Caveolin-1 Is Associated with VCAM-1 Dependent Adhesion of Gastric Cancer Cells to Endothelial Cells

Jaeyoung Shin¹, Jongmin Kim¹, Byungkyu Ryu², Sung-Gil Chi² and Heonyong Park¹

¹Department of Molecular Biology & Institute of Nanosensor and Biotechnology, Dankook University,

²School of Life Sciences and Biotechnology, Korea University

Key Words

Gastric cancer • Metastasis • Cell adhesion • Vascular cell adhesion molecule-1 • Caveolin-1

Abstracts

Background/Aims: Cell adhesion molecules play a critical role in the invasion and metastasis of a variety of human tumors. Abnormal expression of VCAM-1 has been demonstrated to correlate with the malignant progression of gastric tumors, but the molecular mechanism underlying the VCAM-1-dependent metastasis has been rarely investigated. To explore the role for tumor cell-expressing adhesion molecules in the carcinoma-endothelium adhesion, we analyzed expression status of adhesion molecules in gastric cancer cells and its association with tumor cell capability of endothelial adhesion. **Methods:** Endothelial adhesion ability of gastric tumor cells was tested using calcein AM staining assay. Expression of cell surface proteins was determined by Western blot, flow cytometry, and immunofluorescence assays. RNAi-mediated knockdown of gene expression and neutralization with specific antibodies were utilized for functional analysis. **Results:** One of three cell lines tested was identified to be adhesive to endothelial

cells and express VCAM-1. Adherence ability of the cells was dramatically decreased by neutralization of surface VCAM-1. VCAM-1 was co-localized with Caveolin-1 and siRNA-mediated knockdown of Caveolin-1 expression significantly blocked the VCAM-1-dependent cell adhesion. **Conclusions:** Our data imply important roles for VCAM-1 and Caveolin-1 in the regulation of metastatic potential of gastric tumor cells.

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Introduction

Cancer metastasis is a complex process in which tumor cells separate from the primary tumor mass, migrate through the vascular system, extravasate into other tissues and grow new tumors [1-4]. Among these diverse processes, an alteration in adhesive properties of the primary tumor cells is a critical factor for tumor progression [2]. It has been revealed that the cell adhesion is responsible for tumor progression and a variety of molecules for the cell adhesion are elucidated for the cell-

cell adhesion and the cell-matrix adhesion [5-8]. The cell-cell adhesion plays an important role in two different stages of the tumor metastatic process, the detachment from the primary tumor and their adhesion to the circulatory system [1]. To date, a variety of adhesive molecules are known to elicit the tumor cell disassociation from the primary tumor mass. For example, E-cadherin interacts in a homophilic manner with E-cadherin molecule on the surface of adjacent cells in the tumor mass [9].

Loss of cell adhesive molecules induces causally to disassemble free cancer cells from the primary tumor masses, disseminate them to distant sites, and eventually elicit the transition from benign tumors to metastatic tumors [9-10]. The disassembled tumor cells are disseminated to distant sites from their primary tumors via blood vessels and lymphatics. These circulating tumor cells interact with the endothelial cells of blood vessels, which is considered as a preliminary event for the tumor metastasis [1, 11]. The adhesive interactions between cancer cells and the endothelium have been known to be controlled by the elaborate molecular mechanisms [1-5, 11]. Various adhesive molecules, including selectins, integrins, cadherins, and immunoglobulins, have been identified to be responsible for the endothelial adhesion of cancer cells. Some of these adhesive molecules are constitutively expressed while others are induced by many different environmental signals and distributed in the tissue-specific manner as well. In this context, it has been proposed that the expression level and the avidity of the adhesive molecules on the cell surface are likely to be crucial for the tumor progression at distant sites.

Vascular cell adhesion molecule-1 (VCAM-1) is expressed on the surfaces of endothelial cells, mediating cellular adhesion via integrin $\alpha 4\beta 1$. The VCAM-1-dependent adhesion was known to be responsible for lung metastasis [12]. Recently, it has been demonstrated that VCAM-1 is expressed in gastric tumor cells as well as tumor-associated endothelial cells and its expression level is highly related with the invasiveness and advanced stage of gastric tumors [13]. However, the mechanistic basis for the tumor-promoting function of VCAM-1 produced by carcinomas cells has been poorly understood. Integrin $\alpha 4\beta 1$, a partner molecule of VCAM-1, is strongly expressed in the endothelium [14-16]. It is therefore conceivable that endothelial integrin $\alpha 4\beta 1$ and tumor cell-producing VCAM-1 might play a role in the adhesion of the carcinoma cells to endothelium during the malignant progression of tumors. Endothelial layers in blood vessels are exposed to the blood flow, hence cell-cell interactions

are in part regulated by shear stress, a frictional force generated by the blood flow [17-19]. Tumor cell adhesion to endothelium is also controlled by a variety of cell adhesion molecules linked to shear stress [17]. Recently, it was elucidated that membrane microdomains called caveolae are sensing shear stress [18-19]. Caveolin-1 is a principal protein for constructing caveolae, but little is known about roles of Caveolin-1 in the metastasis of tumor cells.

In the present study, we investigated the role of cell adhesion molecules in the carcinoma-endothelium adhesion using gastric tumor cells to gain further understanding of the mechanism of metastatic adhesion of carcinoma cells during tumor progression. We demonstrate first that tumor cell-producing VCAM-1 plays a crucial role in the endothelial adhesion of carcinoma cells and the VCAM-1 role in the adhesion process is exerted through caveolae, suggesting the possible contribution of VCAM-1 and caveolae to the metastatic adhesion of tumor cells during the malignant progression of human gastric tumors.

Materials and Methods

Cell Culture

BAEC obtained from descending thoracic aortas were maintained in a DMEM medium (1 g/liter glucose, Life technologies, Inc.) containing 20% fetal bovine serum (Wel GENE, Inc) with antibiotics at 37 °C and 5% CO₂ [19-20]. Cells used in this study were between passages 3 and 10. The gastric cancer cells, SNU-5, SNU-16 and SNU-620, were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) and 50 µg/ml streptomycin and penicillin in a 5% CO₂ incubator at 37 °C.

Cell Adhesion

Gastric cancer cells (SNU-5, SNU-16 and SNU-620) were grown up to 5-6 x 10⁶ cells/ml and then stained with 15 µM of calcein AM (Sigma) for 45 min at 37 °C and 5% CO₂. While gastric cancer cells were cultured, BAECs were also confluent grown in 6 well plates and then starved in 0.5% FBS-DMEM overnight. Then a million of cancer cells stained with calcein AM were added into a BAEC culture plate and incubated as indicated time points. Then free cells were thoroughly washed away and adhesive cells were observed under a fluorescent microscope (Zeiss Autoplan2). The degree of adherence was quantified by counting the adhesive cells in the representative area as modified Takeda et al.'s method [21]. Degree of adhesion was calculated by the ratio of the number of the adhesive cells to the number of total cells.

For neutralization, the cells were pretreated with mouse anti-integrin antibodies (Santa Cruz) or anti-VCAM-1 antibodies (Santa Cruz) 1 h before performing the adhesion test. For the

treatment of PD98059 (PD, 10 μ M), LY294002 (LY, 1 μ M), wortmannin (WT, 100 nM) and SB202190 or SB202180 (10 μ M) compounds (Calbiochem), cells were pre-incubated with each drug at indicated concentrations 8 h before executing the adhesion test. In case of filipin (Sigma) and nystatin (Sigma), cells were pretreated with 5 μ g/ml of each drug 10 min before experiments. Quantification for neutralization and inhibition tests was also performed as described above.

Transfection of Gastric Cancer Cells with siRNA against Caveolin-1

Double-stranded siRNA (21 nucleotides) were obtained from Dharmacon Research (Lafayette). The target sequence is 5'-AACCAGAAGGGACACACAGUU-3'. A GL2 luciferase oligonucleotide was used as a negative control. SNU-5 cells were grown to 3×10^5 /ml and transfected with annealed siRNA using OligofectAMINE (Invitrogen) for 4 h as the manufacturer recommends [22]. Then the cells were grown for 48 h in RPMI medium containing 10% FBS and 50 μ g/ml streptomycin and penicillin in a 5% CO₂ incubator at 37 °C and cells were used for further assays.

Immunofluorescence

Subcellular localization of Caveolin-1 and VCAM-1 was investigated using an immunofluorescence microscopy. A million of SNU-5 cells were added to BAECs confluent grown on 35-mm plates. For the tight adhesion, two different types of cells were incubated for 8 h and then washed free cells off by phosphate buffered saline (PBS). Then, the cells were fixed in 0.2% glutaraldehyde for 2 min, permeabilized in PBS containing 0.1% Triton X-100 and 50 mM lysine for 30 min, and blocked in PBS containing 2% BSA, 10% goat serum, and 0.1% Triton X-100 for 1 h. The cells were incubated with anti-Caveolin-1 (BD Biosciences) or anti-VCAM-1 (R&D system, Inc.) antibodies overnight at 4 °C and with Cy3-conjugated goat anti-rabbit or mouse antibodies for 30 min. Washed cells were then mounted with Slow-Fade, and fluorescence intensity was quantified by using a fluorescence microscope and an image analysis program (Zeiss Autoplan2) [23].

Western Blots

BAECs were confluent grown in 20% FBS-DMEM and SNU-5 cells were grown in RPMI 1640 medium supplemented with 10% FBS. Cells were washed in ice-cold PBS, scraped in 250 μ l RIPA buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate and 0.1% SDS), solubilized for 15 min to prepare cell lysate as previously described [19]. For Triton-soluble fraction, cells were lysed with 0.25 ml of lysis buffer (50 mM HEPES, pH 7.4, 150 mM NaCl, 1 mM vanadate, 1 mM dithiothreitol, 1.0 % Triton X-100 and 0.1 mM phenylmethylsulfonyl fluoride), solubilized for 15 min and centrifuged for fractionation. Entire solubilization procedures were performed at 4 °C and the protein content of soluble cell lysates was measured by using a Bio-Rad DC assay kit (Bio-Rad). Soluble lysates (10 μ g each) were resolved by 10% SDS-PAGE, transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore), and probed with antibodies specific to VCAM-1 (Santa Cruz), Caveolin-1 (BD Biosciences) and

Fig. 1. Adhesion of gastric cancer cells to endothelial cells. BAECs were confluent grown in DMEM-20% FBS media and incubated with three different gastric cancer cells (SNU-5, SNU-16 and SNU-620). Free floating cells were thoroughly washed with phosphate buffered saline and adherent cells were observed under optic (A) or fluorescent (B) microscope. Adherence degree was quantified as described in Materials and Methods. Quantification data was shown in panel C (means $\pm$ S.E., n=6, * p<0.05).

endothelial nitric oxide synthetase (Cell Signaling Technology). Goat anti-rabbit IgG conjugated to alkaline phosphatase was used as a secondary antibody and the membrane was developed by a chemiluminescent detection method [19].

Flow Cytometry

BAECs confluent grown on the T-75 flask were incubated for 20 min in PBS containing 0.2% EDTA. Then detached cells were harvested by centrifugation (800 rpm, 5 min). After harvesting, the cells (2×10^6 cells/ml) were incubated with 5 μ g/ml rabbit anti-integrin α 4 antibodies or non-immune IgG, washed with PBS by centrifugation (800 rpm, 5 min) and then incubated with 5 μ g/ml of FITC conjugated secondary antibodies (Vector Co.). After washing, stained cells were analyzed with flow cytometry using Becton-Dickinson FACS Caliber [24]. SNU-5 cells (2×10^5 /ml) were grown in 6 well plates. Cells were then harvested, washed and stained consecutively with rabbit anti-VCAM-1 antibodies (Santa Cruz) and FITC conjugated anti-rabbit antibodies as previously described [24]. After washing, stained cells were analyzed with flow cytometry using Becton-Dickinson FACS Caliber.

Fig. 2. Integrin $\alpha 4 \beta 1$ mediated the cell-cell adhesion between SNU-5 and BAECs. Confluent BAECs were pretreated with indicated antibodies and adherences were measured as described above. Panel A represents fluorescence micrographs and Panel B shows their quantification data as bar graphs (means $\pm$ S.E., $n=6$, * $p<0.05$). Flow cytometric analysis of surface expression of integrin $\alpha 4 \beta 1$ in BAECs using integrin $\alpha 4$ antibodies or non-immune immunoglobulin. Panel C exhibits that integrin $\alpha 4 \beta 1$ was expressed on the surface of BAECs.

Fig. 3. Expression of cell adhesion molecules in SNU-5 gastric cancer cells. Gastric cancer cells (SNU-5, SNU-16 and SNU-620) were grown up to $5-6 \times 10^6$ cells/ml and then lysed with RIPA buffer. Proteins in the cell lysates were resolved by SDS-PAGE after applying equal amounts of proteins and Western blots were performed using indicated antibodies (A). To examine whether SNU-5 cells were contaminated with endothelial cells, gastric cancer cells (SNU-5, SNU-16 and SNU-620) and BAECs were lysed with RIPA buffer and immunoblotted with antibodies for Tie-1 and endothelial nitric oxide synthetase (eNOS) (B).

Results

Gastric Cancer Cells Are Adhesive to Endothelial Cells

To investigate the adhesion property of tumor cells to endothelial cells, we used three floating gastric cancer cell lines (SNU-5, SNU-16 and SNU-620), which were derived from the ascites of three cancer patients [25-26]. Floating cancer cells were chosen for adhesion test because endothelial cells, target for adhesion, are adherent to the surface of culture dishes. Two types of gastric cancer cells, SNU-5 and SNU-16 were known to remain homotypically adhesive. We initially tested the adhesion ability of individual tumor cell lines to endothelial cells, and SNU-5 cells were identified to be significantly more adherent compared to SNU-16 and SNU-620 cells by 3-4 fold (Figure 1). Therefore, SNU-5 cells were focused for further assays to dissect the mechanisms of heterotypic cell adhesion.

Fig. 4. VCAM-1 mediated the cell-cell adhesion between SNU-5 and BAECs. SNU-5 cells were pretreated with indicated antibodies as well as calcein AM, and the adherences were measured as described in Materials and Methods. Panel A represents fluorescence micrographs and Panel B shows their quantification data as bar graphs (means $\pm$ S.E., $n=5$, * $p<0.05$). To see the surface expression of VCAM-1 in SNU-5, a flow cytometry was performed using VCAM-1 antibodies or non-immune immunoglobulin. Panel C exhibits VCAM-1 expression on the surface of the SNU-5 cells.

VCAM-1 Is a Key Molecule for Endothelial Adhesion of Cancer Cells

To define the adhesion molecules involved in the SNU-5 adhesion to endothelial cells, we tested whether the cell-cell adhesion is affected by neutralization of adhesion molecules on the endothelial cell surface using various inhibitory antibodies. As shown in Figure 2A and B, the adhesion of SNU-5 cell to endothelial cells was strongly inhibited by antibodies specific for integrin $\alpha 4 \beta 1$ while only a slight inhibition was detected by antibodies for $\alpha 5$ or $\beta 4$. Expression of integrin $\alpha 4 \beta 1$ on the surface of the endothelial cells was verified by flow cytometric analysis (Figure 2C).

To explore whether tumor cell-producing adhesion molecules are also involved in the endothelial adhesion of tumor cells, we analyzed the expression status of various adhesion molecules and its possible association with the adhesion capability of the cells. Compared to other two cell lines, SNU-5 showed expression of diverse adhesion molecules. Intriguingly, VCAM-1, an endothelial cell-specific adhesion protein, was identified to be expressed exclusively from SNU-5 cells. In order to rule out the possibility that VCAM-1 expression results from the endothelial cell contamination of SNU-5, we examined protein expression of Tie-1 and endothelial nitric oxide synthetase (eNOS), endothelial markers, but no detectable expression of Tie-1 and eNOS was observed in SNU-5 cells (Figure 3B). We next dissected whether neutralization of VCAM-1 decreases the adhesion potential of SNU-5

cells. Whereas pretreatment of SNU-5 cells with non-immune IgG or antibodies against integrin $\alpha 4$ had no effect on the endothelial adhesion, VCAM-1 neutralization had a significant effect on their adhesion (Figure 4A and B). These data is consistent with the previous results demonstrating the inhibitory effects of antibodies for integrin $\alpha 4 \beta 1$ in endothelial cells. Surface expression of VCAM-1 in SNU-5 cells was also verified by flow cytometry analysis (Figure 4C).

VCAM-1 Expression and Tumor Cell Adhesion Is Blocked by p38 MAP Kinase Inhibitor

Based on the observation that neutralization of surface VCAM-1 significantly inhibited endothelial adhesion of SNU-5 cells, we further investigated the signaling pathway involved in the VCAM-1-mediated adhesion. SNU-5 cells were treated with various pharmacological inhibitors against signaling molecules and its effect on VCAM-1 expression and endothelial adhesion of the cells were determined. As shown in Figure 5A, the pretreatment of SNU-5 cells with the p38 MAP kinase inhibitor SB202190 significantly blocked their adhesion ability to endothelial cells, whereas the pretreatment of BAECs with the inhibitors revealed no detectable effect on the adhesion. In addition, SB202190 treatment also suppressed expression of VCAM-1 [27-28]. SB202190 has been known to inhibit c-Jun N-terminal kinase (JNK) at high doses [29]. To exclude the possibility that the adhesion-inhibitory effect of SB202190 is associated with

Fig. 5. Inhibitors for p38 MAP kinase decrease VCAM-1 expression and the cell-cell adhesion. SNU-5 cells were pre-incubated with drugs [10 μ M PD98059 (PD), 1 μ M LY294002 (LY), 100 nM wortmannin (WT) and 10 or 50 μ M SB202190 (SB90) and 10 μ M SB202180 (SB80)] for 8 h, and the adhesion test (Panel A) and Western blots with VCAM-1 or actin antibodies (Panel B) were performed.

its JNK inhibition activity, the cells were treated with two different concentrations of SB202190 (10 and 50 μ M). We observed that endothelial adhesion and expression of VCAM-1 are suppressed similarly at both doses of SB202190, strongly supporting that the adhesion-inhibitory effect of SB202190 is mediated mainly through the blocking the p38 MAP kinase cascade. This result is consistent with previous reports that the TNF-stimulated upregulation of VCAM-1 expression is suppressed by p38 MAP kinase inhibition in various cell lines [27-28].

Knockdown of Caveolin-1 Expression Abolishes Endothelial Adhesion of SNU-5

It has been well documented that Caveolin-1 plays an important role in cell adhesion [30-31]. Thus, we tested whether Caveolin-1 is involved in the SNU-5 adhesion to endothelial cells. SNU-5 cells were transfected with siRNA against Caveolin-1 to knockdown its endogenous expression, or with GL2 luciferase oligonucleotide as a control. As seen in Figure 6A, a substantial reduction of Caveolin-1 expression level was detected in Caveolin-1 siRNA-transfected cells compared to GL2 luciferase

Fig. 6. Caveolin-1 is required for the cell-cell adhesion. SNU-5 cells grown to 3×10^5 /ml were transfected with Caveolin-1 siRNA and control RNA as described in Materials and Methods. The cells grown for 48 h in RPMI medium containing 10% FBS were used for further assays. Panel A shows immunoblots with using Caveolin-1, VCAM-1 and actin antibodies. In panel B, adherence for Caveolin-1 siRNA- or control-transfected SNU-5 cells was shown as line graphs (means $\pm$ S.E., $n=3$, * $p<0.05$). To disrupt caveolae, cells were pretreated with vehicle, filipin (5 μ g/ml, 10 min), nystatin (5 μ g/ml, 10 min) and cell adherence was assayed and then shown as bar graphs (means $\pm$ S.E., * $p<0.05$, ** $p<0.01$). Data in bar graphs and line graphs were obtained from at least three independent experiments.

oligonucleotide-transfected controls. VCAM-1 expression was not affected by transfection of Caveolin-1 siRNA or GL2 luciferase oligonucleotide. Interestingly, we found that the adhesion ability of SNU-5 cells is markedly abolished by Caveolin-1 siRNA transfection versus GL2 luciferase

Fig. 7. VCAM-1 was co-localized with Caveolin-1 in SNU-5 cells. SNU-5 cells were immunostained with VCAM-1 and Caveolin-1 antibodies. In 43% of total floating SNU-5 cells, VCAM-1 was detected to be co-located with the Caveolin-1. Arrow represents the cell exhibiting co-localization of two proteins and arrowhead indicates the cell not exhibiting co-localization.

Fig. 8. VCAM-1 translocation from the Triton-insoluble fraction to the Triton-soluble fraction by Caveolin-1 depletion. SNU-5 cells were transfected with Caveolin-1 siRNA (Si-Cav-1). Transfected cells were fractionated by lysis with a buffer containing Triton X-100 and centrifugation. Proteins in the soluble fraction (Tx-sol) and the insoluble fraction (Tx-insol) were resolved by SDS-PAGE and then proteins were detected by western blots. Bar graphs show the mean $\pm$ S.E. (n=3, * p<0.01, **p<0.05).

Fig. 9. Co-localization of VCAM-1 and Caveolin-1 in SNU-5 cells adherent to endothelial cells. SNU-5 cells attached to endothelial cells were immunostained with VCAM-1 and Caveolin-1 antibodies. In the right panel, overlay of two stains were shown.

oligonucleotide transfection (Figure 6B). Furthermore, when caveolae was disrupted by treatment of cholesterol-binding agents, such as filipin and nystatin, the endothelial adhesion of SNU-5 cells was significantly blocked (Figure 6C) [19]. These results thus indicate that Caveolin-1 plays a crucial role in the endothelial adhesion of SNU-5 cells.

VCAM-1 Is Co-localized with Caveolin-1 in Adhesive SNU-5 Cells

Many adhesion molecules including integrins have been known to be co-localized with Caveolin-1 [30-31]. In an attempt to elicit whether the reduced endothelial adhesion of SNU-5 cells by Caveolin-1 knockdown or caveolae disruption results from a blockade of VCAM-1 interaction with Caveolin-1, we first explored the possible

co-localization of these two protein in SNU-5 cells. As shown in Figure 7, a portion of SNU-5 cells was intensively stained with anti-Caveolin-1 antibodies and the cells stained with Caveolin-1 antibodies were also stained with anti-VCAM-1 antibodies at the very similar location. Approximately 43% (63 of 146) of cells we screened displayed the intensive co-staining of VCAM-1 and Caveolin-1 proteins, raising the possibility that localization of VCAM-1 to caveolae via the interaction with Caveolin-1 might be responsible for the VCAM-1-dependent tumor cell adhesion to endothelium. To further define the possible interaction of VCAM-1 with Caveolin-1, we performed *in vitro* binding and immunoprecipitation assays. However, we failed to detect the interaction between these two proteins, leading to the conjecture that VCAM-1 does

not directly bind to Caveolin-1 and other factor(s) might be required for the caveolae localization of VCAM-1 (data not shown).

Since VCAM-1 level was not affected by siRNA-mediated Caveolin-1 depletion in SNU-5 cells, we tested whether the declination of Caveolin-1 influences VCAM-1 amount in the Triton-insoluble fraction that are known as a Caveolin-1-enriched fraction. As shown in Figure 8, the amount of VCAM-1 in the Triton-soluble fraction was increased in Caveolin-1 siRNA-transfected SNU-5 cells, while the amount of VCAM-1 in the Triton-insoluble fraction was decreased in the same cells. This observation suggests that reduction of Caveolin-1 expression could disrupt clustering of VCAM-1 at the Caveolin-1-enriched area, attenuating the adhesion capability of SNU-5 cells to endothelial cells. To confirm that the adherent SNU-5 cells maintain the clustering of VCAM-1 and Caveolin-1, an immunostaining assay of VCAM-1 and Caveolin-1 was carried out for SNU-5 cells attached to endothelial cells. As shown in Figure 9, VCAM-1 was identified to be co-localized with Caveolin-1 in the SNU-5 cells isolated after adhesion. Collectively, these results support that tumor cell-producing VCAM-1 proteins are clustered in the Caveolin-1-enriched fractions in the adhesive SNU-5 cells, and the VCAM-1 localization to caveolae might be crucial for its role in cancer cell adhesion to endothelium.

Discussion

Gastric cancer is one of the most commonly diagnosed malignancies worldwide and a leading cause of cancer mortality in certain areas, such as Korea, Japan, South Africa, and Eastern Europe. Although evidence has accumulated indicating the involvement of the alterations of multiple genes such as p53, K-ras, c-erbB2, K-sam, and E-cadherin, the underlying molecular events that drive the neoplastic process in gastric cancer are largely undefined [13-16]. Cell adhesion molecules (CAMs) are crucial factors to be responsible for tumor angiogenesis, progression and metastasis. In the present study, we investigated the role of CAMs including VCAM-1 in the endothelial adhesion of human gastric carcinoma cells. Here we demonstrate first that tumor-producing VCAM-1 enhances the adhesion capability of carcinoma cells through the collaboration with Caveolin-1. Our study suggests that VCAM-1 expression in carcinoma cells may contribute to the metastatic adhesion of tumor cells, and thus facilitate the malignant progression of human gastric

tumors.

VCAM-1 is a glycoprotein constitutively expressed in various endothelial cells. VCAM-1 has been demonstrated to influence a broad aspect of tumor progression, such as cellular proliferation, angiogenesis and metastasis. VCAM-1 principally mediates intercellular interaction via binding to integrin $\alpha 4 \beta 1$. Interestingly, a recent study demonstrated that VCAM-1 is expressed in tumor cells as well as endothelial cells in primary gastric carcinoma tissues and gastric cancer patients with high level of VCAM-1 expression show poor prognosis compared to healthy controls [13]. Moreover, increased VCAM-1 expression significantly correlates with stage, invasion depth, and lymph node and distant metastasis of gastric cancers, indicating that VCAM-1 plays important roles in gastric cancer progression and metastasis. The carcinoma cell to epithelium adhesion is a critical step for metastatic progression of tumor. In this context, our observation that tumor-producing VCAM-1 is implicated in the increased endothelial adhesion of gastric cancer cells further supports a critical role for VCAM-1 in the malignant progression of gastric tumors. In this work, we observed that VCAM-1 expression levels are variable in three floating gastric cancer cells we tested, and they exhibits different adhesion capability to endothelial cells, suggesting that VCAM-1 expression status could reflect cellular potential for distant metastasis. With a tight association of VCAM-1 level with metastasis observed in tumor patients, it is conceivable that VCAM-1 expression, particularly in carcinoma cells, could be a valuable marker for diagnosis, prognosis and better selection of treatment strategy for gastric cancer patients.

Caveolin-1 is known to be involved in a variety of physiological and pathological functions [30-34]. Tumor-associated functions of Caveolin-1 are controversial depending upon tissue types and stages of cancer [33-34]. Caveolin-1 is implicated as a tumor suppressor in breast and prostate cancers. By contrast, enhanced expression of Caveolin-1 is associated with increased invasive and metastatic potentials of lung and kidney tumor cells [35-36]. In addition, the expression of Caveolin-1 in oral cancer is biphasically changed as tumorigenic stages [37]. Meanwhile, Caveolin-1 is considered as a switching molecule in tumor development by its function as a tumor suppressor as well as a promotor of metastasis [38]. Therefore, Caveolin-1 has diverse effects depending upon stages and types of tumors. Compared with other types of cancers, the expression and functional status of Caveolin-1 in gastric cancer is rarely studied. Our study shows that tumor cell-producing VCAM-1 proteins are

localized to caveolae, probably via its interaction with Caveolin-1, and disruption of caveolae or knockdown of Caveolin-1 expression dramatically abrogated the adhesion-enhancing effect of VCAM-1. Thus, our current results provide an insight on the role of Caveolin-1 in gastric tumorigenesis.

To explore the role of Caveolin-1 in gastric cancer cells, we dissected the adhesion of floating cancer cells to vascular endothelial cells. We observed that Caveolin-1 promotes the endothelial adhesion of gastric cancer cells through clustering of VCAM-1, suggesting that Caveolin-1 may play a role in metastasis or tumor progression. It is worthwhile to note that three different types of floating gastric cancer cells reveal different expression levels of Caveolin-1 (data not shown). It was previously reported that Caveolin-1 modulates cell-cell adhesion through the membrane translocation of adhesion molecules and an increase in E-Cadherin expression [32, 39-40]. Miotti et al. demonstrated that the catenin p120 protein (p120ctn) forms complexes with the E-Cadherin (E-cad) protein in Caveolin-1-positive ovarian carcinoma cells, whereas p129ctn and E-cad are even less associated in Caveolin-1-negative ovarian carcinoma cells [39]. In addition, caveolae recruit other cell adhesion molecules including CEACAM 6 and NrCAM for further cellular functions [31, 41]. Their findings suggest that Caveolin-1 modulates

cell-cell junctions. Consistently, our data also revealed that VCAM-1 is partitioned in Caveolin-1-enriched plasma membrane domain. Additionally, our results address that VCAM-1 is a key molecule for cancer cell adhesion to endothelial cells by the Caveolin-1-dependent fashion, hence providing mechanistic basis for roles of VCAM-1 in gastric cancers. Further studies will be necessary to understand the possible variable function of Caveolin-1 in the development and progression of human gastric tumors.

In conclusion, our study shows that VCAM-1 plays a crucial role in the carcinoma cell to endothelium adhesion by enhancing adhesion capability of tumor cell to endothelial cells through the collaboration with Caveolin-1. Our data thus suggest that elevated expression of VCAM-1 and Caveolin-1 might facilitate the metastatic progression of gastric tumor cells and thus malignant progression of gastric tumors.

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