Jpn. J. Cancer Res. 81, 909–914, September 1990

Purification and Characterization of Tumor Invasion-inhibiting Factors

Atsushi Isoai,[1,3] Yuko Giga-Hama,[1] Kiyoko Shinkai,[2] Mutsuko Mukai,[2] Hitoshi Akedo[2] and Hiromichi Kumagai[1]

[1]*Research Center, Asahi Glass Co., Ltd., 1150 Hazawa-cho, Kanagawa-ku, Yokohama 221 and* [2]*Department of Tumor Biochemistry, Research Institute, The Center for Adult Diseases, Osaka, Higashinari-ku, Osaka 537*

Tumor invasion-inhibiting factors were purified from bovine liver using an *in vitro* system for estimating the tumor invasion ability. The acid-ethanol extract of liver was subjected to ultrafiltration (Amicon PM10 membrane), and the fraction corresponding to the molecular weight range below 10,000 was further fractionated by ion-exchange (DEAE-Toyopearl), gel filtration (Bio-Gel P6), and reverse-phase (C18) chromatographies. Two types of active polypeptides with molecular weights of about 5,000 and 2,000 were purified and named IIF-1 and IIF-2, respectively. Both peptides inhibited tumor invasion with half-maximum concentrations of 2–6 ng/ml *in vitro*. The amino acid compositions of both peptides were determined.

Key words: Tumor invasion — Metastasis — Purification — Invasion-inhibiting factors

It is well known that cancer metastasis appears to proceed by a series of steps including (a) release of tumor cells from the primary site, (b) invasion of surrounding tissues and vasculatures by tumor cells, (c) arrest of the circulating cells in the microvasculatures of distant organs, (d) tumor cell invasion and growth in the secondary sites.[1-3] Among these steps the tumor invasion is the most characteristic process. The capacity of malignant cells to invade surrounding normal structures is of central importance in the dissemination of cancer.

We previously established a method for estimating the invasive capacity of tumor cells *in vitro* to investigate the mechanisms of tumor invasion.[4] When Cl-30 cells were inoculated onto a cultured monolayer of mesothelial cells isolated from rat mesentery, the tumor cells penetrated and grew to form colonies underneath the monolayer. Using this model of invasion, we have demonstrated the presence of anti-invasive activity in the acid-ethanol extract from rat liver.[5]

The present study was performed to elucidate the biochemical and physiological properties of the factors with anti-tumor invasion activity. We report here the purification and some properties of two invasion-inhibiting factors.

MATERIALS AND METHODS

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 (DMEM, GIBCO Laboratory, Chagrin Falls, OH) supplemented with 10% fetal bovine serum (FBS, Boehringer Mannheim, FRG). Mesothelial cells were isolated from normal rat mesentery by trypsin digestion and the cells (4×10^4) were seeded in a 35 mm plastic culture plate (Corning Glass Works, Corning, NY) as described previously.[4] Mesothelial cells in 10% FBS-DMEM grew to a confluent monolayer after 6 days (M-cell sheet).

***In vitro* invasion assay** *In vitro* invasive capacity of Cl-30 cells was estimated essentially as described previously.[6] Briefly, the Cl-30 cells (1.5×10^5) were seeded on the M-cell sheet and cultured in 2 ml of 10% FBS-DMEM. After 20 h at 37°C, the number of penetrated single tumor cells and colonies formed underneath the M-cell sheet was counted under a phase-contrast microscope.

Extraction of anti-invasive activity from bovine liver The anti-invasive activity was extracted from bovine liver by the method described previously.[5] The liver homogenate in acid-ethanol solution (HCl-ethanol) was centrifuged at 10,000*g* at 4°C, and the supernatant was adjusted to pH 5.2 by adding NH_3. After removal of the resultant precipitate, 4 volumes of cold ether and 2 volumes of cold ethanol were added to the supernatant. The precipitate recovered by filtration was dissolved in 1 *M* acetic acid, then the solution was centrifuged to remove insoluble material and lyophilized (acid-ethanol extract). The acid-ethanol extract was dissolved in 10 m*M* sodium phosphate buffer, pH 7.2, containing 0.15 *M* NaCl and filtered through an Amicon PM10 ultrafiltration membrane (molecular weight cut-off 10,000, Amicon Corp., Danvers, MA). The filtrate was lyophilized, dissolved in 10 m*M* ammonium acetate buffer, pH 7.2, dialyzed in Spectrapor 3 dialysis tubing (Spectrum Medical Ind. Inc., Los Angeles) against the same buffer (PM10 filtrate), and used for further purification.

[3] To whom correspondence should be addressed.

Ion-exchange chromatography The PM10 filtrate was applied to a DEAE-Toyopearl 650M (Tosoh Corp., Tokyo) column (5×20 cm) that had been equilibrated with 10 m*M* ammonium acetate, pH 7.2. The unadsorbed material was washed out, then the column was developed with a linear gradient of 10 to 200 m*M* ammonium acetate, pH 7.2, at a flow rate of 40 ml/h. The anti-invasion activity of each fraction was determined using the *in vitro* assay system as described above.

Gel filtration The active fractions from the ion-exchange chromatography were concentrated, applied to a Bio-Gel P6 (Bio-Rad Laboratories Inc., CA) column (1.6×90 cm) that had been equilibrated with 0.1 *M* acetic acid, and eluted at a flow rate of 16 ml/h (1 ml fractions were collected). Two active fractions were obtained with molecular weights of about 5,000 (Pool A) and 2,000 (Pool B).

Reverse-phase chromatography on high-performance liquid chromatography The pooled fractions from the gel filtration were lyophilized and dissolved in water and this solution was applied to a semi-preparative YMC-Pack D-ODS-5 (Yamamura Chemical Laboratory Co., Ltd., Kyoto) column (2.5×25 cm) equilibrated with 0.1% trifluoroacetic acid (TFA). The sample was eluted with a linear 60-min gradient of 0 to 60% acetonitrile in the presence of 0.1% TFA at a flow rate of 10 ml/min. Fractions (10 ml/tube) containing the anti-invasive activity were lyophilized, redissolved in water, and chromatographed by injection into a μBondasphere C18-300A (Nihon Waters Ltd., Tokyo) column (0.39×15 cm) with a linear 60-min gradient of 0 to 20% acetonitrile in the presence of 0.1% TFA at a flow rate of 0.8 ml/min.

Amino acid analysis Amino acid analysis was carried out on an Applied Model 420A apparatus (Applied Biosystems Japan, Tokyo).

Other methods Protein concentration was measured by the method of Lowry *et al.*[7] or estimated by amino acid analysis.

RESULTS AND DISCUSSION

In a previous study, we found the presence of tumor invasion-inhibiting activity in the extract of rat liver.[5] We describe here the purification and some properties of the invasion-inhibiting factors (IIFs) from bovine liver, because the active entity appeared to be present in only a very small amount in rat liver.

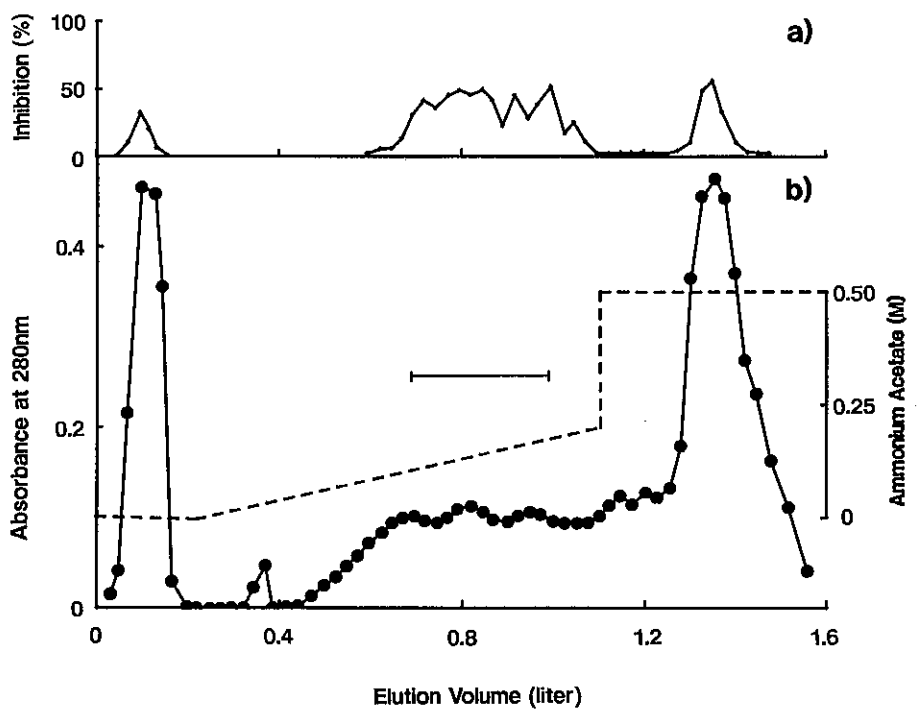
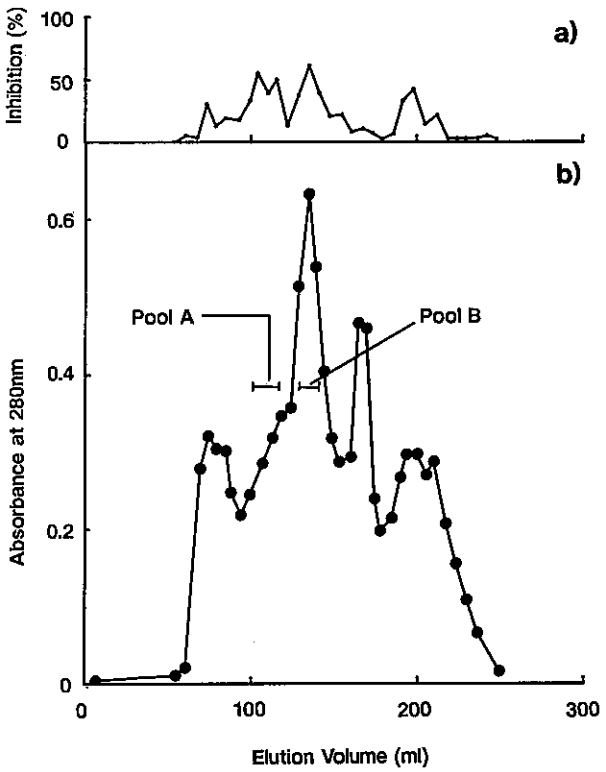


Fig. 1. Ion exchange chromatography of the PM10 filtrate of extract from bovine liver using DEAE-Toyopearl. The column (5×20 cm) was equilibrated with 10 m*M* ammonium acetate, pH 7.2, and was eluted with a linear gradient of 10 to 200 m*M* ammonium acetate, pH 7.2, at a flow rate of 40 ml/h. Anti-invasive activity of each fraction was determined as described in "Materials and Methods." a) *in vitro* invasion-inhibiting activity. b) ●, absorbance at 280 nm; ⊢—⊣, fractions pooled; ----, ammonium acetate concentration.



When a highly invasive clone (Cl-30 cells) obtained from rat ascites hepatoma AH130 was implanted into the rat peritoneal cavity, the cells invaded the peritoneum, and spread to the mediastinal and pararenal lymph nodes. The tumor cells appeared in the bloodstream during the course of tumor growth in the peritoneal cavity. Nevertheless, the cells do not metastasize to other organs, such as the liver, lung and brain. From these observations, we assumed that these organs may have factors which inhibit tumor cell metastasis.

The PM10 filtrate obtained from the acid-ethanol extract of bovine liver was applied to a DEAE-Toyopearl column. Most anti-invasive acitivity was eluted with about 80–170 m*M* ammonium acetate (Fig. 1). Two other bioactive peaks were obtained, but these minor peaks of activity were not further examined in this study.

Fig. 2. Gel filtration column chromatography. The active fractions eluted from the DEAE Toyopearl column were concentrated and chromatographed on a Bio-Gel P6 column (1.6 ×90 cm) as described in "Materials and Methods." a) *in vitro* invasion-inhibiting activity. b) ●, absorbance at 280 nm; ⊢—⊣, fractions pooled.

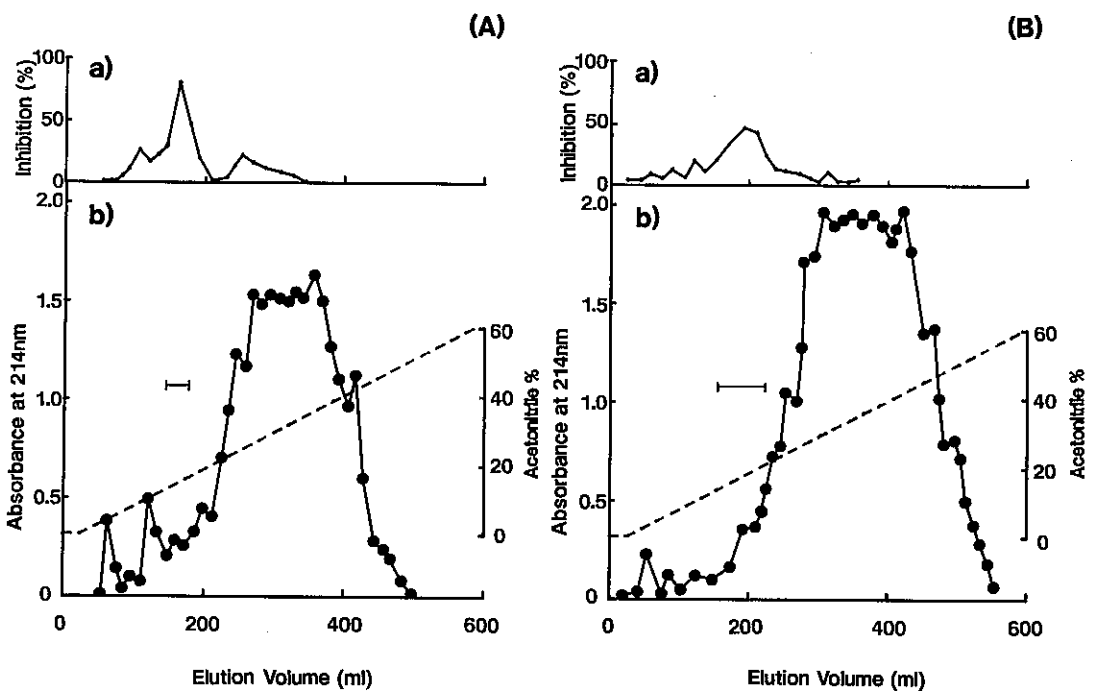


Fig. 3. Reverse-phase chromatography on a semi-preparative HPLC-C18 column. The active fractions obtained from gel filtration were applied on a C18 column (2.5×20 cm) that had been equilibrated with 0.1% TFA and eluted with a linear 60-min gradient of 0–60% acetonitrile in the presence of 0.1% TFA at a flow rate of 10 ml/min. (A) Pool A, (B) Pool B. a) *in vitro* invasion-inhibiting activity. b) ●, absorbance at 214 nm; ⊢—⊣, fractions pooled; ----, acetonitrile concentration.

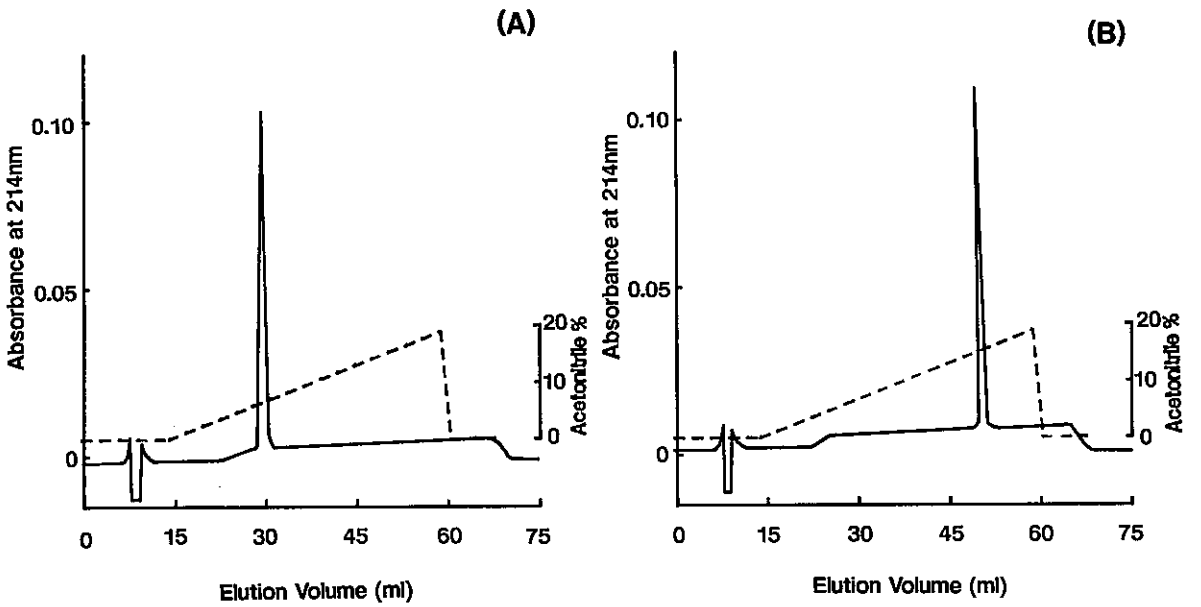


Fig. 4. Purified IIF-1 and IIF-2 on an analytical reverse-phase column. IIF-1 and IIF-2 were each chromatographed on an analytical C18 column (0.39×15 cm) with a linear 60-min gradient of 0–20% acetonitrile in 0.1% TFA at a flow rate of 0.8 ml/min. (A) IIF-1, (B) IIF-2. ——, absorbance at 214 nm; ----, acetonitrile concentration.

The major active fractions from DEAE-Toyopearl chromatography were applied to a gel filtration (Bio-Gel P6) column. As shown in Fig. 2, the activities were eluted at positions corresponding to apparent molecular weights of about 5,000 (Pool A) and 2,000 (Pool B). Though inhibitory activity also appeared in the fractions corresponding to the bed volume of the column, this fraction was found to be cytotoxic, possibly due to the presence of highly concentrated salts. Both fractions (Pool A and B) obtained from the gel filtration were further separated on a semi-preparative C18 column equilibrated with 0.1% TFA, following the elution with 0–60% acetonitrile gradient in the presence of 0.1% TFA. When Pool A was subjected to semi-preparative C18 column chromatography, the anti-invasive activity was eluted with 16–18% acetonitrile (Fig. 3A). In the case of Pool B, the activity appeared in 18–21% acetonitrile (Fig. 3B). Final purification of the IIFs was achieved on an analytical C18 column using a 0–20% acetonitrile gradient. Fig. 4A and B show that each inhibitory activity was eluted as a single peak.

The factor from Pool A of gel filtration was designated as IIF-1 and that from Pool B, IIF-2. The yields of purified IIF-1 and IIF-2 were about 1 μg and 2.5 μg, respectively, from 25 kg of bovine livers. Amino acid compositions of the purified factors are shown in Table I. The possibility that IIF-2 is a fragment of IIF-1 cannot be ruled out at present, but the amino acid compositions of IIF-1 and IIF-2 differ greatly, suggesting that they are unrelated to each other.

Table I. Amino Acid Compositions of IIF-1 and IIF-2 Purified from Bovine Liver

Amino acid	Mol %	
	IIF-1	IIF-2
Asx (Asp/Asn)	8.0	19.0
Glx (Glu/Gln)	12.0	19.0
Ser	8.0	—
Gly	20.0	14.3
Arg	4.0	—
Thr	4.0	4.8
Lys	4.0	14.3
Ala	8.0	28.6
Pro	8.0	—
Tyr	4.0	—
Val	4.0	—
Ile	4.0	—
Leu	12.0	—
Total	100.0	100.0

IIF-1 and IIF-2 inhibited tumor invasion *in vitro* in a dose-dependent manner (Fig. 5A and B) with half-maximum inhibitory concentrations (IC_{50}) of 6 and 2 ng/ml, respectively. Taking their molecular weights into

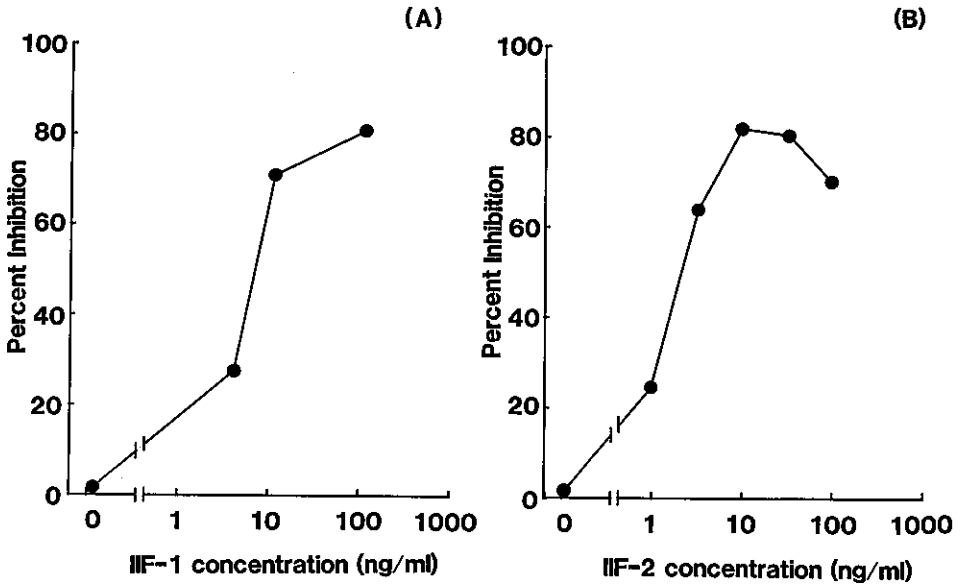


Fig. 5. Effects of purified IIF-1 and IIF-2 on *in vitro* tumor invasion. *In vitro* invasive capacity was determined as described in "Materials and Methods." (A) IIF-1, (B) IIF-2.

account, they appear to have a similar inhibitory activity/mole. The purified factors were not cytotoxic since the viability, as determined by trypan blue exclusion, of Cl-30 cells cultured in the presence and absence of each IIF (up to 100 ng/ml) was identical after 48 h in culture (viability >98%).

It was difficult to estimate the exact recovery of anti-invasive activity, because the homogenate of the liver showed a cytotoxic effect. Moreover, the existence of inhibiting factors other than IIF-1 and -2 was suggested in the step of ion-exchange chromatography. Since PM10 filtrate inhibited the tumor invasion with IC_{50} of about 20 μg/ml, the purification by successive chromatographies resulted in 3,300- and 10,000-fold increase in specific activity for IIF-1 and IIF-2, respectively.

The mechanisms by which purified IIFs inhibit tumor invasion are unknown at present. Tumor invasion seems to involve a complex series of biological events: (1) attachment of tumor cells to extracellular matrix (ECM) components, (2) degradation of ECM by proteases, (3) cell locomotion into ECM. Neither of the IIFs inhibited cell proliferation or the adhesion of tumor cells to fibronectin and laminin (data not shown). Many studies have been done on the relationship of tumor invasion and cell-released or cell-bound proteases which degrade the normal tissue matrix.[8-10] Our preliminary study, however, indicates that IIFs can not suppress the activity of proteases released from tumor cells (data not shown). Since it is unlikely that IIFs inhibit cell adhesion and proteolytic activity as described above, they may inhibit tumor cell invasion by impairing cell movement or locomotion. Shinkai *et al.*[5] previously reported that the anti-invasive activity in the acid-ethanol extract of rat liver is adsorbed on the tumor cell membrane. IIFs may exert their actions by binding to receptors on the tumor cell surface.

It is important to examine whether or not these IIFs do inhibit tumor invasion *in vivo*. Unfortunately, the amount of IIFs we purified from bovine liver was not enough to study their effect *in vivo*. Chemical synthesis of IIFs after the determination of their complete sequences could permit this investigation. The crude material of IIFs has been found to inhibit the peritoneal invasion and metastasis of AH130 cells implanted in rat peritoneal cavity.[5]

Studies are under way to determine the complete structures of IIF-1 and IIF-2. Structural data should allow us to investigate the properties and physiological significance of IIFs, and the mechanisms by which they function.

ACKNOWLEDGMENTS

We thank Prof. Kenji Takahashi, Department of Biophysics and Biochemistry, Faculty of Science, The University of Tokyo, for helpful advice.

(Received March 28, 1990/Accepted June 7, 1990)

REFERENCES

1) Fidler, I. J., Gersten, D. M. and Hart, I. R. The biology of cancer invasion and metastasis. *Adv. Cancer Res.*, **28,** 149–250 (1978).
2) Poste, G. and Fidler, I. J. The pathogenesis of cancer metastasis. *Nature,* **283,** 139–146 (1980).
3) Liotta, L. A., Rao, C. N. and Barsky, S. H. Tumor invasion and the extracellular matrix. *Lab. Invest.*, **49,** 636–649 (1983).
4) Akedo, H., Shinkai, K., Mukai, M., Mori, Y., Tateishi, R., Tanaka, K., Yamamoto, R. and Morishita, T. Interaction of rat ascites hepatoma cells with cultured mesothelial cell layers: a model for tumor invasion. *Cancer Res.*, **46,** 2416–2422 (1986).
5) Shinkai, K., Mukai, M., Komatsu, K. and Akedo, H. Factor from rat liver with antiinvasive potential on rat ascites hepatoma cells. *Cancer Res.*, **48,** 3760–3764 (1988).
6) Shinkai, K., Mukai, M. and Akedo, H. Superoxide radical potentiates invasive capacity of rat ascites hepatoma cells *in vitro. Cancer Lett.*, **32,** 7–13 (1986).
7) Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.*, **193,** 265–275 (1951).
8) Liotta, L. A., Tryggvason, K., Garbisa, S., Hart, I., Foltz, C. M. and Shafie, S. Metastatic potential correlates with enzymatic degradation of basement membrane collagen. *Nature,* **284,** 67–68 (1980).
9) Mignatti, P., Robbins, E. and Rifkin, D. B. Tumor invasion through the human amniotic membrane: requirement for a protease cascade. *Cell,* **47,** 487–498 (1986).
10) Persky, B., Ostrowski, L. E., Pagast, P., Ahsan, A. and Achultz, R. M. Inhibition of proteolytic enzyme in the *in vitro* amnion model for basement membrane invasion. *Cancer Res.*, **46,** 4129–4134 (1986).