < 0.05; ++, P < 0.01, compared with the control by Student’s two-tailed t-test.

by the monolayer assay (cf. Fig. 1). When laminin was used as a chemotactic agent in place of fibronectin, the chemotactic migration of B16F1E7 cells was also suppressed by IIF-2. The number of migrated cells in the presence of 100 μg/ml of IIF-2 was 2575 ± 239/filter as compared with that (4717 ± 309/filter) in the absence of IIF-2 (P < 0.01; mean ± SD of quadruplicate samples).

Since high concentrations of IIF-2 (0.05–0.5 mM) were required to achieve maximum inhibition of chemotaxis and invasion of B16F1E7 cells, it was important to rule out nonspecific effects related to high concentrations of peptides. Therefore, we synthesized a peptide corresponding to residues [1–11] of IIF-2 and examined its effect on chemotactic migration of B16F1E7 cells to fibronectin. This peptide at 1000 μg/ml (1.0 mM), however, showed no significant inhibition (data not shown).

Lack of Effect of IIF-2 on Growth and Adhesion of Tumor Cells. We examined the effect of IIF-2 on the growth of B16F1E7 cells to exclude the possibility that IIF-2 exerts its antiinvasive activity by impairing the cell proliferation. The synthetic IIF-2 at concentrations of 10–1000 μg/ml did not affect cell growth after a 24-h incubation period (data not shown). The inhibition of chemotactic migration and invasion through Matrigel of tumor cells shown in Fig. 2 may be mediated by an inhibition of the binding of the tumor cells to fibronectin and Matrigel. However, the adhesion of B16F1E7 cells to these substrates was not inhibited by the synthetic IIF-2 at concentrations up to 1000 μg/ml (data not shown).

Lack of Effect of IIF-2 on Type IV Collagenase. Degradation of extracellular matrix by proteases secreted from tumor cells or on cell membrane has been shown to be a crucial step in the invasion. The inhibitors of metalloproteases have been shown to suppress tumor invasion and also metastasis (13–16). Therefore, we investigated the effect of IIF-2 on type IV collagenase (gelatinase) secreted from human schwannoma cells, YST-3. IIF-2 at 1000 μg/ml failed to inhibit the enzyme activity estimated by a zymographic analysis (result not shown).

Inhibitory Effect of IIF-2 on Pulmonary Metastasis of Tumor Cells. Since invasion is one of the most important steps in the formation of metastasis, an inhibitor of invasion can be expected to prevent cancer metastasis. Therefore, it was of interest to examine whether IIF-2, which was confirmed to reduce the invasiveness of the tumor cells as described above, might suppress tumor metastasis. We examined the effect of synthetic IIF-2 on pulmonary metastasis of B16F1E7 and 3LL cells in an experimental metastasis model.

The number of metastatic foci on the lung surface was decreased significantly when IIF-2 was coinjected with the tumor cells into the tail vein of mice (Table 1). The reduction of pulmonary colonization of B16F1E7 and 3LL cells was not due to the direct cytotoxic effect of IIF-2 on the tumor cells because the cell viability was more than 95% even after the incubation of the cells with IIF-2.

DISCUSSION

We determined in this work the primary structure of IIF-2, which is one of the tumor invasion-inhibiting factors (8). The amino acid sequence was found to be identical to that of COOH-terminal region [69–89] of HMG17, which is known to be one of the non-histone nuclear proteins (17) and consists of 89 amino acids (18). Although the function of HMG17 remains unclear, it was observed that HMG17 interacts with DNA at its central region and nuclear proteins like histone H2A at its COOH-terminal region (19, 20). Although it is not clear whether HMG17 protein acts as an antiinvasive substance at present, it might show activity similar to that of IIF-2 since it includes a sequence which possesses the invasion-inhibitory activity. In our previous work, we found that pretreatment of tumor cells with partially purified IIF-2 eliminated the invasive ability of the cells and that plasma membranes prepared from the tumor cells did absorb the inhibitory activity (6). These results suggest that IIF-2 binds to the tumor cell surface. If there exists a cell surface component(s) similar to nuclear protein(s) which interacts with COOH-terminal region of the HMG17, IIF-2 could interact with tumor cells via this component(s). Further studies are needed to identify the specific receptor(s) for IIF-2. IIF-2 has the unique repeated sequence,
Gly–Asp–Ala–Lys (residues [4–7] and [18–21]), which may play an important role on its interaction with membrane component(s). The identification of the active site of IIF-2 is now under study.

We confirmed that IIF-2 inhibits the invasion not only of rat hepatoma (Cl-30) cells but also of B16FE7 cells. IIF-2 inhibited the chemotactic migration of B16FE7 cells to fibronectin (Fig. 2) and laminin. We also confirmed that inhibition of tumor invasion by IIF-2 is not due to non-specific effects related to high concentrations (0.05–0.5 mM) of the peptide. Although there are no data which show why IIF-2 is less effective in the high concentrations (0.05–0.5 mM) of the peptide. Although it has been demonstrated that the activity of proteases released from the tumor cells is essential for the completion of tumor invasion (3). There are many reports in which metalloproteases such as type IV collagenases are shown to play a major role in the degradation of extracellular matrix during invasion (13, 14, 24–27). The IIF-2 did not suppress the activity of type IV collagenase (gelatinase) obtained from the conditioned medium of the tumor cells. This protease has been confirmed to be sensitive to metalloprotease inhibitors, such as EDTA and 1,10-phenanthroline, and potently hydrolyzes type IV collagen as well as type I and III collagens. It could not be excluded, however, that IIF-2 acts via an inhibitor of hydrolases responsible for invasion, such as plasminogen activator (13, 14) and glycosaminoglycan hydrolase (28). We have found that penetration by Cl-30 cells in the monolayer assay is not inhibited by protease inhibitors such as the trypsin inhibitor, phenylmethylsulfonyl fluoride, E-64, and phosphoramidon (29). However, further experiments are needed to examine the effect of IIF-2 on the production of proteases in Cl-30 cells.

When we examined the effect of IIF-2 on lung metastasis of B16FE7 and 3LL cells in mice, IIF-2 elicited substantial reduction of the formation of pulmonary metastatic foci of both types of cells. It has been expected that cancer metastasis could be prevented by blocking either one of the sequential steps of metastatic processes including adhesion, degradation of extracellular matrix, and migration (24). For example, synthetic peptides containing the cell-binding site of fibronectin have been reported to suppress cancer metastasis by interfering with the adhesion or the migration of tumor cells (30–33). Tissue inhibitor of metalloproteases is also found to inhibit pulmonary metastasis of melanoma cells in mice by inhibiting the degradation of extracellular matrix by tumor cell-associated proteases (15, 16).

The data thus far obtained suggest that IIF-2 exerts its inhibitory effect on tumor invasion by impairing cell migration without inhibiting cell growth, adhesion to extracellular matrix, and protease activity. Further studies are now in progress to investigate whether IIF-2 would influence cell motility. Although further study is necessary to identify which process of metastasis is inhibited by IIF-2 in vivo, the results of in vitro experiments suggest that IIF-2 inhibits the migration of metastasizing cells through the extracellular matrix (extravasation step) following their arrest in the capillary bed of the lung after i.v. injection.

In summary, IIF-2 is a novel type of antimetastatic substance, which seems to inhibit the migration of tumor cells. It might be therapeutically useful against artificial seeding of tumor cells during surgical removal of primary tumors and prevention of secondary metastasis from established metastases. Production of a large amount of IIF-2 should provide us with a way to examine its specific role in cancer metastasis.

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REFERENCES


IIF-2 INHIBITS TUMOR INVASION AND METASTASIS


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