

Adhesion of Gastric Carcinoma Cells to Peritoneum Mediated by $\alpha 3\beta 1$ Integrin (VLA-3)

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ABSTRACT

The interaction between gastric carcinoma cells and the peritoneal lining is a key step in peritoneal dissemination. In this study, we examined the roles of the $\beta 1$ family of integrin receptors in the adhesion of such cells to the peritoneum. The adhesion of several gastric carcinoma cell lines to peritonea excised from mice was inhibited most by an anti- $\alpha 3$ integrin antibody and to a lesser extent by an anti- $\alpha 2$ integrin antibody. In the peritoneal implantation of NUGC-4 human gastric carcinoma cells in athymic mice, treatment of the cells with anti- $\alpha 2$ or anti- $\alpha 3$ integrin antibody reduced the number of disseminated nodules; suppression by the anti- $\alpha 3$ integrin antibody was stronger than that by the anti- $\alpha 2$ integrin antibody. The cDNAs to human $\alpha 2$ and $\alpha 3$ integrins were introduced into K562 leukemic cells, which were positive for the integrin $\beta 1$ subunit but negative for the $\alpha 2$ or $\alpha 3$ subunit. The $\alpha 3$ integrin-transfected cells adhered to excised peritoneum and to a monolayer of peritoneal mesothelial cells more firmly than did the $\alpha 2$ integrin-transfected cells or the mock transfectant. Reverse transcription-PCR was used to analyze the expression of laminin-5 and laminin-10/11, which have been reported to serve as high-affinity ligands for $\alpha 3\beta 1$ integrin. mRNA for these laminin isoforms was found in mesothelial cells from the diaphragm and parietal peritoneum. These results strongly suggest that $\alpha 3\beta 1$ integrin plays an essential role in mediating the initial attachment of cancer cells to the peritoneum, leading to the formation of peritoneal metastasis.

INTRODUCTION

Peritoneal dissemination frequently occurs in patients with gastric cancer, and it hurts their prognoses. The metastatic dissemination is mediated via exfoliation of carcinoma cells into the peritoneal cavity, followed by adhesion to and invasion through the mesothelium that overlies the contents of the peritoneal cavity. The adhesion of carcinoma cells to the peritoneum is a key step in the initial process of peritoneal dissemination. It has been shown that various cell adhesion molecules expressed on carcinoma cells play crucial roles in their adhesion to the peritoneum. The integrin family of cell adhesion molecules serve as adhesion receptors for extracellular matrix proteins and cellular counterligands. These adhesion receptors are heterodimers of transmembrane glycoproteins (α and β subunits); various combinations of the α and β subunits produce polymorphisms of ligand specificity. A number of reports have demonstrated that alterations in integrin expression profiles in cancer cells are frequently associated with their malignant phenotypes, including invasive and metastatic potentials (reviewed in Refs. 1–5). Integrin receptors expressed on cancer cells contribute directly to the adhesive interactions between metastatic cells and compartments of the host environments. They also contribute to the stimulation of cell growth and motility through intracellular signaling. In the interaction between gastric

carcinoma cells and the peritoneum, the $\beta 1$ integrin family of cell adhesion molecules and CD44 have been suggested to play a crucial role (6, 7). The immunohistochemical analysis of specimens of gastric cancer resected from >100 patients demonstrated that the expression of $\alpha 3\beta 1$ integrin was positively correlated with the occurrence of peritoneal and liver metastases and with increased invasiveness of the tumors (8). Another report showed that $\alpha 2\beta 1$ integrin was significantly expressed on a larger proportion of tumor cells in peritoneal metastases than in primary tumors (9). These observations suggest a causal relationship between the expression of these integrin receptors on carcinoma cells and their peritoneal metastatic potentials. Thus, we attempted to evaluate the possible role of the $\beta 1$ integrin family of adhesion molecules, especially the $\alpha 2\beta 1$ and $\alpha 3\beta 1$ integrins in the adhesion of gastric carcinoma cells to the peritoneum. This study presents evidence showing the importance that these integrin receptors expressed on the carcinoma cell surface have in the cells' interaction with the peritoneum.

MATERIALS AND METHODS

Reagents. A fluorescent dye, 2',7'-bis(carboxyethyl)carboxyfluorescein tetraacetoxymethyl ester (BCECF-AM), was purchased from Dojindo Laboratories (Kumamoto, Japan). Thiol-reactive fluorescent probes, 5-chloromethylfluorescein diacetate (CMFDA; Cell Tracker Green) and 5-[(chloromethyl)benzoyl]amino)tetramethylrhodamine (CMTMR; Cell Tracker Orange) were products of Molecular Probes (Eugene, OR). Matrigel and BSA were purchased from BD Biosciences (San Diego, CA) and Sigma (St. Louis, MO), respectively. Restriction endonucleases and modifying enzymes were purchased from TaKaRa (Osaka, Japan), Toyobo (Osaka, Japan), and Life Technologies, Inc. (Rockville, MD). The monoclonal antibodies used in this study were as follows. Anti- $\alpha 2$ integrin (Gi9) and anti- $\alpha 6$ integrin (GoH3) antibodies were purchased from Beckman Coulter, Inc. (Fullerton, CA). Anti- $\alpha 3$ integrin (SM-T1) antibody was prepared in our laboratory (10). Anti- $\alpha 4$ integrin (SG/73), anti- $\alpha 5$ integrin (KH/33), and anti- $\beta 1$ integrin (SG/19) antibodies were purchased from Seikagaku Corp. (Tokyo, Japan). Anti-CD44 antibody (G44-26) was purchased from BD Biosciences. A polyclonal antibody against human laminin-5 was prepared in a rabbit in our laboratory (11). This antibody cross-reacts with murine laminin-5. Rabbit antimouse laminin-1 was purchased from Progen Biotechnik GmbH (Heidelberg, Germany). These polyclonal antibodies were purified on an immobilized protein A column (ImmunoPure; Pierce, Rockford, IL) according to the manufacturer's instructions. FITC-labeled antimouse IgG and antirat IgG antibodies were purchased from ICN Pharmaceuticals, Inc. (Costa Mesa, CA).

Cell Lines. Human gastric carcinoma cell lines MKN1, MKN28, and GT3TKB were supplied by the RIKEN Cell Bank (Tsukuba, Japan), and NUGC-4 was supplied by the Health Science Research Resources Bank (Osaka, Japan). Stable transfectant cells expressing $\alpha 2$ integrin (K2F10) and $\alpha 3$ integrin (K3-9) were established by transfection of K562 human erythroblastoid leukemia cells with human $\alpha 2$ and $\alpha 3$ integrin cDNA, respectively, that had been cloned in an Rc/CMV mammalian expression vector (Invitrogen, Carlsbad, CA; Refs. 10, 12). Mock transfectant cells (KR) were also prepared by transfection with an Rc/CMV vector alone. These cells were cultured in RPMI 1640 (Life Technologies, Inc.) supplemented with 10% fetal bovine serum (HyClone, Logan, UT) at 37°C under 5% CO₂.

Flow Cytometry. The expression of integrins was measured by a flow cytometer (FACSCalibur; BD Biosciences) as previously described (10), using monoclonal antibodies against human integrin $\alpha 2$, $\alpha 3$, $\alpha 4$, $\alpha 5$, $\alpha 6$, and $\beta 1$ subunits and FITC-labeled secondary antibodies.

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Isolation of Peritoneal Mesothelial Cells. The parietal peritoneum and diaphragm were excised from ICR mice under sterile conditions. Mesothelial cells were isolated essentially as described by Nakashio *et al.* (7). Briefly, the resected parietal peritoneum and diaphragm were washed with serum-free RPMI 1640 and incubated with 0.25% trypsin (Life Technologies, Inc.) in 10 mM PBS (pH 7.3) at 37°C for 20 min. After an equal volume of RPMI 1640/10% FBS was added to the cell suspension, the mixture was passed through nylon mesh and centrifuged at 700 rpm for 5 min. The pelleted cells were suspended in RPMI 1640/10% FBS (1×10^6 cells/ml), and an aliquot (0.1 ml) was placed in a 96-well culture plate, which had been coated with 10% Matrigel solution at 4°C for 16 h. These cells were cultured at 37°C for 2–3 days until growth of a monolayer of polygonal mesothelial cells.

Cell Adhesion to Peritoneum. The excised parietal peritoneum (~1.6 cm²) was placed in a 24-well culture plate, which had been filled with 1.0 ml of 1% BSA/RPMI 1640. Cells were fluorescently labeled with BCECF-AM (3 μ M) at 37°C for 30 min as previously described (13) and were washed twice with 1% BSA/RPMI 1640. A cell suspension (5×10^5 cells/ml in 1% BSA/RPMI 1640; 0.5 ml) was overlaid on the peritoneum in a 24-well plate, and the plate was incubated at 37°C for 40 min. After gentle washing with PBS, the cells adherent to the peritoneum were lysed with 1.0 ml of 1% NP40, and the fluorescence intensity was measured with a fluorescence spectrophotometer (Ex = 490 nm, Em = 520 nm). For the inhibition experiment, the cells were treated with an antibody at 0°C for 30 min before being incubated with the peritoneum.

Cell Adhesion to Mesothelial Cell Monolayer. A monolayer culture of mesothelial cells in a 96-well culture plate was washed twice with 1% BSA/RPMI 1640. To the monolayer, a suspension of BCECF-labeled cells (1×10^6 cells/ml in 1% BSA/RPMI 1640; 0.1 ml) was added, and the plate was incubated at 37°C for 40 min. After the nonadherent cells were removed by gentle washing three times with warm 1% BSA/RPMI 1640, the adherent cells were lysed with 0.2 ml of 1% NP40, and the fluorescence intensity was measured as described above.

Fluorescence Microscopic Observation. To examine the adhesion capacity of $\alpha 3$ integrin-positive and -negative cells to the peritoneum specimen, $\alpha 3$ integrin-transfected cells (K3-9) and mock-transfected cells (KR) were separately labeled, each with a distinct fluorescent probe. K3-9 cells and KR cells were suspended in 1% BSA/RPMI 1640 at a density of 1×10^6 cell/ml and incubated with CMTMR (red fluorescence; 5 μ M) for K3-9 or with CMFDA (green fluorescence; 5 μ M) for KR cells at 37°C for 20 min. The mixture of CMTMR-labeled K3-9 cells and CMFDA-labeled KR cells (5×10^5 cells/ml for each cell type; 0.5 ml) was overlaid on the excised parietal peritoneum in a 24-well plate, incubated at 37°C for 40 min, and washed with PBS. The cells adherent to the peritoneum specimen were observed under a fluorescence microscope (Olympus IX70; Olympus, Tokyo, Japan) equipped with an NIBA filter unit (Ex = 470–490 nm, Em = 515–550 nm) for CMFDA and a WIG filter unit (Ex = 520–550 nm, Em >580 nm) for CMTMR.

Peritoneal Implantation of Gastric Carcinoma Cells. BALB/c *nu/nu* mice (5 weeks old, female) were purchased from Sankyo Lab Service (Tokyo, Japan). NUGC-4 cells (5×10^6 cells; 0.5 ml) suspended in RPMI 1640 were incubated with the antibodies against $\alpha 2$ or $\alpha 3$ integrin subunits at 0°C for 20 min and inoculated into the abdominal cavity of BALB/c *nu/nu* mice. The mice were killed on the 28th day of the study, and disseminated nodules on the mesentery and diaphragm were evaluated. This study was conducted in accordance with the Guidelines for the Care and Use of Laboratory Animals of Hoshi University.

Detection of mRNAs for Laminin Isoforms. mRNAs for laminin isoforms (laminin-5 and laminin-10/11) were detected by the reverse transcription-PCR method. The total RNA was isolated from peritoneal mesothelial cells by the acid guanidinium-phenol-chloroform method (14) using Trizol (Life Technologies, Inc.). Poly A⁺ RNA was purified with Oligotex dT30 (TaKaRa) according to the manufacturer's instructions. cDNA was synthesized by using Moloney murine leukemia virus reverse transcriptase and antisense primers as follows. PCR was performed with a set of primers: 5'-CAGGGGCTGAGTGTGAGCAC-3' (sense primer for laminin-5 $\alpha 3$ subunit) and 5'-GGAGGATGGAACGTAGGTCC-3' (antisense primer for laminin-5 $\alpha 3$ subunit; Ref. 15); 5'-TGTATCTGTCCACCACGACTG-3' (sense primer for laminin-10/11 $\alpha 5$ subunit) and 5'-ACATCTTGAGCCCTGCACGTTCC-3' (antisense primer for laminin-10/11 $\alpha 5$ subunit; Ref. 16). The PCR conditions were as follows: 94°C, 1 min; 61°C, 1 min; 72°C, 1 min for 30 cycles. The

products were separated by 3% agarose gel electrophoresis in 40 mM Tris-acetate buffer (pH 8.0) containing 1 mM EDTA ($1 \times$ TAE), followed by ethidium bromide staining.

RESULTS

Adhesion of Gastric Carcinoma Cells to Peritonea. We first examined how antibodies against the $\beta 1$ family of integrin receptors affected the adhesion of several gastric carcinoma cells to the excised parietal peritonea. The adhesion of MKN1 cells was inhibited most by the antibodies against the integrin $\beta 1$ and $\alpha 3$ subunits and, to a lesser extent, by the antibody against the integrin $\alpha 2$ subunit (Fig. 1A). In contrast, the antibodies against integrin $\alpha 4$, $\alpha 5$, or $\alpha 6$ did not inhibit adhesion. Although a slight inhibition by an anti-CD44 antibody was observed, it was not significant. None of the antibodies completely inhibited the adhesion of these cells to the peritonea. However, the adhesion of MKN1 cells was decreased to 34% in the presence of both anti- $\beta 1$ integrin subunit and anti-CD44 antibodies. These results suggested that $\alpha 2\beta 1$ and $\alpha 3\beta 1$ integrins play a role in the adhesion of MKN1 cells to the peritoneum, although the adhesion is mediated by multiple interactions with various adhesion molecules. Similar inhibitory effects by the antibodies against the $\alpha 2$ and $\alpha 3$ integrin subunits were observed when three additional gastric carcinoma cell lines were subjected to the adhesion assay (Fig. 1B). The anti-integrin $\alpha 3$ subunit antibody inhibited the adhesion of NUGC-4, GT3TKB, and MKN28 cells more effectively than did the anti-integrin $\alpha 2$ subunit antibody.

We then measured the expression profiles of the integrin $\alpha 2$ and $\alpha 3$ subunits on carcinoma cells that had been used for the adhesion assay (Fig. 2). In the flow cytometric analysis, all four cell lines examined were found to express both $\alpha 2$ and $\alpha 3$ subunits, although the expression levels of the individual subunits were somewhat different among these cells.

Suppression of Peritoneal Implantation by Anti-Integrin Antibodies. Because the antibodies against integrin $\alpha 2$ and $\alpha 3$ subunits suppressed the adhesion of the four cell lines to the peritoneum, we examined the effects of these antibodies on peritoneal implantation

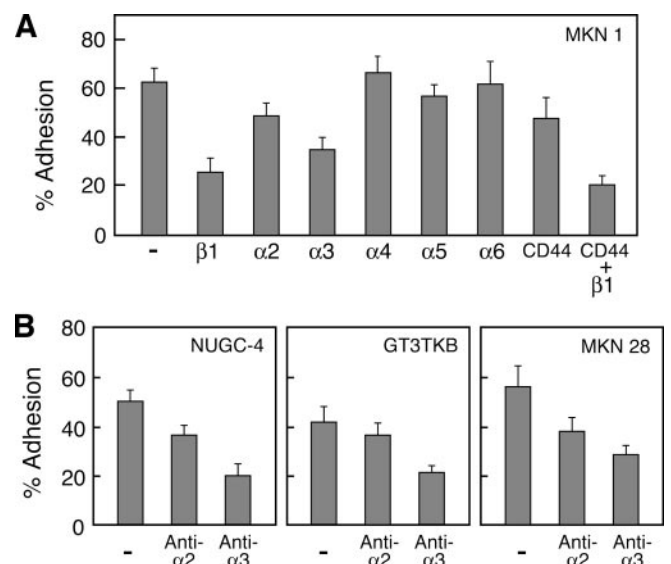


Fig. 1. Adhesion of gastric carcinoma cells to parietal peritoneum. Fluorescently labeled gastric carcinoma cells were overlaid on excised peritoneum and incubated at 37°C for 40 min. After gentle washing to remove nonadherent cells, the adherent cells were lysed with 1% NP40, and the fluorescence intensity was measured with a fluorescence spectrophotometer (Ex = 490 nm, Em = 520 nm). For the inhibition experiment, the cells were treated with an antibody (100 μ g/ml) at 0°C for 30 min before the incubation with peritoneum. The assays were carried out in triplicate, and the error bars indicate the SDs. A, MKN1; B, NUGC-4 (left); GT3TKB (middle); MKN28 (right).

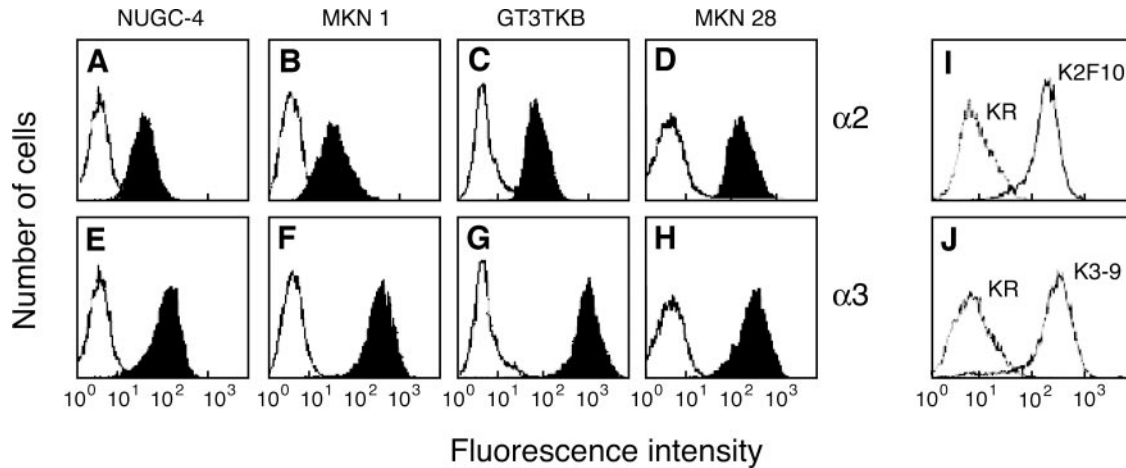


Fig. 2. Flow cytometric analysis of the expression of integrin $\alpha 2$ and $\alpha 3$ subunits in gastric carcinoma and transfectant cells. NUGC-4 (A and E), MKN1 (B and F), GT3TKB (C and G), and MKN28 (D and H) cells were treated with monoclonal antibodies against $\alpha 2$ (A–D) or $\alpha 3$ (E–H) integrin subunits and subsequently with FITC-labeled secondary antibodies. The thin lines represent the profiles of control experiments without anti-integrin antibody. K2F10 ($\alpha 2$ integrin transfectant), K3-9 ($\alpha 3$ integrin transfectant), and KR (mock transfectant) cells were assayed for their expression of integrin $\alpha 2$ (I) and $\alpha 3$ (J) subunits.

Table 1 Effect of antibodies against $\alpha 2$ and $\alpha 3$ integrin subunits on peritoneal implantation of NUGC-4 human gastric carcinoma cells in athymic mice

Antibody	No. of disseminated nodules*
None	127 \pm 39
Anti- $\alpha 2$ integrin	74 \pm 28
Anti- $\alpha 3$ integrin	33 \pm 15
Murine IgG	102 \pm 22

* NUGC-4 cells (5×10^6 cells; 0.5 ml) were treated with anti- $\alpha 2$ or anti- $\alpha 3$ antibody (60 $\mu\text{g/ml}$) or normal IgG and inoculated into the abdominal cavity of athymic mice ($n = 6$), and the disseminated nodules on mesentery and diaphragm were counted on day 28.

after inoculation of NUGC-4 cells into the abdominal cavity of athymic mice. Four weeks after the inoculation, the numbers of disseminated nodules were reduced by the pretreatment of cells with either the anti- $\alpha 2$ or anti- $\alpha 3$ integrin antibody (Table 1), suggesting the involvement of these integrins in the i.p. growth of carcinoma cells. The suppressive effect of the anti- $\alpha 3$ integrin antibody was, however, found to be more prominent than that of the anti- $\alpha 2$ integrin antibody.

Adhesion of $\alpha 2$ and $\alpha 3$ Integrin-Expressing Cells to the Peritoneum. We next evaluated the adhesion capacities of $\alpha 2$ and $\alpha 3$ integrin-expressing cells, which were established from K562 erythroblastoid-leukemic cells by cDNA transfection. Fig. 2, I and J, shows the expression profiles of these integrin molecules in K2F10 cells ($\alpha 2$ integrin-transfectant) and K3-9 cells ($\alpha 3$ integrin-transfectant). Both K2F10 and K3-9 cells adhered to the peritoneum more firmly than did the mock transfectant KR cells (Fig. 3). The increase in the adhesion of K3-9 cells was greater than that of K2F10 cells (a 3.2-fold increase for K3-9 cells versus a 1.7-fold increase for K2F10 cells, when compared with the adhesion of KR cells). The increases in the adhesion of K3-9 and K2F10 cells were inhibited by the addition of corresponding monoclonal antibodies, suggesting that the enhanced adhesion by cDNA transfection was due to the specific function of the integrin molecules expressed on these cells.

K3-9 cells and KR cells were separately labeled, each with a distinct fluorescence probe. The cells were then mixed together and incubated with a peritoneum specimen. By fluorescence microscopic observation, we were able to distinguish K3-9 cells with red fluorescence from KR cells with green fluorescence present in the same field. As shown in Fig. 4, the proportion of K3-9 cells adherent to the peritoneum was much larger than that of KR cells. By comparing the numbers of the two types of attached cells in the same area of the

peritoneum, the possible contribution of heterogeneity in the peritoneal lining to adhesion can be eliminated.

We then assayed the adhesion of K3-9 cells to cultured peritoneal mesothelial cells (Fig. 5). The adhesion of both K3-9 and KR cells to a monolayer of mesothelial cells was increased as compared with their adhesion to Matrigel-coated plates, which were used for the mesothelial cell culture. K3-9 cells, however, adhered to mesothelial cells more firmly than KR cells did. The addition of the anti- $\alpha 3$ integrin antibody decreased the adhesion to the control level, showing that the increased adhesion was $\alpha 3$ integrin dependent.

Expression of Ligands for $\alpha 3\beta 1$ Integrin in Mesothelial Cells.

To assess the expression of ligands for the $\alpha 3\beta 1$ integrin in mesothelial cells, we attempted to detect mRNA for laminin-5 and laminin-10/11, which have been reported to serve as high-affinity ligands for this integrin (17–20). When the products of the reverse transcription-PCR were separated on agarose gel electrophoresis, the expected PCR product (586 bp) for the murine laminin $\alpha 3$ chain (a component of laminin-5) was obtained from mesothelial cells isolated from both the parietal peritoneum and diaphragm (Fig. 6). A PCR with a set of primers for the laminin $\alpha 5$ chain (a component of laminin-10/11) also gave a band with the expected size (330 bp). The identities of these PCR products were confirmed by restriction endonuclease digestion. When the 586-bp product was digested with *MboI*, three fragments

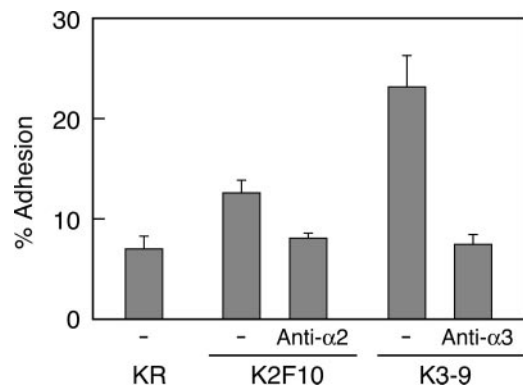


Fig. 3. Adhesion of $\alpha 2$ and $\alpha 3$ integrin-expressing cells to peritoneum. K2F10 ($\alpha 2$ integrin transfectant), K3-9 ($\alpha 3$ integrin transfectant), and KR (mock transfectant) cells were labeled with a fluorescent dye (BCECF-AM) and incubated with excised peritoneum at 37°C for 40 min. The fluorescence intensity associated with the adherent cells was measured with a fluorescence spectrophotometer. The assays were carried out in triplicate, and the error bars indicate the SDs.

Fig. 4. Adhesion of $\alpha 3$ integrin-expressing cells to peritoneum. K3-9 ($\alpha 3$ integrin transfectant) cells were labeled with CMTMR (red fluorescence), and KR (mock transfectant) cells were labeled with CMFDA (green fluorescence). The mixture of these cells was incubated with excised parietal peritoneum at 37°C for 40 min. After the nonadherent cells were removed by gentle washing, the adherent cells were observed under fluorescence microscope. The same field was observed with different filter units: A, CMTMR-labeled K3-9 cells; B, CMFDA-labeled KR cells.

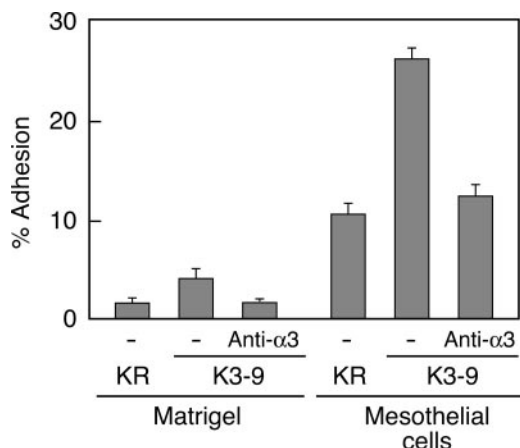
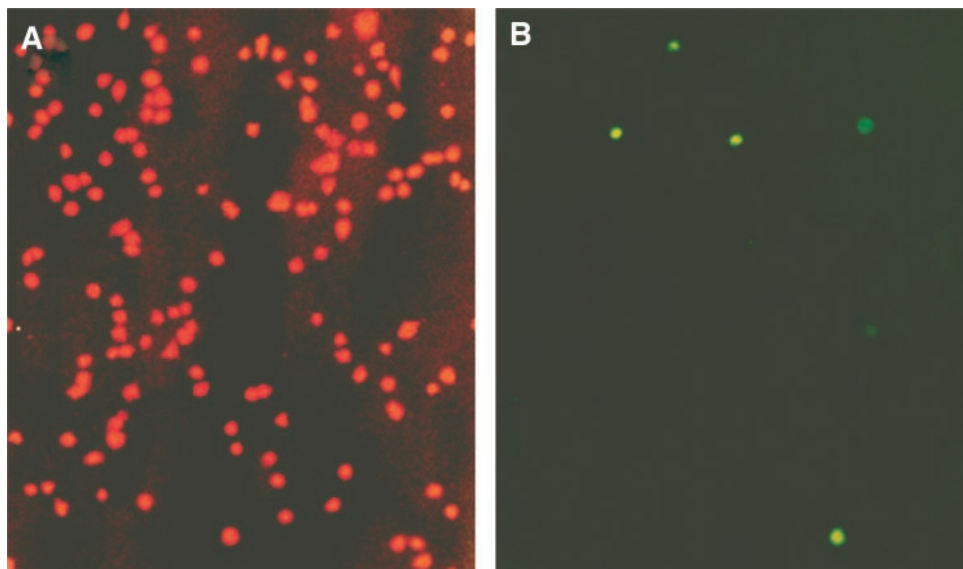


Fig. 5. Adhesion of $\alpha 3$ integrin-expressing cells to peritoneal mesothelial cells. K3-9 ($\alpha 3$ integrin transfectant) and KR (mock transfectant) cells were labeled with a fluorescent dye (BCECF-AM) and incubated with a monolayer of mesothelial cells at 37°C for 40 min. The fluorescence intensity associated with the adherent cells was measured with a fluorescence spectrophotometer. The assays were carried out in triplicate, and the error bars indicate the SDs.

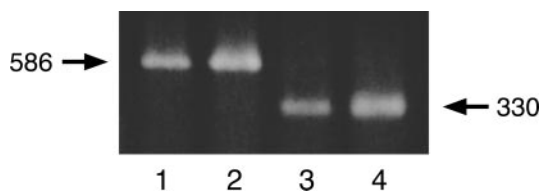


Fig. 6. Analysis of mRNA for laminin $\alpha 3$ and $\alpha 5$ chains by the reverse transcription-PCR method. RNA was isolated from murine peritoneal mesothelial cells and subjected to reverse transcription-PCR by the use of a set of primers for laminin $\alpha 3$ chain (Lanes 1 and 2) and laminin $\alpha 5$ chain (Lanes 3 and 4) under the conditions described in Materials and Methods. Lanes 1 and 3, mesothelial cells from parietal peritoneum; Lanes 2 and 4, mesothelial cells from diaphragm.

(245, 193, and 113 bp) were obtained (15). On the other hand, the digestion of the 330-bp product with *Apal* yielded two fragments (223 and 107 bp; Ref. 16). These results indicate that mesothelial cells from both the parietal peritoneum and the diaphragm express mRNA for laminin-5 and laminin-10/11, which are most likely to mediate the interaction of $\alpha 3$ -expressing carcinoma cells with the peritoneum.

We then examined the effects of antibodies against laminin iso-

forms on the adhesion of MKN1 cells to murine peritoneum. In the presence of anti-laminin-5 polyclonal antibody (120 $\mu\text{g/ml}$), the cell adhesion was decreased to 55% that obtained in the control experiment (Fig. 7). Anti-laminin-1 antibody, however, showed no significant inhibition of the adhesion of MKN1 cells. The inhibition by the anti-laminin-5 antibody was significant but moderate, probably due to the possible contribution of laminin-10/11 to the interaction of carcinoma cells. It is likely that the combination of antibodies against laminin-5 and laminin-10/11 would inhibit cell adhesion more efficiently. This should be examined once anti-laminin-10/11 antibody becomes available, but the results of the present study suggest that laminin-5 plays at least a partial role in the adhesion of $\alpha 3$ integrin-expressing cells to the peritoneum.

DISCUSSION

The attachment of carcinoma cells to the peritoneum is an important step in the initial process of metastatic dissemination of gastric cancer. In this study, we presented evidence that $\alpha 3\beta 1$ integrin plays a crucial role in the adhesion of cancer cells to the peritoneum: (a)

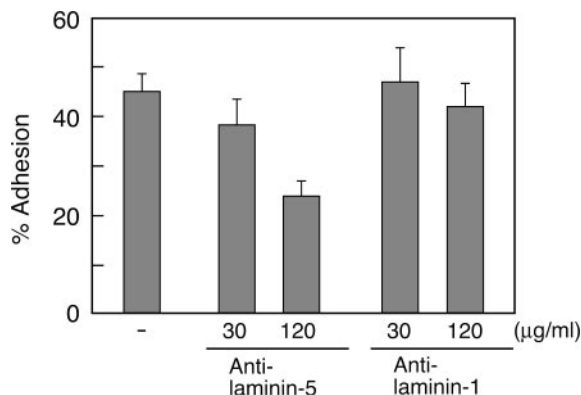


Fig. 7. Effects of antibodies against laminin isoforms on the adhesion of MKN1 gastric carcinoma cells to parietal peritoneum. Fluorescently labeled cells were overlaid on excised peritoneum that had been treated with antibody against laminin-5 or laminin-1 at 0°C for 30 min. After the cells and peritoneum were incubated at 37°C for 40 min, the nonadherent cells were removed by gentle washing three times with a warm medium. The adherent cells were then lysed with 1% NP40, and the fluorescence intensity was measured with a fluorescence spectrophotometer (Ex = 490 nm, Em = 520 nm). The assays were carried out in triplicate. The error bars indicate the SDs.

inhibition of gastric carcinoma cell adhesion to excised peritoneum by specific monoclonal antibodies (Fig. 1); (b) suppression of i.p. growth of NUGC-4 cells by pretreatment with anti- $\alpha 3$ integrin antibody (Table 1); and (c) enhancement of the adhesion of K562 cells to the excised peritoneum and to a monolayer of peritoneal mesothelial cells by cDNA transfection (Figs. 3–5). In peritoneal mesothelial cells, we detected the expression of mRNA for laminin-5 and laminin-10/11, which have recently been identified as high-affinity ligands for $\alpha 3\beta 1$ integrin (Fig. 6). Furthermore, the pretreatment of excised peritoneum with antibody to laminin-5 significantly inhibited the adhesion of carcinoma cells (Fig. 7). With these findings taken together, we can say that $\alpha 3\beta 1$ integrin is a strong candidate as the mediation source in the initial attachment of gastric cancer cells during their peritoneal dissemination processes. These results may provide a biochemical basis for observations in a previous clinical report. In that report, histochemical studies on clinical specimens of gastric cancer associated the expression of $\alpha 3\beta 1$ integrin with peritoneal metastasis (8). Another report suggested the importance of $\alpha 2\beta 1$ integrin by virtue of its significant expression on a larger proportion of tumor cells in peritoneal metastases than in primary tumors (9). The present study also showed $\alpha 2\beta 1$ integrin to play a role in adhesion, although to a lesser extent. Taken together, these findings suggest that the attachment of cancer cells to peritoneum is mediated by multivalent interactions with various adhesion molecules, including $\alpha 2\beta 1$ and $\alpha 3\beta 1$ integrins, and that the individual adhesion molecules responsible for the interaction might depend on the type of carcinoma. Nishimura *et al.* (21) and Kawamura *et al.* (22) also reported that the expression of $\alpha 2\beta 1$ and/or $\alpha 3\beta 1$ integrin on gastric carcinoma cells was correlated with their peritoneal metastasizing potentials, *i.e.*, highly metastatic cells expressed higher levels of these integrins as compared with the less-metastatic cells. These results, along with those of the present study, strongly suggest that the initial attachment of cancer cells to peritoneal lining mediated by $\alpha 2\beta 1$ and/or $\alpha 3\beta 1$ integrin is a key step toward the subsequent process of metastasis formation. Recently, Ishii *et al.* (23) demonstrated the inverse correlation between the expression level of $\alpha 6\beta 4$ integrin and the frequency of peritoneal dissemination of gastric cancer from an immunohistochemical study. The finding is of interest because the expression of $\alpha 6\beta 4$ integrin and $\alpha 3\beta 1$ integrin apparently had opposite effects even though these integrins share a common ligand, laminin-5. One possible explanation is that the intracellular signaling events occurring after the ligand is bound to $\alpha 6\beta 4$ might differ from those occurring after binding to $\alpha 3\beta 1$ integrins.

Our previous reports showed that the expression of $\alpha 3\beta 1$ integrin at both the protein and mRNA levels was increased after the oncogenic transformation of fibroblasts by SV40 or polyoma virus (24, 25). A number of studies have demonstrated that the increased expression of $\alpha 3\beta 1$ integrin in various tumor cells is correlated with their malignant phenotypes, including invasion and metastasis (26–32). Furthermore, the treatment of melanoma and glioma cells with antibodies against $\alpha 3$ integrin was shown to inhibit their ability to migrate and invade (11, 26, 33). These studies suggest that $\alpha 3$ integrin is also involved in the invasion of gastric carcinoma cells into the peritoneum following their attachment to the peritoneal lining and are in agreement with our result that the anti- $\alpha 3$ integrin antibody suppresses the i.p. growth of NUGC-4 cells (Table 1). Our preliminary experiment also showed that the i.p. implantation of $\alpha 3$ integrin-expressing cells (K3-9 cells) in severe combined immunodeficiency mice resulted in a poor survival rate as compared with the implantation of mock transfected cells (KR cells).³

The integrin-extracellular matrix interaction facilitates the transduction of signals into cells. This leads to regulation of the production of extracellular matrix-degrading enzymes. It has recently been reported that the treatment of invasive mammary carcinoma cells with an anti- $\alpha 3$ integrin antibody reduced the production of matrix metalloproteinase (MMP)-9 (34). In human glioma and rhabdomyosarcoma cells, anti- $\alpha 3$ integrin antibodies enhanced their invasion through the Matrigel in association with the increased levels of MMP-2 secretion (35, 36), suggesting that the antibody acted as an agonist. In addition, laminin-5 was shown to induce MMP-9 from human melanoma cells (11). Laminin-5 was also found to be specifically cleaved by MMP-2 and MT1-MMP and the modified laminin-5 induced migration of breast epithelial cells (37, 38). These observations suggest that the expression of extracellular matrix-degrading enzymes is regulated, at least in part, through the integrin-mediated pathways.

Recently, we found that the Ets family of transcription factors regulated the expression of $\alpha 3$ integrin (39). The Ets transcription factors were also reported to be involved in tumor metastasis through the promotion of angiogenesis and the expression of MMPs (40–42). These observations suggest that $\alpha 3\beta 1$ integrin and MMPs cooperatively promote adhesion and invasion of cancer cells during the metastatic processes. In conclusion, the present study strongly suggests that the up-regulation of $\alpha 3\beta 1$ integrin on gastric carcinoma cells facilitates their adhesion to the peritoneum and the subsequent development of peritoneal dissemination.

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REFERENCES

1. Varner JA, Cheresch DA. Integrins and cancer. *Curr Opin Cell Biol* 1996;8:724–30.
2. Ruoslahti E. Fibronectin and its integrin receptors in cancer. *Adv Cancer Res* 1999;76:1–20.
3. Orr FW, Wang HH, Lafrenie RM, Scherbarth S, Nance DM. Interactions between cancer cells and the endothelium in metastasis. *J Pathol* 2000;190:310–29.
4. Holly SP, Larson MK, Parise LV. Multiple roles of integrins in cell motility. *Exp Cell Res* 2000;261:69–74.
5. Felding-Habermann B. Integrin adhesion receptors in tumor metastasis. *Clin Exp Metastasis* 2003;20:203–13.
6. Yashiro M, Chung YS, Nishimura S, Inoue T, Sowa M. Peritoneal metastatic model for human scirrhous gastric carcinoma in nude mice. *Clin Exp Metastasis* 1996;14:43–54.
7. Nakashio T, Narita T, Akiyama S, et al. Adhesion molecules and TGF- β 1 are involved in the peritoneal dissemination of NUGC-4 human gastric cancer cells. *Int J Cancer* 1997;70:612–8.
8. Ura H, Denno R, Hirata K, Yamaguchi K, Yasoshima T. Separate functions of $\alpha 2\beta 1$ and $\alpha 3\beta 1$ integrins in the metastatic process of human gastric carcinoma. *Surg Today* 1998;28:1001–6.
9. Matsuoka T, Yashiro M, Nishimura S, et al. Increased expression of $\alpha 2\beta 1$ integrin in the peritoneal dissemination of human gastric carcinoma. *Int J Mol Med* 2000;5:21–5.
10. Takeuchi K, Tsuji T, Hakomori S, Irimura T. Intercellular adhesion induced by anti- $\alpha 3$ integrin (VLA-3) antibodies. *Exp Cell Res* 1994;211:133–41.
11. Tsuji T, Kawada Y, Kai-Murozono M, et al. Regulation of melanoma cell migration and invasion by laminin-5 and $\alpha 3\beta 1$ integrin (VLA-3). *Clin Exp Metastasis* 2002;19:127–34.
12. Takada Y, Hemler ME. The primary structure of the VLA-2/collagen receptor $\alpha 2$ subunit (platelet GPIa): homology to other integrins and the presence of a possible collagen-binding domain. *J Cell Biol* 1989;109:397–407.
13. Izumi Y, Taniuchi Y, Tsuji T, et al. Characterization of human colon carcinoma variant cells selected for sialyl Le^x carbohydrate antigen: liver colonization and adhesion to vascular endothelial cells. *Exp Cell Res* 1995;216:215–21.
14. Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 1987;162:156–9.
15. Galliano MF, Aberdam D, Aguzzi A, Ortonne JP, Meneguzzi G. Cloning and complete primary structure of the mouse laminin $\alpha 3$ chain. Distinct expression pattern of the laminin $\alpha 3A$ and $\alpha 3B$ chain isoforms. *J Biol Chem* 1995;270:21820–6.
16. Miner JH, Lewis RM, Sanes JR. Molecular cloning of a novel laminin chain, $\alpha 5$, and widespread expression in adult mouse tissues. *J Biol Chem* 1995;270:28523–6.
17. Carter WG, Ryan MC, Gahr PJ. Epiligrin, a new cell adhesion ligand for integrin $\alpha 3\beta 1$ in epithelial basement membranes. *Cell* 1991;65:599–610.

³ H. Takatsuki, S. Komatsu, R. Sano, and T. Tsuji, unpublished observation.

18. Marinkovitch MP, Verrando P, Keene DR, et al. Basement membrane proteins kalinin and nicein are structurally and immunologically identical. *Lab Invest* 1993;69:295–9.
19. Kikkawa Y, Umeda M, Miyazaki K. Marked stimulation of cell adhesion and motility by ladsin, a laminin-like scatter factor. *J Biochem (Tokyo)* 1994;116:862–9.
20. Kikkawa Y, Sanzen N, Sekiguchi K. Isolation and characterization of laminin-10/11 secreted by human lung carcinoma cells. Laminin-10/11 mediates cell adhesion through integrin $\alpha 3\beta 1$. *J Biol Chem* 1998;273:15854–9.
21. Nishimura S, Chung YS, Yashiro M, Inoue T, Sowa M. Role of $\alpha 2\beta 1$ - and $\alpha 3\beta 1$ -integrin in the peritoneal implantation of scirrhous gastric carcinoma. *Br J Cancer* 1996;74:1406–12.
22. Kawamura T, Endo Y, Yonemura Y, et al. Significance of integrin $\alpha 2/\beta 1$ in peritoneal dissemination of a human gastric cancer xenograft model. *Int J Oncol* 2001;18:809–15.
23. Ishii Y, Ochiai A, Yamada T, et al. Integrin $\alpha 6\beta 4$ as a suppressor and a predictive marker for peritoneal dissemination in human gastric cancer. *Gastroenterology* 2000;118:497–506.
24. Tsuji T, Yamamoto F, Miura Y, et al. Characterization through cDNA cloning of galactoprotein b3 (Gap b3), a cell surface membrane glycoprotein showing enhanced expression on oncogenic transformation. Identification of Gap b3 as a member of the integrin superfamily. *J Biol Chem* 1990;265:7016–21.
25. Tsuji T, Hakomori S, Osawa T. Identification of human galactoprotein b3, an oncogenic transformation-induced membrane glycoprotein, as VLA-3 α subunit: the primary structure of human integrin $\alpha 3$. *J Biochem (Tokyo)* 1991;109:659–65.
26. Melchiori A, Mortarini R, Carlone S, et al. The $\alpha 3\beta 1$ integrin is involved in melanoma cell migration and invasion. *Exp Cell Res* 1995;219:233–42.
27. Bartolazzi A, Cerboni C, Nicotra MR, Mottolese M, Bigotti A, Natali PG. Transformation and tumor progression are frequently associated with expression of the $\alpha 3/\beta 1$ heterodimer in solid tumors. *Int J Cancer* 1994;58:488–91.
28. Van Waes C, Surh DM, Chen Z, et al. Increase in suprabasilar integrin adhesion molecule expression in human epidermal neoplasms accompanies increased proliferation occurring with immortalization and tumor progression. *Cancer Res* 1995;55:5434–44.
29. Tawil NJ, Gowri V, Djoneidi M, Nip J, Carbonetto S, Brodt P. Integrin $\alpha 3\beta 1$ can promote adhesion and spreading of metastatic breast carcinoma cells on the lymph node stroma. *Int J Cancer* 1996;66:703–10.
30. Schumacher D, Schaumburg-Lever G. Ultrastructural localization of α -3 integrin subunit in malignant melanoma and adjacent epidermis. *J Cutan Pathol* 1999;26:321–6.
31. Kishima H, Shimizu K, Tamura K, et al. Monoclonal antibody ONS-M21 recognizes integrin $\alpha 3$ in gliomas and medulloblastomas. *Br J Cancer* 1999;79:333–9.
32. Kreidberg JA. Functions of $\alpha 3\beta 1$ integrin. *Curr Opin Cell Biol* 2000;12:548–53.
33. Fukushima Y, Ohnishi T, Arita N, Hayakawa T, Sekiguchi K. Integrin $\alpha 3\beta 1$ -mediated interaction with laminin-5 stimulates adhesion, migration and invasion of malignant glioma cells. *Int J Cancer* 1998;76:63–72.
34. Morini M, Mottolese M, Ferrari N, et al. The $\alpha 3\beta 1$ integrin is associated with mammary carcinoma cell metastasis, invasion, and gelatinase B (MMP-9) activity. *Int J Cancer* 2000;87:336–42.
35. Chintala SK, Sawaya R, Gokaslan ZL, Rao JS. Modulation of matrix metalloprotease-2 and invasion in human glioma cells by $\alpha 3\beta 1$ integrin. *Cancer Lett* 1996;103:201–8.
36. Kubota S, Ito H, Ishibashi Y, Seyama Y. Anti- $\alpha 3$ integrin antibody induces the activated form of matrix metalloprotease-2 (MMP-2) with concomitant stimulation of invasion through Matrigel by human rhabdomyosarcoma cells. *Int J Cancer* 1997;70:106–11.
37. Giannelli G, Falk-Marzillier J, Schiraldi O, Stetler-Stevenson WG, Quaranta V. Induction of cell migration by matrix metalloprotease-2 cleavage of laminin-5. *Science (Wash. DC)* 1997;277:225–8.
38. Koshikawa N, Giannelli G, Cirulli V, Miyazaki K, Quaranta V. Role of cell surface metalloprotease MT1-MMP in epithelial cell migration over laminin-5. *J Cell Biol* 2000;148:615–24.
39. Kato T, Katabami K, Takatsuki H, et al. Characterization of the promoter for the mouse $\alpha 3$ integrin gene. Involvement of the Ets-family of transcription factors in the promoter activity. *Eur J Biochem* 2002;269:4524–32.
40. Sementchenko VI, Watson DK. Ets target genes: past, present and future. *Oncogene* 2000;19:6533–48.
41. Kaya M, Yoshida K, Higashino F, Mitaka T, Ishii S, Fujinaga K. A single ets-related transcription factor, E1AF, confers invasive phenotype on human cancer cells. *Oncogene* 1996;12:221–7.
42. Habelhah H, Okada F, Kobayashi M, et al. Increased E1AF expression in mouse fibrosarcoma promotes metastasis through induction of MT1-MMP expression. *Oncogene* 1999;18:1771–6.