Role of integrin α1 subunits in gastric cancer patients with peritoneal dissemination

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Received July 7, 2011; Accepted October 7, 2011

DOI: 10.3892/mmr.2011.642

Abstract. The interaction between gastric cancer (GC) cells and the peritoneum is a critical event in peritoneal dissemination. The molecular mechanisms of this dissemination, however, remain unclear. Integrins are heterodimeric cell adhesion molecules consisting of α and β subunits that serve as adhesion receptors for extracellular matrix proteins and cellular ligands, and may participate in GC peritoneal dissemination. In this study, we isolated fresh GC cells from a patient with peritoneal metastasis and examined them for integrin expression and investigated the role of integrin α1 subunit molecules in GC. Five clones (KGC1C2, KGC1F3, KGC1H3, KGC1E8, KGC1G10) were established from the clinical GC sample and used in an in vitro adhesion model using a single cell culture method. Each clone was transplanted into the peritoneal cavity of SCID mice, where each clone formed tumors and caused conglutination of organs in the abdominal cavity. We analyzed the expression of integrin subunits for each clone by flow cytometry and found that the expression ratio of α1 subunits paired with β1 subunits was detected at higher levels than other subunits. To verify that anti-integrin α1 subunit (CD49a) antibody inhibits cell adhesion in an in vitro adhesion assay, each clone was treated with anti-CD49a antibody, which significantly inhibited cell adhesion compared to the untreated group. Characterization of α1 subunit expression in GC may be useful in optimizing treatments for different individuals. Having high metastatic abilities, these 5 new GC clones may be beneficial for analyzing integrin function in tumor metastasis.

Introduction

Gastric cancer (GC) is the leading cause of gastrointestinal carcinoma and is the second most common cause of cancer-related mortality worldwide (1). Previous studies have demonstrated that peritoneal dissemination is a unique characteristic of recurrent GC and, more importantly, is related to GC prognosis, since peritoneal dissemination can cause serious clinical complications such as ileus, hydronephrosis and ascites. Thus, controlling peritoneal dissemination in GC patients, particularly in advanced cancer, is critical. Although many studies have attempted to identify the best options for controlling peritoneal dissemination, none have been able to attenuate poor patient prognosis (2,3).

In general, metastatic dissemination is mediated through exfoliation of cancer cells into the peritoneal cavity, with a key step in the process regulated by cancer cell adhesion to the peritoneum that is mediated by various cell adhesion molecules expressed on cancer cells. However, the mechanisms responsible for this disseminated metastasis remain unclear.

Integrins are a large family of heterodimeric transmembrane glycoproteins that attach cells to extracellular matrix (ECM) proteins of the basement membrane or to ligands on other cells and play a decisive role in metastatic dissemination. Integrins contain large (α) and small (β) subunits of sizes 120-170 kDa and 90-100 kDa, respectively, as well as binding sites for Mg²⁺ and Ca²⁺, which are necessary for their adhesive function. Mammalian integrins form several subfamilies, which share common β subunits that associate with different α subunits.

Numerous reports have shown that integrin receptors are highly expressed on cancer cells and help stimulate cell growth and motility through intracellular signaling (4,5). There have also been several studies concerning the role of integrin β1 subunit in peritoneal implantation, although the relationship between integrin αl-α6β1 subunits and peritoneal implantation has not yet been reported. Since the profiles of integrin expression in cancer cells are frequently associated with their malignant phenotypes, as well as invasive and metastatic potentials (6-9), integrins may be important therapeutic targets of peritoneal dissemination.

To study the role of integrins in the progression of peritoneal dissemination, we first isolated fresh GC cells from a patient with type 4 carcinoma (pT4N1H0CY1M0, stage IV) and established clones for the specific model of GC with metastatic dissemination. Five clones were created from the primary GC cells and they possessed tumorigenic properties in the peritoneal cavity of combined immunodeficient (SCID) mice. Moreover, we analyzed the expression of integrin subunits as well as other adhesion molecules of these clones.
and found that the expression ratio of α1 subunits paired with β1 subunits was higher than that for other subunits. These 5 new GC clones with high metastatic ability may be beneficial for analyzing the mechanisms of progression and metastasis.

Materials and methods

Clinical sample. Clinical samples were obtained from the patient with informed consent and agreement (approved by the Ethics Committee of Keio University; no. 17-47). The patient was clinically diagnosed with GC with a pathological diagnosis of type 4 carcinoma (pT4N1H0CY1M0, stage IV). Ascites were collected into 50-mL centrifuge tubes, which were then centrifuged at 800 x g for 5 min. The GC cells were suspended in RPMI-1640 medium (Nacalai Tesque Inc., Kyoto, Japan) containing 10% fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA, USA), penicillin-streptomycin mixed solution (penicillin 10,000 units/ml, streptomycin 10,000 µg/ml; Nacalai Tesque Inc.). In all experiments, cells were cultured at 37°C in a humidified 5% CO2/95% air atmosphere.

Cloning and proliferation assay. Fresh GC cells were detected as CD45- and glycopholin-A-negative cells from the peritoneum sample. The GC cells were then collected and sorted using a FACS Vantage™ SE (Becton Dickinson) with a single cell in each well of a 96-well cell culture plate coated with Matrigel (Becton Dickinson, San Jose, CA, USA) as a basement membrane matrix (Becton Dickinson). Single cells were cultured in the appropriate cell culture medium as described above. Colony formation was observed daily under a microscope. Clones were seeded and cultured in 10% serum-supplemented RPMI-1640. Cells were cultured for 0, 35, 40 and 45 days and then harvested using 0.25% trypsin-EDTA, whereupon the cells were counted by the trypan blue dye exclusion method. Trypan blue is a vital stain used to selectively color dead cells blue, while live cells with intact cell membranes remain uncolored. The growth curve was drawn from cell counts and the population growth rate was calculated.

Transplantation assay. SCID (C.B-17/lcr-scid/scidJcl) mice aged 8-10 weeks (Japan Clea Laboratories, Tokyo, Japan) were used in the experiment. For transplantation, 1x10⁶ cloned cells derived from the GC clinical sample were subcutaneously inoculated intraperitoneally in the mice, which were then sacrificed using diethyl ether 7.4 weeks later and laparotomized. Intrapertitoneal dissemination of the tumor was observed and the tumors weighed.

Flow cytometry. To analyze integrin expression, the cells were removed from the culture dish using 0.25% trypsin and 1 mM EDTA, pelleted by centrifugation, washed with phosphate-buffered saline (PBS) and resuspended at 37°C in Hank’s balanced salt solution (HBSS) containing 2% FBS and 10 mM HEPES (Invitrogen). The cells were then stained with FITC- or PE-labeled monoclonal antibodies against human CD49a (integrin α1), CD49b (integrin α2), CD49c (integrin α3), CD49d (integrin α4), CD49e (integrin α5), CD49f (integrin α6) and CD29 (integrin β1) subunits (Pharmingen, San Jose, CA, USA). The cells were then incubated with human monoclonal antibodies for 30 min at 4°C, and then suspended in HBSS containing 2% FBS, penicillin-streptomycin mixed solution and 10 mM HEPES. The cells were then passed through a 40-µm mesh filter and maintained at 4°C until flow cytometric analysis. The cells were subsequently counterstained with 1 µg/ml propidium iodide (Sigma-Aldrich, St. Louis, MO, USA) to label dead cells. Subsequently, 1x10⁶ viable cells were analyzed and sorted using a FACS Vantage™ SE. The distribution of cells was analyzed using FlowJo software (Tomy Digital Biology, Tokyo, Japan).

Adhesion assay. The adhesion ability of each cell clone was examined by an adhesion assay. Briefly, a 96-well microtiter plate was coated with Matrigel (50 µl/well) (Becton Dickinson). Cells (1x10⁵) were incubated with anti-CD49a (integrin α1) antibody for 30 min at 37°C, and then seeded into a 96-well microtiter plate (Corning Costar, Franklin Lakes, NJ, USA). The cells were allowed to adhere to each well for 12 h at 37°C and were then washed gently twice in PBS. Cancer cells with adhesion capacity were quantified by the WST-8 assay. The value of optical density (OD) was detected using Rainbow Sunrise (Wako Pure Chemical Industries, Ltd, Osaka, Japan). The rate of inhibition was calculated as follows: % inhibition = (OD of treated group - blank)/(OD of control group) x 100.

Statistical analysis. All data were expressed as the mean ± SD. Statistically significant differences were determined using the Student’s t-test. P values <0.05 were considered to denote statistical significance.

Results

In vitro adhesion assay. To select and clone GC cells having adhesion and proliferation capacity, GC cells were evaluated using single cell sorting methods. After 14 days in culture, 5 clones (KGC1C2, KGC1F3, KGC1H3, KGC1E8, KGC1G10) having sphere or colony-like shape appeared in Matrigel-coated 96-well plates (Fig. 1). At day 45, the growth rate (number of cells) of clone KGC1C2 (4.75x10⁶) was ~1.8-fold higher compared to the other three clones (Fig. 2). These results indicate that only 5 of the 96 clones isolated could survive and proliferate, suggesting that 5.2% of GC cells in ascites may possess adhesion and proliferation capabilities.

Evaluation of tumorigenesis in vivo. The tumorigenic ability of the clones in peritoneal tissue was examined using SCID (C.B-17/lcr-scid/scidJcl) mice. Each clone (1x10⁶ cells) was injected into the peritoneal cavity of SCID mice. After 7.4 weeks, each clone was able to generate tumors. Moreover, these metastatic GC cells caused conglutination of surrounding organs in the abdominal cavity, including the stomach, liver and intestine. The weight of tumors derived from the KGC1H2 clone was 1.03±0.21 g (mean ± SD). The KGC1H2 clone formed more tumors than the other clones tested (P<0.05) (Fig. 3). These results indicate that the clones derived from the clinical sample possessed tumorigenic ability in vivo.

Integrin expression profiles determined by flow cytometry. Integrin expression was measured by flow cytometry with...
monoclonal antibodies against human CD49a (integrin α1), CD49b (integrin α2), CD49c (integrin α3), CD49d (integrin α4), CD49e (integrin α5), CD49f (integrin α6) and CD29 (integrin β1) subunits. Notably, the expression levels of 1α integrin subunits were much higher compared to the other integrins evaluated (Table I). These results suggest that the

expression of integrin α1 subunit is correlated with adhesion in the peritoneum.

Suppression of adhesion by anti-integrin antibody. To test the ability of anti-CD49a (integrin α1) antibodies to interfere with cell adhesion in vitro, we examined the effect of anti-CD49a on the adhesion ability of the cloned cells. The inhibition ratio of adhesion was 63.8% in the KGCF3 clone, which appeared to be effectively inhibited by antibodies against integrin α1 (P<0.05) (Fig. 4). This result showed that the adhesive ability of GC cells may be inhibited or controlled by the anti-CD49a (integrin α1) antibody.
Table 1. Flow cytometric analysis of integrin subunit expression in each clone derived from a GC patient with peritoneal dissemination.

<table>
<thead>
<tr>
<th>Integrin subunita</th>
<th>KGC1C2</th>
<th>KGC1F3</th>
<th>KGC1H3</th>
<th>KGC1E8</th>
<th>KGC1G10</th>
</tr>
</thead>
<tbody>
<tr>
<td>α1</td>
<td>18.6±1.23</td>
<td>9.49±0.07</td>
<td>83.5±6.36</td>
<td>36.4±5.35</td>
<td>28.2±5.44</td>
</tr>
<tr>
<td>2</td>
<td>6.07±0.37</td>
<td>4.00±0.36</td>
<td>81.7±5.39</td>
<td>26.6±4.01</td>
<td>18.7±1.08</td>
</tr>
<tr>
<td>α3</td>
<td>0.03±0.04</td>
<td>0.00</td>
<td>0.14±0.01</td>
<td>0.67±0.07</td>
<td>0.32±0.03</td>
</tr>
<tr>
<td>α4</td>
<td>1.66±0.14</td>
<td>5.81±0.17</td>
<td>45.7±5.72</td>
<td>44.2±4.68</td>
<td>12.2±0.61</td>
</tr>
<tr>
<td>α5</td>
<td>0.08±0.01</td>
<td>0.27±0.03</td>
<td>8.87±0.39</td>
<td>8.72±0.25</td>
<td>0.97±0.15</td>
</tr>
<tr>
<td>α6</td>
<td>0.06±0.01</td>
<td>0.53±0.07</td>
<td>0.39±0.04</td>
<td>0.77±0.08</td>
<td>0.98±0.18</td>
</tr>
<tr>
<td>β1</td>
<td>10.2±0.36</td>
<td>3.18±0.24</td>
<td>81.5±9.61</td>
<td>26.5±3.73</td>
<td>19.5±0.82</td>
</tr>
</tbody>
</table>

Cells were treated with monoclonal antibodies against CD49a (integrin α1), CD49b (integrin α2), CD49c (integrin α3), CD49d (integrin α4), CD49e (integrin α5), CD49f (integrin α6) and CD29 (integrin β1). Expression levels were estimated using the fluorescence intensity of FITC or PE in the samples. *Expressed as percentage of expression (mean ± SD).

Discussion

We isolated GC primary cells with adhesion capacity from a patient with peritoneal dissemination using single cell sorting methods, and established 5 clones that were found to adhere to the peritoneum in vivo as well as to the extracellular matrix (ECM) in vitro. The attachment of GC cells to the peritoneum is an important step in the initial phases of metastatic dissemination (10-12). The results of the present study showed that only a small subset of GC cells have potential metastatic ability in the peritoneum.

To elucidate the mechanism of peritoneal dissemination, we analyzed expression of integrins (α1-6 and β1), which act as adhesion molecules. Flow cytometric analysis demonstrated that the level of expression of the α1 subunit paired with β1 subunits was higher compared to other integrins in all of the clones. In addition, the results of the transplantation assay indicated that the integrin expression level was inversely correlated with adhesion.

Integrin α1 (CD49a) is also known as very late antigen 1α subunit (VLA-1α). Integrin α1 associates with integrin β1 subunits (CD29) to form α1/β1 heterodimers, and serves as a receptor for collagen and laminin-1 (13,14). Integrin α1 has been reported to play a role in cell attachment (15-17). Conversely, integrin α1 is expressed at low levels in human breast cancer cell lines (18) and is downregulated in breast and ovarian cancer (19), suggesting that its loss might be associated with malignancy. Furthermore, integrin α1 can negatively regulate EGFR signaling in prostate and cervical cancer cells (20) as well as in mesangial cells (21). In the present study, a cell adhesion blocking assay showed that anti-CD49a (integrin α1 subunit) mAb significantly reduced the adhesiveness of the clones compared to the controls. These data indicate that the α1 subunit is a molecule that is significantly involved in the adhesion of cancer cells to the peritoneum.

Peritoneal dissemination has often been detected in recurrent GC and results from a multistep phenomenon that includes detachment of malignant cells from the primary tumor, transfer to the peritoneal cavity, attachment to the peritoneum, ECM degradation, and migration of adhesive tumor cells into surrounding tissues (22). In a previous study, the expression of integrins in tumor invasion and metastasis, particularly in liver metastasis, was reported (23). Cell adhesion molecules are necessary for many different cellular functions including cell signaling, migration and apoptosis as well as maturation or formation of tight junctions (24-27). By contrast, patients known to have free GC cells in the peritoneal cavity have not always developed peritoneal implantation. It may be that only a minority of cancer cells possess metastatic capability in GC with peritoneal dissemination, so understanding the adhesion of GC cells is important to fully elucidate GC cell functions (28-30).

In conclusion, the interaction of GC cells with the ECM appears to be of considerable importance in peritoneal metastasis as cancer cells that exfoliated into the abdominal cavity were found to attach initially to the mesothelial lining, followed by their invasion through the intercellular gaps of mesothelial cells (31). This study indicated that the adhesion of integrin α1-positive GC cells to the ECM is a critical process in peritoneal dissemination. The clones derived from this patient are available for research and may be useful for defining the biological phenomena involved in GC in metastasis.

Acknowledgements

We thank Sadafumi Suzuki for his invaluable FACS expertise. This work was supported by a Grant-in-Aid from the 21st Century COE program of the Ministry of Education, Science, and Culture of Japan to Keio University and a Grant-in-Aid for Young Scientists (B) from the Ministry of Education, Culture, Sports, Science and Technology (MEXT), Japan.

References


