Jpn. J. Cancer Res. 83, 1317–1326, December 1992

Inhibition of Cell Attachment, Invasion and Metastasis of Human Carcinoma Cells by Anti-integrin β_1 Subunit Antibody

Shin Fujita,[1,2] Hiroko Suzuki, Masao Kinoshita and Setsuo Hirohashi[3]

Pathology Division, National Cancer Center Research Institute, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104

We investigated the expression of β_1 integrins in human carcinoma cell lines, and the anti-metastatic and anti-invasive effects of a newly established anti-human β_1 subunit monoclonal antibody designated NCC-INT-7. All the examined carcinoma cell lines expressed β_1 integrins upon immunoblot analysis. NCC-INT-7 completely inhibited the adhesion of carcinoma cells to laminin, fibronectin, collagens and acetone-fixed tissues including lung, liver and brain. In an *in vitro* invasion model, NCC-INT-7 inhibited the invasion of human bladder carcinoma cell line T24 and human gastric carcinoma cell lines TMK-1, MKN-45 and MKN-74 through an artificially reconstructed basement membrane. In an *in vivo* nude mouse peritoneal dissemination model using MKN-45 and TMK-1, NCC-INT-7 significantly reduced the number of tumor nodules in the mesentery. In an *in vivo* nude mouse liver metastasis model using a serially transplantable human colonic carcinoma, COL-2-JCK, NCC-INT-7 significantly reduced the number of tumor nodules in liver. These results indicate that β_1 integrins play an important role in the tissue attachment, migration, invasion and metastasis of human carcinoma cells, and that this new monoclonal antibody is useful for studies aimed at prevention of metastasis.

Key words: Integrin — β subunit — Attachment — Invasion — Metastasis

The adhesion of cancer cells to extracellular matrix proteins (ECMP[4]) is thought to play an important role in invasion and metastasis.[1)] Integrin molecules, composed of two subunits α and β, are the cellular receptors for binding to ECMP, including laminin, fibronectin, collagens and vitronectin.[2–5)] Fourteen α subunits and 8 β subunits have been reported. Some α subunits associated with more than one β subunit.[6–9)] However, many α subunits associate with only one β subunit. Thus, the integrin superfamily is subdivided into subfamilies according to the β subunits, i.e. the β_1 subfamily, β_2 subfamily, β_3 subfamily and others. The β_1 subfamily, which is identical to the very-late-activation antigens (VLA), includes the fibronectin receptor, laminin receptor and collagen receptor of epithelial cells, fibroblasts and lymphocytes. The β_2 subfamily is composed of LFA-1, Mac-1 and p150,90, and the β_3 subfamily is composed of Gp IIb/IIIa and vitronectin receptor. Others include $\alpha_6\beta_4$ integrin and some recently detected integrins.[10–12)] Epithelial cells express β_1 integrins and $\alpha_6\beta_4$ integrin.[4)] Thus, these integrins are thought to mediate carcinoma cell adhesion to ECMP.

Recently, alterations of expression in the integrins have been reported in carcinoma tissues as well as cultured carcinoma or transformed cells.[13–21)] It is suggested that alteration of integrin expression is associated with alteration of cell adhesiveness and cell mobility, and thus with the invasiveness and metastatic ability of carcinoma cells.

In this study, we established an anti-human β_1 subunit monoclonal antibody designated NCC-INT-7 and used it to investigate the expression of β_1 integrins as a whole in human carcinoma cells. We also examined the roles of β_1 integrins in invasion and metastasis using an *in situ* adhesion assay, in which carcinoma cell adhesion to acetone-fixed target organs was examined, and an *in vitro* invasion assay, in addition to *in vivo* liver metastasis and peritoneal dissemination models. We found that NCC-INT-7 clearly inhibited the attachment of carcinoma cells to both ECMP and to acetone-fixed tissues, and also affected invasion and metastasis in experimental models. This is the first report of anti-invasive and anti-metastatic effects of anti-β_1 subunit antibody in *in vivo* models.

MATERIALS AND METHODS

Cultured cell lines and xenografts The human carcinoma cell lines listed in Table I were maintained in RPMI-1640 supplemented with 10% heat-inactivated FCS at 37°C in

[1] Present address: Department of Surgery, School of Medicine, Keio University, Shinanomachi, Shinjuku-ku, Tokyo 160.

[2] Awardee of a Research Resident Fellowship from the Foundation for Promotion of Cancer Research.

[3] To whom reprint requests should be addressed.

[4] The abbreviations used are: ECMP, extracellular matrix proteins; FCS, fetal calf serum; BSA, bovine serum albumin; PBS, phosphate-buffered saline; PBS(+), PBS containing 100 mg/liter $CaCl_2$ and $MgCl_2$; T-PBS, PBS with 0.5% Tween 20; PMSF, phenylmethylsulfonyl fluoride; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; GRGDSP, Gly-Arg-Gly-Asp-Ser-Pro; GRGESP, Gly-Arg-Gly-Glu-Ser-Pro; RGD, Arg-Gly-Asp.

a humidified atmosphere containing 5% CO_2/95% air. Cell lines PC-1, PC-6, PC-9, PC-14 and H-1 were supplied by Y. Hayata. Cell lines TMK-1, MKN-7, MKN-28, MKN-45, MKN-74 and KATO-III were supplied by T. Suzuki. Cell lines N231 and H69 were supplied by A.F. Gazdar. Cell lines MCF-7, T24, COLO 201, COLO 205, SW837 and SW1116 were obtained from the American Type Culture Collection. Cell lines CCK-81 and A431 were obtained from the Japanese Cancer Research Resources Bank. c-Lu-65A (adherent type), c-Lu-65F (floating type), c-Co-11, c-Li-21, c-Li-24, Okada and PA-1 were established in our laboratory.[22, 23]

A human colon carcinoma xenograft COL-2-JCK was supplied by Y. Onishi. This tumor was maintained by serial transplantation into the subcutaneous tissue of athymic mice (BALB/c *nu/nu*).

Monoclonal antibodies Murine monoclonal antibody NCC-INT-7 (IgG_1, κ) was obtained by immunizing a BALB/c mouse with c-Lu-65F cells and screening antibodies which had the ability to inhibit the binding of cells to type IV collagen-coated wells. The monoclonal antibody was purified from mouse ascites and culture supernatant using a MAPS II Kit (Bio-Rad, Richmond, CA) according to the manufacturer's instructions. NCC-INT-7 immunoprecipitated two subunits of 150 kDa and 130 kDa by non-reducing SDS-PAGE, corresponding to the α and β_1 subunits of integrin, as shown in Fig. 1. An anti-fibronectin receptor monoclonal antibody which recognizes the β_1 subunit, purchased from Iwaki, Tokyo, was used as a control. The inhibition of carcinoma cell adhesion to laminin, fibronectin and type IV collagen by NCC-INT-7 and preclearing analysis by NCC-INT-7 clearly demonstrated that NCC-INT-7 is specific to the β_1 subunit (Fig. 1).

For all the experiments with NCC-INT-7, a monoclonal antibody, NCC-LU-226, raised against lung carcinoma was used as a negative control. This monoclonal antibody has the same isotype as NCC-INT-7, but shows no effect on cell function, including adhesion and growth.

Immunoblot analysis Cultured cells were lysed in lysis buffer consisting of PBS(+), 1 m*M* PMSF (Sigma, St. Louis, MO) and 50 m*M* *n*-octyl β-D-glucopyranoside (Sigma). The total protein concentration in the cell lysates was adjusted to 2 mg/ml using a BIO-RAD Protein Assay (Bio-Rad) according to the manufacturer's instructions. Cell lysates were mixed with Laemmli's sample buffer and denatured at 100°C for 5 min. Samples of 10 μg protein/lane were electrophoresed in 7.5% SDS-polyacrylamide gel[24] and transferred to Immobilon (Millipore, Bedford, MA). The membranes were blocked with blocking buffer for 3 h as described previously,[25] incubated for 2 h with primary antibody and washed 3 times in T-PBS. Then, they were incubated for 30 min with peroxidase-conjugated anti-mouse IgG (IBL, Fujioka) diluted 1:1,000 with the blocking buffer without azide, washed in T-PBS, incubated with ECL reagents (Amersham, Amersham, UK) for 1 min and exposed to X-ray film according to the manufacturer's instructions. Optical density of the detected bands was measured using a Model 620 video densitometer (Bio-Rad).

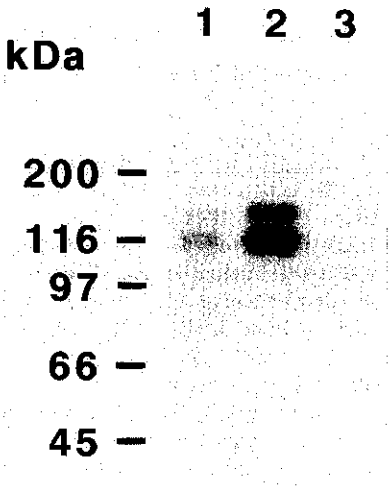


Fig. 1. Immunoprecipitation and preclearing analysis with anti-fibronectin receptor antibody and NCC-INT-7. ^{125}I-surface-labeled c-Lu-65A lysates were subjected to immunoprecipitation using anti-fibronectin receptor antibody (lane 1) and NCC-INT-7 (lane 2) and analyzed by 7.5% SDS-PAGE under non-reducing conditions. Bands of 150 kDa and 130 kDa were immunoprecipitated with anti-fibronectin receptor antibody and NCC-INT-7. The surface-labeled c-Lu-65A lysates were precleared twice with NCC-INT-7, subjected to immunoprecipitation using anti-fibronectin receptor antibody (lene 3) and analyzed by 7.5% SDS-PAGE under non-reducing conditions.

Immunoprecipitation For cell-surface labeling, cultured cells were radio-iodinated witn ^{125}I-sodium iodide for 15 min at room temperature by the iodogen method,[26] washed 3 times in PBS, lysed for 15 min on ice with PBS(+), 1 m*M* PMSF and 0.5% Triton-X-100 and centrifuged at 15,000*g* for 10 min at 4°C. The lysates were incubated with CNBr-activated Sepharose 4B (Pharmacia, Uppsala, Sweden) or Affi-gel protein A (Bio-Rad) conjugated with monoclonal antibody, according to the manufacturer's instructions, for 2 h at room temperature. The precipitates were washed, and the antigens were eluted in Laemmli's sample buffer for 5 min at 100°C, with or without reduction with 10 m*M* dithiothreitol (Sigma). The samples were finally analyzed by SDS-PAGE on 7.5% polyacrylamide slab gels, followed by autoradiography.

***In situ* adhesion assay** Human tissues obtained at autopsy were fixed in acetone at 4°C and stored at −20°C overnight, then embedded in paraffin using the AMeX method.[27] The sections were cut at a thickness of 3 μm and dewaxed with xylene and acetone. Then 2.0×10^5 carcinoma cells/ml were mounted on the sections with monoclonal antibodies at various dilutions, or Gly-Arg-Gly-Asp-Ser-Pro (GRGDSP) peptide (Iwaki, Tokyo) or Gly-Arg-Gly-Glu-Ser-Pro (GRGESP) peptide (Iwaki), and incubated for 1 or 2 h at 37°C in a moist chamber. The sections were washed in PBS, and the attached carcinoma cells were fixed with Cytokeep (Nippon Shoji, Osaka) and stained with hematoxylin and eosin. For quantification, the numbers of attached carcinoma cells on the sections were counted microscopically.

***In vitro* invasion assay** The *in vitro* invasion assay was carried out according to the method reported previously.[28] Filters of Chemotaxicell (Kurabo, Osaka) with an 8.0-μm pore size were coated with 5 μg of fibronectin in a volume of 50 μl on their lower surfaces and with basement membrane Matrigel (Collaborative Research Inc., Bedford, MA) diluted 20-fold with cold PBS on the upper surface of the filters (100 μl/filter). Carcinoma cells were suspended at a final concentration of 2×10^6/ml in D-MEM with 0.1% BSA. Cell suspensions (200 μl) were added to the upper compartment with antibodies, and conditioned medium of the human fibroblast cell line WI-38 (600 μl) was added to the lower compartment and incubated at 37°C for an appropriate time. The carcinoma cells on the upper surface were wiped with a cotton swab. The filters were fixed with methanol, and stained with hematoxylin. The numbers of carcinoma cells on the lower surface were counted using a microscope in 5 fields at a magnification of ×400.

Liver metastasis and peritoneal dissemination models in nude mice In the peritoneal dissemination model, 500 μl of PBS containing gently trypsinized MKN-45 or TMK-1 cells (2×10^7/ml) was injected intraperitoneally into each of five nude mice (BALB/c *nu/nu*, 5 weeks old, male). In MKN-45 experiments, 100 μg/mouse NCC-INT-7 or negative control antibody was administered on days 0, 1, 2, 3 and 4, intraperitoneally. In the TMK-1 experiment, antibodies were administered at 500 μg/mouse only on day 0, and the mice were killed at 21 days after the injection. Peritoneal dissemination was evaluated from the number of tumor nodules in the mesentery.

In the liver metastasis model, nude mice were anesthetized with ethyl ether. The abdominal wall was excised, the spleen was exposed, and 50 μl of PBS containing gently trypsinized MKN-74 (2×10^7/ml) or COL-2-JCK (2×10^6/ml) with 100 μg/mouse NCC-INT-7 or negative control antibody, was injected into each of five nude mice just under the splenic capsule. The spleen was returned to the peritoneal cavity, and the abdominal wall and skin were closed with sutures. The mice were killed at 60 days after the injection. Metastasis to the liver was evaluated from the number of tumor nodules in the liver. The statistical significance of differences between the groups was determined by using the two-tailed *t* test.

RESULTS

Expression of β_1 integrins on cultured carcinoma cells We examined the expression of β_1 integrins on the cultured human carcinoma cell lines using immunoblot analysis (Table I). All the carcinoma cell lines expressed the β_1 subunit to various degrees, and the cells which showed strong expression (++++) were adherent to ECMP as well as the culture dish. This result suggests that carcinoma cells still required the β_1 integrins for binding to ECMP.

Our results revealed minor variations in the molecular weight of β_1 subunits among the cell lines (Fig. 2). The molecular weight of the β_1 subunit of CCK-81 was the highest among the carcinoma cells examined. A lower molecular-weight band corresponded to the pre-β_1 subunit, the precursor of the β_1 subunit.[29] c-Lu-65-A clearly showed the pre-β_1 subunit band. However, the pre-β_1 subunit was not detectable in many carcinoma cell lines.

Inhibition by NCC-INT-7 of carcinoma cell adhesion to the acetone-fixed tissues Attachment of carcinoma cells to normal tissues, e.g. lung, liver, brain, stomach, colon, testis, thyroid, kidney and spleen, was examined by using the *in situ* adhesion assay. All the carcinoma cell lines examined became attached to the acetone-fixed tissue sections. Carcinoma cells became preferentially attached to the stroma and basement membrane, which contain fibronectin, laminin, and collagens (Fig. 3). Carcinoma cells showed good attachment to organs containing a large amount of stroma and basement membranes. This suggested that attachment to the acetone-fixed tissue sections was mediated by ECMP and their receptors. To confirm this, NCC-INT-7 or GRGDSP peptide was added to cell suspensions and their effects on cell attachment were examined. GRGDSP peptide partially inhibited the adhesion of PA-1, SW1116, COLO 205, A431, c-Lu-65A and c-Lu-65F to the sections, whereas GRGESP peptide used as a negative control did not inhibit the adhesion (Fig. 3 D, E). On the other hand, NCC-INT-7 strongly inhibited the adhesion of these cells to the scetions in a concentration-dependent manner (Fig. 4), indicating that attachment to the acetone-fixed tissues was mediated by β_1 integrins. The inhibition was not due to cytotoxicity of the antibody, since NCC-INT-7 was not cytotoxic in a cytotoxicity assay with human complement,[30] and incubation of carcinoma cells with NCC-INT-7 at 100 μg/ml did not affect the viability or proliferation of tumor cells *in vitro* (data not shown).

Table I. Expression of β_1 Integrins on Human Carcinoma Cell Lines

Cell line	β_1 expression	Cell line	β_1 expression
Lung carcinoma		Colonic carcinoma	
Adenocarcinoma		COLO 201	+
Okada	+[a]	COLO 205	+
PC-9	++[b]	SW1116	++
PC-14	+++[c]	SW837	++
Squamous cell carcinoma		CCK-81	++
PC-1	+	c-CO-11	++
Large cell carcinoma		Hepatocellular carcinoma	
c-Lu-65A	++	c-Li-21	+++
c-Lu-65F	+	c-Li-24	+++
Small cell carcinoma		Pancreatic carcinoma	
PC-6	+	PA-1	+++
N-231	+	Cholangiocellular carcinoma	
Gastric carcinoma		H-1	+
MKN-7	+++	Breast carcinoma	
MKN-28	+	MCF-7	++
MKN-45	+	Urinary bladder carcinoma	
MKN-74	++	T24	+++
TMK-1	+	Epidermoid carcinoma	
KATO-III	+	A431	+++

a) The absorbance of the β_1 subunit band is lower than 0.5 times that of c-Lu-65A.
b) The absorbance of the β_1 subunit band is between 0.5 and 1.5 times that of c-Lu-65A.
c) The absorbance of the β_1 subunit band is higher than 1.5 times that of c-Lu-65A.

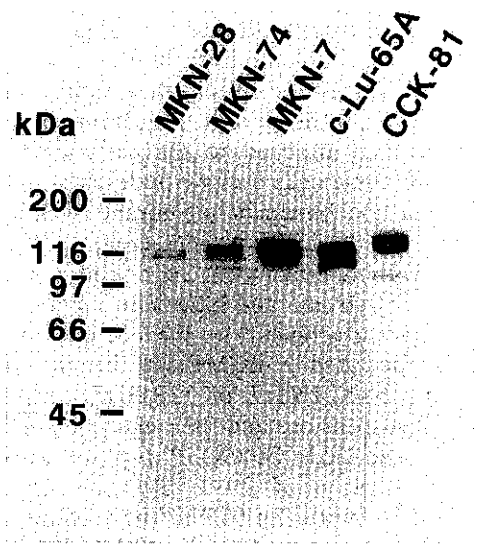


Fig. 2. Immunoblot analysis of β_1 integrin expressed on human carcinoma cell lines. Cell lysates of MKN-28, MKN-74, MKN-7, and c-Lu-65A were subjected to 7.5% SDS-PAGE under non-reducing conditions and blotted onto Immobilon, and the β_1 subunit was detected with anti-fibronectin recepor antibody. The 130-kDa band is β_1 integrin and the lower-molecular-weight band is pre β_1 integrin (c-Lu-65A and MKN-74). Expression of β_1 integrin is weak in MKN-28 (+), intermediate in MKN-74, c-Lu-65A, and CCK-81 (++), and strong in MKN-7 (+++). The molecular weight of β_1 integrin of CCK-81 is higher than that of other carcinoma cells.

Inhibition by NCC-INT-7 of carcinoma cell invasion in *in vitro* invasion assay T24, MKN-74, TMK-1 and MKN-74 cells were added to the upper chamber. After 2 h incubation at 37°C, NCC-INT-7 or negative control antibody (10 μg/ml) was added to the upper chamber. NCC-INT-7 was added to the chamber after carcinoma cells had become attached to the Matrigel, since NCC-INT-7 did not detach cells which had already become attached to ECMP. In this invasion assay, NCC-INT-7 inhibited invasion significantly even after the carcinoma cells had become attached to the Matrigel (Table II, Fig. 5). This result indicated that β_1 integrins play an important role in invasion.

Inhibition by NCC-INT-7 of liver metastasis and peritoneal dissemination of human carcinoma cell lines in *in vivo* nude mouse models TMK-1 and MKN-45 cells were injected into the peritoneal cavity of nude mice with NCC-INT-7 or with negative control antibody, and 3 weeks later, peritoneal dissemination was observed in the mesentery, peritoneal wall, omentum and abdominal lymph nodes. For evaluation, the numbers of tumor nodules in the mesentery were counted (Table III). NCC-INT-7 produced significant decreases in the numbers of tumor nodules in the mesentery of MKN-45 and TMK-1 ($P<0.05$ in Exp. 1 of MKN-45, $P<0.1$ in Exp. 2 of MKN-45, $P<0.01$ in TMK-1) (Fig. 6).

COL-2-JCK and MKN-74 were injected into the nude mice with 100 μg/mouse NCC-INT-7 or with negative

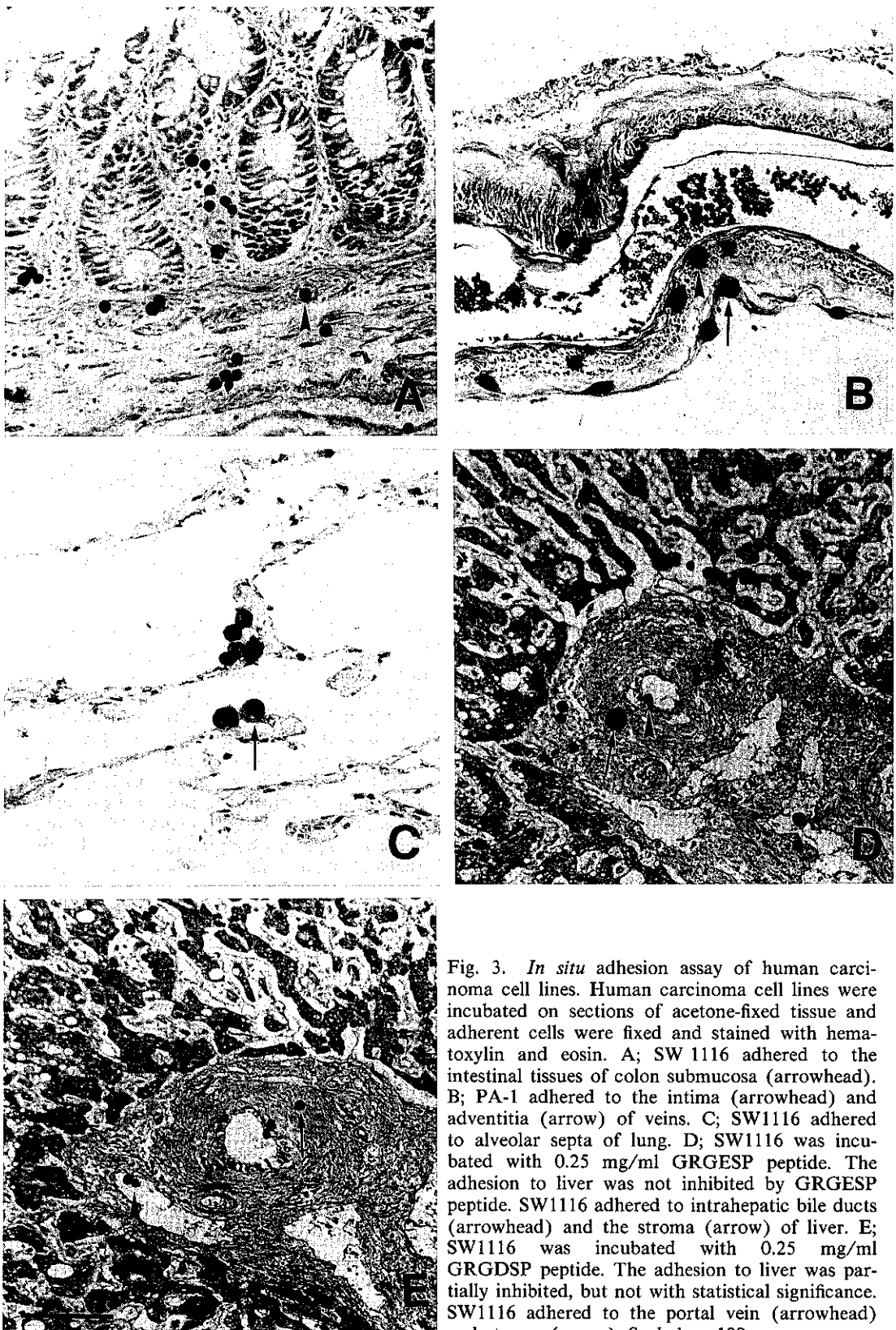
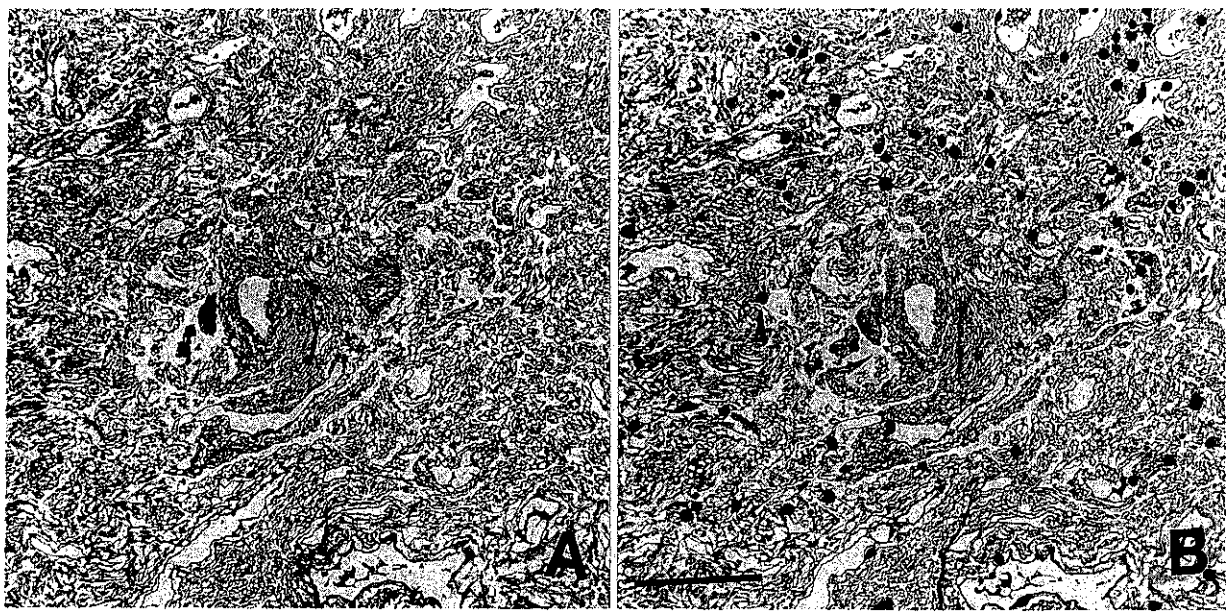


Fig. 3. *In situ* adhesion assay of human carcinoma cell lines. Human carcinoma cell lines were incubated on sections of acetone-fixed tissue and adherent cells were fixed and stained with hematoxylin and eosin. A; SW 1116 adhered to the intestinal tissues of colon submucosa (arrowhead). B; PA-1 adhered to the intima (arrowhead) and adventitia (arrow) of veins. C; SW1116 adhered to alveolar septa of lung. D; SW1116 was incubated with 0.25 mg/ml GRGESP peptide. The adhesion to liver was not inhibited by GRGESP peptide. SW1116 adhered to intrahepatic bile ducts (arrowhead) and the stroma (arrow) of liver. E; SW1116 was incubated with 0.25 mg/ml GRGDSP peptide. The adhesion to liver was partially inhibited, but not with statistical significance. SW1116 adhered to the portal vein (arrowhead) and atroma (arrow). Scale bar, 100 μm.



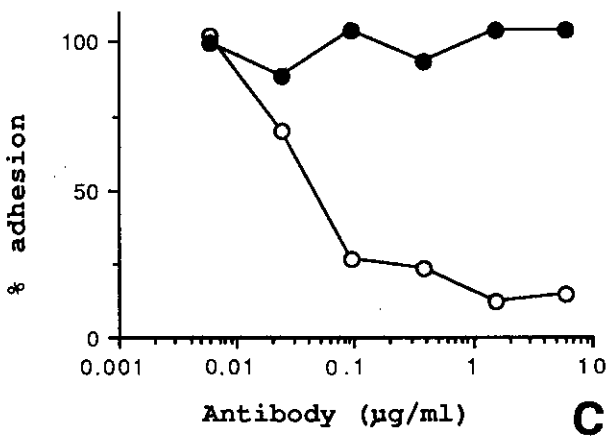


Fig. 4. NCC-INT-7 inhibition of carcinoma cell adhesion to acetone-fixed tissues. (A, B) c-Lu-65-A was incubated on acetone-fixed lung with 10 μg/ml NCC-INT-7 (A) or 10 μg/ml control antibody (B) for 2 h at 37°C. The arrowhead indicates adherent carcinoma cell. Scale bar, 50 μm. (C) c-Lu-65A was incubated on the acetone-fixed lung with serially diluted NCC-INT-7 or control antibody. The cells adherent to the lung were counted by visual observation. Percent adhesion; number of cells on lung incubated with antibody/number of cells on lung incubated without antibody × 100. Each point represents the mean of duplicated examinations

Table II. Effect of NCC-INT-7 on *in vitro* Invasion of Cancer Cells

Cell line	No. of cells (mean ± SD)	
	NCC-INT-7 group	Nagative control group
T24[a]	43.7 ± 18.6**	158.3 ± 32.4**
MKN-74[b]	0*	8.0 ± 6.8*
MKN-45[b]	2.3 ± 2.4*	18.9 ± 11.4*
TMK-1[c]	1.8 ± 1.7**	15.2 ± 6.2**

a) Incubated at 37°C for 6 h.
b) Incubated at 37°C for 2 days.
c) Incubated at 37°C overnight.
** $P < 0.0001$. * $P < 0.005$.

control antibody intrasplenically, and 2 months later, liver metastasis was observed. For evaluation, the numbers of tumor nodules in liver were counted (Table IV). All the carcinoma cells formed tumors at the inoculation site in the spleen and metastasized to liver and, in a few cases, to the lung. NCC-INT-7 produced significant decreases in the numbers of liver nodules in mice injected with COL-2-JCK ($P < 0.1$) (Fig. 7). In the MKN-74 experiment, the number of tumor nodules in liver was reduced in the mice injected with NCC-INT-7. However, this reduction was not significant. These results strongly suggest that β_1 integrins play an important role in liver metastasis and peritoneal dissemination.

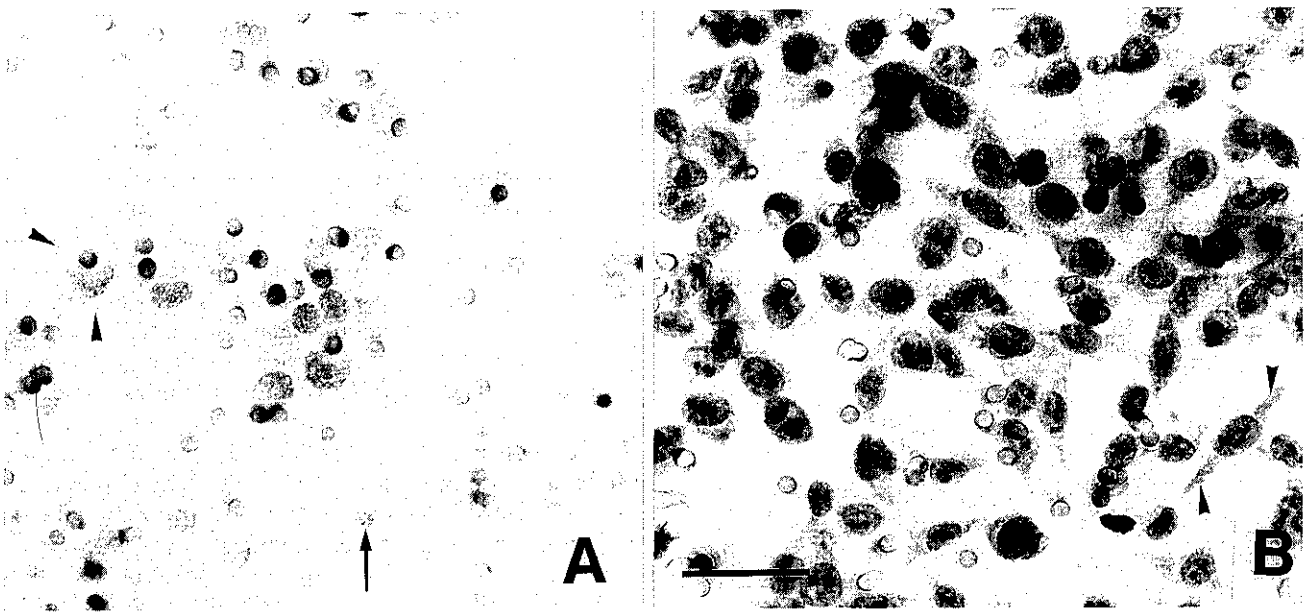


Fig. 5. NCC-INT-7 inhibition of invasiveness of human carcinoma cell lines in an *in vitro* invasion assay. Invasiveness of T24 across an artificially reconstituted basement membrane was examined. (A) Cells were incubated with 10 μg/ml NCC-INT-7, which altered the cell shape from fibroblastoid to round. Arrowheads indicate a round cell. The arrow indicates a pore in the chemotaxis membrane. (B) Cells were incubated with 10 μg/ml negative control antibody. Arrowheads indicate a fibroblastoid cell. Many more cells have invaded in B that in A. Scale bar, 100 μm.

Table III. Effect of NCC-INT-7 on Peritoneal Dissemination of MKN-45 and TMK-1

Cell line	Mean No. of tumor nodules in the mesentery ± SD (range)	
	NCC-INT-7 group	Negative control group
MKN-45 (Exp. 1)[a]	4.2±3.1 (0–9)**	24.4±13.1 (9–43)**
MKN-45 (Exp. 2)[a]	5.8±5.8 (1–13)*	25.2±19.0 (3–49)*
TMK-1	4.2±3.1 (1–8)***	24.4±9.5 (13–35)***

a) These experiments were carried out independently.
* $P<0.1$. ** $P<0.05$. *** $P<0.01$.

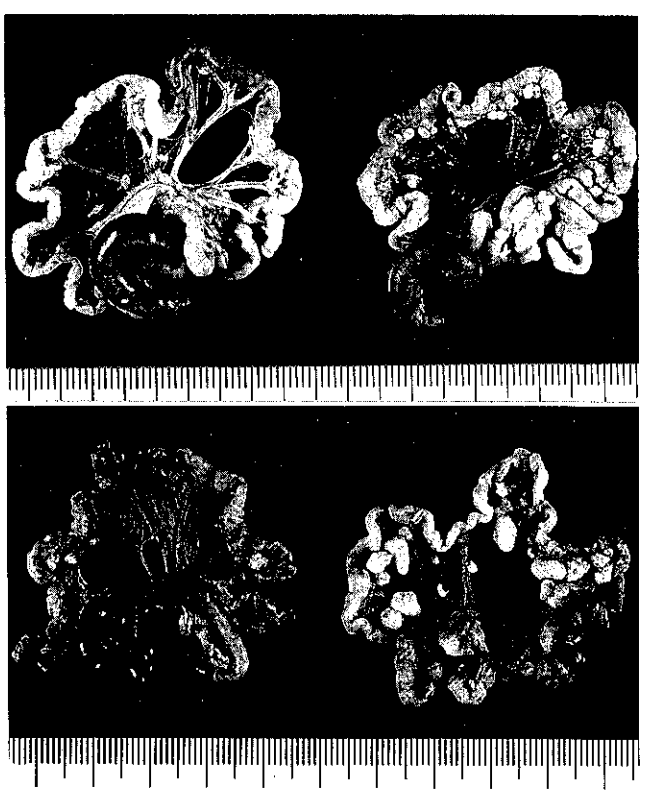


Fig. 6. NCC-INT-7 inhibition of peritoneal dissemination of MKN-45 and TMK-1. 10^7 carcinoma cells/mouse were inoculated into nude mice intraperitoneally with NCC-INT-7 and with negative control antibody, and 3 weeks later, the mice were killed and intestinal mesentery dissemination was examined. (A) Intestines and mesentery from a mouse inoculated with MKN-45 + 100 μg of NCC-INT-7 (left) and those from a mouse inoculated with 100 μg of negative control antibody (right). Arrowheads indicate disseminated tumor nodules in the mesentery. (B) Intestines and mesentery from a mouse inoculated with TMK-1 + 100 μg of NCC-INT-7 (left) and those from a mouse inoculated with 100 μg of negative control antibody (right). Arrowheads indicate disseminated tumor nodules in the mesentery.

Table IV. Effect of NCC-INT-7 on Liver Metastasis of COL-2-JCK and MKN-74

Cell line	Mean No. of tumor nodules in liver±SD (range)	
	NCC-INT-7 group	Negative control group
COL-2-JCK[a)]	0.8±1.1 (0–2)*	8.8±7.7 (0–18)*
MKN-74[b)]	0.6±0.5 (0–1)	4.4±3.9 (1–10)

a) 10^5 cells/mouse were injected with 100 μg/mouse of the antibodies.
b) 10^6 cells/mouse were injected with 100 μg/mouse of the antibodies.
* $P<0.1$.

Fig. 7. NCC-INT-7 inhibition of liver metastasis of human COL-2-JCK. 10^5 carcinoma cells/mouse were inoculated into nude mice with NCC-INT-7 and with negative control antibody, and 2 months later, the mice were killed and liver metastasis was examined. Liver from a mouse inoculated with COL-2-JCK + 100 μg of NCC-INT-7 (left) and that from a mouse inoculated with 100 μg of negative control antibody (right). Arrowheads indicate liver metastatic nodules.

DISCUSSION

Invasion and metastasis of cancer is a complex process. As reported by Liotta *et al.*,[1)] the first step is considered to be tumor cell attachment to the extracellular matrix, mediated by binding of specific attachment factors such as laminin and their receptors. After attachment, local degradation of the matrix is caused by tumor cell proteases (2nd step) and the cells move into the region of the matrix modified by proteolysis (3rd step), eventually leading to the formation of metastasis.[1)] Therefore, carcinoma cell adhesion to the extracellular matrix is very important in invasion and metastasis, and we have investigated the role of β_1 integrins in this process.

All the examined carcinoma cells expressed β_1 subunits upon immunoblotting, and these served as functional ECMP receptors even in carcinoma cell lines, because carcinoma cell lines which expressed β_1 integrin still adhered to wells coated with laminin, fibronectin and collagens, and this adhesion was inhibited by anti-β_1 subunit antibody. It has been reported that the expression of β_1 integrins is reduced immunohistochemically in colonic carcinoma and breast carcinoma tissues.[18–21)] We also investigated the expression of β_1 integrins in gastric and colonic carcinoma tissues immunohistochemically. The expression of β_1 integrins was reduced in some carcinoma tissues. However, total loss of β_1 integrin was detected only exceptionally (unpublished data). These results indicate that β_1 integrins are expressed and are still functional as ECMP receptors in almost all human carcinoma cells.

The molecular weights of β_1 subunits showed minor differences among carcinoma cells. These differences might be caused by differences in post-translational modification. Post-translational modifications of integrins by phosphorylation and glycosylation are reported to be correlated with alterations in cell adhesiveness.[31)] However, no functional difference was clear in our experiments. Thus, the mechanisms of variation in molecular weight and their functional significance remain to be studied.

The *in situ* adhesion assay is a distinctive assay for cell adhesion to actual organs. Cell adhesion to cryostat sections has been reported previously.[32, 33)] Using this method, Netland and Zetter demonstrated organ-specific cell adhesion of B16-F10 melanoma cells and reticulum cell sarcoma cells.[32)] In the present study, we measured cell adhesion to acetone-fixed tissue sections and observed attachment and subsequent cell spreading of carcinoma cells at stroma and basement membrane sites in lung, liver, and other organs, where metastases frequently develop. The finding that addition of NCC-INT-7 completely inhibited carcinoma cell attachment strongly indicated that the initial attachment to actual organs is predominantly mediated by β_1 integrins, and that β_1 integrins play an important role in the first step of invasion and metastasis.

Integrins are divided into two groups: RGD-dependent integrins recognizing Arg-Gly-Asp as a target sequence for binding in ECMP, and RGD-independent integrins. Among the β_1 integrins identified so far, however, only the binding of $\alpha_5\beta_1$ integrin and $\alpha_3\beta_1$ integrin to fibronectin is reported to be RGD-dependent, and the binding of other β_1 integrins to ECMP is reported to be RGD-independent. RGD-independent β_1 integrins are considered to play a more important role than RGD-dependent β_1 integrins in carcinoma cell attachment, because GRGDSP peptide could only partially inhibit the attachment of carcinoma cells to the acetone-fixed tissues. This suggests that GRGDSP peptide alone is unable to inhibit effectively the attachment of carcinoma cells to actual organs. Yamada *et al.* also reported that

peptides containing RGD sequence did not display detectable effects in *in vitro* human cancer cell invasion models.[34] Some investigators, however, have reported that peptide containing RGD sequence inhibits experimental lung metastasis of murine melanoma.[28,35] Therefore, invasion and metastasis of melanoma might depend more on RGD-dependent integrin than on RGD-independent integrin.

We also investigated the effect of NCC-INT-7 on carcinoma cell invasion of reconstituted basement membrane using Matrigel *in vitro*. NCC-INT-7 significantly inhibited the invasion of carcinoma cells even after cancer cells had already adhered to the Matrigel. Another monoclonal antibody against β_1 integrin was reported to inhibit fibroblast migration[29] and the invasion of human fibrosarcoma cell line HT-1080.[34] Thus, the effect of NCC-INT-7 on carcinoma cell invasion may be due to the inhibition of carcinoma cell migration.

These results prompted us to investigate whether this monoclonal antibody inhibits metastasis in an *in vivo* liver metastasis model and a peritoneal dissemination model. In the liver metastasis model, NCC-INT-7 might inhibit the inital arrest of carcinoma cells on the liver, whereas in the peritoneal dissemination model, it might inhibit the initial adhesion and subsequent invasion to the peritoneum. To a greater extent than anticipated, NCC-INT-7 clearly inhibited peritoneal dissemination and liver metastasis of several human carcinoma cells. These results again indicate that β_1 integrins paly an important role in invasion, even *in vivo*.

It is difficult at present to use monoclonal antibodies for cancer therapy, because of their immunogenicity.[36] However, mimetics of the complementarity-determining region[37] of NCC-INT-7, peptides recognized by NCC-INT-7 on β_1 integrins, and cell-binding sequences of ECMP recognized by β_1 integrins are also considered to modulate invasion and metastasis. Studies along these lines may lead to the development of new anti-cancer therapies effective against cancer invasion and metastasis.

ACKNOWLEDGMENTS

We thank Drs. T. Suzuki (Niigata University, Niigata, Japan), Y. Hayata (Tokyo Medidal College, Tokyo, Japan), A.F. Gazdar (NCI-Navy Medical Oncology Branch, Bethesda, MD), and Y. Onishi (Experimental Animal Research Center, Kawasaki, Japan) for providing cell lines and tumors. This work was supported in part by a Grant-in-Aid from the Ministry of Health and Welfare of Japan for the Comprehensive 10-Year Strategy for Cancer Control, and in part by a Special Coordination Funds of the Science and Technology Agency of Japan.

(Received July 1, 1992/Accepted September 12, 1992)

REFERENCES

1) Liotta, L. A., Rao, C. N. and Wewer, U. M. Biochemical interactions of tumor cells with the basement membrane. *Ann. Rev. Biochem.*, **55**, 1037–1057 (1986).
2) Hynes, R. O. Integrins: a family of cell surface receptors. *Cell*, **48**, 549–554 (1987).
3) Hynes, R. O. Integrins: versatility, modulation, and signaling in cell adhesion. *Cell*, **69**, 11–25 (1992).
4) Hemler, M. E. VLA proteins in the integrin family: structures, functions and their role on leukocytes. *Annu. Rev. Immunol.*, **8**, 365–400 (1990).
5) Ruoslahti, E. and Giancotti, F. G. Integrins and tumor cell dissemination. *Cancer Cells*, **1**, 119–126 (1989).
6) Vogel, B. E., Tharone, G., Giancotti, F. G., Gailit, J. and Rouslahti, E. A novel fibronectin receptor with an unexpected subunit composition ($\alpha_V\beta_1$). *J. Biol. Chem.*, **265**, 5934–5937 (1990).
7) Smith, J. W., Vestal, D. J., Irwin, S. V., Burke, T. and Cheresh, D. A. Purification and functional characterization of integrin $\alpha_V\beta_5$. *J. Biol. Chem.*, **265**, 11008–11013 (1990).
8) Hemler, M. E., Crouse, C. and Sonnenberg, A. Association of VLA α_6 subunit with a novel protein. *J. Biol. Chem.*, **264**, 6529–6535 (1989).
9) Sheppard, D., Rozzo, C., Starr, L., Quaranta, V., Erle, D. J. and Pytela, R. Complete amino acid sequence of a novel integrin β subunit (β_6) identified in epithelial cells using the polymerase chain reaction. *J. Biol. Chem.*, **265**, 11502–11507 (1990).
10) Ramaswamy, H. and Hemler, M. E. Cloning, primary structure and properties of a novel human integrin β subunit. *EMBO J.*, **9**, 1561–1568 (1990).
11) Erle, D. J., Ruegg, C., Sheppard, D. and Pytela, R. Complete amino acid sequence of an integrin beta subunit (β_7) identified in leukocytes. *J. Biol. Chem.*, **266**, 11009–11016 (1991).
12) Moyle, M., Napier, M. A. and McLean, J. W. Cloning and expression of a divergent integrin subunit β_8. *J. Biol. Chem.*, **266**, 19650–19658 (1991).
13) Saga, S., Chen, W.-T. and Yamada, K. M. Enhanced fibronectin receptor expression in Rous sarcoma virus-induced tumors. *Cancer Res.*, **48**, 5510–5513 (1988).
14) Plantefaber, L. C. and Hynes, R. O. Changes in integrin receptors on oncogenically transformed cells. *Cell*, **56**, 281–290 (1989).
15) Cheresh, D. A., Smith, J. W., Cooper, H. M. and Quaranta, V. A novel vitronectin receptor integrin ($\alpha_V\beta_X$) is responsible for distinct adhesive properties of carcinoma cells. *Cell*, **57**, 56–69 (1989).

16) Dedhar, S. and Saulnier, R. Alterations in integrin receptor expression on chemically transformed human cells: specific enhancement of laminin and collagen receptor complexes. *J. Cell Biol.*, **110**, 481–489 (1990).
17) Giancotti, F. G. and Ruoslahti, E. Elevated levels of the $\alpha_5\beta_1$ fibronectin receptor suppress the transformed phenotype of Chinese hamster ovary cells. *Cell*, **60**, 849–859 (1990).
18) Koretz, K., Schlag, P., Boumsell, L. and Moller, P. Expression of VLA-α2, VLA-α6, and VLA-β1 chains in normal mucosa and adenomas of the colon, and in colon carcinomas and their liver metastasis. *Am. J. Pathol.*, **138**, 741–750 (1991).
19) Zutter, M. M., Mazoujian, G. and Santoro, S. A. Decreased expression of integrin adhesive protein in adenocarcinoma of the breast. *Am. J. Pathol.*, **137**, 863–870 (1990).
20) Pignateli, M., Smith, M. E. F. and Bodmer, W. F. Low expression of collagen receptors in moderate and poorly differentiated colorectal adenocarcinomas. *Br. J. Cancer*, **61**, 636–638 (1990).
21) Koukulis, G. K., Vitranen, I., Korhoren, M., Laitisen, L., Quaranta, V. and Gould, V. E. Immunohistochemical localization of integrins in the normal, hyperplastic, and neoplastic breast. *Am. J. Pathol.*, **139**, 787–799 (1991).
22) Yamada, T., Hirohashi, S., Shimosato, Y., Kodama, T., Hayashi, S., Ogura, T., Gamou, S. and Shimizu, N. Giant cell carcinomas of the lung producing colony-stimulating factor *in vitro* and *in vivo*. *Jpn. J. Cancer Res.*, **76**, 967–976 (1985).
23) Shimoyama, Y., Gotoh, M., Ino, Y., Sakamoto, M., Kato, K. and Hirohashi, S. Characterization of high-molecular-mass forms of basic fibroblast growth factor produced by hepatocellular carcinoma cells: possible involvement of basic fibroblast growth factor in hepatocellular carcinogenesis. *Jpn. J. Cancer Res.*, **82**, 1263–1270 (1991).
24) Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, **227**, 680–685 (1970).
25) Sakurai, Y., Hirohashi, S., Shimosato, Y., Kodaira, S. and Abe, O. Selection of a monoclonal antibody reactive with a high-molecular-weight glycoprotein circulating in the body fluid of gastrointestinal cancer patients. *Cancer Res.*, **48**, 4053–4058 (1988).
26) Felsted, R. L. and Gupta, S. K. A comparison of K-562 and HL-60 human leukemic cell surface membrane proteins by two-dimensional electrophoresis. *J. Biol. Chem.*, **257**, 13211–13217 (1982).
27) Sato, Y., Mukai, K., Watanabe, S., Goto, M. and Shimosato, Y. The AMeX method: a simplified technique of tissue processing and paraffin embedding with improved preservation of antigen for immunostaining. *Am. J. Pathol.*, **125**, 431–435 (1985).
28) Saiki, I., Murata, J., Matsuno, K., Ogawa, R., Nishi, N., Tokura, S. and Azuma, I. Anti-metastatic and anti-invasive effects of polymeric Arg-Gly-Asp (RGD) peptide, poly(RGD), and its analogues. *Jpn. J. Cancer Res.*, **81**, 660–667 (1990).
29) Akiyama, S. K., Yamada, S. S., Chen, W. and Yamada, K. M. Analysis of fibronectin receptor function with monoclonal antibodies; roles in cell adhesion, migration, matrix assembly, and cytoskeletal orgenization. *J. Cell Biol.*, **109**, 863–875 (1989).
30) Watanabe, M., Oishi, T., Kuzuoka, M., Nudelman, E. D., Stroud, M. R., Kubota, T., Kodaira, S., Abe, O., Hirohashi, S., Shimosato, Y. and Hakomori, S.-I. *In vitro* and *in vivo* antitumor effects of murine monoclonal antibody NCC-INT-421 reacting with dimeric Le^a (Le^a/Le^a) epitope. *Cancer Res.*, **51**, 2199–2204 (1991).
31) Adams, J. C. and Watt, F. M. Changes in keratinocyte adhesion during terminal differentiation: reduction in fibroblast binding precedes $\alpha_5\beta_1$ integrin loss from the cell surface. *Cell*, **63**, 425–435 (1990).
32) Netland, P. A. and Zetter, B. R. Organ-specific adhesion of metastatic tumor cells *in vitro*. *Science*, **224**, 1113–1115 (1984).
33) Shestowsky, W., Fallavollita, L. and Brodt, P. A monoclonal antibody to Lewis lung carcinoma variant H-59 identifies a plasma membrane protein with apparent relevance to lymph node adhesion and metastasis. *Cancer Res.*, **50**, 1948–1953 (1990).
34) Yamada, K. M., Kennedy, D. W., Yamada, S. S., Gralnick, H., Chen, W.-T. and Akiyama, S. K. Monoclonal antibody and synthetic peptide inhibitors of human tumor cell migration. *Cancer Res.*, **50**, 4485–4496 (1990).
35) Humphries, M. J., Olden, K. and Yamada, K. M. A synthetic peptide from fibronectin inhibits experimental metastasis of murine melanoma cells. *Science*, **233**, 466–470 (1986).
36) Foon, K. A. Biological response modifiers: the new immunotherapy. *Cancer Res.*, **49**, 1621–1639 (1989).
37) Saragovi, H. U., Fitzpatrick, D., Raktabutr, A., Nakanishi, H., Kahn, M. and Greene, M. I. Design and synthesis of a mimetic from an antibody complementarity-determining region. *Science*, **253**, 792–795 (1991).