Invasive human pancreatic carcinoma cells adhere to endothelial tri-cellular corners and increase endothelial permeability

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Although adhesive interactions between metastasizing cancer cells and vascular endothelial cells are critical in hematogenous metastasis, the early molecular events of the cancer–endothelial interaction remain largely obscure. Here we investigated the functional impact of cancer cells on endothelial permeability. We examined the binding of human pancreatic carcinoma cells MIA PaCa-2, PANC-1 and PSN-1 to a human umbilical vein endothelial cell (HUVEC) monolayer and the subsequent changes in the transendothelial electronic resistance (TEER) of the HUVEC. We found that MIA PaCa-2 and PANC-1 cells preferentially bound to the tri-cellular corners of HUVEC and induced a rapid and irreversible reduction of TEER. The reduction of HUVEC TEER was associated with the focal disengagement of endothelial junctional adhesion molecules VE-cadherin and CD31. Blocking antibodies to integrin β1, CD44, or CD9 affected neither the MIA PaCa-2 binding to HUVEC nor the reduction of TEER. Specific inhibitors for metalloproteinases, tyrosine-kinases and lipoxigenases, and a neutralizing anti-vascular endothelial growth factor antibody failed to affect the MIA PaCa-2-induced reduction of HUVEC TEER, whereas treatment of the cells with paraformaldehyde or cytochalasin B abrogated the TEER reduction. These findings indicate that the MIA PaCa-2 cells bind selectively to endothelial tri-cellular corners, triggering a reduction of HUVEC TEER, which requires the active metabolism and intact actin cytoskeleton of the carcinoma cells, and is apparently unrelated to previously described cell adhesion and soluble factor pathways. Our data indicate a novel cell-contact-dependent mechanism for the cancer cell-mediated breakdown of endothelial barrier functions, which may be important in hematogenous cancer metastasis. (*Cancer Sci* 2005; 96: 766–773)

The process of cancer metastasis consists of a series of sequential steps, all of which must be successfully completed to give rise to new metastatic foci.[1–3] In hematogenous metastasis, cancer cells that have escaped from the primary sites into the circulation are transported to distant organs and entrapped in the capillary beds by adhering to capillary endothelial cells.[3–5] The metastasizing cancer cells need to extravasate through the vascular wall to invade the surrounding host tissues and establish secondary cancer sites.[4,5] The extravasation process involves the breakdown of impermeable endothelial monolayers and is believed to be one of the major rate-limiting steps in hematogenous metastasis.[1,4,5]

Recent intravital imaging studies visualized the early events of cancer–endothelial cell interactions. In a liver metastasis model, cancer cells entrapped in sinusoidal capillaries induce rapid endothelial cell retraction *in situ*[6] and extravasate with high efficiency.[7,8] In a pulmonary metastasis model, endothelium-attached cancer cells proliferate within the blood vessels for a few days and subsequently penetrate the surrounding host tissues by destroying the vascular walls.[9,10] Endothelial cells lining the blood vessels control the passage of cells attached to their luminal surface by forming physiological barriers using endothelial junctions.[4,5] Although complex interactions between the metastasizing cancer cells and vascular endothelial cells are critical for establishing metastasis,[3–5] the early molecular events of the cancer–endothelial cell interactions remain largely obscure.

The endothelial junctional complexes are comprised of three distinct junctional structures, namely tight junctions, adherens junctions and gap junctions.[11] Tight junctions mainly regulate the paracellular permeability by forming a 'barrier' and 'fence' within the plasma membrane of adjacent endothelial cells. The adherens junctions also regulate vascular permeability and add mechanical strength to the endothelial linkage, whereas gap junctions mediate communication between endothelial cells. Upon interaction with cancer cells, vascular permeability is often down-regulated by modifications to the molecular organization of the endothelial junction complexes.[12,13] Although a large array of cell adhesion molecules,[14–19] metalloproteinases[20,21] and soluble factors[22–26] have been implicated in the active cross-talk between cancer cells and blood vessels, the mechanisms underlying the breakdown of endothelial barrier functions remain to be fully elucidated.

Previous studies demonstrated that the molecular architecture of endothelial junctional zones appears heterogeneous and complex.[11] In particular, at tri-cellular corners, where the borders of three cells converge, the tight junctions are reported to be partially discontinuous,[27,28] yet the barrier properties are preserved. Due to these unique structural and functional

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features, endothelial tri-cellular corners are hypothesized to be potential sites for the opening and closing of paracellular transmigration pathways, which allow the passage of circulating cells to the extravascular tissue.[27,29,30] In support of this hypothesis, Burns *et al*. demonstrated that endothelial tri-cellular corners are the preferred sites for neutrophil firm adhesion and extravasation under physiological flow conditions *in vitro* and *in vivo*.[31–33]

Pancreatic cancers often form hematogenous metastases in the early phase of the disease, resulting in a poor prognosis for patients.[34] In the present study, we used highly invasive human pancreatic carcinoma cells[22,35,36] and human vascular endothelial cells to investigate the influence of cancer cells on the permeability of vascular endothelial cells. Transendothelial electronic resistance (TEER) and cell binding analysis showed that pancreatic carcinoma cells could induce a rapid and irreversible reduction of TEER and preferentially bound to endothelial tri-cellular corners *in vitro*. The cancer cell binding to endothelial cells disrupted the endothelial junction complex at the site of cell contact, which was associated with the focal disappearance of VE-cadherin and CD31. Our results suggest that endothelial tri-cellular corners are preferred sites for invasive human pancreatic cancer cell binding and that the cancer cells binding to these particular sites appear to induce potently the disengagement of endothelial junctions. Our data also indicate a novel cell-contact-dependent mechanism for the cancer-cell-mediated breakdown of endothelial barrier functions, which may be important in hematogenous cancer metastasis.

Materials and Methods

Reagents

Recombinant human vascular endothelial growth factor (VEGF) and goat anti-human VEGF antibody were purchased from R&D Systems (Minneapolis, MN, USA). Tissue inhibitor of metalloproteinase (TIMP)-1 and TIMP-2 were purchased from Daiichi Fine Chemical (Takaoka, Japan). Monoclonal antibodies (mAbs) against human CD31 (hec 7) and CD29/integrin $\beta1$ (Lia1/2) were obtained through the VIth Human Leukocyte Differentiation Antigen Workshop (Kobe, Japan, 1996). Anti-human CD44 mAb (BRIC235) was from the International Blood Group Reference Laboratory (Bristol, UK). Anti-human CD9 mAb (MM2/57) was from Biodesign (Saco, ME, USA). Unless otherwise noted, reagents were obtained from Sigma Aldrich (St Louis, MO, USA).

Cell culture

Human pancreatic carcinoma cell lines MIA PaCa-2 and PANC-1 were obtained from the Cell Resource Center for Biomedical Research, Institute of Development, Aging and Cancer, Tohoku University (Sendai, Japan) and PSN-1 was a kind gift from Dr F. Nakata (Osaka City University Medical School, Osaka, Japan). The pancreatic carcinoma cells were cultured in RPMI-1640 medium supplemented with 10% fetal calf serum, 1% (v/v) $100 \times$ nonessential amino acids, 1 mM sodium pyruvate, 2 mM L-glutamine, 50 µM 2-mercaptoethanol, 100 units/mL penicillin and 100 µg/mL streptomycin. Passage 1 human umbilical vein endothelial cells (HUVEC) were obtained from BioWhittaker (Walkersville, MD, USA). HUVEC cultures were serially passaged, maintained in EGM-2 medium

(BioWhittaker) and used before the fifth passage. CHO cells were cultured in Ham's F-12 medium containing the same supplements as above.

Measurement of TEER

HUVEC (0.5×10^5 cells) were cultured for 5–7 days to form a monolayer on a polycarbonate filter insert (Transwell, pore size 0.4 µm, 6.5 mm diameter; Corning, Corning, NY, USA) coated with human plasma fibronectin (Invitrogen, Carlsbad, CA, USA) (10 µg/mL). To measure TEER, the filter insert with HUVEC was transferred to a resistance measurement chamber (ENDOHM-12; World Precision Instruments, Sarasota, FL, USA) and the upper luminal compartment and lower abluminal compartment were filled with 100 µL and 600 µL of culture medium, respectively. Cancer cells (1.0×10^5 cells) were added to the upper compartment of the resistance measurement chamber, and TEER across the HUVEC monolayer was measured at 37°C with an electrode placed in each compartment, at various time points.[37] The electrical resistance of individual HUVEC monolayers was obtained by subtracting the resistance of a corresponding naked filter coated with fibronectin from that of the filter on which HUVEC were grown. Changes in TEER are presented as a percentage of the control value. Thrombin, which induces a transient reduction of HUVEC TEER, was used as a positive control.[38] For inhibition studies, cancer cells were pretreated for 2 h with TIMP-1 (10 µg/mL), TIMP-2 (10 µg/mL), KB-R7785[39] (10 µM), marimastat[40] (1 µM), or brefeldin A (10 µg/mL), or for 6 h with nordihydroguaiaretic acid (10 µM) and used without washing. Cancer cells pretreated with 1% paraformaldehyde (PFA) (2 h) or 100 µM cytochalasin B (3 h) were used after extensive washing with the culture medium. To study the effects of neutralizing mAbs, cancer cells were pretreated with anti-CD29 mAb (10 µg/mL) and/or anti-CD44 mAb (10 µg/mL) or anti-CD9 mAb (10 µg/mL) for 30 min and used after washing. HUVEC monolayers pretreated with herbimycin A (1 µM, 30 min) were used after washing with the culture medium. All experiments were performed at least three times and the representative data are shown.

Preparation of culture supernatant

MIA PaCa-2 cells (5.0×10^5 cells) were seeded on 75 cm² flasks and cultured for 4 days to reach confluence. After being washed three times with phosphate-buffered saline (PBS) to remove the serum components, the cells were further incubated in 10 mL serum-free RPMI-1640 medium. The culture supernatants were collected at 2, 24 and 48 h, and concentrated 100-fold by ultrafiltration using a Centricon (CentriPlus YM-3; Millipore, Milford, MA, USA). The concentrated culture supernatants were dialyzed against PBS and used in the TEER assay (10 µL/well).

Adhesion assays

Cancer cells (1.0×10^5 cells) were added to the HUVEC monolayer grown on fibronectin-coated transwell inserts and incubated for the indicated times. After unbound cancer cells were removed by gentle washing, the cells were fixed with 0.05% glutaraldehyde in PBS for 10 min at room temperature, washed twice with PBS and incubated for 30 s with 0.25% AgNO$_3$. After being washed with PBS, they were mounted on

a glass slide using Gel/Mount (Biomeda, Foster, CA, USA), and the silver staining was developed under UV light for 5 min. Bound cells were counted manually under the microscope (BX50; Olympus, Tokyo, Japan). In some experiments, MIA PaCa-2 cells were treated with cytochalasin B as above and added to the HUVEC monolayer.

Immunofluorescence microscopy

MIA PaCa-2 cells were labeled with 4 µM 5-chloromethylfluorescein diacetate (CMFDA; Molecular Probes, Eugene, OR, USA) for 10 min at 37°C. The fluorescently labeled MIA PaCa-2 cells (1.0×10^5 cells/well) were added to HUVEC monolayers and incubated for the indicated times. After unbound tumor cells were removed by gentle washing, the cells on the membranes were fixed in 3% PFA/PBS for 15 min at room temperature, followed by treatment with 0.2% Triton X-100/PBS for 5 min, and blocked in PBS containing 10% fetal calf serum for 30 min at room temperature. After being washed with PBS, the cells were incubated with an anti-VE-cadherin antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or anti-CD31 antibody and then with biotin-conjugated donkey anti-goat IgG (Chemicon, Temecula, CA, USA) or biotinylated goat anti-mouse IgG (H + L) antibody (Vector Laboratories, Burlingame, CA, USA), followed by Alexa Fluor 594 Streptavidin (Molecular Probes). After being washed with PBS, the samples were mounted in Prolong Antifade (Molecular Probes) and observed using an LSM 410 confocal laser scanning microscope (Carl Zeiss, Oberkochen, Germany).

Results

Pancreatic carcinoma MIA PaCa-2 and PANC-1 cells induce a rapid and irreversible reduction of the TEER of HUVEC

HUVEC grown to confluence on a polycarbonate transwell insert were placed in a resistance measurement chamber. Invasive human pancreatic carcinoma cells, MIA PaCa-2, PANC-1, or PSN-1 cells, were added to the upper compartment of the transwell to allow direct contact with the HUVEC, and the changes in HUVEC TEER were monitored for 2 h. As shown in Fig. 1, MIA PaCa-2 and PANC-1 induced a strong reduction in HUVEC TEER, whereas PSN-1 induced only a modest reduction. After the addition of MIA PaCa-2 or PANC-1 cells, the reduction of HUVEC TEER was evident within 30 min and reached ~50% of the value for untreated HUVEC after 2 h. Prolonged incubation with MIA PaCa-2 cells further decreased the HUVEC TEER (< 20% of untreated HUVEC at 4 h). In contrast to the effects of MIA PaCa-2 and PANC-1 cells, thrombin elicited only a modest and transient decrease in HUVEC TEER. CHO cells failed to affect the HUVEC TEER. Together, these results indicate that certain pancreatic carcinoma cells rapidly induce a strong and irreversible reduction of HUVEC TEER. In the following experiments, we mainly used MIA PaCa-2 cells to investigate the mechanisms underlying the reduction in TEER.

Direct contact between MIA PaCa-2 cells and HUVEC is important for the reduction of HUVEC TEER

We next sought to determine the relative contributions of direct cell contact and soluble factors to the MIA PaCa-2-
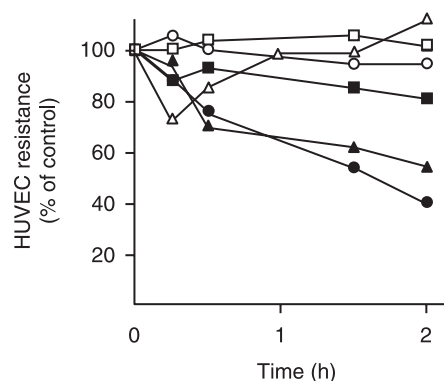

Fig. 1. Certain pancreatic cancer cells decreased the transendothelial electronic resistance (TEER) of a human umbilical vein endothelial cell (HUVEC) monolayer. Human pancreatic cancer cells (MIA PaCa-2 [●], PANC-1 [▲] and PSN-1 [■]) or CHO cells (□) (1×10^5 cells/well) were added to HUVEC monolayers formed on fibronectin-coated polycarbonate membranes in the upper chamber of a transwell chamber. The TEER of the HUVEC monolayer was measured at various time points. Thrombin (△) (3 U/mL) and culture medium (○) were used as a positive and negative control, respectively.

induced reduction of HUVEC TEER. As shown in Fig. 2a, when MIA PaCa-2 cells were added to the lower chamber so that they could not make direct contact with the HUVEC, they failed to decrease TEER. Concentrated MIA PaCa-2 cell culture supernatants also failed to affect HUVEC TEER (Fig. 2b). The pretreatment of MIA PaCa-2 cells with brefeldin A, which inhibits the secretion of various soluble factors,[41] did not affect the MIA PaCa-2 cell-induced reduction of HUVEC TEER (Table 1). Because MIA PaCa-2 cells produce VEGF,[42] which can increase vascular permeability,[25,26] we next tested the effects of a neutralizing anti-VEGF antibody. As shown in Fig. 2c, an anti-VEGF antibody that could abrogate the activities of exogenously added VEGF (10 nM) did not elicit detectable changes in the MIA PaCa-2 cell-induced reduction of HUVEC TEER (Fig. 2c). These observations collectively indicate that the direct cell–cell contact between MIA PaCa-2 cells and HUVEC plays a major role in the reduction of HUVEC TEER and that soluble factors, such as VEGF, which may be produced by MIA PaCa-2 cells, appear to be little involved in the reduction of HUVEC TEER under the conditions used in this study.

MIA PaCa-2 cell-induced reduction of HUVEC TEER is dependent on an active metabolism and intact cytoskeleton of MIA PaCa-2 cells

Cancer cell adhesion to vascular endothelium is regulated by the coordinated actions of cell-adhesion molecules and cytoskeletal components. We found that cytochalasin B, which disrupts the actin cytoskeleton, significantly inhibited the binding of MIA PaCa-2 cells to the HUVEC monolayer (Fig. 3a) and the TEER reduction (Fig. 3b). MIA PaCa-2 cells express high levels of α3β1 and α5β1 integrins, CD44 and CD9 (data not shown), which have been implicated in various cancer–endothelial cell interactions. These adhesion molecules, however, did not appear to play significant roles in the MIA PaCa-2 cell binding to HUVEC, given that neutralizing anti-integrin β1 and anti-CD44 mAbs, used
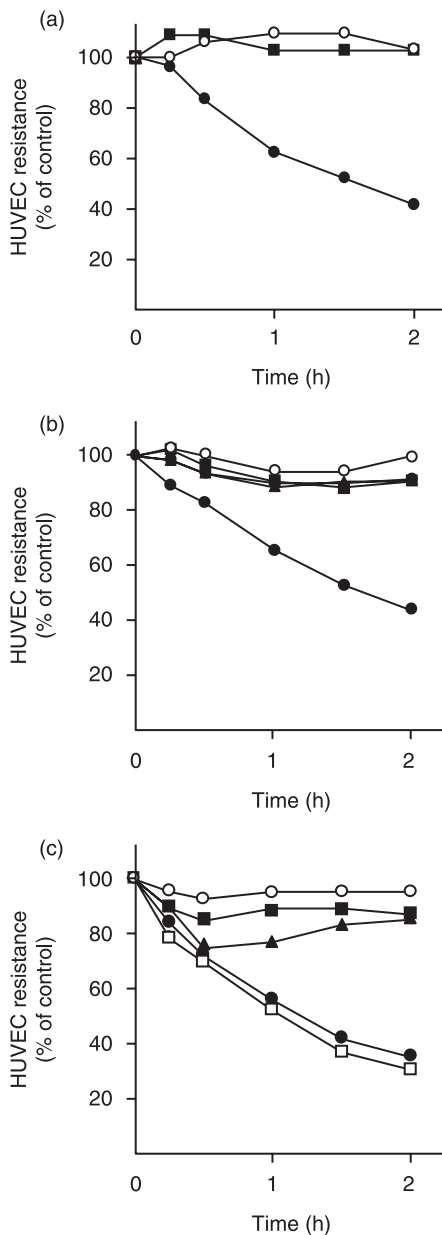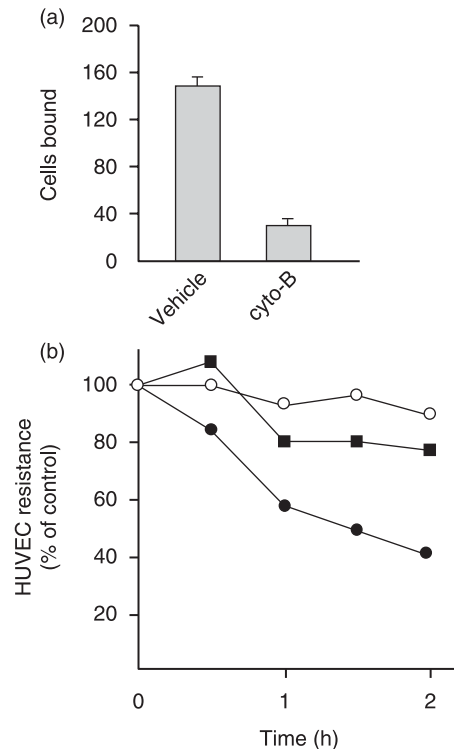
Fig. 2. Direct cell contact between MIA PaCa-2 cells and human umbilical vein endothelial cells (HUVEC) was important for the reduction of HUVEC transendothelial electronic resistance (TEER). (a) MIA PaCa-2-induced reduction of HUVEC TEER. MIA PaCa-2 cells (1×10^5 cells/well) were added either to the upper (●) or lower (■) well of a transwell chamber, and HUVEC TEER was measured at various time points. Culture medium (○) was used as a negative control. (b) Effects of MIA PaCa-2 culture supernatants on HUVEC TEER. MIA PaCa-2 cells (1×10^5 cells/well) (●) and concentrated culture supernatants of MIA PaCa-2 cells harvested at 2 h (□), 24 h (■) and 48 h (▲) were added to HUVEC monolayers and TEER was measured. Fresh culture medium (○) was used as a control. (c) Effects of anti- vascular endothelial growth factor (VEGF) antibody on the MIA PaCa-2 induced reduction of HUVEC TEER. MIA PaCa-2 cells (1×10^5 cells/well) were added to HUVEC in the presence (□) or absence (●) of an anti-VEGF antibody (20 μg/mL), and HUVEC TEER was examined. In parallel, the effects of exogenously added VEGF (10 nM) on HUVEC TEER were tested in the presence (■) or absence (▲) of the anti-VEGF antibody. Culture medium (○) was used as a negative control.

Table 1. Effects of various inhibitors on MIA PaCa-2-induced reduction of human umbilical vein endothelial cell (HUVEC) transendothelial electronic resistance (TEER)

Inhibitors	Concentration	Inhibition of TEER reduction[†]
Paraformaldehyde	1%	+++
Cytochalasin B	100 μM	++
Brefeldin A	10 μg/mL	–
Anti-β1 integrin	10 μg/mL	–
Anti-CD44	10 μg/mL	–
Anti-β1 integrin + Anti-CD44	10 μg/mL 10 μg/mL	–
Anti-CD9	10 μg/mL	–
KB-R7785	10 μM	–
Marimastat	1 μM	–
TIMP-1	10 μg/mL	–
TIMP-2	10 μg/mL	–
Herbimycin A	1 μM	–
Nordihydroguaiaretic acid	10 μM	–

[†]TEER of HUVEC was measured at 2 h after the addition of MIA PaCa-2 cells.
+++ 70–100% of the reduction of TEER was inhibited; ++, 30–70%; –, 0%.

Fig. 3. The intact actin cytoskeleton of MIA PaCa-2 cells was required for both binding to human umbilical vein endothelial cells (HUVEC) and the reduction of HUVEC transendothelial electronic resistance (TEER). (a) MIA PaCa-2 cell binding to the HUVEC monolayer. Cytochalasin B-treated (100 μM, 3 h) or vehicle-treated MIA PaCa-2 cells were added to the HUVEC monolayer, and incubated for 2 h at 37°C. After washing, the number of MIA PaCa-2 cells that bound to HUVEC was counted. The number of bound MIA PaCa-2 cells was scored for three 200× microscopic fields at the indicated time points. The data are presented as the number of bound cells in a 0.658 mm² field (mean ± SD). (b) Effects of treating MIA PaCa-2 cells with cytochalasin B on the reduction of HUVEC TEER. Cytochalasin B-treated (■) or vehicle-treated (●) MIA PaCa-2 cells were added to the HUVEC monolayer, and HUVEC TEER was measured. Culture medium was added as a negative control (○).

either alone or in combination, or a neutralizing anti-CD9 mAb, showed no apparent effects (Table 1). Other adhesion molecules implicated in tumor–host interactions, such as $\alpha 1\beta 1$, $\alpha L\beta 2$, $\alpha M\beta 2$, $\alpha 4\beta 1$, $\alpha 4\beta 7$, $\alpha v\beta 3$ integrins, nectin-2 and DNAM-1, were not detectable on the surface of MIA PaCa-2 cells (data not shown).

We next asked if MIA PaCa-2 cells require an intact metabolism to reduce HUVEC TEER. Pretreatment of MIA PaCa-2 cells with PFA abrogated the TEER reduction, although it affected MIA PACA-2 cell binding only marginally (Table 1 and data not shown). We also investigated the potential contribution of metalloproteinases, tyrosine-kinases and lipoxigenases in the reduction of TEER using specific inhibitors (KB-R7785, marimastat, TIMP-1, TIMP-2, herbimycin A and nordihydroguaiaretic acid) at concentrations sufficient to block their target enzymes,[24,40,43–45] but no significant effects were observed (Table 1). Together, these results indicate that an active metabolism of MIA PaCa-2 cells is indispensable for the reduction of HUVEC TEER and that an intact actin cytoskeleton of the MIA PaCa-2 cells is also required for this process.

MIA PaCa-2 cells and PANC-1 cells preferentially adhere to HUVEC at tri-cellular corners

Previous studies by Burns *et al*.[31–33] demonstrated that neutrophils selectively adhere to the tri-cellular corners of HUVEC and transmigrate thereafter. We thus examined whether invasive cancer cells also preferentially bind to endothelial tri-cellular corners. In this experiment, the binding of MIA PaCa-2 cells to HUVEC was differentially scored as binding to tri-cellular corners or to the region between two adjacent endothelial cells, and the results were compared with those obtained using PANC-1 and PSN-1 cells. As shown in Fig. 4a, silver nitrate staining allowed us to detect the precise position of the bound cancer cells in the HUVEC cell junction. Although MIA PaCa-2, PANC-1 and PSN-1 showed comparable levels of binding (Fig. 4b), MIA PaCa-2 and PANC-1 cells, but not PSN-1 cells, preferentially bound to HUVEC tri-cellular corners; such binding accounted for approximately 75% and 70% of the MIA PaCa-2 and PANC-1 binding, respectively, but only 30% of the PSN-1 binding (Fig. 4c). These observations indicate that MIA PaCa-2 and PANC-1 cells, which could induce a large reduction of TEER, preferentially bound to the tri-cellular corners of HUVEC.

Fig. 4. MIA PaCa-2 cells and PANC-1 cells preferentially adhered to human umbilical vein endothelial cells (HUVEC) at tri-cellular corners. (a) MIA PaCa-2 cells adhering to HUVEC were scored as adhering at tri-cellular corners (asterisks), or between two adjacent endothelial cells (arrows). The photograph represents results obtained at 60 min. (b) Quantitative analysis of the MIA PaCa-2, PANC-1 and PSN-1 cell binding to the HUVEC monolayer. The number of bound tumor cells was scored for three 200× microscopic fields at the indicated time points. The data are presented as the number of bound cells in a 0.658 mm² field (mean ± SD). (c) MIA PaCa-2 and PANC-1 cells preferentially bound to the HUVEC monolayer at tricellular corners. MIA PaCa-2, PANC-1, and PSN-1 cells were added to the HUVEC monolayer and incubated for 1 h. After the unbound tumor cells were removed by gentle washing, the bound tumor cells and endothelial cell borders were stained with silver nitrate. The bound tumor cells were easily distinguishable from HUVEC due to their round shape. The bar graph displays the percentages of the tumor cells that bound to tri-cellular corners or between two adjacent HUVEC. At each time point, at least 80 bound tumor cells were examined.

MIA PaCa-2 cell binding induces the focal disappearance of VE-cadherin or CD31 from HUVEC junctions

We next investigated whether MIA PaCa-2 cell binding affects the integrity of the endothelial junctions of HUVEC. To this end, CMFDA-labeled MIA PaCa-2 cells were added to confluent HUVEC monolayers, and incubated for 30, 60 and 90 min. After unbound MIA PaCa-2 cells were removed, the HUVEC
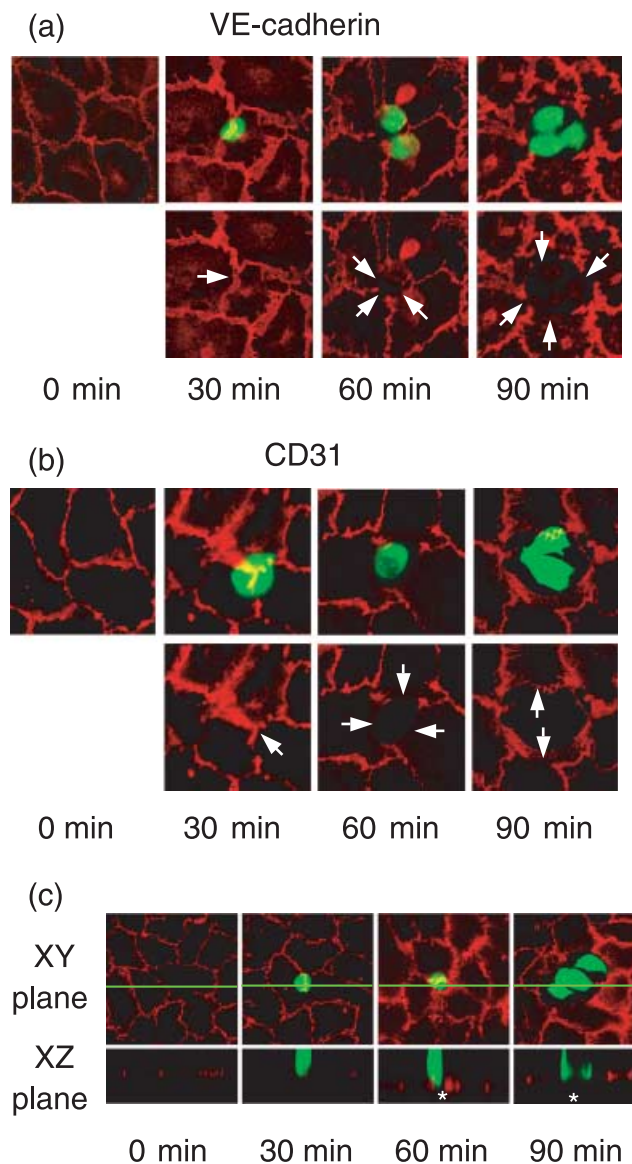
(a)

(b)

(c)
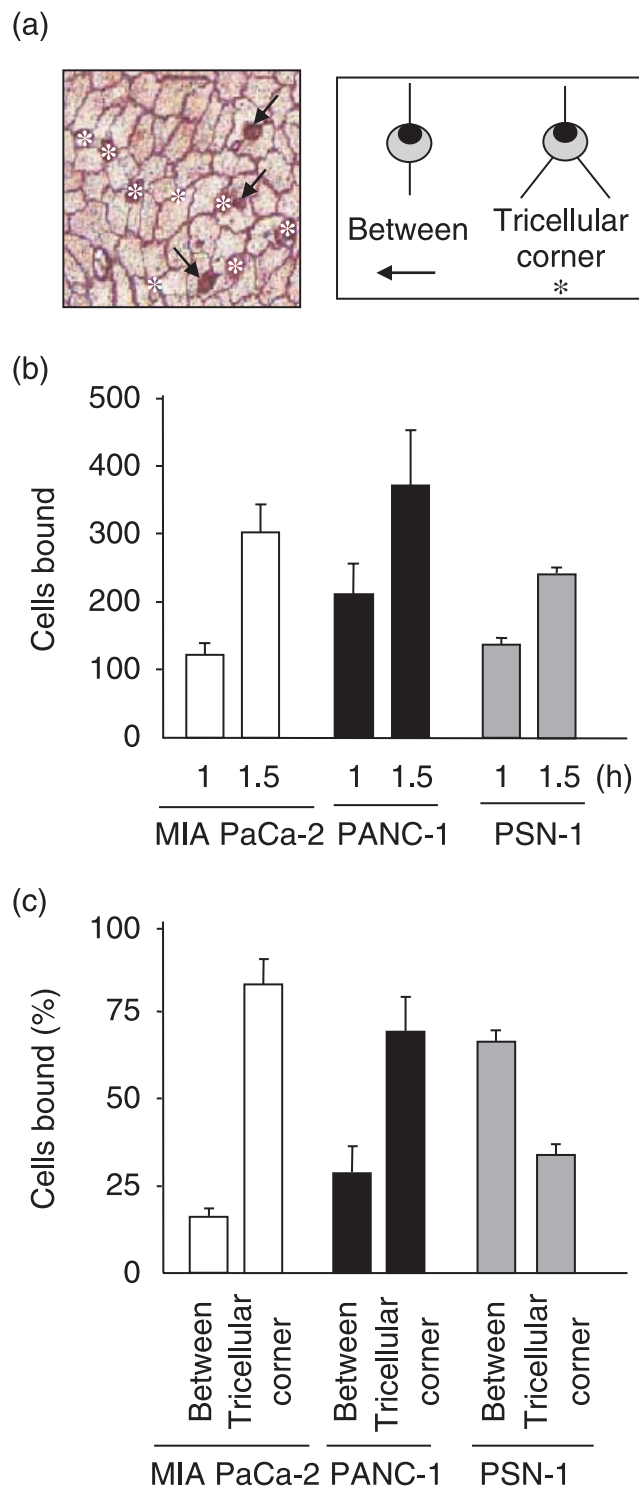
monolayers were stained with an anti-VE-cadherin or anti-CD31 mAb and analyzed by confocal microscopy. Before the addition of MIA PaCa-2, the HUVEC retained well-developed endothelial junctions and showed no gaps at the tri-cellular corners as revealed by immunofluorescence staining with anti-VE-cadherin (Fig. 5a) or anti-CD31 (Fig. 5b) antibodies. As shown in Fig. 5, 30 min after their addition, MIA PaCa-2 cells showed preferential binding to the tri-cellular corners of the HUVEC monolayers, but the HUVEC junctions remained occluded. At 60 min, focal losses of anti-VE-cadherin and anti-CD31 staining were observed in the immediate vicinity of the MIA PaCa-2 cell-binding sites (arrows), whereas the VE-cadherin and CD31 staining was apparently unaffected around the neighboring endothelial cells. At 90 min, the disruption of cell junctions was seen in the HUVEC monolayer exclusively around the adherent MIA PaCa-2 cells but not at sites distant from the tumor cell adhesion. Disengagement of the HUVEC junctions was confirmed by the examination of vertical confocal images. As shown in Fig. 5c, bound MIA PaCa-2 cells were observed within the gap formed between HUVEC at 60 min or later, and appeared to be in direct contact with the subendothelial matrices. These observations indicate that MIA PaCa-2 cells that had bound to the tri-cellular corners of HUVEC induced the focal disappearance of endothelial VE-cadherin and CD31 at an early stage of cell adhesion and subsequently disrupted the junctions of HUVEC to infiltrate the subendothelial layer.

Discussion

In hematogenous metastasis, cancer cells bind to the vascular wall and disengage the endothelial junctions to invade the surrounding tissues by mechanisms as yet unknown. In the present study, we showed that highly invasive human pancreatic carcinoma cells, such as MIA PaCa-2 and PANC-1, preferentially bound to the tri-cellular corners of HUVEC and induced a rapid and irreversible reduction of TEER. The reduction of HUVEC TEER required direct cell adhesion between the tumor cells and HUVEC and the active metabolism and intact cytoskeleton of the tumor cells, which apparently induced the focal disengagement of endothelial junctional adhesion molecules, including VE-cadherin and CD31. Although cancer cell attachment may occur randomly to the cell surface and cell-to-cell junctions of HUVEC, our results collectively indicate that the binding of certain pancreatic cancer cells to endothelial tri-cellular corners plays a critical role in increasing endothelial permeability.

Certain cell-adhesion molecules, such as β1 integrins, CD44, CD9 and selectins,[14,15,18,19] have been implicated in cancer–endothelial interactions; however, these adhesion receptors did not seem to play important roles in the binding of MIA PaCa-2 cells to HUVEC under our experimental conditions. Blocking mAbs to the integrin β1 chain and/or CD44 or CD9 did not show any detectable effects on the MIA PaCa-2 cell binding to HUVEC (data not shown). In addition, the MIA PaCa-2 cells and HUVEC used in this study were devoid of the expression of sialyl Lewis[x] epitopes and E-/P-selectins. Other adhesion molecules implicated in tumor–host interactions, such as αLβ2, αMβ2 α4β7 and αvβ3 integrins,[15–17] nectin-2[46] and DNAM-1[47] were also undetectable in the MIA PaCa-2

(a) VE-cadherin

0 min   30 min   60 min   90 min

(b) CD31

0 min   30 min   60 min   90 min

(c)

XY plane

XZ plane

0 min   30 min   60 min   90 min

Fig. 5. MIA PaCa-2 cell binding induced the redistribution of VE-cadherin and CD31 between adjacent human umbilical vein endothelial cells (HUVEC). (a, b) Immunohistochemical analysis of VE-cadherin and CD31 in the HUVEC monolayer after the addition of MIA PaCa-2 cells. MIA PaCa-2 cells labeled with 5-chloromethylfluorescein diacetate were added to a HUVEC monolayer and incubated for the indicated times. After the unbound MIA PaCa-2 cells were removed by gentle washing, the HUVEC monolayers were stained with an anti-VE-cadherin antibody (a, red) or anti-CD31 antibody (b, red) and observed under a confocal microscope. Overlaid images of MIA PaCa-2 cells (green) and VE-cadherin or CD31 (red) staining are shown in the upper panels. VE-cadherin or CD31 staining alone is shown in red (lower panels). At 60 and 90 min, MIA PaCa-2 cells (green) adhered to the HUVEC monolayer and induced the redistribution of VE-cadherin and CD31 (red) between adjacent HUVEC (lower panels, arrows). Arrows indicate the positions of MIA PaCa-2 cell binding. (c) Cross-sectional view of MIA PaCa-2 cells bound to the HUVEC monolayer. After the addition of MIA PaCa-2 cells (green), HUVEC monolayers were stained with anti-CD31 (red) as in Fig. 5b. The XZ plane cross-sectional views (lower panels) according to the green line in the XY plane (upper panels) were obtained at the indicated time points. At 60 and 90 min, bound MIA PaCa-2 cells were observed in the gaps (asterisks) formed between HUVEC.

cells. The cell adhesion molecules mediating the MIA PaCa-2 cell binding to HUVEC need to be identified in future investigations.

The MIA PaCa-2 cell-induced reduction of HUVEC TEER appeared to be cell-contact-dependent, and endothelial gap formation was selectively observed at the sites of MIA PaCa-2 cell binding. These observations appear incompatible with those reported by Nakamori et al.,[22] in which both MIA PaCa-2 and PSN-1 cells could induce an increase in endothelial permeability by secreting humoral factors. However, induction of the endothelial retraction in their study was observed 3 h or more (most significantly at 48 h) after stimulation with tumor-derived factors, whereas in our study the disruption of endothelial junctions was already detectable at 30 min and prominent 2 h after the tumor cells were added. Therefore, it is likely that these two studies examined distinct phenomena at different time points, and pancreatic carcinoma cells may have two distinct mechanisms for disrupting endothelial junctions: one operating early using a contact-dependent mechanism; the other operating late using a humoral mechanism. Previous studies using other types of cancers have reported a similar contact-dependent disengagement of endothelial junctions and suggest that VEGF[25,26] or eicosanoid 12(S)-hydroxyeicosateraenoic acid[23,24] produced by the cancer cells plays a critical role in the endothelial disengagement. In contrast, our data appear to negate the involvement of at least these soluble factors in the MIA PaCa-2-induced reduction of HUVEC TEER under the conditions and in the time frame we examined, because neither a neutralizing anti-VEGF antibody nor the lipoxygenase inhibitor nordihydroguaiaretic acid affected the reduction of TEER. We found, instead, that pretreatment of MIA PaCa-2 cells with PFA, but not with specific inhibitors for metalloproteinases and tyrosine-kinases, abrogated the reduction of TEER. Thus, while the potential contribution of cancer-cell-derived soluble factors cannot be ruled out formally, our results favor a model in which MIA PaCa-2 cells transmit signals to the HUVEC by way of PFA fixation-sensitive mechanisms in a cell-contact-dependent manner, leading to the disengagement of endothelial junctions at early time points after cell adhesion.

An unexpected finding of this study was that invasive human pancreatic carcinoma MIA PaCa-2 and PANC-1 cells preferentially bound to HUVEC tri-cellular corners. Previous studies reported that neutrophils[31–33] as well as melanoma A375 cells[14] also preferentially bind to the tri-cellular corners of HUVEC. Unlike MIA PaCa-2 cells, these cells migrated across the HUVEC monolayers without, apparently, disrupting the endothelial junctions.[14,31,33] Thus, cell binding to the tri-cellular corners per se is not sufficient to trigger the irreversible dissociation of cell junctions in HUVEC but probably requires one or more additional factors. Although the mechanisms underlying the selective binding to tri-cellular corners remain unclear, one possible explanation is that the tight junction strands are partly discontinuous at tri-cellular corners, exposing the pro-adhesive subendothelial matrices, resulting in the matrix binding of the pancreatic carcinoma cells at these specific regions. Alternatively, the endothelial tri-cellular corners may selectively express certain cell-adhesion molecules that support the selective binding of the pancreatic cancer cells. This possibility is supported by recent studies showing that the epithelial cells of developing Drosophila express a cell adhesion molecule that is preferentially localized to tri-cellular corners.[48,49]

In the present study, we showed that certain highly invasive human pancreatic cancer cells preferentially bound to tri-cellular corners and reduced TEER. The reduction of TEER was accompanied by the local disjunction of VE-cadherin and CD31, thus facilitating the cancer cells' access to the subendothelial matrix and invasion into surrounding tissues. Our data provide a novel cell-contact-dependent mechanism for the cancer cell mediated breakdown of endothelial barrier functions, which deserves further investigation.

Acknowledgments

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